Microbial sulfate reduction in the tissue of the cold-water sponge *Geodia barretti* (Tetractinellida, Demospongiae)

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EXTENDED ABSTRACT

Metabolic rates, community structure and the chemical micro-environment of sulfate-reducing bacteria (SRB) within the tissue of the boreal demosponge *Geodia barretti* were examined, and implications for biological-chemical and sponge-bacteria interactions are discussed. To enable these investigations, histological, molecular biological and biogeochemical methods were combined, new methods were developed and established methods were modified.

Sponges represent the very base of metazoan evolution and can be regarded as the oldest animal phylum still alive. Fossil records indicate that the family Geodiidae has been existing since the early Cambrian. *Geodia barretti* hosts vast amounts of associated microorganisms in its intercellular matrix ("bacteriosponge"). This species is extremely rich in siliceous spicules, with a pronounced cortex of microscleres at the sponge surface. Various methods in sponge histotechnology were developed and evaluated which allow preparation of tissue sections without removing the spicules. These sections are applicable for examination of the microbial community by fluorescence in situ hybridization (FISH) as well as for histological investigations.

A cultivation method for fragments of *G. barretti* was also developed. Cultivated fragments served as experimental units for microelectrode studies. Histological investigations over a cultivation period of 8 months showed the ability of this sponge to grow and regenerate from a random piece of tissue.

FISH on tissue sections showed that *G. barretti* contains 2.23 $*10^{10}$ SRB cm^{-3} tissue, which represent 7.6 % of the bacterial community. The SRB community was found to be different from that in marine sediments. The predominant genus was *Desulfovibrio*, which comprises specialized feeders favoured by high substrate concentrations. SRB were evenly distributed throughout the choanosomal tissue, but were absent in and directly beneath the cortex. Repeated measurements with oxygen-sensitive microelectrodes showed that in a pumping *G. barretti*, the cortex and the subcortical spaces were well oxygenated. In the choanosome, oxygen decreased rapidly and was always depleted 4-6 mm below the sponge surface. When the sponge stopped pumping, diffusive oxygen consumption in the overlying water could be observed. Oxygen profiles in cultivated fragments showed a nearly parabolic shape and anoxic conditions already 500µm below the surface, indicating that oxygen supply was solely due to molecular diffusion with no pumping activity involved. Sulfate reduction rates (SRR) in sponge tissue ranged from 1-1200 nmol SO_4^{2-} cm^{-3} d^{-1} with strong variations within the same sponge. Time series with tissue slurry showed strong dependence of SRR on incubation time with $^{35}SO_4^{2-}$. This indicates a rapid sulfide reoxidation, and thus a direct coupling between sulfate reduction and sulfide oxidation. Even the highest rates measured may still underestimate true SRR, which were estimated to be up to 5000 nmol cm^{-3} d^{-1}. From these rates, cell specific SRR (csSRR) up to 0.22 fmol $cell^{-1}$ d^{-1} were calculated, which are in a reasonable range for natural environments.

My results indicate the presence of an endosymbiotic sulfur cycle in the tissue of *G. barretti*, driven by the activity of SRB and by oxic/anoxic cycles in the tissue due to varying pumping activity of the sponge. Tissue anoxia may arise due to the active metabolism of sponge cells and aerobic associated bacteria under low pumping activity. Under these conditions, sponge cells will switch their metabolism to fermentation. Fermentation products serve as substrates for SRB, which can be consumed by sponge cells ("bacterial farming"). In the presence of oxygen, the sulfur cycle within the sponge tissue is completed by oxidation of sulfide to sulfate. The involvement of sulfide-oxidizing bacteria in this step is very likely.

My results indicate the relevance of anaerobic metabolic processes in the intercellular matrix of *G. barretti*, with sulfate-reducing bacteria as putative key players in sponge metabolism and nutrition. The possibility of an ancient origin of the symbiosis between SRB and sponges or sponge precursors remains a matter of discussion. However, this efficient system of internal nutrient recycling by symbiotic partners may be the reason for the success of *Geodia* and other bacteriosponges through earth history until today.

ERWEITERTE ZUSAMMENFASSUNG

Umsatzraten, Gemeinschaftsstruktur und das chemische Mikromilieu sulfatreduzierender Bakterien (SRB) im Gewebe des borealen Schwammes *Geodia barretti* (Demospongiae) wurden untersucht, und biologisch-chemische sowie Schwamm-Bakterien-Wechselwirkungen diskutiert. Dafür wurden histologische, molekularbiologische und biogeochemische Methoden kombiniert, neue Methoden entwickelt und bewährte modifiziert.

Schwämme stehen am Anfang der Metazoenentwicklung und können als ursprünglichster aller jetzt lebender Tierstämme bezeichnet werden. Fossilienfunde zeigen, dass die Familie Geodiidae seit dem frühen Kambrium existiert. *Geodia barretti* beherbergt eine große Zahl assoziierter Mikroorganismen in seiner interzellulären Matrix. Solche Schwämme werden auch als „bacteriosponges“ („Bakterienschwämme“) bezeichnet. Diese Art besitzt zahlreiche Nadeln aus Silikat, die an der Oberfläche eine dichte Cortex aus Mikroskleren bilden. Verschiedene histotechnologische Methoden wurden erprobt, um Gewebeschnitte ohne vorherige Herauslösung der Skleren zu erhalten. Solche Schnitte wurden für die Untersuchung der Bakteriengemeinschaft mit Fluoreszenz in situ Hybridisierung (FISH) benötigt, sowie auch für histologische Untersuchungen.
Ferner wurde eine neue Methode zur Kultivierung von Gewebefragmenten von *G. barretti* entwickelt. Kultivierte Schwammfragmente dienten als experimentelle Einheiten für Untersuchungen mit Mikroelektroden. Histologische Beobachtungen über einen Kultivierungszeitraum von 8 Monaten hinweg zeigten die Fähigkeit dieses Schwammes, sich aus einem beliebigen Gewebestück zu regenerieren.
Bakterienzählungen mit FISH an Gewebeschnitten ergaben $2{,}23*10^{10}$ SRB cm^{-3}. Das sind 7,6% der gesamten Schwammbakterien. Die Zusammensetzung der SRB-Gemeinschaft in *G. barretti* unterschied sich von der in marinen Sedimenten. Die Gattung *Desulfovibrio* war am zahlreichsten vertreten. Diese Gattung beinhaltet Sulfatreduzierer, die auf bestimmte Substrate spezialisiert sind und bevorzugt bei hohen Substratkonzentrationen wachsen. SRB waren gleichmäßig im choanosomalen Gewebe verteilt, fehlten jedoch in und direkt unterhalb der Cortex. Wiederholte Messungen mit Sauerstoff-Mikroelektroden zeigten eine gute Sauerstoffversorgung in Cortex und subcorticalen Kammern eines aktiv pumpenden Schwammes. Im Choanosom nahm der Sauerstoff rapide ab, und war 4-6 mm unter der Oberfläche nicht mehr messbar. In nicht-pumpenden Individuen wurde diffusiver Sauerstoffverbrauch im überliegenden Wasser beobachtet. Sauerstoffprofile an kultivierten Fragmenten zeigten eine nahezu parabolische Form, was darauf hinweist, dass die Sauerstoffversorgung allein durch Diffusion gedeckt wird und die Fragmente nicht aktiv pumpen können. Sulfatreduktionsraten (SRR) im Schwammgewebe lagen zwischen 1-1200 nmol SO_4^{2-} cm^{-3} d^{-1}, mit starken Variationen innerhalb eines Individuums. Zeitserien mit homogenisiertem Schwammgewebe zeigten eine starke Abhängigkeit der SRR von der Inkubationszeit mit $^{35}SO_4^{2-}$. Dies deutet auf eine schnelle Reoxidation des Sulfids hin, was bedeutet, dass Sulfatreduktion direkt mit Sulfidoxidation verknüpft ist. Auch die höchsten gemessenen Raten unterschätzen möglicherweise die wahren SRR, welche auf bis zu 5000 nmol cm^{-3} d^{-1} geschätzt wurden. Von diesen Raten wurden zellspezifische Sulfatreduktionsraten bis zu 0,22 fmol $cell^{-1}$ d^{-1} kalkuliert, welche vergleichbar sind mit solchen in marinen Sedimenten.
Meine Ergebnisse lassen auf einen endosymbiontischen Schwefelkreislauf im Gewebe von *G. barretti* schließen, angetrieben von der Aktivität der SRB und von oxischen/anoxischen Kreisläufen im Gewebe aufgrund von variierender Pumpaktivität des Schwammes. Sind Schwammzellen und aerobe assoziierte Bakterien metabolisch aktiv in einem schwach pumpenden Schwamm, können Gewebebereiche anoxisch werden. Unter diesen Bedingungen betreiben Schwammzellen Fermentierung. Fermentationsprodukte sind Substrate für SRB, welche von Schwammzellen konsumiert werden können. In der Gegenwart von Sauerstoff wird der Schwefelkreislauf im Schwammgewebe durch Oxidation von Sulfid zu Sulfat vervollständigt. Die Beteiligung von sulfidoxidierenden Bakterien an diesem Schritt ist sehr wahrscheinlich.
Meine Ergebnisse weisen auf die Bedeutung anaerober Prozesse in der interzellulären Matrix von *G.* barretti hin, wobei den SRB möglicherweise eine Schlüsselrolle für den Metabolismus und die Ernährung des Schwammes zukommt. Ob diese Symbiose ein Relikt aus der frühen Entwicklungsgeschichte der Tiere ist, bleibt spekulativ. Allerdings könnte dieses effiziente System des internen Nährstoffrecyclings zwischen Symbiosepartnern der Grund für das lange Überleben der Gattung *Geodia* und anderer „Bakterienschwämme“ sein.

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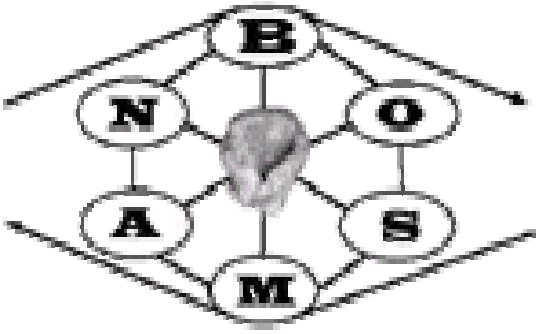

DEFINITIONS AND ABBREVIATIONS

FISH: Fluorescence in situ hybridization

SAB: Sponge associated bacteria

SAM: Sponge associated microorganisms

SRB: Sulfate-reducing bacteria

SRR: Sulfate reduction rate/s

Sulfide, free sulfide: Sum of H_2S, HS^-, S^{2-}

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1 INTRODUCTION

1.1 Sponges on the base of metazoa evolution

Sponges are sedentary filter-feeding organisms, characterized by an unusual body plan built around a system of water canals and chambers. Within the chambers, special flagellated cells called choanocytes produce a water current that enters the sponge body through surface pores (Porifera = bearing pores), and leaves it through larger openings called osculi. 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 such a high degree of independence that the sponge body resembles a protozoan colony in some respect. However, the mode of sexual reproduction and embryogenesis, production of an extracellular matrix with collagen fibrils, possession of adhesion molecules and receptors for cell-cell contact as well as simple processes for signal transduction and a functional immune system (Ax 1995; Müller 1998) characterize sponges as true metazoa. Although the monophyly of the Porifera is still a matter of debate (Reitner and Mehl 1996; Zrzavy et al. 1998; Borchiellini et al. 2001; Schuetze et al. 1999) they can be grouped into three classes: Calcarea, characterized by calcareous spicules – this class has recently been suggested to be upgraded to the phylum level (Borchiellini et al. 2001); Hexactinellida, characterized by siliceous spicules of hexactine structure and syncytial tissue organization; and Demospongiae, the most numerous and diverse class. They generally have a mineral skeleton made of silceous spicules, but several lineages, like the common bath sponge, have no mineral skeleton at all. The sketch of a typical Demosponge body plan is shown in Fig. 1, Plate 1. In spite of their simple structure, sponges have been succeeding in nearly all contemporary aquatic environments and have developed an exciting variety of strategies for competing even under unfavourable ecological conditions.

Sponges represent the very base of metazoan evolution and can be regarded as the oldest animal phylum still alive. The spicule record for sponges starts in the Late Proterozoic. The oldest spicules with demosponge affinities were found in ca. 750 Ma old Noon Day Dolomite in Nevada, and in the Neoproterozoic *Cloudina*-Reefs (ca. 555 Ma) of southern Namibia (Reitner and Wörheide 2002). The fossil record documents that all classes of the Porifera have been present at least since the lowermost Cambrian (Reitner and Mehl 1995; Reitner and Mehl 1996). This implies that the evolution of the Porifera must have taken place in the Precambrian, well before the so called “Cambrian Explosion”. 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 good biomarkers for sponges, were found in 1800 Ma old stromatolites (McCaffrey et al. 1994; Moldowan et al. 1994).

1.2 Sponge-bacteria associations

A common strategy among sponges is their close association with prokaryotes. Whereas the intercellular matrix of higher animals is usually sterile, the sponge tissue may host vast amounts of microbes. Sponges harbouring large symbiotic bacterial populations have been termed as "bacteriosponges" (Reiswig 1981).

Symbiosis is defined as "a close and usually obligatory association of organisms of different species living together" (Lawrence 1989). Symbiosis is often confused with mutualism, defined as a symbiosis with mutual benefit for both partners. Other possibilities of symbioses are commensalism (one partner benefits, the other is not harmed) and parasitism (one partner receives advantage to the detriment of the other). Multifaceted host-symbiont interactions undoubtedly affect the biology and biochemistry of the sponge animal as a whole. Despite over 20 years of research on this topic, the particular functions of the microbes within the eukaryotic and the prokaryotic cell populations largely remain undetermined and the nature of this symbiosis is still a matter of discussion.

The first approaches were phenotypic studies of sponge associated microorganisms with light and electron microscopy. These investigations revealed species-specific microbial communities different from those in the ambient sea water (Vacelet 1970; Vacelet 1975; Vacelet and Donadey 1977; Wilkinson 1978a; Wilkinson 1978b; Wilkinson 1978c). The cultivation of sponge microbes showed a surprising physiological and phylogenetic variety of associated microorganisms (Wilkinson et al. 1981; Santavy et al. 1990; Hentschel et al. 2001). However, the culturable microbial community represents only a small percentage of the sponge associated microbes. Estimates of bacterial recoverability from environmental samples range from 0.01% to 12.5% of the existing community (Olson et al. 2000). The application of modern molecular biological methods such as fluorescence in situ hybridization (FISH) and 16S rRNA sequencing improved the actual knowledge about the phylogenetic affiliation of sponge symbionts (Schumann-Kindel et al. 1997; Friedrich et al. 1999; Manz et al. 2000; Schmidt et al. 2000; Friedrich et al. 2001; Webster et al. 2001a; Webster et al. 2001b; Webster and Hill 2001; Hentschel et al. 2002; Margot et al. 2002) and facilitated the detection of specific groups of prokaryotes without cultivation. Recently, microorganisms known to prefer anaerobic conditions such as Archaea (Preston et al. 1996; Margot et al. 2002; Webster

et al. 2001a) and sulfate-reducing bacteria (Schumann-Kindel et al. 1997; Manz et al. 2000) have been detected in sponges. Microbiological and molecular biological studies support the view that sponge-microbe associations are obligatory and species-specific.

Immunological studies (Wilkinson 1984) and biomarker investigations (Thiel et al. 1999; Thiel et al. 2002) indicate that sponge associated bacteria are passed on from generation to generation and over geological time. However, a comparative phylogenetic analysis of microbial symbionts from different sponges (Hentschel et al. 2002) suggests that the sponge microbial consortium contains a mixture of evolutionary ancient permanently associated bacteria and those that are acquired horizontally from the water column.

Associated bacteria undoubtedly benefit from the protected and nutrient-rich environment within the sponge tissue. Several lines of evidence indicate that some sponges obtain a significant portion of their nutrition from the symbionts making the symbiosis a true mutualism. A variety of marine shallow-water sponges show cyanobacteria as autotrophic symbionts which are known to contribute to host nutrition through extracellular lysis and phagocytosis (Wilkinson 1978a; Wilkinson and Garrone 1980). Moreover, symbiotic cyanobacteria appear to be able to fix nitrogen (Wilkinson and Fay 1979) and control the redox potential within the sponge tissue by photosynthesis (Arillo et al. 1993). Heterotrophic bacterial symbionts may also contribute to the nutrition of their hosts. Ultrastructural evidence of intracellular digestion of methanotrophic symbionts in a new species of *Cladorhiza* has been reported by Vacelet et al. (1996). In *Ceratoporella nicholsoni* and *Stromatospongia novae*, the numerous symbiotic heterotrophic bacteria are phagocyted in some parts of the sponge (Vacelet and Donadey 1977). Similar observations were made with *Biemnia ehrenbergi* (Ilan and Abelson 1995). Since microbial nutrition is based on dissolved organic matter (DOM), whereas sponge cells feed on particles, there is no food competition between symbiotic partners. “Bacterial farming” rather enhances the food spectrum of the host either by making DOM from the water column available for sponge cells or by DOM recycling within the sponge tissue. Indeed, the food spectrum of bacteriosponges appears to be overwhelmingly dominated by DOM (Reiswig 1981). Another possibility for a mutualistic symbiosis is the involvement of associated bacteria in the production of sponge secondary metabolites.

