Eco-physiological, chemotactic and taxonomic characterization of hypersaline *Beggiatoa* originating from microbial mats

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Zusammenfassung

Die Gattung *Beggiatoa* kommt weltweit an diversen Standorten mit verschiedenen Salinitäten vor. Obwohl mikrobielle Matten und andere Lebensräume, z. B. hydrothermale Öffnungen in der Tiefsee, schon in der frühen Erdgeschichte als potentielle Umgebung für die farblosen Schwefelbakterien existierten, haben sie sich wahrscheinlich erst in Zusamenhang mit dem Anstieg von Sauerstoff während des späten Proterozoischen Zeitalters enwickelt (vor 1.05 – 0.64 Milliarden Jahren). Die erst kürzlich als *Thiomargarita* interpretierten Mikrofossilien in 0.6 Milliarden alten Gesteinen unterstützen diese Annahme. *Beggiatoa* Filamente sind auf entgegengesetzte Gradienten eines Elektronen-Spenders (Sulfid) and eines Elektronen-Akzeptors (Sauerstoff) angewiesen, solange kein alternativer Elektronen-Akzeptor verfügbar ist (Nitrat). In mehreren Studien wurde das Autreten, die Physiologie and die Phylogenie von *Beggiatoa* in ihrer natürlichen Umgebung untersucht und beschrieben. Gleichwohl existiert nur ein begrentztes Wissen über *Beggiatoa* in hypersalinen mikrobiellen Matten. In dieser Doktorarbeit wurden hypersaline *Beggiatoa* aus mikrobiellen Matten hinsichtlich ihrer Öko-Physiologie, ihres chemotaktischen Verhaltens und ihrer Taxonomie in einer umfassenden Studie untersucht.

Die Hypothese zur vertikalen Tages-Migration von *Beggiatoa* Filamenten aus hypersalinen mikrobiellen Matten und die begleitende Physiologie wurden im 2. Kapitel untersucht. Phylogenetische Analysen auf der Basis von 16S rRNS Genen belegten die Zugehörigkeit zu einem schmalen, nicht-vakuoliertem und marinen Stamm, MS-81-6, welcher aus Salzmarschen stammt. Mit der Kombination von stabiler Isotopen Technik und Mikroskopie konnte eine dominante, Matten bewohnende Art ermittelt werden, welche Filamentdurchmesser zwischen 6 und 8 µm aufwies, und Nitrat-Speicherungs-Kapazitäten von ungefähr 40 mM hatte. Nitrat wird mit hoher Wahrscheinlichkeit in Vakuolen gespeichert. Das Vorhandensein von Vakuolen in hypersalinen *Beggiatoa* konnte durch den Fluoreszenzfarbstoff Fluourescein Isothiocyanat und Konfokale Laser Mikroskopie bestätigt werden. Mittels Messungen durch Mikrosensoren und stabilen Isotopen wurden die vorherrschenden *In situ* Bedingungen ermittelt. Die vertikalen Verteilungsmuster im Licht zeigten ein hohes *Beggiatoa* Vorkommen am Übergang von Sauerstoff- und Sulfidgradienten bei Licht (8 mm Tiefe), während sich die Filamente im Dunkeln hauptsächlich im anoxischem Bereich, in der Nähe des sulfidischen Grenzbereiches (7 mm Tiefe), aufhielten. Die hypersalinen *Beggiatoa* können durch ihre Nitrat-Speicherung den nächtlichen Sauerstoffmangel überdauern, obwohl die Nitratwerte im Porenwasser niedrig waren (5-10 µM). Unter *In situ* Bedingungen oxidieren *Beggiatoa* also Sulfid mit Sauerstoff bei Licht und mit intern gespeicherten Nitrat im Dunkeln. Mit der Speicherng von Nitrat zeigen *Beggiatoa*

eine optimale Strategie, um in suboxischen Zonen in sulfidischen Sedimenten zu leben und um Dunkel-Perioden in phototrophischen mikrobiellen Matten zu überleben.

In Kapitel 3 wird eine neue Methode vorgestellt, welche eine Messung zur zeitaufgelösten Analyse von niedrigen pH Werten in Zellen von lebenden *Beggiatoa* möglich macht. Sie basiert auf Fluoreszenzsättigung angeregter Zustände und ermöglicht eine indirekte Fluoreszenzlebensdauermessung. Durch die Lebensdauer des Anregungszustandes von dem Farbstoff Fluorescein Isothiocyanat (FITC) konnten pH Werte zwischen 2 und 5 dargestellt werden. Die Lebendauer war abhängig von dem pH Wert des Farbstoff umgebenden Mediums und diese wurde wiederum in Relation zum Verhältniswert der Anregungszustandes-Lebenszeit gesetzt. Nachdem theoretische und praktische Tests durchgeführt worden sind, konnte die Methode erfolgreich auf vakuolierte hypersaline *Beggiatoa* übertragen werden. Die gleichzeitige Messung von verschiedenen internen pH Werten zeigte das Cytoplasma im neutralen pH Wert Bereich, die Vakuole hatte saure pH Werte (2-4). Ein saurer pH Wert in Vakuolen könnte eine wichtige Rolle für die Nitrat-Speicherung darstellen.

Da der Mechanismus der Fortbewegung und das chemotaktische Verhalten wichtige Eigenschaften sind, die es *Beggiatoa* ermöglichen sich den fluktuierenden Gradienten in mikrobiellen Matten anzupassen, wurden diese im 4. Kapitel untersucht. In ihrer natürlichen Umgebung können unter Umständen mehrere Parameter die Verteilung der hypersalinen *Beggiatoa* beeinflussen, z. B. Sulfid, Sauerstoff, Nitrat, Licht. Um zu ermitteln welcher der auslösende Faktor ist, der ein chemotaktisches Verhalten bedingt, wurden zwei experimentelle Ansätze im Labor durchgeführt. Die verschiedenen Parameter wurden zu Gradientenkulturen hinzugefügt, welche mit einer größeren Menge von hypersalinen *Beggiatoa* angereichert wurde. Transparente, mit Flüssig-Medium gefüllte Kapillaren, enthielten wenige Einzel-Filamente, welche unterschiedlichen Konzentrationen von Sulfid ausgesetzt wurden. Durch Mikrosensor-Messungen, visuelle Positionsbestimmung von Filamenten, automatisierte Filmkamera-Analyse und statistische Variations Partitionierungs Analyse konnte gezeigt werden, dass Sulfid der hauptsächliche Faktor ist, der die Bewegungsrichtung beeinflusst. Niedrige Konzentrationen von Sulfid (5-10 μM) zogen die Filamente an, hohe Sulfidkonzentrationen (>25 μM) stießen sie ab. Allerdings schien Nitrat die Filamente anzuziehen, hingegen Sauerstoff und blaues Licht resultierten in eine negative Reaktion. Diese Ergebnisse konnten das *In situ* Migrationsverhalten von *Beggiatoa* im Zusammenhang mit den vorhandenen Faktoren in den mikrobiellen Matten erklären, was eine gerichtete chemotaktische Fortbewegung auch in Sulfid-freien Zonen erlauben würde, die *Beggiatoa* auf der Suche nach Elektronen-Spendern oder Elektronen-Akzeptoren durchqueren

müssen. Der Fortbewegungsmechanismus, der sich aus der Produktion und Exkretion von Schleimstrahlen aus Poren an der Filament-Oberfläche ergibt, konnte über Lektinanfärbung und Konfokale Laser Mikroskopie aufgeklärt und gezeigt werden.

Morphologische und physiologische Charakteristika von *Beggiatoa* Filamenten aus hypersalinen mikrobiellen Matten von verschiedener Herkunft wurden verglichen, und ihre phylogenetische Verwandtschaft auf 16S rRNS Sequenz-Basis zu limnischen und marinen *Beggiatoa* wurde im 6. Kapitel untersucht. Als nächste Verwandte (~90% Sequenz Ähnlichkeit) fanden sich die marinen Stämme MS-81-6 und MS-81-1c, welche aus Salzmarschen isoliert wurden. Ein neuer Gattungs-Name, „*Kandidat* Halobeggiatoa“, wurde vorgeschlagen, um die Gruppe der hypersalinen *Beggiatoa* zusammen zufassen, da diese untereinander phylogenetisch sehr ähnlich sind (95-100% Sequenz Ähnlichkeit), Übereinstimmungen in Filamentdurchmessern, im Vorhandensein von Vakuolen und hohe Salzbedürfnisse haben. Die neu vorgeschlagene Gattung besteht aus zwei Untergruppen, die eine umfasst Arten aus mexikanischen, die andere aus diversen spanischen mikrobiellen Matten. Die Arten dieser zwei Untergruppen konnten erfolgreich durch speziell hergestellte FISH Sonden (Fluoreszenz *In situ* Hybridisierung) unterschieden werden. Die Gemeinschaft der Filament-assoziierten Bakterien erschien phylogenetisch sehr divers, und obwohl dominiert durch gamma-Proteobakterien, konnte eine spezifische Assoziierung von kleinen Bakterien mit *Beggiatoa* nicht festgestellt werden.

Summary

The genus *Beggiatoa* occurs worldwide in diverse habitats with a wide range of salinities. Although microbial mats and other habitats, i.e. hydrothermal vents in the deep sea, were present on early earth as potential environment for these colorless sulfur bacteria, they evolved probably in the late Proterozoic concomitant with the rise of oxygen (1.05 – 0.64 billion years ago). The recent interpretation of Neoproterozoic microfossils (dated 600 million years old) as the giant vacuolated sulfur bacterium of the genus *Thiomargarita* provided evidence of this assumption. *Beggiatoa* filaments depend on counter-gradients of electron-donor (sulfide) and electron-acceptor (oxygen), unless an internal storage of an alternative electron-acceptor is present (nitrate). Several studies have illuminated the occurrence, physiology and phylogeny of *Beggiatoa* in their natural habitats. Nevertheless, only limited knowledge exists of *Beggiatoa* in hypersaline microbial mats. In this thesis, hypersaline *Beggiatoa* originating from microbial mats were comprehensively studied, with respect to their eco-physiology, chemotactic behavior and taxonomy.

The supposed vertical diel migration and the accompanying physiology of *Beggiatoa* filaments from hypersaline microbial mats were investigated in chapter 2. Phylogenetic analysis on 16S rRNA gene basis showed closest affiliation to a narrow, non-vacuolated marine strain, MS-81-6, originating from salt marshes. By combining stable isotope and microscopy techniques, the most dominant mat inhabiting species with filament diameters between 6 and 8 µm, revealed nitrate storage capacities of about 40 mM. Nitrate is most probably stored in intracellular vacuoles, the presence of the latter was confirmed by fluorescein isothiocyanate staining and confocal laser microscopy. Microsensor measurements and stable isotope analyses were used to determine prevalent *In situ* conditions. The vertical distribution pattern showed highest *Beggiatoa* densities at the depth zone where during illumination gradients of oxygen and sulfide overlap (8 mm depth) whereas under dark conditions the filaments were most abundant in the anoxic zone of the mat, close to the sulfide boundary layer (7 mm depth). The survival of hypersaline *Beggiatoa* under anoxia during dark periods could be explained by their nitrate storage capacities, even though nitrate porewater concentrations were low in the mats (5-10 µM). Sulfide oxidation by *Beggiatoa In situ* was thus apparently conducted with oxygen under illuminated conditions and with internally stored nitrate under dark conditions. Nitrate storage of *Beggiatoa* was suggested as an optimal strategy to both occupy suboxic zones in sulfidic sediments and survive dark periods in phototrophic microbial mats.

A novel method for the measurement of time-resolved analysis of low pH ranges in cells of live *Beggiatoa* was presented in chapter 3. The new methodology is based on excited

state saturation and was developed to enable indirect fluorescence lifetime estimation. The fluorophore fluorescein isothiocyanate (FITC) could display pH values between 2 and 5 if the lifetime of excited states' of FITC was employed. A theoretical derived saturation ratio function was set into relation of the fluorophore lifetime and pH of the fluorophore surrounding medium. After theoretical and practical tests, the method was applied successfully on vacuolated hypersaline *Beggiatoa* filaments with simultaneously measured different internal pH values, near neutral in the cytoplasm and acidic in the vacuoles (2-4). Acidic pH values in vacuoles could play a role in internal nitrate accumulation.

In chapter 4 locomotion mechanism and chemotactic behavior were investigated since they are essential tools for *Beggiatoa* to thrive in environments with fluctuating gradients as occur in microbial mats. Several parameters can possibly influence the distribution pattern of hypersaline *Beggiatoa* filaments in their natural habitat, i.e. sulfide, oxygen, nitrate, light. In order to determine the key triggers that result in a chemotactic behavior, two experimental laboratory approaches were conducted. Gradient agar tubes with bulk enrichments of hypersaline *Beggiatoa* were subjected to the different key parameters, as well as liquid medium-based transparent capillaries with single filaments and different concentrations of sulfide. Microsensor measurements, visual filament position determination, automated camera analyses, and statistical variation partitioning analysis revealed sulfide as the key trigger determining motility, attracting filaments at low (5-10 μM) but repelling them at high (>25 μM) sulfide concentrations. However, also nitrate appeared to attract filaments, while in contrast oxygen and blue light resulted in a negative response. These findings could explain the *In situ* migration behavior of *Beggiatoa* in relation to the present triggers in microbial mats, also allowing directional movement in sulfide free regions, through which *Beggiatoa* have to pass in search for either electron donors or electron acceptors. The gliding locomotion mechanism resulting from the production and excretion of slime jets from pores at the filaments surface could be clarified and visualized by lectin-staining and confocal laser microscopy.

Morphological and physiological characteristics of *Beggiatoa* filaments of diverse origins of hypersaline microbial mats were compared and their 16S rRNA sequence-based phylogenetic relationship to freshwater and marine *Beggiatoa* was studied in chapter 5. Highest similarities to the marine strains MS-81-1c and MS-81-6, which were previously isolated from saltmarshes, were detected (~90% sequence homology). A new genus name, '*Candidatus* Halobeggiatoa', was proposed to accommodate the studied group of hypersaline *Beggiatoa* as these feature close phylogenetic affiliations (95-100% sequence similarities), similarities in filament diameter, vacuole presence, and high salt requirements. The newly

proposed genus comprises two phylogenetic sub-branches, one composed of species from Mexican and the other of species of different Spanish microbial mat origins. Species of the two sub-branches could successfully be discriminated with specifically designed FISH (fluourescence In situ hybridization) probes. The community of filament-associated bacteria, however, appeared phylogenetically highly diverse and although dominated by gamma-Proteobacteria *Beggiatoa* did not seem to support specific associations of strains or groups of small bacteria.

Chapter 1
Introduction

A brief introduction to *Beggiatoa*

The genus *Beggiatoa* comprises a specific group of colorless, filamentous proteobacteria, and one species (*Beggiatoa alba*, wrongly interpreted as *Oscillatoria alba*) was the first described organism (Vaucher 1803) of the larger group of morphological conspicuous sulfur bacteria, namely *Thioploca*, *Thiomargarita* and *Beggiatoa*. Colorless sulfur bacteria are involved in oxygen, nitrogen, sulfur and carbon cycles (Nelson et al. 1982; Fossing et al. 1995; Jørgensen & Gallardo 1999; Middelburg 2000), as for energy generation they oxidize reduced sulfur compounds with either oxygen or nitrate as electron acceptor. In addition to the utilization of simple organic compounds, they can also fix carbon dioxide via the Calvin-cycle for biomass generation (Nelson & Castenholz 1981a; Nelson & Jannasch 1983; Nikolaus et al. 2003). *Beggiatoa* and other sulfide oxidizers of the group of morphological conspicuous sulfur bacteria produce and store elemental sulfur granules when grown in the presence of reduced inorganic sulfur compounds such as hydrogen sulfide, polysulfide, sulfite, thiosulfate or polythionates. Also inclusions of poly-ß-hydroxybutyrate and polyphosphate can be found, for energy storage. By the internal storage of nitrate, large marine sulfur bacteria can bridge the absence of oxygen as electron acceptor for the oxidation of sulfide or internal sulfur (McHatton et al. 1996; Hüttel et al. 1996). These organisms may have had and still have significant impacts on ecosystems and biogeochemical cycles throughout the oceans and landlocked habitats. This will be outlined in more detail in the following thesis introduction.

Earth history and evolution of colorless sulfur bacteria

Earth originated about 4.6 billion years ago during the generation of our solar system. In the Hadean Eon (4.6 to 3.9 billion years ago) Earth was bombarded by chunks of material of the early solar system. A thin and solid crust was built, preserved in oldest known crustal material, mineral zircons of Western Australia (4.4 billion years old) (Wilde et al. 2001), and the oldest body of continental crust in Northwest Canada, the Acasta Formation (between 4.0 and 3.8 billion years old) (Stern & Bleeker 1998). There is evidence that liquid water existed on Earth's surface during its first 600 million years, suggesting that the planet cooled down rapidly. The Archean (time span between 3.9 to 2.5 billion years ago) continents remained small, due to Earth's hot interior, but by the end of the Archean Eon the total volume of crust approached that of the present state (Stanley 2005). Yet, only 7% of Archean continental crusts are still present in the modern rock record, due to erosion and crust subduction processes. Chemical analysis of the oldest terrestrial material indicates that most Archean rocks formed in the presence of water (Grotzinger et al. 2007). An atmosphere formed immediately after the Moon-forming impact (~4.5 billion years ago), both the gaseous

atmosphere and liquid water are thought to have gassed out from the hot core of Earth, where it was present in elemental form (Press & Siever 1995). Other geologists think that most of the air and water today originated from volatile-rich matter which impacted early Earth during the bombardment in the Hadean Eon. Solar luminosity was markedly less then than at present (85–90% of current levels); however, the mildly reducing and anoxic prebiological atmosphere was filled with greenhouse gases, mainly composed of carbon dioxide (CO_2) and water vapor (H_2O), less abundant methane (CH_4) and nitrogen (N_2), which probably sustained warm temperatures (60-73°C) (Lowe & Tice 2007). Subaerial and subaqueous volcanoes, weathering of the crust, as well as hydrothermal vents were probably very active and supplied the atmosphere and the oceans with potential electron donors (H_2, H_2S, S^0, Fe^{2+}, CH_4, CH_2O, NH_4^+) and electron acceptors (CO_2, CO, SO_4^{2-}, S^0, NO (NO_3^- and NO_2^-)) (Canfield et al. 2006). These compounds may have been involved in fuelling the anaerobic metabolisms of early life forms based on the carbon isotope record, as suggested by Canfield et al. (2006). During the Archean Eon oxygen was virtually absent from the atmosphere and anoxic oceans, the latter being enriched in dissolved Fe^{2+} but depleted (<200μM) in SO_4^{2-} concentrations (Canfield et al. 2000; Habicht et al. 2002). Possible organic, isotopically 'light' (probably biologically reduced), carbon burial was determined in 3.8 old rocks from the Isua belt, Greenland (Rosing 1999), the oldest evidence of life. The rock record of mid- and late Archean successions (between 3.6 and 3.0 and 3.0 and 2.5 billion years ago) harbors many, of what is claimed, fossil microbial biofilms and stromatolites (organosedimentary structures produced by microbial trapping, binding and precipitation; the origin of these is usually, but not always photosynthetic) (Awramik 1992). Negative $\delta^{13}C$ isotope values support the biogenicity of ancient organic matter, proven for stromatolites samples originating from the Warrawoona Group, Australia (3.45 by), Strelley Pool Chert, Australia (3.3 by), Moodies Group, South Africa (3.2 by), Pongola Supergroup, South Africa (~3 by), Steep Rock, Canada (2.8 by), Fortescue Group, Australia (2.7 by), Yellowknife Supergroup, Canada (2.65 by), and the Transvaal Supergroup, South Africa (2.56 by). Archean microfossils composed of carbonaceous matter of biological origin and identifiable biological morphologies were found in the Onverwacht Group, South Africa and the Pilbara Group in Western Australia (between 3.4 and 3.2 by) and these were presumably deposited under shallow marine conditions. Some other filamentous microfossils found in a 3.2 billion years old volcanogenic massive sulfide (deep water) deposits in Western Australia and other sources likely originated from hydrothermal settings at mid-ocean ridges (Schopf 2006; Rasmussen 2000; Nisbet 2000). Fossils of the Dresser formation (~3.5 by) showing broad filamentous (20 μm in diameter) morphologies were thought to resemble present day chemotrophic beggiatoa-like gliding

bacteria and/or photoautotrophic oscillatoriacean cyanobacteria (Ueno et al. 2001; Schopf 2006). Although Jannasch & Wirsen (1981) suggested a present day occurrence of filaments in microbial mats associated with deep-sea thermal vents to represent non-photosynthetic cyanobacterium-like organisms, and the fact that cyanobacteria are capable of sulfide-dependent anoxygenic photosynthesis as well as fermentation under dark conditions (Stal 2000), more reliable data than morphology alone of cyanobacterial appearance in older than 2.7 billion years old rocks are needed to prove cyanobacterial oxygen production in Earth early history (Fig. 1). Some evidence is found in molecular fossils such as preserved biological lipids like 2α-methylhopanes, which are characteristic hydrocarbon biomarkers for cyanobacteria in 2.7 billion years old rocks from the Pilbara Group, Western Australia (Brocks et al. 1999; Summons et al. 1999). The isoptopic signature of these hopanes show enrichment in the lighter $\delta^{12}C$ isotope which is thought to be due to the discrimination against the $\delta^{13}C$ isotope by the enzyme RuBisCO (ribulose biphosphate carboxylase/oxygenase) (Nisbet & Fowler 2003). Steranes, a chemical characteristic of Eukaryotes were also present (Nisbet & Sleep 2001).

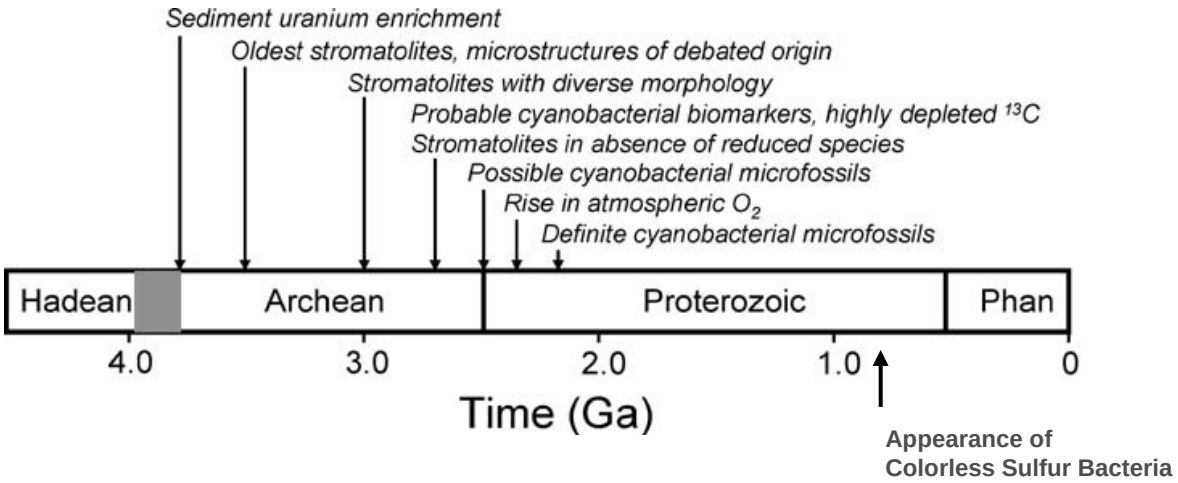

Figure 1: Modified after Canfield et al. (2005) and Knoll (2003): A summary of the geologic evidence pointing to the early emergence of cyanobacteria and the likely late appearance of colorless sulfur bacteria.

RuBisCO is an enzyme used by aerobic, microaerobic as well as anaerobic microorganisms for CO_2 fixation. As its use is not limited to photosynthetic bacteria, e.g. also it is found in *Beggiatoa* its presence is not necessarily an indicator for photosynthesis (Nisbet & Fowler 1999). The possible metabolism of the earliest life form is speculated upon as no direct (fossil) evidence is currently available. The earliest self-sustaining metabolism was probably rooted in chemolithoautotrophy (Wächtershäuser 1988), as most of the deeply rooted lines of Bacteria and Archea are hyperthermophiles, and many of the present day ones are autotrophic

(Woese 1987; Stetter 1996; Nisbet & Sleep 2001). The suggested archaic autotrophic mechanism for carbon fixation was a prototype of the reductive citric acid cycle, which was thought to function as a non-enzymatic surface reaction system comprising heavy metal sulfides and resulted in an exergonic reaction of organic carbon and pyrite formation ($4CO_2$ + $7H_2S$ + 7FeS -> $(CH_2\text{-}COOH)_2$ + $7FeS_2$ + $4H_20$ and $4HCO_3^-$ + $2H^+$ + $7H_2S$ + 7FeS -> $(CH_2\text{-}COO^-)_2$ + $7FeS_2$+$8H_2O$) (Wächtershäuser 1990). Hydrothermal vents would have allowed chemolithotrophs to thrive, using compounds such as H_2S and Fe^{2+} to gain energy or H_2 and CO_2 for methanogenesis (Nisbet & Fowler 2003). Anaerobic photoautotrophs mostly carried out primary oceanic production by utilizing Fe^{2+} and/or H_2S as electron donors for CO_2 reduction, and methanogens mostly performed organic matter recycling. The native S^0 and SO_4^{2-} generated by UV photolysis of volcanic SO_2 strongly influenced the sulfur cycle, as well as the rain-out of nitrate aerosols, both introducing oxidation power for anaerobic respiration, e.g. by sulfate reducers as is suggested by isotopic evidence from 3.5 billion years-old fossil samples (Shen et al. 2001). Microbial mats around shallow-level and subaerial hydrothermal systems were probably composed of anoxygenic photosynthetic bacteria, underlain by sulfate reducers and methanogens. Anoxygenic photosynthesis was likely to evolve before oxygenic photosynthesis, as it uses a variety of electron donors and probably evolved from infrared thermotaxis, allowing the bacterium to leave deep hydrothermal vent sites and thrive in sunlit, mesothermophilic habitats. Additionally, green and purple sulfur bacteria carry two different photosystem reaction centers, of which structural analogs appear to be combined in oxygenic phototrophs, indicating an origin from genetic transfers between cooperating or closely juxtaposed cells, each using anoxygenic photosynthesis (Blankenship 1992). Therefore, the evolution of structured microbial mats may have paralleled the evolution of photosynthesis. With the rise of cyanobacteria about 2.7 billion years ago and the accompanied development of oxygenic photosynthesis, organisms became independent from hydrothermal settings where locally strong redox gradients could support chemosynthetic life, allowing organisms to spread across the planet (Knoll 2003). Oxigenic phototrophs in early microbial mats (2.3 billion years old) could have supported an oxic micro-environment with available electron donors (H_2S) and electron acceptors (O_2 and NO_3^-) even in an otherwise anoxic world, which could sustain chemolithoautotrophs such as *Beggiatoa*. From 2.2 to 2.3 billion years ago, ^{34}S-depleted sulfides become a continuous feature of the geologic record, which reflect sulfate concentrations of 1 mM or higher (Canfield 1998; Canfield & Raiswell 1999). Independent lines of geochemical evidence point to the first accumulation of oxygen in the atmosphere around this time (Holland 2004). The time delay between first evidence of cyanobacterial oxygenic photosynthesis and first accumulation of oxygen (between 2.1 and

1.8 billion years ago) was probably due to oxygen consumption by reduced compounds and aerobic respiration, exceeding the net production of oxygen. When the net oxygen production exceeded the consumption, oxygen accumulated in the atmosphere and resulted in oxidized surface waters, but still reduced deep oceans, which remained anoxic until about 0.8 billion years ago (in the Proterozoic) (Fennel et al. 2005) (Fig. 2). A deep oxidized ocean may have been initiated by a steep decrease in reductant supply and a further increase in oxygen concentrations (Holland 2006).

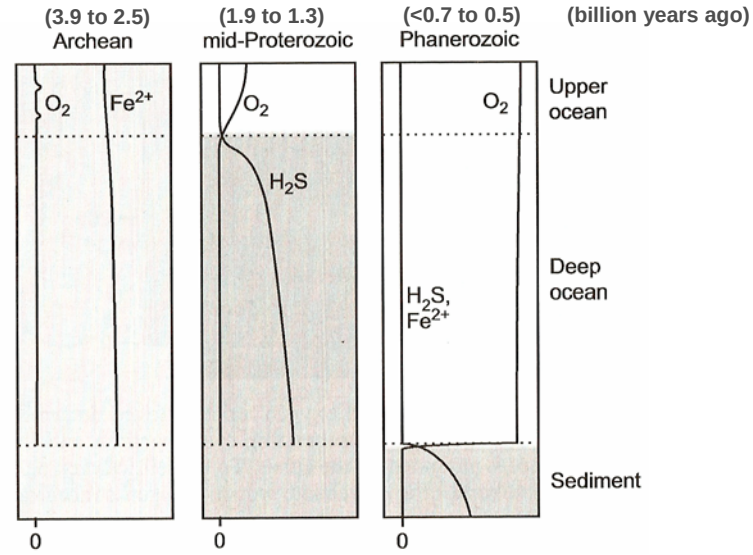

Figure 2: Modified after Anbar & Knoll (2002): vertical oxygen distributions in the ocean during major stages of Earth history. Anoxia and the presence of ferrous iron or hydrogen sulfide are indicated by grey shading. During the Archean, oxygen was restricted to oases of oxygenic photosynthesis possibly associated with cyanobacterial blooms and benthic microbial mats.

As non-photosynthetic sulfide-oxidizing bacteria could have transformed sulfide to sulfate by anaerobic respiration with nitrate, a complex sulfur cycle could have been operational on the early Earth even in the total absence of free oxygen (Shen & Buick 2004). However, the evolution of these sulfide oxidizers have been dated so far to the late Proterozoic (between 1.05 and 0.64 billion years ago), when the expression of the oxidative part of the sulfur cycle was made possible by increasing atmospheric oxygen levels to >10 percent of present levels during the period of enhanced organic carbon burial (Canfield & Raiswell 1999; Canfield & Teske 1996). These rather modern bacteria likely emerged from the latest major evolutionary radiation of bacteria, the beta- and gamma-Proteobacteria. The fact that large sulfur bacteria of the genera *Beggiatoa*, *Thiotrix*, *Thioploca* and *Thiomargarita* require a partly oxidized environment and moreover depend on gradients of sulfide with at least the occasional presence of oxygen or nitrate reflects their evolutionary origin in a time of change from a reducing to an oxidizing marine biosphere (Teske et al. 1995). The recent interpretation of Neoproterozoic microfossils (dated 600 million years old) as the giant vacuolated sulfur bacterium of the genus *Thiomargarita* provided evidence of the presence of the colorless

sulfur bacteria (Bailey et al. 2007), but this finding doesn't exclude an earlier time of first appearance.

The colorless sulfur bacteria

Phylogenetic relationships, Morphology, Motility, Physiology, Habitats

The colorless sulfur bacteria have been described first by Winogradsky (1887), comprising a very heterogeneous group of non-pigmented organisms which share the ability to oxidize reduced or partially oxidized inorganic sulfur compounds. The use of this ability as a taxonomic criterion has linked many genera that have limited or no phylogenetic relationship. Nevertheless, they share the characteristic of being gram-negative bacteria and the lack of bacteriochlorophyll-production. Virtually all known morphological bacterial shapes and types of motility occur among the colorless sulfur bacteria, e.g. both filamentous as well as unicellular types occur. Colorless sulfur bacteria are found both in the domains of Eubacteria and Archea, however, a physiological distinction can be made as one group of genera appears to accumulate elemental sulfur, an intermediate oxidation product of reduced sulfur components, inside the cell (*Thiovulum*, *Thiospira*, *Thiobacterium*, *Macromonas*, *Achromatium*, *Thiomargarita*, *Thiotrix*, *Thioploca*, *Beggiatoa*), while in the other group sulfur accumulates outside of the cells (*Arcobacter*, *Thiobacillus*, *Thiomicrospira*, *Thioalkalivibrio*, *Thiosphaera*, *Sulfolobus*, *Acidianus*, *Thermotrix*, *Thioclava*, *Paracoccus*, *Pseudomonas*, *Alcaligenes*) (Friedrich 1998; Robertson & Kuenen 2006; Sorokin et al. 2006; Sorokin et al. 2007). Endosymbiotic sulfur-oxidizing bacteria living in invertebrates, i.e. in the trophosome of tube worms (*Riftia pachyptilia*), or in the gill of several mollusks (*Thyasira flexuosa*, *Myrtea spinifera*, *Bathymodiolus thermophilus*, *Calyptogena magnifica*) have been found at hydrothermal vent sites (Cavanaugh et al. 1981; Wood & Kelly 1989; Nelson et al. 1995) and other habitats (mangrove swamps, sea grass beads, anoxic marine basins, sewage outfalls, rotting whale carcasses) (Distel 1998). Chemoautotrophic symbiosis provides the endosymbiotic bacteria with sulfide and oxygen for energy generation and carbon dioxide fixation, whereas the invertebrate species benefit from the organic compounds excreted by the bacteria (Dahl & Prange 2006). In the trophosome of *Riftia pachyptila* (Vestimenifera) large, spherical cells were found to be structurally similar to the marine sulfur-oxidizing bacterium *Thiovulum*, belonging to the epsilon-Proteobacteria (Bright & Sorgo 2003). A closest relationship on the 5S and 16S rRNA basis has been suggested between symbiotic colorless sulfur bacteria and the genus *Thiomicrospira* (Lane et al. 1985), the latter is found to be most abundant also as free-living community at hydrothermal vents (Ruby & Jannasch 1982; Jannasch et al. 1985; Wirsen et al. 1998). This genus and the symbiotic forms belong to the

group of gamma-Proteobacteria. Other and morphological conspicuous sulfur bacteria from genera such as *Macromonas*, *Thiobacterium*, *Thiospira* and *Thiovulum* and large bacteria from genera like *Achromatium*, *Thiomargarita*, *Thioploca*, *Thiotrix*, and *Beggiatoa* are also affiliated to the gamma-Proteobacteria.

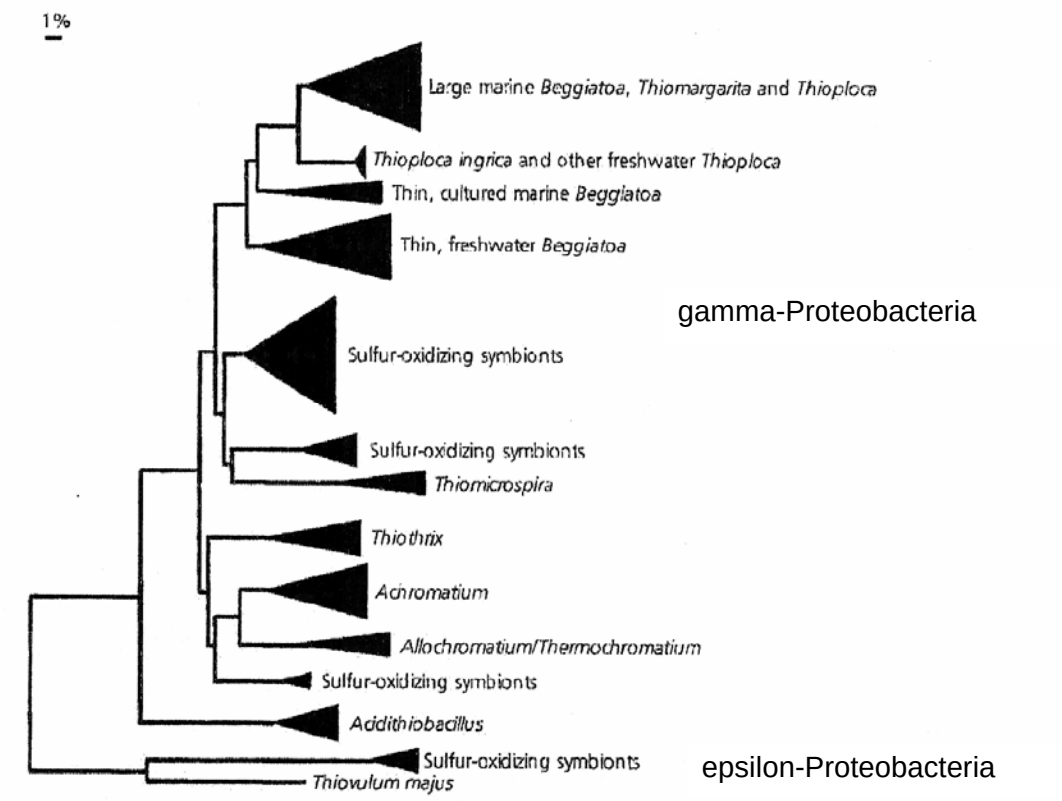

Figure 3: Modified after Gray & Head (2005): Phylogenetic tree of respresentatives of the sulfur-oxidizing bacteria from the gamma-Proteobacteria and epsilon-Proteobacteria

On the basis of 16S rRNA sequence analysis the genera *Beggiatoa*, *Thioploca* and *Thiomargarita* appear to form a monophyletic group within the gamma-Proteobacteria (Fig. 3) (Gray & Head 2005). Four distinct lineages from this group can however be differentiated with respect to their morphology and physiology (Teske & Nelson 2006). Multicellular, filamentous forms, *Beggiatoa* and *Thioploca*, but also pearl-chain-associated cells like *Thiomargarita* can be defined by the diameter of the cells (<200 µm for *Beggiatoa* and *Thioploca*, <750 µm for *Thiomargarita*), cell inclusions such as nitrate-storing vacuoles and the presence of cell-surrounding sheath material, the latter only found within the genus of *Thioploca*. As these morphological features can vary, e.g. cell inclusions depend on the growth conditions, and different cell diameters can be found in the same environments (Mußmann et al. 2003), physiological criteria are additionally taken in consideration for the discrimination of the various multicellular sulfur bacteria. The first group of the four lineages comprises large-vacuolated, autotrophic, marine *Beggiatoa* and *Thioploca* species,

Thiomargarita namibiensis and a so far uncharacterized attached, rosette-forming and vacuolated sulfur bacterium (Kalanetra et al. 2004). Freshwater *Thioploca* isolates form the second and heterotrophic freshwater *Beggiatoa* the third group of organisms. The fourth lineage comprises thin (~ 4µm), non-vacuolated autotrophic marine strains of *Beggiatoa*. This last lineage builds a clade of its own, branching deeply within the larger monophyletic group, and have been claimed to form the root of the large-vacuolated sulfur bacteria clade (Ahmad et al. 2006).

The genus *Thiotrix* comprises attached, filamentous and partly vacuolated species, and forms a deep branch with the gamma-Proteobacteria. They are phylogenetically distinct from the monophyletic group of the genera *Beggiatoa*, *Thioploca* and *Thiomargarita*. Different strains also feature physiological variations related to the carbon metabolism which are thought to be an ecological adaption to their environment (Rossetti et al. 2003; Engel et al. 2004). The genus *Achromatium* is represented by a large (125 µm length), unicellular bacterium (Gray et al. 2004; Head et al. 1996) which falls within the gamma-Proteobacteria and belongs together with the genus *Thiobacterium* to the family Thiotrichaceae (La Rivière & Schmidt 1999). *Thiovulum* has members in the epsilon-Proteobacteria (Lane et al. 1992), which are highly motile unicellular organisms, forming conspicuous veils on top of sulfidic sediments (Jørgensen & Revsbech 1983). Another autotrophic sulfur oxidizer falling into the group of epsilon-Proteobacteria is a member of the genus *Arcobacter*, a small, motile vibrioid with large accumulations of external elemental sulfur excretion (<500 µm) (Wirsen et al. 2002). One member of the genus *Macromonas* (La Rivière & Schmidt 1999), a unicellular beta-Proteobacterium, has calcite inclusions and sulfur globules, similar to *Achromatium* cells.

