Growth and nitrate reduction of *Beggiatoa* filaments studied in enrichment cultures

Von der naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität Hannover
zur Erlangung des Grades einer

Doktorin der Naturwissenschaften
Dr. rer. nat.

genehmigte
Dissertation

von
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geboren am 06.05.1968 in Bremen

2007

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Tag der Promotion: 28.11.2007

CONTENTS

SUMMARY

In this thesis, several aspects of the gliding, filamentous, colourless sulphur bacteria *Beggiatoa* were investigated. The first part of this thesis addressed the growth mode, breakage of filaments for multiplication, and movement directions of filaments of *Beggiatoa*. Marine *Beggiatoa* were enriched in oxygen-sulphide gradient tubes, and observed with a camera system. Growth mode, breakage, and movement directions of *Beggiatoa* filaments were documented via time-lapse video recording. The initial doubling time of cells was 15.7 ±1.3 h (mean ±SD; room temperature). Filaments grew up to an average length of 1.7 ±0.2 mm, but filaments of up to ca. 6 mm were also present. First breakages of filaments occurred ca. 19 h after inoculation, and time-lapse movies illustrated that a parent filament could break into several daughter filaments within a few hours. In many cases, filament breakage occurred at the same point at which the tip of a forming loop of the filament had been observed before. As it is known that filament breakage is accomplished by the formation of a sacrificial cell, it was hypothesised that sacrificial cells interrupt the communication between two parts of one filament. As a consequence, the two parts of one filament can move towards each other forming the tip of a loop at the sacrificial cell.

The second part of this thesis focused on the physiology of *Beggiatoa*. The sulphur bacteria *Beggiatoa* can reach high biomass in many aquatic habitats, e.g. in and on freshwater and marine sediments, and affect the benthic sulphur cycle. In addition, *Beggiatoa* may influence the nitrogen cycle when they use nitrate anaerobically as an alternative electron acceptor in place of oxygen. The ability of freshwater and marine *Beggiatoa* to oxidise sulphide anaerobically with nitrate has been studied for some time. However, for freshwater *Beggiatoa*, the anaerobic sulphide oxidation with nitrate has not been unequivocally documented, and was therefore a special focal point of this thesis.

In a first study, the general ability of freshwater *Beggiatoa* to oxidise sulphide with nitrate as alternative electron acceptor was investigated. A freshwater *Beggiatoa* strain was highly enriched in oxygen-sulphide gradient tubes. The gradient tubes contained different nitrate concentrations, and the chemotactic response of the *Beggiatoa* mats was observed. The effects of the *Beggiatoa* on vertical gradients of nitrate, sulphide, oxygen, and pH were determined with microsensors. The more nitrate that was added to the agar, the deeper the *Beggiatoa* filaments glided into anoxic agar layers, suggesting that the *Beggiatoa* used nitrate to oxidise sulphide at depths below the depth that oxygen penetrated. In the presence of nitrate, *Beggiatoa* formed thick mats (>8 mm), compared to the thin mats (ca. 0.4 mm) that

were formed when no nitrate was added. These thick mats spatially separated oxygen and sulphide, but not nitrate and sulphide, and therefore nitrate must have served as the electron acceptor for sulphide oxidation. This interpretation is consistent with a fourfold-lower oxygen flux and a twofold- higher sulphide flux into the nitrate-exposed mats compared to the fluxes for controls without nitrate. Additionally, a pronounced pH maximum was observed within the *Beggiatoa* mat; such a pH maximum is known to occur when sulphide is oxidised to elemental sulphur with nitrate as the electron acceptor.