1.3 Secondary metabolites and sponge cultivation

Porifera are one of the richest phyla concerning the biosynthesis of bioactive natural products. Some of these secondary metabolites, e.g. sterols, have been used for chemotaxonomic classification of sponges (Bergquist et al. 1980; Bergquist and Wells 1983; Bergquist et al. 1986). The antiviral, antimicrobial and anti-tumor properties of many sponge secondary metabolites have received increasing attention of organic chemists and pharmacologists (Faulkner et al. 1994; Garson 1994; Schmitz 1994; Munro et al. 1999). It still remains a matter of discussion whether secondary metabolites are produced by sponge cells, associated microorganisms or through an interplay of both. Several studies suggest or demonstrate secondary metabolite production by sponge symbionts, (e.g. Molinski 1993; Oclarit et al. 1994; Bewley et al. 1996; Hentschel et al. 2001), whereas others indicate the production by sponge cells (e.g. Uriz et al. 1996b; Uriz et al. 1996a; Garson et al. 1998; Turon et al. 2000). Even within the same species, some metabolites may be produced by sponge cells and others by symbionts (Flowers et al. 1998; Debitus et al. 1998). There is also the very interesting possibility that the sponge cells produce the inactive precursors and the symbionts produce the enzymes that activate them (Ebel et al. 1997).

As the demand for pharmacologically potent natural products is constantly increasing, attempts for biotechnological production of sponge tissue have been made. Sponges are known to possess strong regenerative capacities (reviewed in Simpson 1984), and pieces of living sponge tissue are able to grow and regenerate into healthy sponges. This potency has been used for cultivation of sponge tissue samples in both half-open systems and open sea aquaculture on a broad range of sponge species (reviewed in Osinga et al. 1999). Another possibility is to use bioreactors containing sponge cells either with or without associated bacteria (Pomponi and Willoughby 1994; Osinga et al. 2001). The poor success of the latter approach may reflect our sparse knowledge on (micro-) biological-chemical interactions in sponge tissue and its impact on the metabolic capacities of the sponge animal as a whole.

1.4 *Geodia barretti* (Bowerbank, 1858) – a „living fossil“ with biotechnological potential

Geodia barretti (Geodiidae, Astrophorida, Tetractinellida, Demospongiae) is a massive, usually globular sponge which can grow up to 50 cm in diameter (Fig. 2, Plate 1). The tissue of *G. barretti* is divided into the cortex, a superficial region reinforced by special spicules, and the medulla, the interior part of the sponge where choanocyte chambers are located within

the choanosome. The cortex is about 500 µm thick and consists of star- and ball-shaped microscleres, small spicules called euasters and sterrasters, respectively. Megascleres, larger spicules, are radially arranged directly beneath the cortex as well as chaotically in the choanosome.

The Geodiidae are probably one of the most ancestral of the demosponge groups. The oldest remains of geodiid spicules are known from archaeocyathid mounds of the Mount Scott Range near Flinders range, Australia, indicating that the family Geodiidae exists since the early Cambrian (Gruber and Reitner 1991; Reitner and Mehl 1995).

Geodia barretti is one of the most common species in Norwegian fjords and in offshore mass occurrences of sponges along the North East Atlantic shelf and slope (Klitgaard et al. 1997). It is a strong producer of antibacterial and antiviral secondary metabolites, with the brominated alkaloid „Barretin“ as the most prominent compound (Lidgren and Bohlin 1986; Sölter et al. 2002). The tissue of *G. barretti* hosts vast amounts of associated microorganisms (Plate 1, Fig. 3 and 4).

1.5 Aim of this study: Examining the role of sulfate-reducing bacteria in the bacteriosponge *G. barretti*

Sulfate-reducing bacteria (SRB) are organoheterotrophs which gain energy by oxidation of fermentation products as simple organic acids, some alcohols and H_2. They use sulfate as a terminal electron acceptor and reduce it to sulfide. A symbiosis between SRB and higher animals seems unlikely because sulfide is toxic to most areobic organisms. However, sulfate reducers have been described from termite guts (Kuhnigk et al. 1996), the intestines of some mammals (Morvan et al. 1996) and as epibionts on a marine nematode (Polz et al. 1999). SRB as endobionts have been described from a hydrothermal vent polychaet (Cottrell and Cary 1999) and from a marine oligochaet (Dubelier et al. 2001). Sulfate reducers of the genus *Desulfovibrio* have been detected in *Chondrosia reniformis* (Hadromerida) (Manz et al. 2000) by means of FISH. This is – until now – the first and only report of sulfate-reducing bacteria in sponge tissue. However, delta-Proteobacteria, which the sulfate reducers belong to, were detected in many sponges with molecular biological methods, as in *Theonella swinhoei* (order “Lithistida”) (Schmidt et al. 2000), *Rhopaloeides odorabile* (Dictyoceratida) (Webster et al. 2001b), *Aplysina aerophoba* and *A. cavernicola* (Verongiida) (Friedrich et al. 1999; Friedrich et al. 2001). In *Aplysina cavernicola*, delta-Proteobacteria were the most abundant symbionts. No previous attempt had been made to investigate the microbial community of *G. barretti.*

Previous approaches to sponge-bacteria interactions were indirect studies on fixed specimens. Physiological properties of associated microorganisms were investigated in isolates, but never within the living sponge. For a thorough understanding of the bacteriosponge-system, time has come to focus on the interactions between sponge cells and associated microorganisms, as well as on the interactions in the microbial community within the living sponge. The aim of the following study is to elucidate the role of sulfate-reducing symbionts within the "bacteriosponge" *G. barretti*. To enable these investigations, new methods in sponge histology and cultivation were developed and established methods in histology and biogeochemistry were modified. These will be described and discussed in chapter 2. Combining these methods, I examined community structure, distribution, activity and the micro-environment of sulfate-reducing bacteria associated with *G. barretti* (chapter 3). This represents a novel approach in sponge science.

The role of sulfate-reducing bacteria for biological-chemical and sponge-bacteria interactions in the sponge tissue, as well as possible implications of these results for the understanding of sponge biology and evolution will be discussed in chapter 4.

2 DEVELOPMENT OF METHODS

2.1 Histological methods

2.1.1 Introduction

To investigate the impact of sponge associated microorganisms (SAM) on the metabolism of the host sponge, a combination of classical histological and modern molecular biological methods has become necessary. Recently, fluorescence in situ hybridization (FISH) on sponge sections (e.g. Manz et al. 2000; Webster et al. 2001b) has become a popular method for classification and localization of SAM within the sponge tissue. In the following chapter I will elucidate those methods which allow histological investigations and FISH on *Geodia barretti* and other cold-water sponges rich in siliceous spicules.

Paraffin is the classical embedding technique for light microscopic examination of any kind of tissue (Romeis 1989) and is commonly used for sponge histology. A faster and easier way is the use of cryotechniques which involve the cutting of frozen tissue blocks on a special cryomicrotome. For both techniques, siliceous spicules must be removed with hydrofluoric acid (HF) in order to obtain sections of good quality. This procedure includes several

disadvantages: First, it makes the observation of the skeletal arrangement of the sections impossible. Second, if rich in siliceous spicules, as most cold-water sponges are (Barthel 1995), the tissue may collapse after the removal of the spicules. Furthermore, after HF-treatment, FISH is no longer possible (own observations). For sponge species lacking a skeleton of siliceous spicules, HF-treatment can be omitted and then FISH is possible on both paraffin sections (Manz et al. 2000) and cryosections (Webster and Hill 2001; Webster et al. 2001a; Friedrich et al. 1999).

Embedding in resin offers an alternative technique. Some methylmetacrylat (MMA) resins are used in histology, usually in kits combined with benzoylperoxid (Romeis 1989). Two common trade names are e.g. Technovit and Histocryl. MMA resins were applied on sponges lacking a siliceous skeleton for histology and FISH (Manz et al. 2000; Böhm et al. 2001; Wagner et al. 1998a). Magnino and co-workers (1999) treated the siliceous sponge *Theonella swinhoei* with HF prior to embedding in Technovit 8100.

Resins originally designed for transmission electron microscopy (TEM) have also been used in light microscopy. Calcified biofilms were stained *en bloc* prior to embedding, either with lipophilic dyes after dehydration or with water-soluble dyes on hydrated samples, and cut with a circular saw using a diamond knife (Reitner 1993; Arp 1999). A similar method was described by Bhattacharyya et al. (1999) for undecalcified rat tibia. Embedding in LR White resin combined with *en bloc* - staining was successfully applied on sponges with calcareous basal skeleton and on siliceous sponges (Reitner 1992; Reitner 1993; Reitner and Gautret 1996; Wörheide 1997; Hoffmann et al. 2003).

A very easy method to obtain sections without HF-treatment is to cut the fixed, but not embedded sponges into sections of less than 1mm using a sharp scalpel and let them air-dry (Soest et al. 2000; Hooper 2000). Sections obtained this way are useful for the examination of skeleton structure for species determination, but the tissue structures will be destroyed. However, Margot et al. (2002) used this method for FISH on Mediterranean axinellid demosponges.

In the following study, different methods for embedding, cutting and staining were tested on a wide range of sponges from the Norwegian Sea. The aim of this study is to evaluate various methods which allow preparation of tissue sections applicable for histological investigation and FISH of siliceous sponges without removing the spicules. Although some of the described embedding techniques may also be used for TEM, I will focus on the application in light- and fluorescence microscopy.

2.1.2 Material and methods

2.1.2.1 Sampling and fixation of sponge species

The specimens in this report were collected from 1999 to 2001 from different localities in the Norwegian Sea. Species from the Sula Ridge region (64°05'N; 08°05'E) were sampled in May 1999 with the Norwegian research vessel "Johan Hjort" using a triangular dredge, as well as in July/August 1999 with the German research vessel "Poseidon" and the manned submersible "Jago". Sponges from the Korsfjord near the city of Bergen, Norwegian West Coast (60°09'12"N; 05°08'52"E) were sampled in August and September 2000, and in October 2001 with the Norwegian research vessels "Hans Brattström" and "Aurelia", using a triangular dredge. Sponges from Sula Ridge were growing in association with living and decaying coral reefs of *Lophelia pertusa*, on drop stones or in soft bottom sediments near the reefs in 200 - 300 m depth (Freiwald et al. 2002). In Korsfjord, the sponges were growing on a hard bottom slope in 100 – 300 m depth. Species used in this report are listed in table 1. All specimens were fixed in 2% formaldehyde + 0,04% glutardialdehyde in filter sterilized sea water (FSSW). Fixed samples were subsequently dehydrated in ethanol series (15%, 30%, 50% EtOH in artificial seawater) and stored in 70% ethanol.

Table 1: sponge species used in this study

	Species	**Sampling site**
Hexactinellida		
	Sympagella n. sp.	SR
Demospongiae		
Homoscleromorpha		
	Plakortis simplex SCHULZE, 1880	SR
Tetractinomorpha		
	Dragmastra normani SOLLAS, 1880	SR, KO
	Geodia barretti BOWERBANK, 1858	SR, KO
	Geodia macandrewii BOWERBANK, 1858	SR, KO
	Isops phlegraei SOLLAS, 1880	SR, KO
	Thenea muricata BOWERBANK, 1858	SR, KO
	Tentorium semisuberites SCHMIDT, 1870	SR, KO
Ceractinomorpha		
	Forcepia forcipis BOWERBANK, 1866	SR, KO
	Mycale lingua BOWERBANK, 1866	SR, KO
	Axinella infundibuliformis LINNAEUS, 1759	SR, KO
	Phakellia ventilabrum PALLAS, 1766	SR; KO
	Phakellia robusta BOWERBANK, 1866	KO
	Oceanapia robusta BOWERBANK 1866	SR

SR = Sula Ridge, KO = Korsfjord

2.1.2.2 Embedding, cutting and staining

Protocols as described below are suitable for tissue blocks of 1-2 cm^3. For larger samples, times for dehydration, staining, infiltration and hardening must be longer. Embedding techniques evaluated in this chapter are listed in table 2.

Table 2: Overview of different embedding techniques evaluated in this study

	Paraffin	**Cryomatrix**	**Histocryl**	**Technovit 7100**	**LR White**
Cutting	Rotary microtome	Cryo-Rotary microtome	Rotary microtome Circular saw	Rotary microtome Circular saw	Circular saw
Mounting	Silanized slides, heat	Not necessary	Heat	Glue, heat	Mounting medium
Removing of embedding medium	Xylol	Thawing	Not necessary	Not necessary	-

Paraffin

Tissue blocks were embedded in paraffin as described (Romeis 1989). To remove any air, blocks soaked with paraffin were placed in a paraffin bath at 60°C in a desiccator for a few minutes. Paraffin blocks were cooled on a –5°C plate prior to cutting. Paraffin sections of 5µm thickness were obtained using a rotary microtome HM 340 E (Microm, Walldorf, Germany) with single-use blades. The slices were mounted on silanized glass slides, dewaxed by xylene treatment and dried for at least 1h at 60°C prior to staining or FISH. Staining techniques according to Goldner and Giemsa (Romeis 1989) attained good results. Sections were mounted with Biomount balsam (Plano W. Plannet, Wetzlar, Germany) and viewed under a light microscope. For DAPI staining, the sections were covered with a 4', 6-diamidino-2-phenylindole (DAPI) solution of 1-3 µg/ml for 5-10 minutes in the dark, rinsed with distilled water, mounted with Citifluor antifading agent (Plano W. Plannet, Wetzlar, Germany) and viewed under an epifluorescence microscope.

Cryotechniques

Tissue blocks were embedded in Cryomatrix (Shandon Inc, Pittsburgh, USA) as described by the manufacturer. Frozen blocks were stored at -20°C, sectioned with a cryomicrotome (Mikrom, Walldorf, Germany) at -20°C, and placed on coated glass slides (CryoFrost, Fisher). Sections were air-dried at room temperature for 20 minutes prior to staining and FISH.

Methylmetacrylat resins: Technovit, Histocryl

Tissue blocks were embedded in Technovit 7100 (Plano W. Plannet, Wetzlar, Germany) as described (Manz et al. 2000). Sections of 5-10µm thickness were obtained using a rotary microtome as described above. Sections of 20µm and more were obtained using a circular saw (Leica 1600) with diamond knife.

For embedding with Histocryl (Plano W. Plannet, Wetzlar, Germany), dehydrated tissue blocks where infiltrated with Histocryl and benzoyl peroxide as described by the manufacturer. Samples were transferred into the polymerization solution (10 ml infiltration solution + 15-30 µl accelerator) and immediately cooled with running tap water for 5 minutes. Samples remained in the water bath for a few hours until hardening was completed. Sections of 5-10µm thickness were obtained using a rotary microtome as described above.

Sections were fixed on glass slides by heat (60°C for at least 1 h) for staining and FISH.

LR White resin

If staining is desired, tissue blocks have to be stained before embedding in LR White. We developed protocols for Toluidin blue O, Calcein and DAPI as follows: For staining with Toluidin blue O and Calcein, tissue samples were dehydrated in ascending Ethanol series. After dehydration, samples were placed in the staining solution over night (Toluidin blue: 1g/100 ml EtOH; Calcein: saturated solution in EtOH). Samples were washed 3-4 times in Ethanol before embedding. For DAPI staining, tissue blocks were placed in a staining solution of 10 µg/ml DAPI in 90% EtOH over night. Samples were washed in 99% EtOH, and dehydrated in 99% Ethanol for a few hours. Tissue blocks from all staining techniques were infiltrated with LR-White:EtOH 1:2 and 2:1 for at least 18h, finally embedded in LR White (Plano W. Plannet, Wetzlar, Germany) and hardened for 20h at 60°C. Since all described dyes are soluble both in EtOH and LR White, infiltration solutions should contain a minor amount of the used dye. To reduce unintentional extraction of dye during the embedding steps, it is favourable to use EtOH from the washing steps to prepare infiltration solutions. Tissue sections from 20µm to 1mm were cut using a circular saw with a diamond knife. Sections were mounted on glass slides with Biomount balsam.

2.1.2.3 FISH and image processing

Fluorescene in situ hybridization (FISH) was performed on sponge tissue sections as described (Manz et al. 2000). Samples were viewed under a Zeiss axioplan microscope (Zeiss,

Oberkochen, Germany). Microscopical pictures were taken using a CCD Camera and processed with MetaMorph imaging software.

2.1.3 Results and discussion

Comparison of the described methods with respect to embedding operation, section qualities and staining techniques is shown in table 3. Embedding process of the cryotechnique is quick, easy and reliable. The other methods take more time and are more complex with many pitfalls which may cause unexpected trouble. However, most of these difficulties can be overcome by some training and standardizing of the methods.