Other species with the ability to oxidize reduced sulfur compounds are also found in other sub-divisions of Proteobacteria and Eubacteria (i.e. anoxygenic phototrophic green and purple sulfur bacteria, some species of cyanobacteria), but also in the domain of thermophilic Archaea bacteria within the genus *sulfolobales*. Although all of these organisms perform the same metabolic reaction (oxidation of reduced sulfur compounds), the mechanism of reduced sulfur compound oxidation shows a high level of variation in the metabolic pathway, indicating a convergent evolution of the colorless sulfur bacteria (Robertson & Kuenen 2006). Not only the enzymes and electron carriers can differ for the sulfur/sulfide oxidation pathway, but also their localization in the membranes appears to be different.

Reduced sulfur compounds, the energy source of the colorless sulfur bacteria, occur in many forms and oxidation states, hydrogen sulfide, $H_2S/HS^-/S^{2-}$ and organic polysulfanes R-S_n-H (-2), polysulfides S_n^{2-}(-1), elemental sulfur mainly as cyclo-octa-derivate S_8 (0),

thiosulfates $S_2O_3^{2-}$ (+5), sulfite SO_3^{2-} (+4) and sulfate SO_4^{2-} (+6). Most commonly used as electron donors are hydrogen sulfide, elemental sulfur and thiosulfate, whereas the final sulfur oxidation product is sulfate, involving the transfer of 8 electrons to an appropriate electron acceptor, i.e. oxygen or nitrate. Oxidation of hydrogen sulfide occurs in stages, the first oxidation step ends with elemental sulfur, S^0, which can be used as an energy reserve if the hydrogen sulfide supply has been depleted.

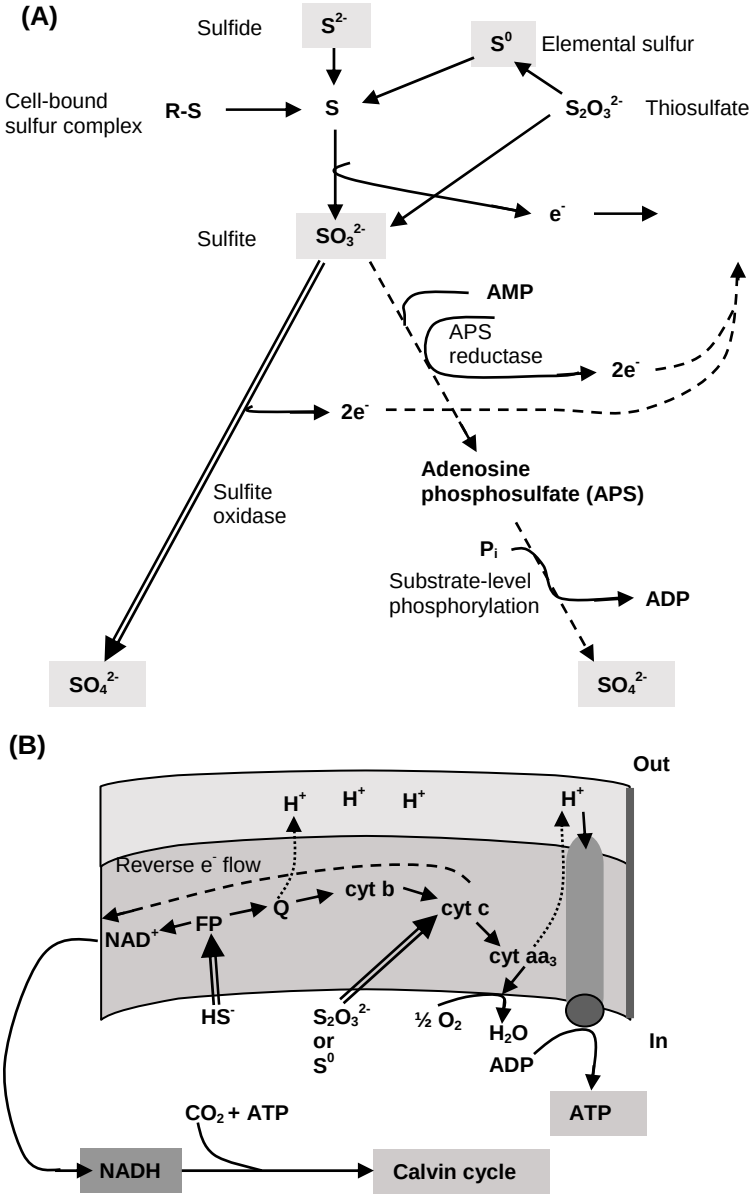

Figure 4: Modified after Madigan et al. (2003): Oxidation of reduced sulfur compounds by sulfur chemolithotrophs. (A) Oxidation steps of different compounds. The majority of sulfite is oxidized via the sulfite oxidase pathway. (B) Electrons of sulfur compounds are transferred into the electron transport chain and result in proton motive force; electrons from thiosulfate and elemental sulfur enter the chain at the cytochrome c level. NADH is generated by a reverse electron flow (electron donors are more electropositive than NAD^+/NADH). Cyt, cytochrome; FP, flavoprotein; Q, quinone.

For chemolithotrophic and phototrophic bacteria the first step from sulfide to elemental sulfur is either mediated via reduction of a c-type cytochrome and a flavocytochrome c-sulfide dehydrogenase, or via reduction of a quinone and a sulfide-quinone-reductase. In the heterotrophic, freshwater *Beggiatoa alba* the electrons from sulfide enter the electron-transport chain via a flavocytochrome (Schmidt et al. 1987). If this process also occurs in the big chemolithoautotrophic sulfur bacteria remains to be investigated. The produced sulfur can either be stored or directly oxidized further to sulfite, mediated possibly by a sulfur oxygenase. If nitrate is the alternative electron acceptor under anoxic conditions, the transferred electrons could be linked to a respiratory chain, as shown for *Thiobacillus denitrificans* (Kelly 1999). The final oxidation step of sulfite to sulfate can be processed via two biochemical pathways, indirect by a reverse adenosine phosphosulfate (APS) reductase, known from the metabolism of sulfate reducing bacteria, or directly by sulfite:acceptor oxidoreductase (Fig. 4). The indirect pathway produces a high-energy phosphate bond by the conversion of AMP (adenosine monophosphate) to ADP (adenosine diphosphate), the direct way yields energy by transporting the electrons from sulfite to cytochrome c via the sulfite:acceptor oxidoreductase, what generates ATP (adenosine triphosphate) and proton motive force (PMF). Although the APS reductase pathway has been shown for some *Thiotrix* and *Achromatium* species (Grabovich et al. 1999; Head et al. 2000b), only some *Beggiatoa* species exhibit this kind of substrate-level phosphorylation (Hagen & Nelson 1997). Consequently, the pathways of dissimilatory sulfur metabolism involved in the electron transport display considerable variations, even between closely related organisms (Friedrich et al. 2001). However, all electrons enter the electron transport system at either the flavoprotein or cytochrome c, resulting in a different energy yield, and get transported to either oxygen or nitrate, generating a PMF which results in ATP synthesis (Madigan et al. 2003). As the sulfur-oxidizing bacteria often live at the interface of oxic and anoxic layers, many of them are equipped with survival mechanisms for anaerobic energy metabolism, in which nitrate can serve as alternative electron acceptor. Alternatively, even intracellulary stored elemental sulfur may substitute oxygen or nitrate as the terminal electron acceptor under anoxic conditions, resulting in reduction to sulfide and the oxidation of organic compounds. It represents a temporary form of anaerobic respiration for metabolism maintenance, which was shown for freshwater *Beggiatoa* (Nelson & Castenholz 1981b; Schmidt et al. 1987). Nitrate is reduced to ammonium (DNRA, dissimilatory reduction of nitrate to ammonium) rather than to dinitrogen gas (Otte et al. 1999; Sayama et al. 2005), due to the inhibition of denitrification in highly sulfidic environments (Brunet & Garcia-Gil 1996; Burgin & Hamilton 2007), although other studies suggested *Beggiato*a capable of

denitrification (Fossing et al. 1995; McHatton et al. 1996; Sweerts et al. 1990). Nevertheless, the cytochrome c-nitrite reductase complex, which mediates the reduction step of nitrite to ammonium, also produces ATP when coupled to sulfide oxidation (Simon 2002). The coenzyme NAD (Nicotinamide adenine dinucleotide) gets reduced to NADH via the reversed electron flow of sulfide oxidation pathway driven by PMF, the resulting reducing power is used for autotrophic growth (Robertson & Kuenen 2006). Mostly the marine species of the big sulfur bacteria are capable of chemolithoautotrophic growth on sulfide (H_2S) and carbon dioxide (CO_2) using the Calvin cycle for CO_2 fixation. The carbon metabolism of the colorless sulfur bacteria subdivides them into different physiological groups: 1. The obligate chemolithotrophs require an inorganic energy source and carbon dioxide to assimilate cell carbon via the Calvin cycle, as mentioned above for the colorless sulfur bacteria (Schlegel & Fuchs 2006). 2. The facultative chemolithotrophs grow either chemolithoautotrophically as obligate chemolithotrophs or heterotrophically with organic compounds as energy and carbon source. Some *Beggiatoa* species can use two or more metabolic pathways for energy and carbon gain, termed as mixotroph, growing on reduced sulfur compounds as well as organic substrates (Nelson & Jannasch 1983). 3. Chemolithoheterotrophic organisms like some thiobacilli generate energy from the oxidation of reduced sulfur compounds, but cannot fix carbon dioxide. 4. Sulfur-oxidizing chemoorganoheterotrophs, such as some freshwater *Beggiatoa* (Larkin & Strohl 1983), *Thiobacterium*, *Macromonas* and *Thiotrix* (Dubinina & Grabovich 1984), can oxidize sulfide without gaining energy. The beneficial effect of this reaction would be the detoxification of metabolically produced hydrogen peroxide, H_2O_2, which reacts with hydrogen sulfide and elemental sulfur can be deposited.

The metabolic diversity of sulfur bacteria and their high abundances in life-supporting environments where reduced sulfur compounds are available, plays a significant role in numerous processes: the oxidation of reduced sulfur compounds, the deposition of elemental sulfur, the fixation of inorganic carbon, the utilization of organic compounds as electron donors or carbon sources, consumption of oxygen and reduction of nitrate and nitrite to ammonium. Some species may fix dinitrogen (N_2) and mobilize insoluble metals and other minerals (bioleaching). Colorless sulfur bacteria occur worldwide in very diverse habitats and with a wide range of salinities, but always in a restricted niche in efficient competition with chemical sulfide oxidation, at the aerobic/anaerobic interface (Jørgensen & Nelson 2004). To thrive at the diffusive boundary layer between their electron donors, reduced sulfur compounds, and their electron acceptor, oxygen or nitrate, these bacteria make use of one or more characteristic features: their extreme size, the storage of sulfur and the oxidizing agent nitrate, motility and chemotaxis. Fast swarming *Thiovulum* cells (~600 µm/s) showed

chemotactic responses towards critical concentrations of oxygen and sulfide, and due to aggregation in form of a veil and their large size (<25 μm) they are visible by the unaided eye. Through these veils *Thiovulum* bacteria actively increase their food supply by creating an advective flagella-induced flow of oxygen-rich water towards the sulfidic sediment through this spatially organized structure (Jørgensen 1988; Schulz & Jørgensen 2001). *Beggiatoa* and *Thioploca* are also typical species that inhabit the specialized niche between the oxygen and sulfide interface, both in freshwater and marine sediments. These filamentous, gliding forms of sulfur bacteria use their chemotactic properties to adjust their position with respect to the opposed gradients of oxygen and sulfide. Whereas some *Beggiatoa* species rely on and follow the simultaneously presence of oxidant and reductant, like in phototrophic microbial mats (Nelson et al. 1986b; Jørgensen & Des Marais 1986a; Garcia-Pichel et al. 1994), *Thioploca* can overcome spatially separated pools of oxygen or nitrate and reduced sulfur compounds by ascending from their sheaths through the diffusive boundary layer into the oxidant-rich (oxygen or nitrate) water column (Hüttel et al. 1996; Zopfi et al. 2001). Large, vacuolated forms of *Thioploca* and *Beggiato*a, as well as the largest known prokaryote *Thiomargarita*, can survive temporal fluctuations of sulfide, nitrate and oxygen with the decomposition of their storage compounds, elemental sulfur and nitrate (Schulz et al. 1999; Schulz & Jørgensen 2001). Applications of sulfur bacteria in manmade artificial habitats such as waste-water and microbial leaching treatments are improving, in order to convert the toxic hydrogen sulfide to elemental sulfur and to extract various metals from sulfide ores, respectively (La Rivière & Schmidt 2006).

Big sulfur bacteria have important implications on the cycling on many elements, especially if occurring in high biomasses (Gray & Head 2005). The carbon cycle is predominantly influenced by heterotrophic bacteria and their conversions, both in lake and sea sediments (Fenchel et al. 1998). Chemolithoautotrophic bacteria, for which CO_2 is the primary source of carbon, can enhance the carbon burial in some microhabitats, such as in the eutrophic Conceptión Bay, Chile (Graco et al. 2001). Dense layers of sulfur bacterial mats have also been observed at shallow or deep sea hydrothermal vent sites, including *Beggiatoa* filaments (Nelson et al. 1989), and unicellular organisms such as *Thiobacillus*, *Thiovulum*, *Thiomicrospira* and *Macromonas* (La Rivière & Schmidt 2006). These mats can retain the emitted carbon from methane seeps within the sediment by the associated activities of anaerobic methane-oxidizing archaeal-sulfate-reducer consortia and the sulfur-oxidizing bacteria (Joye et al. 2004; Boetius & Suess 2004). *Thioploca* mats in the Gulf of Mexico seemed to influence even higher trophic levels as these appeared to support communities of diverse foraminifera (Robinson et al. 2004; Panieri & Sen Gupta 2008). Some of the big

sulfur bacteria such as *Beggiatoa*, *Thioploca* and *Thiomargarita* can influence the nitrogen budget and the productivity of a marine system, as nitrogen fixation, denitrification and the dissimilatory reduction of nitrate to ammonium (DNRA) have been observed (Polman & Larkin 1988; Fossing et al. 1995; Sweerts et al. 1990; Otte et al. 1999). These genera can accumulate vast amounts of nitrate intracellulary, and *Thioploca* cells were suggested to monopolize the available nitrate as they thrive out of the sediment in the overlying water outcompeting other sulfur bacteria by separating the simultaneous supply of nitrate and sulfide (Fossing et al. 1995; Zopfi et al. 2001). Increased organic matter inputs to the sediment are followed by an increased sulfate reduction; the resulting higher sulfide concentrations inhibit denitrification and favour DNRA of the sulfur-oxidizing bacteria (Brunet & Garcia-Gil 1996; Burgin & Hamilton 2007). The greater amount of nitrogen retention due to ammonium reoxidation in the system can induce eutrophication, even though nitrogen is an important limiting factor in marine environments (Nixon 1981). In nutrient-rich upwelling systems along the Pacific coast of South America and at the Namibian coastline, high rates of sulfate reduction in sediments have been reported, but only low concentrations of sulfide (Ferdelman et al. 1997). Reactive iron was considered as sulfide sink, enhanced by bacterial sulfur disproportionation (Zopfi et al. 2008), but also the extensive mats of *Thiomargarita*, *Thioploca* and *Beggiatoa* cells were held responsible for 16-91% of sulfide reoxidation (Jørgensen 1977; Gallardo 1977; Gallardo & Espinoza 2007) and reducing the concentrations of toxic sulfide (Otte et al. 1999; Bargarinao 1992). Along the continental shelf from South Chile to Peru over a distance of more than 3000 km, the discontinuous occurrence of *Thioploca* mats represents the largest sulfur bacteria community on the planet (Jørgensen & Gallardo 1999). In Namibian sediments the non-motile, large *Thiomargarita namibiensis* cells with high storage capacities of up to 0.8 M nitrate and 1.7 M elemental sulfur intracellulary (Schulz et al. 1999), were responsible for more than 50% of the sulfide reoxidation and represented a major repository of elemental sulfur in these sediments (Brüchert et al. 2003). Although the concentrations of sulfate and dissolved sulfide in freshwater habitats is much lower than in marine sediments, large accumulations of big sulfur bacteria such as *Thioploca*, *Beggiatoa* and also the unicellular *Achromatium* influence the sulfur cycle on the oxidative side (Gray et al. 1997; Head et al. 2000a). Already at the beginning of the 20th century, Lauterborn (1907) discovered *Thioploca* filaments in Lake Constance, Germany, and found them in greater water depths and less sulfidic sediments than *Beggiatoa* filaments.

The genus *Beggiatoa*

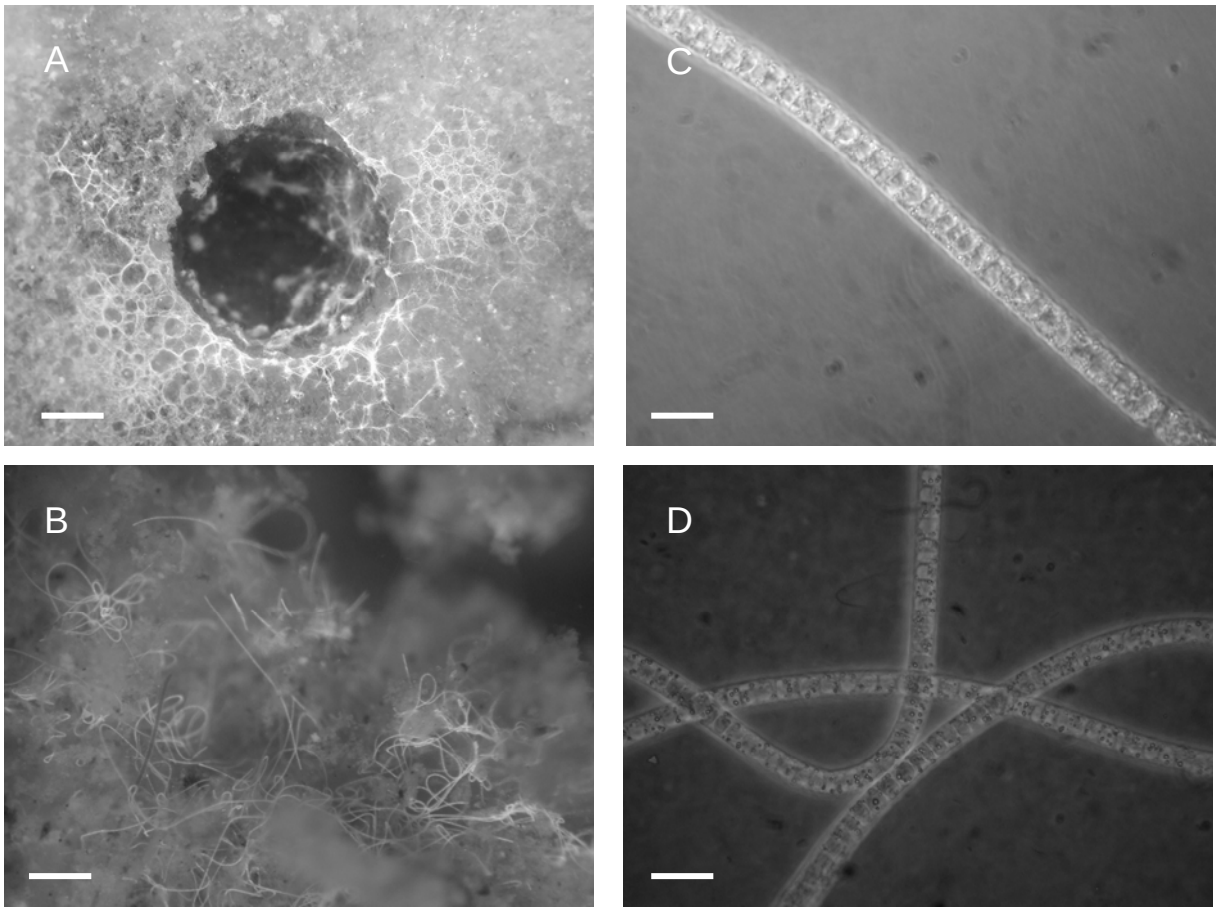

Figure 5: Hypersaline *Beggiatoa* filaments from microbial mats, (A – C) images of filaments of mesocosm mats originated from Chiprana, Spain with 8-9% salinity; (D) light micrograph of filament of saltern pond with 6% salinity, Ibiza, Balearic Islands, Spain; (A+B) photographs of filaments on top of the mat or mat pieces, respectively, (C+D) light micrographs of single filaments with intracellular vacuoles; scale bar: (A) 10 mm, (B) 1 mm, (C+D) 10 µm

The genus *Beggiatoa* contains filamentous, multicellular, gliding prokaryotic organisms belonging to the gamma-Proteobacteria. They are named after the Italian physician F. S. Beggiato (Trevisan 1842), who identified the previously characterized *Oscillatoria alba* (Vaucher 1803) as *Beggiatoa alba*, and included this species within the genus. Deriving from the former assignment to cyanobacteria, *Beggiatoa* species are named colorless sulfur bacteria. It distinguishes them from pigmented filamentous bacteria, and characterizes the white appearance due to the intracellular storage of elemental sulfur (Strohl 2005). These morphologically conspicuous sulfur-oxidizing bacteria are motile, and react chemotactically to environmental compound gradients. Further morphological key criteria are the filament diameters (ranging from 1 to 200 µm) and the presence of internal vacuoles per cell. Marine species with filament diameters larger than 10 µm possess vacuoles as a storage compartment, most likely for nitrate, which is also true for large, marine *Thioploca* and *Thiomargarita*. *Beggiatoa* filaments do not form a polysaccharide sheath around a bundle of trichomes like

the closely related *Thioploca* sp., but each single trichome of *Beggiatoa* sp. can produce a slime matrix consisting of polysaccharide and protein conjugates (Mußmann et al. 2007). The phylogenetic remotely related filamentous *Thiotrix* sp. differs from *Beggiatoa* species by the presence of attachment holdfasts at terminal cells. *Beggiatoa* species occur worldwide in various habitats, all rich in sulfide. The metabolic spectrum includes both obligate and facultative chemoautotrophy (Nelson & Jannasch 1983; Hagen & Nelson 1996), as well as chemoheterotrophy (Nelson & Castenholz 1981a; Mezzino et al. 1984). Freshwater *Beggiatoa* sp. are mainly heterotrophic and non-vacuolated, with thin filament diameters and poly-ß-hydroxybutyrate or polyphosphate inclusions and intracellulary storage of elemental sulfur (Teske & Nelson 2006).

Morphology and Motility of *Beggiatoa*

Beggiatoa can easily be recognized by their multicellular, filamentous morphology, composed of cylindrical cells with lengths from 1.5-8 × their width (Faust & Wolfe 1961; Scotten & Stokes 1962) for thin freshwater and marine strains, and disk-shaped cell lengths of 0.1-0.9 × their width for large marine *Beggiatoa* (Teske & Nelson 2006). Filaments usually have consistent cell widths over the entire length and rounded terminal cells. The length of the whole filament varies between several mm (Jørgensen 1977; Gundersen et al. 1992; Larkin & Henk 1996) and up to 10 cm (McHatton et al. 1996). Freshwater species have thin filament diameters from 1-7µm, marine filament widths can vary between 2-200 µm (Macalady et al. 2006; Larkin & Henk 1996), and represent one of the largest bacteria in nature (Schulz & Jørgensen 2001). The cell cytoplasm is surrounded by a membrane and a first cell wall layer, the latter presumably a murein layer. Tested *Beggiatoa* strains turned out as gram negative, however, the outer cell wall structure is unusually complex for gram negative bacteria. It consists of at least two layers of different composition, peptidoglucan and lipopolysaccharide-like, the most external one showed a fibrillar pattern (Strohl 2005). Reproduction, growth and dispersion of *Beggiatoa* filaments by septation of only the inner two membranes closing like the iris of a diaphragm, results in transverse binary fission of cells (Strohl & Larkin 1978a); the outer wall layers do not take part in septation (Strohl 2005). Hormogonia can also be produced by this way of filament breakage and sacrificial cell death via necridia formation (Teske & Nelson 2006). Cell inclusions of *Beggiatoa* are conspicuous and serve energy and substrate storing purposes: poly-ß-hydroxybutyrate (PHB) (Pringsheim 1964; Strohl & Larkin 1978b), polyphosphate (Strohl & Larkin 1978b) and elemental sulfur (Winogradsky 1887; Strohl et al. 1981b). PHB accumulation appears to be restricted to the heterotrophic, freshwater strains, and is depending on the amount of available acetate in the medium

(Kowallik & Pringsheim 1966). Deposition of PHB seemed to be induced by high aeration and can account for up to 50% of dry weight in the absence of sulfide (Güde et al. 1981). A freshwater *Beggiatoa alba* strain containing PHB inclusions was able to survive several days in a medium lacking carbon and energy sources (Strohl 2005). Inorganic polyphosphate, a linear polymer of inorganic phosphate moieties linked by high energy phosphoanhydride bonds, can be visualized intracellularly by staining with 4'-6'-diamino-2-phenylindole (DAPI) (Seufferheld et al. 2004). It is proposed to serve as energy supply and ATP substitute, and can have a role in cation storage, as chelator of metals, a buffer against alkali, a channel for DNA entry, as a structure of channels and pumps, and as regulator of responses to stress in the stationary phase (Kornberg 1995). Elemental sulfur is stored in globules, enclosed in a single or multiple protein layer (the latter found in *Beggiatoa alba*; Strohl et al. (1982)), an extension of the cytoplasmic membrane, and an inner sulfur inclusion envelope, separating the elemental sulfur from the cytoplasm. The location of sulfur in the periplasmatic space seemed to be of useful consequences: the oxidation of sulfide at the outer surface of the sulfur inclusion membrane may establish a proton gradient necessary for ATP generation and the potential of sulfide toxicity can be reduced within the cytoplasm (Dahl & Prange 2006). Elemental sulfur in *Beggiatoa* strains is mainly present in the common, stable S_8 ring configuration, cyclo-octasulfur, proven for marine species from the Guaymas Basin (Nelson et al. 1989). However, the sulfur speciation can differ according to the different sulfur depositions pathways (Pasteris et al. 2001; Prange et al. 2002; Teske & Nelson 2006). *Beggiatoa* cells grown in the absence of sulfide contained small, rudimentary sulfur envelopes, indicating that a primer envelope was always present (Strohl et al. 1982).

Another structure at the outer cell wall layer, the fibrillar pattern, might play an essential role in the gliding process, the way *Beggiatoa* filaments move. *Beggiatoa* revealed average gliding velocities ranging between 1-3 $\mu m\ s^{-1}$ (Nelson et al. 1989). The mechanism of gliding requires the contact of the cell to a solid surface. This contact is established by extracellular slime excretion, identified for *Beggiatoa alba* as mainly consisting of polysaccharides (Strohl & Larkin 1978b). Parallel rows of pore-like structures on the outer filament surface were discovered by electron microscopy and are thought to secrete slime which allows a forward and rotary motion. The latter is achieved by the fibrillar pattern on the surface of the filament, which creates specific surface topographies influencing the mode of rotation (Larkin & Henk 1996; Hoiczyk & Baumeister 1998; Hoiczyk 2000). Wolgemuth et al. (2002) demonstrated the propulsive force of slime thrust in a mathematical model: dehydrated slime (a charged polyelectrolyte gel) became hydrated at the nozzle (pore) opening, expanded and pushed the cell forward against the substrate. Carbohydrate

polymerization processes and the compression of polymer chains inside the nozzle could already be a driving force for gliding motion (Carrillo et al. 2007). The ecophysiological advantage of motility for *Beggiatoa* is obvious, as they need to overcome diffusion limitations to thrive between opposing and fluctuating gradients of sulfide and oxygen or nitrate (Schulz & Jørgensen 2001), but tolerate only low concentrations of electron donor (sulfide) and electron acceptor (oxygen or nitrate). Therefore, *Beggiatoa* filaments not only compete with chemical oxidation of sulfide but also follow the narrow layer of the oxygen or nitrate/sulfide interface and can separate the compounds efficiently from each other (Teske & Nelson 2006).

Chemotaxis of *Beggiatoa*

Beggiatoa, as well as other motile colorless sulfur bacteria, are capable of chemotactic behavior, their movement is dependant on chemical stimuli (Armitage 1997), which allows them to position themselves at the optimal nutrient concentrations and move as environmental conditions change. The phobic response to oxygen values higher than 5% of atmospheric saturation enables the microaerophilic *Beggiatoa* filaments to escape from oxidative damage to essential enzymes and even large filaments can retract from oxic zones and accumulate in a thin film at the oxic/anoxic interface (Møller et al. 1985; Schulz & Jørgensen 2001). The sharp lower boundary of bacterial plates in oxygen/sulfide gradient cultures also reflects an avoidance response to high sulfide values (Nelson et al. 1986b). Such a narrow microhabitat at the interface of oxidant and reductant is not only found in marine and brackish sediments, but also in phototrophic microbial mats, where the counter gradient of oxygen and sulfide has a diel up and down movement (Jørgensen & Des Marais 1986a). *Beggiatoa* filaments migrate to follow this interface while maintaining a safe distance from fully oxic conditions, their average gliding speed of up to 3 μm s^{-1} can be faster than that of cyanobacteria in the same phototrophic mat (Garcia-Pichel et al. 1994; Møller et al. 1985). Additionally, light is controlling the migration pattern of *Beggiatoa* in these systems, the step up phobic response is already induced at low light intensities (2% of full summer sunlight) and exposure results in retraction back into the mats (Nelson & Castenholz 1982). The phobic response to light may override the phobic response to oxygen, and filaments find their way back into deeper sediments even through a rising oxygen peak during the first morning light (Schulz & Jørgensen 2001). Nelson & Castenholz (1982) speculated that cytochromes may be responsible for sensing blue light, since these chromoproteins have a major absorption maximum in the blue light region, at 430 nm. Nitrate as alternative electron acceptor is also likely to play a role as chemical stimuli as it was proven as an attractant for Thioploca filaments, a closely related organism of *Beggiatoa* (Hüttel et al. 1996; Zopfi et al. 2001).

Kamp et al. (2006) observed a downward migration and the development of thicker bands upon nitrate addition to enrichment cultures of freshwater *Beggiatoa*. Large, marine *Beggiatoa* spp. are not tied to the thin interface of oxygen and sulfide, but can tolerate fluctuating levels of oxidant and reductant (Gundersen et al. 1992; Teske & Nelson 2006), likely due to their ability to store nitrate as electron acceptor intracellulary (McHatton et al. 1996). Furthermore, a negative chemotactic response to sulfide prevents these organisms to not get lost at deeper sediments depths where sulfide values increase dramatically, such as in suboxic zones of marine sediments (Jørgensen & Postgate 1982; Preisler et al. 2007). Large, vacuolated *Beggiatoa* filaments can occur in high biomasses on the surface of hydrothermal vent sediments and of cold seeps (Jannasch et al. 1989; Nelson et al. 1989; Mills et al. 2004). They can accumulate high concentrations of nitrate (McHatton et al. 1996; Mußmann et al. 2003), which is used as an oxidant most likely by reduction to ammonia, thus providing a link between the biochemical cycles of nitrogen and sulfur (Teske & Nelson 2006).

Physiology of *Beggiatoa*

The sulfur metabolism of *Beggiatoa* strains is not yet fully understood with respect to specific enzymatic steps. Particularly the degradation of sulfur globules needs further investigation (Dahl & Prange 2006). Freshwater *Beggiatoa* are mostly chemoorganotrophic, using acetate and other short-chained organic acids (C_2, C_3, and C_4) as sole carbon and energy source (Larkin & Strohl 1983; Strohl et al. 1986). Certain strains, however, may also use acetate and sulfide as combined energy sources (Güde et al. 1981), but this form of 'mixotrophic' growth was questioned later (Nelson 1989; Nelson 1992). However, two of the latter, *Beggiatoa alba* B18LD and *Beggiatoa* strain OH-75-2a, show constitutive sulfide oxidation to elemental sulfur, promoted by a sulfite:acceptor oxidoreductase, which could in principle allow an energy gain (Schmidt et al. 1986). Thus, an increase in growth rate or protein synthesis could not be observed (Nelson & Castenholz 1981b). Electron transport inhibition experiments suggested the coupling of sulfide oxidation and oxygen reduction by an electron transport system (Schmidt et al. 1987). It occurs most likely via the respiratory chain and cytochrome c as electron acceptor, since the necessary enzymes for sulfide oxidation are membrane associated as well as the respiratory chain (Teske & Nelson 2006). Sulfur oxidation in heterotrophic strains does not proceed efficiently beyond the stage of elemental sulfur, i.e. the well studied strain OH-75-2a revealed no activity of sulfite-oxidizing enzymes, which would make the oxidation of sulfite to sulfate possible (Hagen & Nelson 1997). Nevertheless, oxidation of reduced sulfur compounds was probably used for other purposes than energy conservation: thiosulfate as catalase replacement for the detoxidation of

peroxides and elemental sulfur globules as electron acceptor reserve, allowing a rudimentary anaerobic respiration with sulfur and leading to the production of sulfide to survive short periods of anoxia (Nelson & Castenholz 1981b). *Beggiatoa alba* BL18LD, as well as strain OH-75-2a, were capable of reducing elemental sulfur back to sulfide. However, as oxygen is needed for sulfide and acetate oxidation for synthesis of cell material, this led to the assumption that sulfide and acetate compete for oxygen (Nelson & Castenholz 1981b; Schmidt et al. 1987). In autotrophic growth of mostly marine *Beggiatoa* strains sulfide provides electrons for energy generation and for carbon fixation. Filaments without intracellular nitrate storage as alternative electron acceptor, depend on the concurrent availability of oxygen and sulfide in opposed overlapping gradients (Nelson & Jannasch 1983; Jørgensen & Revsbech 1983). Two thin, non-vacuolated, marine *Beggiatoa* strains, MS-81-6 and MS-81-1c, showed high growth rates in sulfide gradient cultures while positioned in a horizontal plate at the oxygen-sulfide interface (Nelson et al. 1986a). Nonetheless, these strains revealed physiological differences in their different enzyme equipment and ecological consequences. The obligate autotrophic strain MS-81-1c oxidized sulfur to sulfate by either the adenosine phosphosulfate pathway with APS (adenosine phosphosulfate) reductase and ATP (adenosine triphosphat) sulfurylase, the substrate-level phosphorylation, or by a highly regulated sulfite:acceptor oxidoreductase. In contrast, the facultative autotrophic strain MS-81-6 used the membrane associated sulfite:acceptor oxidoreductase for sulfide oxidation and possibly the ATP sulfurylase for assimilative growth with acetate on sulfate (Nelson & Jannasch 1983; Hagen & Nelson 1997). The latter strain was capable in adjusting the two sulfide oxidation steps by depositing elemental sulfur if sulfide was overabundant, enhancing the removal of toxic sulfide. However, in case sulfide was the limiting compound, it was completely oxidized to sulfate (Nelson et al. 1986a). This kind of flexibility depending on ecological conditions is also represented in the anaerobic nitrate respiration of large, vacuolated and nitrate storing marine *Beggiatoa* species. Elemental sulfur as well as nitrate can be stored intracellulary in high concentrations of approximately 300-400 mM (Mußmann et al. 2003; Preisler et al. 2007). Sulfide oxidation with nitrate as electron acceptor results most likely in ammonium production via dissimilatory nitrate reduction to ammonium, DNRA (Sayama et al. 2005; Preisler et al. 2007). Sulfide is oxidized with nitrate to elemental sulfur in the suboxic zones of the sediment, the second oxidation step to sulfate can occur within the suboxic zone with nitrate or at the sediment surface with oxygen, the latter would result in a higher energy yield. The aerobic electron-transport pathway is more efficient in gaining energy because the involved cyctochrome

creates a steeper electrochemical gradient by delivering protons as well as electrons to the cytoplasmic membrane side, where oxygen and protons are combined (Gray & Head 2005).

With respect to the carbon metabolism of *Beggiatoa*, autotrophy and heterotrophy can clearly be distinguished, whereas 'mixotrophy', obtaining carbon from acetate and carbon dioxide and energy from sulfide oxidation (Pringsheim 1970; Güde et al. 1981; Strohl & Schmidt 1984) couldn't be demonstrated experimentally (Nelson et al. 1989; Nelson 1992). Autotrophic growth was demonstrated in axenic cultures of thin marine strains, MS-81-1c and MS-81-6, the first obligate, the second being a facultative autotroph (Nelson et al. 1982; Nelson & Jannasch 1983). The assimilative carbon fixation occurs via the Calvin cycle, indicated by the activity of Ribulose-1,5-biphosphate carboxylase/oxygenase (RubisCo). Strain MS-81-1c showed no regulation of this enzyme by additions of acetate, the facultative *Beggiatoa* type however does down-regulate its RubisCo enzyme activity upon acetate addition and expressed 2-oxoglutarate dehydrogenase, a key enzyme of the citric acid cycle, which is not active under autotrophic growth (Hagen & Nelson 1996). Both strains accumulate autotrophically fixed carbon into cellular biomass, instead of oxidizing acetate to carbon dioxide. Large, marine *Beggiatoa* spp. have been proven to show autotrophic potential, the carbon dioxide fixation by RubisCo is enhanced upon addition of sulfide (Nelson et al. 1989). Hydrothermal vent organisms of the Guayama Basin, cold seep *Beggiatoa* in the Monterey Canyon and from the Gulf of Mexico had significant levels of RubisCo activity (Nelson et al. 1989; McHatton et al. 1996; Larkin et al. 1994). In *Beggiatoa* mats associated with sulfate-reduction/methane-oxidation, filaments fixed carbon dioxide derived from methane oxidation (Orphan et al. 2002). In this methane and hydrocarbon-rich environment *Beggiatoa* mats can occur in white and orange color, the latter induced by high cytochrome contents, which is referring to their different carbon metabolism, the non-pigmented cells being chemoautotrophic and the pigmented organoheterotrophic (Nikolaus et al. 2003). Freshwater *Beggiatoa* grow mostly heterotrophically and can use acetate as the sole carbon and energy source (Nelson & Castenholz 1981a; Larkin & Strohl 1983), except strain *Beggiatoa* D-402, which also revealed high activities of RubisCo and had a similar sulfide-oxidizing enzyme system as MS-81-6 (Grabovich et al. 1998; Grabovich et al. 2001; Patritskaya et al. 2001). *Beggiatoa alba* B18LD increased the assimilation of carbon dioxide by the addition of acetate, indicating an anaplerotic reaction of the citrate cycle (Strohl et al. 1981a). Nelson and Castenholz showed heterotrophic nutrition of the freshwater strain OH-75-2a on acetate, ethanol, lactate, and pyruvate with enzymes of the glyoxylate and tricarboxylic acids cycles (Nelson & Castenholz 1981a). Nevertheless, mixotrophic growth couldn't be proven for heterotrophic, freshwater *Beggiatoa* filaments; only the facultative

autotrophic, marine strain MS-81-6 might be assignable to mixotrophy with respect to carbon and energy metabolism (Hagen & Nelson 1996).

Heterotrophic nutrition of freshwater strains can also include the growth on various nitrogen compounds, such as nitrate, nitrite, ammonia, urea, aspartate, asparagines, alanin, and thiourea (Vargas & Strohl 1985a, b). Ammonia assimilation of *Beggiatoa alba* follows the glutamine synthetase-glutamate synthase pathway. If *Beggiatoa* use nitrate or nitrite as sole nitrogen source, it results in the production of ammonia by an assimilatory nitrate reductase, which is not membrane associated in freshwater species. Denitrification was suggested by Sweerts et al. (1990) for a freshwater mat of *Beggiatoa* on a lake sediment. Dinitrogen fixation was shown for nine strains of freshwater and marine *Beggiatoa* grown in gradient cultures of oxygen and sulfide (Nelson et al. 1982). Several heterotrophic freshwater strains and the autotrophic strains MS-81-1c and MS-81-6 repressed nitrogenase activity if nitrate or ammonia were available in the growth medium (Nelson & Castenholz 1981a, b; Nelson et al. 1982). In contrast to thin freshwater and marine strains, large marine *Beggiatoa* species have the ability to accumulate nitrate intracellulary, most likely in their vacuoles, and use it as a respiratory electron acceptor for sulfide oxidation. The latter organisms showed that nitrate reductase enzyme complexes are most likely membrane bound within the respiratory chain (McHatton et al. 1996). By their internal storage of nitrate as oxidant and elemental sulfur as reductant, the filaments can survive a period of days to weeks (Preisler et al. 2007). In reduced marine sediments, nitrate would be the limiting factor, rather than sulfide. Therefore, the dissimilatory nitrate reduction to ammonium (DNRA) is favored because dentrification is repressed by high sulfide concentrations (Brunet & Garcia-Gil 1996; Burgin & Hamilton 2007). Additionally, only 1 mol of nitrate is necessary to oxidize 1 mol sulfide in DNRA, but 1.6 mol nitrate is required to oxidize 1 mol sulfide if nitrate is reduced to dinitrogen. The produced ammonia can be reassimilated or reoxidized to nitrate by other bacteria in the sediment, whereas denitrification would cause a nitrogen loss for the system (Teske & Nelson 2006). If large vacuolated marine *Beggiatoa* filaments occur in high biomasses, they can be of high significance in the balance of nitrogen in the specific system.

