Beggiatoa - the ecophysiological significance of large, white, filamentous sulfur oxidizers within the nitrogen and the sulfur cycle

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Zusammenfassung

Die Bedeutung von *Beggiatoa* für die Ökophysiologie des Stickstoff- und Schwefelkreislaufes wurde in dieser Arbeit untersucht. *Beggiatoa* sind auffällige Mikro-Organismen, die mit bloßem Auge gesehen werden können, die aber oft unter der Sedimentoberfläche verborgen bleiben. Da vakuolenhaltige *Beggiatoa* Sulfid mit Nitrat oxidieren, könnten sie sowohl für den Schwefel- als auch für den Stickstoffkreislauf von entscheidender Bedeutung sein. In ihrer Vakuole können die *Beggiatoa* Nitrat in hohen Konzentrationen speichern, während sie elementaren Schwefel als globuläre Partikel in der Zelle einlagern. Die Ökophysiologie dieser internen Nitrat- und Schwefelanreicherung und deren Transport durch die gleitenden *Beggiatoa* wurde in einem marinen Küstensediment (Eckernförder Bucht) untersucht.

Unsere Daten zeigten, daß der interne Nitratpool von *Beggiatoa* der wichtigste Nitratpool des gesamten Sedimentes ist und mehrere Zentimeter tief in das Sediment hineintransportiert werden kann. Im Vergleich zum großen gesamten Schwefelpool des Sediments ist dagegen der interne Schwefelpool von *Beggiatoa* nur von geringer Bedeutung. *Beggiatoa* können mehrere Wochen anhand von diesen internen Pools leben und daher ist ihre Vertikalverteilung wahrscheinlich nicht auf Nitratlimitierung, sondern auf die vertilkale Sulfidverteilung im Sediment zurückzuführen. Hohe Sulfidkonzentrationen wurden von *Beggiatoa* im wesentlichen gemieden und sie blieben daher auf die sulfidabgereicherte Zone beschränkt. Es kam aber auch vor, daß *Beggiatoa*-Filamente in Regionen gefunden wurden, wo Sulfidkonzentrationen im millimolaren Bereich gemessen wurden. Daher scheint das Vermeiden des Sulfids weniger auf die giftige Wirkung des Sulfids zuruckzuführen zu sein, als vor allen Dingen der Orientierung zu dienen. Es ist möglich, daß das Sulfid in den oberen Schichten der BIZ (Beggiatoaenthaltenden Zone) komplett von *Beggiatoa* Filamenten verbraucht wird, aber das Meiste des produzierten Sulfids wird durch andere Prozesse entfernt. Daher bestimmt die Sulfidverteilung viel eher die *Beggiatoa*-Verteilung als umgekehrt.

Innerhalb der BIZ können mehrere verschiedene *Beggiatoa*-Unterarten koexistieren und sind auch mehr oder weniger gleichmäßig über die BIZ verteilt. FISH-Untersuchungen zeigten, daß mindesten 4 der im Sediment der Eckernförder Bucht gefundenen Größenklassen auch phylogenetisch unterschiedlich waren. Die Dynamik der *Beggiatoa*-

Gemeinschaft schien unabhängig von umweltabhängigen Faktoren zu sein. Wahrscheinlich sind Fraßdruck und Viren wichtiger für die Zusammensetzung dieser Gemeinschaft. Die Eckernförder Bucht mit ihren leicht zugänglichen *Beggiatoa* Populationen ist ein ideales natürliches Labor, daß für ökologische Populations-Untersuchungen an koexistierenden, nicht kultivierten Bakterien genutzt werden kann.

Thesis abstract

The ecophysiological significance of *Beggiatoa* within the nitrogen and sulfur cycle was investigated. *Beggiatoa* are conspicuous organisms occurring in many marine sediments occasionally visible with the naked eye, sometimes hidden below the surface in the sediment. The vacuolated *Beggiaota* could contribute significantly to both the nitrogen and the sulfur cycle, as they oxidize sulfide with nitrate. *Beggiatoa* can store high concentrations of nitrate in intracellular vacuoles and elemental sulfur in sulfur globules. The ecophysiology of this internal nitrate and sulfur accumulation and the transport by gliding *Beggiatoa* spp. was investigated in a coastal marine sediment (Eckernförde Bay). Our data showed that the internal nitrate of *Beggiatoa* is the most important nitrate pool and can be transported several centimeters (2-6 cm) down into the sediment. The internal sulfur pool is only of low importance in comparison to the large external elemental sulfur pool in the sediment.

Beggiatoa can live for several weeks using these internal pools and their vertical distribution is probably not nitrate limited but related to the vertical free sulfide distribution in the sediment. *Beggiatoa* avoided high sulfide concentrations and were normally restricted to the sulfide depleted zone. This mechanism seems rather for orientation than to escape the toxicity of sulfide as they were occasionally found in regions with mM sulfide concentrations. The sulfide of the upper layers of the BIZ (*Beggiatoa* Inhabiting Zone) could be removed by *Beggiatoa* but most of the produced sulfide in the sediment is removed by other processes. Therefore the sulfide determines the distribution of *Beggiatoa* in the sediment rather than vice versa.

Within the BIZ different *Beggiatoa* strains can coexist and members of all size classes are more or less evenly distributed over this horizon. FISH investigations showed that at least 4 size classes in the sediment of Eckernförde Bay are phylogenetically different. The community dynamics of *Beggiatoa* seem not to be related to environmental parameters. Possibly grazing or viruses are more important for the community composition. The location Eckernförde Bay with their conspicuous *Beggiatoa* forms an ideal natural laboratory that could be used to investigate the ecology of coexisting populations of uncultured bacteria.

Contents

1. Introduction

1.1 *Beggiatoa* –conspicuous organisms in marine sediments

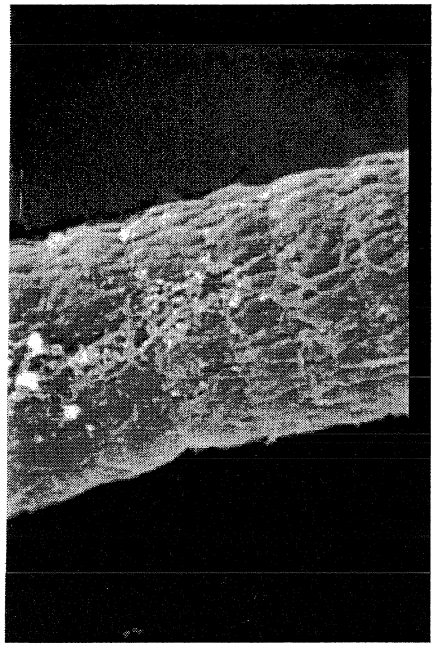


Fig. 1: REM picture by Anke Tolz (Group: Prof. W. Heyser) and A.Preisler. *Beggiatoa* filament: width ca 20 µm.

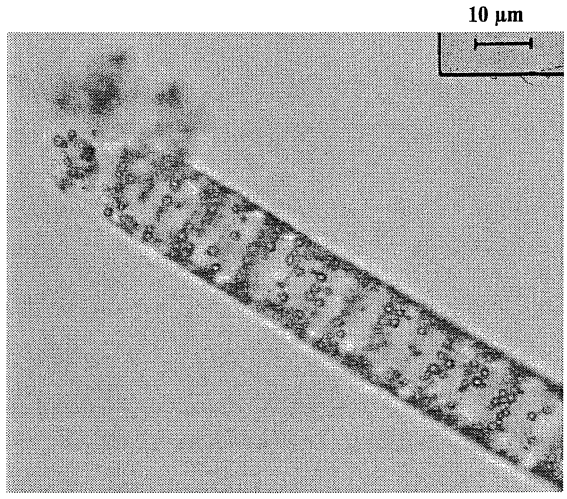


Fig.2: *Beggiatoa* unterm Lichtmikroskop. Foto: E. Wieringa

Beggiatoa and *Thioploca* are among the largest known prokaryotes with a cell diameter of up to 160 µm. They form multicellular filaments (Fig. 1) with a length of up to 1-2 cm. Both genera are motile and form dense mats on sediments in estuarine, shelf, seep, and deep sea hydrothermal vent environments [1]. They can also glide deep within the sediment and do not always form conspicuous mats on the sediment surface [16, 26, 42]. Because of their size and internal sulfur globules (Fig. 2) they are visible to the naked eye and were discovered more than 100 years ago [46, 21]. At the same time *Thiotrix* was discovered, which is morphologically similar but not able to glide and attached to surfaces. Spherical non-motile relatives of *Beggiatoa* and *Thioploca* are *Thiomargarita* (400 µm) [35] and *Achromatium,* (100 µm) [12] the latter was discovered as early as 1875.

The most common form of marine giant bacteria from coastal sediments of Germany belong to the genus *Beggiatoa* [23], [26]

1.2 Phylogeny

Filamentous sulfide oxidizing bacteria of the genus *Beggiatoa* belong to the *Gammaproteobacteria* [13]. The *Gammaproteobacteria* is a large phylum, including a wide variety of bacteria dominated by facultative anaerobic organisms. Many of them, but not all, use reduced sulfur compounds as electron donor. A large subgroup of sulfide oxidizing bacteria are the *Chromatiaceae*. These include anoxygenic phototrophic bacteria that comprise a number of physiologically and phylogenetically distinct groups, including purple sulfur, purple non-sulfur bacteria, heliobacteria and aerobic anoxygenic phototrophic bacteria [2]. The giant sulfur bacteria include *Beggiatoa*, *Thioploca*, *Thiotrix*, *Thiomargarita* and *Achromatium* [42, 12]. *Beggiatoa*, *Thioploca* and *Thiomargarita* form a monophyletic group whereas *Thiotrix* and *Achromatium* belong to other branches (Fig. 2). All strains can oxidize sulfide to elemental sulfur which can be stored intracellulary [20, 12]. Some marine members have a vacuole and appear hollow. This characteristic, together with the absence of photosynthetic pigments morphologically distinguishes the vacuolated genera *Beggiatoa*, *Thioploca,* and *Thiomargarita* from other filamentous bacteria, such as *Cyanobacteria* [42]. They form a tight cluster (Fig. 2) within the *Gammaproteobacteria* based on 16S rRNA analysis [17]. The large vacuolated *Beggiatoa* are more closely related to the vacuolated *Thioploca* and *Thiomargarita* than to nonvacuolated freshwater *Beggiatoa.* Thus the genus *Beggiatoa* is not a homogeneous phylogenetic group [26]. The genus of *Beggiatoa* comprises many strains with similar morphology but different physiology, as they occur in freshwater, brackish, marine and hypersaline environments. *Beggiatoa* include strains that are heterotrophic (e.g. *Beggiatoa* culture OH-75-B), mixotrophic (e.g. *Beggiatoa* alba strain B18 LD) and autotrophic (e.g. *Beggiatoa alba* strain MS-81-6) [10, 29]. The size of *Beggiatoa* is unaffected by environmental conditions [36,26] and can vary, depending on the strain, between <1 μm and up to 200 μm [37,26]. For that reason cell diameter is a common feature to distinguish different species in *Beggiatoa* and in *Thioploca* [14, 16].

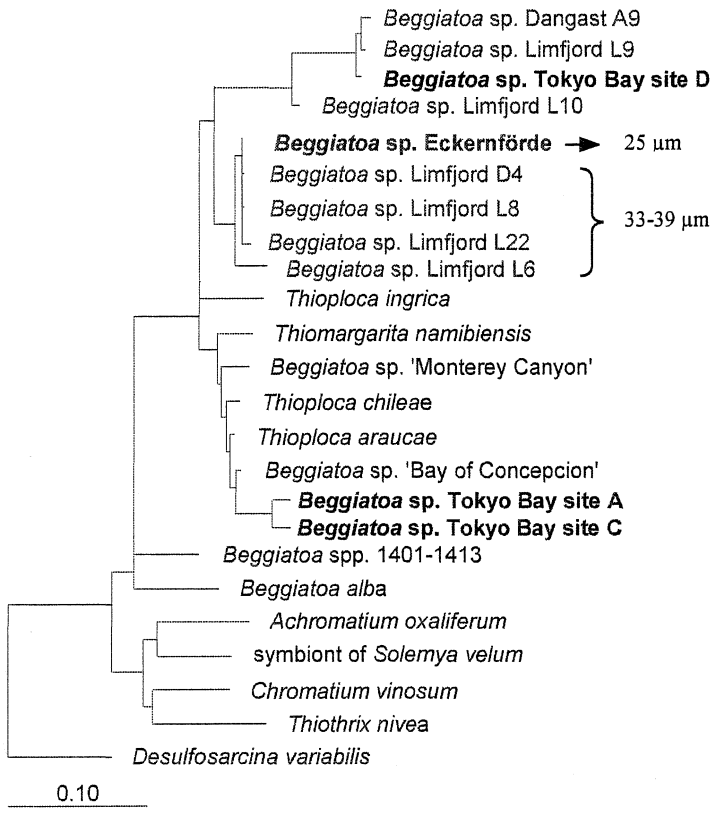


Fig. 2: From Mußmann 2004. Phylogenetical reconstruction of 16S rRNA Sequences of some filamentous sulfur oxidizers of the Gammaproteobacteria. The bar reflects 10% difference in sequenzes.

1.3 Physiology

Physiology of the sulfur oxidizing bacteria

It is well known that big bacteria are able to oxidize sulfide and that they store elemental sulfur in the vacuole, which gives them a white appearance [46, 21]. As these sulfur oxidizing bacteria can occur in high numbers in marine sediments they were thought to play an important role in the sulfur cycle [16]. However, it was not certain why the big bacteria oxidize sulfide. Two possibilities were discussed. One possibility was that the sulfide oxidation is a detoxification mechanism [47]. Alternatively sulfide is oxidized to channel electrons into the respiration chain to gain energy [19]. The option of

detoxification was supported by the fact that elemental sulfur was stored outside the cytoplasm and did not promote an increase in growth rates of the freshwater strain *Beggiatoa alba* [10]. When pure cultures of thin marine *Beggiatoa* strains became available chemoautotrophic growth on sulfide was shown [29, 28, 11]. It was proven that sulfide in the marine strains is used as e-donor for respiration, although a detoxification (removing peroxides with sulfide or removing sulfide attached to enzymes) effect cannot be excluded. Oxygen was thought to be the sole oxidant until very high nitrate concentrations were measured in some members of *Thioploca*, *Beggiatoa* and *Thiomargarita* [22, 26,35]. In purified *Beggiatoa* samples of Monterey Bay nitrate reductase was measured [24] leading to the conclusion that the nitrate was used as oxidant for sulfide respiration. It is not clear whether nitrate is used in addition to the oxygen or instead. Indeed, many nitrate reducing bacteria can also use oxygen. *Thiomargarita,* which also stores nitrate, uses oxygen as well as nitrate [38]. Experiments in *Thioploca* showed that the final product of nitrate reduction is ammonium, although N_2 was also measured in small amounts [32]. Since, at present, no pure cultures of nitrate storing *Beggiatoa* are available there are many uncertainties. In spite of many positive indications, the Dissimilatory Nitrate Reduction to Ammonium as final product (DNRA) is not certain.