In a second study, a freshwater *Beggiatoa* strain was phylogenetically analysed and investigated with respect to the capability of intracellular nitrate storage and the dissimilatory use of nitrate. The 16S rDNA sequence retrieved from the filaments revealed the affiliation with the genus *Beggiatoa* and with a confined cluster of freshwater strains. Intracellular nitrate storage was found with concentrations of 35 ±32 mmol L^{-1}. To follow the metabolic pathway of the possible dissimilatory use of nitrate by this freshwater *Beggiatoa* strain, the filaments were highly enriched in oxygen-sulphide gradient tubes in which experiments with ^{15}N-labelled nitrate were carried out. The ^{15}N-labelling experiments showed that the freshwater *Beggiatoa* used the intracellular nitrate for DNRA (dissimilatory nitrate reduction to ammonium), whereas denitrification was not detected. This study revealed for the first time that a freshwater *Beggiatoa* strain was capable of intracellular accumulation of nitrate, and that the nitrate was used to perform DNRA.

Keywords: *Beggiatoa*, sulfur bacteria, nitrate reduction

ZUSAMMENFASSUNG

In dieser Doktorarbeit wurden verschiedene Aspekte der gleitenden, filamentösen Schwefelbakterien *Beggiatoa* untersucht. Der erste Teil der Doktorarbeit beschäftigt sich mit der Wachstumsweise, den Filamentteilungen zur Vermehrung und den Bewegungsrichtungen von *Beggiatoa*. Marine *Beggiatoa* wurden in Sauerstoff-Sulfid Gradientenkulturen angereichert und mit einem Kamerasystem beobachtet. Die Wachstumsweise, Teilungen und Bewegungsrichtungen wurden mit Zeitrafferaufnahmen dokumentiert. Die anfängliche Verdopplungszeit der Zellen betrug 15,7 ±1,3 h (Mittelwert ±Standardabweichung; Raumtemperatur). Durchschnittlich wuchsen die Filamente zu einer Länge von 1,7 ±0,2 mm an, allerdings waren auch Filamente mit Längen von ca. 6 mm nachweisbar. Erste Filamentteilungen fanden ca. 19 h nach der Beimpfung statt, und die Zeitrafferaufnahmen zeigten, dass sich ein Elternfilament innerhalb weniger Stunden in mehrere Tochterfilamente teilen kann. In vielen Fällen fand die Filamentteilung genau an der Stelle statt, an der zuvor die Spitze einer sich bildenden Filamentschlaufe beobachtet worden war. Da bekannt ist, dass Filamentteilungen durch Opferzellen ermöglicht werden, wurde die Hypothese aufgestellt, dass diese Opferzellen die Kommunikation zwischen zwei Filamentabschnitten unterbrechen. Infolgedessen ist es möglich, dass die zwei Abschnitte eines Filaments sich aufeinander zu bewegen können, wobei die Spitze der Schlaufe an der Opferzelle gebildet wird.

Der zweite Teil der Doktorarbeit beschäftigt sich mit der Physiologie von *Beggiatoa*. Die Schwefelbakterien *Beggiatoa* können in vielen aquatischen Systemen große Biomassen erreichen, z.B. auf und in marinen und limnischen Sedimenten, in welchen sie den benthischen Schwefelkreislauf beeinflussen. Außerdem könnten *Beggiatoa* Einfluss auf den Stickstoffkreislauf nehmen, wenn sie unter anaeroben Bedingungen Nitrat als alternativen Elektronenakzeptor statt Sauerstoff benutzen könnten. Die Fähigkeit von limnischen und marinen *Beggiatoa* unter anaeroben Bedingungen Sulfid mit Nitrat zu oxidieren wurde bereits früher untersucht, allerdings konnte dieser Prozess speziell bei Süßwasser-*Beggiatoa* bisher nicht einwandfrei nachgewiesen werden, und bildete daher einen besonderen Schwerpunkt dieser Doktorarbeit.