The possession of a large central vacuole is consistent with the accumulation of nitrate, although a freshwater strain was found to accumulate nitrate in low amounts without the presence of a vacuole (Kamp 2007), and marine, attached filaments resembling *Thiotrix* own vacuoles with other functions than nitrate storage (Kalanetra et al. 2004). Nevertheless, cells with vacuoles of *Thioploca*, *Thiomargarita* and *Beggiatoa* were found to contain extremely high concentrations intracellular nitrate of up to 800 mM (Fossing et al. 1995; McHatton et al. 1996; Schulz et al. 1999). Sulfide oxidizing bacteria are able to use nitrate as

an alternative or the only electron acceptor when thriving in anoxic sediments at the sulfidic boundary and can outcompete other chemolithotrophs by oxidizing sulfide under anoxic conditions. As the vacuolated bacteria feature large diameters, but as the biovolume contains only down to 2% active cytoplasm, the cells are not diffusion limited (Schulz & Jørgensen 2001). Vacuoles are separated from the cytoplasm by a bilayered membrane, the tonoplast, which might originate from a cytoplasmic membrane invagination (Maier et al. 1990). Therefore, the tonoplast might be an inverted version of a cytoplasmic membrane. The proteins integrated within this hydrophobic membrane are responsible for the selective transport of hydrophilic and charged molecules, only water and apolar gases such as oxygen, nitrogen and methane can diffuse through this barrier (Lehninger et al. 1994).

These membrane-associated enzyme complexes induce an electrochemical gradient of protons (proton potential) by proton translocations across the membrane, and make use of the provided energy by the return flow of the protons (White 2007). The energy can be used for other membrane purposed such as ATP synthesis, reversed electron transport, motility processes and solute transport. In yeasts, the vacuolar ATPase (V-type ATPase) is localized within the tonoplast and actively pumps protons across the membrane into the vacuole resulting in acidification of this intracellular compartment (Graham et al. 2003). ATPases can translocate two protons per ATP molecule hydrolyzed, but also a vacuolar pyrophosphatase (PPase) has the potential to contribute to the vacuole acidification, as well as secondary transport systems, i.e. proton symport with an anion (H^+/R^-) or proton antiport with a cation in exchange (H^+ for R^+) (Kurkdjian & Guern 1989). The low pH in the vacuole is required to support the activity of acid hydrolases for the degradation of macromolecules, and the generated proton motive force drives the coupled transport of small molecules into the lumen (Stevens & Forgac 1997; Forgac 1999). In yeasts, the vacuole is responsible for a variety of physiological processes including pH regulation, ion regulation and storage, amino acid storage, and metal detoxification (Graham et al. 2003). For vacuolated plant cells the ion storage in vacuoles plays an important role, and proton translocation is proposed via vacuolar ATPases and PPases and an exchanging antiport of protons and Ca^{2+} cations (Hirschi et al. 2001). As plants are also able to store nitrate up to 50 mM in their vacuoles (van der Leij et al. 1998; Crawford & Glass 1998), it seems possible that comparable transport systems are responsible for the accumulation of high nitrate concentrations, presumably within the vacuoles of large sulfur bacteria, against the concentration gradient. Nitrate and nitrite uptake in bacteria is known to be mediated by two different systems: ABC transporters, comparable to the mitochondrial respiratory chain, that are driven by ATP hydrolysis, and secondary transporters reliant on the proton motive force (Moir & Wood 2001). The latter transporter

types revealed protein homologues which are involved in nitrate and nitrite transport mechanisms. A nitrate/proton symport or a nitrate/hydroxide antiport was suggested by Moir and Wood for *Escherichia coli* (Moir & Wood 2001). A recent genome study of *Beggiatoa* filaments proposed that the vacuolar nitrate uptake and accumulation is linked to proton translocation and pH gradient generated by vacuolar-type ATPases and PPases (Mußmann et al. 2007) (Fig. 6). The resulting energy can be used to exchange the accumulated protons in the vacuole and nitrate in the cytoplasm, similar to the widely distributed H^+/Cl^- antiporter systems (De Angeli et al. 2006).

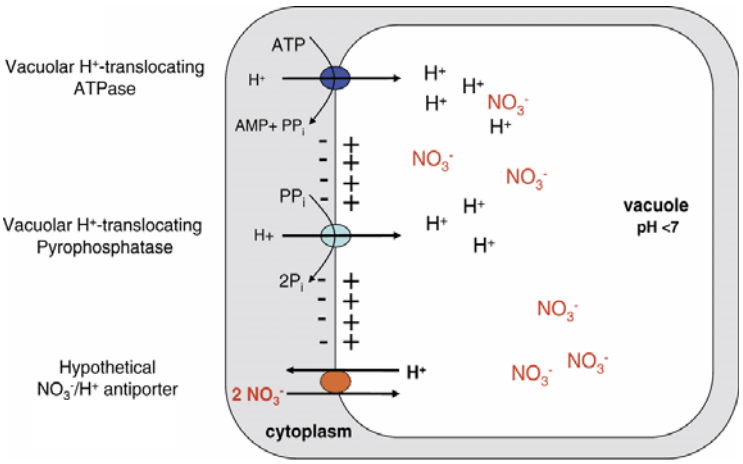

Figure 6: Modified after Mußmann et al. (2007): Hypothetical model of nitrate accumulation in the vacuole of Beggiatoa.

The presence of an electric potential and a corresponding acidic pH within the vacuole was detected in preliminary pH measurements and by a fluorescent cation stain for *Beggiatoa* sp. and *Thiomargarita namibiensis* (Mußmann et al. 2007). The membrane-bound nitrate reductase receives electrons from an ubiquinol pool in an electron transfer process that is coupled to the generation of a transmembrane proton electrochemical gradient (Berks et al. 1995). This correlation underlines the connection of transmembrane ABC transporters for nitrate respiration and the generated proton gradient of the vacuole membrane. Additionally to this respiratory membrane-bound enzyme, a periplasmic dissimilatory nitrate reductase was characterized for Proteobacteria (Bru et al. 2007). Whereas the membrane-bound nitrate reductase is expressed only under anaerobic growth conditions, the periplasmic nitrate reductase is also active in the presence of oxygen and thought to function as a switch in the transition between aerobiosis and anaerobiosis (Zumft 1997; Bru et al. 2007). Further investigation is needed to clarify the role of the acidic vacuole and the corresponding transport systems of the tonoplast.

Phylogeny of *Beggiatoa*

Beggiatoa filaments had originally been identified as unpigmented cyanobacteria "*Oscillatoria alba*" (Vaucher 1803), but despite some morphological resemblances none of the *Beggiatoa* species is phylogenetically related to cyanobacteria (Reichenbach et al. 1986; Stahl et al. 1987). The gliding motility and other morphological similarities, i.e. differentiation of the terminal cells of the filament and intimate connections between cells within the trichome led to this assumption (Pringsheim 1949). Properties such as the absence of pigments, internal sulfur and nitrate storage, extensive vacuolated forms, and sulfide oxidation capabilities discriminated *Beggiatoa* species from cyanobacteria. Gliding motility was then considered as an invaluable taxonomic marker (Strohl 2005). Nevertheless, a recent genome analysis of *Beggiatoa* revealed high similarities between gene fragments of *Beggiatoa* and of some cyanobacterial species, i.e. filamentous *Nostoc*, gliding *Anabaena variabilis* and *Gloeobacter violaceus*, suggesting an extensive horizontal gene exchange between (filamentous) cyanobacteria and *Beggiatoa* (Mußmann et al. 2007).

The formation of a robust polysaccharide sheath around aggregated filaments is the defining, but phylogenetically weak characteristic of *Thioploca*, which distinguishes them from *Beggiatoa* filaments (Teske et al. 1999; Ahmad et al. 1999). This morphological feature didn't match the 16S rRNA gene sequence-based phylogeny, since large, vacuolated, nitrate-storing, marine, autotrophic *Beggiatoa* are more closely related to both large, highly-vacuolated, nitrate-accumulating, autotrophic marine and narrow, non- or moderate vacuolated, freshwater or brackish *Thioploca* species, rather than to narrow freshwater, heterotrophic *Beggiatoa* strains (Strohl 2005). The sheath formation could be an adaptive feature depending on micro-environmental conditions, since individual *Beggiatoa*-like filaments can occur in *Thioploca* mats and diameters often matched between these two genera (Schulz et al. 1996).

The classification of individual *Beggiaota* species on the basis of filament diameters has been shown to be a conservative character, but also revealed higher genetic diversities than the morphologically descriptions would suggest, i.e. natural populations occurred in overlapping filament diameters (Jørgensen 1977; Mußmann et al. 2003), similarily to *Thioploca* trichomes, where several diameters can coexist in the same sheath (Teske et al. 1996). Additionally, the genome analysis of two phylogenetically different *Beggiatoa* species with similar diameters of 30 µm pointed to the potential genomic microdiversity among closely affiliated filaments (Mußmann et al. 2007). The genome size of these marine *Beggiatoa* strains was estimated to more than 7.6 Mb, whereas the genomes size of *Beggiatoa alba* was determined with 3 M base pairs (Genthner et al. 1985). Another molecular

characterization is reflected in the GC content, which can differ between organisms of the same phenotype, and can indicate a genetically distinct relationship (Strohl 2005).

Regarding vacuole formation of large *Beggiatoa* species and the concomitant ability to store nitrate intracellularly, these morphological and physiological features seemed to map onto the 16S rRNA data as it places the highly vacuolated forms of *Beggiatoa* into a monophyletic cluster, separated from the smaller, non-vacuolated, autotrophic and heterotrophic species (Teske & Nelson 2006).

The metabolic spectrum between members of the genus *Beggiatoa* is reflected in a diverse carbon metabolism. This phenotypic diversity can even be observed at species level, i.e. related filaments of the same origin being either a facultative (MS-81-1c) or obligate autotroph (MS-81-6), and is revealing the ecological and evolutionary diversification (Hagen & Nelson 1996). Nevertheless, three morphologically and physiologically different groups originating from various habitats can be distinguished (Teske & Nelson 2006): 1) Narrow (non-vacuolated) heterotrophic filaments from freshwater origins; 2) Narrow (non-vacuolated) facultative and obligate autotrophic *Beggiatoa* from marine environments (Nelson et al. 1986b; Hagen & Nelson 1996) and 3) Large (vacuolated) autotrophic marine strains (Nelson et al. 1989; McHatton et al. 1996; Gallardo & Espinoza 2007). Generally, a predominance of heterotrophic growth is given for freshwater filaments, and autotrophic metabolism for the marine *Beggiatoa* strains, discriminating also between the preferred habitats. Only recently a freshwater isolate has been shown to be capable of lithoautotrophic metabolism (Grabovich et al. 2001). The metabolic versatility of freshwater and marine strains (MS-81-6) could reflect a potentially low and/or variable supply of sulfide in these environments and the need for supplementary growth on organic carbon (Gray & Head 2005).

Habitats of *Beggiatoa*

Beggiatoa spp. are known to occur worldwide in diverse habitats with wide salinity ranges, on freshwater, brackish, marine and hypersaline sediments (Jørgensen 1977; Sweerts et al. 1990; Garcia-Pichel et al. 1994; Sayama 2001). Ecological niches in freshwater habits can be sulfur springs, streams, organic-rich wetlands, lake sediments and rice paddies (Scotten & Stokes 1962; Joshi & Hollis 1977; Strohl & Larkin 1978b; Fukui et al. 1999; Engel et al. 2004). In the brackish and marine environments, *Beggiatoa* can be found in an even wider variety of habitats, i.e. organic-rich coastal sediments, eutrophic and oxygen-depleted basins, upwelling regions, caves, hydrothermal vents, cold sulfide or hydrocarbon seeps, gas hydrates, black band disease of corals and whale falls (Jannasch et al. 1989; Nelson et al. 1989; Sassen et al. 1993; Larkin et al. 1994; Kuever et al. 1996; Richardson 1996; Deming et al. 1997; Mattison

et al. 1998; Graco et al. 2001; Sahling et al. 2002; Brüchert et al. 2003; Mußmann et al. 2003; De Beer et al. 2006; Macalady et al. 2008). As marine and hypersaline habitats, saltmarshes and phototrophic microbial mats are known (Nelson et al. 1982; Garcia-Pichel et al. 1994; Hinck et al. 2007).

Specific physiological characteristics define the ecological niche of *Beggiatoa* filaments. They are oligotrophic, respiratory organisms, which oxidize organic acids (heterotrophic strains) and/or sulfide (autotrophic strains) by the reduction of oxygen or nitrate (Nelson & Castenholz 1981b; Hagen & Nelson 1996, Hagen & Nelson 1997; McHatton et al. 1996). As microaerophilic organisms they display phobic responses to oxygen concentrations above 4% air saturation (Jørgensen & Revsbech 1983; Møller et al. 1985), but also negative chemotactic reactions to sulfide have been observed (Jørgensen & Postgate 1982; Preisler et al. 2007). These features characterize *Beggiatoa* as gradient organisms and enable them to track the interface of their substrates, electron donor (sulfide and other reduced sulfur compounds) and electron acceptor (oxygen or nitrate). Photophobic responses guide the filaments through microbial mats, when they need to escape from the mat surface through a developing oxygen peak, produced by phototrophs (Nelson & Castenholz 1982; Møller et al. 1985). Small, non-vacuolated *Beggiatoa* species rely on the simultaneous presence of reductant and oxidant in steep opposing gradients, whereas large, nitrate accumulating filaments can endure alternating exposures of sulfide and oxygen or nitrate (Gundersen et al. 1992; Jørgensen & Revsbech 1983; Nelson et al. 1986b). If occurring in high biomasses, filaments can form visible white mats on top of the sediment, the mats being either thin and compact or more diffuse and thick, and encompassing 30 to 60 cm thick layers, associated with tube worms at marine hydrothermal vents (Nelson et al. 1989; McHatton et al. 1996; Gundersen et al. 1992; Jørgensen & Revsbech 1983). Thick monocultures of *Beggiaota* mats in nature can separate sulfide from oxidant, compete with the chemical oxidation of sulfide, create their own microoxic niche and linking the biochemical cycles of the sulfur and nitrogen (Sayama et al. 2005; Teske & Nelson 2006). *Beggiatoa* filaments can also be dispersed within a suboxic zone, the main biomasses located deeper in the sediment and only a small fraction represented as sediment cover (Preisler et al. 2007). In photosynthetic microbial mats filaments often are not detectable on the surface, but constitute a part of the mat matrix and follow to a certain degree the diel cycling of the oxic-anoxic interface (Garcia-Pichel et al. 1994; Hinck et al. 2007).

Microbial mats in hypersaline environments

The term microbial mat is also used for benthic microbial communities that are so called "monocultures" of i.e. *Beggiatoa* or *Thioploca* filaments, masses of filaments in and on soft, organic-rich sediments (Teske & Nelson 2006). *Beggiatoa* can occur in association with other filamentous and non-filamentous bacteria and form complex microbial mats as discussed in the following section. Phototrophic or photosynthetic microbial mats are dense, cohesive communities with a visible laminated pattern due to the vertical zonation of diverse metabolic groups with different pigmentations, growing typically at the sediment-water interface. The mat-inhabiting microorganisms excrete extracellular polysaccacharides (EPS) which form a polymer matrix embedding the bacteria and contributing to the stabilization of unconsolidated sediments (Pierson & Castenholz 1992). These benthic organo-sedimentary mats can reach extensions from very few square centimeters (thermal springs) to several square kilometers (hypersaline mats, intertidal flats) and thickness of more than one meter (Des Marais et al. 1992; Stal & Caumette 1994). Microbial mats are considered the oldest structured and smallest ecosystems in the world, as they represent modern analogues of ancient 3 – 3.5 billion years old stromatolites, laminated and lithified carbonate build-ups preserving evidence of Earth's earliest biosphere (van Gemerden 1993; Karsten & Kühl 1996; Reid et al. 2000). Few microbial mats still form stromatolites that are metabolically active (Laval et al. 2000; Reid et al. 2000). Studies of the latter and modern microbial mat communities may provide insights into early stages of life and their environment in ancient atmospheres and oceans (Castenholz 1994; Des Marais 1995). Microbial mats are distributed all over the world in a wide range of environments, including marine sediments and intertidal flats, marine salterns, thermal springs, hypersaline lakes and lagoons, ice-covered Antartic lakes, hot and dry deserts, deep sea hydrothermal vents, alkaline lakes, freshwater lakes and streams (Pierson & Castenholz 1992; D'Antoni D'Amelio et al. 1989). Most locations are characterized by extreme conditions of temperature, salinity, moisture or pH, being responsible for the absence of efficient grazers and enhancing the development of cyanobacterial mats (Cohen & Rosenberg 1989; Farmer et al. 1992; Castenholz 1994).

The term cyanobacterial mat indicates the substantial role of filamentous cyanobacteria in the development of a microbial mat. The mats are inhabited by diverse functional groups of microorganisms, such as cyanobacteria and diatoms as oxygenic phototrophs, chemolithotrophic colorless sulfur bacteria, anaerobic phototrophic purple sulfur bacteria, anaerobic sulfate-reducing bacteria and aerobic heterotrophs, which are positioned in a typical zonation pattern (van Gemerden 1993). Numerically less important groups are the nitrifying and denitrifying bacteria and methanogenic bacteria. Cyanobacteria play a key role

in most microbial mats as pioneer species and often represent the dominant phototrophic group, providing physical strength and growth substrates for other mat organisms (Krumbein 1977; Jørgensen et al. 1983; D'Antoni D'Amelio et al. 1989; Des Marais et al. 1992; van Gemerden 1993; Stal 2000).

Hypersaline microbial mats can be found in sheltered costal lagoons, tidal channels, salterns, evaporation ponds and inland lakes. Best studies examples of hypersaline mats are those from the salt ponds in Guerrero Negro, Baja California Sur, Mexico and the Solar Lake, Sinai, Egypt (D'Antoni D'Amelio et al. 1989; Des Marais et al. 1992; Stal 2000). The only permanent hypersaline natural inland lake "La Salada de Chiprana" in Western Europe, Spain, gave reason for numerous publications on the structure and function of the inhabiting microbial mats (Vidondo et al. 1993; Diaz et al. 1998; Valero-Garces et al. 2000; Vila et al. 2002; Jonkers et al. 2003; De Wit et al. 2005; Ludwig et al. 2005; Bachar et al. 2007; Hinck et al. 2007). Steep physico-chemical gradients, i.e. of light, oxygen, sulfide, carbon dioxide and pH, have been observed within a few millimeters of the upper hypersaline mat due to high metabolic activities of the densely packed microorganisms (Revsbech et al. 1983; Des Marais 1995). The steep, small scale gradients in these cohesive ecosystems make diffusion the predominant mode of solute transport within the mat and its environment (van Gemerden 1993). The mat overlying diffusive boundary layer constitutes a physical barrier, inducing adaptational behavior such as chemotaxis and mobility of diatoms and cyanobacteria living in the uppermost layers of the mat (Karsten & Kühl 1996). Oxygenic photosynthesis by cyanobacteria and diatoms is the driving force of hypersaline mats producing an oxygen supersaturation in the top mat layers (van Gemerden 1993). Cyanobacteria are also capable of sulfide-dependent anoxygenic photosynthesis and fermentation as a further adaptation to the changing environmental conditions (Cohen et al. 1986; De Wit & Van Gemerden 1987). The carbon fixed by the primary producers, cyanobacteria and diatoms, is decomposed by a variety of chemotrophic organisms (Stal 2000). Also nitrogen compounds are released into the mats during theses processes, particularly if the decomposed species were diazotrophs, able to fix atmospheric nitrogen. Under oxic conditions the excreted and decomposed carbon is respired by heterotrophic bacteria, under anoxic conditions the organic substrates are fermented by heterotrophs and cyanobacteria. The fermentation products serve as substrates for sulfate-reducing bacteria. Dissimilatory sulfate reduction is the dominant anaerobic process of carbon mineralization in hypersaline mats (Jørgensen et al. 1992; Canfield & des Marais 1993). The sulfide produced is used for the reduction of carbon dioxide to cellular carbon by anoxygenic photosynthesis of anaerobic purple sulfur bacteria which are often seen as a pink layer below the cyanobacteria. Green sulfur bacteria, as obligate anaerobic

organisms, grow underneath the population of purple sulfur bacteria (Stal 2000). The deeper layers of hypersaline microbial mats often appear black or grey, due to the presence of iron sulfide (FeS) or pyrite (FeS_2). Sulfate reducing bacteria were thought to inhabit this anoxic layer, but recent research showed abundances and activities even in the oxic top mat layers (Canfield & des Marais 1991; Stal 2000; Jonkers et al. 2005). Denitrifiation, fermentation and methanogenesis are still considered to be confined to the anoxic zone of the microbial mats, while aerobic heterotrophs and nitrifiers are active in the oxic part of the mat (Fig. 7).

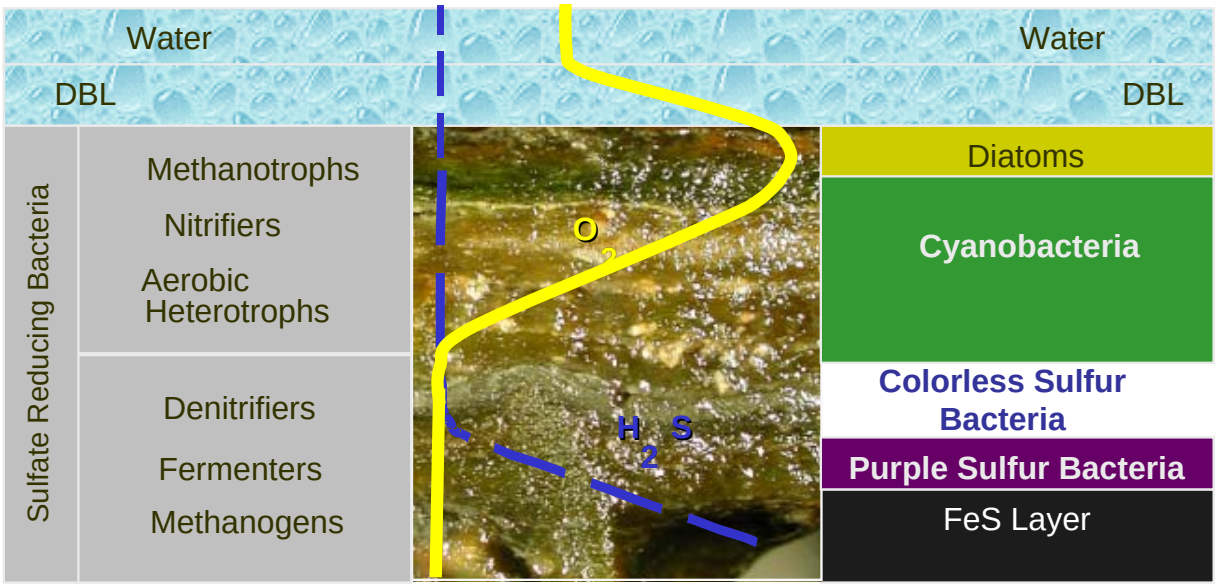

Figure 7: Modified after van Gemerden (1993) and Karsten & Kühl (1996): Simplified scheme of the orientation of microbial mat organisms in relation to O_2 and H_2S gradients in the light. The visible lamination of organisms is depicted on the right; the orientation of organisms that do not contribute to the visible layering is shown on the left.

Colorless sulfur bacteria as chemolithoautotrophs utilize sulfide both as energy source and as electron donor for carbon dioxide reduction. Sulfide gets oxidized by either using oxygen or nitrate as electron acceptor. Their presence in microbial mats and the high affinities for their substrates outcompetes the chemical oxidation of sulfide and causes a highly dynamic oxygen-sulfide interface (Revsbech et al. 1983). *Beggiatoa* filaments can be found at a narrow layer of the oxic/anoxic interface, and migrate with the fluctuating diel up-and-down movement of this interface zone (Garcia-Pichel et al. 1994). The competition for reduced inorganic sulfur compounds of colorless sulfur bacteria and purple sulfur bacteria is regulated by the availability of oxygen and light (Jørgensen & Des Marais 1986a). Both oxygen and sulfur cycle are strongly regulated by the natural light regime. During illumination, oxygen supersaturation occurs in the top mat layers, shifting the oxygen/sulfide interface from the surface to greater depths within the mat. During darkness, oxygen consumption by respiration

and sulfide oxidation results in oxygen depletion, whereas sulfide accumulates and rises to the mat surface (Jørgensen et al. 1979; Jørgensen et al. 1983; Revsbech et al. 1983). If the interface of oxygen and sulfide remains outside the photic zone, purple sulfur bacteria are excluded and *Beggiatoa* spp. colonize this physiological niche (Jørgensen & Des Marais 1986a). However, low oxygen concentrations at the interface can lead to incomplete sulfide oxidation and favor the coexistence of both groups (van Gemerden 1993; van den Ende et al. 1996). The migration of *Beggiatoa* filaments is not only regulated by the combined effects of oxygen and sulfide, but also controlled by light as already referred in the physiology and chemotaxis section. Photophobic responses to light intensities above 2% of summer sunlight and wavelengths between 400-500 nm prevent the filaments to get lost at the mat surface when initial morning light induces a rapid oxygen build-up (Nelson & Castenholz 1982; Schulz & Jørgensen 2001). Metabolic versatility, sulfide tolerance, UV resistance and motility are necessary physiological and behavioral adaptations of all mat-inhabiting organisms. Due to high mineralization rates, the annual biomass accretion in microbial mats is low. Complex interactions between the different physiological groups of microorganisms cause almost closed substrate cycles (Des Marais 2003) (Fig. 8).

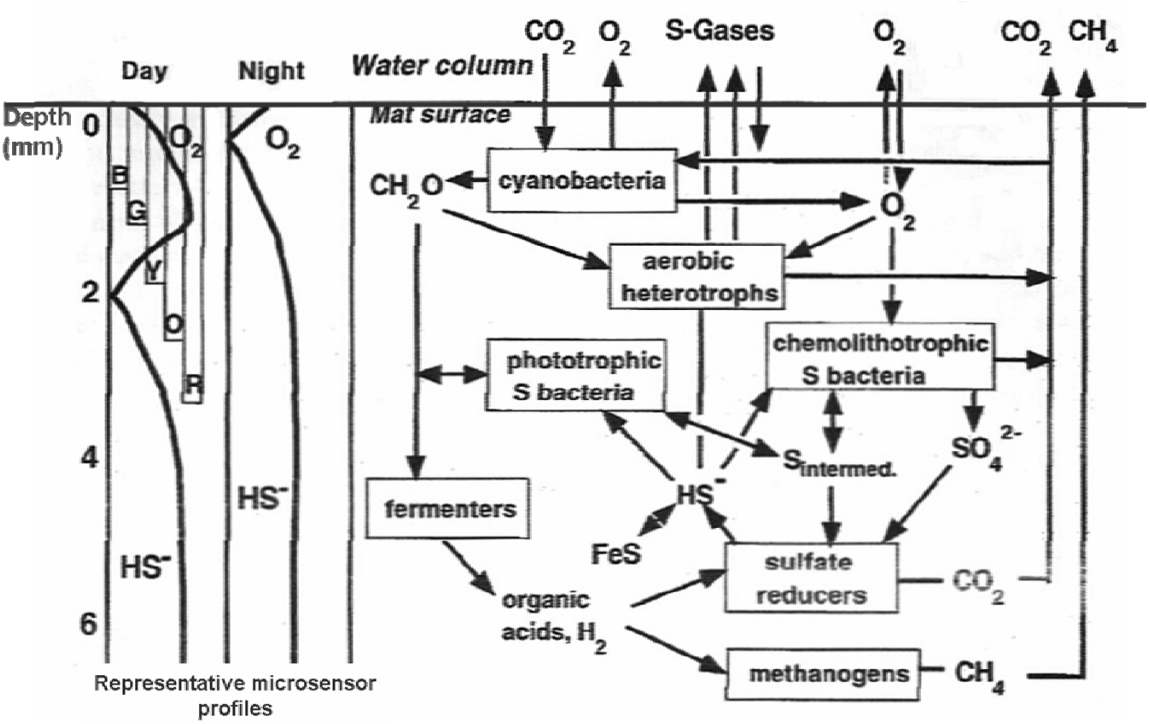

Figure 8: Modified after Des Marais (2003): Flow diagram of a cyanobacterial microbial mat with depth-related light and chemical gradients. The vertical bars on the left represent the relative depth penetration of blue (B), green (G), yellow(Y), orange (O), and red (R) light.

The reduction of sulfate and the oxidation of sulfide are decisive processes of the sulfur cycle while the carbon cycle is dominated by photosynthesis and respiration processes. The

nitrogen cycle within mats can include all steps of oxidation stages of the nitrogen cycle, with the nitrogen-fixing organisms as key players (van Gemerden 1993; Stal 2000). Anaerobic ammonia oxidation (anammox) is ubiquitous for marine sediments, sediments colonized by microalgae were found to be unable to sustain anammox activity due to periodically occurring N-limitations (Risgaard-Petersen et al. 2005; Revsbech et al. 2006). *Beggiatoa* depend on the temporal availability of nitrate as an alternative electron acceptor for sulfide oxidation if distributed in the suboxic zone of the mat (Hinck et al. 2007). The habitats that host microbial mats are typically oligotrophic (Javor 1983), yet microbial mats are highly productive aquatic ecosystems and reflect the efficient recycling of key nutrients within the mat (Des Marais 2003). Bacterial production of low molecular weight nitrogen and sulfur compounds is important as these represent the center of energy and electron flow in anaerobic ecosystems. Mostly non-heterocystous cyanobacteria occur in microbial mats, nitrogen fixiation depending mainly on oxygen avoidance or temporal separation from oxygenic photosynthesis, since oxygen exerts a negative effect on the nitrogenase (Stal 2000). Highest nitrogenase activities were obtained during sunrise and sunset, in the lowest layer of the cyanobacterial mat (2-3 mm). The required high energy and the need for low potential reducing equivalents to fix nitrogen are provided below the top layer, where oxygenic photosynthesis and carbon dioxide fixation occurs. The degradation of organic matter deposited at the surface is the driving force for benthic nitrogen cycling (Herbert 1999), and 99% of the organic matter is recycled in the mat (Krumbein 1977). The by ammonification liberated ammonium gets assimilated during the day by phototrophs in the upper mat layers, during the night it is conserved in these photic layers, reflecting a high production rate (Jørgensen et al. 1979). Nitrification of ammonium to nitrite and further to nitrate could be in fact spatially separated, with ammonia oxidizers in the photic/oxic zone and nitrite oxidizers in the suboxic zone, but the mechanism of nitrification in suboxic zones is not clarified yet (Revsbech et al. 2006). Nitrification is also depending on diurnal variations as the enzyme of the first nitrification step is inhibited by wavelengths < 480 nm (Guerrero & Jones 1996). Nevertheless, nitrification is considered the main source of nitrate available in microbial mats (Bonin & Michotey 2006). Nitrate is consumed in the photic zone due to uptake mechanisms, but below this layer and above the oxic/anoxic interface a maximum in nitrate production was observed (Francis et al. 2007) (Fig. 9). In the anoxic layer at a depth of 6 mm, nitrate decreased due to respiratory processes, the mat location where main abundances of *Beggiatoa* filaments were detectable during darkness (Hinck et al. 2007).

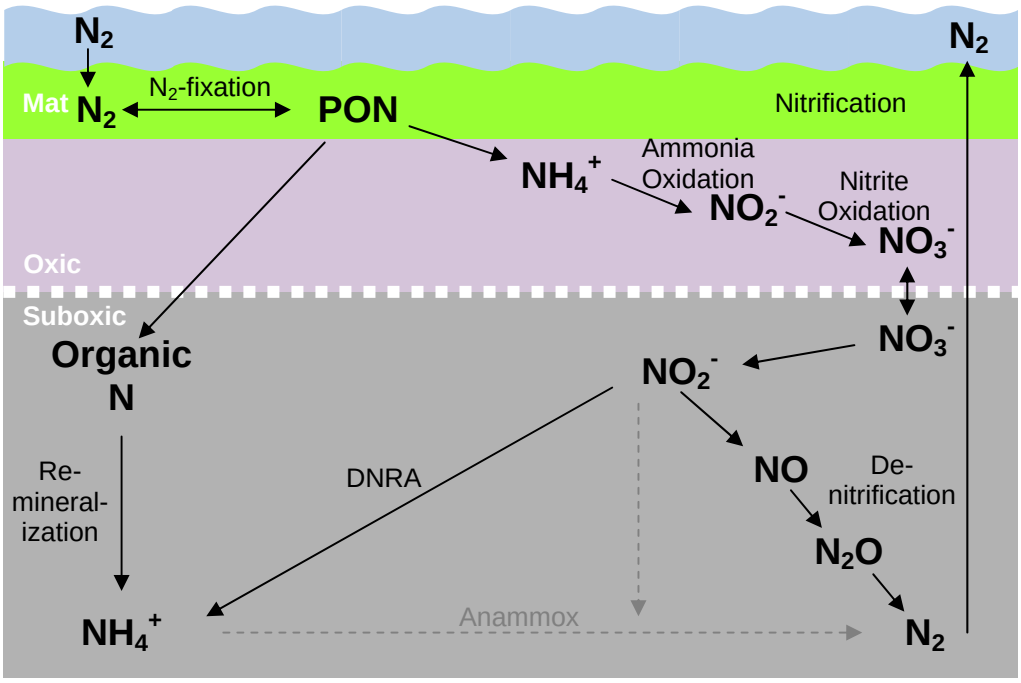

Figure 9: Modified after Francis et al. (2007) and Stal (2000): Microbial nitrogen transformations above, below and across an oxic/suboxic interface in a cyanobacterial mat. Nitrite plays a central role as metabolic intermediate/product within and between N-cycling pathways.

Denitrification as an anaerobic process would lead to a major loss of nitrogen to the atmosphere, in microbial mats during spring and summer high rates of 25-50% of produced nitrate was denitrified (Bonin & Michotey 2006). Nevertheless, the uptake of nitrate in certain mat layers as well as the varying sulfide concentrations limits the denitrification rates (Brunet & Garcia-Gil 1996). The dissimilatory reduction of nitrate to ammonium (DNRA) may be favored in environments where nitrate–availability is variable because also sulfate-reducing bacteria have DNRA capacity as secondary metabolism (Jørgensen & Revsbech 1989; Rysgaard et al. 1996). Sayama et al. (2005) observed that *Beggiatoa* separated the oxic, nitrate containing surface layers from the deeper sulfidic layers, and showed that DNRA could only be attributed to the presence of *Beggiatoa* filaments. Therefore, *Beggiatoa* could have an important impact on the nitrogen cycle if present in high biomasses in sulfidic environments, as the preferred process of DNRA would result in greater nitrogen retention in the system. Effective recycling of nitrogen can lead to enhanced sulfate regeneration, and consequently, carbon mineralization may also be increased (Gray & Head 2005).

Microsensor measurements

The application of microsensors in the early 1980's advanced the understanding of microbial mats, because it enabled fine-sale measurements and quantification of the steep physico-chemical gradients created by limited diffusion transport and high metabolic activities of

these systems. Microsensors have been useful in the characterization of diverse microenvironments, as they allow simultaneous measurements of several chemical or physical parameters with high spatial resolution and negligible disturbance of the sample (Revsbech & Jørgensen 1986; Amann & Kühl 1998; Kühl & Revsbech 2001). Due to their small and fragile tip sizes (typically smaller than 20 µm) a micromanipulator is needed for small scale positioning *In situ*. Microsensors used in environmental studies can be divided into three groups:

1) Electrochemical sensors (microelectrodes) which convert a chemical into an electrical signal, 2) optical sensors (optodes) which are based on measuring optical property changes (light signals) when the analyte interacts with an indicator and 3) bio-microsensors where cultures of microorganisms or enzymes are immobilized behind a membrane and convert the analyte into a product that can be measured by an associated electrochemical sensor (Glud et al. 1999; Kühl & Revsbech 2001). A large number of sensors and measurable parameters exist, i.e. O_2, H_2, H_2S, CO_2, Ca^{2+}, pH, NO_2^-, NO_x, Fe^{2+}, temperature, glucose, diffusivity, DOC, CH_4 and irradiance (for more information the reader is referred to Kühl & Revsbech (2001) or Gieseke & de Beer (2004)). Depending on the measurement principle, three different types of microelectrodes can be distinguished: potentiometric, amperometric and voltametric sensors (Taillefert et al. 2000; Kühl & Revsbech 2001). In the present study microelectrodes of the amperometric, i.e. oxygen and sulfide, and potentiometric type, i.e. pH, have been used as well as optical microsensors.

Optical microsensors are based on the collection and direction of light signals through a single-stranded optical fiber between the tip and the electronical measurement device. Sensors of this type sense light from the tip surroundings (Holst et al. 2000). The used optic fiber light microsensor had tip diameters between 20 and 30 µm and the tip was rounded (Jørgensen & Des Marais 1986b). These simple tapered optical fibers collect light from a certain angle and have detection limits for UVB-NIR light lower than 1 µmol $m^{-2}s^{-1}$. They have been applied to measure microscale spectral light distributions in sediments and biofilms, in order to study zonations of phototrophic microorganisms (Kühl & Revsbech 2001).

The two amperometric microsensors widely used are the oxygen sensor, which is a miniaturized Clark-type oxygen electrode, and the sulfide, H_2S, electrode. A Clark-type sensor has a gold-coated cathode situated behind a silicon membrane and is immersed in an electrolyte solution (Clark et al. 1953) (Fig. 10-I). The reduction of oxygen at the cathode is transferred into a current being linearly proportional to the concentration of the analyte and being measured by sensitive amplifiers (Picoampermeters). The zero current was minimized by introducing a guard cathode, which consumes oxygen present in the electrolyte (Revsbech

1989). With tip sizes of 1-100 µm and detection limits of 0.1 µM the sensor is very stable, insensitive to calcium and magnesium ions and pH independent. The oxygen sensor can last for months.

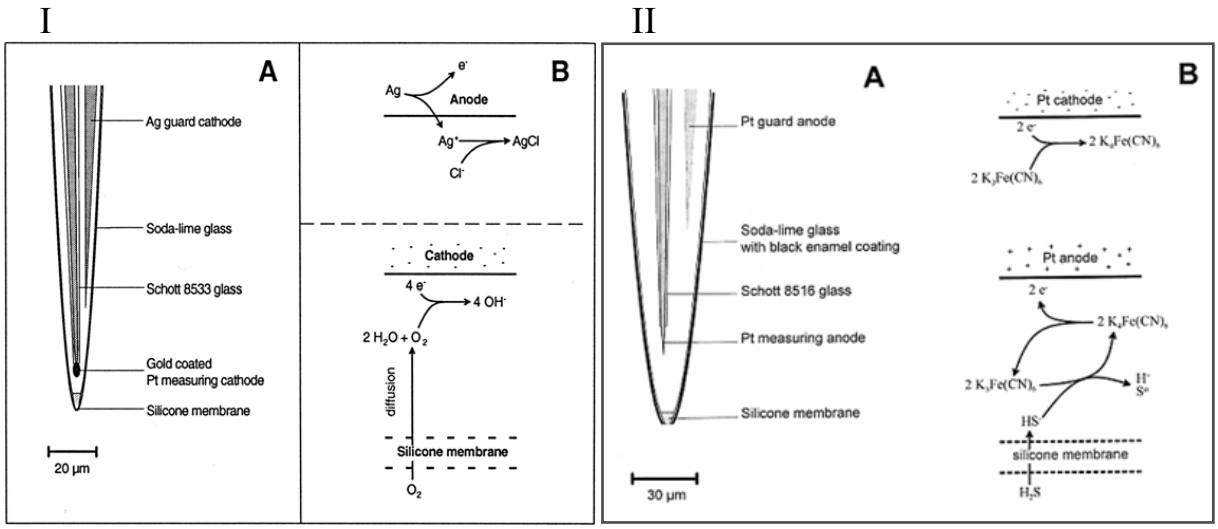

Figure 10: After Kühl & Revsbech (2001): (I) O_2 microsensor, (II) H_2S microsensor
(A) Detailed drawing of microsensor tip. (B) Measuring principle and reactions.