The internal nitrate stores allow *Beggiatoa* filaments to oxidize sulfide in anoxic sediments. This discovery gave credence to the hypothesis that *Beggiatoa* is responsible for the suppression of sulfide concentrations down to depths greater than 2 centimeter [16]. Recent estimates indicate that *Beggiatoa* and *Thioploca* could be responsible for a major fraction of the sulfide removal in the sediment [26, 32]. However, it also iron is important for binding or oxidizing sulfide in *Thioploca* dominated sediments [8]. For *Beggiatoa* there are few data on this topic, leaving uncertainty on their importance for sulfide removal in sediments.

Storage compounds

All big marine *Beggiatoa* and *Thioploca* (> 10 µm) tested so far are known to store nitrate. In their vacuole nitrate concentrations of up to 370 mM were found [24, 26, 1]. The unique vacuole in Giant Bacteria functions as a storage pool for nitrate, allowing respiration during conditions of nitrate depletion [13, 17]. Vacuolated sulfur bacteria that do not store nitrate have also been found recently [17]. In this case other functions were discussed such as density regulation or oxygen storage. The surface to volume ratio is unfavorable in big cells due to diffusion limitations leading to insufficient supply of metabolites in the prokaryotic cell [37]. However, the vacuole reduces the cytoplasm to a thin layer and improves the surface to volume of active biomass ratio considerably. *Thiomargarita* consist of 98 % vacuole [37] and in *Thioploca* the vacuole takes about 90 % of the cell volume [32] which is probably similar in *Beggiatoa*. In addition to the sulfur inclusions the big bacteria accumulate nitrate and PHB (poly-β-hydroxybutyric acid) as storage products [19]. Recently polyphosphates, that function as energy storage, were observed in *Thiomargarita* [39].

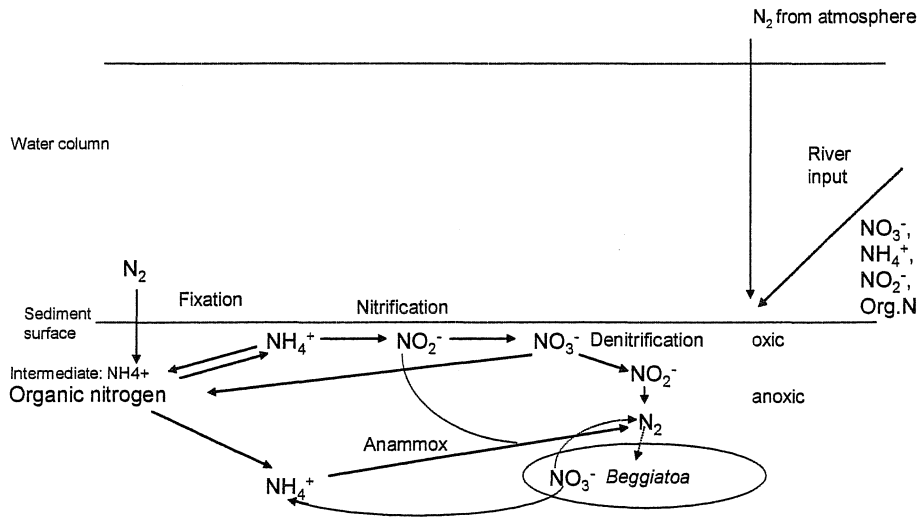
1.4 Ecology

The sulfide oxidizing capacity of large sulfide oxidizing bacteria may well be important for the sulfur cycle [16, 26]. Considering the nitrate storing capacity of sulfur oxidizing bacteria [44] they may also be important in the nitrogen cycle.

Nitrogen cycle

Nitrogen occurs in marine environments as dissolved gas (N_2, N_2O) and fixed nitrogen (nitrate, nitrite, ammonium and nitrogen bound in organics). Most of the nitrogen is N_2 gas that is dissolved as well in the water column as in the sediment [45]. N_2 is an inert gas for most organisms but it can be fixed by specialized bacteria into ammonium and eventually into organic matter [4]. In some marine habitats nitrogen fixation can be the major source of nitrogen uptake [5]. Other important nitrogen sources for microorganisms are nitrate and ammonium that are transported into the ocean by rivers

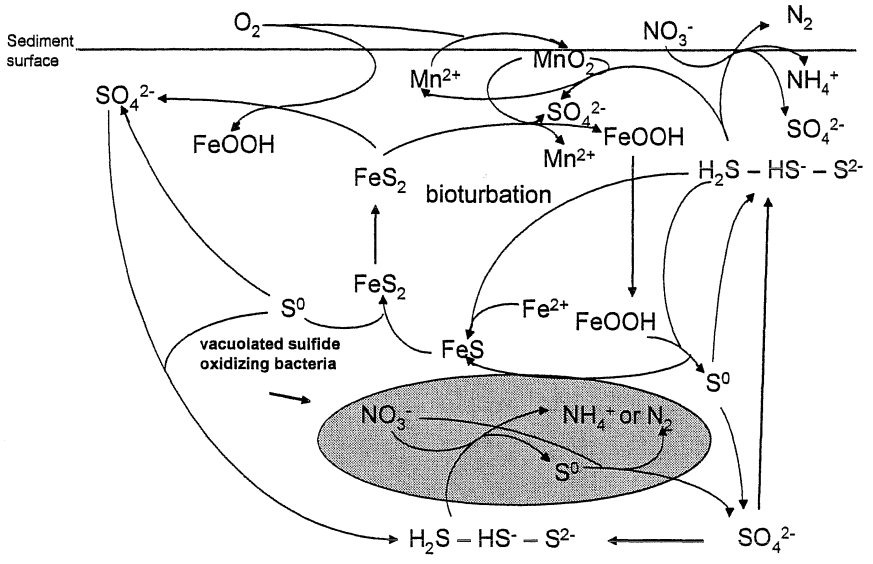
[9]. They are not only used as nutrient but also as electron acceptors and donors, respectively [6, 18]. Nitrate is also produced by aerobic ammonium oxidation in a two step process known as nitrification. Ammonium is released by the anoxic decomposition of organic matter or is produced by DNRA. The loss of nitrogen out of the sediment is mainly due to the production of N_2 gas as final product of denitrification (reduction of nitrate to N_2). The nitrogen could be also removed from the system by anaerobic ammonium oxidation with nitrite (anammox). This process contributes significantly to the nitrogen budgets in some anoxic oceanic environments as well in the water column [19] as in the sediment [45, 7]. In general the nitrate concentrations in coastal sediments are in the range of several µM [25, 22]. However, in sediments containing high amounts of nitrate storing sulfur oxidizing bacteria millimolar concentrations of nitrate were measured in the pore water leading to the conclusion that these bacteria contribute significantly to the nitrogen cycle in the sediment [44]. As nitrogen fixation also occurs in some *Beggiatoa* strains [30] the contribution of *Beggiatoa* to the N-cycle is varied and complex.



Nitrogen cycle in a coastal marine sediment based mainly on Kuypers et al. 2003 including *Beggiatoa*. Additional literature see in the text.

Sulfur Cycle

The marine sulfur cycle is also very complex. Many sulfur intermediates occur in marine sediments due to reduction and oxidation of sulfur compounds [31] The sulfur cycle is very important as sulfate is the most abundant electron acceptor (ca. 28 mM) in standard (33-35 ‰ salinity) seawater [27]. Sulfate reduction is an important pathway of anaerobic mineralization in most continental shelf sediments [15]. The product of sulfate reduction is free sulfide that is released into the sediment where under certain conditions it may accumulate to mM concentrations which can be toxic for the biotic community in the sediment [26]. Nevertheless, many prokaryotes and eukaryotes are able to live in the sulfide enriched zone [3]. Of the prokaryotes the sulfate reducing and the sulfide oxidizing bacteria are very abundant in highly sulfidic environments. The electron acceptors for microbial sulfide oxidation include mainly oxygen and nitrate. These oxidants do not penetrate very deep into the sediment due to relatively low water column concentrations, mass transfer resistance and the activity of aerobic and nitrate reducing bacteria. Sulfide oxidation also takes place with oxidized Fe and Mn as e-acceptor. MnO_2 reduction by sulfide is a mainly biotic pathway whereas Fe(III) probably oxidizes sulfide abiotically [8, 43]. Complete oxidation of sulfide to sulfate by MnO_2 occur only in the uppermost 0-1 cm of the sediment, whereas Fe(III) reduction can take place at least in the upper 4 cm of the sediment [22]. The oxidation of sulfide back to sulfate occurs in a complex web of competing chemical and biological reactions [48]. The intermediates of the oxidation of free sulfide are mostly S^0, S_2O_3, $S_4O_6^{2-}$and SO_3^{2-} with S^0 having the slowest turnover times [48]. The intermediates can disproportionate into sulfate and sulfide [13]. Besides oxidation by Fe/Mn, sulfide below the penetration depth of oxygen and nitrate can be oxidized by the giant bacteria. In an environment free of nitrate these bacteria use their internal nitrate pool for oxidation of the sulfide to sulfate with elemental sulfur as an intermediate [47].



Based on Fig.4 in Jorgensen and Nelson (2004) with supplements of nitrate and *Beggiatoa* according to the current state of knowledge (see text). Sulfate is mentioned several times to show the different pathways.

1.5 The aim of this study

Sulfur oxidizing big nitrate storing bacteria are assumed to be significant for the sulfur cycle. According to several estimations, these bacteria can be responsible for most sulfide removal from the sediment [16, 26, 32]. Therefore it was assumed that *Beggiatoa* could control the sulfide availability in the sulfide depleted zone. However recent investigations showed that this is probably not always the case. In sediments containing high biomass of *Thioploca* it was calculated that most of the sulfide is removed by iron [8]. Our hypothesis is that the sulfur cycle controls *Beggiatoa* distributions, whereas *Beggiatoa* has little influence on the distribution of sulfide and on the sulfur cycle in general. We compared the sulfide production and input with the sulfide oxidation potential of *Beggiatoa* in all sediment layers of the *Beggiatoa* inhabited zone (BIZ).

Another important point was to focus on the contribution of *Beggiatoa* to the nitrogen cycle. A big nitrate pool is stored in *Beggiatoa* that possibly transports this pool to several centimeters below the surface [34, 9]. One aim was to elucidate whether indeed *Beggiatoa* transports nitrate deep into the sediments. To evaluate this we determined the

cellular nitrate content and the biomass distribution and compared this with the measured nitrate pool in the sediment. We also investigated for how long *Beggiatoa* can live with this storage pool. This may cause a restricted distribution of *Beggiatoa* to the top of the sediment due to limitations in nitrate supply.

Finally, we identified naturally occurring *Beggiatoa* populations by fluorescence in situ hybridization (FISH) to determine whether phylogeny is correlated with the filament width. We studied temporal and spatial dynamics of *Beggiatoa* subpopulations in attempt to explain the coexistence of physiologically and phylogenetically similar organisms.

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2 Manuscripts

2.1 Coexistence and community dynamics of different *Beggiatoa* strains in sulfidic marine sediments

André Preisler, Marc Mußmann and Bo Barker Jørgensen

The vertical distribution of the big filamentous sulfide oxidizing bacteria was investigated in sediments from Eckernförde Bay (Germany, Baltic Sea). All strains of the nitrate storing sulfide oxidizing bacteria that were found belonged to the genus *Beggiatoa*. The close relatives *Thioploca* and *Thiomargarita* were not found.

Based on FISH staining at least 4 different strains of *Beggiatoa* were found that can be divided in size classes of 14±4 µm, 20±3 µm, 27±5 and 37±4 µm. Detailed observations showed that the size class 27±5 was probably composed of two different strains (30±3, 24±3). *Beggiatoa* inhabited the sulfide- and oxygen-free habitat in between the oxic and the sulfidic zone. Members of all size classes were more or less evenly distributed over this horizon. No seasonal changes in abundance and community composition were observed. The community compositions, however, differed between years. The stability in spatial and temporal distribution of the *Beggiatoa* community over a year, and the differences between years, suggests that they coexist because of a top down control by grazers and viruses. As *Beggiatoa* are conspicuous organisms and easily to sample in the environment they may serve as model organisms to investigate the ecology of coexisting populations of uncultured bacteria.

Introduction

The filamentous, colorless sulfur bacteria *Beggiatoa* spp. belong to the *Gammaproteobacteria* [7] and often grow abundantly at the surface of sulfide-rich sediments [10]. Because of internal sulfur globules they are visible to the naked eye and were discovered more than 100 years ago [25]. Different size classes of *Beggiatoa* can occur at the sediment surface [12] or they are distributed in a toplayer of several centimeter thick [9, 11], similar to *Thioploca* [19]. Which physiological or ecological circumstances enable coexistence of different *Beggiatoa* strains is unclear. The concept of niche separation has proven useful to explain species diversity and community structure [14, 16]. Based on the trade-off theories different species can coexist if species that are better at dealing with one environmental constraint are necessarily worse at dealing with another [21, 22]. One general tenet to explain diversity is that species occupying the same niche cannot coexist and the number of coexisting species cannot exceed the number of limiting resources, but many observations showed that it is not that simple [24, 23, 6, 20, 18]. In addition to the different features in resource uptake a top-down control of the community must also be considered. This is probably also the case in bacteria communities as bacteriovorous organisms are present in all marine sediments [26] and control the bacteria diversity [13]. For instance nematodes have an effect on the bacterial community composition as they might prefer the faster growing bacteria [15]. The high abundance of viruses found in aquatic environments suggests that virus infection could also be an important factor controlling bacterial numbers [2]. For the big sulfur oxidizing bacteria it is known that different genera can coexist in the sediment. For instance a coexistence of *Thiomargarita* and *Beggiatoa* was observed in sediments of Namibia [17], and off the coast of Chile [19]. All of them belong to the same cluster among the *Gammaproteobacteria* but are clearly different in physiology as *Thiomargarita* are not able to move, *Thioploca* move in sheets and *Beggiatoa* without sheets. The notion that different *Beggiatoa* strains coexist was mainly based on the diameter but also on 16S rRNA data [11], indicating a genetically fixed filament diameter. In contrast to strains without nitrate accumulation, in nitrate storing *Beggiatoa* physiological differences are not yet documented.