Table 3: Comparison of different embedding methods for siliceous sponges without spicule removing

	Paraffin	Cryomatrix	Histocryl	Technovit 7100	LR White
Embedding operation	+	++	+	+	+
Section quality	+	o	++/++*	+/++*	++
Section min. thickness (µm)	3	?	5/20*	5-10/20*	20
FISH on section	++	++	++	++	-
DAPI staining on section	++	++	++	++	-
Goldner, Giemsa staining on section	++	?	o	o	-

++ very good; + good; o bad; - not possible; ? no data; * cut with circular saw

Highest quality of sponge tissue sections including siliceous spicules are gained with LR White blocks cut on a circular saw. Valuable information about skeleton structure and tissue-skeleton-arrangement can be achieved with this method. Sections of 20 – 1000 µm are obtained, which can be ground to ca. 5µm. Even ultramicrotome sections for TEM can be obtained from spiculated tissue blocks (Uriz et al. 2000), but the knife is easily damaged if spicules are thick and numerous (personal observation). Prior DAPI staining allows visualization of different sponge cell types and of associated bacteria (Plate 2, Fig. 1A and B). The calcium-binding fluorochrome Calcein is commonly used in growth-since-labelling experiments of calcareous skeletons (Rahn 1976a; Rahn 1976b; Reitner and Gautret 1996; Wörheide 1997). Applied on calcified biofilms, Calcein greatly enhances the contrast, e.g. of small non-phototrophic bacteria, and labels the surfaces of carbonate crystals (Reitner 1993; Reitner et al. 1995; Arp 1999). In siliceous sponges, contrast in the tissue is enhanced and different cell types and tissue structures are clearly visible (Plate 2, Fig. 2A). *En bloc* staining with Toluidine blue O cannot be recommended for sponges with dense tissue and numerous SAB, for these tissues are easily overstained. The protocol can be adjusted to the species of interest by inserting more and longer washing steps or by dilution of the staining solution. High-contrast light microscopic images can be obtained by this method (Plate 2, Fig. 2B). Due to the hydrophobic character and the high density of this resin, FISH on LR White

sections is impossible. Oligonucleotide probes used for FISH are water soluted and will not penetrate the resin. The same applies to DAPI and histological stains based on dyes in water solution.

Using a rotary microtome with sharp steel knife, best section qualities were gained with Histocryl. This was also true for species and tissue parts containing many spicules, e.g. the cortex of *Geodia* species. Sections down to 5 µm were suitable for FISH. From Technovit blocks, sections of varying thickness and quality were obtained, but usuable parts for FISH could be found on most sections. Sections of better quality were obtained using a circular saw. These sections were relatively thick (> 20 µm), which made interpretation of FISH difficult.

Because of the incompatibility of MMA resins with ethanol, only stains in water solution can be used for Histocryl and Technovit sections. Unfortunately, for both MMA resins, I observed incomplete and irregular staining with Goldner and Giemsa. (Gerrits 1992) describes good results for different histological stainings on soft tissue embedded in Technovit 7100. The failure of these attempts may be due to the rough and uneven surfaces of sections from spiculated tissue. In Histocryl sections, I sometimes observed massive stain accumulations between the spicules, whereas the surrounding tissue remained largely unstained.

Paraffin sections without prior dissolution of the siliceous skeleton were of bad quality in tissue parts which contained numerous, thick or long spicules, e.g. the cortex of *Geodia*. For tissue parts containing few or small spicules, results were better, and even though tissue sections were partly torn, useable areas for FISH and histological staining could be found on most sections. Careful cooling of the paraffin blocks prior to cutting and the use of a new blade for each cutting event helped to improve section quality.

Extremely bad sections were achieved from cryoblocks: the sponge tissue was entirely torn and no tissue structures were visible. This was probably due to differences in toughness between the spicules and the embedded tissue. No attempt was made on histological staining of these sections, whereas application of FISH probes and DAPI staining achieved clear signals. However, information gained from these cryosections is comparable to that from homogenized sponge tissue. We assume that the quality of cryosections could probably be optimized by varying the cutting temperature and by better training of the cutting procedure.

For all described techniques on rotary microtomes, section quality is never quite as good as with prior acid treatment. Although some histological stainings could successfully be applied on sponge sections with siliceous spicules, we recommend the classical way of spicule removing for histological investigations where sections < 20 µm are needed, e.g. for the study of fine tissue structures. Methods for embedding and cutting of siliceous sponges should be

chosen according to the aim of investigation and the properties of the examined species. Until now, there is no standard method available which fits all needs.

Conclusions and recommendations:

1. For overview of tissue and skeleton arrangement: LR White embedding combined with *en bloc* staining, cutting with circular saw.
2. For FISH on siliceous sponges: Histocryl embedding for species or tissue parts with high spicule content, paraffin embedding if spicule content is low. Cutting with rotary microtome.
3. For detailed tissue examination: classical histological methods (spicule removing by acid prior to embedding, cutting and staining).

2.2 Cultivation and regeneration of sponge fragments

2.2.1 Introduction

Most research on sponge cultivation was and still is directed towards tropical and Mediterranean species. Previous cultivation experiments on boreal species have been short-time scale studies for fundamental research, as for *Thenea muricata* (Witte 1995) or *Halichondria panicea* (Barthel and Theede 1986). No attempt has been made so far on cultivation of *Geodia barretti.*

In the following chapter, I describe a cultivation technique in half-open systems for tissue samples of *G. barretti,* which were used as experimental units for microelectrode studies described in chapter 3. Valuable information about healing and regeneration processes and their coordination in the sponge tissue was gained by parallel microscopic surveying of tissue, skeleton and canal system over a cultivation period of 8 months. This represents an important step towards a thorough understanding of sponge biology.

2.2.2 Material and methods

1.1.1.1 Sampling

Sponges were sampled near the city of Bergen on the west coast of Norway, between 100 and 150 m depth on a hard bottom slope in Korsfjord at 60°09’12’’N; 05°08’52’’E (Fig. 1). Samples were taken in July 2000 and in March and May 2001 with the Norwegian research

vessels “Hans Brattström” and “Aurelia” by dredging with a triangular dredge. Sampling is one of the most critical factors in sponge cultivation experiments, as many sponges are sensitive to air exposure and shifts in water temperature. To minimize exposure to air, the wire was stopped when the dredge appeared at the water’s surface. An outboard-working person placed the sponges in buckets under water, which were subsequently transported to deck and emptied into larger vessels filled with running seawater. Later experience in the lab showed that *G. barretti* tolerates short exposures to air, and sampling in 2001 was done by emptying the dredge directly in water-filled vessels. The described sampling method should, however, be applied to extremely air-sensitive species if sampling by SCUBA diving or submersibles is not possible.

After sampling, the sponges were transported immediately to the cultivation site.

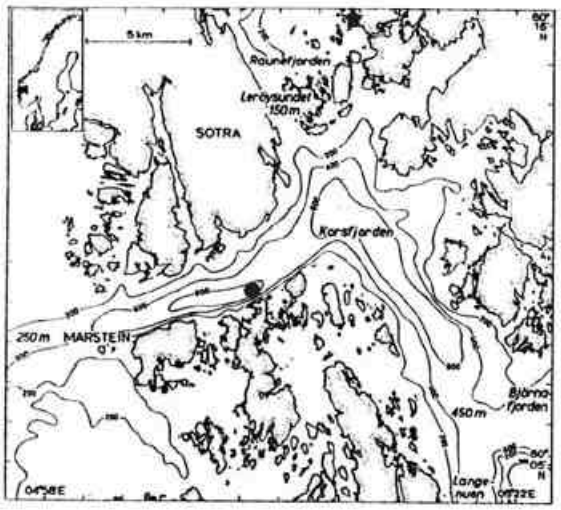

Fig. 1: Sampling and cultivation sites in Korsfjord on the Norwegian west coast near the city of Bergen. ● Sampling site, ★ cultivation site: Marine Biological Station of Bergen University. After Bakke and Sands (1977).

2.2.2.1 Cultivation of tissue samples

The cultivation experiments were performed at the Marine Biological Station of the University of Bergen, Norway. For this experiment, only tissue from the choanosome, at least 2 cm below the cortex, was used. Tissue samples of 2-4 cm^3 (approx. 3-7 g) were placed in cultivation tanks of 50 l, which were connected in an open circulation system with seawater from 40 m depth. Chemical and physical conditions in both sampling site and cultivation site water were determined by standard methods (Grasshoff 1983) and are shown in table 4. No food except that from the unfiltered seawater was added to the cultivation tanks. The explants were placed on fine mesh plastic grids (3 mm mesh) without any artificial connection. The weight of the samples (n = 30 for batch1, n = 300 for batch 2 and n = 100 for batch 3) was

determined as drip dry wet weight at day 0, 15, 45, 100 (batch 1-3), and 350 (batch 1). Only the explants that were still alive at the time of measurement were included.

Table 4: Water analysis

	Temperature °C.	**Salinity ppm**	**Phosphate µM**	**Silicate µM**	**Oxygen ppm**
Sampling site	7-10[a]	34.5-35[b]	0.77	2.79	7.5
Cultivation site	6-15	32.5-33.5	0.64 – 0.69	3.6 – 4.6	7.5

[a] Bakke and Sands 1977
[b] Matthews and Sands 1973

2.2.2.2 Unsuccessful cultivation methods

Different approaches were made to find a successful cultivation technique. Tissue samples considerably smaller than 2 or larger than 4 cm^3 died within the first weeks, as well as samples including old cortex. Fragments placed on stones (natural substrate), hung on plastic-coated threads, or glued to the substrate with silicone also failed to survive. New explants died when the water temperature was raised above 10 °C, while explants with a partly developed cortex survived temperatures up to about 15 °C.

2.2.2.3 Histology

For histological examination, sponge fragments were fixed in 2% formaldehyde + 0,04% glutardialdehyde in filter sterilized seawater at day 0, 2, 6, 10, 20, 27 and after 8 months. Fixed samples were subsequently dehydrated in ethanol series (15%, 30%, 50% EtOH in artificial seawater) and stored in 70% EtOH.

To obtain microscopical sections including silicate spicules, tissue samples were embedded in LRWhite as described in chapter 2.1.2.2. Sections from 500 to 20 µm were cut with a circular saw (Leica 1600), mounted in Biomount balsam (Plano) and viewed under a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany).

To obtain thinner sections, the spicules were removed with 5% hydrofluoric acid, and tissue samples were embedded in paraffin after standard protocol (Romeis 1989). Sections of 5 µm were cut with a steel knife (Microm microtome HM 340 E). After removal of the paraffin, sections were stained according to Goldner (Romeis 1989).

Microscopical pictures were taken with a CCD Camera and processed with MetaMorph.

2.2.3 Results

2.2.3.1 Growth

The explants showed a decrease in weight the first weeks after transplantation into the cultivation tanks. However, after approximately six weeks the weight equaled the original weight, and within one year it increased by approximately 40% compared to the original weight (Fig. 2).

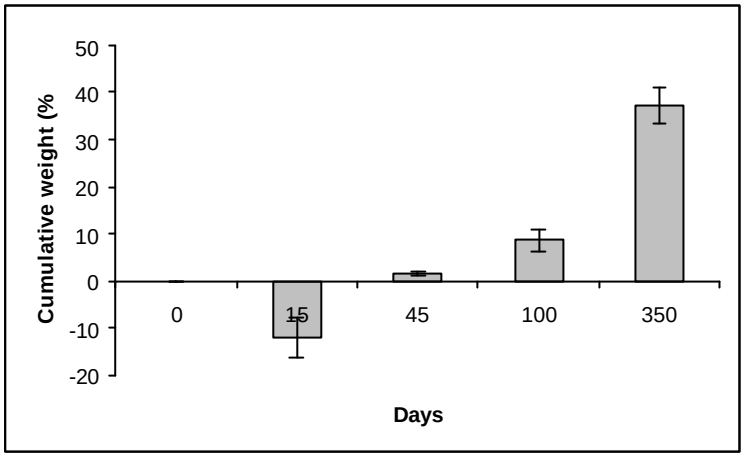

Fig. 2: Cumulative weight of cultivated fragments of *Geodia barretti* over 350 days of cultivation, based on batch 1-3 (day 0: n = 430, day 15: n = 430, day 45: n = 288, day 100: n = 43, day 350: n = 10). Error bars show standard deviation.

From batch one, 33% of the explants were still alive after 1 year, while in batch two and three, 89% and 97% respectively, died within the first three months (table 5).

Table 5: Survival with time of explants of *Geodia barretti* from batch 1-3 (in %).

Batch	Day 0	Day 15	Day 45	Day 100	Day 350
1 (n = 30)	100	100	33	33	33
2 (n = 300)	100	100	89	11	-
3 (n = 100)	100	95	5	3	-

2.2.3.2 Reproduction

In explants of batch 2, fixed in April 2001, both oogenesis (Plate 4, Fig. 5) and mature oocytes (Plate 4, Fig. 6) could be observed. The sponges used for initiation of batch 3 (sampled in May 2001) contained numerous spermatocysts (not shown). No reproductive cells, however, could be found in samples from batch 1.

2.2.3.3 Cicatrisation and regeneration

During cultivation, the shape of the explants changed from cubic to spherical (Plate 3). Already after two days, the canals at the surface were closed (observation by eye and microscopic control), the edges of the fragments were rounded, and the entire surface was covered by a transparent "skin". After 8 months, the canals within the fragments were considerably smaller in diameter, and a new cortex had developed.

Closing of the canals at the cut surface was due to initiative growth at the canal walls (Plate 4, Fig 1 and 2) by a growing front of motile sponge cells, followed by cells with inclusions in an otherwise cell- and bacteria-poor mesohyl. We observed the same process at canal walls in the endosome, diminishing the diameter of the canals. Torn tissue at the cut surfaces was covered by a closed line of motile sponge cells (Plate 4, Fig 3). Remodelling, defined as spontaneous disorganization and reorganization of the tissue (Simpson 1984) occurred in the superficial region of the explants (ectosome), where choanocyte chambers had degenerated, and cells with inclusions accumulated (Plate 4, Fig 4). After 10 to 20 days, sterrasters began to accumulate in the ectosome, and after 8 months, both sterraster and euaster layers of the new cortex had developed (Plate 5). Microscleres of all age classes in the subcortical tissue showed that at this time, the new cortex was still under construction. Broken choanosomal megascleres were incorporated in the new cortex, whereas radially arranged triaene megascleres were lacking. Subcortical spaces and ostia started to form.

2.2.4 Discussion

2.2.4.1 Cultivation methods

In contrast to previous studies on *Geodia cydonium* (Müller et al. 1999c), *Tethya lyncurium* (= *Tethya aurantium* Pallas) (Connes 1966a), and *Chondrosia reniformis* (Bavestrello et al. 1998) our explants derived from choanosomal tissue regenerated into healthy sponges, while explants including old cortex failed to survive. Connes (1966a) demonstrated that choanosomal fragments of *T. lyncurium* died within a few days. The other studies only regarded ectosomal fragments. Cultivation methods which have been successfully applied to a broad range of sponges (e.g. Verdenal and Vacelet 1985; Duckworth et al. 1999; Pronzato et al. 1999) did not succeed with *Geodia barretti.* These contrasting results show once more that "there will probably never be a standard method for sponge cultivation" (Osinga et al. 1999).

2.2.4.2 Growth rates

The weight increase of 40% within one year is lower than described for cultivation of warm-water species. Explants of the commercial bath sponge *Spongia officinalis* double their weight in about one year in open-sea aquacultures in the Mediterranean Sea (Verdenal and Vacelet 1985). For *Geodia cydonium*, Müller et al. (1999c) observed weight increases of 53% and 90% after 3 and 6 months, respectively, both in open-sea aquacultures and half-open systems in the North Adriatic Sea. The highest growth rates ever reported (5000% in one month for *Lissodendoryx* sp.) were found in an aquaculture in New Zealand by Battershill and Page (1996).

So far, almost nothing is known about growth rates of *G. barretti* in the field. A specimen of *G. barretti* has been observed in situ by scuba diving in the Trondheimsfjord, Mid-Norway, over a two year period, but no measurable change in size or shape was noticed (Rapp, personal observation).

Since weight increase starts parallel to intensive spicule production in the explants of *G. barretti*, it remains an open question how much of the increase is due to the production of organic material. However, though not measured, an increase in size of the explants was observed during the cultivation period.

2.2.4.3 Reproduction and seasonality

The development of egg cells shows that cultivated fragments of *G. barretti* are able to continue their reproductive cycle. This observation may be used for harvesting of egg cells to raise this species in culture, as first described by Wilson (1898). The reproductive cycle for *G. barretti* is unknown. Tetractinellid sponges are oviparous: they release eggs and sperm to the water, where free-swimming larvae develop (Bergquist 1978). *Geodia cydonium* from the Mediterranean (Adriatic, Italy) is described as a gonochoristic, oviparous species with reproductive period from June to October (Liaci and Sciscioli 1969 in Simpson 1984). Boreal and arctic deep-water sponges usually have seasonal reproduction (Witte 1996; Ereskovsky 2000) with reproductive periods between February and July. We did not find any gametes in *G. barretti* sampled in March, July and August from the Korsfjord, and assume that *G. barretti* is a seasonal breeder with reproductive period in early summer (April-June). The high mortality of explants from batch 2 and 3 may imply that the reproductive period is a bad time for initiation of a cultivation experiment. Tissue regeneration, spicule production, and sexual reproduction are energy-consuming processes, and may be difficult to perform at the same

time. Fröhlich and Barthel (1997) showed seasonal variations in rates of silica uptake in *Halichondria panicea* from the Baltic Sea. During the most intense phase of reproduction activity, female specimens showed a significant drop in their silica uptake, and obviously did not produce spicules during this time. However, more experimental data are needed to elucidate a correlation between reproductive season and cultivation success.