H_2S microelectrodes are also Clark-type electrodes with a platinum anode behind a silicone membrane. It contains ferricyanide as redox mediator, which is reduced by H_2S to ferrocyanide and then reoxidized at a polarizing voltage of 0.08 V (Jeroschewski et al. 1996; Kühl et al. 1998) (Fig. 10-II). The lifetime of the sensor is relatively short (weeks) and its sensitivity to light in the UV region is hampering in situ measurements at high irradiances. It has tip diameters between 10-100 µm and a detection limit of 1 µM H_2S. Since the dissociation of H_2S depends on the pH, pH needs to be recorded simultaneously to allow calculations of the total dissolved sulfide concentration.

In potentiometric microelectrodes, a potential difference proportional to the analyte concentration is created by concentration differences of ions between the analyte and the electrolyte over an ion-permeable membrane. These membranes can include ion-specific glass (glass pH electrodes) or are comprised of a liquid membrane in LIX sensors (LIX = liquid ion exchangeable membrane). Whereas the glass pH electrodes has a long lifetime (weeks-months) but due to large tip sizes (20-200 µm) limited spatial resolutions, the LIX microsensors (Hinke 1969) are easy to build but feature short lifetimes (days) (De Beer 2000). The gradients in microbial mats which can thus be measured with microsensor techniques are the result of intense microbial activity and solute diffusion. Although bioirrigation in hypersaline microbial mats is insignificant, as higher organisms are mostly excluded, these systems are very heterogeneous. Nevertheless, microsensors are a useful tool to obtain the *In*

situ microbial activity. The linear concentration gradient of a solute in the diffusive boundary layer (DBL) can be used to calculate diffusive solute production or uptake rates in diffusion dominated systems using Fick´s 1st law (Jørgensen & Revsbech 1985; Kühl et al. 1996). Conversion rates can be obtained by measuring concentration changes of chemicals involved in the reactions. From oxygen profiles in the light, the net oxygen production can be evaluated by calculating downward and upward fluxes from the photosynthetic active layer. From measured concentration profiles, process rates and the depth distribution of processes can be estimated by application of diffusion-reaction models, which requires the knowledge about the solute diffusivity in the system (Revsbech & Jørgensen 1986; Epping & Jørgensen 1996; Berg et al. 1998).

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Aims of the thesis

This work has been carried out to investigate the physiology (function) and phylogeny (structure) of filamentous sulfur bacteria originating from hypersaline phototrophic microbial mats, using a set of *In situ* and laboratory techniques. Main subject of this study was to unravel the *In situ* diel migration patterns of *Beggiatoa* in relation to changing pore water components. Laboratory applications with selected pore water components (e.g. oxygen, nitrate, sulfide) were analyzed with respect to the resulting specific motility behavior of the filaments, and conclusions were drawn concerning their *In situ* behavior. Phylogenetic relationships of hypersaline *Beggiatoa* from different origins to other filamentous sulfur bacteria were obtained, and morphological features such as intracellular vacuoles were visualized by diverse fluorochrome and confocal laser scanning microscopic techniques.

In the first manuscript (Hinck et al. 2007, chapter 2 of this thesis) the behavioral pattern of *Beggiatoa* from a natural permanent hypersaline inland lake were investigated and compared to published data of *Beggiatoa* migratory behavior in other environments. Measurements of pore water components, intracellular nitrate and elemental sulfur concentrations revealed a predominantly sulfide concentration-dependent migration behavior. Highest filament abundances were found at the zone which features during the diel cycle highly dynamic oxygen and sulfide concentrations. Obtained results suggested a behavioral strategy similar to *Beggiatoa* filaments occurring in the suboxic zone of sulfidic deep sea sediments. Both perform sulfide oxidation with oxygen or nitrate, and storage nitrate in intracellular vacuoles during periods when both components are available (day-light period in phototrophic mats). Stored nitrate can than be used for anaerobic sulfide oxidation during periods of oxygen absence (dark period in phototrophic mats). The most dominant morphotype occurring in the investigated hypersaline mat (6-8 µm filament diameter) was analyzed with respect to morphological characteristics (presence of vacuoles) and phylogenetic relation to other *Beggiatoa* strains. This study increased knowledge on the structure and function of hypersaline *Beggiatoa*.

The second manuscript (Beutler et al., chapter 3 of this thesis) describes a novel technique to measure pH within vacuoles and cytoplasm of living *Beggiatoa* filaments based on fluorescent properties of compound specific dyes. Estimation of pH can be made as it influences the fluorophore's lifetime. The value of the method was demonstrated by the simultaneous estimation of pH within vacuole and cytoplasm of living *Beggiatoa* filaments. The expected pH difference across the vacuole membrane was dissipated by nigericin, a Na^+/H^+ antiporter. The expectations of an acidic vacuole content, possibly in correlation with nitrate storage capacities, needs future verification.

The third manuscript (Hinck et al., chapter 4 of this thesis) represents a follow-up of the *Beggiatoa* migration behavior study described in chapter 2 where it was concluded that *In situ* migration was mainly controlled by the sulfide gradient, both under light and dark conditions. However, since several triggers interact (i.e. oxygen, sulfide, nitrate, pH, light) it appeared previously difficult to pinpoint the single most determining effect causing the observed distribution patterns of *Beggiatoa* under *In situ* conditions. Therefore in this study, two different sets of experiments were conducted to investigate which single factor most influences the migration behavior of *Beggiatoa*. Gradient agar tubes inoculated with several filaments were subjected to different key factors (oxygen, sulfide, nitrate, light), and the migration of filaments in relation to compound concentration was determined over a 5 week period and subsequently evaluated using variation partitioning statistical analysis. Secondly, the chemotactic response of single filaments to low and high sulfide concentrations was filmed in glass-capillaries. The interpretation of the statistics and single responses gave important information on an active chemotacic behavior of hypersaline *Beggiatoa* towards optimal sulfide concentrations, and confirmed sulfide to be the key trigger affecting *In situ* migration patterns.

The fourth manuscript (Hinck et al., chapter 5 of this thesis) deals with the clarification of phylogenetic relationships between the investigated hypersaline *Beggiatoa* of this PhD study, and those originating from other mat habitats. Additionally, the morphological and physiological characteristics of the various hypersaline *Beggiatoa* strains were compared with respect to filament diameter, vacuole presence, internal nitrate and sulfur storage, and salinity requirements. To date, few hypersaline *Beggiatoa* species have been well characterized, particularly due to difficulties in obtaining axenic cultures of these slow-growing organisms due to the often presence of firmly attached ‘satellite’ bacteria. The latter were also phylogenetically characterized in order to determine whether these represent a specific group of bacteria. Future studies must reveal whether the specifically developed FISH primers for the investigated hypersaline *Beggiatoa* of this study can discriminate suffienciently between hypersaline *Beggiatoa* species and other phylogenetically closely related larger marine vacuolated sulfur oxidizers.

Contributions to the manuscripts

The thesis comprises four manuscripts, presented here as chapters.

Chapter 2: Physiological Adaptation of a Nitrate-Storing *Beggiatoa* sp. to Diel Cycling in a Phototrophic Hypersaline Mat

by Susanne Hinck, Thomas R. Neu, Gaute Lavik, Marc Mußmann, Dirk de Beer and Henk M. Jonkers

The concept was developed by S. H., D. de B. and H. M. J.. S. H. measured and evaluated all data with contribution of G. L. and M.M.. T. R. N. carried out the confocal laser microscopy, with help of S. H.. S. H. wrote the manuscript with editorial help of all co-authors.

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Chapter 3: A method for the estimation of pH in life cells based on excited state saturation

by Martin Beutler, Susanne Hinck and Dirk de Beer

The study was initiated by M. B.. Experiments were carried out by M. B. and S. H., the data were analyzed by M. B. with contribution of S. H. and D. de B.. M. B. wrote the manuscript with editorial help of all co-authors.

The manuscript is submitted to Journal of Microbiological Methods.

Chapter 4: Locomotion mechanism and chemotactic behavior of hypersaline *Beggiatoa*

by Susanne Hinck, Alban Ramette, Thomas R. Neu, Martin Beutler, Melissa B. Duhaime, Dirk de Beer and Henk M. Jonkers

D. de B., H. M. J. and S. H. developed the concept of this study. S. H. measured and evaluated all data, with contribution of M. B. D.. A. R. and M. B. evaluated the data statistically and analyzed the movement of individual filaments in the capillaries with a computer programm, respectively. T. R. N. carried out the confocal laser microscopy, with help of S. H.. S. H. wrote the manuscript with editorial help of all co-authors.

This manuscript is submitted to Aquatic Microbial Ecology.

Chapter 5: Cultivation and identification of a monophyletic cluster of hypersaline *Beggiatoa* and their associated bacteria

by Susanne Hinck, Marc Mußmann, Thomas R. Neu, Sabine Lenk, Dirk de Beer and Henk M. Jonkers

S.H. and H. M. J. initiated this study, with contribution of M. M.. S. H. conducted the experiments and evaluated the data, except the following: The *Beggiatoa* concensus tree was calculated by M. M., FISH images were performed by S. L.. T. R. N. carried out the confocal laser microscopy, with help of S. H.. S. H. wrote the manuscript with editorial help of all co-authors.

This manuscript is in preparation for submission.

Chapter 2

Physiological Adaptation of a Nitrate-Storing *Beggiatoa* sp. to Diel Cycling in a Phototrophic Hypersaline Mat

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Physiological Adaptation of a Nitrate-Storing *Beggiatoa* sp. to Diel Cycling in a Phototrophic Hypersaline Mat▽

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The aim of this study was to investigate the supposed vertical diel migration and the accompanying physiology of *Beggiatoa* bacteria from hypersaline microbial mats. We combined microsensor, stable-isotope, and molecular techniques to clarify the phylogeny and physiology of the most dominant species inhabiting mats of the natural hypersaline Lake Chiprana, Spain. The most dominant morphotype had a filament diameter of 6 to 8 μm and a length varying from 1 to >10 mm. Phylogenetic analysis by 16S rRNA gene comparison revealed that this type appeared to be most closely related (91% sequence identity) to the narrow (4-μm diameter) nonvacuolated marine strain MS-81-6. Stable-isotope analysis showed that the Lake Chiprana species could store nitrate intracellularly to 40 mM. The presence of large intracellular vacuoles was confirmed by fluorescein isothiocyanate staining and subsequent confocal microscopy. In illuminated mats, their highest abundance was found at a depth of 8 mm, where oxygen and sulfide co-occurred. However, in the dark, the highest *Beggiatoa* densities occurred at 7 mm, and the whole population was present in the anoxic zone of the mat. Our findings suggest that hypersaline *Beggiatoa* bacteria oxidize sulfide with oxygen under light conditions and with internally stored nitrate under dark conditions. It was concluded that nitrate storage by *Beggiatoa* is an optimal strategy to both occupy the suboxic zones in sulfidic sediments and survive the dark periods in phototrophic mats.

Beggiatoa spp. are large, filamentous, gliding, colorless sulfur bacteria and are known to occur worldwide in diverse habitats with a wide range of salinities. They form visible white mats on the surfaces of organic-rich freshwater sediments (39, 46, 63, 64) and in sulfur springs (36), marine caves (37), marine eutrophic coastal zones (22, 58, 59), upwelling regions (7, 15), whale falls (11), cold seeps (10, 43, 48), and gas seeps (4, 28). These filamentous bacteria migrate in daily cycles in microbial mats (16, 23, 67). Microbial mats are dense, cohesive communities with a visible laminated pattern due to the zonation of different metabolic groups with different pigmentations. Within a few millimeters of the upper mat, steep physicochemical gradients occur due to high metabolic activities of the densely packed microorganisms. The main functional groups are cyanobacteria, purple sulfur bacteria, colorless sulfur bacteria, and sulfate-reducing bacteria (67). Most mats are characterized by a microbiologically controlled rapid sulfur cycle. In phototrophic mats, oxygenic photosynthesis and dissimilatory sulfate reduction result in opposing gradients of oxygen and sulfide, which sometimes overlap in a narrow transition zone. The sulfide and oxygen distributions underlie diel cycles (54), which induce migratory behavior of phototrophic and nonphototrophic organisms (8, 55). Gliding motility and a tactile response to diverse parameters, e.g., light, oxygen, and sulfide (41, 44, 45), enable *Beggiatoa* spp. to follow the movement of the dynamic transition zone in phototrophic mats. *Beggiatoa* spp. have been reported to exist at the oxic-anoxic interface in hypersaline microbial mats (16, 23), but none of these organisms have been characterized for phylogeny and function. The preference for a niche where oxygen and sulfide co-occur has been shown for both marine and freshwater *Beggiatoa* species. For some filaments, the capability of anaerobic sulfide oxidation with nitrate has been demonstrated, and the larger relatives can store nitrate, presumably in intracellular vacuoles (30, 47, 64, 65). Although narrow nonvacuolated *Beggiatoa* spp. proliferate in zones of <1 millimeter thick where both oxygen and sulfide co-occur (26, 45), wider, vacuolated forms of *Beggiatoa* and *Thioploca* are found in broader (up to 10 cm wide) suboxic zones where neither oxygen nor sulfide occurs (1). Especially larger filamentous species (9 to 375 μm in diameter) and other representatives of big colorless sulfur bacteria, such as *Thioploca* spp. and *Thiomargarita namibiensis*, possess internal vacuoles (24, 32, 38, 42, 60), which are presumably the main location of nitrate storage. These organisms can use internally stored nitrate as an electron acceptor for anaerobic sulfide oxidation in deep suboxic sediment layers (1, 38, 58). Therefore, it is thought that sulfur bacteria play an important role in and form a link between the sulfur and nitrogen cycles (7, 33, 40). In a recent study, 21 *Beggiatoa* spp. from various freshwater and marine habitats were characterized phylogenetically by 16S rRNA gene sequencing (2). Fourteen of the 21 studied *Beggiatoa* relatives are known to be

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vacuolated and able to store nitrate. Species from freshwater (5 species) and marine (16 species) habitats form phylogenetically separate subclusters, with vacuolated forms being restricted to a subcluster within the marine cluster of larger (>9 μm) *Beggiatoa* spp. Species with smaller filament diameters (1 to 6 μm) have not been shown to be vacuolated, including some *Beggiatoa* members originating from phototrophic microbial mats.

The purpose of our study was to understand the behavior and metabolism of currently uncultured hypersaline *Beggiatoa* spp. in intact microbial mats and, furthermore, to clarify their phylogenetic relationship to other filamentous sulfur bacteria. We characterized for the first time a hypersaline *Beggiatoa* sp. which originates from the natural hypersaline Lake Chiprana, Spain. The vertical distribution of this morphotype and its physiology in relation to environmental parameters were studied in intact mat samples by using direct methods, i.e., microsensor, stable-isotope, and microscopy techniques. In addition, the 16S rRNA gene of this species was sequenced. Morphology was documented in a detailed microscopic study, with emphasis on the presence of vacuoles for nitrate storage.

MATERIALS AND METHODS

Sampling site and mesocosm description. The permanent hypersaline natural inland Lake Chiprana in northeastern Spain (41°14′30″N, 0°10′50″W) has a total surface area of 31 ha and a maximum depth of 5.6 m. The lakebed is composed of tertiary paleochannels with fossilized sands and silt deposits (66). Through permanent groundwater inflow, the ionic composition of the lake water is dominated by magnesium sulfate (SO_4^{2-}, 0.5 mol liter^{-1}; Mg^{2+}, 0.35 mol liter^{-1}), and it has an average salinity of ~80‰ (21). Intact microbial mat samples (15 by 15 by 4 cm) were collected in September 2004 approximately 1 m from the shoreline at a 50-cm water depth and were transferred to Bremen, Germany, for further analysis and laboratory experiments. The mat samples were kept in the laboratory in an open plastic basin (1.2 by 1.2 by 0.5 m) with a continuous flowthrough of aerated artificial lake water (seawater plus 80 g/liter $MgSO_4 \cdot 7H_2O$). Illumination was set as close as possible to natural conditions, with a light intensity of 500 μmol photons m^{-2} s^{-1} and an illumination regimen of 14 h of light and 10 h of darkness. During the whole incubation period of 5 months, and specifically at the times that experiments with these laboratory-incubated mats were performed (after 3 months of mesocosm incubation), oxygen and sulfide profiles were measured for mat characterization to establish that major physiological processes, i.e., oxygen and sulfide production, persisted in the mat. Most *Beggiatoa* filaments were found in microbial mats inhabited by the charophyte *Lamprothamnium papulosum*. These mats were subsampled by coring, with a diameter of 5 cm and a thickness of 2 cm, for further detailed microsensor measurements.

Microsensor measurements of oxygen, sulfide, pH, and light. Subsamples of the laboratory-incubated mat were taken for respective experimental procedures, and these were subsequently characterized for oxygen and sulfide dynamics by microsensor analysis. These microbial mat subsamples were exposed to a 14-h light (500 μmol photons m^{-2} s^{-1})–10-h dark diel cycle at room temperature (20°C) within a small aquarium (15 by 15 by 5 cm). The overlying water had a similar salinity and composition to those of the original Lake Chiprana water and was aerated by an air jet over the water surface. The mats were illuminated by a fiber-optic halogen light source (KL 1500; Schott, Germany), and the irradiance at the mat surface was determined with an underwater scalar irradiance meter (LI-250; LiCor). To ensure steady-state conditions, microsensors for oxygen (O_2), sulfide (H_2S), and pH were applied at the end of each experimental light or dark setting. Since repeated profiles measured at the same spot varied <2% (results not shown), we assumed steady-state conditions. The mat profiles were taken by mounting all microsensors on a motorized micromanipulator (Oriel) stabilized by a heavy stand. Sensors were operated and their signals were recorded via a computer acquisition system (LabView; National Instruments). The sediment surface relative to the sensor tips was determined with the aid of a dissection microscope (SV6; Zeiss, Germany). Profiles of O_2, H_2S, and pH were recorded in steps, with a vertical depth resolution of 200 μm. The Clark-type O_2 microsensor (53) had a tip diameter of 15 μm and a response time of less than 3 s. It was two-point calibrated by using the signals in the overlying air-saturated brine (100% air saturation) and the anoxic zones of the mat (0%). Dissolved oxygen concentrations at a given salinity (80‰) and temperature (20°C) were determined by the method of Sherwood et al. (62). The pH glass-microelectrode (54) and the H_2S microsensor (20) were calibrated before their application to microbial mats. The pH microsensor had a tip diameter of ~15 μm and response times of less than 20 s and was calibrated with standard solutions (Mettler-Toledo, Switzerland). The H_2S microsensor had a tip diameter of 20 μm, was coated with black enamel paint to avoid light interference (34), and had response times of less than 20 s. It was calibrated in anoxic phosphate buffer (0.2 M; pH 8.2) by adding Na_2S from a stock solution, and exact concentrations were determined spectrophotometrically by the Pachmeyer method (52). Calculations of total sulfide derived from the local pH and H_2S concentration (20) were done according to the procedure described by Wieland and Kühl (68). Where mentioned below, total sulfide denotes the sum of H_2S, HS^-, and S^{2-}. Spectral light gradients in intact mat pieces illuminated with a light intensity of 500 μmol photons m^{-2} s^{-1} were measured using an optic fiber light microsensor (27) with a tip size of approximately 30 μm. The sensor was connected to a USB2000 Ocean Optics spectrometer. The measurements were started from below at a depth where no light penetration could be detected, and the sensor tip was moved upwards in steps of 500 μm until the mat surface was reached. At each depth, a light spectrum between 400 and 800 nm was recorded, and all spectra were normalized to the light spectrum taken at the mat surface.

Depth distribution of *Beggiatoa* filament densities and biomass determination. The vertical distribution of *Beggiatoa* filament densities in the mat was determined under both light and dark conditions, directly after finishing the microsensor profiling. The microbial mat subsamples were cut perpendicular to the mat surface at former microsensor measurement spots, placed immediately within a petri dish while still wet, and kept in darkness. With the aid of a dissection microscope at a magnification of ×16, images of the cross section were taken with a Nikon Coolpix digital camera mounted on a camera adapter. To minimize the effect of retraction of *Beggiatoa* filaments back into the mat, less than 10 min passed between cross-sectioning and digital imaging. However, due to the motility of *Beggiatoa* filaments, typically up to 2.5 μm s^{-1}, an unknown part of the population may have retracted into the microbial mat during the mat handling time. Although this may have led to an underestimation of the total population size, this potential effect did not likely affect the relative depth distribution of the *Beggiatoa* population. Several images of three different mat sections were analyzed. Images were processed using Adobe Photoshop 9.0 for Windows to enhance the contrast and the brightness of the white filaments. The depth distribution of filament densities was analyzed by counting the numbers of filaments in 1-mm-wide depth zones from the surface down to a depth of 10 mm. Percentages of filaments were determined as the number of filaments per depth zone against the total filament number for the mat cross section (average of three samples), and corresponding standard errors were calculated. *Beggiatoa* filament biovolume was estimated from the determined filament densities and a determined average filament volume value, which was obtained by measuring the lengths and widths of 100 individual filaments and calculating the value with the formula for a cylindrical geometrical shape. A *Beggiatoa* filament-specific weight density of 1 g cm^{-3} was assumed, which enabled calculation of biomass per 1-mm depth interval.

Total extractable nitrate and elemental sulfur. For analysis of nitrate (NO_3^-) and elemental sulfur (S^0), triplicate samples of the microbial mats were frozen at −20°C directly after determination of the *Beggiatoa* filament depth distribution. The frozen samples were sliced horizontally with a cryomicrotome (Microm, GmbH, Walldorf, Germany) into 1-mm-thick slices from the surface down to a depth of 10 mm. Each slice was transferred to a glass vial containing 0.5 ml of a 20% zinc acetate solution. After being freeze-dried for porosity and wet bulk density determinations, gastight vials (6 ml; Labco) were filled with demineralized water, spiked with a 10 μM $^{15}NO_3^-$ solution, and subsequently flushed with helium gas to reduce the N_2 background and to introduce a 1-ml headspace. In order to reduce the NO_3^- to NO gas, 0.5 ml of titanium(III) chloride was injected directly afterwards, and vials were immediately vigorously shaken (9, 56). Gas samples of the headspace were injected into a gas chromatograph-mass spectrometer which was equipped with a reduction oven (630°C), reducing the NO gas to N_2. The stable-isotope composition of the N_2 gas was analyzed by mass spectrometry. Due to the high $^{15}NO_3^-$ background (10 μM), nearly all (>99%) of the $^{14}NO_3^-$ formed an excess $^{14}N^{15}N$ ($^{29}N_2$)/$^{15}N^{15}N$ ($^{30}N_2$) ratio compared to the $^{15}NO_3^-$ medium (99% pure). Based on the known amount of $^{15}NO_3^-$, we determined the $^{14}NO_3^-$ content by using the principle of the isotope pairing method (49).

For zero-valent sulfur analysis (S^0), mat pieces fixed with 0.5 ml 20% zinc acetate in 10-ml glass vials were extracted with 10-ml 100% high-performance

liquid chromatography (HPLC)-grade methanol aliquots for 2 days. Subsequently, samples were completely homogenized by sonication and vigorous shaking. The methanol phase was then separated from the mat slurry by centrifugation (1 min at 3,000 × *g*). Elemental sulfur was quantified by HPLC and UV detection at 265 nm as described by Zopfi et al. (69).

Measurements of *Beggiatoa* internal nitrate and elemental sulfur concentrations. One *Beggiatoa* type, based on its morphological characteristics, was found to dominate the Lake Chiprana *Beggiatoa* community. For internal nitrate concentration determination, *Beggiatoa* filaments were picked out of intact mat samples with the aid of a glass needle and transferred to a droplet of artificial saline water (80 g/liter NaCl) on a microscope slide. Filament biovolume (V) was calculated from length (l) and diameter (d) determinations according to the equation $V = \pi(0.5d)^2 \times l$. The biovolume of the entire filament was taken for calculations of internal nitrate and elemental sulfur. Single filaments were then placed into 300-μl glass vials filled with 150-μl aliquots of deionized water and frozen at −20°C to disrupt the cells. After thawing, the samples were centrifuged (1 min at 3,000 × *g*), and 100-μl supernatant aliquots were taken and transferred into 6-ml glass vials filled with 10 μM $^{15}NO_3^-$. The $^{14}NO_3^-$ content was determined by the isotope method as described above for the mat samples. The elemental sulfur concentration was determined on the same samples by 100% HPLC-grade methanol extraction of the remaining air-dried pellet.

In an incubation experiment with an enriched *Beggiatoa* population for the determination of *Beggiatoa* nitrate storage potential, filaments were picked out of intact mat pieces and placed in oxygen-free artificial lake water on top of 50 μM NO_3^--containing agar. Filaments were then picked at two sampling times with a 24-h time interval. Samples were analyzed for internal nitrate and elemental sulfur concentrations as described above.

16S rRNA gene analysis of Lake Chiprana *Beggiatoa* spp. General bacterial primers (Interativa, Ulm, Germany) were used to amplify almost-full-length 16S rRNA genes from single filaments. The master mix for the PCR was prepared as follows: 50 pmol each of primers 8f (19) and 1492r (31), 2.5 μmol of each deoxyribonucleoside triphosphate, 1× Super-Taq buffer (HT Biotechnology Ltd., Cambridge, United Kingdom), and 1 U of *Taq* DNA polymerase (Eppendorf, Germany) were amended with sterile water to a total volume of 100 μl. Live *Beggiatoa* filaments taken directly out of intact mat pieces were rinsed in artificial seawater before the addition of the universal primers. The PCR was started with an initial denaturation step of 4 min at 94°C, followed by 32 cycles of 0.5 min at 94°C, 0.5 min at 48°C, and 1.5 min at 72°C, and was terminated with a final step of 10 min at 72°C.

PCR products were purified (PCR purification kit; QIAGEN, Hilden, Germany), ligated using a TOPO-TA sequencing kit (Invitrogen, Carlsbad, CA), and then transformed according to the company's specifications. Clones with inserts were extracted from the clone library and sequenced by *Taq* cycle sequencing performed with vector primers and a model ABI sequencer (Applied Biosystems). The retrieved *Beggiatoa* sequences were analyzed with the ARB software package (35). The alignment was corrected manually, and the phylogenetic tree was constructed by maximum parsimony, neighbor joining, and maximum likelihood analyses with different filter sets. For tree reconstruction, only full-length sequences were taken into account. The tree was constructed by including subsets of data and an outgroup reference sequence. The tree structure was computed by these different approaches to build a consensus tree. All partial trees were checked for branching incongruities. Multifurcation in specific branching orders was used for different results by the applied methods. Analyses included closely related BLAST matches (3).

Confocal laser scanning microscopy. For visualization of intracellular vacuoles and other morphological cell characteristics, several specific fluorochromes were applied to freshly picked *Beggiatoa* filaments. Fluorescein isothiocyanate (FITC; Research Organics, Cleveland, OH), Newport Green (Molecular Probes, Invitrogen), and a vacuole membrane marker (MDY-64; Molecular Probes, Invitrogen) were used to stain intracellular constituents (29). Nucleic acids were marked with SYBR green (Molecular Probes, Invitrogen). SYPRO orange (Molecular Probes, Invitrogen) was used for protein staining. Some filaments were double stained with SYBR green and SYPRO orange as described previously (70). *Beggiatoa* filaments were transferred to and stained on microscope slides. The stained filaments were examined by means of a TCS-SP1 confocal microscope controlled by Leica confocal software, version 2.61, build 1537 (Leica, Heidelberg, Germany). The TCS-SP1 microscope was attached to an upright microscope and equipped with three different lasers (488, 561, and 633 nm). For microscopy, a 63×, 1.2-numerical-aperture water immersion lens corrected for coverslips was used. Images were projected with the microscope software and Imaris (Bitplane, Switzerland). Images were printed from Photoshop without any corrections.

Determination of migration speed. Freshly picked *Beggiatoa* bacteria from intact microbial mats were placed under different conditions (in medium or artificial seawater and in gradient tubes or microcapillary tubes, but always at 20°C) to visualize movement and determine gliding velocities. Movements were recorded with a charge-coupled-device camera under red light illumination, which presumably does not affect the movement of these bacteria (44). Image sequences were analyzed, and movement speeds were determined. All values for several measurements in the same data set were averaged.

Nucleotide sequence accession number. The nucleotide sequence of the *Beggiatoa* morphotype described here (Chiprana, mesocosm mat [MM]) has been deposited in GenBank under accession number EF 428583.

RESULTS

Comparison of intact Lake Chiprana and mesocosm-incubated mats. The composition of the microbial mats kept in the mesocosm was similar to that of mats obtained directly from Lake Chiprana. Vertical cuts of mats from the mesocosm system showed similarly colored depth layers, with each inhabited by similar species to those in the natural microbial mat (21). The surface layers of both mats were composed mainly of diatoms (*Nitzschia* spp.), *Chloroflexus* spp., and unicellular cyanobacteria. The underlying green and brown layers, with whitish embedded calcium carbonate crystals, included several types of filamentous cyanobacteria, dominated by species of the genera *Oscillatoria* and *Microcoleus*. Below a depth of 1 cm, a dark gray zone of iron sulfide (FeS) precipitates emerged. In the green and brown mat layers, several white filaments appeared, with enough to detect them easily by the naked eye but too few to form a distinct layer of *Beggiatoa* sp.-like organisms. Microscopic analysis of these filaments revealed a dominant morphotype of 6 to 8 μm in diameter, with filament lengths varying between 1 and 10 mm. Differential interference contrast microscopy (6, 50) revealed sulfur inclusions as well as internal vacuoles. Filament ends were always rounded. There was no visible difference in filament morphotype in distinct mat layers.

***Beggiatoa* distribution dynamics in intact mats.** The total estimated *Beggiatoa* biomasses in the 0- to 10-mm depth zone for the illuminated and dark mats were 11.5 μg mm^{-2} and 10.1 μg mm^{-2} (wet weight), with total biovolumes of 0.012 and 0.010 μl mm^{-3} mat, respectively. The vertical distributions of *Beggiatoa* filaments were slightly but significantly different in dark- and light-incubated mats (Fig. 1). The maximum filament abundance in light-incubated mats was at a depth of 8 mm, and after 10 h of dark incubation, the maximum filament abundance was at a depth of 7 mm. In the light, the maximum abundance was found in the depth zone where oxygen and sulfide co-occurred (Fig. 2A). Upon darkening of the environment, oxygen retreated close to the surface, whereas the sulfide front hardly moved. In mats incubated in the dark, a gap of more than 5 mm existed between the oxic and sulfidic zones, and the *Beggiatoa* filaments remained in the anoxic layer close to the sulfidic zone (Fig. 2B). Thus, the vertical distribution of *Beggiatoa* seemed more related to the distribution of sulfide than to that of oxygen. Routine oxygen and sulfide profiling of the mesocosm-incubated intact Lake Chiprana microbial mat showed that although the mat expanded in thickness over the incubation period, oxygen and sulfide production in the mat persisted (Fig. 2C). The presence of sulfide in the mesocosm-incubated mats suggests that active sulfate reduction in the surface layer continued during the incubation period. Previous

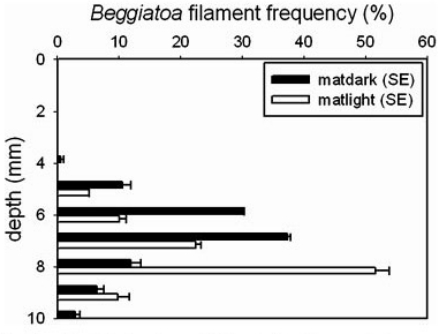

FIG. 1. Depth distribution of *Beggiatoa* filaments in a microbial mat after 10 h of dark and 14 h of light incubation (500 μmol photons m^{-2} s^{-1}). The data shown are frequencies (%) with standard errors (SE). Black bars, incubation in the dark; white bars, incubation in the light.

studies concerning Lake Chiprana microbial mats showed that the concentrations of dissolved organic carbon (21, 21a) as well as sulfate reduction rates (34a) were indeed highest in the photic zone of the microbial mat, indicating that excreted photosynthates may primarily fuel the process of sulfate reduction in these mats.

Some *Beggiatoa* species show a phobic response to wavelengths between 400 and 500 nm (41, 44). Microprobe light measurements revealed, however, that light of these wavelengths was already completely absorbed within the top 2 mm of illuminated mats (Fig. 3), which is much shallower than the main *Beggiatoa* biomass depth distribution (between 5 and 9 mm). However, in the latter depth zone, significant intensities of infrared light (800 nm) were still measurable (i.e., about 15% of the mat surface intensity at a depth of 6 mm). In additional laboratory tests, we could not find any indices for either a phobic or a philic response of Lake Chiprana *Beggiatoa* organisms subjected to infrared light (results not shown). These results show that light itself likely does not directly affect the *Beggiatoa* depth distribution.

Microsensor pH measurements of light- and dark-incubated mats (Fig. 2) showed pH maxima. The pH maximum in illuminated mats was positioned close to the depth where the oxygen concentration reached its maximum, while in dark mats it occurred in the depth zone with the highest *Beggiatoa* filament abundance and increasing sulfide values. The pH maximum in the illuminated mat was likely mostly due to CO_2 fixation by oxygenic phototrophs (cyanobacteria and diatoms) and other autotrophic bacteria (*Beggiatoa*), which resulted in a shift in the carbonate equilibrium. The maximum in the dark mat could be explained by the anaerobic metabolism of *Beggiatoa*, i.e., sulfide oxidation to elemental sulfur with concomitant nitrate reduction to ammonia, as this is a proton-consuming process, as also shown by Sayama et al. (59).

Total nitrate concentrations of between 1 and 15 nmol cm^{-3} were determined within the mat (Fig. 4) and included porewater nitrate as well as intracellular nitrate that would be released from the *Beggiatoa* filaments during freezing and thawing. Due to the small sample size (~10 μg mm^{-2}), the lower concentrations (1 to 2 nmol cm^{-3}) measured in the lower part of the mat (between 9 and 10 mm deep) might be caused by minimal contamination and therefore indistinguishable from zero. In the illuminated mat, nitrate concentrations were below 5 nmol cm^{-3} throughout the profile, and no clear trend was recognizable. The dark-incubated mat revealed a nitrate maximum at a depth of 4 mm, just below the disappearance of oxygen and at the same depth as the first *Beggiatoa* filaments. Below this maximum, nitrate values decreased toward the detection limit at a 10-mm depth, where only small numbers of *Beggiatoa* filaments were found.

The elemental sulfur microbial mat profiles (Fig. 5) reflected the *Beggiatoa* filament depth distribution, with the highest values for depths below 4 mm and above 9 mm for both light and dark incubation. Similar to the nitrate results, the measured profiles of S^0 in the dark mat showed higher values than the profiles for the illuminated mat. The dip in S^0 concentration at the depths with the highest filament abundances may be explained by *Beggiatoa* S^0 oxidation by oxygen and internally stored nitrate under light and dark incubation conditions, respectively, although external sulfur deposition or oxidation by other mat community members cannot be excluded.

Intracellular nitrate and sulfur storage. Stable-isotope gas chromatography-mass spectrometry and elemental sulfur HPLC analysis revealed that intracellular concentrations of both nitrate and elemental sulfur were variable (4 to 42 mM for nitrate and 26 to 252 mM for S^0) in *Beggiatoa* organisms picked directly out of intact mats (Table 1). In an incubation experiment with an enriched *Beggiatoa* population in water containing 50 μM nitrate, filaments were able to increase internal nitrate concentrations about fivefold during a 24-hour period. The longer that filaments were exposed to nitrate, the more internal nitrate was detectable. After an initial 24-hour incubation period, *Beggiatoa* internal nitrate values amounted to 8 mM. An additional 24-hour incubation period with excess nitrate resulted in an increased internal nitrate concentration of 44 mM. The values for internally stored sulfur remained relatively unchanged (26 to 34 mM) during this experiment.

Morphology and phylogeny. Microscopic analysis revealed a dominant morphotype with a mean diameter of 6 to 8 μm, while the length varied from <1 mm to >10 mm. Differential interference contrast microscopy showed the presence of vacuoles (Fig. 6B), which was further verified by specific staining procedures with diverse fluorochromes and confocal laser scanning microscopy (Fig. 6C to F). Images of *Beggiatoa* filaments stained with FITC, an amine-reactive dye, clearly showed the presence of vacuolar structures (Fig. 6C) by positively staining the central vacuole. Intracellular sulfur granules were also recorded in white due to their reflection signals (Fig. 6C, D, E, and F). In addition, the black gaps which appeared within filaments could be interpreted as lysed (empty) cells, which may indicate potential filament breaking points (Fig. 6C). Some cells seemed to be packed with sulfur inclusions only (Fig. 6C). MDY-64, a known yeast vacuole membrane marker, was employed here to test its applicability for *Beggiatoa* vacuole and other membrane-coated cell constituents. This fluorochrome is known to distribute throughout the cell but becomes enriched within membrane structures (57). It showed a negative imprint of the vacuole lumen (Fig. 6D) but apparently also stained other small membrane-coated particles within the cytoplasm. With SYBR green, multiple areas of highly enriched nucleic acid accumulations could be demon-

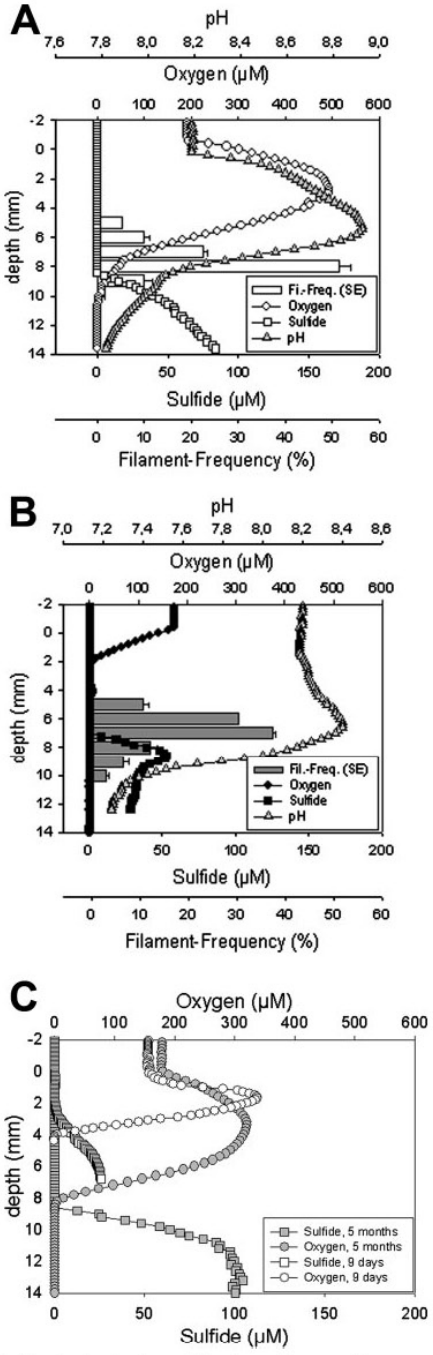

FIG. 2. Depth distribution of *Beggiatoa* filaments (frequency), oxygen and total sulfide concentrations, and pH in illuminated (A) and dark (B) Lake Chiprana microbial mats incubated for 3 months in a laboratory mesocosm system. Illuminated mats were incubated for 14 h at a light intensity of 500 µmol photons m^{-2} s^{-1}, while dark mats were incubated in the dark for 10 h prior to measurements. Note the different scale for pH. Measured profiles of oxygen and sulfide concentrations in mats incubated in the mesocosm for 9 days and 5 months (C) show that although the mats expanded vertically, both oxygen and sulfide production in the mats persisted during the incubation period.

strated within single cells (Fig. 6E and F). SYPRO orange, a protein-specific fluorochrome, stained the cell surface as well as a thin cytoplasmic layer surrounding the vacuole (Fig. 6F).