Therefore we investigated whether several different strains can coexist and which factors influence their spatial distribution. It may be hypothesized that different strains occupy different ecological niches and coexist due to chaotic spatial and temporal oscillations that can occur due to irregular and unpredictable changes [18]. The different niches could allow them to flourish at different depths or different times of the year. Another possibility is that viruses and grazers are the main controlling mechanisms.

Methods:

Sampling

The study sites are located in Eckernförde Bay (Germany, Baltic Sea) and the harbour of Eckernförde. We sampled in March 2002 (4 stations), June 2002 (2 stations), January 2003 (3 stations) and June 2003 (2 stations). All these stations were very similar in species composition and the obtained data were therefore pooled. Two sediment cores with an inner diameter of 10 cm were collected from each station by a small multiple corer, based on the construction described by Barnett et al. 1984. The core was sectioned into 5 mm (0-20 mm sediment depth) and 10 mm depth intervals (20-100 mm sediment depth) depth intervals for biomass analyses.

Biomass determination

Defined sediment subsamples (ca. 300-500 mg) from each depth were resuspended in filtered sea water (10 ml), of which 300-400 mg were investigated microscopically. Filament width and length of all *Beggiatoa* filaments were determined for each depth interval [9]. The biomass was quantified using the filament volume (cylindrical shape) and assumed density of 1 g cm^{-3} [11]. The diameter of all *Beggiatoa* strains was used to determine the different size classes. The total biomass per unit area was obtained by integrating biomass densities over depth.

Fluorescence in situ hybridization (FISH)

To distinguish the different size class fluorescence in situ hybridization was performed as described in Mußmann et al. 2003. Three different probes were used for the dominant clone sequences of *Beggiatoa* spp. from Eckernförde harbour (Blim575 5'CTA GCC GCC TAC ATA CGC-3', Blim193 5'-AAA AGA CGC CCT TCC- 3' and Bdan193 5'AAA CAG GCG CCC TCT TTC-3'.

Results

Species composition

In samples from the harbour, filament width of *Beggiatoa* was measured with brightfield microscopy and epifluorescence microscopy after situ hybridization with probes Blim575, Blim193 and Bdan193. None of the probes stained filaments of 27±5 µm diameter. Filaments of the biomass size class 14±4 µm were stained by the probe Bdan193 targeting nitrate storing bacteria. Not all filaments of this size class were stained by probe Bdan193 thus this size class could be composed of different strains. Filaments of 20±3 µm diameter were stained by probe Blim193. Probe Blim575 stained the size classes of a broad spectrum of 37-50 µm diameter. The filaments are probably artificially flattened by the used method. From measurements in well preserved filaments the range of the filaments is assumed to be 37±4 µm. No significant change of filament width occurred in the other size classes as the range was very similar to the non-fixed samples. From these FISH investigations we conclude that at least 4 different nitrate storing size classes coexist in the sediment that can be divided in size classes 14±4 µm, 20±3 µm, 27±5 and 37±4 µm. As the same size peaks were measured in Eckernförde Bay, the strains were assumed to be the same as in the harbour. As width of the filaments is probably constant and genetically inherited [11], the shift of the peak from 30 µm to 25 µm in 2003 (Fig. 1) suggests that the size classes 27±6 could be composed of two different strains (24±3, 30±3). Filaments of 6±3 width were observed only very rarely.

Fig. 1

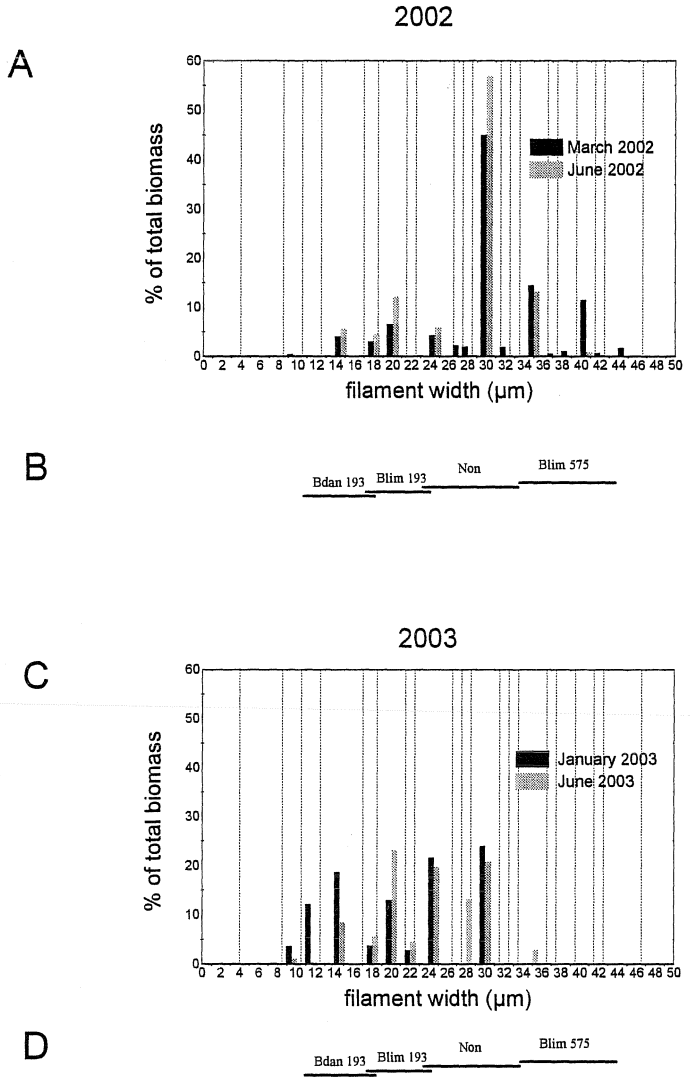

Fig.1A,C: Eckernförde Bay: Pool of 4 (March 2002), 2 (June 2002), 3 (January 2003), 3 (June 2003), respectively. Importance in % of different filament width integrated over all sediment depth. Fig. 1 B,D: diameter range that stained the different probes

Distribution of biomass in general

Beggiatoa biomass was distributed heterogeneously, with significant variations within a distance of less than 1 m. The heterogeneity depended mainly on variations in the horizon of the oxidized and the reduced zone of the sediment (light zone was defined as oxidized zone, dark zone as reduced zone). *Beggiatoa* biomass was 16 g m^{-2} (Station 1, Fig 2A,B),

and 27.9 g m^{-2} (Station 2, Fig 2C,D), respectively. At the harbor, patchiness was even visible to the naked eye as the white *Beggiatoa* mats were restricted to certain areas. Here the amount of biomass ranged from 27 (Fig. 2E,F) to>60 g m^{-2}.

Also the vertical distribution of *Beggiatoa* biomass within a given depth zone was variable. At 4 stations the biomass peaked at 5-10 mm (e.g. Fig. 2A). At 2 stations the biomass peaked at 15-20 mm sediment depth. At 6 stations biomass decreased gradually with depth with main biomass at the top and low biomass in the deepest sediment layer. At two stations it was rather evenly distributed over the whole horizon containing *Beggiatoa* biomass (e.g. Fig. 2C). In the harbour, *Beggiatoa* biomass appeared as a mat at the surface and the sediment surface appeared snowy white. In addition *Beggiatoa* biomass was found down to 20 mm sediment depth (Fig. 2E) in >3 mM sulfide concentrations.

Biomass integrated over depth from Fig. 2C was ca 27 g m^{-2} but it reached more than 60 g m^{-2} in another core from the harbour (not shown). At a station with *Beggiatoa* filaments distributed over 6 centimeter sediment depth biomass was 63 g m^{-2} (data not shown) indicating that high amounts of *Beggiatoa* biomass can be hidden in the sediment.

Fig. 2

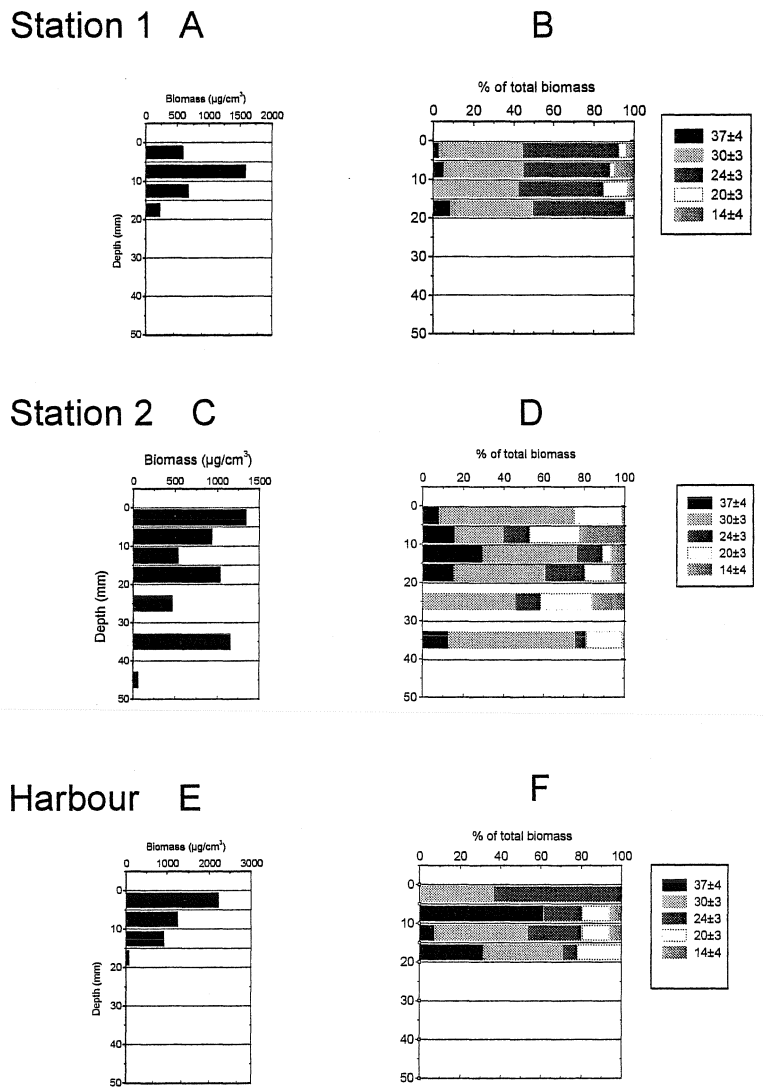

Fig. 2 A,C,E: *Beggiatoa* biomass. Fig. 2 B,D,F: Importance in % of different filament width in different depth intervalls.

Seasonal dependence of species composition

The species composition in the sediment was very similar at all stations in Eckernförde Bay. In 2002 filaments of 30±3 µm width had the highest biomass in all sediment depths.

To investigate the seasonal dependence of species composition the biomass of each filament width was integrated and compared to total biomass (Fig. 1). A seasonal change in species distribution was not observed as there was no difference between winter and summer as well in 2002 (Fig. 1A) as in 2003 (Fig. 1B). From June 2003 to January 2002 the biggest species (37±4 µm) almost disappeared and the abundance of the 30±3 µm size class decreased considerably. In June 2003 a similar distribution was observed.

Vertical distribution of different size classes.

The different size classes were rather evenly distributed over the whole sediment (Fig.2C, F, I; Fig. 3). All size classes between 10 µm and 40 µm were present at each horizon down to 20 mm (Station E3-2, Fig. 2D) or 40 mm sediment depth (e.g. station E6-3, Fig. 3). *Beggiatoa* biomass of 5-9 µm filament width also occurred in negligible amounts down to 20 mm sediment depth (not shown). In 2002 filaments of 30±2 µm in width accounted for the majority of *Beggiatoa* biomass at all depths, whereas no dominant *Beggiatoa* population could be identified in 2003 (Fig. 2A-F).

Discussion

Spatial and temporal variations of Beggiatoa community

Different size classes of *Beggiatoa* are assumed to belong to different phylogenetical clusters (Mußmann et al. 2003) and are used as a criterium by which species of the genus *Beggiatoa* and *Thioploca* were designated [9, 12, 8]. Our results from FISH staining confirmed this assumption. We showed that at least 4 different size classes and strains (filament width between 10 µm and 45 µm) can coexist in the sediment and are rather evenly distributed over the whole horizon of *Beggiatoa* biomass occurrence. No consistent correlation between community composition and depth in the sediment or season could be found. Due to the surface to volume ratio larger *Beggiatoa* might be better adapted to the deeper layers as they have more space for the storage of nitrate. This can then be utilized as an oxidant below the free nitrate penetration depth in the sediment.

However, a clear correlation between size and vertical distribution was not found as the narrower strains were also found in the deepest horizon of *Beggiatoa* distribution. No sediment layer was found from which one of the observed strains was generally excluded. In 2002 the size classes of 30±3 µm width was dominant and the species composition did not depend on the season as follows from the data collected in winter and summer. However, from June 2002 to January 2003 the widest size class almost disappeared and the narrower members became more abundant in biomass. This pattern was also observed in June 2003. The question was what caused this observed fluctuation. In the time between June 2002 and January 2003 bottom water temperature arose up to 14 °C in October 2002. Observations (unpublished) in culture studies indicated that survival of *Beggiatoa* of Eckernförde Bay could be effected by temperature of >15 °C. Temperature shifts may affect the fluctuations in the composition of the *Beggiatoa* community. In addition, anoxic bottom water conditions arose in September and October and nitrate concentrations were less than 0.3 µM in August, September and October (one measurement per month; H.P. Hansen pers. comm.). A salinity increase of more than 6 ‰ from June to July 2002 was also observed in the bottom water of Eckernförde Bay by H.P. Hansen and it stayed higher in 2003 than in 2002 over the whole year.

In summary no population of *Beggiatoa* seemed to have a preference for sediment depth or season. All *Beggiatoa* having filaments wider than 10 µm store nitrate and are assumed to have rather similar physiological features. As they probably compete for more than one resource (CO2, sulfide, organics, nitrate, phosphor, vitamins, trace elements etc.) coexistence of at least 4 different strains is not in contradiction to the hypothesis that the number of coexisting species cannot exceed the number of limiting resources [24, 23, 6, 20]. As their physiology might be rather similar it is nevertheless improbable that four different resources are limiting. In plankton communities a very high diversity of species using a small number of limiting resources were observed which is called the "paradox of the plankton" and is mainly explained by oscillation and chaotic behaviour of the whole ecosystem [18]. However, chaotic oscillation seems to be rather improbable as the community was rather stable over a period of several months. Thus, the slow changes in community compositions could be due to grazers. In addition, viruses could be an important factor [15] in controlling species diversity of *Beggiatoa* filaments.