2.2.4.4 Healing and regeneration

Only few studies are available about healing and regeneration processes in sponges. Cicatrisation has been described in cultivated fragments of *Hippospongia communis*, *Spongia officinalis*, *Agelas oroides*, *Axinella damicornis* and *Petrosia ficiformis* in the Mediterranean Sea (Pronzato et al. 1999). Regenerative processes started immediately after transplantation. Within 2-3 days the sponges rebuilt their external protective layer, and after 24 hours a thin, transparent cell layer covered the cut surfaces. After one month, the sponge fragments developed a rounded shape. In the studies on *T. lyncurium* (Connes 1966a and b) and *C. reniformis* (Bavestrello et al. 1998), the old cortex was observed to surround the entire surface of the explants after a few weeks. Unfortunately, none of these studies related cicatrisation to growth rates. The "presence of a cuticle" on explants of *Spongia officinalis* is suspected to limit further growth (Verdenal and Vacelet 1985).

Cells with inclusions, also called spherulous cells, possess nutritive-metabolic or storage functions, and are found in a broad range of sponge species (see Simpson (1984) for review). Such cells are often connected to growth and secretion processes (Reitner and Gautret 1996). The generally high amount of these cells in the tissue of *G. barretti* may be the reason for the good regenerative capacity of this sponge.

It was suggested by Simpson (1984) that the formation of a highly structured, definitive cortex may completely limit further growth and thus may act as a signal for senescence. We demonstrated that tissue from an adult *Geodia*, though the whole sponge may have reached senescence, never looses its ability to grow and regenerate. These findings may encourage starting successful aquacultures of other sponge species with pronounced cortex.

The decrease in weight during the first weeks is most probably due to remodelling of the tissue, i.e. degeneration of choanocyte chambers and torn tissue in the ectosome, and by consumption of storage material. Weight increases when cicatrisation is finished, and the new cortex starts to develop.

Regeneration of all functional units from a random piece of the choanosome requires totipotency of cells, information transfer, and orientation in the given piece of sponge tissue.

Cultivation experiments with parallel histological examinations may be most enlightening for fundamental research on these still poorly understood aspects of sponge biology.

2.3 Biogeochemical methods

2.3.1 Examination of microbial sulfate reduction in sediment biogeochemistry

Activities of sulfate-reducing bacteria have been investigated intensively in marine sediments (e.g. Jörgensen 1977; Jörgensen 1982; Jörgensen 1989b; Canfield 1993; Devereux et al. 1996; Ferdelman et al. 1997; Habicht and Canfield 1997; Hines et al. 1999; Knoblauch et al. 1999; Boetius et al. 2000; Thamdrup et al. 2000) and microbial mats (Canfield and Marais 1991; Cypionka 1994; Jörgensen 1994; Habicht and Canfield 1996; Teske et al. 1998) with radioactively labelled sulfate. Recently, the application of this method on a marine oligochaete has achieved the first report on endosymbiotic microbial sulfate reduction in a marine invertebrate (Dubelier et al. 2001).

Anoxic sampling of pore water for determination of sulfide, the metabolic end product of microbial sulfate reduction, is a difficult approach even in sediment biogeochemistry. The development of a special sampling device was necessary to obtain sponge pore water that had not been in contact with oxygen during the sampling.

Studies with oxygen sensitive microelectrodes and recent physiological studies on SRB report frequent occurrence and activity of SRB in oxic environments (e.g. Dilling and Cypionka 1990; Ramsing et al. 1993; Teske et al. 1998; Minz et al. 1999b; Minz et al. 1999a; Okabe et al. 1999; Schramm et al. 1999; Sigalevich et al. 2000). From these findings, sulfate reducers should be perceived as microaerophiles rather than obligate anaerobes, which makes their association with aerobic organisms more reasonable. However, data on chemical conditions in sponge tissue are sparse. The use of microsensors to investigate oxygen concentrations in sponge tissue represents a novel approach in sponge science and has been applied only to a few species (Gatti et al. 2002; Schönberg et al. submitted). I used oxygen-sensitive microelctrodes to examine oxygen microprofiles within the sponge tissue. The thick cortex and the high spicule content of *G. barretti* caused problems for application of the fragile microelectrode.

Modifications of established methods in sediment biogeochemistry for the application to living *Geodia barretti* are described in the next section.

2.3.2 Modified methods

2.3.2.1 Microelectrodes

Oxygen profiles in the tissue of *G. barretti* were measured with oxygen sensitive Clark-type microelectrodes (Clark et al. 1953; Revsbech and Jörgensen 1986). The cortex of *G. barretti* defied insertion of the electrode. This problem was circumvent by inserting the electrode tip into pore openings (ostia) or by piercing the cortex with a canula and sliding the sensor in through the cut. Insertion of the sensor in 10 days old fragments caused no problems, because the cortex was not regenerated at this state (see chapter 2.2). To avoid any horizontal tension on the electrode tip during profiling, the examined sponge specimen or fragment had to be fixed carefully.

2.3.2.2 Sulfate reduction rates

In order to determinate sulfate reduction rates in the tissue of *G. barretti*, the whole core incubation method with $^{35}SO_4^{2-}$ (Jörgensen 1978) was modified. Tissue cores were taken from cortex to cortex of pumping sponges, incubated with 300 or 600 kBq $^{35}SO_4^{2-}$ for 30 minutes, sliced, fixed in 20% zinc acetate and frozen. The high amounts of tracer and the short incubation times were chosen because previous experiments had indicated that immediate reoxidation of the tracer obscured sulfate reduction rates in the tissue of *G. barretti* (unpublished results).

To calculate sulfate reduction rates, reduced sulfur species were analysed with the single-step chromium reduction method (Fossing and Jörgensen 1989).

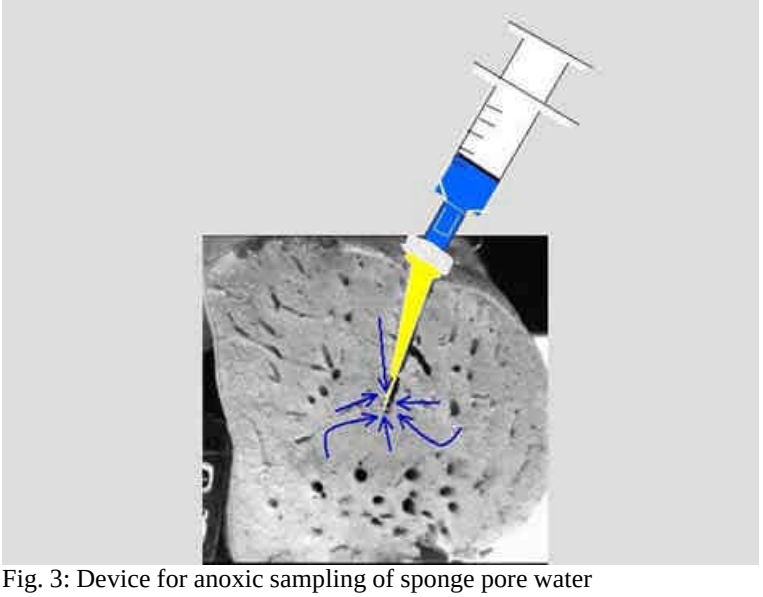

Fig. 3: Device for anoxic sampling of sponge pore water

2.3.2.3 Anoxic sampling of sponge pore water

Sponge pore water from the choanosome for hydrogen sulfide analysis was sampled with a newly developed setup as shown in Fig. 3. A yellow micropipette tip was fixed on a silicon tube, which was attached to a 10 ml plastic syringe. The tip was pushed into the sponge tissue, flushed once with the pore water, until 1-5 ml from approximately 5 cm depth were sampled. The tip with the attached tube could even remain in the sponge tissue for a few days to allow repeated sampling. With this technique, pore water can be sampled from a living sponge, and any contact of the pore water with atmospheric oxygen is avoided.

3 THE ROLE OF MICROBIAL SULFATE REDUCTION IN THE TISSUE OF *G. BARRETTI*: EXPERIMENTS AND RESULTS

3.1 Introduction

In the following study, I combined the histological, molecular biological and biogeochemical methods outlined in the last chapter in order to investigate phylogenetic affiliation, metabolic activity and the chemical micro-environment of sulfate-reducing bacteria in the tissue of *G. barretti*. On the basis of these results, the role of sulfate reducers for the metabolism of *G. barretti* will be elucidated. Biological-chemical and sponge-bacteria interactions in sponge tissue will be discussed in chapter 4.

3.2 Experiments

3.2.1 Sampling and maintaining of sponges

Different specimens of *Geodia barretti* were sampled as described in chapter 2.2. Whole sponges were maintained in cultivation tanks with running sea water from 40 m depth until experiments started (max. 1 week). Chemical and physical parameters in the water from sampling and cultivation site, respectively, are shown in table 4. Filtration activity was checked regularly with fluorescein dye (Pile et al. 1997). The fragments were cultivated as described in chapter 2.2, and, after 10 days of cultivation, they were used for oxygen profiling.

3.2.2 Biogeochemical studies

Profiles of 50 µm steps up to 1 cm into the tissue were measured both in freshly retrieved whole sponges and in 10 days old cultivated fragments of *Geodia barretti* as described in chapter 2.3. Before and after every application of the sensor, the pumping activity of the examined specimen was checked qualitatively by application of fluorescein dye into the osculum. Cultivated fragments, which could not be checked for pumping activity, were observed for signs of decay for two days after the experiment. After that, they were fixed for histological investigation as described in chapter 2.1.

To determinate sulfate reduction rates, tissue cores from two actively pumping specimens were incubated with $^{35}SO_4^{2-}$ as described in chapter 2.3. To evaluate the effect of reoxidation on rate determination, homogenized tissue of a freshly retrieved sponge was incubated with $^{35}SO_4^{2-}$ and recurrently sampled over a time course of 2 hours.

For determination of sulfide in sponge pore water, approximately 50 water samples from different sponge specimens showing different pumping activities were taken as described in chapter 2.3. Each water sample was directly filtered into 5% zinc acetate (ZnAc) solution and immediately determined spectrophotometrically (Cline 1969). Detection limit for this method is 1 µM of sulfide.

The tissue samples (ca. 1 g wet weight) for analysis of elemental sulfur were taken from one individual directly from beneath the cortex (n=1), as well as from the choanosome 2 cm (n=2) and 3 cm (n=1) away from the cortex. Sulfur was extracted by different solvents with ascending lipophilies (dichlormethan/methanol (1:1), dichlormethan, n-hexan), and determined by gas chromatography – mass spectroscopy (GC – MS; Varian CP-3800) as described (Gryglewicz and Gryglewicz 2001).

3.2.3 Phylogenetic determination and quantification of associated microorganisms

Sponges were fixed and Paraffin, Technovit and Histocryl sections for fluorescence in situ hybridization (FISH) were prepared as described in chapter 2.1. FISH was performed on sponge sections as described (Manz et al. 2000). Fluorescent-labelled oligonucleotide probes were used which were complementary to 16S and 23S rRNA characteristic for Archaea, Eubacteria as well as alpha-, beta- and gamma subclasses of Proteobacteria. Sulfate-reducing bacteria were examined with 12 oligonucleotide probes specific on genus level or for phylogenetic subbranches. The combined use of these probes enables the in situ characterization of 84% of the family *Desulfovibrionaceae* (Manz et al. 1998) and for more than 72% of the *Desulfobacteriaceae* (Manz et al. 1998; Katrin Ravenschlag, pers. comm.),

for which 16S rRNA data are currently available. For probe sequences and formamide concentrations see table 6. Results of microscopical counting were always corrected by substracting signals observed with probe NON338 and signals not observed with 4', 6' – diamidino-2-phenylindole (DAPI) staining. For quantification of sulfate-reducing bacteria, mean values were calculated by using 10-20 randomly chosen fields on 5-6 tissue samples from 3 sponge specimens.

Table 6: Oligunucleotide probes used in this study

Probe	Specificity	Sequenze (5' – 3')	rRNA target site (Position)[a]	FISH FA concn (%, vol/vol)[b]	Reference
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	16S, 338-355	20-60	(Amann et al. 1990)
NON338		ACTCCTACGGGAGGCAGC	16S, 338-355	20	(Amann et al. 1990)
ARCH915	Archaea	GTGCTCCCCCGCCAATTCCT	16S, 915-934	20	(Stahl and Amann 1991)
ALF1b	α-Proteobacteria	CGTTCG(C/T)TCTGAGCCAG	16S, 19-35	20	(Manz et al. 1992)
BET42a	β-Proteobacteria	GCCTTCCCACTTCGTTT	23S, 1027-1043	35	(Manz et al. 1992)
GAM42a	γ-Proteobacteria	GCCTTCCCACATCGTTT	23S, 1027-1043	35	(Manz et al. 1992)
DSMA488	Desulfarculus sp./ Desulfomonile sp./ Syntrophus spp.	GCCGGTGCTTCCTTTGGCGG	16S, 488-507	60	(Manz et al. 1998)
DSV698	Desulfovibrio spp.	GTTCCTCCAGATATCTACGG	16S, 698-717	35	(Manz et al. 1998)
DSV214	Desulfomicrobium	CATCCTCGGACGAATGC	16S, 214-230	10	(Manz et al. 1998)
DSV407	Desulfovibrio spp.	CCGAAGGCCTTCTTCCCT	16S, 407-424	50	(Manz et al. 1998)
DSV1292	Desulfovibrio spp.	CAATCCGGACTGGGACGC	16S, 1292-1309	35	(Manz et al. 1998)
DSR651	Desulforhopalus spp.	CCCCCTCCAGTACTCAAG	16S, 651-668	35	(Manz et al. 1998)
DSS658	Desulfosarcina spp./ Desulfofaba sp./ Desulfococcus spp. / Desulfofrigus spp.	TCCACTTCCCTCTCCCAT	16S, 658-685	60	(Manz et al. 1998)
DSD131	Desulfovibrio sp.	CCCGATCGTCTGGGCAGG	16S, 131-148	20	(Manz et al. 1998)
660	Desulfobulbus sp.	GAATTCCACTTTCCCCTCTG	16S, 660-679	60	(Devereux et al. 1992)
221	Desulfobacterium spp.	TGCGCGGACTCATCTTCAAA	16S, 221-240	35	(Devereux et al. 1992)
DSB985	Desulfobacter spp./ Desulfobacula spp.	CACAGGATGTCAAACCCAG	16S, 985-1003	20	(Manz et al. 1998)
DRM432	Desulfuromonas spp./ Pelobacter sppp.	CTTCCCCTCTGACAGAGC	16S, 432-449	40	(Ravenschlag et al. 2000)

[a] *E. coli* numbering
[b] Formamide (FA) concentration in the hybridization buffer

3.3 Results

3.3.1 Oxygen profiles

Three oxygen profiles of whole sponges (2 specimens) and four profiles of cultivated fragments (2 individuals) were obtained. Fig. 4 shows two profiles of whole sponges related to a histological sponge section (LR White embedding) of the same scale. In pumping individuals, the cortex and the subcortical spaces were well oxygenated. In the choanosome, oxygen decreased dramatically and was depleted between 4-6 mm below the sponge surface. Pumping activity was always low, i.e. a weak and hardly detectable water stream from the osculum could be made visible with fluorescein dye. When the sponge stopped pumping, oxygen was depleted directly beneath the cortex and diffusive oxygen consumption could be observed in the overlying water (Fig. 5). No oxygen could be measured in the oscular opening, but it was not clear if the sponge was pumping while this measurement was taken.

Profiles measured in 10 days old cultivated fragments were different (Fig. 6). A gradual oxygen decrease in the overlying water and a total depletion 500µm below the surface could be observed. The histological section (LR White) shows that no cortex or structures of the canal system were developed at the fragment's surface at that time.