Several single filaments of the dominant *Beggiatoa* morphotype were picked from intact mat pieces for the construction of a 16S rRNA gene clone library. One of 80 clones contained a nearly full-length 16S rRNA gene sequence related to the *Beggiatoa* sequence, while the other sequences were related to contaminant, i.e., nontarget, bacteria. The *Beggiatoa*-related sequence was assumed to correspond to the dominant morphotype observed in the mat, as only one single filament was applied in the PCR. To avoid *Beggiatoa* DNA autolysis, the picked filaments were immediately transferred into the PCR vial without a previous rigorous cleaning step. This procedure, however, apparently caused the dominance of sequences of contaminant bacteria in the clone library. The obtained target sequence grouped with a cluster of marine *Beggiatoa* isolates (Fig. 7). So far, all characterized larger *Beggiatoa* species (>9 µm) in this group contain vacuoles. The hypersaline morphotype from Lake Chiprana appeared to be most closely related (91% sequence similarity) to the marine, narrow (4 µm), and reportedly nonvacuolated *Beggiatoa* sp. strain MS-81-6 (AF 110277), which originated from the Sippewissett salt marshes (Massachusetts) (47).

Gliding motility. The gliding movement of filamentous, multicellular *Beggiatoa* organisms proceeds with a reported average speed of 3 µm s^{-1} (41). The measured gliding speed of the hypersaline *Beggiatoa* strain from Lake Chiprana incubated under diverse conditions was between 1 and 2 µm s^{-1}. The slowest gliding movement, 0.94 µm s^{-1} (±0.44 µm s^{-1} [standard deviation]), was detected in microcapillaries containing artificial anoxic seawater and a nitrate concentration of 50 µM. Filaments moving in medium in gradient tubes with 20 mM H_2S added from one side were slightly faster (1.49 ± 0.11 µm s^{-1}). The highest gliding speed, 2.00 ± 0.29 µm s^{-1}, was recorded for microcapillaries filled with anoxic medium only. With an average speed of 2 µm s^{-1}, filaments could have covered a distance of 72 mm in 10 h, the time of the dark incubation period, if they would have followed a straight line.

DISCUSSION

The retrieved sequence of the hypersaline nitrate-storing *Beggiatoa* sp. obtained in this study clearly showed a phylogenetic relationship to three (*Thioploca*, *Thiomargarita*, and *Beggiatoa*) of the four genera of the large sulfur bacteria, which group within the γ-subclass of the *Proteobacteria*. Only representatives of *Beggiatoa* and *Thiothrix*, the fourth genus of the large sulfur bacteria, are maintained in pure culture to date. The classification of uncultured morphotypes of *Beggiatoa* sp.-like bacteria has been based on filament diameter, the presence of intracellular vacuoles, nitrate storage capacity, and some metabolic properties. The dominant *Beggiatoa* sp.-like morphotype obtained from the hypersaline Lake Chiprana had an average filament width of 6 µm and clearly recognizable intracellular vacuoles. The closely affiliated marine strain MS-81-6 has been grown in pure culture and was studied by Nelson et al. (46, 47) and Hagen and Nelson (17, 18). This strain is characterized by a narrow filament width (4 µm) and the apparent absence of intracellular vacuoles. Although the mor-

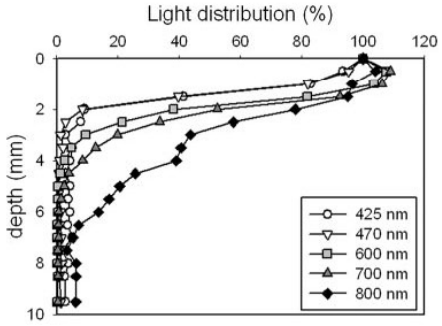

FIG. 3. Light profiles in the studied mat, illuminated at 500 μmol photons m^{-2} s^{-1}. The shorter wavelengths (<500 nm) were virtually completely absorbed in the first 2 mm, while the longer wavelengths penetrated deeper into the mat.

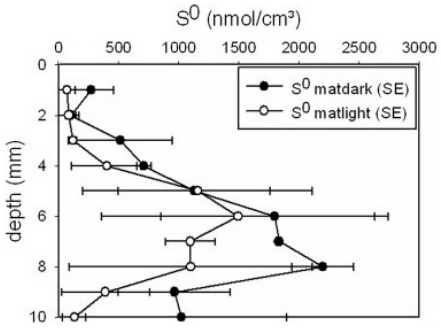

FIG. 5. Microbial mat profiles of total extractable elemental sulfur after light (14 h of 500 μmol photons m^{-2} s^{-1}) and dark (10 h) incubation.

phological characteristics of these two morphotypes differ in at least two aspects (filament width and vacuolation), they both appear to be tolerant to salinities above those found in marine water: Lake Chiprana has an average salinity of 8%, and strain MS-81-6 was isolated from a salt marsh where salt concentrations during low tide can also increase strongly due to evaporation. Ahmad et al. (2) defined strain MS-81-6, together with another cultivated, nonvacuolated, but narrower (2 μm) strain (MS-81-1c) from the same salt marsh (47), as the root of the large vacuolated sulfur bacterium clade. Interestingly, the Lake Chiprana isolate seems to be an intermediate between both groups, as its cells are narrow but vacuolated. The *Beggiatoa* morphotype from Lake Chiprana appears to be more closely related phylogenetically to representatives of the marine narrow nonvacuolated *Beggiatoa* than to those of larger marine and vacuolated sulfur bacteria. Therefore, vacuolation and nitrate accumulation capability appear not to be restricted to wide-diameter *Beggiatoa* spp., as previously assumed (29).

The vertical diurnal migration behavior of hypersaline but further uncharacterized *Beggiatoa* organisms from photosynthetic mats from Guerrero Negro, Mexico, was studied before (16). In that study, it was observed that during the day, *Beggiatoa* bacteria were concentrated in the zone where oxygen and sulfide co-occurred. At night, however, this population was split into two subpopulations. One part rose to the surface of the mat, while another part of the population remained in the deeper, now anoxic, part of the mat. A similar behavior to that of the latter group was found for Lake Chiprana *Beggiatoa*. During the light (daytime) period, filaments were most abundant in the narrow oxygen and sulfide transition zone. Here they most likely oxidized sulfide by using oxygen as an electron acceptor, as the observed pH minimum in this zone reflects this acidic process. Simultaneously, the filaments could concentrate nitrate, presumably in the intracellular vacuoles, while at the oxic-anoxic transition zone other microbial mat community members produce nitrate during aerobic ammonium oxidation (nitrification). This scenario seems possible, as the ability for nitrate storage of the studied Lake Chiprana strain was confirmed by stable-isotope techniques. Although measured total extractable nitrate values were low, nitrate can be concentrated strongly, 1,000- to 10,000-fold, by *Beggiatoa*, as shown by the incubation experiment, similar to data reported for other strains (13, 38). The fact that the determined total (internal plus pore-water) extractable nitrate profiles did not reflect the *Beggiatoa* depth distribution can be explained by the ratio of relatively low *Beggiatoa* volume to total microbial mat volume, as this amounted to less than 1%. The analyzed microbial mat elemental sulfur concentration profiles, however, appeared similar to the *Beggiatoa* depth distribution, but this might be coincidental. Although internal sulfur concentrations were found to be significantly higher than internal nitrate concentrations, the actual impact of the rather low total *Beggiatoa* biomass (3.1 to 5.9 g m^{-2}) and the related intracellular stored sulfur contribution to total (internal plus external) sulfur concentrations in the

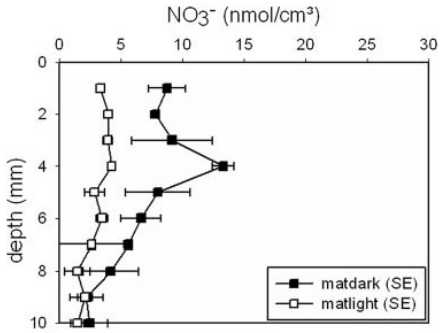

FIG. 4. Microbial mat profiles of total extractable nitrate after light (14 h at 500 μmol photons m^{-2} s^{-1}) and dark (10 h) incubation.

TABLE 1. *Beggiatoa* sp. intracellular nitrate and sulfur storage

Filament sample group	Internal concn (mM)[a]	
	Nitrate	Sulfur
Filaments from fresh, intact mats	4 ± 0.8	252 ± 0.5
Filaments from dark-incubated mats	42 ± 1.9	26 ± 0.4
Filaments in incubation expt		
1 day	8 ± 0.7	26 ± 2.4
2 days	44 ± 3.4	34 ± 5.3

[a] Data are means ± standard deviations.

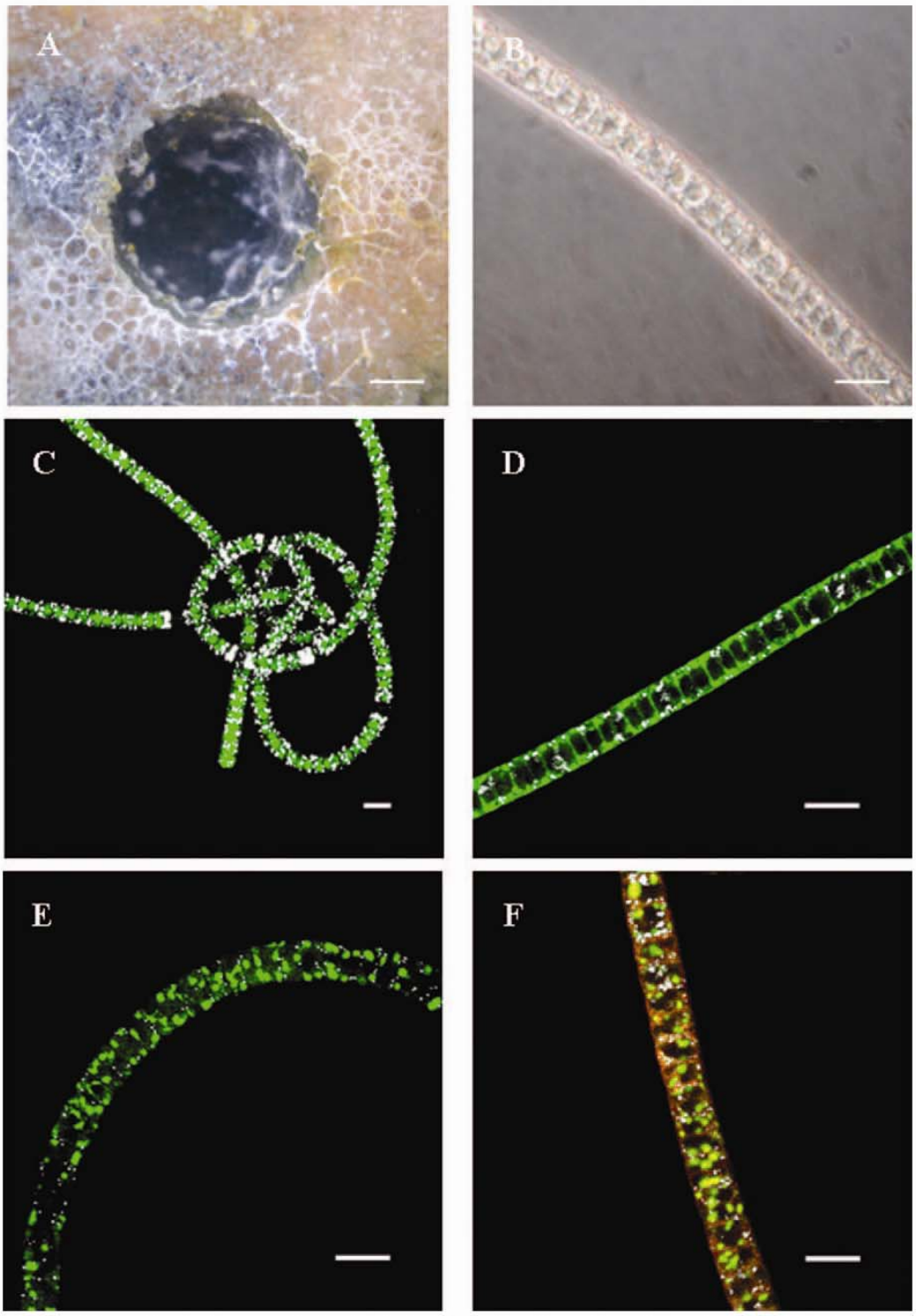

FIG. 6. Images of hypersaline *Beggiatoa* sp. originating from Lake Chiprana. Bar, 10 mm (A) or 10 μm (B to E). (A) Top view of a cored anoxic microbial mat covered by *Beggiatoa* bacteria. (B) Differential interference contrast micrograph of a vacuolated and sulfur globule-containing *Beggiatoa* filament. (C) FITC-stained filament. White areas, reflection of sulfur globules. (D) Staining with yeast vacuole membrane marker MDY-64. White areas, reflection of sulfur globules. (E) SYBR green nucleic acid stain. White areas, reflection of sulfur globules. (F) Red, protein stain with SYPRO orange; green, SYBR green stain for nucleic acids; white, reflection of sulfur globules.

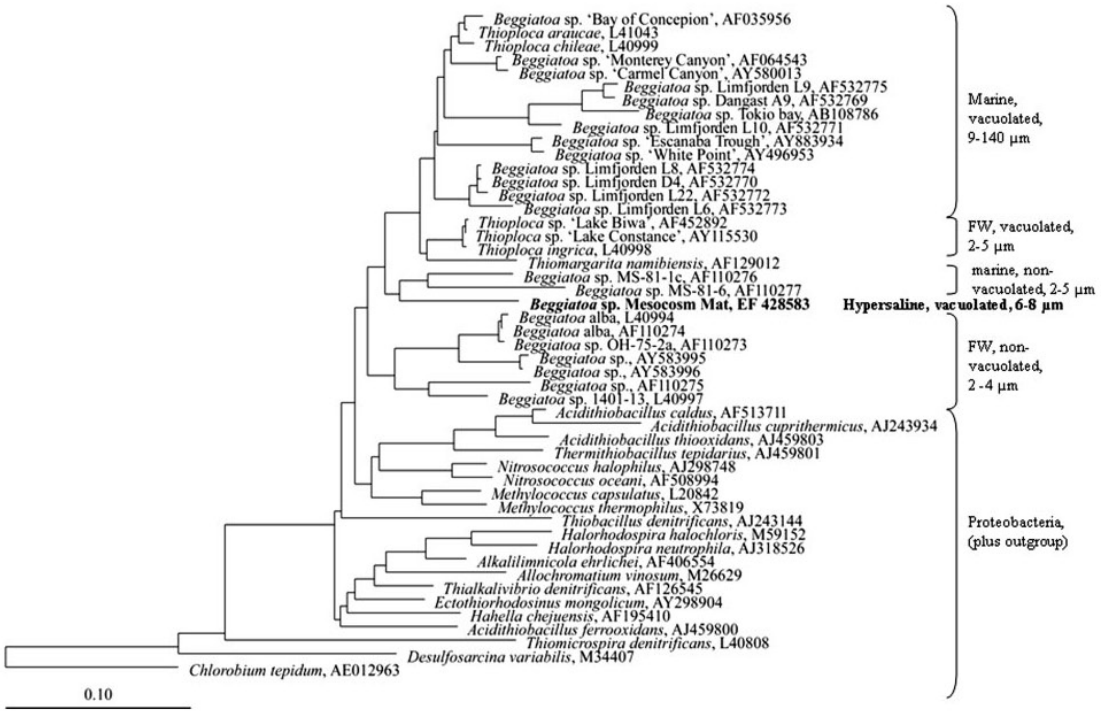

FIG. 7. 16S rRNA gene-based phylogenetic reconstruction showing the positions of the hypersaline nitrate-storing *Beggiatoa* strain originating from Lake Chiprana and other representatives of the gamma subdivision of the *Proteobacteria*. Bar, 10% estimated sequence divergence.

mat remain to be investigated. The relatively low areal biomass values actually suggest that the impact of Lake Chiprana *Beggiatoa* on the system's sulfur cycle may be rather low. Analogous examinations have been conducted in different habitats and have shown very low intracellular elemental sulfur concentrations in comparison to a large amount of extracellular elemental sulfur in the bulk sediment (25). Various authors made widely different estimations of the importance of nitrate-storing bacteria for S cycling. With generally 1 order of magnitude higher cell densities, estimations ranged from 3 to 91% of all produced sulfide being oxidized by these bacteria (12, 42, 51). While the importance of *Beggiatoa* bacteria for mat ecology is debatable, their physiological adaptation to the diel cycles typical for microbial mats is, without a doubt, highly interesting. Their nitrate storage capacity allows *Beggiatoa* bacteria to remain active during the night, when most mats are anoxic and thus exclude the metabolic activity of aerobic sulfide oxidizers. *Beggiatoa* bacteria are most likely repelled by steep oxygen and sulfide gradients, allowing them to find the overlapping zone. Nevertheless, the *Beggiatoa* filaments seemingly do not use their tactic responses to follow the retracting oxygen front during evening darkening. Nitrate storage also allows them at night to remain close to the position they found during the day. A similar strategy was proposed for the immotile large sulfur bacterium *Thiomargarita namibiensis*, which must overcome long periods (weeks to months) of anoxic conditions, as in its natural habitat dark marine sediments, oxygen, and nitrate are only incidentally introduced during turbulent mixing events (60, 61). *Beggiatoa* organisms from cold seeps and other sulfidic marine sediments preferably inhabit the suboxic zone (59), the zone that separates the oxic and sulfidic zones, where neither sulfide nor oxygen is present in detectable amounts (5, 14). Whereas marine filamentous strains use a characteristic suite of tools (gliding motility, negative tactic responses to sulfide and oxygen, and nitrate storage) to migrate through a permanent anoxic sediment, the hypersaline *Beggiatoa* organisms of this study apparently use the same tools to position themselves optimally in a dynamic environment. How far salinity itself, in terms of both quantity and quality, influences the *Beggiatoa* survival strategy remains to be investigated. We can only hypothesize at this stage that not salinity but, rather, oxygen and nitrate concentration dynamics of a specific sulfidic ecosystem are the determining factors influencing *Beggiatoa* migration behavior. The internally stored nitrate can thus be used to overcome spatially or temporally separated zones of sulfide and nitrate availability. This unique example shows that one set of characteristics can be applied in two different strategies and thereby allow a competitive advantage in two entirely different habitats.

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Chapter 3

A method for the estimation of pH in life cells based on excited state saturation

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Abbreviations:

FITC: fluorescein-iso-thio-cyanate
BCEF: bis(carboxyethyl)-carbonyl fluorescein

Keywords: pH**,** *Beggiatoa*, confocal microscopy, FITC, saturation, fluorescenc

Summary

Imaging techniques that allow intracellular pH determination in ranges below pH 3 employ costly equipment and can have a long data acquisition time (minutes). Here, we describe a new methodology based on excited state saturation employing the fluorophore fluorescein-iso-thio-cyanate for confocal microscopy allowing a fast data acquisition in live organisms. To develop the method a model description of the fluorophore's molecular states was developed that lead to a ratio function dependant on the excited states' lifetime. Due to the lifetime dependence on the pH of fluorophore surrounding medium for fluorescein-iso-thio-cyanate this ratio function was useful for pH determination. The model was tested theoretically and the pH dependence of the ratio function was verified experimentally with an artificial dye-bead system. Finally, a simple measuring protocol was developed allowing the automatic determination of the ratio function in images of live cells under the confocal microscope. This procedure was applied successfully to vacuolated *Beggiatoa* filaments with different internal pH values, near neutral in the cytoplasm and acidic in the vacuoles.

Introduction

Imaging of intracellular pH of live cells is instrumental for obtaining insights into intracellular physiological processes. A range of indicator dyes is available that cover a wide pH-range (Invitrogen, 2008, Martin and Lindqvist, 1975, Rink et al., 1982, Roos, 2000, Whitaker et al., 1991). The use of fluorescein-iso-thio-cyanate (FITC) or bis(carboxyethyl)-carbonyl fluorescein (BCEF, (Paradiso et al., 1984, Rink et al., 1982)) allow a pH determination based on a spectral fluorescence ratio. Aslan et al. (2005) enhanced ratiometric pH-sensing using SNAFL-2 dye on silver island films. But the determination of the ratio in all of these methods is very erratic as one of the spectral peaks involved shows only a low fluorescence quantum yield at pH below 4. Therefore, fluorescence lifetime imaging was employed to measure pH (first in the near neutral region) to solve this (Lin et al., 2003, R. Sanders, 1995). Lin et al. (1999) reported of lifetime sensors useful between pH 3-5. French et al. (1997) employed fluorescence lifetime measurements of FITC in a two photon mode to image pH below 3 in live cells. The disadvantage of fluorescence lifetime imaging is the high acquisition time of several minutes to get appropriate low-noise images, and that very costly equipment is necessary. In this paper, we describe a new method that has an acquisition time of approximately 1 second and which can be used on all commercially available confocal microscopes. It allows high spatial resolution measurements of pH in acid environments and in different cell organelles by using the excited state saturation technique previously tested for

the estimation of Förster resonance energy transfer (Beutler et al., 2008, Jares-Erijman and Jovin, 2003, Jares-Erijman and Jovin, 2006).

Fluorescent molecules can be driven into excited state saturation by intense excitation light (Hanninen et al., 1996, Jares-Erijman and Jovin, 2003). Davis et al. (2005) have modelled and used saturation effects in fluorescence correlation spectroscopy. The principle of excited state saturation is that at low light intensities, the fluorescence emission of a fluorophore population is proportional to the excitation intensity (Heintzmann, 2003, Heintzmann et al., 2002), while when exposed to increasing excitation light intensities, an increasing fraction of the fluorophores becomes excited until a saturation level is reached. At saturation, the fluorescence intensity is defined by the intrinsic radiative rate (Jares-Erijman and Jovin, 2003). In this limiting case, most fluorophores are in the excited state since re-excitation is virtually instantaneous after fluorescence emission. The shape of the fluorescence intensity over excitation intensity is influenced by the lifetime of a fluorophore (Jares-Erijman and Jovin, 2003). From this measured curve or at least of two points of this curve the fluorescence lifetime of the fluorophore population can be determined. As the apparent lifetime of fluorophores can be dependent on the H^+ or other ion concentrations the measurement of the fluorescence saturation characteristics allows a determination of H^+ or other ion concentrations, by choosing specific fluorophores.

Here, we tested the theoretical concept and gained a ratio function that is related to the fluorescence lifetime of a fluorophore and by that in FITC to the pH of the fluorophore surrounding medium. We also tested the concept with an artificial fluorophore dye system in a confocal microscope and developed a simple measuring protocol that allowed the estimation of pH in life cells under the confocal microscope. Finally, this protocol was applied to life *Beggiatoa* filaments allowing the simultaneous estimation of pH within vacuole and cytoplasm. There is evidence that the vacuoles of these organisms are acidic, based on microsensor studies (Mußmann et al., 2007). Imaging of the pH in life *Beggiatoa* was not possible so far, thus we tested the new technique with *Beggiatoa* filaments.

Material and methods

Molecular state models

To derive a pH dependant ratio function α of fluorescence intensities under different excitation light intensities the system of differential equations applying to the transitions in a FITC fluorophore molecule is given in Figure 1.

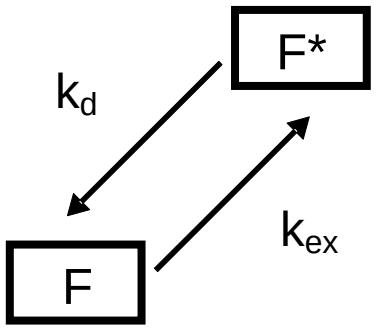

Figure 1: State diagram of a fluorophore (in this case FITC molecule). Asterisks indicate an excited (S) state of the fluorophore. *k*'s are rate constants. Before light absorption, the FITC molecule is in its ground state, denoted as F. By absorption of a photon (k_{ex}) it passes to the first excited singlet state *F**. From there it can deactivate via light emission (fluorescence) and non-radiative means (combined rate, $k_d = \tau^{-1}$). Triplet states are neglected in this model.

The system given in Figure 1 leads to the differential equation:

$$\frac{dF(t)}{dt} = k_d F^*(t) - k_{ex} F(t)$$
$$\frac{dF^*(t)}{dt} = -k_d F^*(t) + k_{ex} F(t) \qquad (1)$$

The system of equations can be solved for the steady-state condition (all derivatives set to 0 and the conservation condition: $F + F^* = 1$), yielding for the excited S-state population:

$$F^* = \frac{k_{ex}}{(k_d + k_{ex})} \qquad (2\ A)$$

The excitation of a state is denoted by an asterisk in superscript. A superscript "ex" means an excitation rate and "d" means a de-excitation rate.

The steady-state fluorescence I_f is given by

$$I_f \propto k_d F^* = F^* \tau^{-1} \quad (2\ B)$$

Using two different excitation intensities $k_{ex,1}$ & $k_{ex,2}$ leads to expression for τ

$$\tau \propto \alpha = \frac{k_{ex,2} I_{f,1} - k_{ex,1} I_{f,2}}{-k_{ex,1} k_{ex,2} (I_{f,1} - I_{f,2})} \quad (3)$$

To model the pH dependence on ratio α in Figure 2, FITC lifetime to pH-conversion were taken from French et al. (1997).

Beggiatoa cultivation and preparation

The *Beggiatoa sp.* filaments (Hinck et al., 2007) were enriched in lithotrophic agar gradient tubes and modified as described by Kamp et al. (2006) & Nelson and Jannasch (1983). Before the measurements were carried out the filaments were incubated in 250 µM FITC for 1 h or additionally in 50µM nigericin to dissipate the ΔpH across the membranes.

Cover slip coating

The cover slips were coated by Cell-Tak (BD, US, (Allen et al., 1992, Eygensteyn et al., 1997)) using the adsorption technique (Allen et al., 1992, Eygensteyn et al., 1997). The method is based on the observation that Cell-Tak comes out of solution as the pH is raised and spontaneously adsorbs to the first surface it contacts. The resulting coating is quite thin (probably close to a protein mono-layer) and more uniform than that achieved by other methods.

Bead preparation

A suspension of commercially available biotin-coated polystyrene beads (Invitrogen, diameter 1 µm) was diluted 10-fold with phosphate buffered saline (PBS), pH 7 buffer. Aliquots of the resulting suspension were incubated for 1 h with FITC-streptavidin (0.022 mg ml^{-1}). The suspension was put in a centrifuge (Eppendorf 5415C) at 14000 rpm for 15 min and washed with PBS. The beads were then immobilized on a cover slip by incubation for 45 min. Excess liquid was removed from the cover slips and dried in air. For test of different H^+

concentrations artificial seawater (same salinity as the *Beggiatoa* enrichment tube medium) was adjusted to different pH and was added to the sample before measurement in the confocal microscope.

Microscopy

Measurements were carried out in a Zeiss 510 confocal laser scanning microscope with a C-Apochromat 40×, NA 1.2 water objective lens using one photomultiplier detector. Excitation light intensities were determined in front of the objective lens with a power-meter (Biospherical Instruments OSL). From the measurements of power levels the mean excitation rate constants (k_{ex}) were estimated with the help of calculated point spread functions. From the estimated power density and the absorption cross section for the different dyes excitation rate constants were calculated.

The beads or organisms were excited at a wavelength of 488 nm (Argon ion laser of the confocal microscope). The beamsplitter in front of the laser was HFT 488. The detector was equipped with a longpass filter LP 505. The confocal pinhole was closed to 1 Airy unit. Images were recorded with a pixel-dwell-time of 2.56 □s.

For the excitation intensity series of measurements, the laser intensities were adjusted with the built-in acousto-optical transmission filter. Transmission images were recorded on the inbuilt photodiode. An image using linear excitation intensity (k_{ex} = 0.025 ns^{-1}) was recorded before and after the actual image with the intensity to be tested (different intensities can be seen in Figure 3). This lead to a final measuring protocol (applied to live *Beggiatoa* used in Figure 5 for ratio α estimation according to equation 3) where a series of three images was collected. First and third image using k_{ex} = 0.025 ns^{-1} ($k_{ex,1}$ in equation 3) and second image employing k_{ex} = 0.25 ns^{-1} ($k_{ex,2}$ in equation 3). All images were corrected for photobleaching of the fluorophores according to Beutler et al. (2008). Image processing as described above was carried out with Matlab (Mathworks, US), dipimage (TU Delft, The Netherlands) and imagej (National institutes of Health, US).

Results

The model described in equation 2 and 3 give the fundamental principle for deriving fluorophore's lifetimes from saturation measurements. To test the model, F^* were plotted (Figure 2A&B) against different excitation rates k_{ex}. For all different pH (τ) the fluorescence intensities (s-states (F^*)) are driven into saturation with higher excitation rates k_{ex} (Figure 2 A). The fluorescence intensities are highest for pH 10 and lowest for a low pH 1. For higher

intensities all curves obtained for different pH approach the same maximal value and saturate (Figure 2B). The ratio α (Eq. 3) was calculated for $k_{ex,1}$ = 0.05 ns^{-1} and $k_{ex,2}$ = 0.2 ns^{-1} indicated in Figure 2A as horizontal dotted lines, and found to increase with pH (Figure 2C) and changes between pH 1 and pH 10 by a factor of 5.

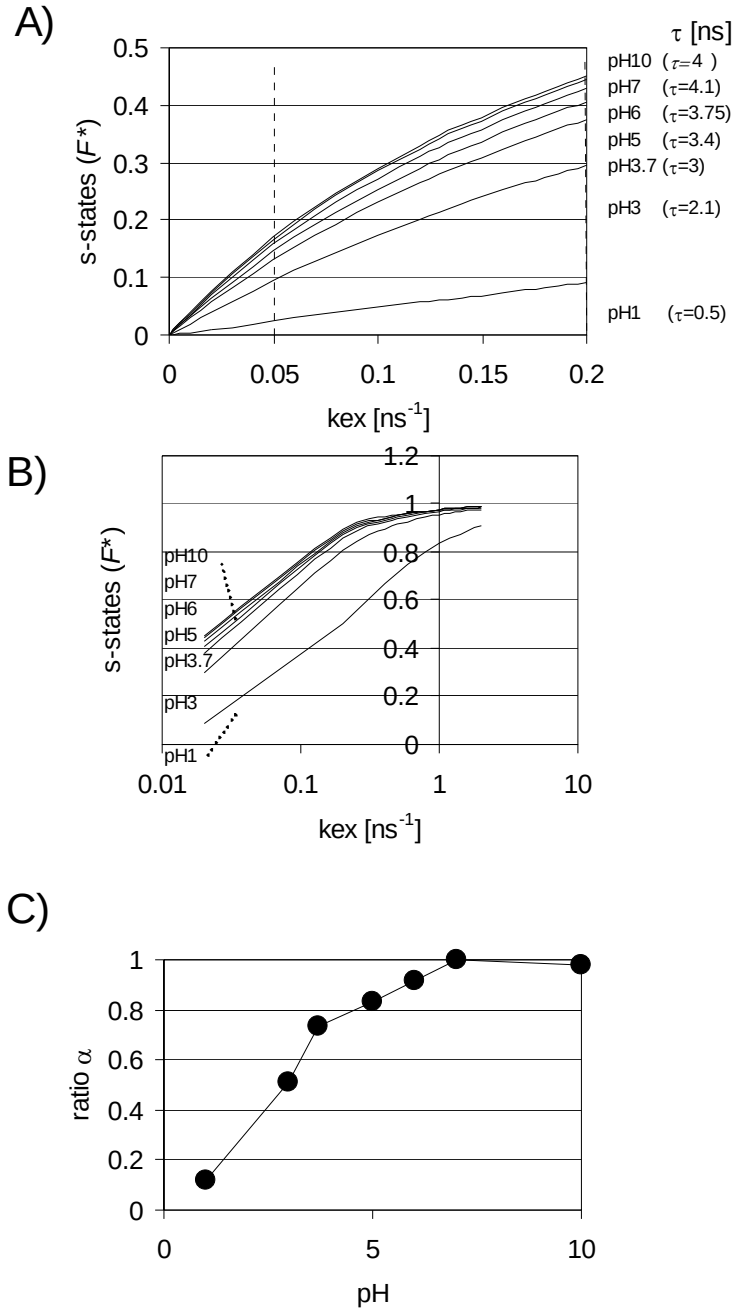

Figure 2: simulation of fluorescence intensities (*F** according to equation 2) and their ratio α (according to equation 3) for FITC. τ to pH conversions were taken from French et al. (1997). A) Fluorescence intensities (*F**) in dependence on excitation intensities and for different pH (τ). B) same as A) but logarithmic scaling of x-axis. C) ratio α calculated according to equation 3 in dependence on the pH (τ).

To proof the principle of pH determination by excited state saturation and to use as calibration standard for the experiments in Figure 5, a test was carried out using FITC–coated beads as a sample incubated in medium with different pH (2-10). In Figure 3 the mean fluorescence intensities of the beads measured at different excitation intensities in the confocal microscope and incubated in medium with different pH are shown. The curves follow a similar trend as those calculated theoretically from equation 2. All curves show a linear increase in fluorescence intensity when low excitation intensities are applied. They approach saturation with higher excitation intensities. A low pH value (pH 2) had lowest fluorescence intensities and highest pH value (pH 10) gave highest fluorescence intensities.

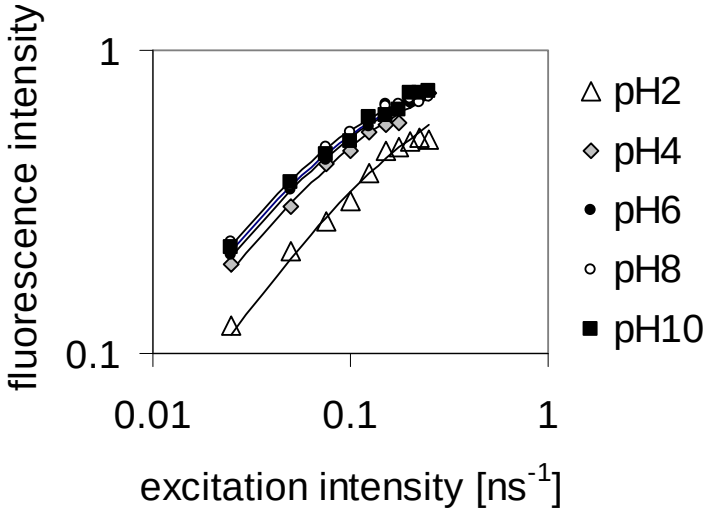

Figure 3: Mean fluorescence intensities of FITC-coated-beads excited by different excitation intensities and incubated in medium with 5 different pH-values. Lines were fitted according to equation 2.

In Figure 4, the ratio α (according to equation 3) is shown. It was calculated for highest ($k_{ex,2}$ in equation 3) and lowest excitation intensities ($k_{ex,1}$ in equation 3) of the curves shown in Figure 3. It changes between pH 2 and 10 by a factor of two. This met the same range as the theoretical evaluation shown in Figure 2C.

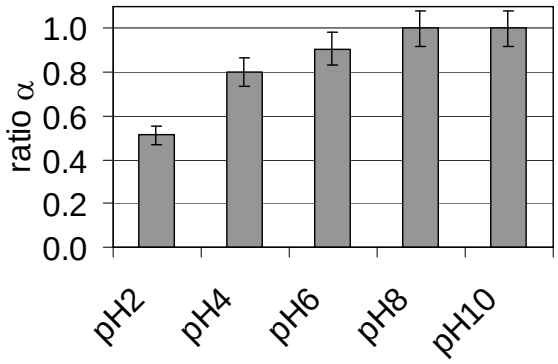

Figure 4: Ratio α calculated according to equation 3 obtained from images of FITC-coated-beads excited by different excitation intensities and incubated in medium with 5 different pH-values.

A fast measuring protocol enabling the acquisition of ratio α (equation 3) was then applied to live *Beggiatoa* filaments. In Figure 5 the transmission images and the calculated ratio α (according to equation 3 from two excitation intensity measurements) are shown. In Figure 5A two filaments can be seen that were incubated in FITC and artificial seawater only. Within the vacuoles a ratio α of 0.4~pH 2 was measured. The surrounding region which was the cytoplasm has ratios α that are between a range of 1 and 0.8 (pH 10 - 6.5). In a second experiment (Figure 5B) filaments were treated with nigericin (Pressman et al., 1967, Sze, 1980), a Na^+/H^+ antiporter that dissipates the pH difference across cell membranes. Thus, the pH inside the cell and the vacuole will obtain the pH value of the medium outside the cell (pH 8). The measured ratios α within the filament were in the range of 1 and 0.8 indicating an increase of the internal pH to the external pH value.

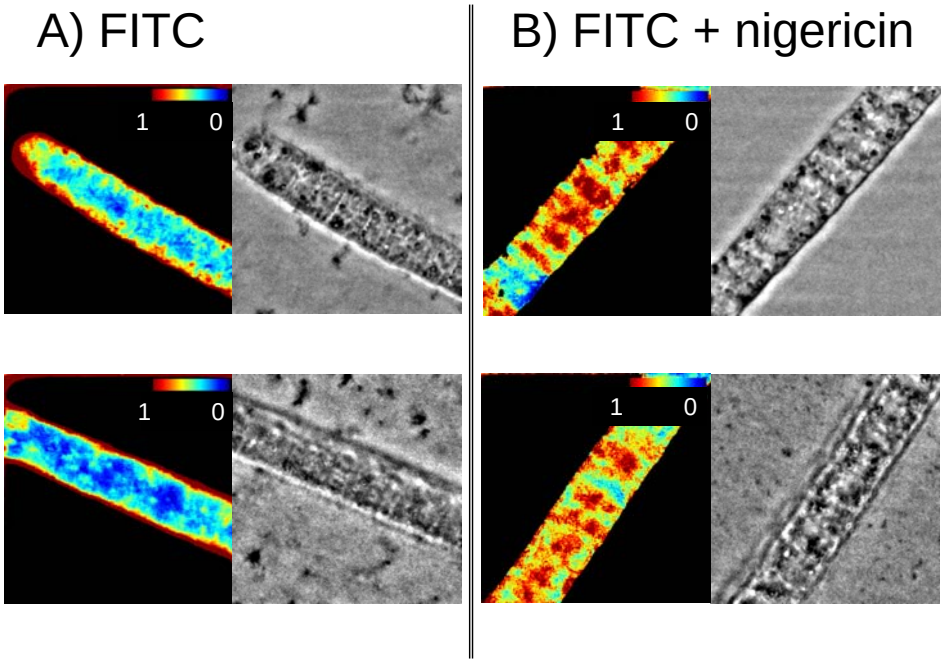

Figure 5: Transmission images and ratio α (calculated according to equation 3). A) of two *Beggiatoa* filaments incubated in FITC and B) of two *Beggiatoa* filaments incubated in FITC and nigericin.

Discussion

In this paper we describe a new method useful in confocal intracellular pH determination in acid environments. The model calculation of equations 2 & 3 (Figure 2) clearly showed that the concept of fluorophore saturation recently applied to estimate Förster resonance energy transfer rates (Beutler et al., 2008, Jares-Erijman and Jovin, 2003) can be employed to estimate pH(τ) in the case of FITC.

Indeed, the fluorescence intensity saturation curves differed in ranges with low excitation rates for different pH values (Figure 2A and B) but approached similar values for high excitation rates. This was used to calculate the ratio function α (equation 3) that is independent of intracellular fluorophore concentration gradients but dependent on the fluorophores lifetime and pH (Figure 2A). The ratio α is therefore a measure for pH or lifetime of a fluorophore. The concept could be proven with an artificial dye bead system showing the usefulness of the ratio α estimation. The needed light intensity could be easily reached by a conventional argon laser to drive the fluorophores population into ~70% saturation (Figure 3, pH 10). These lasers are implemented in many confocal microscopes and make the ratio α estimation therefore broadly accessible.