Fuhrman suggested in 1999 [4] that viruses could be an explanation for the paradox of the plankton due to different impacts on the different species. The importance of viruses for population dynamics has been rarely mentioned in the reviews concerning this well investigated topic. Viruses are responsible for about 50-100% of the total bacterial mortality in environments that are unfriendly to protists [4]. Although grazing on *Beggiatoa* also occurs [3], *Beggiatoa* are rather inconvenient food due to the low ratio of usable carbon to water, nitrate and sulfur. In addition, the anoxic *Beggiatoa* inhabiting zone (BIZ) is unfriendly to many grazers and mortality due to virus infection is possibly higher than grazing [4]. Further investigation will be needed to reveal how viruses can control *Beggiatoa* abundance.

Conclusion

The investigations showed that filament width is a good morphological characteristic to distinguish different strains of *Beggiatoa*. An unpublished partial 16S rRNA analysis showed that at least one strain was identical in Eckernförde and in Limfjorden (Denmark). Our FISH investigation showed that one strain was even distributed over Eckernförde, Limfjorden and the Wadden Sea (Germany). The species community was not influenced by seasonal changes. However, episodic events like temperature changes can have an effect on the species composition. Other events which could have influence in the *Beggiatoa* community are currents. By this the *Beggiatoa* filaments are supposed to be swept away and transported from one region to the other [5]. The observed dynamics of the *Beggiatoa* community are probably mainly controlled by grazing and virusses. Further investigations are needed to confirm this. In contrast to most other bacteria *Beggiatoa* are very conspicuous and can be easily distinguished morphologically. Therefore this genus could serve as a model organism for ecological studies, in particular on coexistence and niche partitioning of phylogenetically and physiologically related organisms.

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2.2 Ecophysiology of *Beggiatoa* in a coastal marine sediment (Eckernförde Bay)- the importance of internal nitrate- and sulfur storage.

André Preisler, Gaute Lavik, Elze Wieringa, Lubos Polerecky, Sybille Zitzmann, Michael Nielsen & Bo Barker Jørgensen

Abstract

The vertical distribution of intracellular nitrate and sulfur in *Beggiatoa* at different depths was investigated in Eckernförde Bay (Germany-Baltic Sea). The main biomass of *Beggiatoa* was found in sediment layers without detectable oxygen, nitrate or free sulfide in the porewater. After freezing and thawing of the sediment a large nitrate pool was detected down to 5 cm sediment depth. This nitrate pool is transported down in vacuoles of *Beggiatoa* by gliding motion. Whereas *Beggiatoa* is largely responsible for the nitrate pool in the sediments, they are not important for the sedimentary sulfur content. Only 2-9% of the sulfur in the *Beggiatoa* inhabited zone is intracellular and a large sulfur pool exists below the *Beggiatoa* zone. The nitrate reduction rates were in the range of 13-15 $\mu mol\ cm^{-3}_{Begg}\ d^{-1}$. The pools of sulfur and nitrate allow *Beggiatoa* to survive up to 3 weeks in the anoxic sediments, and allow them to travel several meters without external supplies.

Introduction

Beggiatoa spp. are multicellular, filamentous, colorless sulfur bacteria inhabiting freshwater and marine sediments. Intracellular nitrate storage was recently discovered in the large marine *Beggiatoa*, *Thioploca* and *Thiomargarita* which sets these prokaryotes apart from the rest of the sulfur oxidizing bacteria [10, 43]. Inclusions of elemental sulfur are much more widespread among the sulfur bacteria and have been observed in *Beggiatoa* and *Thioploca* already around 1900 [4, 26]. The sulfur inclusions give the *Beggiatoa* a white colour and conspicuous snowy white mats can form at the sediment surface [18, 32].

In marine sediments O_2 and free sulfide are generally separated by an intermediate zone where neither is present in detectable concentrations [16]. In such cases no visible mat may appear at the sediment surface but the sediment might still harbor high *Beggiatoa* biomass. Up to 20 g fresh weight per m^2 was found in Limfjorden and in Dangast (Wadden Sea, Germany), where *Beggiatoa* was found down to more than 4 cm depth [22, 34]. Nitrate penetration in coastal sediments is usually in a range of a few millimeter, as shown with microsensors [34, 30]. It is, however, unclear whether nitrate is available for *Beggiatoa* in the anoxic but oxidized layers where neither oxygen nor free sulfide are detectable [19].

All marine representatives of *Beggiatoa* with a filament diameter >10 µm examined so far were found to store nitrate up to a concentration of 370 mM [29, 34, 1]. In sediments containing *Beggiatoa* which were frozen, thawed and centrifuged (2000 x g) previous to pore water analysis, nitrate concentrations can be 100-fold the concentration in the overlying seawater [42]. After such treatment, a large nitrate pool of up to 3 mM nitrate was measured in continental margin sediments of Chile containing *Thioploca* [46].

Small (ca 5 µm wide) marine *Beggiatoa* strains grow chemoautotrophically on oxygen and free sulfide [35, 12]. Since McHatton et al. [29] found high concentration of nitrate reductase in purified Monterey *Beggiatoa,* it is believed that these vacuolated *Beggiatoa* can grow chemoautotrophically by free sulfide oxidation with nitrate [16]. The product of nitrate reduction in *Beggiatoa* is not known, but experiments with purified *Thioploca* bundles revealed ammonium to be the reduced product, although N_2 was also produced in

small amounts [38]. Due to the close phylogenetic relationship and similar morphology of *Thioploca* and *Beggiatoa* [34, 1], both groups might produce ammonium, but the versatility among different species with respect to end products of nitrate reduction needs still to be investigated [16].

Autotrophic marine *Beggiatoa* in gradient cultures accelerate the turnover of free sulfide 1000 to 2000-fold relative to the chemical sulfide oxidation with oxygen, resulting in their significant effect on the free sulfide profile [36]. Free sulfide is assumed to be the electron donor for the reduction of oxygen or nitrate in sulfur oxidizing bacteria [29, 8]. Sulfide is probably oxidized first to elemental sulfur [36] and, in a second step, the stored elemental sulfur is further oxidized to sulfate [38]. For Limfjorden sediments, Jørgensen [22] estimated that *Beggiatoa* has the potential to oxidize almost all of the produced free sulfide. Mussmann et al. [34] calculated for the same habitat that the contribution of *Beggiatoa* to the total free sulfide oxidation was about 50 %. For *Thioploca* communities off the coast of Chile it was estimated that these sulfur bacteria have the potential to oxidize in some regions of the continental shelf 25-91 % of the free sulfide produced by sulfate reduction [38]. Calculations for the shelf sediments of the central Chile upwelling area suggest that the dense mats of *Thioploca* were oxidizing a maximum of 16-34 % of sulfide production [8]. So the actual importance of nitrate storing bacteria for the sulfur cycle is rather uncertain.

There are several indications of intracellular nitrate storage in *Beggiatoa* living in deeper layers [42, 41,15], like *Thioploca* does [17, 49]. Because of the intracellular nitrate and sulfur storage, a vertical shuttling between the nitrate-rich bottom water and the deeper sediment layers is suggested for *Thioploca* [10, 49]. This is confirmed by the observation of nitrate containing filaments that were found down to 13 cm depth [49, 12].

Thioploca produce a dense network of sheaths in the sediments, which may facilitate the vertical gliding. *Beggiatoa* is only found as free living filaments, also deeper into sediments. Only few data have been published on vertical nitrate and sulfur transport by gliding *Beggiatoa*. Our aim was to assess whether intracellularly stored nitrate and sulfur are significant pools in *Beggiatoa* containing sediments and to investigate how long *Beggiatoa* can survive and maintain motility using its internal nitrate stores.

Methods

Sampling

Two stations in Eckernförde Bay (Germany, Baltic Sea) were used for this study and sampled during early summer (Station 1 (E4-1): June 2002 and Station 2 (E8-1): June 2003). Two parallel sediment cores with an inner diameter of 10 cm were taken at each station by a small multiple corer, described previously [2]. One core was sectioned into 5 mm (0-2 cm sediment depth) and 10 mm depth intervals (2-10 cm sediment depth) for biomass, nitrate and sulfur analyses, while the second core was used for microsensor measurements. The vertical distributions of the lighter (oxidized) and dark (reduced) zones were the same in both parallel cores. The porosity of the sediment at station 1 was determined in a separate core two months later.

Microsensor measurements

Profiles of nitrate plus nitrite were measured with a microbiosensor [27], with a 90% response time of 2 min, a tip diameter of 100 µm, and an electrophoretic sensitivity control of +400 mV [23]. A four-point calibration was done at in situ temperature and in seawater from the sampling site. The nitrate concentrations used for calibration were immediately measured with an NOx analyzer. Calibration was linear in the range from 10 µM to 112 µM nitrate. Oxygen was measured with a Clark-type oxygen micro-electrode with a guard cathode [40]. The pH profiles were measured with glass microelectrodes [39]. Dissolved hydrogen sulfide was measured using a H_2S microsensor [25, 14]. The total sulfide profiles were calculated from the measured H_2S and pH microprofiles as described previously [14]. The value for pK_1^* used was corrected for temperature and salinity according to [31].

The microsensors were mounted on a motorized micromanipulator. During the measurements, the cores were cooled in a thermostated waterbath to a temperature of 15° C (in situ temperature: ca 6° C) and the salinity of the overlying water was 22 ‰. Net sulfide consumption and production rates and the diffusive flux were evaluated by fitting the microprofiles by a diffusive model implemented in a Matlab program. The rates were compared with sulfate reduction rates (SRR) measured in March and September 2002 at

the same station (E8-1) to calculate the total (gross) free sulfide consumption. It is known that SRR depends on temperature [48, 24]. However, since no data were available for SRR in June 2003 (bottom water temperature ca. 6° C), we assumed that the rates were similar to those in March (bottom water: 4°C) rather than those measured in September (bottom water: 12°C).

Sulfate reduction (SR)

Three (March 2002) replicate sub-cores were taken out of one MiniMuc core. Carrier-free $^{35}SO_4^{2-}$ radiotracer was dissolved in water and injected into the replicate push-cores at 1 cm intervals according to the whole core injection method of [21]. The sub-cores were incubated at 4°C for 48 hrs in the dark. After incubation, the sediment cores were sectioned into 1 cm intervals and transferred into 50 ml plastic centrifuge vials filled with 20 ml zinc acetate (20% w/w). SR rates were determined using the single step acid Cr-II method according to [9].

Nitrate and elemental sulfur measurements in the sediment

Sayama [42] described a method in which the total nitrate concentrations of *Beggiatoa* can be determined by comparing frozen samples with unfrozen ones (both centrifuged at 2000 x g). We used a similar approach, comparing the values obtained from frozen samples to those from bottom water (E4-1, E8-1) or from microsensors (E8-1). In samples that were frozen and thawed, highly scattered nitrate concentrations in sediment layers that harbored high *Beggiatoa* biomass were measured. Reproducibility and accuracy was strongly improved when the sediment samples were fixed in ZnAc (20 % solution) prior to freezing. After thawing porewater was extracted by centrifugation at 3000 x g for 10 min. Nitrite plus nitrate were measured using a NOx-Analyser (Thermo Environmental Instruments, Franklin, USA) based on NO_3^- and NO_2^- reduction to NO by $V(III)Cl_3$ (in 1M HCL) [4]. As the nitrite concentration was insignificant as compared to nitrate (checked at one station) in these environments [27] NOx will be written as nitrate. Sulfur was extracted from the pellet of the centrifuged samples with methanol (100 %). Elemental sulfur (S^0) was determined by HPLC, as described by Zopfi et al. [50].

Biomass determination

Beggiatoa biomass was determined within one day after sampling. Defined subsamples of a core (300-500 mg) were resuspended in filtered sea water (10 ml). From this suspension 300-400 mg were taken and placed on a microscope slide. For each depth interval the *Beggiatoa* filaments were counted under a microscope [22]. The biomass, quantified using the filament volume (cylindrical shape) and assumed density of 1 g cm^{-3} [34], was integrated over all depths to get the total biomass per unit area. Biomass in the intervals that were not analysed (1.5-2 cm, 2-3 and 4-5 cm) in E4-1 was estimated by interpolation.

Intracellular nitrate and intracellular sulfur determination

Intracellular nitrate was measured in *Beggiatoa* filaments immediately after sectioning of the core. Filaments from the different sediment depths were transferred into artificial sea water (22 ‰) using a glass needle. Their length and width were determined under the microsocope and the biovolume was calculated assuming a cylindrical shape [34]. The whole procedure took about 1-2 hours per sediment layer. During this procedure, the availability of free nitrate for *Beggiatoa* for accumulation was negligible.

Several filaments (7-11) of the same diameter (18 and 30 µm) were subsequently transferred into 250 µl of deionized water (purified H_2O for inorganic trace analysis, Fluka) and frozen at – 20 °C, causing cell rupture. After thawing, the samples were centrifuged and 200 µl of the supernatant was used to measure nitrate by the NOx-analyser (see above). Based on the biovolume and the dilution factor, the average intracellular nitrate concentrations were determined. The remaining pellet was dried in air and the intracellular elemental sulfur grains of *Beggiatoa* were dissolved in methanol (100 %) over 2-3 days. The elemental sulfur was measured by HPLC (see above).

Intracellular nitrate and sulfur concentrations in sediment intervals that were not analysed were estimated by interpolations. For the calculations we assumed that other size classes, for which the intracellular nitrate and sulfur concentrations were not measured, had intracellular concentrations equal to the average of the 18 and 30 µm size classes (77 % of the total biomass).

Cell specific nitrate reduction and sulfide oxidation rates

An aquarium was filled with sediment from Eckernförde Bay containing *Beggiatoa* and left to develop for several months at 10° C with overlying oxic seawater. From this sediment, 10 samples of the top 3 cm were transferred into glass vials. The glass vials were topped with sea water and sealed by a rubber stopper. The vials contained 0.8 ml sediment and 0.2 ml overlaying water. One vial was left open to serve as oxygenated reference. After 12, 20 and 42 days two vials were used for intracellular nitrate, biomass and motility measurements.