In einer ersten Studie wurde die grundsätzliche Fähigkeit von Süßwasser-*Beggiatoa* untersucht, Sulfid mit Nitrat als alternativem Elektronenakzeptor zu oxidieren. Ein limnischer *Beggiatoa*-Stamm wurde in Sauerstoff-Sulfid Gradientenkulturen stark angereichert. Die Gradientenröhrchen enthielten Nitrat in verschiedenen Konzentrationen, und die chemotaktische Reaktion von *Beggiatoa* wurde beobachtet. Die Effekte von *Beggiatoa* auf

vertikale Nitrat-, Sulfid-, Sauerstoff- und pH-Gradienten wurde mit Mikrosensoren bestimmt. Je mehr Nitrat dem Agar zugegeben wurde, desto tiefer glitten die *Beggiatoa*-Filamente in anoxische Agarschichten, was den Schluss nahelegt, dass *Beggiatoa* unterhalb der Sauerstoffeindringtiefe Nitrat zur Sulfidoxidation verwenden. Wenn Nitrat anwesend war, bildete *Beggiatoa* dicke Matten (>8 mm), im Vergleich zu dünnen Matten (ca. 0,4 mm) in Abwesenheit von Nitrat. Diese dicken Matten führten zu einer räumlichen Trennung von Sauerstoff und Sulfid, nicht aber von Nitrat und Sulfid. Daher muss Nitrat als Elektronenakzeptor für die Sulfidoxidation fungiert haben. Diese Interpretation steht in Einklang mit einem vierfach niedrigeren Sauerstoffflux und einem zweifach höheren Sulfidflux in die nitrat-exponierten Matten, verglichen mit den entsprechenden Fluxen in Kontrollansätzen ohne Nitrat. Zusätzlich wurde ein ausgeprägtes pH-Maximum innerhalb der *Beggiatoa*-Matte gefunden. Diese pH-Maxima treten bekanntermaßen dann auf, wenn Sulfid mit Nitrat als Elektronenakzeptor zu elementarem Schwefel oxidiert wird.

In einer zweiten Studie wurde ein limnischer *Beggiatoa*-Stamm phylogenetisch analysiert und hinsichtlich seiner intrazellulären Nitratspeicherfähigkeit und dissimilatorischen Verwendung von Nitrat untersucht. Die aus den Filamenten gewonnene 16S rDNS Sequenz belegte die Zugehörigkeit des untersuchten Stammes zur Gattung *Beggiatoa* sowie zu dem distinkten Cluster der Süßwasserstämme. Intrazelluläre Nitratspeicherung wurde in Konzentrationen von 35 ±32 mmol L^{-1} nachgewiesen. Um den Stoffwechselweg einer möglichen dissimilatorischen Verwendung von Nitrat durch diesen Süßwasserstamm zu verfolgen, wurden die Filamente in Sauerstoff-Sulfid Gradientenkulturen stark angereichert und Experimente mit ^{15}N-markiertem Nitrat durchgeführt. Die ^{15}N-Markierungsexperimente zeigten, dass die Süßwasser-*Beggiatoa* intrazelluläres Nitrat für DNRA (Dissmilatorische Nitrat-Reduktion zu Ammonium) benutzten, wohingegen Denitrifikation nicht nachgewiesen werden konnte. Diese Studie zeigt damit erstmalig, dass ein limnischer *Beggiatoa*-Stamm Nitrat intrazellulär akkumulieren kann, und dass dieses Nitrat für DNRA verwendet wird.

Schlagworte: *Beggiatoa*, Schwefelbakterien, Nitrat-Reduktion

Chapter 1

General introduction

INTRODUCTION

This introduction leads the way to the studies presented in the following chapters/manuscripts of this thesis (Kamp *et al*., chapter 2 to 4 of this thesis). The first part of the introduction provides some fundamental background knowledge on the bacterium *Beggiatoa*. The history of research on *Beggiatoa*, its morphology, life cycle, habitats, and gliding motility are addressed. The cultivation of *Beggiatoa* is described and the genus *Beggiatoa* is set in the phylogenetic context. In addition to published knowledge, some further own observations are provided, which are not included in the following chapters of this thesis. The second part of the introduction focuses on the ecophysiology of *Beggiatoa*. The ecological role of *Beggiatoa* in the benthic sulphur and nitrogen cycles is discussed with respect to the contemporary state of research.