The experiment in Figure 5 with *Beggiatoa* filaments showed that the method is applicable to living organisms. An easy measuring protocol was developed that can be used on a wide range of confocal microscopes. The procedure allowed an image acquisition within

a second and could be used in time-resolved analysis of life organisms. As we know this could not be achieved by any other method in this pH-range.

Theoretically, fluorescence lifetime estimation by fluorescence saturation could be applied to wide range of ion and pH indicators. For example, the ion indicator Fluo-4 (Ca^{2+}-sensitive), MagFluo-4 (Mg^{2+} sensitive) are intensity based. This means their fluorescence lifetime changes due to the Ca^{2+} or Mg^{2+} concentration in the fluorophore's environment. These dyes are based on fluorescein like FITC and have similar lifetimes and lifetime-changes. Therefore, the method here can be applied to other dyes allowing quantitative determination of a range of other ions. The method allows determination of a wide range of compounds with high spatial resolution, and seems well suited for intracellular physiological studies.

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Chapter 4

Locomotion mechanism and chemotactic behavior of hypersaline *Beggiatoa*

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Abstract

In this study the locomotion mechanism and chemotactic behavior of a filamentous hypersaline *Beggiatoa* strain was investigated by confocal laser scanning microscopy and experimental techniques. Microscopic examination of lectin-stained filaments revealed the presence of slime jets at the filament's surface. We assume that the production of these slime jets are for locomotion purposes as they occur as lines circulating the filaments with 2-4 μm spacing, suggesting that this results in the observed characteristic spiraling movement of filaments. To identify the key triggers that determine chemotactic behavior of *Beggiatoa* in gradient systems such as natural microbial mats, motile response towards sulfide, oxygen, nitrate and blue light was studied in both agar-based reagent tubes (static gradients) and liquid medium-based transparent capillaries (dynamic gradients). Microsensor measurements, visual filament position determination, automated camera imaging analyses, and statistical variation partitioning analysis revealed that sulfide was the prime parameter determining motility, attracting filaments at a low (5-10 μM) but repelling them at a high (>25 μM) sulfide concentration. Nitrate, which can be used besides oxygen as electron acceptor by *Beggiatoa*, was found to attract filaments while oxygen and blue light, like higher sulfide concentrations, repelled filaments. The results of this study can explain observed migratory behavior of *Beggiatoa*, not only in natural sulfidic environments, but also in sulfide-free regions through which *Beggiatoa* have to migrate in search for either electron donors or acceptors.

Introduction

Bacteria of the genus *Beggiatoa* are filamentous and have the ability to move by gliding. *Beggiatoa* typically proliferate in sulfidic environments which are further characterized by the presence of a suitable electron acceptor, either oxygen or nitrate, with which they can oxidize present reduced sulfur compounds for energy generation and production of reducing equivalents needed for carbon dioxide fixation (Schulz & Jørgensen 2001). The simultaneous presence of both electron donor and acceptor usually results in rather steep and opposing gradients of both components. In some environments such as phototrophic microbial mats, the opposing gradients may overlap (Jørgensen & Revsbech 1983; Møller et al. 1985; Nelson et al. 1986b), while in others such as shallow or deep sea sediments, an intermediate zone where none of the compounds (sulfide and oxygen or nitrate) occur, may be present (De Beer et al. 2006). In all these natural environments vertical migration of *Beggiatoa* has been observed, and specifically in phototrophic mats, migration was found to be related to the present light regime (Garcia-Pichel et al. 1994). In the latter phototrophic mats, *Beggiatoa* were found to

be concentrated in the specific zone where sulfide and oxygen overlap during the daylight period (Teske & Nelson 2006; Hinck et al. 2007). At the nighttime period, however, when oxygen is absent and sulfide concentrations increase, *Beggiatoa* were found to follow the upwards moving sulfide front (Hinck et al. 2007). Although it is clear from previous observations that *Beggiatoa* can likely sense chemical gradients and are possibly able to position themselves in zones of optimal concentrations, it is still not unambiguously resolved what the roles of individual components are, as in natural environments gradients of various compounds (e.g. chemicals) (Nelson & Jannasch 1983; Møller et al. 1985) or other potential triggers (e.g. light) (Nelson & Castenholz 1982) are simultaneously present. Alternatively, as in the above described example of a phototrophic mat during nighttime, it is not clear whether a single compound such as sulfide actually serves as chemical attractant or rather as repellent. It appears here that migratory behavior in relation to a certain chemical may also be concentration dependent.

Besides the to-be clarified role of individual physico-chemical triggers in locomotion behavior, also the actual locomotion mechanism of filamentous *Beggiatoa* is still unclear. Gliding motility for filamentous cyanobacteria has been explained in previous studies by the mechanism of polysaccharide extrusion through junctional pore complexes at the cell surface (Hoiczyk & Baumeister 1998; Hoiczyk 2000). In some earlier studies, also slime excretion by *Beggiatoa*-like filamentous organisms was observed (Faust & Wolfe 1961; Scotten & Stokes 1962; Burchard 1980, Møller et al. 1985), and in one study slime excreted by *Beggiatoa alba* was found to consist mainly of sugars (Strohl & Larkin 1978b). In a more recent study parallel rows of pore-like structures on the outer filament surface of *Beggiatoa* were discovered by electron microscopic analysis (Larkin & Henk 1996). This observation combined with the previously discovered ability of *Beggiatoa* to excrete slime suggests that the locomotion mechanism of these organisms may be similar to those of filamentous cyanobacteria.

The goal of this study was to clarify and quantify the effect of individual potential physico-chemical stimuli on motility behavior and, moreover, to find further evidence for the locomotion mechanism of specific hypersaline *Beggiatoa* strains. To unravel the role of individual potential stimuli on motility behavior, defined single- and multiple stable gradient as well as dynamic gradient experiments were performed. Furthermore, detailed confocal laser microscopic analysis was performed on specific fluorescent marker-stained filaments to clarify their locomotion mechanism.

Material and Methods

Origin of *Beggiatoa* and phylogenetic characterization

The hypersaline *Beggiatoa* strain used in the laboratory-based experiments originated from photosynthetic microbial mats growing in an active saltern (38°51'15 N, 1°23'09 E) of the Balearic island Ibiza, Spain. Sampling took place at the end of October 2005, when the salinity of the saltern pond reached two-fold seawater salinity (~6 %). The *in situ* water temperature at the time of sampling was 18° C. *Beggiatoa* filaments picked from these mats were enriched in agar gradient cultures, using a slightly modified version of (Nelson & Jannasch 1983). The basal medium components of both top and bottom agar were prepared according to Kamp (Kamp et al. 2008). Slight modifications were made in terms of salinity (~56 g/L NaCl) and sulfide concentration of the bottom agar (6 mM Na_2S) in order to adjust to *in situ* conditions. Furthermore, 50 µM nitrate was added to the top agar layer serving as a potential electron acceptor. The cultures were incubated at room temperature (~20° C) and kept in the dark. A specific enrichment obtained from single filaments was chosen as representative strain for the laboratory experiments as this *Beggiatoa* strain grew relatively fast. Analysis of 16S rRNA gene sequences of selected filaments revealed that this *Beggiatoa* strain showed 99 % sequence similarity to a previously characterized hypersaline strain originating from a Spanish hypersaline lake microbial mat, which was incubated in a mesocosm system in our laboratory (Hinck et al. 2007). Both strains are characterized by a typical filament diameter of 7-10 µm, and contain a central vacuole, as well as the ability to store both nitrate and elemental sulfur intracellularly.

Confocal laser scanning microscopy of lectin-stained *Beggiatoa* filaments

The hypersaline *Beggiatoa* were stained with various lectins and studied by confocal laser scanning microscopy (CSLM) for clarification of their locomotion mechanism. Firstly, over 70 different fluorescently-labeled lectins were tested on freshly isolated *Beggiatoa* filaments to select ones that best stained *Beggiatoa* glycoprotein excretions, as we expected that these glycoconjugates are involved in the locomotion mechanism. The lectin DBA (*Dolichos biflorus*-Agglutinin; Sigma-Aldrich), a fluorescein-isothiocyanate conjugate (FITC), showed reproducible fluorescence binding patterns. DBA was previously applied for discriminating A_1 and A_2 blood types as it has an affinity for terminal N-acetyl-α-D-galactosaminyl residues. The stained filaments were analyzed with a TCS-SP1 confocal microscope, controlled by Leica software (version 2.61, build 1537; Leica, Heidelberg). The TCS-SP1 mounted in an upright position was equipped with three different visible lasers. From the laser lines

available, the 488 nm line of the Argon laser was employed for both reflection (480-500 nm) and emission (500-550 nm) imaging of the FITC lectin. A water immersion lens with a 1.2 numerical aperture and a magnification of 63×, corrected for cover slips, was used for imaging. The images were processed with the microscope software, 3-dimensional projections were prepared with Imaris version 5.7.2 (Bitplane, Switzerland), and printed with Photoshop without application of any corrections.

Stable gradient incubations: Qualitative migration experiment

An initial impression of *Beggiatoa* migration behavior was obtained by incubating the filaments in soft agar culture tubes containing either a single- or multiple gradients of potential motility triggers (oxygen, sulfide and nitrate). The soft agar tubes were prepared anoxically in a N_2-flushed glove bag. Anoxic and reduced conditions were further established by the addition of cysteine (2 mM) to the agar. Resazurine, a redox indicator, was supplemented to the agar to visualize possible oxygen introduction during inoculation of the soft agar with *Beggiatoa* filaments. Different sets of gradients were applied in this experiment, either single parameter (set 1-3; introduced to the top-side of the agar tubes) or combined parameters (set 4-5; introduced to the top- and bottom-side of the agar tubes):

1) Oxygen (tubes' top-side exposed to air)
2) Nitrate (2 ml $NaNO_3$ solution added to tube on top of the agar in concentrations of 10 µM, 100 µM and 1000 µM)
3) Sulfide (2 ml Na_2S solution added to top-side of tube in concentrations of 10 µM, 100 µM, 1000 µM)
4) Opposing gradients of oxygen (tubes' top-side exposed to air) and sulfide (final concentration of 6 mM Na_2S in bottom agar plug)
5) Opposing gradients of nitrate (100 µM $NaNO_3$ solution added to top-side of agar tube) and sulfide (final concentration of 6 mM Na_2S in bottom agar plug)
6) Negative biological control (anoxic agar without additions but inoculated with *Beggiatoa*)

Approximately 10-20 *Beggiatoa* filaments were inoculated at a depth of 1 cm below the agar surface, and vertical migration of filaments was visually followed over the following 80 day period, i.e. number and position of filaments relative to the agar surface was determined.

Stable gradient incubations: Quantitative migration experiment

In a follow-up experiment vertical migration of filaments as well as the development of parameter gradients in soft agar culture tubes were quantified over a 40-day period. Again, different sets of soft agar tubes, this time consisting of 3 replicates, were prepared, without additions of cysteine or resazurine. Sets 1-4 represented single parameter gradients:

1) Oxygen (tubes' top-side exposed to air)
2) Blue light (as narrow band imposed from the side)
3) Nitrate (2 ml of 1 mM $NaNO_3$ solution in artificial seawater added to tube on top of the agar)
4) Sulfide (final concentration of 6 mM Na_2S in bottom agar plug)
Sets 5 and 6 represented combined parameter gradients:
5) Opposing gradients of oxygen (tubes' top-side exposed to air) and sulfide (final concentration of 6 mM Na_2S in bottom agar plug)
6) Opposing gradients of nitrate (1 mM solution on top) and sulfide (6 mM in agar plug)

Non-inoculated tubes served as abiotic controls for each set of parameters applied. Like in the first qualitative experiment, the soft agar tubes were again inoculated at a depth of 1 cm below the agar surface with 10 to 20 *Beggiatoa* filaments at the start of the experiment. Vertical filament distribution was determined by visual inspection every 5 to 10 days using a dissection microscope (SV6; Zeiss, Germany). During inspection, number and position of filaments relative to the agar surface was determined. During the incubation period, gradient establishment of parameters was determined after 10, 25, and 40 days incubation. Parameter concentration profiles in tube-replicates were determined only once, i.e. immediately after removal of the tube stopper to minimize effect of exposure to the oxygen-containing atmosphere. Thus, measured tubes were terminated after microsensor analysis as introduced oxygen may have affected further filament migration behavior.

Measurement of compound concentration profiles in stable gradient incubations

Concentration gradients of oxygen, sulfide and pH in soft agar culture tubes were measured using microelectrodes. Compound concentrations profiles of non-inoculated agar tubes (abiotic controls) were measured in parallel with tubes inoculated with *Beggiatoa* filaments. For profile determination, microsensors were mounted on a motorized micromanipulator (Faulhaber; Märzhäuser GmbH) and vertical compound concentration was determined at 500 µm depth intervals. Sensor-operation and signaling-processing was handled by a computer acquisition system (Lab View; National Instruments). Sensor position relative to the agar

surface was determined with the aid of a dissection microscope (SV6; Zeiss, Germany). Microsensors for oxygen (Revsbech 1989), pH (Revsbech et al. 1983), and H_2S (Jeroschewski et al. 1996) had tip diameters of 10-15 µm, response times of less than 20 seconds, and were calibrated according to standard protocols (Kühl et al. 1998; Wieland & Kühl 2000).

Concentration profiles of nitrate was determined according to the VCl_3 (Vanadium (III) chloride) reduction method (Braman & Hendrix 1989) using a chemiluminescence analyzer (Model 42C, Thermo Environmental Instruments Inc., Franklin, Mass., USA). Prior to measurements, nitrate-containing *Beggiatoa* filaments were carefully separated from the agar which was then removed in 10 mm layers from the tubes. The semi-solid slush-agar was subsequently transferred to 5 ml glass vials containing 0.1 ml of 20 % (w/v) ZnAC and closed with a rubber septum (Exetainers, Labco). Slush-agar tubes without nitrate served as negative control in the VCl_3 assay. In order to better compare obtained course profiles with the other compound profiles, nitrate values determined for 10 mm layers were intrapolated to 1-mm layer values by moving average.

To study the potential effect of blue light as a trigger for *Beggiatoa* filament migration, a series of inoculated agar tubes were illuminated from the side at the depth of inoculation (10 mm below agar surface) in a narrow 5-mm band using a blue light LED (wavelength of 445 nm, intensity of 130 µmol photons m^{-2} s^{-1}).

Dynamic gradient incubations

In the above-described soft agar culture tube experiments, compound gradients developed slowly in time (days). However, as in reality some compounds such as sulfide show a much more dynamic, i.e. faster (minutes), change in local concentration in time, dynamic gradient incubation experiments were additionally performed to investigate *Beggiatoa* motility behavior under such conditions. For these experiments flat micro slide glass capillaries (internal dimensions: 8 x 0.8 x 40 mm; VitroCom, Inc., N.J., USA) were used as incubation chambers. Capillaries were filled with anoxic artificial seawater and both open sides were closed with a 1.5% agar plug. The artificial seawater with a salinity of 6% was buffered (50 mM HCO_3^-) to prevent pH changes after addition of sulfide. Several *Beggiatoa* filaments were subsequently inoculated in the central liquid medium by puncturing one agar-filled side of the incubation chamber with a needle through which the filaments were released. Following inoculation, an aliquot sulfide solution (250 µl of a 100 mM Na_2S solution) was introduced at one (left) side into the capillary, however, at the outer side of the 1.5% agar plug. The outer open part of the capillary was again sealed with another 1.5% agar plug (see Figure 6A for a schematic drawing of the capillary set-up). Sulfide, from the concentrated

sulfide solution sandwiched between the two agar plugs, diffuses through the agar plug into the buffered artificial seawater solution, passing in time the location where the *Beggiatoa* filaments were situated after inoculation. The sulfide diffusion process in this case is rather fast (minutes to hours) due to the concentrated sulfide solution applied. The moving sulfide front (and concentration) was monitored by a sulfide microsensor which sensing tip was positioned close to the *Beggiatoa* filaments in the centre of the capillary. The whole setup was placed in a nitrogen-flushed glove bag to maintain oxygen-free medium conditions during all stages of incubation chamber preparation and monitoring of the dynamic sulfide gradient. Chemotactic behavior of several individual filaments in response to a dynamic sulfide gradient was continuously monitored by a CCD (charge-coupled-device) camera attached to a dissection microscope, and was used to record time-lapse videos. The induced migration behavior was analyzed through Image J processing by calculating the center of mass of all filaments in the capillary in single images, choosing the most representative result out of 3 parallels.

Statistical analyses of experimental data

Migration behavior of *Beggiatoa* filaments in the soft ager culture tube experiments (stable gradient incubations) was statistically analyzed by variation partitioning in order to determine which of the applied parameters predominantly affected directional movement. Filament counts at 1-mm depth intervals were square-root transformed to normalize their distribution prior to performing statistical analyses. All measured parameters which potentially affect *Beggiatoa* migration behavior (i.e. the explanatory variables) were determined with one decimal precision. To understand the effects of each individual factor, and combinations thereof, multivariate procedures were applied. For each explanatory variable and its corresponding quadratic value, a stepwise selection procedure was therefore performed to retain only the terms that significantly explain changes in filament counts. Quadratic terms were also considered in the study to make sure that the typical hump-shaped depth distribution of filaments could be modeled by a quadratic transformation of linear relationships (Legendre & Legendre 1998). The ecological interpretation of a significant quadratic term is that the filament distribution would tend to display a local optimum for a specific variable, whereas a linear term would indicate a gradual increase or decrease in the filament distribution. Z-transformations (null mean and unit variance) were applied to all variables before including them in the models and qualitative variables were transformed into a set of dummy variables (Ramette 2007). Variation partitioning was then used to quantify the amount of filament variation that could be attributed to each factor and to factor co-variation,

and to what remained unexplained by the models (i.e. residuals) (Legendre & Legendre 1998; Ramette & Tiedje 2007). The method enables the assessment of respective effects of explanatory variables when other variables in the experiment are controlled for (i.e. taken into consideration as co-variables). The significance of the partial effects of each explanatory variable was assessed by 1000 permutations of the data under the full-model assumption. Co-variation values were quantifiable, but not testable for significance (Legendre & Legendre 1998). All statistical analyses were done with the R package *vegan* (http://cran.r-project.com).

Nucleotide Sequence accession number

The nucleotide sequence of the *Beggiatoa* species described here (Ibiza, 6%, enrichment culture [I6C]) has been deposited in GenBank under accession number EU 919200.

Results

Locomotion mechanism

Confocal laser scanning micrographs of the DBA-FITC lectin-stained *Beggiatoa* revealed patterns of glycoconjugates excreted from the surface of the filaments (Figure 1). The fluorescently labeled exoglycoconjugates appeared as dots or slime jets positioned in spiral patterns on the filaments surface (1A-D). The dots likely indicate pore openings for slime secretions. Spacing between the slime jets and pores was approximately 2 to 4 µm. The angle of the spiral pattern was approximately 100° and 120° for filaments visualized in Figure 1A and 1B and between 130° and 150° for those depicted in Figure 1C. Glycoconjugate accumulation at the opposite end of the filament probably reflects the direction of movement. In Figure 1D, a slime trail left behind on the microscopic slide is visible above the filament. Figures 1B and 1C-2 depict 3-dimensional reproductions of Figure 1A and 1C-1 respectively.

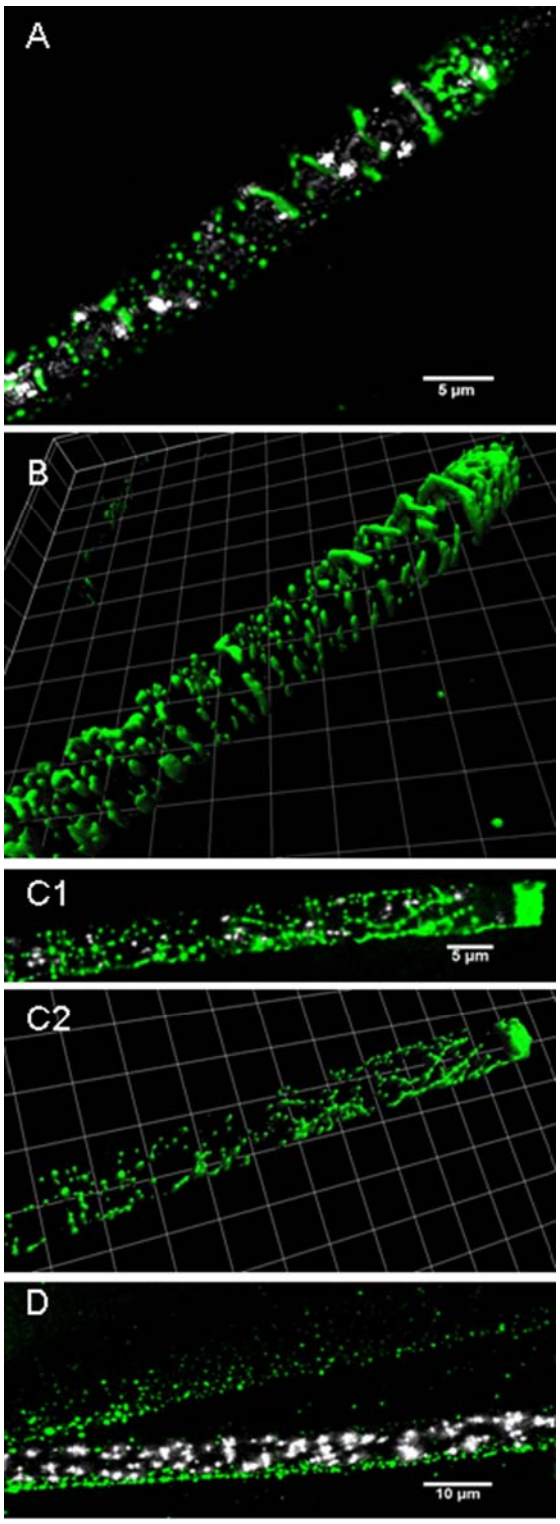

Figure 1: Confocal laser scanning micrographs of the hypersaline *Beggiatoa* strain used in this study fluorescently stained with DBA-FITC lectin (green). White coloration: reflection of sulfur globules. (A): DBA-FITC lectin showing spiral pattern of exoglycoconjugates on filament surface. (B): Movement direction indicated by accumulation of exoglycoconjugates at filament's end. 3-dimensional image by imaris of same filament as (A); length of one square equals 5 µm. (C): Different filament with DBA-FITC lectin pattern showing accumulation of exoglycoconjugates at filament end. (C1): overlay image. (C2): 3-dimensional image. (D): Gycoconjugates footprint (upper part of picture) left by moving *Beggiatoa* filament (lower part of picture).

Migration behavior

In a previous study, vertical migration behavior of a phylogenetically closely related hypersaline *Beggiatoa* strain during a light/dark diurnal period in an intact microbial mat was followed (see Figure 2 for a summary of those results). It was observed in that study that during illumination the majority of the population was positioned at a depth of 8 mm, at the interface of the overlapping oxygen and sulfide profiles. In dark mats, however, filaments

were found positioned 2 mm closer to the sediment surface, still being at the front of the sulfide profile where sulfide concentration was between 0-10 µM, but away from oxygen what was retracted almost completely to the sediment surface.

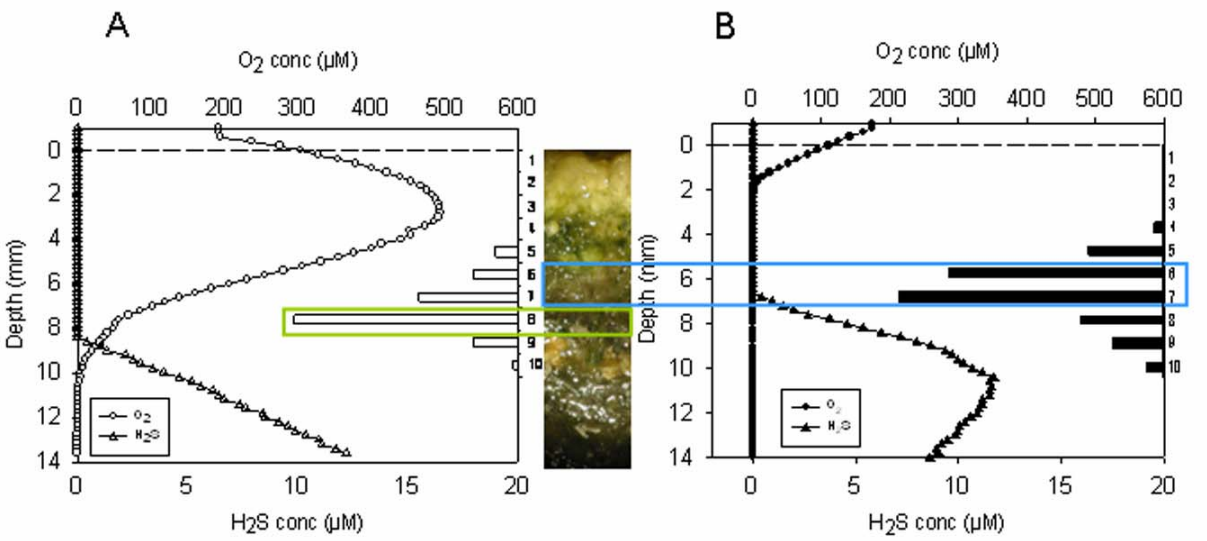

Figure 2: Depth distribution of *Beggiatoa* filaments (frequency in %, without scale) and oxygen and hydrogen sulfide concentrations in an intact illuminated (A; 14h at a light intensity of 500 µmol photons m^{-2} s^{-1}) and dark (B; 10h in the dark) incubated microbial mat. Highest filament frequencies in illuminated mat occur at 8 mm depth (50% of total), while dark incubated mats showed highest filament abundances at a depth of 7 mm (40% of total). The green rectangle in the cross-section of a mat sample indicates the location of main *Beggiatoa* abundances in illuminated mats. The blue rectangle shows the position of highest filament frequencies in dark mats, showing that *Beggiatoa* migrated 1-2 mm upwards during the dark period.

Static gradient experiments: Qualitative migration experiments

The long-term experiment in which vertical filament migration over an 80 day period in soft agar was followed revealed different responses to oxygen, nitrate and sulfide when added as single parameter. Downward diffusing oxygen always resulted in a negative (repelling) response, while downward diffusing nitrate caused a positive (attracting) response to all concentrations tested (10, 100 and 1000 µM). Sulfide, however, resulted in a bivalent response as filaments appeared attracted, i.e. moved upwards, to a low (10 µM) sulfide concentration, but were repelled, i.e. moved downwards, by higher downward diffusing sulfide concentrations (100 µM and 1 mM). When sulfide was diffusing upwards from the bottom agar plug, migration patterns were reversed, indicating that migration was influenced by sulfide, rather than simply by gravity. Opposing gradients of oxygen and sulfide or nitrate and sulfide resulted both in the establishment of a confined band of filaments at a specific depth of the soft agar tube. In control tubes (no parameter introduced) filament distribution over time appeared random.

Static gradient experiments: Quantitative migration experiments

In follow up experiments migration behavior in relation to compound diffusion direction and concentration profiles was quantified. The establishment of compound gradients in soft agar tubes was followed during a 40-days incubation period.

Oxygen, as single parameter applied, induced a pronounced impact on migration behavior, as it resulted in a clear negative (repelling) response (Figure 3A). At the end of the incubation period, filaments had accumulated near the bottom of the agar tube, at depths between 40 and 50 mm, still exposed to an oxygen concentration of about 40 µM.

Blue light introduced at the inoculation position also acted as a repellent, as filaments migrated to depths comparable to those of the oxygen treatment (Figure 3B).

In the anoxic control tubes some oxygen intrusion during the experimental period was observed, i.e. oxygen was found down to a maximal depth of 5 mm (Figure 3C). This unintended but minimal oxygen intrusion was likely a methodological artifact caused by opening of tubes needed for microsensor measurements. In all replicates, however, before microsensor measurements started all filaments were located below the top 5-mm zone in the deeper anoxic zone of the tubes, and this zone their distribution appeared random.

In the soft agar to which nitrate was applied as single parameter, nitrate appeared homogeneously dispersed over the medium and thus did no longer formed a gradient after the 40-day incubation period (Figure 3D). Here, like in the control treatment, filament distribution was random over the tube. During intermediate periods of this treatment (10 and 25 days incubation), however, nitrate gradients were detectable with filaments located mainly at concentrations between 130 and 150 µM (data not shown). A steady decrease in nitrate concentration in the soft agar medium was observed over the incubation period, resulting in only relatively low nitrate values after 40 days.

An important observation, in the control and all the latter three single-parameter incubations, was that the majority of the filaments of the initial inoculate disintegrated and died during the incubation period. This is depicted in the graphs (Figures 3-5) by small cross sizes, which indicate low abundances.

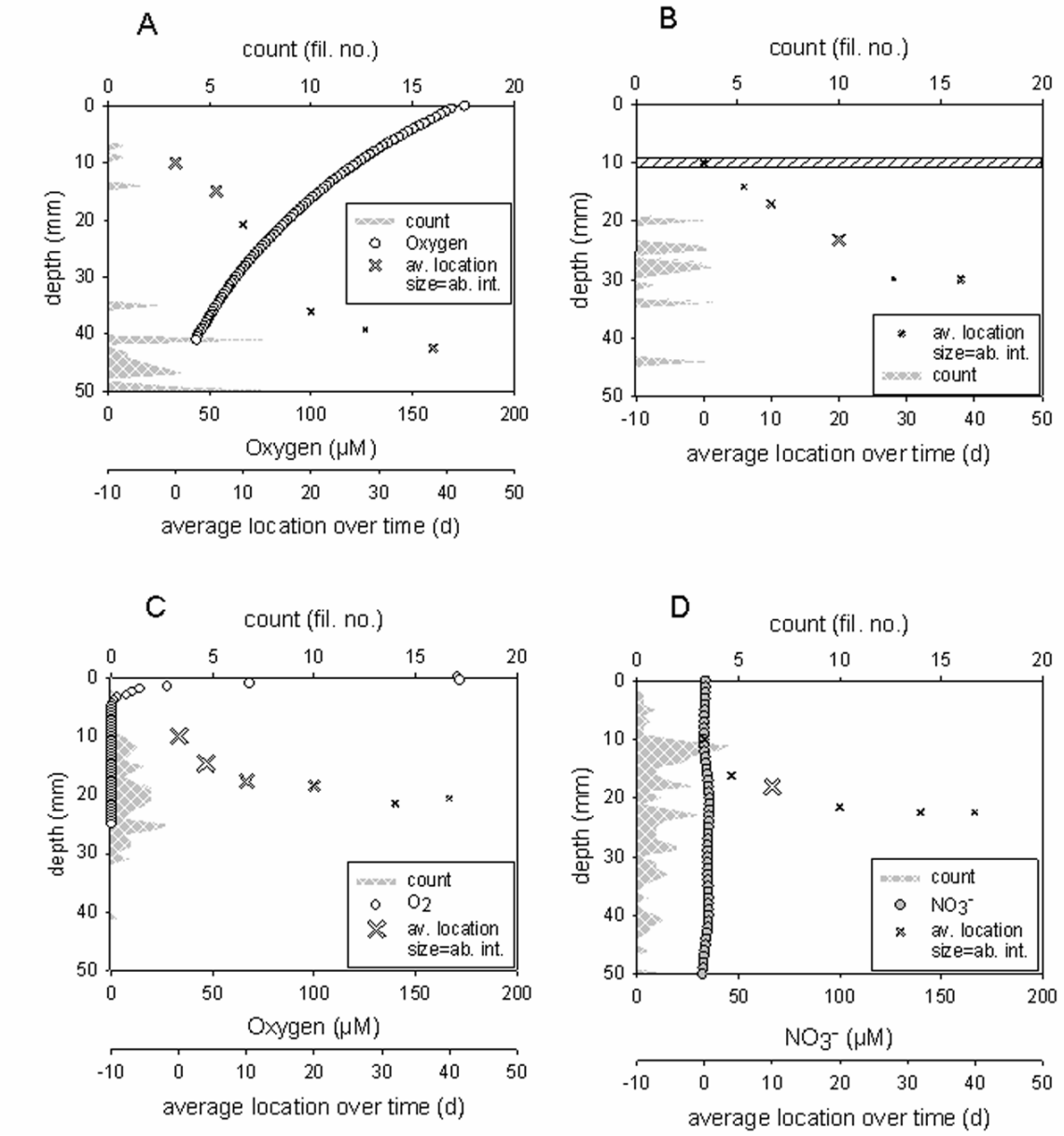

Figure 3: Single gradient incubation experiments in agar tubes. Parameters determined after 40 days inoculation with *Beggiatoa* filaments. Shaded area represents the vertical distribution of filaments after 40 days. Crosses represent the average depth of filaments and their abundance intensity (indicated by relative cross size) in time. (A) Effect of oxygen. (B) Blue light (445 nm, 130 µE). (C) Control (no trigger applied / anoxic medium) (D) Nitrate.

The filament migration behavior in the sulfide treatment (applied via the bottom agar plug) was different. During the first 10 days of incubation filaments migrated downwards after which they formed a confined band at a depth of 20 mm (Figure 4). Microsensor measurements revealed that the H_2S concentration at the exact position of the band was low, i.e. between 0 and 15 µM. With increasing incubation time, sulfide concentration decreased, resulting in a further downward migration of filaments. Final observations and microsensor measurements after 40 days incubation showed that most filaments were still aggregated but in a somewhat wider layer where sulfide concentrations amounted to 10 µM. Interestingly,

also filament abundance had increased by that time. The latter phenomenon can be explained by growth on sulfide, what can be oxidized by filaments using intracellular-stored nitrate (already present at the time of inoculation) as electron acceptor.

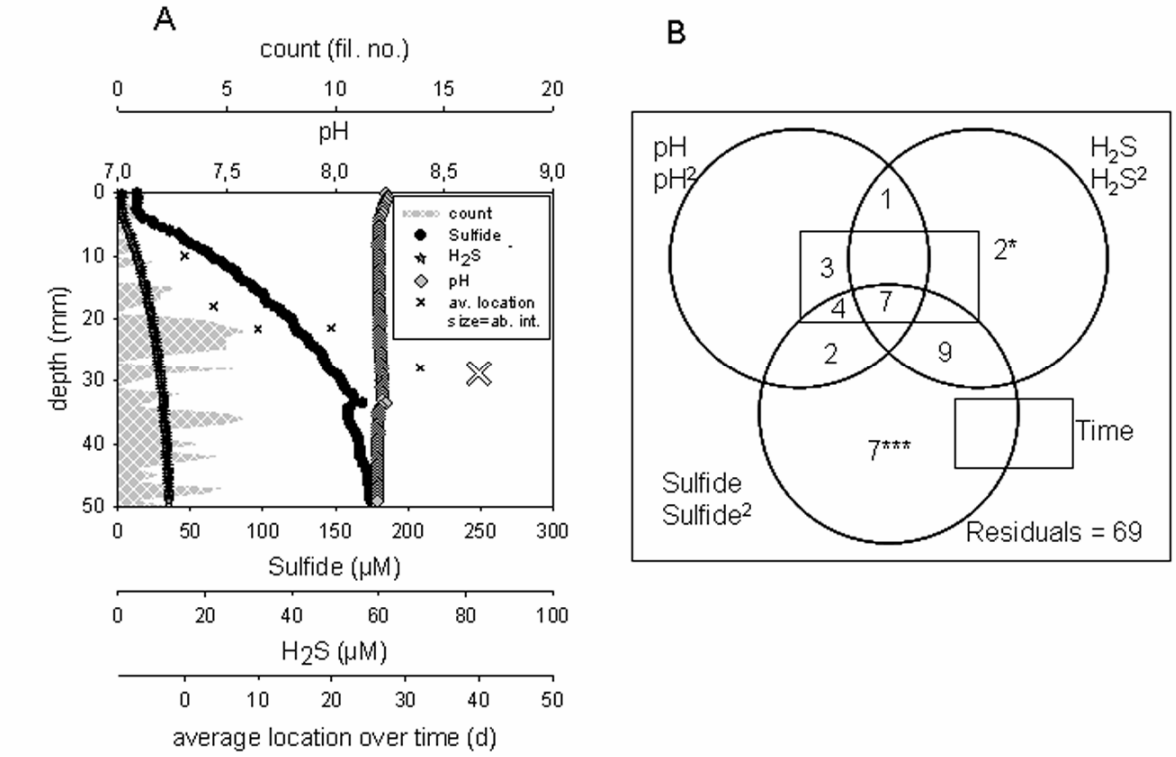

Figure 4: (A) Single gradient incubation experiments in agar tubes: effect of sulfide after 40 days incubation. Sulfide was added to the bottom agar plug (symbols as in Fig. 3). Both H_2S and pH were determined to allow calculation of total sulfide ($H_2S + HS^- + S^{2-}$). (B): Statistical analysis of sulfide by variation partitioning. The graph represents 31% of the biological variation that is partitioned into the respective parts of each factor and of their covariation (overlapping circles). The fourth factor, time, is indicated by two squares, representing the overlap between its pure fraction (not significant for this calculation) and its covarying fractions with the other factors of the analysis. Significance of the variation of pure fractions were tested by 1000 permutations of the data, and are represented for $P<0.05$ (*) and $P<0.001$ (***).

When combined parameters were applied, migration behavior and growth of filaments appeared similar as in the sulfide treatment. When sulfide was supplied via the bottom agar plug and oxygen diffused downwards from the top of the tube, filaments formed a band in the 10 to 30 mm depth zone, while their number simultaneously increased (Figure 5A). Microsensor measurements showed that H_2S concentration in this zone was low, with values only up to 5 µM, corresponding to a total sulfide concentration of about 50 µM.

When nitrate (from above) and sulfide (via the bottom agar plug) were simultaneously applied, filament migration behavior was comparable to that of the oxygen-sulfide treatment (Figure 5C). Over the entire incubation period, filaments positioned themselves at a depth (descending over time) where H_2S concentration was between 1 and 15 µM. H_2S and total sulfide decreased to concentrations below 5 µM and 20 µM respectively after 40 days incubation.