The intracellular nitrate per volume of *Beggiatoa* was calculated from the biomass and the nitrate released after freezing and thawing, measured using the procedures described above. For the calculation we assumed a porosity of 0.88 and a density of 1.3 g cm^{-3}. Cell motility, as measure for viability, was determined from the parallel vial by microscopy. For the initial values, two sediment samples from the aquarium were used.

The intracellular nitrate reduction rate was determined from the decrease of the intracellular nitrate.

Results

Biogeochemistry in the sediment

Vertical profiles of nitrate, oxygen, free sulfide, sulfur and *Beggiatoa* biomass were determined in sediment cores with well preserved sediment surface (station E8-1, Fig.1.) Nitrate and oxygen penetration was 2.5 mm (Fig. 1A) and 1.25 mm (Fig. 1B), respectively. Nitrate rapidly declined below the oxic zone, indicating the activity of nitrate reducing bacteria.

Areal oxygen consumption of the sediment calculated from the oxygen microprofile, using Fick's law and diffusion coefficient in water of $D=1.75*10^{-9}$ m^2 s^{-1} (Revised Gastables by Ramsing and Gundersen – Unisense), was ca 75 mmol m^{-2} d^{-1}.

Free sulfide was below the detection limit (ca. 1 µM) in the upper 13 mm. Thus the sulfidic zone was spatially separated from oxygen and nitrate. Between 13 mm and 30

mm the gradient of free sulfide increased gradually. The zone of highest sulfide consumption was below 30 mm sediment depth.

Total elemental sulfur concentration (Fig. 1C) showed a peak at 30-40 mm depth and a slight increase at the deepest measured interval (90-100 mm) (data not shown).

The total nitrate concentration in the sediment, obtained after freezing and thawing, was ca 200 µM in the top layer (0-0.5 cm) (Fig. 1C), then decreased steeply with depth. The nitrate concentration in the overlying water was 8-10 µM. The total nitrate concentrations in the sediments closely correlated with the *Beggiatoa* biomass distribution. Total *Beggiatoa* biomass was 6 g m^{-2} at station E8-1 and filament widths varied between 10 and 30 µm. The main biomass of *Beggiatoa* was rather evenly distributed over the top centimeter and decreased rapidly with depth (Fig. 1C). In Eckernförde Bay, however, *Beggiatoa* biomass can occur much deeper in the sediment. For example up to 420 µg cm^{-3} of biomass was observed several centimeters (50-60 mm) deep in the sediment at another station (data not shown).

Fig.1 A) B) C)

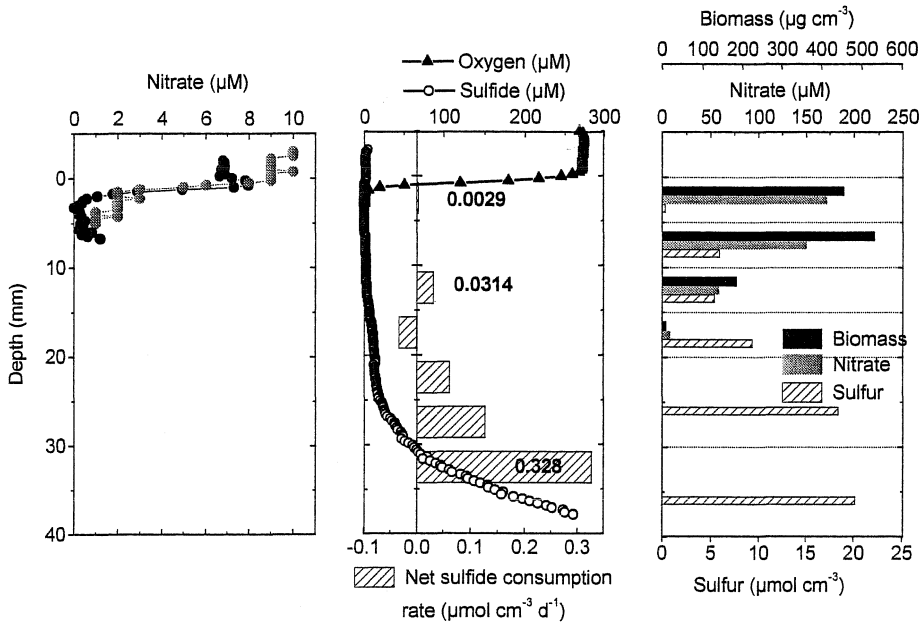

Figure 1: Microsensor profiles of (A) free pore water nitrate and (B) oxygen and free sulfide measured in a core from station E8-1 (54°31.152N, 10°01.278E). Net sulfide consumption rates calculated from the fit of the sulfide profile are also shown. (C) Profiles of *Beggiatoa* biomass, total nitrate (per volume of pore water) and elemental sulfur concentrations (per volume of sediment) measured in a parallel core. Nitrate and sulfur present in the sediment and stored in *Beggiaota* are included. Dashed lines indicate the measured sediment intervals.

The intracellular nitrate pool

The free nitrate concentrations (Fig. 1A) are very low as compared to the total sedimentary nitrate pool obtained after freezing and thawing. The vertical *Beggiatoa* biomass distribution was closely correlated with the profile of total nitrate concentration, as demonstrated in Figs. 1C and 2A, C. In order to verify that this nitrate pool is originating from the intracellular nitrate of *Beggiatoa*, we determined intracellular nitrate concentrations of the filaments from different depths. From these values, together with the *Beggiatoa* biomass distributions, we calculated the intracellular nitrate pool. The nitrate profile calculated from the biomass profile (Fig. 2A) and intracellular nitrate concentrations (Fig. 2B) is similar to the directly measured total nitrate in the sediment

(Fig. 2C), indicating that *Beggiatoa* can account for the entire amount of nitrate in the anoxic part of the sediment. Only in the top layer a significant deviation was observed, where a much higher total nitrate was found than the sum of the free nitrate and the pool inside *Beggiatoa* vacuoles.

Intracellular sulfur

At station E4-1, *Beggiatoa* biomass was detected down to 60 mm sediment depth. Distribution of sulfur at station E4-1 (Fig. 2E) was similar to the distribution at station E8-1 (Fig. 1C). A peak occurred between 20 and 40 mm sediment depth and a strong increase was measured at 90-100 mm. *Beggiatoa* filaments stored ca 10 times more elemental sulfur than nitrate. The cellular sulfur content was the same at all depths (Fig. 2E). The total sulfur distribution did not reflect the distribution of *Beggiatoa* in the sediments (Fig. 2E). The sulfur pool inside *Beggiatoa* is only a small fraction of total elemental sulfur in the sediments.

Fig.2 A) B) C)

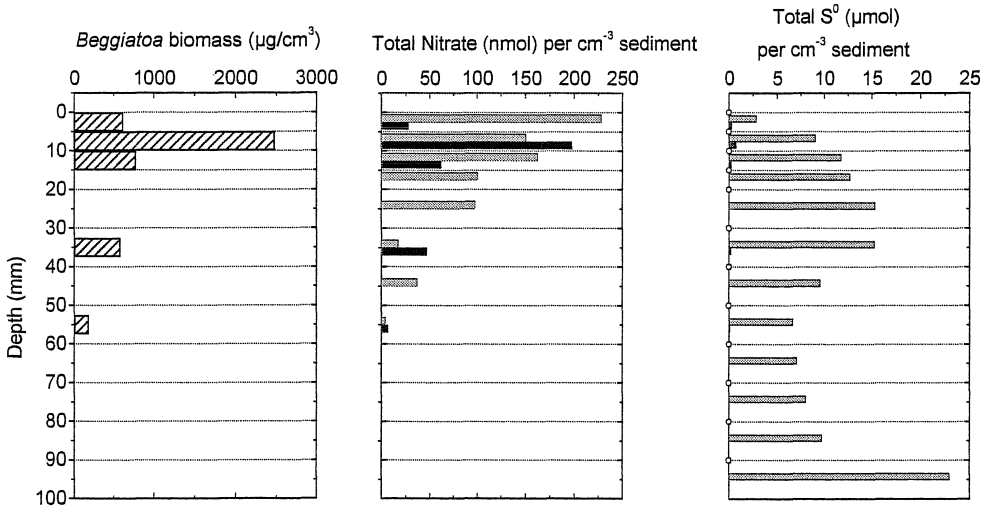

Figure 2. Vertical profiles of (A) *Beggiatoa* biomass, (B) nitrate in the sediment stored inside *Beggiatoa* (black bars), total nitrate concentration in the sediment (grey bars), (C) elemental sulfur in the sediment stored inside *Beggiatoa* (black bars) and total elemental sulfur in the sediment (grey bars). All profiles were measured in a core from station E4-1 (54°31.30N, 10°02.18E). Profiles of nitrate (C) and sulfur (F) stored in *Beggiatoa* were calculated from the biomass distribution (A) and the respective intracellular concentrations. The subscripts S, B and PW referred to sediment, *Beggiatoa* and pore water, respectively.

Survival of *Beggiatoa* under anoxic conditions

After 20 days in an oxygen and nitrate free environment, 90 % of the *Beggiatoa* filaments still showed gliding activity under the microscope (Fig. 3). After 42 days the *Beggiatoa* biomass was comparable to the initial value, however, only one *Beggiatoa* out of 25 filaments showed gliding motility after placement on a microscope slide. After 56 days only a few filaments were visible and no gliding activity was observed.

After 20 days the intracellular nitrate was below detection limit of ca 1-2 μmol cm^{-3} (Fig. 3). The nitrate reduction rates calculated from the slope of the intracellular nitrate decrease (Fig. 3), assuming porosity of 0.88, was ca. 13 μmol cm^{-3} (*Beggiatoa*) d^{-1}. In a control sample exposed to oxygen for 8 weeks, the intracellular nitrate did not decrease considerably.

Fig.3

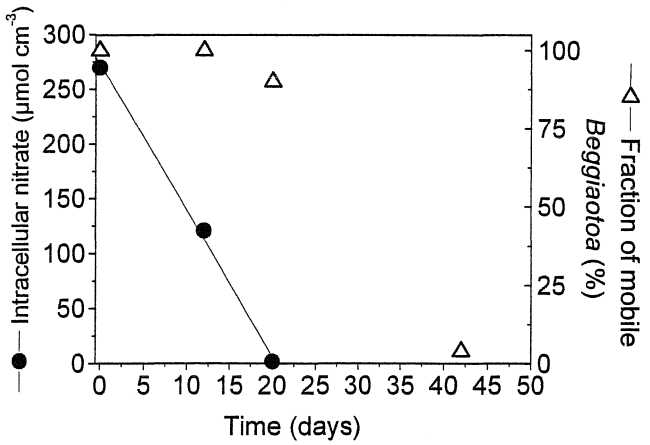

Fig. 3. Concentration of intracellular nitrate and fraction of mobile *Beggiatoa* as a function of time during which *Beggiatoa* were stored under anoxic conditions.

Discussion

To further explore the finding of Sayama [42] we compared the biomass and internal nitrate amounts of *Beggiatoa* to the extracted nitrate pool. Indeed, the total extracted nitrate in the sediments originated from nitrate, stored in *Beggiatoa* vacuoles, as suggested by Sayama. No data on the turnover rates, thus on the actual importance of *Beggiatoa* nitrate metabolism for the sulfur geochemistry in deeper sediments, were available. Related to this, it is of interest what the survival time of *Beggiatoa* in the starvation conditions in the deeper sediment, and what their metabolic products are.
We could extract nitrate from sediment several cm below the oxic zone. Nitrate diffusing from the water column into the porewater does not penetrate that deep. The distribution of extracted nitrate overlaps with the distribution of *Beggiatoa* in the sediments, thus we can conclude that *Beggiatoa* transports nitrate deep in the sediments. *Beggiatoa* stored nitrate can completely account for the deeper subsurface nitrate pool, below the oxic zone. Nitrate measurements in the sediment surface (0-0.5 cm) after freezing and thawing of the samples revealed an additional nitrate pool in or near the oxic zone that could not be explained by the presence of *Beggiatoa.* This could be due to diatoms that are also known to store nitrate intracellularly [28]. The additional intracellular nitrate pool in the oxic zone is less than 20% of the total sedimentary nitrate pool.
Beggiatoa cannot obtain nitrate in deeper layers from the porewater, as it was simply not detected. In addition to the nitrate microsensor measurements, in several thawed pore water samples from Eckernförde Bay that were gently centrifuged to avoid *Beggiatoa* cell breakage the extracellular pool of nitrate was also below the detection limit (data not shown). In theory, nitrate can be produced anaerobically through ammonium oxidation by Mn-oxides [6, 33]. However, the process was not found in sediments from the Skagerrak, that are rich in Mn-oxide [45]. The intracellular nitrate detected in the filaments in deeper sediment layers must therefore originate from the water column or the sediment surface, and is transported down in the vacuoles of *Beggiatoa.* This can be either an active transport, similar to *Thioploca,* or a passive transport by storms and currents [41], as it is suggested for the immobile *Thiomargarita* occurring in deep sediment layers (> 12 cm) [43]. However, the gliding motility of *Beggiatoa* is well documented. The velocity can be mm-cm h^{-1} [5], which was confirmed by our

observations (not shown), thus the gliding motility of *Beggiatoa* is very likely the main mechanism of nitrate transport in deeper sediment layers.

Elemental sulfur – sediment and intracellular pools

Mats of *Beggiatoa* accelerated turnover of free sulfide 1000 to 2000-fold in gradient cultures in comparison to the chemical oxidation and thus have a significant effect on the free sulfide profile [36]. The intermediate of free sulfide oxidation to sulfate by *Beggiatoa* is elemental sulfur, which is stored in the periplasm. In sediments covered by dense populations of *Beggiatoa*, these bacteria were found responsible for almost the entire elemental sulfur on top of the sediment [47]. However, in Eckernförde Bay sediments exists a large extracellular elemental sulfur pool, similar to Chilean sediments containing *Thioploca* [49]. Most of the elemental sulfur was found below the *Beggiatoa* containing layer, so most elemental sulfur is not stored in *Beggiatoa.*

The high external elemental sulfur pool suggests that most of the free sulfide was oxidized outside *Beggiatoa*, by Fe(III) and Mn(IV) [50]. This was also confirmed by the sulfide profiule which also revealed that most of the sulfide was oxidized below *Beggiatoa.* Unfortunately, Fe(III) and Mn(IV) data are not available in this study. Reactive Fe(III) can be present several centimeters deep in the sediment by physical reworking through bioturbation, waves, and currents [13]. Free sulfide can also be precipitated by Fe(II) [e.g. 16,19,3]. Also in *Thioploca* dominated sediments the majority of free sulfide oxidation involves the formation and reoxidation of Fe sulfide minerals [8]. This evidence makes the importance of *Beggiatoa* debatable, however, pool sizes not necessarily reflect the importance of the producing or consuming process. The importance of *Beggiatoa* for the sulfur geochemistry is topic of another study (next chapter).