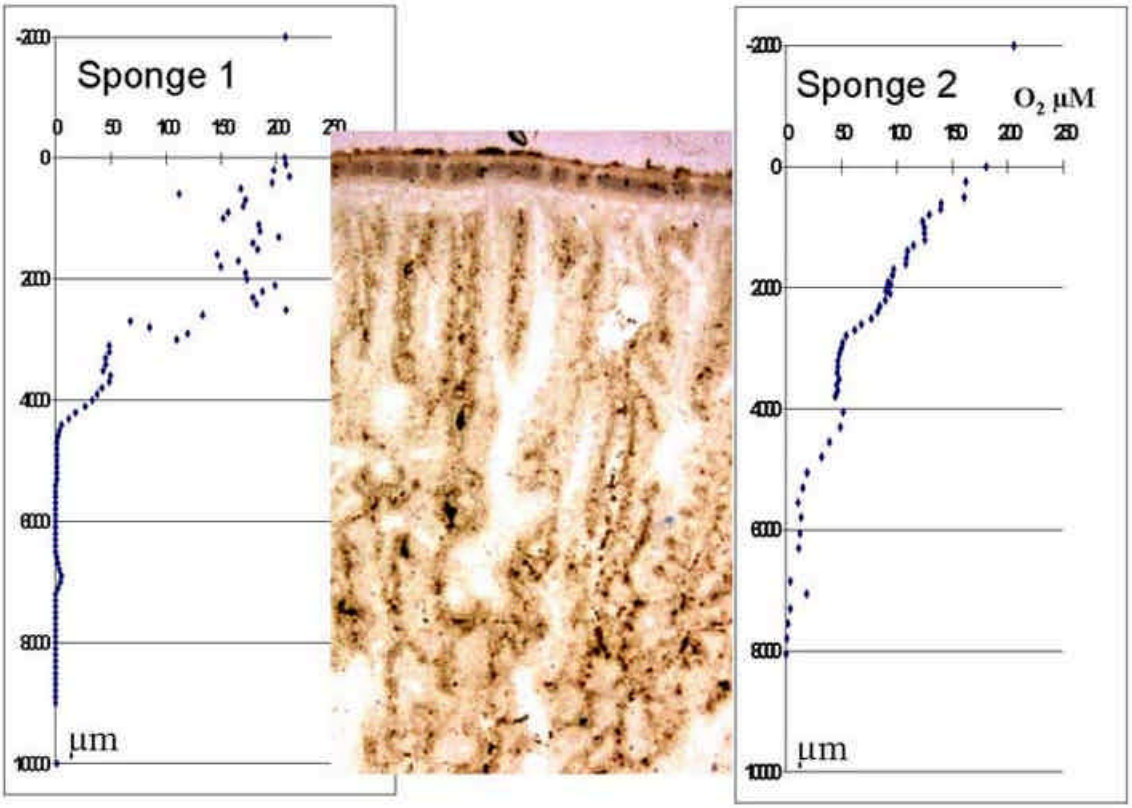

Fig. 4: Oxygen profiles 1 cm into the tissue of two pumping *G. barretti* related to a microscopical sponge section of the same scale.

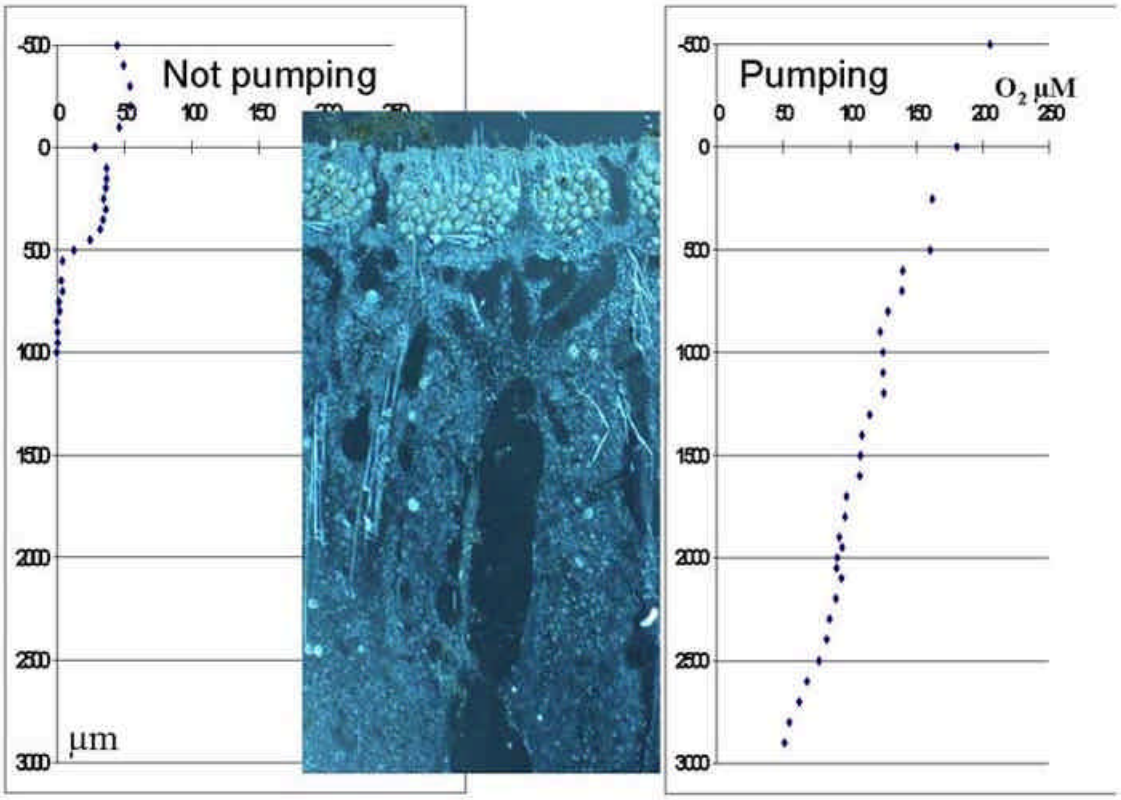

Fig. 5: Oxygen profiles 3 mm into the tissue of sponge 2, related to a microscopical sponge section of the same scale. Left, not pumping; right, pumping.

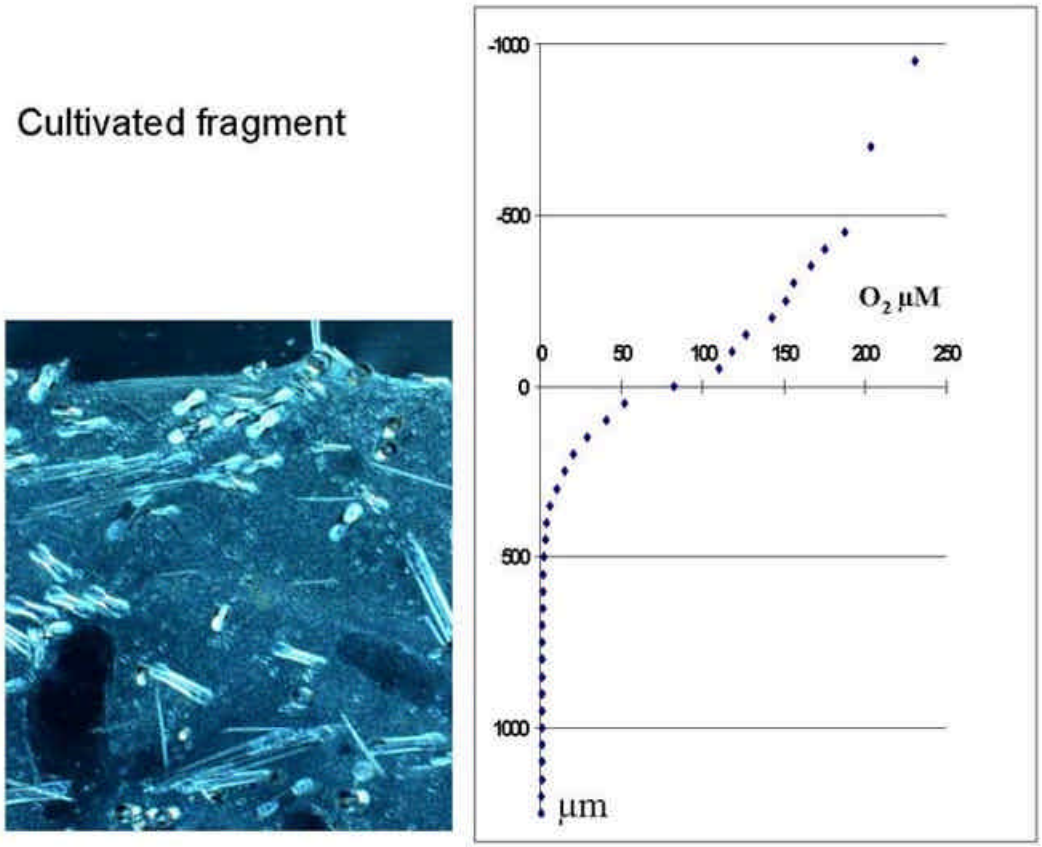

Fig. 6: Oxygen profile in a 10 days old cultivated fragment of *G. barretti* related to a microscopical tissue section of the same scale.

3.3.2 Pore water and solid phase analysis

Hydrogen sulfide was always under detection limit.

Directly beneath the cortex, elemental sulfur was 1.3 nmol cm^{-3} tissue. In the choanosome 2 cm away from the cortex, sulfur concentrations of 1.6 and 1.9 nmol cm^{-3} were measured; and 3 cm away from the cortex, the sulfur concentration was 2.5 nmol cm^{-3}.

Table 7: Community structure of associated microorganisms obtained by FISH

Probe	Specificity	Cells cm^{-3}	% of DAPI
Domain/upper group level			
ARCH 915	Archaea	no signal	
ALF 1b	α-Proteobacteria	not countable	> 50 (1)
BET42a	β-Proteobacteria	no signal	
GAM42a	γ-Proteobacteria	ca. $2.5 * 10^{10}$ (2)	ca. 8.5
Sulfate-reducing bacteria (genus level):			
DSMA488	Desulfarculus sp./ Desulfomonile sp./ Syntrophus spp.	$0.53 * 10^{10} \pm 0.2$	1.8
DSV698	Desulfovibrio spp.	$1.7 * 10^{10} \pm 0.4$	5.7
DSR651	Desulforhopalus spp.	no signal	
DSS658	Desulfosarcina spp./ Desulfofaba sp./ Desulfococcus spp. / Desulfofrigus spp.	no signal	
DSV214	Desulfomicrobium	no signal	
DSV407	Desulfovibrio spp.	no signal	
DSV1292	Desulfovibrio spp.	no signal	
DSD131	Desulfovibrio sp.	no signal	
660	Desulfobulbus sp.	no signal	
221	Desulfobacterium spp.	no signal	
DSB985	Desulfobacter spp./ Desulfobacula spp.	no signal	
DRM432	Desulfuromonas spp./ Pelobacter sppp.	no signal	
Sum sulfate-reducing bacteria:		$2.23 * 10^{10}$ (3)	7.6

(1) based on repeated optical estimation
(2) based on 10 counts on one sponge section, and on repeated optical estimation
(3) sum of mean values

3.3.3 Phylogenetic determination and quantification of associated microorganisms

Phylogenetic affiliation of the bacterial community in the choanosome of *G. barretti* as determined by FISH counts is shown in table 7. For the three sponge specimens examined, signals were identical. All types of SAB were more or less evenly distributed throughout the entire choanosome (Plate 2, Fig. 3 for DSV698). Within the cortex, only few sponge associated bacteria (SAB) and no sulfate reducers were present. In the first millimeters

beneath the cortex, the number of SAB increased gradually. The first sulfate reducers appeared in tissue patches 1-4 mm away from the cortex, gradually increasing in number towards the choanosome. The total number of associated bacteria in the choanosome of *G. barretti* is $2.45*10^{11}$ cells per gram wet weight (Ines Kaesler, pers. comm.), or $2.94*10^{11}$ cells cm^{-3} sponge (sponge density = 1.2 ± 0,11 g cm^{-3}; n=33). The total number of SRB (sum of signals obtained by specific probes) is 2.23 $*10^{10}$ cells cm^{-3}, or 7.6% of the bacterial community. The percentage for each microbial group is given in table 7. Alpha-Proteobacteria were too numerous to be counted microscopically. The number given here represents an estimation based on repeated microscopical observations.

3.3.4 Sulfate reduction rates

Sulfate reduction rates in sponge tissue ranged from 1-1200 nmol cm^{-3} d^{-1} with strong variations within the same sponge, but generally increasing from the cortex towards the medulla (Fig. 7b). Time series with tissue slurry (Fig. 7a) showed strong dependence of sulfate reduction rates on incubation time with $^{35}SO_4^{2-}$.

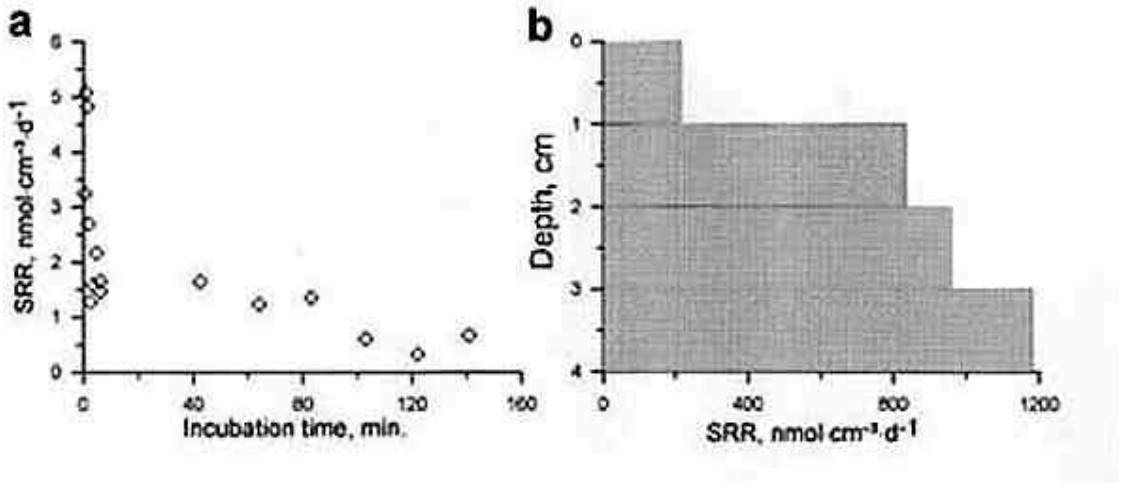

Fig. 7 a: Time series of sulfate reduction rates in a tissue slurry indicating strong dependence of sulfate reduction rates on incubation time with $^{35}SO_4^{2-}$. b: Sulfate reduction rates in a tissue core of *G. barretti*, increasing from the cortex (0 cm) towards the medulla (4 cm).

4 MICROBIAL SULFATE REDUCTION IN SPONGE TISSUE: DISCUSSION AND APPLICATIONS

4.1 Sulfate reduction, oxygen concentration, and sulfide reoxidation

The rapid decrease of sulfate reduction rates with incubation time in the slurry experiment indicates an immediate reoxidation of ^{35}sulfide, indicating that sulfate reduction is directly

coupled with sulfide oxidation. The lower rates in the slurry compared to rates in core incubations may be explained by the higher content of oxygen in the slurry. However, the effect of time is obvious. If oxygen is present, the $^{35}S^{2-}$ will rapidly be reoxidized to $^{35}SO_4^{2-}$, which will lead to a dramatic underestimation of SRR (Jörgensen and Bak 1991). To keep this effect as small as possible, high amounts of tracer and short incubation times were used for core incubations.

The shape of oxygen profiles in whole sponges indicates turbulent oxygen transport in cortex and subcortical spaces, probably due to the pumping activity of the sponge. The rapid decrease at ca. 4 mm indicates rapid oxygen consumption, and oxygen transport by molecular diffusion. This may indicate the moment when the electrode enters the sponge tissue. Oxygen profiles of cultivated fragments show a nearly parabolic shape as known from the surface of undisturbed marine sediments (Revsbech et al. 1980), indicating that no pumping activity is involved in oxygen supply. Obviously, cultivated fragments are completely dependent on strong water currents to maintain sufficient supply with oxygen by diffusion gradients. This is consistent with observations from cultivation experiments (chapter 2.2). Within the first months of cultivation, the canal system of the fragments, especially at the ectosome, is under rearrangement, without any inhalant or exhalant openings at the surface present until the cortex is regenerated. Gatti and co-workers (2002) examined oxygen concentrations in the tissue of *Suberites domuncula* and in sponge primmorphs (well defined sponge cell aggregations surrounded by a dermal membrane; Müller et al. 1999b) with oxygen sensitive microoptodes. They observed that oxygen concentrations were strongly dependent on the surrounding flow regime, as well as on culturing conditions of the sponges. Oxygen concentrations in the tissue of adult sponges were between 50 – 60% of the surrounding water, and the oxygen content in primmorphs was never below 2.3% of the surrounding water.

I showed that the oxygen concentration in the tissue of *G. barretti* was strongly dependent on its pumping activity (Fig. 5). It is reported from many sponges in situ to reduce or even stop pumping for several hours at irregular intervals (Reiswig 1971; Vogel 1977; Gerodette and Flechsig 1979; Pile et al. 1997). These patterns were found out to be related to the sediment load of the water (Gerodette and Flechsig 1979), ambient water flow (Vogel 1977; Pile et al. 1997) or sponge growth form (Reiswig 1971). In *G. barretti*, reduced pumping activity leads to anoxia in parts of the tissue and even in the canal- and exhalant water. However, during the experiments, pumping activity was always low. It can be assumed that *G. barretti* may show much higher pumping activities under more favourable conditions, which will lead to oxic

conditions at least in the pore water and the exhalant water stream. The tropical bacteriosponge *Verongia fistularis* removes only 2.8 to 8.3 (mean 5.33%) of available oxygen during a single transit of water through the sponge body (Reiswig 1974). Fragments of *G. barretti* can be considered to be permanently anoxic both in pore water and tissue, but can survive with this consistent tissue anoxia for several months. From these results I conclude that tissue anoxia is a common feature in living *G. barretti*, although the metabolism of the sponge is based on oxic respiration and is dependent on regular input of oxygen.