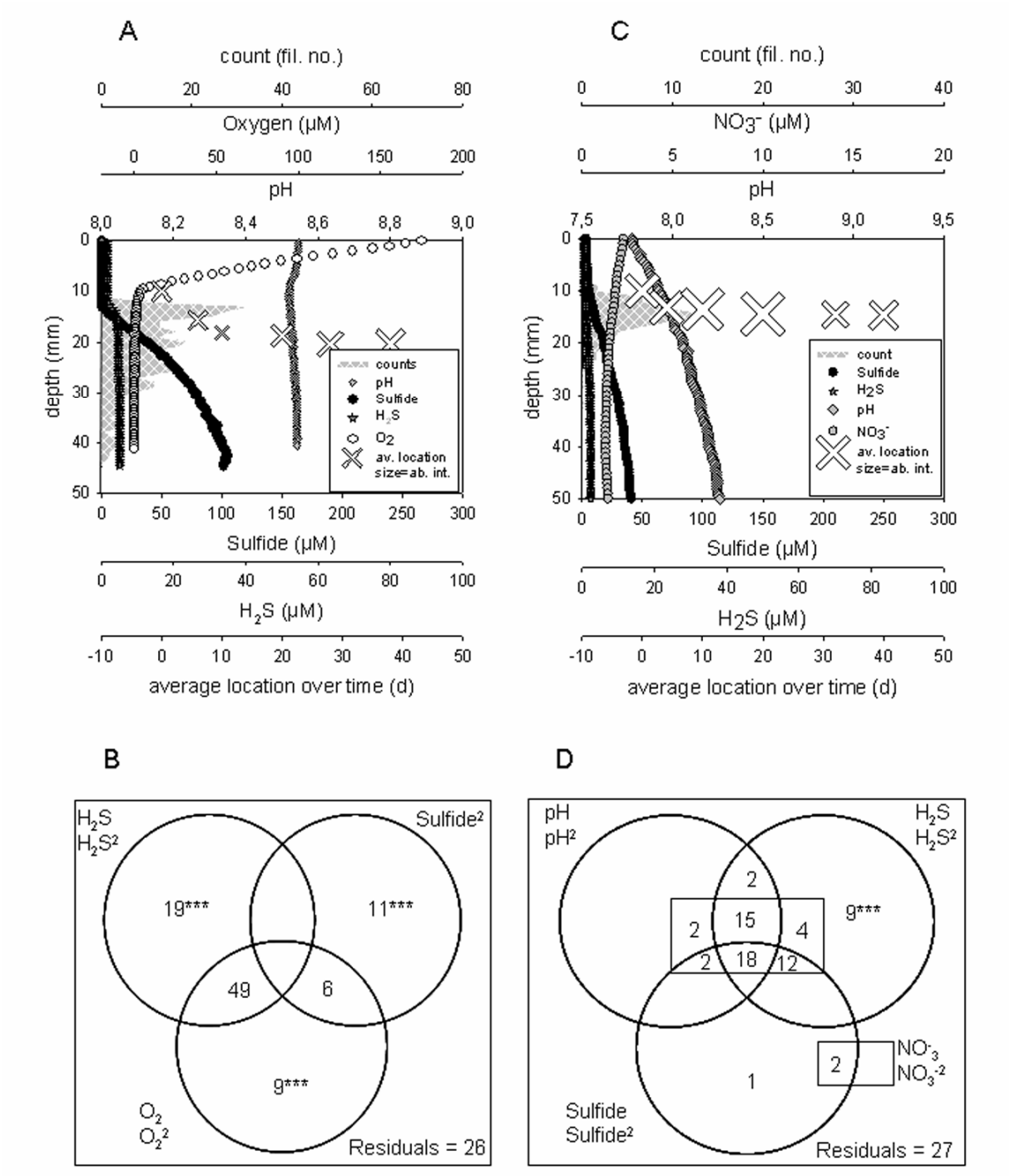

Figure 5: Combined gradient incubation experiments in agar tubes: sulfide, oxygen and nitrate concentration profiles determined after 40 days incubation (symbols as in Fig. 3). (A) Sulfide added from below, and oxygen diffusing downwards from above. (B) Statistical analysis done by variation partitioning of incubation conditions in (A). (C) Sulfide added from below, and nitrate added from above. (D) Statistical analysis done by variation partitioning of incubation conditions in (C). *P* values for each respective factor are indicated when $P<0.001$ (***).

Statistical analyses

To clarify the respective roles of each applied factor in directional movement, the variation of filament spatial distribution was partitioned as a function of potential environmental triggers (Figures 4B, 5B and 5D). A variation partitioning graph represents the

theoretical 100 % of the biological variation that is partitioned into the respective parts of each factor and of their co-variation (overlapping circles). If a fourth factor is present, it is indicated by two squares representing the overlap between its pure fraction and its co-varying fractions with the other factors of the analysis (e.g. Figure 4B and 5D).

When applied as a single parameter, sulfide in the form of H_2S appeared to have a small but significant impact on migration as it could explain 2% of the filament distribution pattern, whereas total sulfide ($H_2S + HS^- + S^{2-}$) could explain 7% (Figure 4B). In the oxygen plus sulfide treatment, however, 74% of the variation in filament distribution could be attributed to the combined presence of oxygen and sulfide (Figure 5B). Here, H_2S appeared the most significant trigger affecting chemotactic response (19%), followed by total sulfide (11%), and oxygen (9%). In the nitrate plus sulfide treatment, H_2S also appeared the main factor influencing migration behavior. Although nitrate, pH, and total sulfide were individually selected along with their quadratic terms, when the factors were all conjointly analyzed with H_2S, they did not show significant effects (Figure 5D). Most of the variation was significantly attributed to H_2S, suggesting H_2S as the main factor affecting *Beggiatoa* distribution pattern among the four factors considered in this treatment. Noticeably, the high level of co-variation between pH, H_2S, sulfide and nitrate (57%) does indicate that filament distribution was likely also influenced by the other factors besides H_2S.

Dynamic gradient experiments

The microslide glass capillary incubation chamber system appeared particularly useful to determine the threshold concentration that induced motility reaction and direction of *Beggiatoa* filaments. Figure 6A represents a schematic drawing of the set-up and indicates the direction of movement (towards sulfide source: -µm; away from sulfide source: +µm) of filaments relative to the sensor tip, which was set to position 0 µm. A relatively fast moving sulfide front with concomitant higher sulfide concentrations resulted, 3 hours after the addition of sulfide, in a movement of filaments away from the sulfide source (Figure 6B). In contrast, a slower moving sulfide front with lower sulfide concentrations resulted, during the first 1.5 hour incubation period, in an immediate movement of filaments towards the sulfide source (Figure 6C), where after filament migration rate slowed down substantially. The position of the microsensor in the incubation chamber allowed tracking of the sulfide concentration increase over time. The direct observation of migration direction and speed of *Beggiatoa* in relation to changing sulfide concentrations revealed a concentration dependent switch in migration direction. A H_2S concentration below 5 µM induced movement towards the sulfide source while a concentration above 25 µM caused movement away from the

source. This experiment thus convincingly showed that sulfide can act, depending on its concentration, both as chemo-attractant and chemo-repellent.

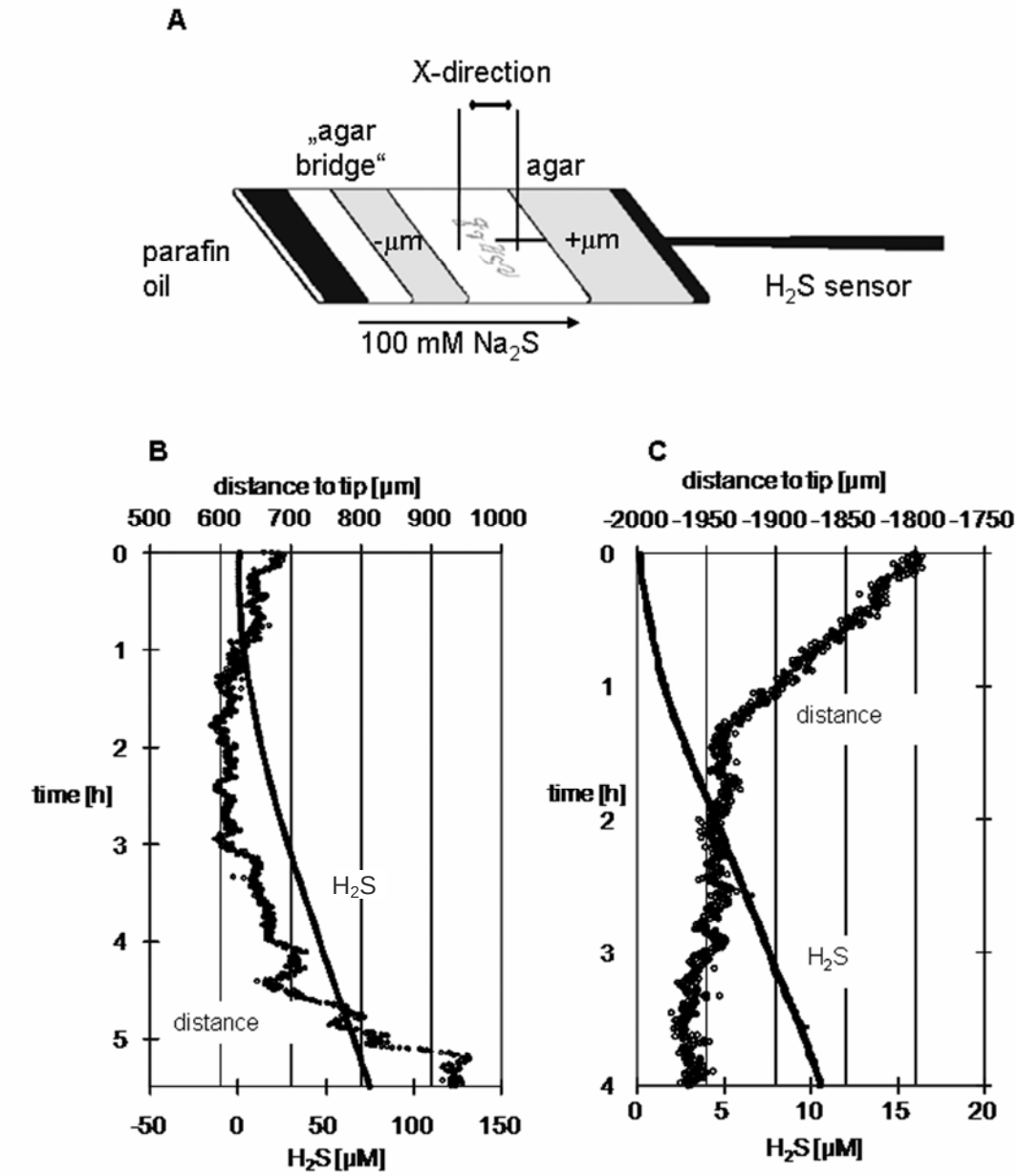

Figure 6: Image analysis of dynamic gradient experiment. (A) Schematic drawing of experimental setup; H_2S sensor tip set to position 0 µm. The distance of the filaments to the tip is defined by the x-direction. (B and C) Measured H_2S concentration at sensor tip and position of *Beggiatoa* filaments in time relative to tip position. Positive distance means that *Beggiatoa* are positioned to the right of the sensor tip, away from the moving sulfide front (see figure A). As sulfide front approaches sensor tip from the left (see figure A), the actual sulfide concentration experienced by *Beggiatoa* is somewhat lower in (B) but higher in (C) than recorded at the location of the sensor tip. (B) Relatively fast moving sulfide front due to relatively high H_2S concentration applied. (C) Relatively slow moving sulfide front due to relatively low H_2S concentration applied.

Discussion

In a previous study it was hypothesized that slime excretion through specific pores found on the surface of *Beggiatoa* filaments and which appeared to be placed in a spiral manner around the filaments could provide the driving force needed for directional movement (Larkin &

Henk 1996). In the present study we found exoglycoconjugate-based slime spirals on the surface of hypersaline *Beggiatoa* filaments after successful staining with fluorescently labeled *Dolichos biflorus*-Agglutinin lectins (Figure 1A, B and C). These results represent the first visual evidence of extracellular deposition by *Beggiatoa* gliding on a solid surface which until now has only be shown for filamentous cyanobacteria (Hoiczyk & Baumeister 1998) and unicellular myxobacteria (Wolgemuth et al. 2002). In a recent meta-genomic research (Mußmann et al. 2007) found homologous parts in the genome of a *Beggiatoa* strain and that of several filamentous cyanobacteria (*Nostoc* sp., *Anabaena* sp., *Trichodesmium* sp., *Hahella chejuensis*) which may encode for proteins involved in the extrusion of extracellular glycoconjugates. In cyanobacteria, the thrust for translocation is presumably generated by steady secretion of mucilage, whereas the surface protein oscillin creates specific surface topographies influencing the mode of rotation (Hoiczyk & Baumeister 1997). The directional motion of the cyanobacterium *Anabaena variabilis* caused by sideways bending and looping (Hoiczyk 2000) was also observed for hypersaline *Beggiatoa* in this study. The footprint stain image (Figure 1D) shows lateral secretion of mucilage by the filament, presumably in preparation for side-way bending. Genetic parallels to filamentous cyanobacteria (Mußmann et al. 2007) suggest that glycoconjugate production and extrusion by *Beggiatoa* is driven by proton motive force.

While the mechanism of motility in *Beggiatoa* is thus likely based on exoglycoconjugate-based slime extrusion, the reason for migratory behavior is likely the need to follow the often moving oxygen/sulfide interface zone in sediments as both, sulfide as electron donor, and oxygen as electron acceptor, can support growth (Jørgensen & Revsbech 1983; Nelson et al. 1986a; Nelson et al. 1986b; Richardson 1996; Macalady et al. 2006). However, if nitrate is present as an alternative electron acceptor, either in sediment pore water (Sayama 2001; Mußmann et al. 2003; Preisler et al. 2007) or internally stored in vacuoles (McHatton et al. 1996), *Beggiatoa* can oxidize the upward diffusing sulfide independently of fluctuating oxygen gradients. As migratory behavior has also been observed in anoxic sediments where *Beggiatoa* commute between deeper positioned sulfidic zones and shallower positioned nitrate-containing zones, we hypothesized in this study that besides sulfide and oxygen probably also other environmental parameter gradients can guide directional movement. As a model environment we studied intact photosynthetic microbial mats originating from a permanent hypersaline lake in Spain (La Salada de Chiprana) as here, besides oxygen and sulfide (Figure 2), also nitrate and light as potential migratory triggers are present. To determine the potential and to identify the relative importance of the four aforementioned parameters, the motile behavior of *Beggiatoa* in artificial gradients of

particularly single parameters was experimentally determined, as the presence of multiple parameters (as in the environment) may hamper identification of the role of individual parameters. The *Beggiatoa* strain used in this study appeared phylogenetically closely related (99% 16S rRNA sequence similarity) to the dominant one in the lake Chiprana microbial mat previously investigated (Hinck et al. 2007). Moreover, as both types featured similar filament diameters and internal sulfur and nitrate storage capacities, we assumed that chemotactic responses of both *Beggiatoa* types are similar and, consequently, that the results of the laboratory experiments of this study can be used to explain migration behavior observed in the intact microbial mat.

Oxygen as a single parameter provoked a strong negative response, as it repelled filaments, causing downward migration. In a previous study, the tolerated oxygen tension of *Beggiatoa* was shown to be less than 5% of atmospheric saturation (Møller et al. 1985). Although oxygen can act as an electron acceptor during oxidation of reduced sulfur compounds, a too high concentration is apparently not tolerated. Oxygen, or concomitantly occurring high concentrations of oxygen radicals, may inhibit physiological processes, classifying these *Beggiatoa* as micro-aerophilic organisms. A similar negative response in our study was observed when blue light was applied as a single factor. As visible light in the blue region is unlikely to pose direct negative effects on physiological processes, it seems more likely that *Beggiatoa* use it for orientation or alternatively as indicator for possible simultaneous presence of damaging UV radiation. When sulfide and/or oxygen are absent for orientation, *Beggiatoa* may use the blue light gradient in the search for electron donors or acceptors, e.g. deeper positioned sulfide or shallower positioned nitrate or oxygen. Nelson & Castenholz (1982) suggested that cytochromes may be likely candidates responsible for sensing blue light, as these feature major absorption peaks at 430 nm in the blue light range.

That nitrate can play a role as chemo-attractant was also previously shown for *Thioploca* sp., i.e. filamentous sulfur bacteria phylogenetically closely related to *Beggiatoa* (Zopfi et al. 2001). Analogous to blue light, nitrate could also act as a single guiding parameter in sediment zones where other triggers are absent. This situation occurs typically in subsurface zones of deep sea sediments where *Beggiatoa* proliferate, in between oxic surface- and deeper positioned sulfidic zones (De Beer et al. 2006).

The distinct role of sulfide as chemo-tactic trigger has been proposed in previous studies. However, in studies where filaments occurred in an environment with a steep sulfide gradient, sulfide was defined as a chemo-repellent for *Beggiatoa* as well as for *Thioploca* species (Hüttel et al. 1996; Preisler et al. 2007). Other studies showed however that sulfide could potentially also act as a chemo-attractant as filaments inhabited zones with an

oxygen/sulfide counter-gradient (Nelson & Jannasch 1983; Nelson et al. 1986a). In order to study the exact role of sulfide, we analyzed migration behavior of *Beggiatoa* filaments in both static and dynamic gradients. Applied variation partitioning analysis on data obtained from static gradient experiments indicated sulfide as the most important migration-determining factor among the ones tested. Moreover, the latter experiments also suggested a concentration dependent behavior towards sulfide, i.e. movement towards low sulfide concentrations and away from high sulfide concentrations. This effect could successfully be quantified in the dynamic gradient experiments as filaments were attracted to concentrations up to 5 μM but repelled by concentrations over 25 μM H_2S. Particularly the latter experiments clearly revealed that sulfide can act both as attractant and repellent and that the effect on *Beggiatoa* movement is thus concentration dependent.

The clarification of the role of the individual physico-chemical parameters oxygen, sulfide, nitrate and blue light as triggers to induce directional movement now helps to further explain the migration behavior of *Beggiatoa* in various natural environments. In the intact hypersaline mat depicted in Figure 2, *Beggiatoa* remained positioned during both the day and night at the sulfide front in a concentration range between 0 and 10 μM H_2S. In this zone, sulfide acted as electron donor while either oxygen (day) or internally stored nitrate (night) acted as electron acceptors. Sulfide was thus the most important chemo-tactic trigger, enabling the filaments to position themselves at the optimum depth that would allow growth during both day and night. However, in other natural environments, a suitable electron donor and acceptor may not be simultaneously present. This would force *Beggiatoa* to migrate to overcome the spatial distances between the two. In the intermittent zone where neither sulfide nor oxygen is present for orientation, another trigger is needed. Nitrate can play such a role in deep sea sediments (De Beer et al. 2006) and blue light in illuminated environments (Nelson & Castenholz 1982).

The conclusion of the present study is that the tested parameters, oxygen, sulfide, nitrate and blue light, can individually affect *Beggiatoa* migration behavior. In the presence of only one of these parameters, filaments can still orientate themselves and migrate in the direction of the missing parameter needed for their metabolism. If oxygen and sulfide co-occur as opposing gradients, filaments are likely repelled by both substrates when they appear in high concentrations. In such situations, *Beggiatoa* would form a small but spatially restricted band in the zone where both parameters overlap and occur at lowest concentrations.

Acknowledgements

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Chapter 5

Cultivation and identification of a monophyletic cluster of hypersaline *Beggiatoa* and their associated bacteria

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This manuscript is in preparation for submission

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Abstract

In this study we compared morphological and physiological characteristics of several hypersaline filamentous sulfur-oxidizing *Beggiatoa* spp. from various habitats and established their 16S rRNA sequence-based phylogenetic relationship. All species were morphologically very similar, with filament diameters between 6 and 14 µm, and all had intracellular vacuoles. However, isolates grown in enrichment culture revealed different requirements for salinity in accordance with their respective origins which varied from moderate (6 %) to highly (15 %) hypersaline. Filaments contained internal nitrate and elemental sulfur concentrations of 4 and 250 mM, respectively. These concentrations were strongly depending on the microenvironment, and in artificial gradient cultures increased drastically. The investigated strains are phylogenetically closely related at the 16S rRNA gene level (> 96 % sequence identity) and form a distinct cluster within the phylum of sulfur-oxidizing gamma-Proteobacteria comprising genera such as *Beggiatoa*, *Thioploca*, *Thiotrix* and *Thiomargarita*. We propose the genus name '*Candidatus* Halobeggiatoa' for the hypersaline members of this monophyletic cluster. One of the two sub-branches of which the monophyletic cluster is composed includes strains originating from the Mexican Guerrero Negro microbial mats, while the other comprises strains from diverse Spanish microbial mats. Fluorescence *In situ* hybridization (FISH) using specific probes could discriminate between members of the two sub-branches and confirmed the *In situ* occurrence of the isolated strains in the original mat. We also investigated the diversity of bacteria that live attached to *Beggiatoa* filaments. The community of these associated bacteria was highly diverse and dominated by gamma-Proteobacteria.

Introduction

Members of the genus *Beggiatoa* and *Thioploca* are among the largest and most conspicuous bacteria in nature (Schulz & Jørgensen 2001). These multicellular, filamentous gamma-Proteobacteria often form extensive mats with a whitish appearance due to intracellularly stored elemental sulfur (Gallardo 1977; Jannasch et al. 1989; McHatton et al. 1996; De Beer et al. 2006). *Thioploca* and *Beggiatoa* filaments can be morphologically distinguished from each other by the characteristic polysaccharide sheath surrounding several individuals of *Thioploca* trichomes. Whereas large marine *Beggiatoa* often form dense mats at the surface of organic rich sediments, they are also found embedded in the matrix of photosynthetic, microbial mats where they experience diurnal dynamics of oxygen and sulfide concentrations (Garcia-Pichel et al. 1994; Hinck et al. 2007). Although *Beggiatoa* were previously classified

into individual species based on filament diameter size (Leadbetter 1974), natural populations with overlapping filament diameters have shown that this characteristic alone is probably not sufficient to allow species differentiation (Jørgensen 1977; Mußmann et al. 2003). Therefore, in other studies also characteristics like presence of intracellular vacuoles, the ability to oxidize sulfide with concomitant intracellular sulfur storage, and capability to use nitrate in addition to oxygen as terminal electron acceptor were considered (Nelson et al. 1986b; McHatton et al. 1996; Sayama et al. 2005; Kamp et al. 2006). Particularly vacuole formation and the ability to accumulate nitrate in these vacuoles, appeared as a suitable discriminative feature. Vacuolated nitrate-accumulating, large (>9 µm) marine autotrophic *Beggiatoa* species are abundant; however, these remain uncultured so far. In contrast, non-vacuolated, narrow (<9 µm) autotrophic and heterotrophic freshwater strains have been studied in culture (Nelson & Castenholz 1981a; Mezzino et al. 1984; Kamp et al. 2006; Nelson & Jannasch 1983; Hagen & Nelson 1996). Ahmad et al. (2006) recently suggested that two non-vacuolated narrow facultative and obligate autotrophic marine strains (MS-81-6 and MS-81-1c), which have been isolated before (Nelson & Jannasch 1983; Hagen & Nelson 1996), form the root of the *Beggiatoa* clade from which larger and also vacuolated strains evolved.

Hypersaline *Beggiatoa* have been observed in a variety of environments (Garcia-Pichel et al. 1994; Santelli et al. 2006; Sorokin et al. 2006), however, only few are described in more detail (Hinck et al. 2007). Axenic cultures are difficult to obtain due to associated bacteria that are firmly attached to the slow-growing *Beggiatoa*. These attached bacteria hamper the physiological characterization of picked and cleaned filaments thus observed biochemical processes (such as denitrification, dissimilatory nitrate reductase to ammonium, DRNA, under anoxic conditions) could in fact have been mediated by the associated bacteria. In this study both *Beggiatoa* and their satellite bacteria from a variety of hypersaline environments have been phylogenetically characterized. Main goal besides establishing phylogenetic relationships was to morphologically characterize hypersaline *Beggiatoa* originating from distinct locations and to determine whether these support a specific group of satellite bacteria. As the investigated hypersaline *Beggiatoa* formed a phylogenetic coherent and monophyletic cluster we here propose the new genus name '*Candidatus* Halobeggiatoa' for its members.

Material and Methods

Characterization of strains

Individual *Beggiatoa* filaments from different hypersaline habitats were picked, enriched in culture, and analyzed. The denomination of obtained enriched strains used for this study is

listed in Table 1. The strains 'Mesocosm 8%' (M8) and 'Mat 10' (M10) were obtained from intact microbial mat samples (named M8M and M10M) and originated from the hypersaline lake 'La Salada de Chiprana' located in Northeastern Spain. The mat termed M8M consisted of multiple layers of different functional groups of microbes interspersed with calcium carbonate crystalline precipitates. Mat M10M was located near the inflow of a freshwater channel and was highly sulfidic, as indicated by blackening by FeS, and overlaid with a dense layer of filamentous cyanobacteria. Lake Chiprana had an average salinity of ~8%, with an ionic composition dominated by magnesium sulfate (magnesium sulfate equated 70% of total salinity). *Beggiatoa* filaments were also picked from intact microbial mats (mat I6M and mat I15M), which are located in an actively operated saltern on the Spanish Balearic Island, Ibiza, with different salinities, 6 and 15 %, respectively. Mat I6M and mat I15M were sampled from two different ponds of the saltern, at different stages of evaporation. The neighboring island of Ibiza, Formentera also harbors an inactive saltern. Here, *Beggiatoa* were picked from microbial mats located close to 'Playa de Levante' in the North of Formentera. *Beggiatoa* originating from the Mexican Guerrero Negro saline ponds (Baja California Sur) were recovered from a microbial mat mesocosm system at the NASA Ames Research Center (CA, USA). Filaments from all mats were hand-picked in the laboratory using a dissection microscope and a sterile needle. The diameter of each filament was determined with the aid of a microscope. Internal nitrate and elemental sulfur content of selected strains was determined by stable isotope analysis and high-performance liquid chromatography (HPLC) as previously described by (Hinck et al. 2007).

Table 1: Habitat characteristics and description of vacuolated hypersaline *Beggiatoa* species used in this study. All hypersaline organisms were extracted from phototrophic microbial mats and were grown in a similar gradient media with adapted salinity values.

Bacterium	Filament width (µm)	origin		internal
		site of isolation	salinity (%)	NO_3^- / S^0 (mM)
M8M M8C	6-8 10	mesocosm mat (Chiprana, Spain) (41°14'30N 0°10'50W)	8	4 / 252 (M) [a] 430 / 6 (C) [a] +/- (90 / 3)
M10M [b] M10C	NA [c] 9-10	microbial mat Chiprana, Spain (41°14'27N 0°11'05W)	8	ND [c]
GN8M GN8C	9-10 7-8	microbial mat Guerrero Negro, Mexico (27°57'24N 114°04'26W)	8	ND [c]
I6M I6C	6-8 8-9	solar saltern, Ibiza, Spain (38°51'15N 1°23'09E)	6	470 / 11 [a] +/- (280 / 6)
I15M I15C	9 10	solar saltern, Ibiza, Spain (38°50'51N 1°23'56E)	15	650 / 25 [a] +/- (190 / 9)
F15M F15C	NA [c] 10-14	inactive saltern, Formentera, Spain (38°44'21N 1°26'09E)	15	ND [c]

[a] Internal nitrate and elemental sulfur concentrations are available for specific enrichment cultures (Ibiza 6%, Ibiza 15%), and for mesocosm mats and cultures thereof; values in brackets show the standard deviation for internal nitrate/ elemental sulfur values.
[b] First letter indicates the origin: M=mesocsom, GN=Guerrero Negro, I=Ibiza, F=Formentera; the number designates the salinity, and 'M' or 'C' after the number denotes for mat or culture, where the filaments have been extracted. 'M10' specifies a certain location at the lake Chiprana, Spain, which has 8% salinity.
[c] ND, not determined. NA, not available.

Growth media, isolation, and enrichment culture

Beggiatoa filaments were picked from intact mats using a sterile needle and inoculated in gradient media for enrichment cultivation. The agar gradient media was composed of two medium components, the top and bottom agar respectively, prepared with slight modifications of the recipe described by Nelson & Jannasch (1983). Adjustments comprised changes in salinity, sulfide concentrations in the dense bottom agar (6 mM Na_2S), and 50 µM nitrate addition to the top slush agar. The gradient medium was prepared in screw-cap tubes according to Kamp et al. (2008) and the pH of the medium was 8.2. The individually picked *Beggiatoa* filaments were rinsed in artificial seawater and inoculated 10 mm below the top slush agar surface. The cultures were incubated at room temperature (~20°C) in the dark. Filament-associated bacteria were isolated from *Beggiatoa* enrichment cultures by streaking sub-samples on agar (1%) plates, based on the same two-component media as described before. Obtained colonies were re-streaked on fresh plates and resulting colonies were phylogenetically classified using the 16S rRNA gene sequence.

PCR amplification, cloning, and sequence analysis of 16S rRNA genes

Live and individual *Beggiatoa* filaments were harvested from intact mats and enrichment cultures, rinsed in sterile artificial seawater, and directly used as template for PCR amplification. Almost full-length (1400 base pairs) 16S rRNA genes from single filaments were amplified by general bacterial primers (Interativa, Ulm, Germany). The master mix for the PCR was prepared shortly before addition of the template: 50 pmol of primer 8f (Hicks et al. 1992) and primer 1492r (Kane et al. 1993), 2.5 µmol of each desoxyribonucleoside triphosphate, 1× Super-Taq buffer (HT Biotechnology Ltd., Cambridge, United Kingdom), and 1 U of Taq DNA polymerase (Eppendorf, Germany) were combined with sterile water to a total volume of 50 µl. Amplification started with an initial denaturation step of 4 min at 94°C, followed by 32 cycles of 0.5 min at 94°C, 0.5 min at 48°C, and 1.5 min at 72°C. The PCR was terminated by the final step of 10 min at 72°C. Amplified DNA was purified (PCR purification kit; QUIAGEN, Hilden, Germany), and cloned by using a TOPO-TA sequencing kit (Invitrogen, Karlsruhe, Germany) according to the manufactures' instructions. Clones with inserts were selected from the established clone libraries and sequenced by Taq cycle sequencing using vector primers and a model ABI sequencer (Applied Biosystems). The same procedure as described above was used to obtain sequences of satellite bacteria. A direct sequence approach of purified PCR products was conducted for some hypersaline strains (referred to as I15M and C, and I6M), indicated by the abbreviation "seq" instead of "clone" in the tree.

Phylogenetic reconstruction

The retrieved sequences of *Beggiatoa* and associated bacteria were analyzed using the ARB software package using the SILVA dataset (Pruesse et al. 2007). The 16S rRNA gene sequences were aligned and manually checked for sequencing errors. Phylogenetic trees for hypersaline *Beggiatoa* filaments were constructed using only nearly full-length sequences, and calculated by maximum parsimony, neighbor joining, and maximum likelihood analyses with different filter sets. The maximum parsimony calculation was subjected to bootstrap analysis (100 replicates). Partial trees were checked for branching incongruities, branching orders that revealed different results were shown as multifurcation. Subsets of the data and out-group reference sequences were included. The phylogenetic tree for associated bacteria was calculated by maximum likelihood approach (RAxML, (Stamatakis et al. 2005)), by using only nearly full-length sequences. Subsequently, the obtained sequences were inserted into the calculated tree by applying parsimony criteria, without allowing changes in the overall tree topology. Furthermore, related Blast matches were included in the analyses (Altschul et al. 1990).

Probe design for fluorescence in situ hybridization (FISH)

Three different oligonucleotides probes for FISH and competitors were designed by the PROBE_DESIGN tool of the ARB package to target sequences of the various strains of hypersaline *Beggiatoa* characterized in this study (Table 2). Probe HSBegg224 (5'-CGGTCATGGGCTCATCCG-3') was designed to match all retrieved sequences of hypersaline *Beggiatoa* sp. Probe HSBegg1241 (5'- GCTTCCCATTGTTCTTAC-3') was designed to match all sequences of hypersaline *Beggiatoa*, except those originating from Guerrero Negro (GN). The competitor HSBegg1241c was applied to discriminate against non target organisms (i.e. *Beggiatoa* from GN) of the corresponding labeled probe. The general bacterial probe EUBI-III was used as a positive control, the Non EUB probe as negative control for unspecific binding. All oligonucleotide probes were labeled with Cy3-fluorochrome at the 5'-end. The filaments were rinsed and fixed for 1 hour at room-temperature in formaldehyde (3.6% in artificial seawater with adequate salinity). Fixed samples were placed on membrane filters (GTTP, 0.2 µm, Millipore, Eschborn, Germany), and covered with a thin film of 0.2% agarose. Hybridization and microscopy of the 4', 6'-diamidino-2-phenylindole (DAPI)-stained filaments were done as described previously (Snaidr et al. 1997; Manz et al. 1998).

Table 2: Ribosomal-RNA-targeted oligonucleoties fluorescent primers designed for all '*Candidatus* Halobeggiatoa' species (HSBegg224), and for *Candidatus* Halobeggiatoa, except Guerrero Negro species (HSBegg1241).

Primer	Positions (16S) [a]	Oligonucleotide Sequences (5' – 3')	Primer specificity	Ref.
HSBeg224	224 - 241	-CGGTCATGGGCTCATCCG-	all hypersaline Beggiatoa	this study
HSBeg1241	1241 - 1258	-GCTTCCCATTGTTCTTAC-	all hypersaline Begg. except Guerrero Negro	this study
HSBeg1241c	1241 – 1258	-GCTTCCCATTGTTCTTAC-	Nonsense competitor to probe HSBeg1241	

[a] Based on *E. coli* 16S rRNA base pair numbering.

Confocal laser scanning microscopy (CLSM)

The morphological traits of the hypersaline *Beggiatoa* from different locations, their vacuoles and the distribution of nucleic acids within the filaments were observed by confocal scanning microscopy. Several specific fluorochromes were applied for direct staining of freshly picked *Beggiatoa* filaments fixed on microscopic slides. Intracellular vacuoles were marked by the membrane markers DCFDA, CMFDA, FM4-64 and MDY-64 (Molecular Probes, Invitrogen), and fluorescein isothiocyanate conjugate (FITC; Research Organics, Cleveland, OH, USA), Nile Red was used as a lipophilic marker and SYPRO orange was applied as protein stain (Molecular Probes, Invitrogen). Newport Green, a metal-ion response indicator, was tested for staining the internal vacuoles of *Beggiatoa* (Molecular Probes, Invitrogen). Nucleic acids were stained by SYBR Green and SYTO 9 (Molecular Probes, Invitrogen). Double staining with SYPRO Orange, FM4-64 or Nile Red and SYTO 9, fitc or CMFDA was applied to some filaments. Stained filaments were visualized by means of a TCS-SP1 confocal microscope controlled by Leica software, version 2.61, build 1537 (Leica, Heidelberg, Germany). The confocal microscope was mounted to an upright microscope and equipped with tree different lasers (Argon, Laserdiode, Helium/Neon). For imaging the laser lines at 488, 561, and 633 nm were employed. Microscopy was conducted by a 63×, 1.2 high-numerical-aperture water immersion lens, corrected for cover slips. Projection of the images was done with the microscope software and Imaris ver. 6.1.5 (Bitplane, Switzerland). The images in Figure 1 were filtered using Gauss 3x3x3. Final images were printed from Photoshop without any corrections.

Results

Morphological characterization of hypersaline strains

All characterized strains featured average filament diameters in the range from 6 to 9 µm in mats and from 8 to 14 µm in enrichment cultures, indicating enhanced growth in gradient cultures (Nelson et al. 1982). However, filaments from Guerrero Negro microbial mats showed the opposite trend, i.e. diameters of mat-grown filaments were larger (9-10 µm) than of those which were incubated in gradient media (7-8 µm) (Table 1). Microscopic analysis revealed the presence of at least two size classes in intact Guerrero Negro mats. The diameters of the dominant larger size class were between 9 and 10 µm, while the few encountered filaments of the smaller size class were between 1 and 2 µm (Figure 1, G). The latter filaments appeared as compact bundles in the mat, but these, unfortunately, could not be maintained in enriched state in gradient cultures. Further detailed microscopic observation of all investigated hypersaline strains revealed gliding motility, the presence of sulfur inclusions and, except for the small 1-2 µm diameter-sized Guerrero Negro filaments, intracellular vacuoles. Three strains (M8, I6, and I15) picked from mats and enriched in gradient cultures were further analyzed for internal nitrate- and elemental sulfur content, and appeared to contain comparable nitrate (430-650 mM) and elemental sulfur (6-25 mM) concentrations. Mesocosm mat-incubated filaments, however, were found to contain a substantially lower internal nitrate (4 mM) but higher internal elemental sulfur concentration (250 mM).

CLS micrographs proved the existence of vacuoles, which were stained with the membrane tracers CMFDA, DCFDA, MDY-64 or FM4-64 as well as with FITC, an amine reactive dye that targets proteins associated with the cytoplasmic layer around the vacuole (Figure 1, A1-F1). Other protein stains, SYPYRO orange or Nile red showed, in combination with vacuole dyes, the cytoplasm. The mechanism of filament breakage through a sacrificial cell (Figure 1, B1) was detected by the Newport green stain, which also unraveled the presence of vacuoles of filaments originating from M10C. In Guerrero Negro mats also unicellular sulfur oxidizing bacteria could be visualized after staining with MDY-64, a yeast vacuole membrane marker (Figure 1, G). Filaments picked out of enrichment cultures showed even cell sizes as well as an even distribution of intracellular vacuoles. The spatial distribution of nucleic acids in cells was detected by SYBR green and SYTO9 (Figure 1, A2-F2) and a projection of this is visualized in Figure 1, H, showing several enriched nucleic acids accumulations in a single cell.

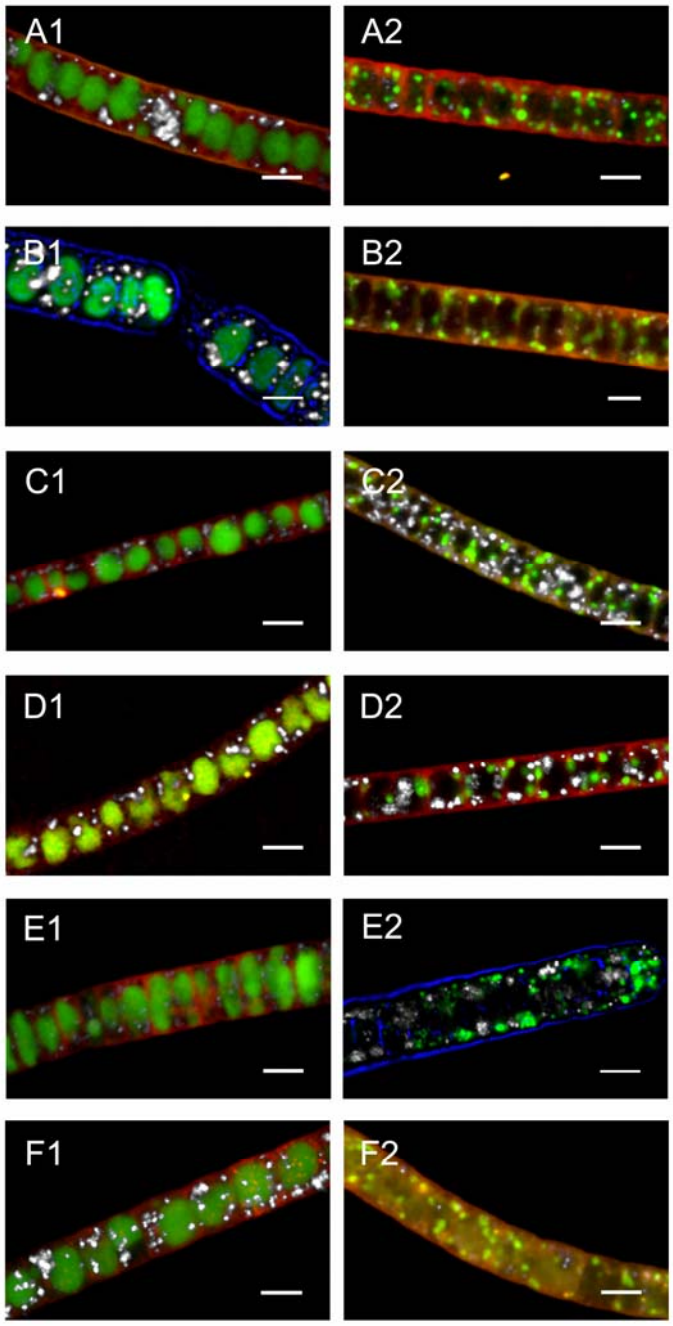


Figure 1A-F: *Beggiatoa* filaments stained for visualization of vacuoles (left column) and nucleic acid distribution (right column). Reflection signals of intracellular sulfur granules are shown in white. Scale bar = 5 µm.
A from M8 mesocosm 8% salinity, A1 – FITC (green), Sypro Orange (red), A2 – Syto 9 (green), FM4-64 (red)
B from M10 8% salinity, B1 – Newport Green (green), transmission signal (blue), B2 – Syto 9 (green), Sypro Orange (red)
C from GN8 Guerrero Negro 8% salinity, C1 – CMFDA (green), Nile Red (red), C2 – Syto 9 (green), Nile Red (red)
D from I6 Ibiza 6% salinity, D1 – DMFDA (green), Sypro Orange (red), D2 – Sybr Green (green), Nile Red (red)
E from I15 Ibiza 15% salinity, E1 – DMFDA (green), Sypro Orange (red), E2 – Sybr Green (green), transmission signal (blue)
F from F15 Formentera 15% salinity, F1 – CMFDA (green), Nile Red (red), F2 – Syto 9 (green), Nile Red (red)

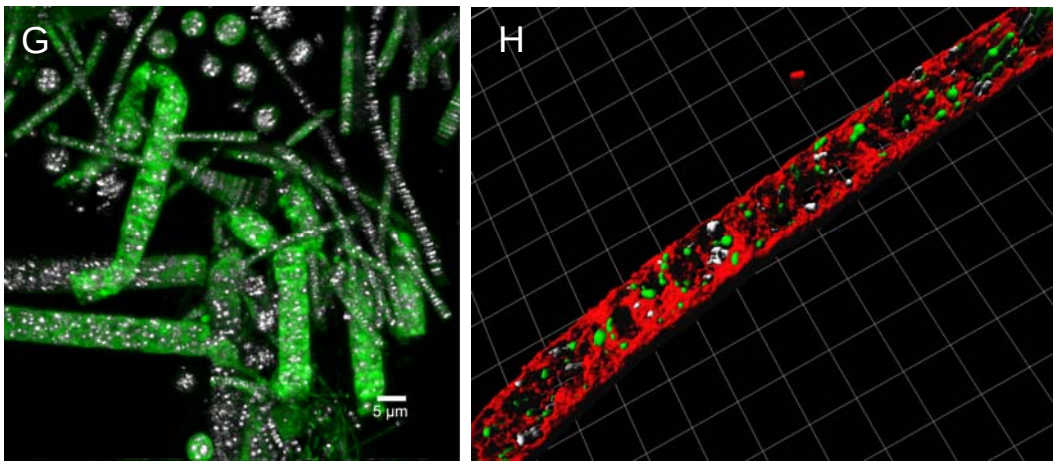


Figure 1G-H: Reflection signals of intracellular sulfur granules in both images are shown in white.
G, Filamentous and unicellular sulfur bacteria of different morphology from Guerrero Negro mats, 8% salinity (GN8M), MDY-64 (green). H, *Beggiatoa* filaments from mesocosm mats, 8% salinity (M8M), isosurface projection, grid size 5 µm, Syto 9 (green), FM4-64 (red).