Survival in the environment free of nitrate and oxygen

From experiments with purified samples, different authors suggest that the intracellular nitrate is used as terminal electron acceptor for respiration [29, 38]. By reducing the

nitrate, *Beggiatoa* can live under anoxic conditions and may be a key species responsible for channeling nitrate to either ammonia or N_2 [42, 49, 11].

High *Beggiatoa* biomass occurs down to several centimeter depth, and thus the bacteria must be able to glide in an environment without available free nitrate (Fig. 1C, 2A). Moreover, in some ecosystems (e.g., Limfjorden), *Beggiatoa* have to survive oxygen depletion in the bottom water that can occur for several weeks [20]. Under these conditions *Beggiatoa* can survive on intracellularly stored nitrate. McHatton et al. [29] estimated that, the if the nitrate is used for autotrophic respiration, the strain from Monterey Bay could glide about 6 cm depth until the intracellular nitrate is depleted. For *Thioploca* it is estimated that the intracellular nitrate is sufficient for about 200 h [38]. Our data show that *Beggiatoa* is indeed well adapted to an environment free of nitrate and oxygen as the intracellular nitrate of *Beggiatoa* was consumed in ca 440 hours (Fig. 6a). Two independent experiments gave nitrate reduction rates of ca. 13 and 15 µmol cm^{-3}_{Begg} d^{-1}. These values are comparable to the nitrate reduction rates (19 µmol cm^{-3} d^{-1}, recalculated) estimated for *Thioploca* by Otte et al. [38]. It is worth mentioning that most of the *Beggiatoa* were still living when intracellular nitrate was below the detection limit (<1-2 µmol cm^{-3}). After 6 weeks almost all *Beggiatoa* were immotile, i.e., presumably dead. Whether the cells simply die or change in some dormant stage is an ecologically relevant question that could lead to understanding of the colonization of new environments.

In comparable sediments Sorensen et al. [44] measured denitrification rates during summer to less than 0.2 mmol m^{-2} d^{-1}. From the *Beggiatoa* biomass and the nitrate reduction rates in *Beggiatoa* (13-15 µmol cm^{-3} d^{-1}) the turnover of nitrate and the corresponding nitrate uptake in the sediment can be calculated. Considering *Beggiatoa* biomass of 63 g m^{-2}, areal nitrate consumption or uptake by *Beggiatoa* would be 0.82-0.95 mmol m^{-2} d^{-1} suggesting that *Beggiatoa* could contribute significantly to the total nitrate flux into the sediment. In this study, we show that nitrate is transported several centimeters deep into the sediment by *Beggiatoa* filaments, thereby enhancing considerably the N-flux into the sediment that would be otherwise mainly governed by diffusion. The intracellular nitrate is sufficient for *Beggiatoa* to glide several meters (assuming a gliding velocity of 1.2 cm h^{-1} [46]). Even if they would glide without any

orientation, which is probably not the case, they might not come in danger of nitrate starvation in the zone in which they are present. The restriction to the upper few centimeters is therefore most likely not due to the depletion of the intracellular nitrate but due to a negative response to high free sulfide concentrations. A negative tactile response towards 150 µM sulfide was reported for *Thioploca* [12]. The negative response to sulfide could thus be used as a means to stay in the upper sediment layer.

The high intracellular nitrate pools therefore will also function to survive periods of nitrate depletion in the bottom water. *Beggiatoa* can probably contribute significantly to sedimentary nitrate reduction, by transporting considerable amounts of nitrate several centimeters into the anoxic sediment depleted in nitrate. The migratory behaviour and active nitrate accumulation by *Beggiatoa* gives obvious advantages that are limited by downward diffusion [37].

Beggiatoa, whose diameter is in the same range as the size of diatoms, could provide an easy and accessible food source. Some bacteriovorous ciliates are known to feed on the sulfur bacteria containing sulfur granules [7]. However, the sulfur granules are not usable for grazing organisms. Moreover *Beggiatoa* is mainly composed of vacuole and poor in organic material. The high amounts of nitrate and sulfur need to be separated from the organics, which increases the efforts or, in food-web terminology, the handling time for the grazers. The handling time is known to influence the prey preference of the predators (Badii et al. 2004). Nitrate and sulfur could therefore serve not only as electron acceptor and donor, respectively, but also as a protection from bacteriovorous organisms.

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2.3 The distribution of *Beggiatoa* is rather a response to- than a determinant of the sulfide profile.

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The contribution of *Beggiatoa* to the removal of sulfide was investigated in sediments from Eckernförde Bay. Microsensor measurements showed that oxygen penetrates 1-2 mm, while free sulfide is present below 30-100 mm depth. *Beggiatoa* was exclusively found in the anoxic and sulfide free intermediate zone. Clearly, sulfide produced in and diffusing into the *Beggiatoa* inhabited zone (BIZ) is consumed efficiently by anaerobic processes.

The sulfide removal rate in the BIZ was calculated as the sum of the local sulfate reduction and diffusional free sulfide influx from deeper sediments. The sulfate reduction rates were determined by a radiotracer method. The diffusional influx from deeper layers was determined from sulfide microprofiles obtained with microsensors.

The total sulfide consumption in the BIZ was at two stations 0.29 and 2.8 μmol $cm^{-2}d^{-1}$. The total sulfide oxidation rate of *Beggiatoa* at these stations was an order of magnitude lower (0.01-0.042 and 0.02-0.081, respectively). The sulfide produced by sulfate reduction and diffusing in from deeper layers must be removed by other anaerobic processes, e.g. binding to Fe(II) or oxidation by Fe(III), which is supported by pH microprofiles. It was concluded that the distribution of *Beggiatoa* is rather a response to- than a determinant of the sulfide profile.

Introduction

Filamentous sulfur-oxidizing nitrate storing bacteria of the genera *Beggiatoa* are among the largest bacteria in nature, inhabiting the oxidized anoxic surface layer of organic rich marine sediments.

Conspicuous white *Beggiatoa* mats occur where sulfide and oxygen overlap in a zone of 50 μm thickness [11]. However, in many marine sediments there is a distinct separation of O_2 and H_2S by an intermediate zone of several centimeter thickness where neither is present in detectable concentration [10,17]. Since 1888 it was thought that *Beggiatoa* can grow lithotrophically at the expense of sulfide [27] and subsequent investigations showed that this could be a general feature in the small strains [19,20,6] and the marine nitrate storing strains [15,13]. All wide (cell diameter >10 μm) marine representatives of *Beggiatoa* examined to date were found to store nitrate up to a concentration of 370 mM [10,12]. McHatton et al. [10] found high concentration of nitrate reductase in purified Monterey *Beggiatoa,* which indicates that nitrate could be used for the oxidation of sulfide [15]. Because of the oxidation of sulfide with nitrate in the anoxic zone, *Beggiatoa* are assumed to have a strong impact on the sediment sulfur chemistry [20]. In sediments containing high biomass it is believed that big sulfur oxidizing bacteria could be responsible for the sulfide depletion zone in the sediment [13, 20, 10]. Sulfide oxidation of *Beggiatoa* is, however, in concurrence with other sulfide oxidation processes. For instant precipitation and oxidation by iron is known to play a large part in many marine sediments. Reactive Fe(III) can be present several centimeters deep in the sediment by physical reworking through bioturbation, waves, and currents [8] and can oxidize the sulfide chemically [11]. Precipitation of sulfide and subsequent oxidation can also occur in the sediment [28]. In Aarhus Bay on the East coast of Jutland most of the H_2S produced precipated as iron sulfide and S^0 by reaction with iron [26]. Even in sediments with high biomass of the closely related relative *Thioploca* sulfide oxidation is mainly due to formation and reoxidation of Fe sulfide minerals [4]. The aim of this study is to investigate the hypothesis that *Beggiatoa* is the important sulfide oxidizer in marine sediments and are responsible for the sulfide free zone in anoxic marine sediments.

Methods:

Sampling

Sediments were sampled in Eckernförde Bay (Germany, Baltic Sea – Fig.1) in March 2002 (E3-2a) and January 2003 (E6-5). For sampling, two sediment cores with an inner diameter of 10 cm were collected per station by a small multiple corer (MiniMuc), as described by [1]. The first core was sectioned into 5 mm (0-20 mm sediment depth) and 10 mm depth intervals (20-100 mm sediment depth) depth intervals for biomass, nitrate and sulfur analyses, while the second core was used for microsensor measurements and subsequently for *Beggiatoa* biomass determination. The vertical distributions of the lighter (oxidized conditions) and dark (reduced conditions) zones were the same in both parallel cores.

Biomass determination

Sediment subsamples (300-500 mg) from each depth were resuspended in 10 ml filtered sea water, ca. 300-400 mg of the suspension was placed on a microscope slide. Filament width, length and total number of the *Beggiatoa* filaments were determined for each depth interval [13]. The biomass was quantified using the filament volume (cylindrical shape) and assumed density of 1 g cm^{-3} Mußmann et al. [17].

Sulfate reduction rate (SRR)

Three (March 2002) and two (January 2003) replicate push-cores, respectively, were taken out of a MiniMuc core. SRR was quantified using the whole core injection method [12]. Carrier-free $^{35}SO_4^{2-}$ dissolved in water was injected into replicate push-cores at 1 cm intervals and the push cores were incubated at 4°C for 48 hrs in the dark. To stop bacterial activity after incubation, the sediment cores were sectioned into 1 cm intervals and transferred into 50 ml plastic centrifuge vials filled with 20 ml zinc acetate (20% w/w). SRR rates were measured using the single step acid Cr-II method according to [5].

Microsensors

Microsensors for O_2, H_2S and pH were made and used as described previously [9,24]. The tip diameters were ca 20 µm, the response time (t_{90}) less than 3 seconds. The microsensors were mounted on a motor driven micromanipulator. Motor control and data acquisition were done using a computer. The sediment surface was determined with a dissection scope.

Analysis of microsensor profiles

Net sulfide consumption and production rates and the diffusive flux were quantified by fitting the microprofiles with a diffusion-reaction model implemented in a Matlab program. The sulfide diffusion coefficient was calculated from $D_s=ø/\theta^2 D_w$ [3], resulting in $8.11*10^{-10}$ m^2/s(station E6-5) to $8.53*10^{-10}$ m^2/s (station E3-2). The total sulfide consumption in the BIZ was calculated by adding the flux of free sulfide diffusing from below and the sulfate reduction rate. The cell-specific nitrate reduction by *Beggiatoa* were calculated to be 13 µmol cm^{-3} d^{-1} (see Chapter 3). The coupled sulfide oxidation rates were calculated to be between 13-52 µmol cm^{-3} d^{-1}. This is based on the assumption that the ratio of sulfide oxidation/nitrate reduction is between 1 and 4.

If the internal nitrate is used for oxidation of sulfide and in addition to further oxidize the internal sulfur to sulfate the stochiometry is 1:

$$HS^- + NO_3^- + H^+ + H_2O \rightarrow SO_4^{2-} + NH_4^+ \text{ [10]}$$

If the internal nitrate is only used for oxidation of sulfide to sulfur the stochiometry is 4:

$$2H^+ + 4H_2S + 1\ NO_3^- \rightarrow 4S^0 + NH_4^+ + 3\ H_2O$$

Results

Vertical distribution of biomass

Beggiatoa were found in the top 15-60 millimeters of the sediment. The vertical distribution of *Beggiatoa* biomass was highly variable, between stations and season. At 6 out of 14 stations we observed a gradual decrease of the biomass with depth (e.g. Fig.

2A). At 4 stations the *Beggiatoa* biomass peaked in the anoxic zone at 5-10 mm depth (e.g. station E3-2a, Fig. 3A) and at 2 stations the biomass peaked at 15-20 mm sediment depth. *Beggiatoa* biomass was rather evenly distributed over the upper 20 and 40 mm in stations E3-4 and E4-2, respectively (data not shown).

Sulfide profiles and *Beggiatoa* biomass

Measurements with microsensors showed that oxygen penetration into the sediment was only about 1 mm at station E6-5 (Fig. 2B) and it was 2.5 mm maximum at other stations. The sulfide concentrations were around or below the detection limit (1 μM) down to 20 mm sediment depth and increased rapidly below. The main *Beggiatoa* biomass occurred in the zone where neither oxygen nor sulfide were present in detectable concentrations. The deepest sediment horizon containing *Beggiatoa* biomass was correlated with the zone where upward diffusing sulfide disappeared (e.g., Figs. 2A and 3A) and very little or no biomass was found below. This typical distribution was observed in 9 of the 11 stations in Eckernförde Bay.

Fig.1 E6-5

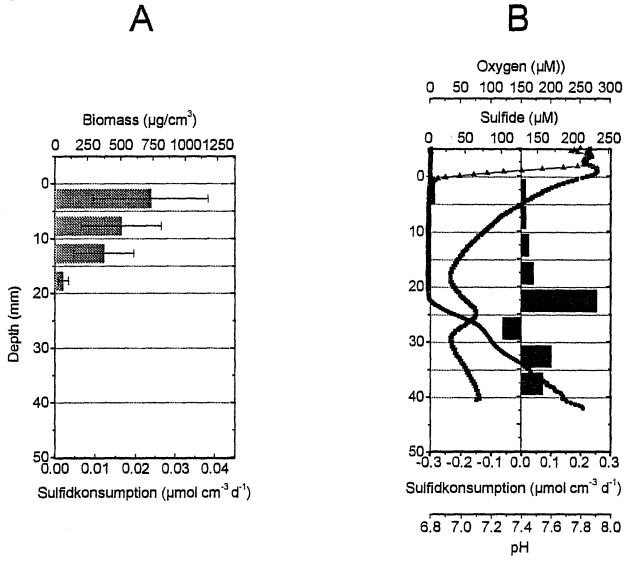

Fig.2 E3-2

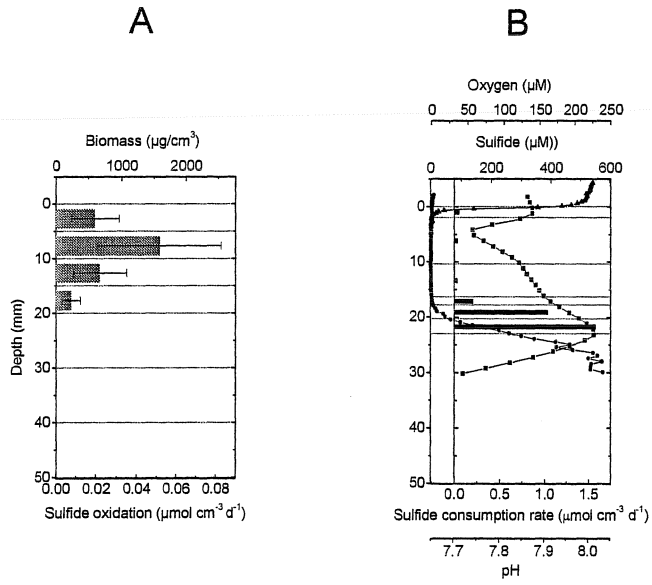

Fig. 1-2; A: *Beggiatoa* biomass and the sulfide oxidation rates (grey bars). Error bars for description of the range: sulfide oxidation/ nitrate reduction 1 and 4. B: Depth profile of oxygen (down triangle), pH (squares), free sulfide (dots) and the calculated total sulfide removal rates (calculated from SRR and sulfide influx into the BΓZ) of the sediment.