It can be assumed that oxygen present in the pore water of incubated tissue cores rapidly reoxidizes ^{35}sulfide and still leads to underestimation of SRR. It is not possible to decide whether the increase of SRR from the cortex to the medulla is due to lower sulfate reduction or higher sulfide reoxidation in the cortex region. In a decaying sponge, SRR were considerably higher (data not shown); the lowest rates measured being 5000 nmol cm^{-3} d^{-1}. I assume that true SRR in a living sponge are in between the highest rates measured in core incubations and this value, i.e. between 1000 and 5000 nmol cm^{-3} d^{-1}. These are among the highest rates of sulfate reduction measured in a natural system, only comparable to those of microbial mats (Canfield and Marais 1991; Habicht and Canfield 1996; Teske et al. 1998). Sulfate reduction rates in continental margin sediments from the coast of Denmark and Norway are lower by 1-3 orders of magnitude (Canfield et al. 1993).

4.2 Sulfate-reducing bacteria within the microbial community

The results obtained by FISH on sponge sections indicate that associated bacteria mainly belong to the alpha- and gamma-Proteobacteria. This is consistent with phylogenetic investigations on pure cultures of microorganisms isolated from *G. barretti* (Ines Kaesler, pers. comm.). Alpha- and/or gamma-Proteobacteria are reported to dominate the microbial communities of many marine sponges (Schumann-Kindel et al. 1996; Friedrich et al. 2001; Hentschel et al. 2001; Webster et al. 2001b; Webster and Hill 2001).

At least two strains of sulfate reducers could be detected in the tissue of *G. barretti*. One was affiliated to the *Desulfosarculus/Desulfomonile/Syntrophus* group of the family *Desulfobacteriaceae*. The other positive signal codes for a *Desulfovibrio sp*. The only other sulfate-reducing symbiont described from a marine sponge (Manz et al. 2000) belongs to the same genus, though affiliated to another strain. This symbiont was detected with the oligonucleotide probe DSV1292, which gained no signals in *G. barretti* (compare table 7).

Based on SRB cell numbers as detected by FISH, average cellular SRR were calculated. Assuming SRR between 1000 and 5000 nmol cm^{-3} d^{-1}, cell specific SRR (csSRR) for

associated sulfate reducers in *G. barretti* range from 0.05 to 0.22 fmol $cell^{-1}$ d^{-1}. These rates are slightly higher than those from arctic marine sediments, where csSRR were 0.14 fmol $cell^{-1}$ d^{-1} in the uppermost sediment layer and decreased steeply with depth to 0.02 fmol of sulfate per day (Ravenschlag et al. 2000). Rates of SRB of *G. barretti* were lower, by factors of 3.6 to 28, than the specific SRRs of mesophilic *Desulfovibrio* grown in pure cultures at 8°C (Knoblauch et al. 1999). The higher csSRR of SRB of *G. barretti* compared to those of arctic sediments may indicate more favourable growth conditions within the sponge mesohyl than in a strongly substrate-limited marine sediment (Glud et al. 1998). However, higher csSRR may also be due to higher in situ temperatures in Korsfjord. Though calculated rates from SRB of *G. barretti* are far below those from pure cultures, they are nevertheless in a reasonable range for natural environments.

The community structure of SRB in *G. barretti* is different from that in marine sediments. SRB belonging to the *Desulfosarcina-Desulfococcus* group (probe DSS658), which gained no signals in *G. barretti,* are described as the predominant group of sulfate reducers in coastal sediments (Sahm et al. 1999), arctic sediments (Ravenschlag et al. 2000) and salt marshes (Edgcomb et al. 1999; Rooney-Varga et al. 1997). This group is known to be nutritionally versatile with respect to potential electron donors and capable of complete oxidation of organic carbon to CO_2 (Widdel and Bak 1991). *Desulfovibrio spp*, which were detected in *G. barretti* as well as in *Chondrosia reniformis* (Manz et al. 2000), can use only a few simple organic acids, hydrogen, and (in some cases) ethanol as electron donor. They do not grow well in the presence of low substrate concentrations but were found to be favoured by higher substrate concentrations (Trimmer et al. 1997; Widdel and Bak 1991). This group may be well adapted to the highly specific ecosystem of the sponge mesohyl with specific substrate molecules in high concentrations.

Sulfate-reducing bacteria of the *Desulfosarculus/Desulfomonile/Syntrophus* group were also detected in *G. barretti*, though in lower numbers than *Desulfovibrio spp*. Different strains of *Desulfomonile* possess dehalogenating properties (Weerdt et al. 1990; Ni et al. 1995; Sun et al. 2001). It might be possible that these sulfate reducing symbionts are involved in the Barettin metabolism, a brominated alkaloid which is the main secondary metabolite of *G. barretti.* Bacterial symbionts, cyanobacteria or eubacteria of the genus *Vibrio*, respectively, were shown to be the producer of brominated biphenyl ethers in two species of the Dysideidae (Elyakov et al. 1991; Unson et al. 1994).

It can be concluded that *G. barretti* hosts a specific, metabolically active community of at least two different strains of sulfate reducers. Associated SRB are able to show high sulfate reduction rates in situ in spite of possible oxic conditions.

4.3 Evidence of an endosymbiotic sulfur cycle

In the dense tissue of *G. barretti*, hypoxia or anoxia will arise due to reduced pumping activity and the active metabolism of sponge cells and aerobic SAB. Fermentation products such as succinate, propionate and acetate accumulate during anaerobic metabolism under low oxygen concentration in many marine invertebrates (Grieshaber et al. 1994). In *G. barretti*, not only sponge cells, but also associated fermenting bacteria may contribute to this process.

Facultative anaerobic bacteria fermenting sucrose and fucose represent the majority of symbionts within the carribean sclerosponge *Ceratoporella nicholsoni* (Santavy et al. 1990). The presence of fermenting bacteria within the diverse microbial community of *G. barretti* is likely.

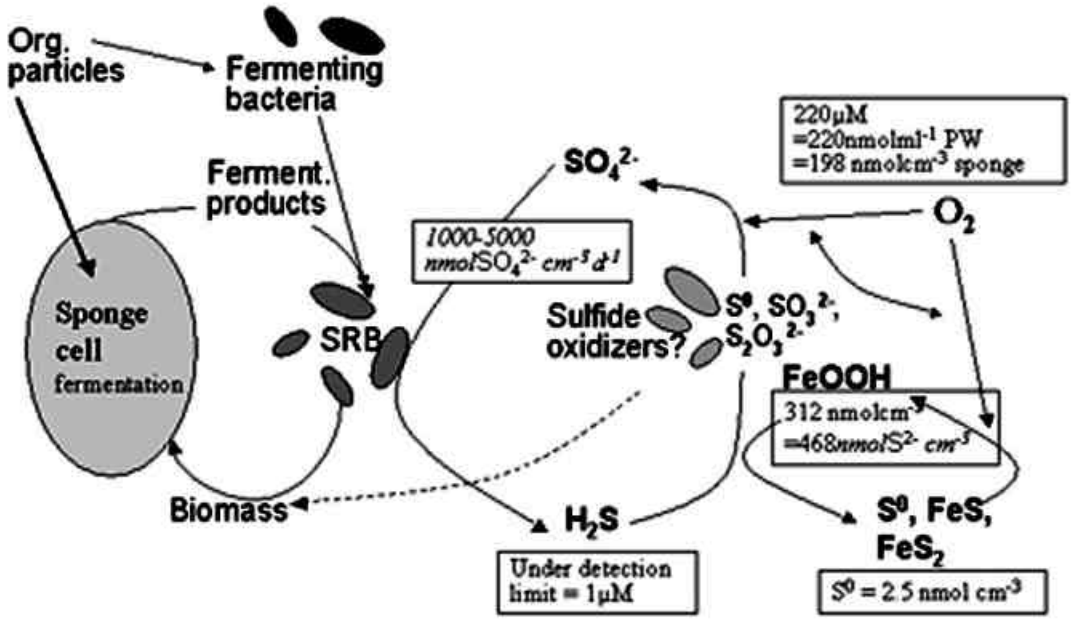

Fig. 8: Biological-chemical and sponge-bacteria interactions in the tissue of *G. barretti*.

Fermentation products will serve as carbon and electron sources of associated SRB (Fig. 8). As discussed above, I assume that under normal conditions, the canal system of the sponge is flushed by oxygenated water. In the few milli- or even micrometers of choanosomal tissue

between the canals, all niches will be present from oxic via microoxic to anoxic. Niches are variable in time and space and strongly dependent on pumping rate and metabolic activity of the sponge. Anoxic zones may enlarge if pumping activity is low and sponge metabolism is high, and may even disappear if the conditions are opposite. Sulfate reducers are known to tolerate or – in some cases – seem to prefer aerobic situations and show high rates of sulfate reduction under these conditions (Canfield and Marais 1991; Santegoeds et al. 1998; Teske et al. 1998; Minz et al. 1999a; Minz et al. 1999b; Schramm et al. 1999; Sigalevich et al. 2000; Wang et al. 2000). Therefore, they are found evenly distributed throughout the choanosome, only missing in and immediately beneath the cortex.

Free sulfide reacts spontanously with O_2 to produce mostly S^0, $S_2O_3^{2-}$, and SO_4^{2-}. Elemental sulfur has been implicated as an important reaction intermediate of sulfide oxidation (Canfield and Thamdrup 1994; Pyzik and Sommer 1981), usually present as a small but highly dynamic pool (Howarth 1984). Considerable amounts of elemental sulfur in the tissue of *G. barretti* indicate the presence of an oxidative sulfur cycle. The increase of sulfur concentrations towards the medulla is well in accordance with the number of SRB and indicates higher sulfide production and reoxidation in the choanosome compared to the cortex region. In many natural environments, sulfide-oxidizing bacteria are present at the O_2/H_2S interface to utilize the energy gained by this oxidative reaction for the fixation of CO_2 (Jörgensen 1989a). Many sulfide oxidizers store elemental sulfur intracellularly. Mutual symbiosis between sulfide-oxidizing bacteria and gutless marine invertebrates is the basis for ecosystems at hydrothermal vents based on chemoautotrophy (Cavanaugh 1994). The ability to oxidize reduced sulfur species is not restricted to a specific phylogenetic group of bacteria (Kelly 1991), which makes it difficult to detect sulfide oxidizers by FISH. Symbiotic sulfide oxidizers, however, are frequently ascribed to the *Gammaproteobacteria* by means of phylogenetic 16S rRNA analysis (e.g. Dubelier et al. 1995; Haygood and Davidson 1997). In the bacterial community of *G. barretti*, gamma-Proteobacteria were present showing approximately the same number as sulfate reducers.

An endosymbiotic sulphur cycle based on the activity of sulfate-reducing and sulfide-oxidizing symbionts has recently been described in a marine oligochaete (Dubelier et al. 2001). In this example, symbiotic bacteria served as a food source for the gutless host organism, making this symbiosis a true mutualism. As mentioned in chapter 1.2, many bacteriosponges consume their associated microorganisms (“bacterial farming”). Mid chain branched fatty acids, which are assumed to derive from a yet unknown sulfate reducer, have been detected in the tissue, as well as incorporated in demospongic acids of *G. barretti* (V.

Thiel and T. Pape, pers. comm.). This may indicate a biomass transfer from associated SRB to sponge cells.

My data indicate the presence of an endosymbiotic sulfur cycle in the tissue of *G. barretti* allowing effective nutrient utilization within the sponge–microbe community.

4.4 Mass balances and rates

According to the equation:

(1) $S^{2-} + 2\ O_2 \rightarrow\ SO_4^{2-}$

2 mol of O_2 are necessary to oxidize 1 mol of sulfide. An oxygen concentration of 220 µM in the ambient water reveals approximately 170 nmol O_2 cm^{-3} sponge (sponge porosity = 0.77 ± 0.26 ml H_2O cm^{-3} sponge; n=33). This would oxidize 85 nmol S^{2-}, which represents 1.7 - 8.5% of the daily SRR. So if the sponge stopped pumping, sulfate reduction could carry on for 30 min to 2 h before sulfide would be released. This explains why it is extremely difficult to measure SRR in living sponges.

To balance sulfide production from sulfate reduction, a pumping rate of 50 ml oxygen saturated H_2O cm^{-3} sponge d^{-1} is needed. In situ pumping rates of *G. barretti* are not known. In situ pumping rates of different marine and freshwater sponge species range from 3.8 to 23.8 l H_2O cm^{-3} d^{-1} (see table 8). From these data it can be assumed that *G. barretti* will easily exhibit in situ pumping rates that will balance sulfate reduction and even lead to excess of oxygen in sponge pore water.

Table 8: In situ pumping rates of marine and freshwater sponges

	l water cm^{-3} sponge d^{-1}	**Reference**
Mycale sp	18.14 – 23.32	(Reiswig 1974)
Tethya crypta	3.8	(Reiswig 1974)
Verongia gigantea	4.32 – 8.6	(Reiswig 1974)
Verongia fistularis	10.67	(Reiswig 1981)
Baikalospongia bacillifera	3.45 – 5.18	(Pile et al. 1997)

However, I showed that consistent anoxia appear in the tissue of *G. barretti*, and do obviously not harm the sponge animal as a whole. How does the sponge prevent sulfide toxification under these conditions?

The presence of considerable amounts of biologically available iron (312 nmol cm^{-3}) indicates another process of efficient sulfide removal even in the absence of O_2. Biologically available iron describes the pool of iron which is extractable with 6 M HCl, i.e. FeS and different

species of Fe(III), either amorphous, mineralised or bound to biological structures. Most of this pool was due to Fe(III) (Ole Larsen, pers. comm.).

According to the equation:

(2) $2\ FeOOH + 3H_2S \rightarrow 2\ FeS + S^0 + 4\ H_2O$ (e.g. Pyzik and Sommer 1981)

2 mol of Fe(III) are needed to remove 3 mol of sulfide, either as FeS or S^0. Thus, 312 nmol cm^{-3} Fe(III) would remove 468 nmol cm^{-3} sulfide. This accounts for 10 – 50% of the daily SRR or 2.5 – 12 h of full sulfate reduction before sulfide will be released. Genes coding for ferritin, a protein binding and transporting iron, have been described from the hadromerid sponge *Suberites domuncula* (Pennec et al. 2003). Localisation of ferritin with in situ hybridization gained rare signals in the cortex region of the sponge. In the medulla, cells were frequently stained, especially endopinacocytes which surround the canals. This indicates an uptake of Fe(III) from pore water and storage within the choanosome. Ferritin would allow transportation of Fe(III) to zones of intensive sulfate reduction within anoxic tissue parts. Until now, no oxygen binding and transporting structures in sponges have been described. Oxygen profiles indicate that oxygen supply into dense tissue parts is dependent on molecular diffusion.

Reduced iron species can be reoxidized in the presence of O_2, and serve again as a sulfide scavenger if oxygen is absent. The iron pool in *G. barretti* may represent an efficient buffer system similar to that in marine sediments (e.g. Jörgensen 1989a) to prevent any accumulation of toxic H_2S if oxygen is not present.

4.5 Sponges and sulfate reducers – an ancestral symbiosis?

Several lines of evidence indicate a long-standing symbiosis between sponges and sulfate reducing bacteria. SRB were among the oldest organisms on earth (Wagner et al. 1998b; Shen et al. 2001), dominant players in the Proterozoic ocean and ubiquitous witnesses of the evolution of eukaryotes and higher organisms. Phylogenetic analyses of associated bacteria in different sponges showed a deep branching of a sponge-specific sequence cluster within the division of *Deltaproteobacteria*, which sulfate reducers belong to (Hentschel et al. 2002). This means that these symbionts are phylogenetically far away from their free-living relatives, which might reflect a long-standing existence within sponge tissue. The other sponge-specific sequence clusters are not deeply branching within their divisions, which indicates that these groups may have joined the symbiosis later in the evolutionary history of the sponges.

The presence of complex isomeric mixtures of specific mid-chain branched carboxylic acids (MBCA) in the tissue of *G. barretti*, as mentioned in chapter 4. 3, also gives evidence for an ancient origin of this symbiosis. These MBCA, which have been detected in different sponge species (Thiel et al. 1999; Thiel et al. 2002), are potential lipid precursors for complex mixtures of mid-chain branched monomethylalkanes often observed in fossil sediments and oils (e.g. Fowler and Douglas 1987; Summons 1987). Thiel and co-workers (1999) suggest that the bacterial source organisms of the MBCA have been widespread in the geological past, and are found "inherited" in the protective environment of sponges in recent marine ecosystems. These biomarkers are unknown in any modern marine environment, but distinctive isomers of MBCA have been found in strains of sulfate-reducing bacteria (Ueki and Suto 1979; Kaneda 1991; Parkes and Taylor 1983). Summons (1987) suggests that mid-chain methylation in fatty acids may be a vestigial strategy for controlling membrane fluidity which originated prior to the development of an oxic biosphere and the evolution of the now dominating oxygen-dependent desaturating pathways in the biosynthesis of lipids. It can be assumed that the characteristic MBCA assemblage in sponges is derived from ancient strains of sulfate reducing symbionts in the sponge mesohyl. Interestingly, the same MBCA have recently been discovered in anoxic parts of biofilms from the highly alkaline Walker Lake in Nevada (Volker Thiel, pers. comm.). Assuming a higher alkalinity of the Early Proterozoic compared to recent oceans (Kempe and Kazmierczak 1994), microbial mats in modern soda lakes could serve as a protective environment for proterozoic bacteria, similar as "living fossil" sponges.