Phylogenetic reconstruction of hypersaline *Beggiatoa* and proposition of genus '*Candidatus* Halobeggiatoa'

Single filaments of all selected hypersaline environments were sequenced in order to construct a 16S rRNA gene clone library. Twenty-seven almost full-length sequences were retrieved and phylogenetically analyzed. All obtained sequences grouped within the *Beggiatoa-Thioploca-Thiomargarita* cluster (Figure 2), and appeared most closely related to the strain MS-81-1c (86-90% sequence homology), originating from Sippewissett salt marshes (Massachusetts) (Nelson et al. 1982), and to uncultured species originating from submarine caves, EF 467566 and 467528, and AB 250581 (87-90% sequence homology). Since several sequences originating from the same location revealed sequence homologies of >99.8%, only one representative sequence from one location was selected for the final consensus tree (Figure 3) (Stackebrandt & Ebers 2006). The resulting 16 sequences of hypersaline *Beggiatoa* shown represent one monophyletic cluster (15 sequences) accommodating sequences from Spain and Mexico, and a separate single sequence originating from Guerrero Negro, Mexico (GN8). Three other sequences of filaments from Guerrero Negro mats and those originating from the different Spanish mats formed two respective sub-branches within the monophyletic cluster. Within these sub-branches sequence similarities were high (98-100%), while between the sub-branches sequence similarities were lower (95-98%). The Spanish sub-cluster included a previously characterized strain originating from a Lake Chiprana microbial mat (Hinck et al. 2007). The one sequence that fell outside the monophyletic cluster, GN8M clone number 285, appeared affiliated to an uncultured gamma proteobacterium (93% sequence similarity). All hypersaline sequences were distantly related to large, marine *Beggiatoa* spp. from the Bay of Conceptión, Monterey, and Carmel Canyon,

USA (88% sequence similarity). The 16 S rRNA genes from the hypersaline *Beggiatoa* species within the proposed monophyletic cluster were more than 96% similar to each other, and more than 7% different from all other *Beggiatoa* and *Thioploca* species, which is greater than the postulate range for a coherent genus (Devereux et al. 1990). Sequences of the monophyletic clade cluster tightly within less than 4% sequences divergence, corresponding to distances contained within many bacteriological genera (Devereux et al. 1990; Stackebrandt & Goebel 1994). As there is so far no other phenotypic trait than high-salt tolerance, which would set the out-grouped *Beggiatoa* sequence apart from other non-hypersaline *Beggiatoa*, the genus '*Candidatus* Halobeggiatoa' is proposed (Murray & Schleifer 1994; Euzeby 1997).

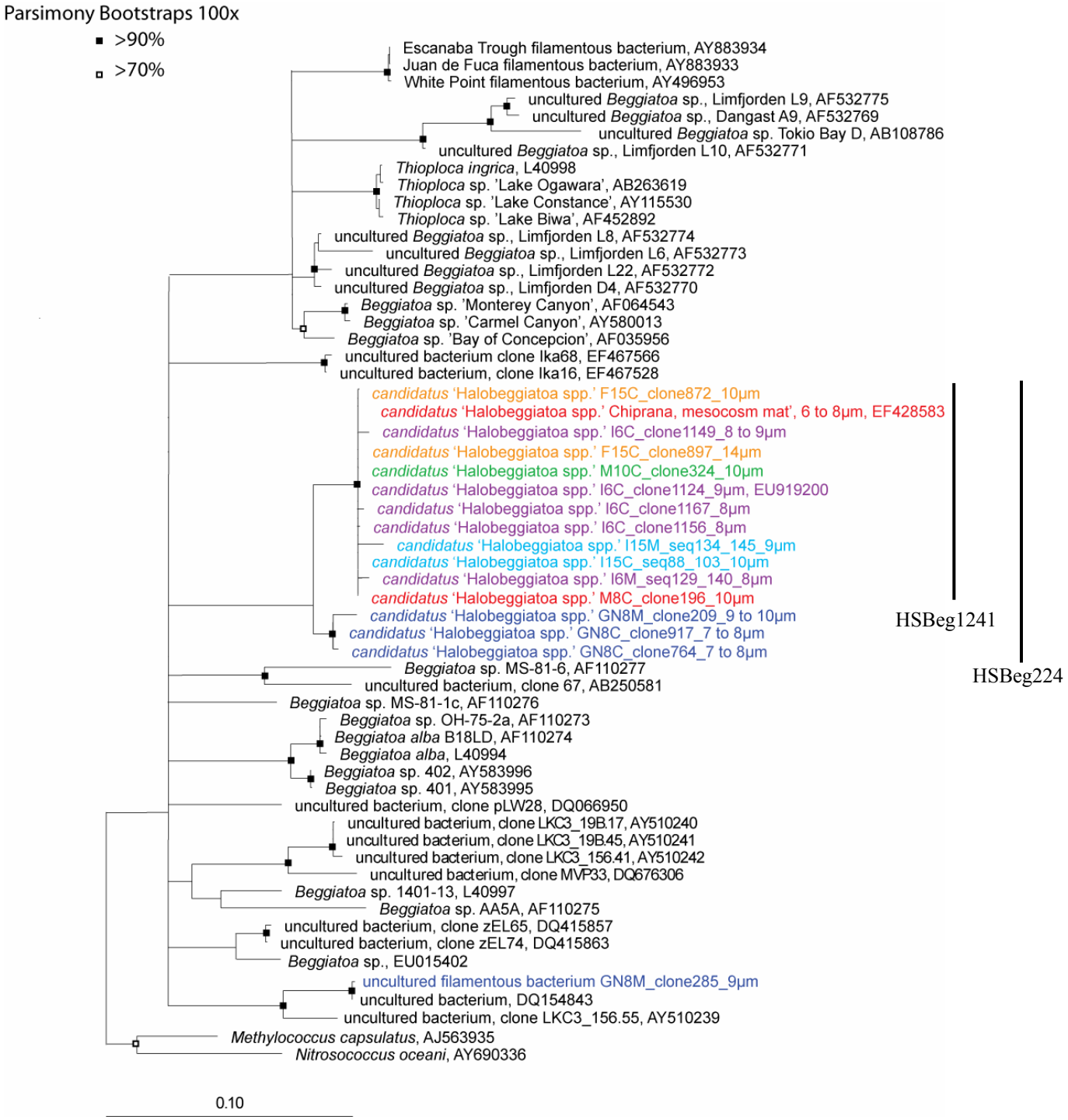
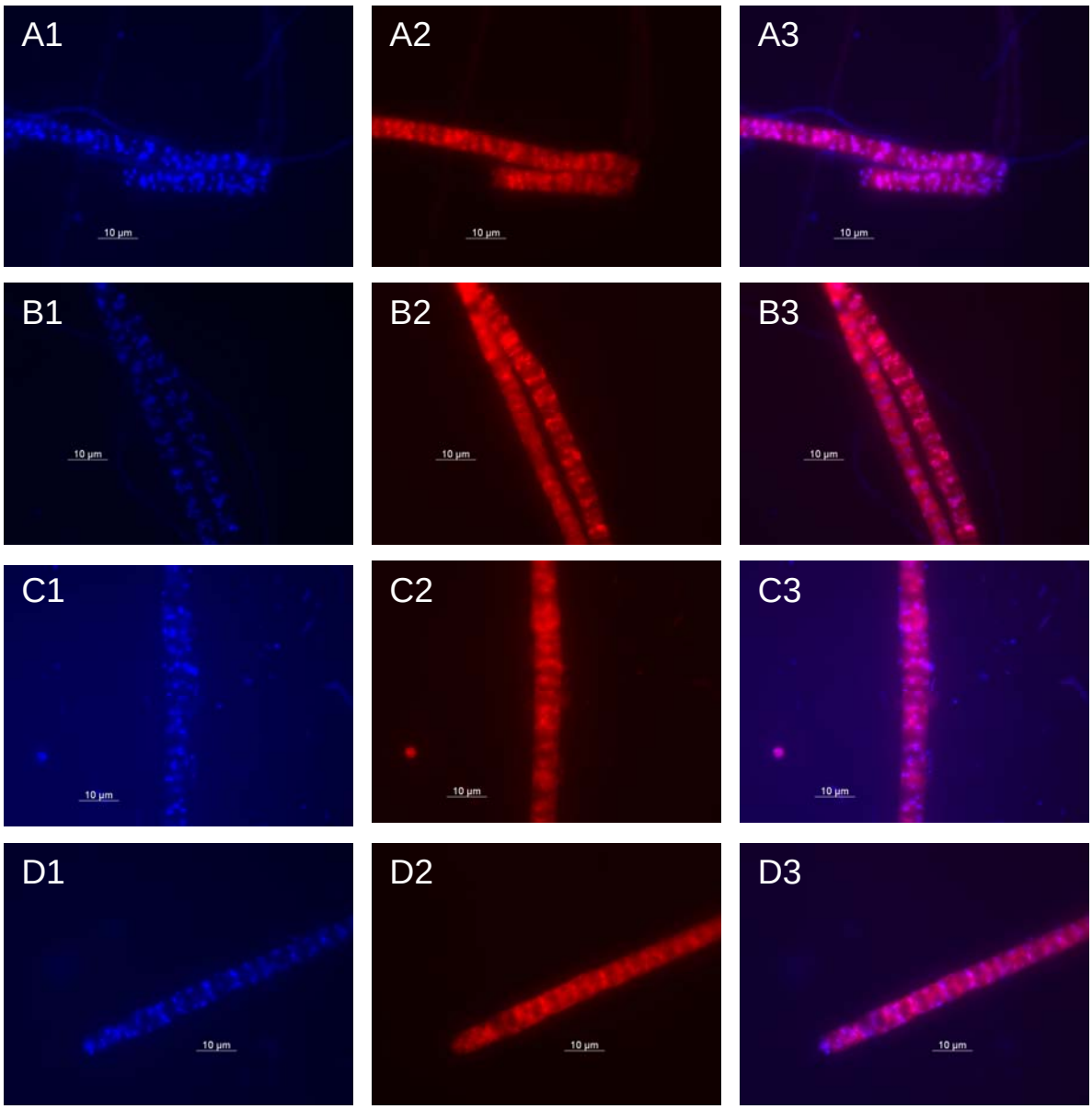


Figure 2: Phylogenetic relationships of the '*Candidatus* Halobeggiatoa' spp. cluster, exclusively involving almost complete gene sequences. Three organisms represented by partial sequences, indicated by „seq" in the name were retrieved by direct sequencing without the cloning procedure („clone"), and subsequently inserted into the concensus tree by applying parsimony criteria without changing the tree topology. Species investigated in this study are colored after habitat affiliation, both for enrichment culture and microbial mat, abbreviated with ‚C' and ‚M', respectively, after the number, which indicates the salinity in %:
Red: ‚Chiprana, mesocosm mat', 8% salinity, abbreviation ‚M8'
Orange: ‚Formentera', 15% salinity, abbreviation ‚F15'
Green: ‚Mat10', abbreviation ‚M10' (salinity is 8%, 10 refers to spec. sample location at Chiprana lake)
Purple: ‚Ibiza, 6% salinity, abbreviation ‚I6'
Turquoise: ‚Ibiza, 15% salinity, abbreviation ‚I15'
Blue: ‚Guerrero Negro', 8% salinity, abbreviation ‚GN8'
The bar corresponds to 10% estimated sequence divergence.

Fluorescence *In situ* hybridization (FISH) of hypersaline *Beggiatoa*

The novel FISH probes for targeting hypersaline *Beggiatoa* and marine, vacuolated *Beggiatoa* are displayed in Table 2. The probe HSBegg224 targeting all investigated hypersaline species revealed positive hybridization signals with strains I6M (Figure 3A-C) and GN8M (Figure 3D-F). Probe HSBegg1241 targeting all '*Candidatus* Halobeggiatoa' sequences, except those of the filaments originating from Guerrero Negro, showed a positive signal on filaments of Ibiza microbial mats with 6% salinity, but no signal for filaments originating from Guerrero Negro mats. The probes HSBegg224 and HSBegg1241 gave a signal comparable to the EUB I-III probe. The general probe EUB I-III also showed the filament associated bacteria, whereas the specifically designed probes HSBegg224 and HSBegg1241 stained the filaments only.



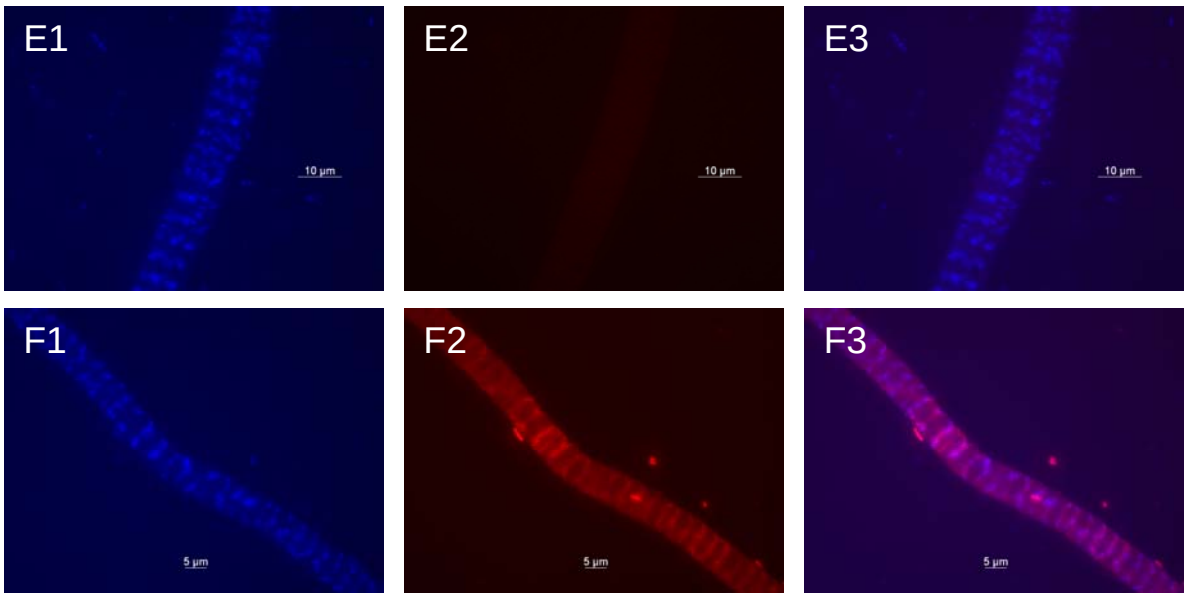


Figure 3: Epifluorescence micrographs of '*Candidatus* Halobeggiatoa' from Ibiza mats, 6% (I6M) (A-C) and from Guerrero Negro mats, 8% (GN8M) (D-F). One row (1-3) shows the same microscopic field, 1: DAPI stained filament, 2: Cy3 fluorescence, 3: combined, Dapi and Cy3;
A, I6M, FISH with HSBegg224 at 50% formamide;
B, I6M, FISH with HSBegg1241 at 20% formamide;
C, I6M, FISH with EUB at 10% formamide;
D, GN8M, FISH with HSBegg224 at 10% formamide;
E, GN8M, FISH with HSBegg1241 at 10% formamide - negative for GN8 (E2);
F, GN8M, FISH with EUB at 10% formamide

Identification of *Beggiatoa*-associated bacteria

Approximately only one out of 20 clones in the library obtained from mat-picked filaments contained a *Beggiatoa* related 16S rRNA sequence. The other sequences appeared to originate from contaminant bacteria firmly attached to *Beggiatoa* filaments. In order to avoid autolysis of *Beggiatoa* DNA before or during the PCR procedure, filaments were processed fast and filament attached bacteria were apparently not completely removed from the filament surface. In addition to these directly obtained sequences, unicellular bacteria growing in specific zones in the gradient media cultures were taken and streaked on agar plates to obtain pure colonies, which were also phylogenetically analyzed (Figure 4). The majority of the obtained sequences of attached bacteria from directly mat-picked filaments could be unambiguously assigned to known phyla, i.e. subdivisions of Proteobacteria, Spirochaeta, Acidobacteria, Verrucomicrobia, Firmicutes, Bacteroidetes, Planctomycetales, and Chloroflexi. Sequences of attached bacteria from mat-picked filaments appeared typically affiliated to the phyla Chloroflexi, Acidobacteria, and genera *Pseudomonas* and *Halothiobacillus* of the gamma-Proteobacteria. The culturable fraction of organisms co-occurring with *Beggiatoa* in the gradient cultures affiliated with the Planctomycetales, Spirochaeta, Firmicutes, and to hypersaline members of the proteobacterial genera *Marinobacter* and *Halomonas*, as well as

members of the genus *Thalassospira* of the Rhodospirillaceaea. No overlap was found in the diversity of sequences obtained from the gradient cultures and from the clone libraries recovered from mat-picked *Beggiatoa* filaments. The majority of co-occurring bacteria isolates as colonies from gradient cultures grouped within the Gamma-Proteobacteria. Regarding the habitats, associated bacterial sequences from the mesocosm (M8) and mat 10 (M10), both originating from Lake Chiprana, Spain, seemed to be mainly limited to the Proteobacteria, whereas sequences from Formentera (F15), Ibiza (I6), and Guerrero Negro (GN8) were more divers. Associated bacteria from Ibiza (I15) mats were only present as one sequence obtained from a colony, not more sequences of co-occurring organisms were obtained from this habitat.

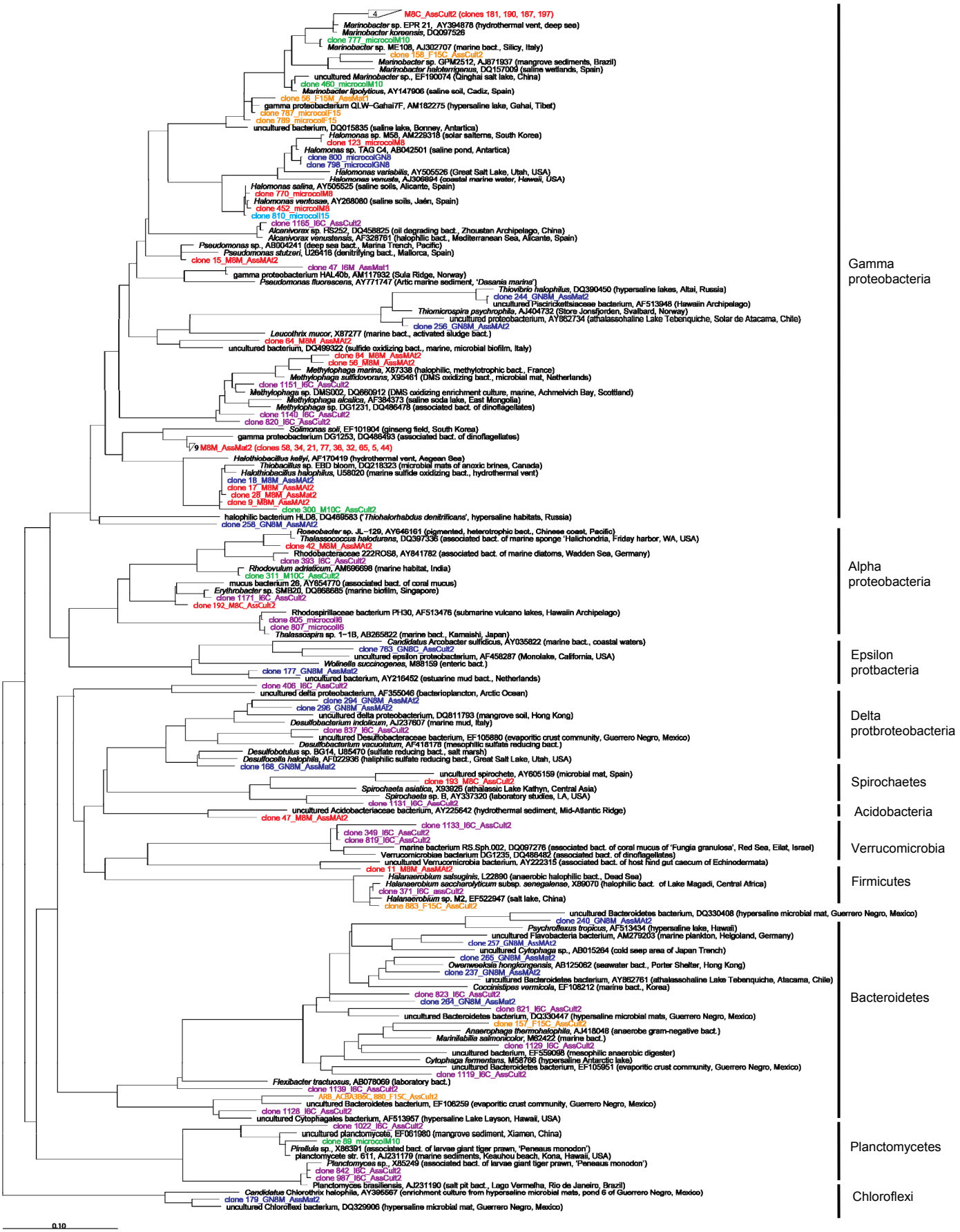


Figure 4: Maximum-likelihood phylogenetic tree (raxML) based on 16S rRNA gene sequences, partial sequences of associated bacteria were inserted by applying parsimony criteria after tree reconstruction without

changing the topology. Species investigated in this study are colored after habitat affiliation, as indicated in Figure 1. Bar, 10% estimated sequence divergence.

Discussion

Despite the early discovery of *Beggiatoa* and their detailed morphological and physiological description (Winogradsky 1887), the phylogenetic diversity within this genus needs further analysis and correlation to phenotypic and ecophysiological properties (Teske & Nelson 2006). In this study we expand the described phylogenetic and phenotypic diversity of halophilic *Beggiatoa.* The retrieved 16S rRNA genes from almost exclusively all recovered hypersaline *Beggiatoa* species formed a monophyletic cluster showing more than 96% sequence identity and more than 7% difference from all other *Beggiatoa* and *Thioploca* species. This exceeds the threshold value defining a coherent genus (Devereux et al. 1990). Sequences of the monophyletic clade cluster tightly within less than 4% sequences divergence, corresponding to distances contained within many bacteriological genera (Stackebrandt & Goebel 1994). The clear phenotypic trait of high-salt tolerance, sets the out-grouped *Beggiatoa* sequence apart from non-hypersaline *Beggiatoa*, therefore the genus '*Candidatus* Halobeggiatoa' is proposed (Murray & Schleifer 1994; Euzeby 1997).

Most likely the GN8M clone 285 sequence is derived from a *Beggiatoa*-like organism as indicated by the affiliation with the obtained sequence by Dillon (sequence DQ154843 in GenBank, August 2005), however, an affiliation with single celled, yet unknown bacteria can not be completely excluded (Figure 2). In general, the multifurcation in the phylogenetic reconstruction does not confirm a single origin of a monophyletic origin of both, vacuole formation or halophily. Furthermore, the well supported multifurcation of cultivated and non-cultivated representatives of the entire phylum contradicts earlier findings by (Ahmad et al. 2006) that propose cultivated *Beggiatoa* spp. as the phylogenetic root of morphologically diverse, non-cultured, vacuolated relatives of this phylum.

Besides phylogenetic relationships, also phenotypic and physiological properties of the investigated hypersaline *Beggiatoa* strains were characterized (Figure 1). The filament diameters of the halophilic '*Candidatus* Halobeggiatoa' appear to be in the same range as for previously described narrow marine and freshwater strains of 2-9 µm in diameter (Macalady et al. 2008; Ahmad et al. 2006; Nelson et al. 1986b; Canganella et al. 2007; Grabovich et al. 2001). The fact that narrow filamentous *Beggiatoa* from habitats with different salinities appear phylogenetically only distantly related, do therefore not support a distinct phylogenetic lineage for thin-filamentous *Beggiatoa* as was suggested before (Strohl 1989). That the filament diameter size may also be influenced by environmental conditions is also shown in

this study as filaments grown in culture featured different diameters than those directly picked from mats (Table 1).

Similar to filament diameter, also the presence of vacuoles, internal storage compartments for compounds such as nitrate, has been used in previous studies as a distinctive morphological feature to characterize filamentous sulfide oxidizers (Schulz-Vogt 2006). This trait, however, does also not appear to be a phylogenetic distinctive feature as large marine and vacuolated *Beggiatoa* strains phylogenetically cluster with both large marine and narrow freshwater non-vacuolated *Thioploca* species (Teske et al. 1995; Teske et al. 1999; Ahmad et al. 1999; Jørgensen & Gallardo 1999). Previously taken scanning electron microscopic images of *Beggiatoa*-like filaments of a sulfataric spring in the 'Grotta Azzurra', Cape Palinuro Massif, Italy, did not reveal any vacuolar structures (Canganella et al. 2007). However, all '*Candidatus* Halobeggiatoa' species, whether of thalassic (solar saltern) or athalassic (lake Chiprana) origin, characterized in the present study did feature vacuoles.

The morphological characteristics filament widths and vacuolation do thus not appear to be distinctive for phylogenetic relationships (Mußmann et al. 2003). A further characteristic is the metabolic diversity, which appears to be high within the group of sulfide oxidizing *Beggiatoa*. Teske & Nelson (2006) defined three morphologically and physiologically different groups originating from various habitats: 1) Narrow (non-vacuolated) heterotrophic filaments from freshwater origins; 2) Narrow (non-vacuolated) facultative autotrophic and obligate autotrophic *Beggiatoa* from marine environments (Nelson et al. 1986b; Hagen & Nelson 1996) and 3) Large (vacuolated) autotrophic marine strains (Nelson 1989; McHatton et al. 1996; Gallardo & Espinoza 2007). An exception has already been found in a freshwater *Beggiatoa* strain (Grabovich et al. 2001), which was capable of autotrophic metabolism. To these, a fourth group, i.e. narrow (6-9 µm threshold) vacuolated autotrophic *Beggiatoa* from marine and hypersaline environments can be added ((Sayama 2001) and this study).

Another important criterion for ecophysiological bacterial characterization is the differential salt requirement, which could reflect their adaptation to specifically high- or low-salt habitats (Imhoff et al. 1998). As cultured marine *Beggiatoa* were isolated from salt marshes with fluctuating salinities their salt tolerance is presumingly high, although not yet tested (Nelson et al. 1986b). However, growth of the latter salt marsh strains occurred at salinity ranges of 1.8 to 2.7%, those from submarine caves experienced changing salinities with an average of 3.4% (Canganella et al. 2007) and the ones originating from the Italian mountain cave system (Macalady et al. 2008) lived at approximately 1.75% salinity. The '*Candidatus* Halobeggiatoa' species in this study grew at hypersaline salt concentration

ranges between 6% (Ibiza I6) and 15% (Formentera F15 and Ibiza I15). These strains probably own high adaptation capacities to different salinity values, since the saltern ponds of Ibiza are still actively operated and salinities within ponds vary between 3 and 14% and between 14 and 28% in the evaporation ponds. The tolerance for high salinities of hypersaline filaments is an argument for designating these as members of the proposed '*Candidatus* Halobeggiatoa' genus. This study suggests that halophily might be reflected in the phylogenetic tree by the presence of a specific phylogenetic lineage of hypersaline *Beggiatoa* in contrast to marine- and freshwater strains. Further biogeographic and phylogenetic studies are necessary to confirm the hypothesis of one coherent lineage of hypersaline *Beggiatoa*.

Fluorescence *In situ* hybridization (FISH) of cultivated and mat-picked hypersaline '*Candidatus* Halobeggiatoa' spp. indicated that we covered a substantial diversity of *Beggiatoa*-like organisms in the mat by both cultivation and direct picking of single filaments. The bacteria attached to '*Candidatus* Halobeggiatoa' filaments are not confined to a specific taxon as can be concluded from the encountered diversity throughout various phyla. The enrichment for *Beggiatoa* in gradient cultures selected for a limited number of associated organisms compared to the large diversity of naturally occurring bacteria living attached to *Beggiatoa*. Whether *Beggiatoa*-associated bacteria significantly interact with their hosts in a syntrophic lifestyle or are mere commensalists, needs further clarification.

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Chapter 6
Conclusions and outlook

Conclusions and outlook

Many *Beggiatoa* species from various habitats have been described since their discovery in the early 19th century (Vaucher 1803; Trevisan 1842), but only a few studies dealt with hypersaline environments and *Beggiatoa* occurrences (Jørgensen & Des Marais 1986; Garcia-Pichel et al. 1994). In this thesis, hypersaline *Beggiatoa* spp. were characterized with respect to their physiology (function) and their phylogeny (structure) in their natural habitat (*In situ*), phototrophic microbial mats, and in specifically designed laboratory set-ups (*Ex situ*). For the laboratory experiments, *Beggiatoa* filaments were isolated from microbial mats and brought into gradient-enrichment-cultures (modified after Nelson & Jannasch (1983) and Kamp et al. (2008)). Further investigations concentrated on the cell structures (inclusions and extrusions) and the associated functions, i.e. the chemistry of the vacuole liquids (*In vivo*), stressing the uniqueness and specialization of these bacteria.

The main focus of this thesis was the determination of diel distribution pattern of the dominant morphotype of *Beggiatoa* filaments (6-8 µm diameter) and the correlating porewater compounds within the hypersaline microbial mats (chapter 2 of this thesis). The significant but minor migratory distance observed *In situ* might be influenced by their rather slow gliding velocities with respect to the fast moving oxygen-sulfide front upon darkening or illumination. The survival of *Beggiatoa* in a sulfidic environment during darkness could be explained by the finding of high internal nitrate storage capacities (1000 to 10000 times of ambient concentrations), most likely in vacuoles, which allows a physiological flexibility in a dynamic mat environment and independency of the fluctuating oxygen gradients. During illumination, *Beggiatoa* oxidize sulfide with oxygen, being present as steep, narrow overlapping, opposing gradients, such as in marine mats (Jørgensen & Postgate 1982; Jørgensen & Revsbech 1983). During darkness, when oxygen becomes depleted in the zone where *Beggiatoa* resides, filaments use their internal oxidant storage (nitrate) to oxidize sulfide or internally stored elemental sulfur anaerobically (McHatton et al. 1996; Mußmann et al. 2003; Preisler et al. 2007). A study on hypersaline phototrophic microbial mats revealed a diel migration pattern, *Beggiatoa* moving down during the day, and forming whitish layers on top of the mat during the night (Jørgensen & Des Marais 1986). Similar findings were observed by Garcia-Pichel et al. (1994) in mats of the same origin (Guerrero Negro, Mexico), with the notable difference that at night one population of *Beggiatoa* rose to the surface whereas another population remained in the deeper, anoxic part of the mat. Whether the latter population could store nitrate, and was taxonomically different from the first, was not resolved. Nitrate-storing *Beggiatoa* in suboxic zones can outcompete other sulfide oxidizers which do not store nitrate. Nitrate production by nitrification could serve as oxidant supply

for mat inhabiting *Beggiatoa* filaments (Bonin & Michotey 2006; Francis et al. 2007). Total extractable concentrations of nitrate were low (5-10 μM), but under dark conditions a peak (15 μM) was observed below the photic zone and above the sulfidic boundary layer, where highest abundances of filaments could be responsible for efficient nitrate uptake. Whether nitrification is possible at the oxic/anoxic interface and if a nitrate source is solely derived from aerobic ammonium oxidation, remains to be investigated. The development of a NOx micro-biosensor operating in hypersaline medium would be necessary to obtain fine scale nitrate concentration profiles, as the stable isotope pairing method (Nielsen 1992) used in this study on discrete depth intervals resulted only in a relatively coarse profile. Nevertheless, the ^{15}N-isotope technique could be useful to resolve the possible role of sulfide in the nitrate reduction pathway in *Beggiatoa*, i.e. nitrate reduction to either ammonium (DNRA) or N_2 (denitrification).

Chemotactic responses obtained in two different laboratory set-ups (*Ex situ*) were used to explain observed *In situ* migration behavior (chapter 4 of this thesis). As the distribution pattern of hypersaline *Beggiatoa* was thought to be mainly controlled by the sulfide gradient both under light and dark conditions, it was previously hypothesized that sulfide is the key trigger determining *In situ* behavior of filaments. However, as in fact multiple potential triggers interact (e.g. oxygen, sulfide, nitrate, light, pH) it appears impossible to pinpoint solely based on observations alone the single most determining factor influencing distribution patterns. Gradient culture experiments done in this study with bulk enrichments of filaments revealed that in fact several different parameters can influence migration behavior. Both oxygen and blue light resulted in a negative (repelling) while nitrate resulted in a positive (attracting) response, enabling the organisms to use these triggers for orientation when other factors are absent. Statistical analyses identified H_2S as the factor with the highest impact on distribution pattern, confirming the previous assumption. The further experimental application of single filaments in micro-capillaries revealed a bivalent response to sulfide: low sulfide concentrations (0-20 μM) acted as an attractant, while higher sulfide concentrations (>20 μM) acted as repellent. These findings can be used to explain observed *In situ* migration behavior of *Beggiatoa*. The responses of hypersaline *Beggiatoa* in natural mats seemed therefore to be correlated to present concentration ranges of sulfide. Another possibility was proposed by Fenchel (2002), who argued that a kind of short term memory may also play a role, allowing *Beggiatoa* to compare current and past chemical environments during migration, resulting in a movement towards the source of an attractant. Further micro-capillary experiments with single filaments, pre-incubated under different but defined conditions of chemical stimuli, would be necessary to definitely clarify the matter of concentration dependent behavior.

Confocal laser micrographs of lectin stained filaments showed the *Beggiatoa* gliding mechanism by slime jets, which was until now only proven for cyanobacteria (Hoiczyk & Baumeister 1998; Hoiczyk 2000). Although Larkin & Henk (1996) observed parallel rows of pore like structures on the filament surface by electron microscopy, the excretion of glycoconjugates had not been shown for *Beggiatoa* so far. A previous genome study found exoprotein domains similar to those from filamentous cyanobacteria, supporting the hypothesis that these proteins have a function in gliding motility (Mußmann et al. 2007). The visualization of glycoconjugate-excretions and the simultaneous observation of behavioral responses in micro-capillaries could possibly elucidate the fine tuned mechanism of gliding, e.g. how partly reversals, bending and looping function, and what kind of reaction is featured with respect to a certain chemotactic trigger.

The study on vacuoles of the narrow hypersaline *Beggiatoa* investigated in this study yielded interesting new insights. The presence of vacuoles as well as the ability to store nitrate internally was unknown in hypersaline *Beggiatoa*, which appeared phlogenetically positioned between narrow, non-vacuolated, and larger (>9 µm diameter) vacuolated *Beggiatoa* species (chapter 5 of this thesis). The tolerance for higher salinities could explain the close phylogenetic affiliation to thin (2-4 µm), non-vacuolated filaments, MS-81-6 and MS-81-1c (Nelson et al. 1986; Hagen & Nelson 1996; Hagen & Nelson 1997), as these strains originated from salt marshes, where fluctuations in salinity are possible. However, neither the proposed new type of '*Candidatus* Halobeggiatoa' nor the previously characterized marine *Beggiatoa* originating from saltmarshes have been tested for halotolerance or halophily. Nevertheless, several hypersaline *Beggiatoa* spp. from various origins (Spain mainland, Balearic Islands, Mexico) build a monophyletic group on the 16 S rRNA gene basis in the monophyletic phylum of gamma-Proteobacteria that accommodates the genera *Thioploca*, *Thiomargarita*, and *Beggiatoa*. Moreover, additional morphological characteristics (filament diameter range and presence of vacuoles) supported the occurrence of a coherent genus. However, it remains to be investigated whether the proposed genus '*Candidatus* Halobeggiatoa' is supported by higher resolving phylogenetic markers such as 16 and 23 S rRNA intergenic spacer or functional gene analysis. Hypersaline *Beggiatoa* did not seem to support specific phylogenetic groups of attached or even associated bacteria *In situ*, whereas enrichment cultures of *Beggiatoa* appeared selective for a limited number of associated organisms within the gamma-Proteobacteria. Future studies on cultivated *Beggiatoa* in minimal media should resolve whether they do support a specific population of associated bacteria as these may be masked by other bacteria sticking to filaments that are directly picked from mats.

A new methodology of time-resolved analysis in living *Beggiatoa* was developed in order to determine whether pH values in vacuoles are indeed as low it as was previously assumed (chapter 3 of this thesis). This technique allowed for the first time imaging of very low pH inside the vacuoles of live *Beggiatoa*. An acidic pH in vacuoles had been detected in preliminary measurements of *Beggiatoa* sp. and *Thiomargarita namibiensis*. This acidification is of major importance for trans-membrane gradients of electrochemical potential and pH, which affect nitrate transport into cells (De Angeli et al. 2006; Miller et al. 2007; Siverio 2002; Moir & Wood 2001). A nitrate/proton antiport for nitrate accumulation in vacuoles was previously detected in *Arabidopsis thaliana* by De Angeli et al. (2006), similar to a chloride/proton antiport system. Mußmann et al. (2007) found evidence for chloride/proton exchangers and chloride channels, which could display weak similarities to a nitrate/proton antiporter in a marine *Beggiatoa* genome. However, Moir & Wood (2001) suggested either a nitrate/proton symport mechanism or a nitrate/nitrite antiporter system for bacterial cells. The fact that vacuoles could originate from an intrusion of the cytoplasmic membrane, as mentioned in the introduction section, might help to visualize the direction of proton transfer across the membrane and the type of transport systems used for nitrate uptake. Further applications of our studied FITC lifetime pH determination and specific inhibitors could target the function of vacuolar transport and metabolism systems in *Beggiatoa*. Double staining with other ion indicators could unravel the mechanisms of the membrane potential build-up and maintenance, responsible for transport and metabolism.

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Hinck, S., de Beer, D. and Jonkers, H. M. (2005) In situ physiology and phylogenic diversity of *Beggiatoa* in hypersaline microbial mats. EGU - European Geosciences Union, General Assembly 2005, Vienna, Austria

Hinck, S., de Beer, D. and Jonkers, H. M. (2006) Eco-physiological characterization of a *Beggiatoa* sp. from the hypersaline Lake Chiprana, Spain. ISME – 11th International Symposium on Microbial Ecology, Vienna, Austria

Erklärung

Hierdurch erkläre ich, dass die Dissertation „Eco-physiological, chemotactic and taxonomic characterization of hypersaline *Beggiatoa* originating from microbial mats ” selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden. Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

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