Sulfide consumption in the sediment

We compared the total rates of sulfide production and sulfide influx in the BIZ with the rates of sulfide oxidation by *Beggiatoa.*

Separately, the total sulfide consumption rates were calculated in the BIZ from the sulfide influx and SRR (Fig. 2B, 3B). The highest sulfide consumption rates occurred at the depth where upwards diffusing sulfide disappeared. In the BIZ, the sulfide produced by the local sulfate reduction was completely consumed (Fig. 2B, 3B).

At station E6-5 (Fig. 2A) *Beggiatoa* biomass was distributed in the upper 2 cm of the sediment. The main sulfide consumption occurred at 2-2.5 cm sediment depth. The total sulfide consumption in the upper 2.5 cm was 0.288 μmol cm^{-2} d^{-1} and *Beggiatoa* was consuming 0.011-0.042 μmol $cm^{-2}d^{-1}$ (Fig. 1D, Tab.1). *Beggiatoa* biomass was rather low at the deeper horizon of *Beggiatoa* distribution near the highest rates of sulfide consumption and most sulfide was consumed below the *Beggiatoa* containing layers (Fig. 2A, B). At station E3-2 the total free sulfide consumption in the upper ca. 2 cm was 2.8 μmol cm^{-2} d^{-1} whereas free sulfide consumption due to *Beggiatoa* was only 0.081 μmol cm^{-2} d^{-1} maximum (Tab.1). The total sulfide consumption rates of *Beggiatoa* were comparable to the sulfide produced by local sulfate reduction (Fig. 2A,3A, Tab.1). The sulfide diffusing upwards from below is consumed in much higher rates than can be explained by the activity of *Beggiatoa.*

Tab.1

	Beggiatoa	sulfide influx	SRR	total (influx+SRR)
E3-2	0.020-0.081	2,74	0,085	2,825
E6-5	0.011-0.042	0,217	0,071	0,288

Tab.1: Sulfide consumption rates (μmol cm^{-2} d^{-1}) in the BIZ (Beggiatoa inhabiting zone).

pH profiles

In Eckernförde sediments remarkable pH-profiles were measured. At four investigated stations with high *Beggiatoa* biomass densities (10.9-36.6 g m^{-2}) a pH dip was observed at about 0.4-0.6 cm sediment depth in the BIZ and a pH peak in the zone where upward diffusing sulfide disappears (e.g. station E3-2, Fig. 3B). In a reference station without *Beggiatoa* (E6-9) pH also decreased down to the sulfide enriched zone where it exhibited a small peak (data not shown).

In a core with sediment from Eckernförde Bay oxygen was removed from the water column by sparging with nitrogen. This removal of oxygen had no effect at all on the sulfide and pH profiles in the sediments.

The addition of sulfide to the water column under anoxic conditions increased the pH within minutes (Fig. 4A,B). When sulfide was consumed after 14 h the pH decreased again to the previous situation (not shown). Thus the pH dip in the deeper sediments, coinciding with the zone where sulfide disappeared, seems indeed correlated with the sulfide distribution.

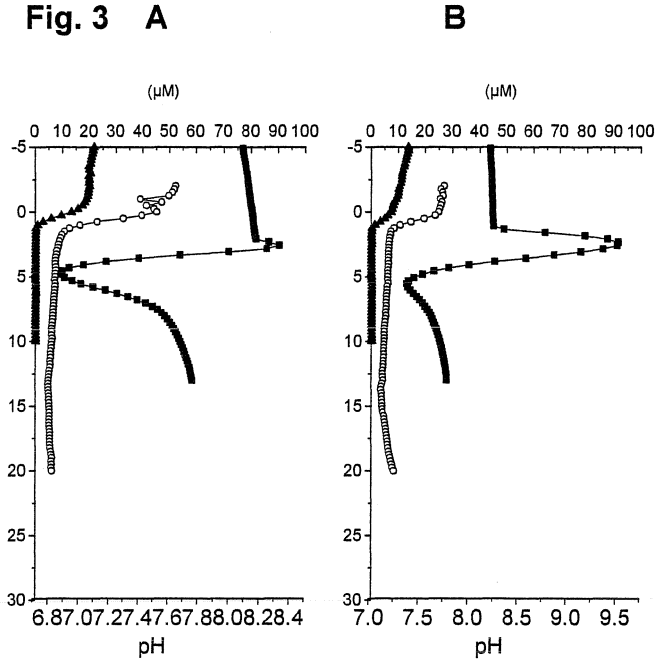

Fig. 3. Depth profile in a sediment core after bubbling with nitrogen and addition of sulfide. Oxygen (down triangle), pH (squares), free sulfide (dots) are shown. A: Begin of sulphide incubation, B: ca 0.5 hours later.

Discussion

Vertical *Beggitoa* biomass distribution and the sulfide profile

Generally, *Beggiatoa* filaments were distributed evenly throughout the sulfide depleted zone and are not present in the highly sulfide enriched zone. Occasionally, biomass decreased with depth or reached maximum in the center of the zone.

Beggiatoa distributions did not correlate with the highest sulfide removal zones, i.e. no peak abundance was observed where the upward diffusing sulfide disappeared. At most of the stations the main biomass was observed above this horizon. Our data show that in general *Beggiatoa* avoid high sulfide concentrations similar to *Thioploca* [7]. In most of

the investigated stations *Beggiatoa* filaments were present in the zone where neither oxygen nor sulfide was present in detectable amounts. This is in good accordance to the assumption that sulfide from sulfate reduction of the sediment could be sufficient, although no sulfide is measured in the upper centimeters of the sediment [13, 17].

The contribution of *Beggiatoa* to total sulfide consumption

The deepest occurrence of *Beggiatoa* was strongly correlated with the onset of detectable sulfide concentrations. This correlation can either be due to the fact that the sulfide is removed by the present *Beggiatoa* biomass or that *Beggiatoa* is distributed in this zone because sulfide concentrations are sufficiently low. Our data show that the free sulfide distribution dictates the distribution of the *Beggiatoa*, while *Beggiatoa* has no significant effect on the sulfide profiles. In order to oxidize all produced sulfide in the sediment, *Beggiatoa* of deeper layers would need cell-specific sulfide oxidation rates of about 2 orders of magnitudes higher than those of the upper layers (Fig. 2B, 3B). Of course, this is impossible, in contrast it has been assumed that *Beggiatoa* use nitrate and sulfide for respiration with constant rates in all sediment layers [10, 15]. Furthermore, we also observed that the highest sulfide consumption occasionally occurred in the sediment layers where no *Beggiatoa* were present. Therefore, we conclude that most of the free sulfide diffusing upwards from deeper sediment layers is not consumed by *Beggiatoa*, as thought previously [13,17], but by some other processes. The only realistic alternative process to nitrate reduction is the removal of sulfide by Fe(III) reduction and FeS precipitation. No iron data for the presented stations are available, but it is known that reactive Fe(III) can be present several centimeters deep in the sediment by physical reworking through bioturbation, waves, and currents [8,10]. Therefore the contribution is similar to the situation observed in a *Thioploca*-dominated sediment off the coast of Chile. In this environment most of the sulfide is be removed by iron [4] and not by the sulfur oxidizing bacteria.

However, within the BIZ the sulfide consumption rates of *Beggiatoa* were in the same range as the sulfate reduction rates (Tab.1) and the total sulfide consumption rates of the sediment (Figs. 2, 3 A-B).

Therefore *Beggiatoa* can account for the sulfide consumption in the upper layers but the contribution to total sulfide consumption is probably rather low.

Vertical pH profile and *Beggiatoa*

The pH profiles confirm that most likely in addition to *Beggiatoa* other processes are involved in the oxidation of sulfide. At many stations a pH minimum was observed within the BIZ and a peak in the zone where sulfide diffusing upwards from below disappeared.

The pH minimum was observed around 40-60 mm depths and. *Beggiatoa* could be involved by reduction of nitrate by internal sulfur [10], that would lead to a proton release:

$$7\ H_2O + 4S^0 + 3\ NO_3^- \rightarrow 4\ SO_4^{2-} + 3\ NH_4^+ + 2\ H^+.$$

Another possibility could be the bacterial oxidation of Fe^{2+} with nitrate [2], which would also release protons [21].

$$Fe^{2+} + NO_3^- + 13H_2O \rightarrow 8\ FeOOH + NH_4^+ + 14H^+$$

Since the dip occurred below the penetration depth of nitrate, which is usually less than 5 mm in coastal sediments [16, 14], reduction of pore water nitrate is rather unlikely.

MnO_2 reduction by iron is the most likely process:

$$2\ Fe^{2+} + MnO_2 + 2\ H_2O \rightarrow Mn^{2+} + 2\ FeOOH + 2\ H^+ \text{ [22,23]}$$

A geochemical process is more likely than a biological explanation because of the amazing stability of the pH profiles to perturbations. In another bay of the Baltic sea (Aarhus Bay) manganese reduction occured also between 50 mm and 10-20 mm depth [26]. Oxidation of FeS and FeS_2 or H_2S by MnO_2 would be in concurrence to the oxidation of Fe^{2+} as this reaction would increase the pH:

$FeS_2 + 7.5\ MnO_2 + 11\ H^+ \rightarrow Fe(OH)_3 + SO_4^{2-} + Mn2^+ + 4\ H_2O$ (Schippers and Jørgensen 2001)

$FeS + 1.5\ MnO_2 + 3\ H^+ \rightarrow Fe(OH)_3 + SO_4^{2-} + 1.5\ Mn2^+$ (Schippers and Jørgensen 2001)

$4\ MnO_2 + 4\ H_2S \rightarrow 4\ Mn^{2+} + 4\ S^0 + 8\ OH^-$ (Jørgensen and Nelson 2004).

This process cannot explain the stable pH dip near the interface. Both Fe^{2+} and free sulfide can be oxidized by Mn oxides rapidly [18] with opposite effects on the pH. The availability of Fe^{2+} (decrease of pH) and free sulfide (increase of pH) controls the pH profiles in the BIZ. Since *Beggiatoa* could deplete the sulfide in the upper layers of the BIZ (see above) these bacteria favor the observed pH minimum by sulfide oxidation using the internal nitrate.

The increase of pH below the mentioned dip could be explained by anoxic sulfide oxidation with MnO_2 (see above) or Fe(III).

$2\ FeOOH + H_2S \rightarrow 2Fe^{2+} + S^0 + 2H_2O + 4OH^-$ (Johnson und Hallberg 2005)

This was confirmed by an experiment in a core of Eckernförde Bay (Fig. 4). The incubation with sulfide resulted in an anoxic alkaline peak indicating that sulfide oxidation under anoxic conditions can indeed result in consumption of protons.

The oxidation of sulfide to sulfur by *Beggiatoa* could also contribute to the sulfide oxidation in the sediment:

$7\ H_2O + 4S^0 + 3\ NO_3\text{-} \rightarrow 4\ SO_4^{2-} + 3\ NH_4^+ + 2\ H^+$

Since these bacteria are in general more abundant in the upper sediment regions the oxidation of sulfide by iron is probably more important in this region. Further investigations are needed to understand the pH profiles in the sediments.

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3 Discussion

3.1 The influence of *Beggiatoa* on the nitrogen and sulfur cycle

The Physiology of sulfur oxidation in *Beggiatoa*

More than 100 years ago it was observed that *Beggiatoa* can oxidize reduced sulfur compounds and store sulfur globules internally [34]. Subsequent investigations showed these organisms to perform lithotrophic respiration [22, 21]. The elemental sulfur grains are enclosed in invaginations of the cell membrane outside the cytoplasm [30]. In the vacuolated sulfur oxidizing bacteria sulfide is oxidized by using internally stored nitrate [19]. In a first step the sulfur is probably oxidized to elemental sulfur and in a second independent step the sulfur is further oxidized to sulfate [24]. But there are still a lot of open questions. For instance, why is the intermediate of elemental sulfur internally stored and not immediately further oxidized to sulfate? The accumulation of elemental sulfur globules probably costs energy and in the *Beggiatoa* inhabited zone it seems not necessary to store an intermediate product. Our sulfate reduction profiles and *Beggiatoa* biomass distribution indicates that enough sulfide is produced for the *Beggiatoa* although in the zone where they live sulfide is undetectable with microsensors [16]. According to our calculations *Beggiatoa* can glide several meters in the anoxic environment, before its internal energy stores are depleted. Thus they can easily reach the sulfidic zone that usually occurs only a few centimeters below the sediment surface. An advantage of storing elemental sulfur could be that it is further oxidized in the top layer with oxygen or nitrate. This is also confirmed by several experiments which showed that *Beggiatoa* can survive for days in an environment without free sulfide (data not shown). At the oxic surface sulfide production due to sulfate reduction can also occur [14] but here *Beggiatoa* have to compete for sulfide with many bacteria using oxygen or nitrate for respiration. Therefore a considerable difference of elemental sulfur storage in the upper layers in comparison to the deeper layers should be expected as the sulfur should accumulate in the

anoxic sediment layers and be consumed on the top of the sediment surface. However, our results did not confirm this hypothesis. In contrast, the whole topic appears far more complex. Older hypotheses still have to be considered. For instance instead of being further oxidized elemental sulfur could be also reduced back to sulfide by H_2 or acetate [15]. This physiological property would further improve the ability to survive in an anoxic environment. In the freshwater strains *Beggiatoa cf. leptomitiformis* [23] and *Beggiatoa alba* [26] reduction of the internal sulfur under anoxic conditions was demonstrated. Another advantage of the sulfur storage that has not been discussed in publications up to now could be considered. For many heterotrophic organisms seeking for organic carbon the elemental sulfur is useless and has to be removed after grazing on *Beggiatoa*. This does not completely prevent *Beggiatoa* from being grazed [6] but the time and energy consuming process improves the ability of *Beggiatoa* to compete with fast growing organisms. This is not the only reason for producing elemental sulfur but it is most likely of selective advantage.