Combined sulfur isotope and molecular biological studies provide indirect evidence for a connection between the evolution of the microbial sulfur cycle and the metazoans (Habicht and Canfield 1996; Canfield and Teske 1996; Canfield 1998). These authors suspect an intimate linkage between the history of atmospheric oxygen and seawater sulfate and a parallel evolution of bacteria of the oxidative sulfur cycle and metazoan life some time between 640 and 1050 Ma. Although this pre-dates the appearance of the Ediacaran fauna (e.g. Seilacher 2000), it is consistent with the hypothesis for evolution of the first metazoans as part of a major eukaryotic radiation, as indicated by "molecular clock" studies (Morris 1993; Wray et al. 1996; Brohmann et al. 1998; Gu 1998; Suga et al. 1999).

The origin of multicellular animals still remains one of the most enigmatic of all phylogenetic problems (Willmer 1990; Müller 2001). Monophyly of the Metazoa is now widely accepted (e.g. Ax 1995; Müller 1995; Westheide and Rieger 1996; Wägele and Rödding 1998; Schuetze et al. 1999), and sponges definitely are metazoans (compare chapter 1.1). Thus, a

common ancestor of all Metazoa, which possessed all typical metazoan characters, must have existed. A recent molecular biological study suggests that the common ancestor of the Metazoa was a sponge, or a sponge-like organism (Borchiellini et al. 2001). Although many hypotheses try to explain the transition from unicellular organisms to multicellular organization, only three basic models seem possible (Willmer 1990; Westheide and Rieger 1996): Cell divisions of an unicellular ancestor living within a common extracellular matrix; cellularization of a multinucleated cell; or aggregation of cells attracting each other through chemical signals.

The nature of this unicellular ancestor is still a matter of debate. Choanoflagellates, which show morphological similarities to sponge choanocytes and do even form planktonic colonies, have been proposed for a long time; this view was supported by molecular sequence data (Wainright et al. 1993; Kumar and Rzhetsky 1996; King and Carroll 2001; Lang et al. 2002). However, ultrastructural studies did not support these findings (reviewed in Ax 1995; Rieger and Weyrer 1998).

Reitner and co-workers (1999) and Reitner and Wörheide (2002) offered the hypothesis that sponges derived from a union of choanoflagellate colonies and different bacteria, in which the eukaryotic partners evolved mechanisms to control an assemblage of prokaryotes. This hypothesis is mainly based on morphological similarities between non-phototrophic biofilms and simple, incrusting (bacterio-)sponges in extreme environments (Reitner 1993). The results of my study also show noticeable similarities between the tissue of *G. barretti* and microbial mats with respect to sulfate reduction rates, chemical gradients and their fluctuations (day/night vs. pumping/not pumping), bacterial density and the complex interactions of biochemical processes (Ramsing et al. 1993; Canfield and Marais 1991; Jörgensen 1994; Habicht and Canfield 1996; Karsten and Kühl 1996; Teske et al. 1998; Minz et al. 1999b). But different from a microbial mat, all processes and features described from *G. barretti* are under biological control of the sponge. Though lacking a central nervous system, sponges show surprising abilities to coordinate processes within the sponge body, as I showed for regeneration processes in chapter 2.2. Moreover, sponges are provided with a natural, innate immune system, acting on both cellular and molecular level (Müller et al. 1999a). This means that sponges are able to control their microbial population with phagocytosis and the production of secondary metabolites, directly influencing bacterial number and activity. Another possibility for active control of chemical gradients within the sponge tissue is the modulation of the pumping rate.

Was the first metazoan a sponge-like organism, an assemblage of different prokaryotes under biological control of a – yet undefined - protist colony, arisen as part of a major eukaryotic radiation? In the Proterozoic ocean, where organic carbon can be assumed to be mainly available in dissolved form, an association of different bacteria would be able to metabolise a wide array of simple molecules and transfer them into biomass, which would serve as a better food resource for the eukaryotic partner. A symbiosis with facultative anaerobic or microaerophilic microorganisms as the ubiquitous sulfate reducers might have been advantageous under low and unstable oxygen conditions.

Although the establishment of a stable oxygen atmosphere and major changes in ocean chemistry gave rise for the evolution of other animal phyla with different body plans and ecological strategies, it would not be surprising if *Geodia barretti*, the "living fossil", had preserved this ancestral symbiosis within its tissue until today.

4.6 Implications for sponge biotechnology and ecology

It is still not decided if the complex interactions between sponge cells, SRB and other sponge bacteria are preserved features from the origin of metazoan life or if they evolved later in time. But my results undoubtedly prove the presence and metabolic activity of SRB in the tissue of present *G. barretti*. They further indicate the importance of anaerobic processes for the sponge metabolism, with SRB as putative key players for sponge nutrition. It is possible that metabolic processes, probably also the production of sponge secondary metabolites, are dependent on certain chemical conditions in the sponge tissue. This may be an explanation for the hitherto poor success of in vitro cell cultures of sponges (see chapter 1.3).

Mass settlements of giant *Geodia* and related sponges around Iceland and the Faroe islands have been reported by local fishermen for many years. The local name for these sponge accumulations is "ostur" or "ostebund", meaning "cheese bottom". Klitgaard and co-workers (1997) suggested that at these sites, suspension feeders were favoured by special hydrographic patterns causing elevated bottom currents and particle resuspension. However, this does not explain the predominance of *Geodia, Isops* and *Stryphnus* species, nor their extraordinary sizes of up to 72 cm and body weights of more than 20 kg (Tendal et al. 1993; Klitgaard et al. 1997). These sponges are grouped within the same family (*Isops*) or order (*Stryphnus*) as *Geodia*, and show similar growth forms and tissue structures (own observations). *Stryphnus* and *Isops* species collected in the Norwegian Sea contain high amounts of a mid-chain branched C-18-carboxylic acid (T. Pape, pers. comm.), which may indicate symbiotic sulfate reducing bacteria. These sponges may benefit directly from elevated bottom currents which

relieves their active pumping for tissue re-oxidation (Vogel 1977). Thus, an efficient utilization of nutrients by sponge/bacteria symbiosis using different pathways of carbon mineralization with oxic/anoxic cycles in the tissue becomes possible. This efficient system of internal nutrient recycling may be the reason for the success of *Geodia* and other bacteriosponges through earth history until today.

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Plate 1

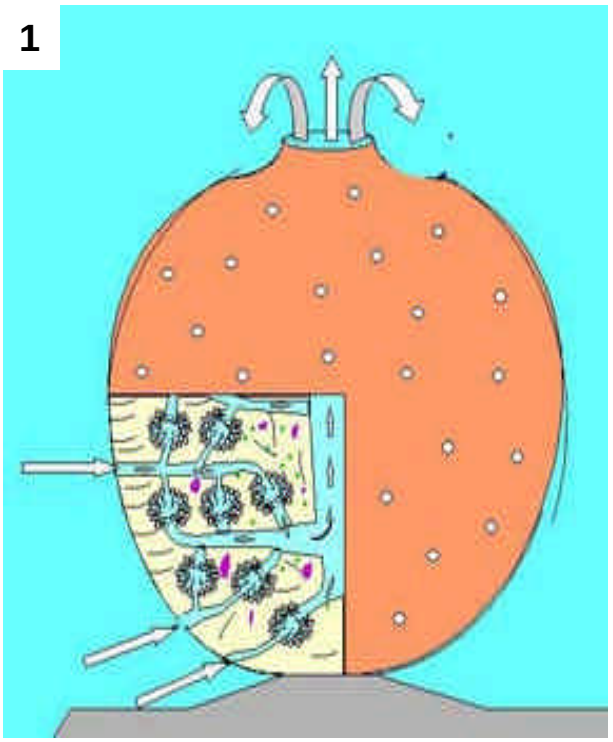
Fig. 1. A typical Demosponge body plan built around a system of water canals. Within the choanocyte chambers, special flagellated cells (choanocytes) produce a water current that enters the sponge body through surface pores, and leaves it through larger openings called osculi. Direction of the water current through the sponge body is indicated by arrows. The sponge mesohyl contains several cell types (purple dots), sponge spicules and associated bacteria (green dots).
(After Ronald Osinga, unpublished)

Fig. 2. *Geodia barretti* , sampled in the Korsfjord, Western Norway, with associated brittle star. Osculi are collected in deep sunken holes.

Fig. 3. The tissue of *G. barretti* hosts vast amounts of associated microorganisms, visible as tiny blue dots in the mesohyl (arrows). Fluorescence micrograph, DAPI staining. Bar=10 µm
Cc = choanocyte chamber, Ca = canal.

Fig. 4. Associated microorganisms of different morphotypes (arrows) are dispersed in the mesohyl of *G. barretti.*
TEM micrograph; Bar = 2,5 µm
Ce = sponge cell

1



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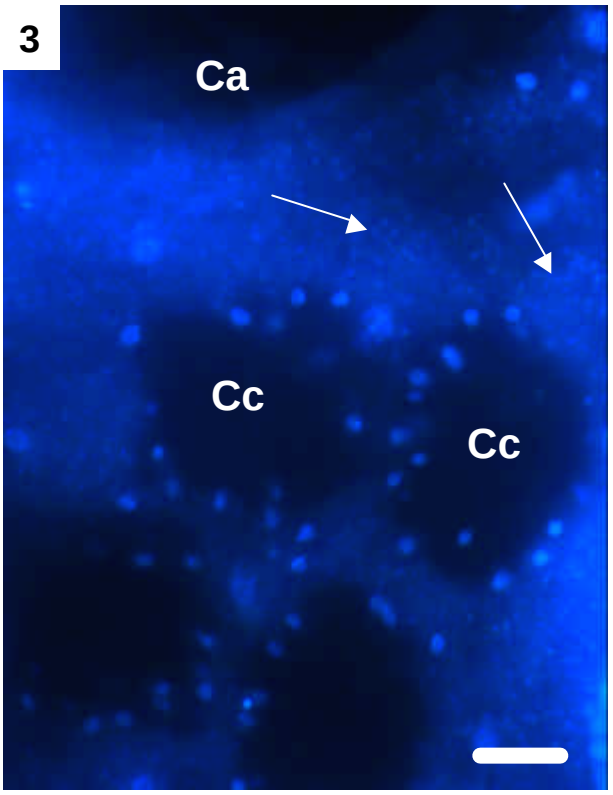

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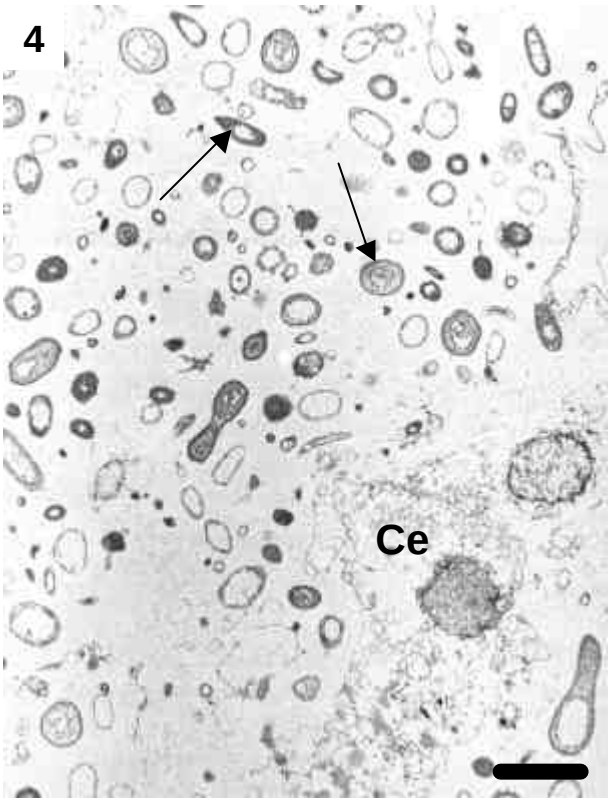
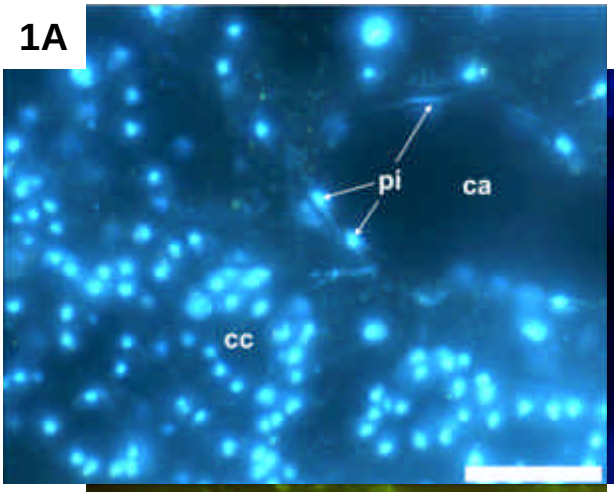


Plate 2

Fig. 1. DAPI stained LR White sections (20µm) of *Phakellia ventilabrum* (A) and *Plakortis simplex* (B). Choanocyte chambers (cc), canals (ca), and endopinacocytes lining canal walls (pi) are visible. This technique clearly shows differences in tissue structure and density of associated microorganisms (tiny dots in the tissue) in this two sponge species. Bar = 30 µm

Fig. 2. Fluorochrome image (Calcein, A) and light microscopic image (Toluidin blue O, B) of *Phakellia ventilabrum*. In this LR White sections (100 µm), bundles of spicules (sp) typical for this species with accompanying cells (ac) are visible. Please notice choanocyte chambers (cc) and canals (ca) in the choanosome, and different cell types in the ectosome (ec) at the sponge surface. Bar = 100 µm