In spite of the numerous open questions there is no doubt that *Beggiatoa* is able to oxidize sulfide and to store sulfur in concentrations of several mM. Therefore the contribution of *Beggiatoa* to the sulfur cycle was investigated in this study and the results showed that its contribution was less than expected until now.

Ecophysiology of *Beggiatoa* sulfur oxidation in the sediment

It was assumed that *Beggiatoa* could be the reason for the suppression of the sulfide profile [16]. This was a hypothesis that has not yet been investigated in all details up to now. In *Thioploca* dominated sediments the contribution of metals to sulfide removal was investigate and was compared to the potential sulfide removal rates of the big sulfur bacteria [7]. Until now the sulfide removal rates were not compared with the *Beggiatoa* biomass in the different locations of the *Beggiatoa* inhabiting zone. Thus, to investigate the contribution of *Beggiatoa* in more detail we tried to figure out, whether the different sulfide removal rates in the sediment were correlated to the biomass distribution. There are two different sources of sulfide in the BIZ (*Beggiatoa* Inhabiting Zone). One source is the sulfide locally produced by sulfate reduction and the other is the sulfide produced in the deeper part of the sediment that diffuses upwards according to the concentration

gradient. The sulfide removal rates in different layers of the *Beggiatoa* inhabiting zone can be calculated. Biomass and sulfide removal profiles were not similar at all as the *Beggiatoa* in deeper layers should have to oxidize the sulfide in 2-3 orders of magnitude higher rates. Such high rates are not possible, thus we believe that the sulfide diffusing from deeper sediments is oxidized by abiotic processes. The extremely high rates could confirm the detoxification hypothesis, i.e. that the sulfide in the cells is oxidized, the thus produced sulfur is transported out of the cytoplasm in the globules. But, we calculated that *Beggiatoa* cannot be responsible for the extremely high sulfide oxidation rates in a part of the sediment. There are three arguments favoring that most of the sulfide diffusing upwards from below is not consumed by *Beggiatoa.* Firstly, the averaged sulfide removal rates in *Beggiatoa* in a sulfidic environment (calculated from the nitrate reduction rates) were much too low to be responsible for this (see above). Secondly, a lot of sulfide removal occurred in a zone where *Beggiatoa* were not present. Thirdly, there is a large pool of elemental sulfur in the sediment that is not associated with *Beggiatoa.* As sulfate reduction does not produce elemental sulfur as an intermediate, this pool has to be produced by sulfide oxidation. Most sulfur in the sediment is extracellular, thus not produced by *Beggiatoa.* Our investigations showed that *Beggiatoa* can oxidize sulfide at a much lower rate than the sulfide production rate in the sediment. *Beggiatoa* is only of low importance for the sulfide distribution but the occurrence of sulfide determines the distribution of *Beggiatoa* biomass. Consequently, *Beggiatoa* can be used as an indicator organism for free sulfide concentrations in the sediment. This is clearly not because they need free sulfide for energy, but probably because they use the steep sulfide gradient for locating the bottom sediments, i.e. for navigation back to the surface. Due to the negative tactile response to sulfide they will normally frequent the zone where sulfide concentrations are around zero, but they can survive high sulfide concentrations well: occasionally we found healthy *Beggiatoa* in highly sulfidic regions of the sediment (Zitzmann, pers. comm.).

Impact of Sulfide on Beggiatoa:

Although *Beggiatoa* are assumed to oxidize the sulfide for respiration they tend to avoid

it. *Beggiatoa* may use sulfide for orientation, which does not exclude that sulfide could be toxic for them [2].

The biogeochemical properties of the BIZ are not remarkable, with respect to sulfate reduction rates, organic content or grain size. The only remarkable feature of the areas where *Beggiatoa* resides is the presence of free sulfide below it. *Beggiatoa* can travel several meters on their internal energy stores, thus without a signal to return to the surface they may easily get lost deep in the sediments and perish. Their tactile responses to oxygen are well documented, our data indicate a negative tactile response to sulfide. A negative response to both oxygen and sulfide would constrain their presence to the non-sulfidic and anoxic zone 'in between' for which they are physiologically ideally equipped.

Free sulfide occurs as H_2S, HS^- and S^{2-}. HS^- is the most common form in marine sediments [2]. At higher levels it can be very toxic. Cytochrome c oxidase complex is well conserved in prokaryotes and eukaryotes and there are no substantial differences in the sensitivity to sulfide [2]. Sulfide inhibits the cytochrome c oxidase and therefore could be also toxic for sulfide oxidizing bacteria containing cytochrome c oxidase as last enzyme of the electron transport system [1]. High sulfide concentrations cause reduction of the Cu^{2+} atom forming a partially reduced enzyme-sulfide complex [2]. Cytochrome oxidase of the cbb_3-type is present in the big sulfide oxidizing bacteria of the genus *Beggiatoa* [9]. Furthermore many other enzymes are known to be inhibited by sulfide including common enzymes like ATPase [2]. There have to be mechanisms in *Beggiatoa* to withstand toxic sulfide concentrations. In general the protection mechanisms against high sulfide concentrations are poorly understood until now and there are probably many different mechanisms. One mechanism is to oxidize the sulfide inhibiting certain enzymes or proteins. For that reason it was thought for a long time that the oxidation of sulfide to elemental sulfur is rather a detoxification mechanism than a respiration [18]. The best protection against high sulfide concentrations is just to avoid them. Since only low sulfide concentrations are sufficient for metabolic energy generation, this is an obvious way for gliding organisms in a sulfide gradient. However, we observed that *Beggiatoa* could survive mM concentrations of sulfide in harbor sediments, which might mean that actually protection against sulfide is not required. Therefore *Beggiatoa* is

resistant to very high sulfide concentrations but in general avoid high sulfide concentrations which could also be used as an opportunity to orientate.

Nitrate

In contrast to sulfur storage and PHB storage the discovery of nitrate storage in the big bacteria is rather new [19, 12, 8]. Phylogenetical analysis based on 16S rDNA showed that all vacuolated sulfur oxidizing bacteria form a tight cluster within the gamma-Proteobacteria [20]. *Thioploca* and *Beggiatoa* are very closely related and they probably can only be distinguished by the fact that *Thioploca* live in sheaths and *Beggiatoa* without. Therefore the nitrate storage and use could have likewise very similar ecophysiological reasons. Since nitrate reductase has been found in purified *Beggiatoa* filaments of Monterey Bay [19] it is assumed that nitrate reduction is used for respiration. Nitrate is a good oxidant and could therefore be the explanation for the occurrence of *Beggiatoa* below the oxic zone. However, it has to be considered that respiration with nitrate is not yet proven in a pure culture and there are also disadvantages of the use of the internal nitrate pool. The accumulation of nitrate against a very high gradient (ca. 10 000 fold) costs energy. It was calculated that roughly 15 % of energy liberated from respiratory reduction of a mole of nitrate driven by sulfide oxidation is needed to accumulate the nitrate [13]. One possibility that could improve the accumulation of nitrate is that the nitrate is produced in *Beggiatoa* by ammonium oxidation with oxygen. Instead of losing energy they would gain energy by this process. It is not yet known how the nitrate accumulation occurs and it will be of special interest in future to investigate this.

Recently vacuolated big sulfur bacteria closely related to vacuolated *Beggiatoa* were found without internal nitrate storage [17]. This shows that the vacuole can have other functions than nitrate storage. Secondly, survival for big sulfur bacteria without internal nitrate can occur, showing that nitrate is not the sole terminal electron acceptor in all vacuolated strains. In *Thiomargarita* oxygen is used in addition to nitrate [27] and also in *Beggiatoa* culture experiments survival was much better with oxygen supply (data not shown). Metals (iron and manganese) could also be involved in the respiration as it was suggested for Achromatium [11]. Other advantages of the nitrate storage still have to be

considered as it could also serve as a nutrient or protection mechanism against high sulfide concentrations in addition or maybe even instead of serving as terminal electron acceptor for respiration. This would be not in contradiction to the finding of nitrate reductase [19] as this enzyme would be needed in all of those possibilities.
Regardless of how the nitrate is used our data show that it is consumed in an environment free of nitrate and oxygen. Our data show that the nitrate storage pool is a very efficient adaptation to the anoxic environment. The nitrate was not depleted within days or even weeks in a sulfidic environment, although the nitrate must be kept against a very high gradient. Therefore the nitrate storing bacteria can sustain nitrate depletion for a long period of time having an advantage over other bacteria depending on nitrate supply.

Contribution of *Beggiatoa* to the nitrogen cycle

Many data indicated that nitrate storing bacteria form the main nitrate pool in the sediment. Nitrate storing bacteria can store nitrate up to 370 mM [8] and a large nitrate pool is measured in the sediment after freezing or squeezing [25, 33]. The conclusion was that cell breakage of nitrate storing bacteria could be responsible for this nitrate pool. This conclusion was confirmed by the comparison of internal nitrate, biovolume and the external nitrate pool, which showed that *Beggiatoa* could entirely account for the large nitrate pool in sediments. The principal means of removing the nitrogen out of the system is the production of N_2 gas [31, 29]. If the internal nitrate would be reduced to N_2 *Beggiatoa* would participate in the nitrogen removal of the sediment. However, the final product of nitrate reduction is probably ammonium and the nitrate storing bacteria could contribute to eutrophication by accumulation of ammonium in the sediment. Heterotrophic bacteria produce a large pool of ammonium in the sediment by amino acid oxidation [10]. Whether the reduction of nitrate is important for the environment was often discussed. In *Thioploca* dominated sediments it was calculated that sulfide oxidation by *Thioploca* could lead to an 83 % increase of the sedimentary ammonium production [35]. In Eckernförde Bay the sulfate reduction rates and the internal nitrate reduction rates by *Beggiatoa* were in the same range and the situation could be similar. Simultaneously to the release of ammonium *Beggiatoa* probably can contribute significantly to the nitrate uptake in the sediment. This is also the case if *Beggiatoa*

accumulates nitrate by nitrification as the thereby produced nitrate pool is transported into the sediment instead of being released by other nitrifying bacteria. The form of available nitrogen has an effect on the phytoplankton and microphytobenthos communities. Cyanobacteria can fix nitrogen [3], the phytoplankton of the planktonic community using ammonium are called "regenerated producers" [4] and are different than the primary producers using nitrate called the "new producers" [5]. As *Beggiatoa* could increase ammomium- and decrease nitrate concentrations in the water column high biomass of these nitrate storing bacteria could lead to a shift in the communities of primary producers.

Beggiatoa distribution

The diameter of *Beggiatoa* is a common feature to distinguish different *Beggiatoa* strains [28]. At least two [16] or three [21] different size classes can live together in the same sediment. The investigation of 16S rDNA and FISH clearly confirmed that different size classes belong to different strains [20] indicating that width could indeed be a good possibility to distinguish the different strains. Our investigation showed that probably even more strains can live in the same sediment as we found at least 4 different strains rather evenly distributed over all sediment layers in Eckernförde Bay. In the special situation of the harbor with sulfide reaching the surface the same size classes were found. In 2002 the biomass in Eckernförde Bay was very high (ca. 63 g m^{-2}) comparable to the biomass in mats of the Hydrate Ridge. In the mats of Hydrate Ridge near Orgeon diversity was lower than in Eckernförde Bay showing only 2-3 size classes. The sulfate reduction rates were rather low in Eckernförde Bay and sulfide was 2-4 cm below the surface. Thus high sulfide concentrations reaching the surface are a condition for development of a mat but high biomass can occur without this condition.
The sulfide distribution seems not to have a great influence on the diversity of *Beggiatoa*. This is a very interesting observation as *Beggiatoa* is assumed to use sulfide for respiration. We found the same size classes and similar diversity in completely different environments concerning the vertical sulfide profile. Additionally we found in rather similar looking sediments very low *Beggiatoa* biomass not far away from stations with high *Beggiatoa* biomass. Nitrate and oxygen penetration is also mostly in the same range

in coastal sediments. Therefore we cannot draw conclusions from the measured microsensor profiles on diversity of *Beggiatoa*. We suggest that other factors for instance temperature and viruses (as discussed in chapter 2.1) could be the main factors influencing *Beggiatoa* diversity. *Beggiatoa* are surely responding to temperature effects as survival in enrichment cultures were much better at low temperature than at high temperature. The effect of viruses should be further investigated in future. Distribution of *Beggiatoa* biomass is poorly understood until now. Further investigations are needed to understand the environmental factors controlling *Beggiatoa* diversity. This is especially interesting as *Beggiatoa* is much easier to identify than other bacteria and could be a model organism for future investigations of biodiversity in the bacterial community.

3.2 Conclusion and Outlook

Beggiatoa could be an important member of the nitrogen cycle by changing the form of available nitrogen for the environmental community. *Beggiatoa* are indicator organisms for sulfide reaching the surface as they probably use the sulfide for orientation. High biomass of *Beggiatoa* is not only found in such environments but also in environments with sulfide depletion at the top 2-4 cm sediment depth. The contribution of *Beggiatoa* to the whole sulfur cycle seems to be less than expected until now and it will be interesting to investigate how exactly the sulfide removal occurs and how the pH profiles are affected. Although *Beggiatoa* can tolerate high sulfide concentrations they rather avoid sulfide. The many open questions that were found during this study show that further investigations are needed especially to understand the physiology. Although culture experiments were not successful, it was at least possible to purify Eckernförde *Beggiatoa* by let them gliding through the Agar. This was already used for genome analysis and can help to get a better understanding of *Beggiatoa* physiology. However, to clarify the respiration of *Beggiatoa* a lot of prework has to be done as nothing is known about sulfide oxidizing genes until now. There is no good alternative to getting a pure culture of vacuolated *Beggiatoa* and therefore it is important to further concentrate on this point.

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„Das Wissen wandelt sich stets, aber die Weisheit veraltet nie."
(Murshida R.F. v.Scholtz)