Fig. 3. In situ identification of sponge associated bacteria with fluorescently labelled rRNA-targeted oligonucleotide probes on a tissue section from the choanosome of *G. barretti.* Epifluorescence micrographs of different bacterial groups were visualized using specific filter sets. Eubacteria (green) were hybridised with Oregon Green-labelled probe EUB338. Sulfate reducers, which appear orange by image overlap, were hybridised with CY3-labelled probe DSV698 specific for *Desulfovibrio* spp. Sulfate reducers of the genus *Desulfovibrio* are evenly distributed throughout the choanosome. Bar = 20 µm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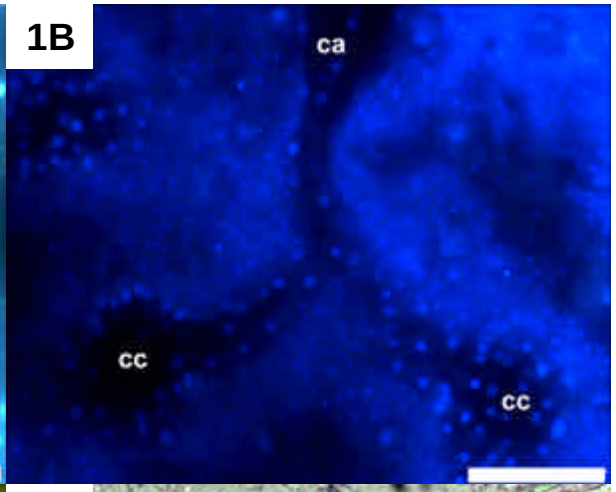
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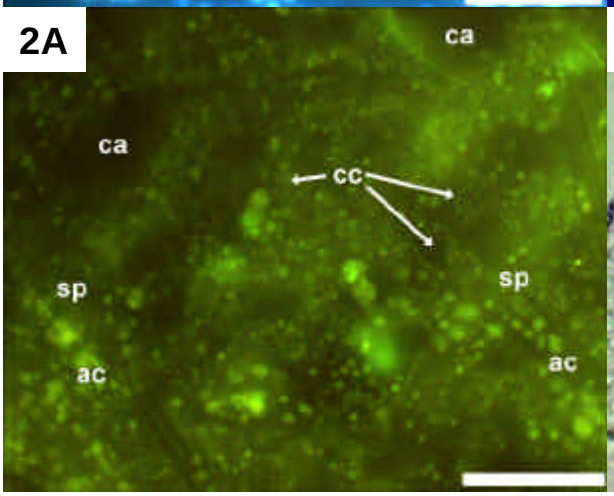
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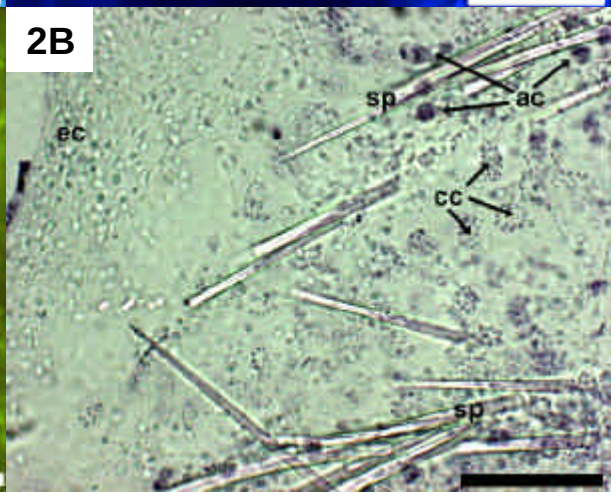
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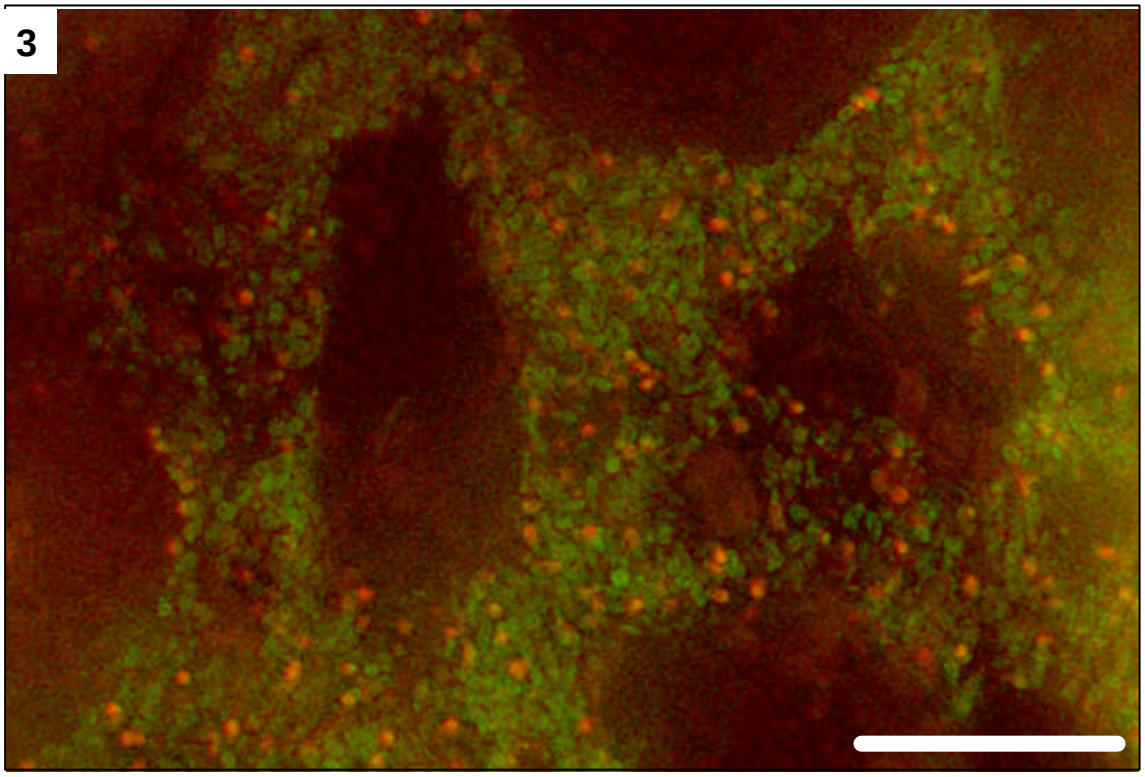
3

Plate 3

During cultivation, explants of *G. barretti* changed their shape from cubic to spherical. Canals at the cut surface were already closed after 2 days. Choanosomal canals in 8 months old explants were smaller in diameter than in the original sponge. Bar = 1 cm

Fig. 1. Start of cultivation

Fig. 2. After 2 days

Fig. 3. After 20 days

Fig. 4 and 5. After 8 months of cultivation

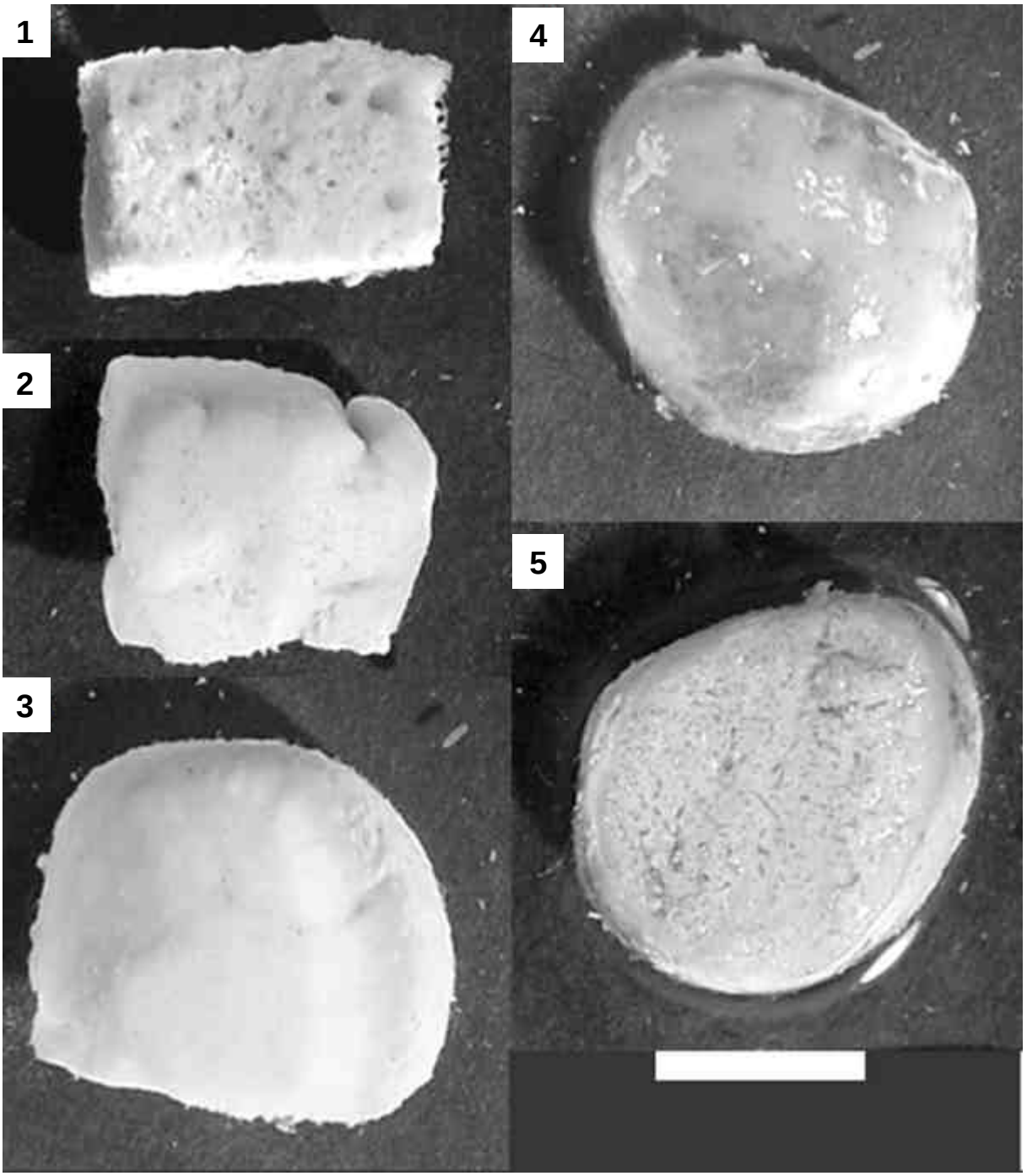
1
2
3
4
5

Plate 4

Histological sections of cultivated fragments of *G. barretti.*

Fig. 1. Cut surface of a two days old explant of *G. barretti.* A growing front of motile sponge cells (Cm) is accompanied by cells with inclusions (Ci) in an otherwise cell-and bacteria-poor mesohyl. Bar = 500 µm

Fig. 2. Detailed view of 4a.

Fig. 3. Torn tissue at the cut surface of a two days old explant is covered by a closed line of motile sponge cells (Cm). Bar = 30 µm

Fig. 4. During cultivation, the (new) ectosome is remodelled: choanocyte chambers are regenerated, and cells with inclusions (Ci) accumulate (27 days old explant). Bar = 100 µm

Fig. 5. Oogenesis in cultivated explants sampled 2nd of April 2001. Bar = 30 µm

Fig. 6. Mature oocytes in cultivated explants sampled 10th of April 2001. Bar = 30 µm

Paraffin embedding, Goldner staining, spicules removed (Fig. 1-5)

LR-White embedding (Fig. 6)

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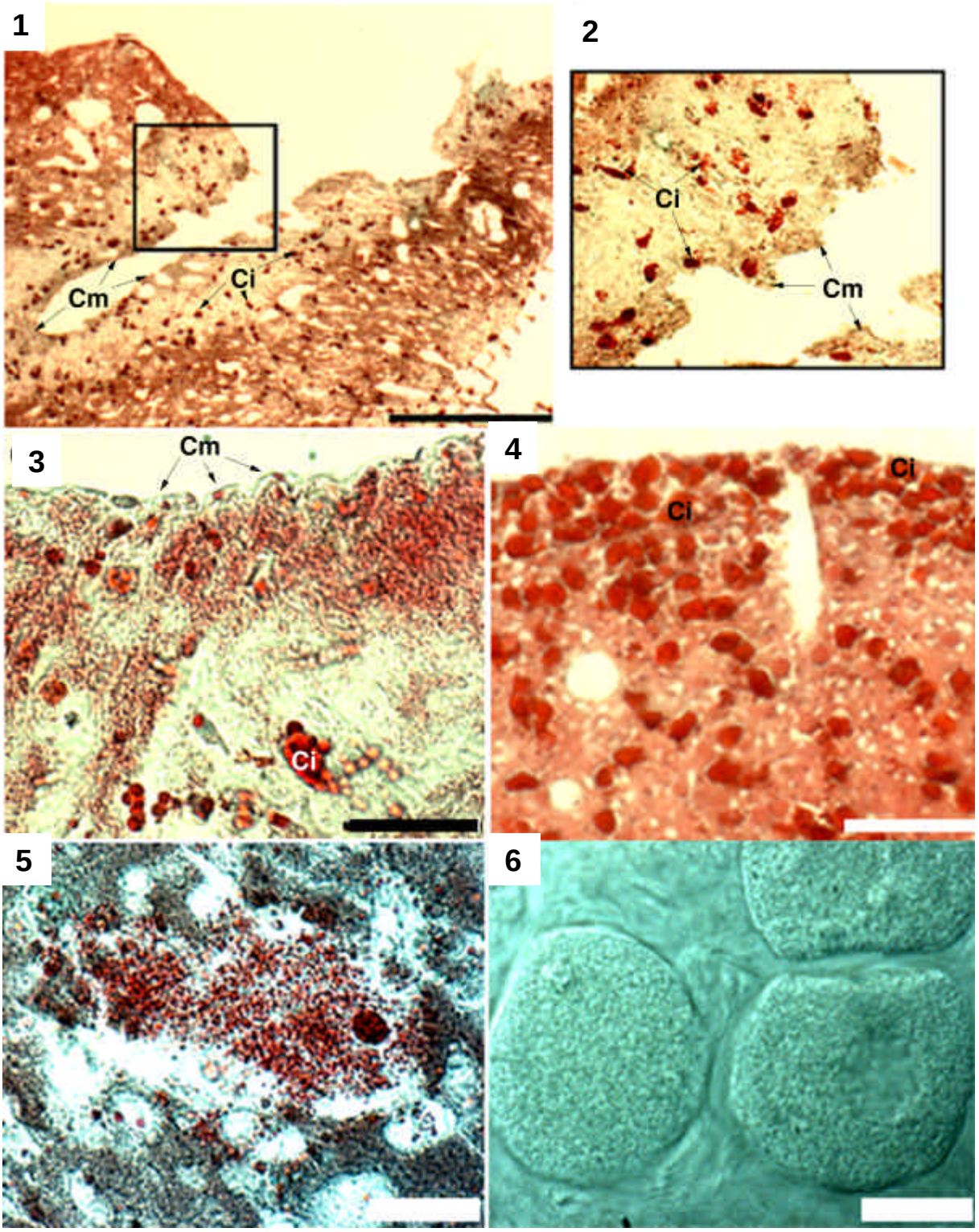
1
Cm
Ci
2
Ci
Cm
3
Cm
Ci
4
Ci
Ci
5
6

Plate 5

Cortex of an adult *G. barretti* (Fig. 1,2) compared to the cortex of an 8 months old explant (Fig. 3,4). In cultivated fragments, the sterraster (st) layer is considerably thinner, and subcortical spaces (sp) are smaller. Old choanosomal megascleres are occasionally included in the new cortex, but radial triaene megascleres are lacking. Both sterraster (st) and euaster (eu) layers are developed in the new cortex. Ostia (os) begin to form.

Bar = 200 µm (1,2); bar = 50 µm (3,4)

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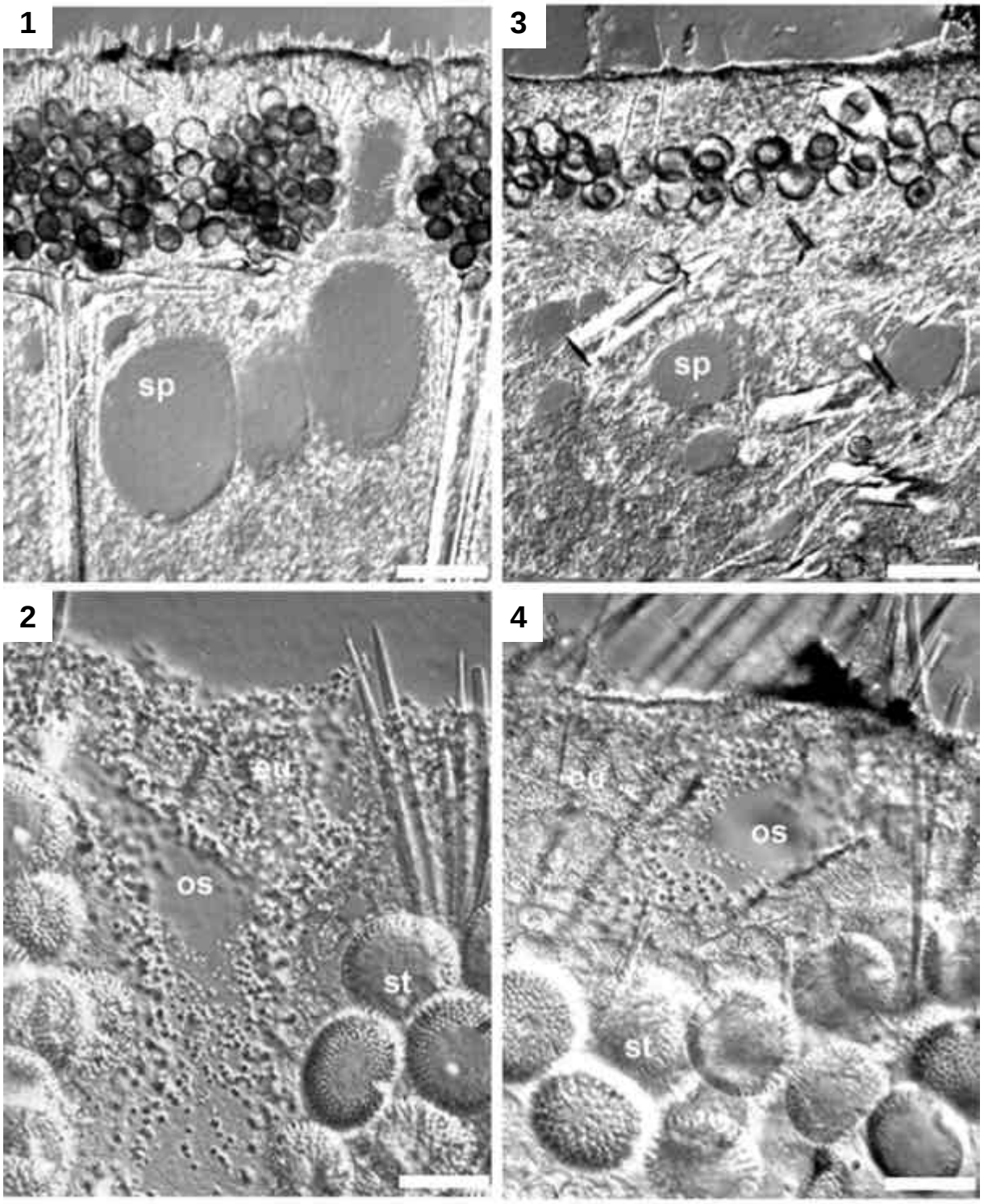
1
sp
3
sp
2
ed
os
st
4
ed
os
st

Lebenslauf

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19.11.1970	Geboren in Kassel, Staatsangehörigkeit: deutsch
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September 1996 – Oktober 1997	Diplomarbeit am Max-Planck-Institut für Marine Mikrobiologie in Bremen
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