THE BACTERIUM *BEGGIATOA*

History of research on *Beggiatoa*

The gliding filamentous, colourless sulphur bacteria of the genus *Beggiatoa* (Thiotrichales) were named in remembrance of the Italian medic and botanist F. S. Beggiato (Trevisan, 1842). Originally, they had been described as the colourless cyanobacterium "*Oscillatoria alba*" nearly 40 years earlier (Vaucher, 1803). The filamentous genera *Oscillatoria* and *Beggiatoa* have some morphological resemblances. However, an important morphological contrast to *Oscillatoria* is that *Beggiatoa* deposit elemental sulphur globules within each cell (Strohl, 2005; Teske and Nelson, 2006) and does not contain green pigments. Studies on these sulphur globules of *Beggiatoa* led to a major significant finding in microbiology: the origin of the concept of chemolithotrophy (Winogradsky, 1887). Winogradsky (1887) observed in slide cultures of *Beggiatoa* that the intracellular sulphur granules disappeared in the absence of sulphide while sulphate was produced. Thus, he concluded that *Beggiatoa* oxidised sulphide to elemental sulphur and subsequently to sulphate for energy generation.

Morphology of *Beggiatoa*

Both freshwater and marine *Beggiatoa* species are known (Fig. 1A to D). The multicellular *Beggiatoa* belong to the biggest bacteria in nature (Schulz and Jørgensen, 2001). They are visible with the naked eye and appear white because of the internally stored sulphur globules that can comprise ca. 20% of the cell dry mass of *Beggiatoa* (Nelson and Castenholz, 1981). The filament widths of different freshwater *Beggiatoa* species vary from ca. 1-7 µm; marine species have filament widths from ca. 2-200 µm (Larkin and Henk, 1996; Macalady *et al.*, 2006; Teske and Nelson, 2006). Individual cells in narrow filaments are typically cylindrical, with lengths that exceed their width; individual cells of the wider marine *Beggiatoa* are disk-shaped, with widths that exceed their length. The latter contain a large, central vacuole that comprises >80% of the cellular biovolume (McHatton *et al.*, 1996). *Beggiatoa* filaments can grow up to several mm in length (Jørgensen, 1977; Gundersen *et al.*, 1992; Larkin and Henk, 1996) and may contain some hundreds and up to a few thousands of cells (Kamp *et al.*, unpublished data). The longest filaments were found among the wide marine *Beggiatoa* and reach 10 cm in length (McHatton *et al.*, 1996).

Life cycle of *Beggiatoa*

Reproduction of *Beggiatoa* is by transverse binary fission of cells within the filament. The divisions occur by septation, in which inner membranes close like the iris of a diaphragm (Strohl and Larkin, 1978; Strohl, 2005). The filament dispersion of *Beggiatoa* is accomplished by formation of sacrificial cells (necridia) at various points within the filament. Upon dying, the sacrificial cells lyse, dividing the filament into daughter filaments (Strohl and Larkin, 1978; Kamp *et al.*, chapter 2 of this thesis). This process is comparable with filament breakage in the filamentous cyanobacterium *Oscillatoria* (Lamont, 1969). Filament dispersion and growth may also occur via separation of one or a few end cells to produce a hormogonium (Strohl, 2005). The separation of an end cell can be observed with a light microscope and proceeds within minutes (Fig. 2; Kamp *et al.*, unpublished data). The division of a filament can be also associated with filament death (Strohl, 2005). For example, dying or dead fragmented filaments can be found in old *Beggiatoa* cultures, as well as in *Beggiatoa* cultures in which essential nutrients are missing (Fig. 3A; Henze, 2005). However, in that case the filament width is mostly not consistent over the entire fragments; even massive local extensions and round structures may be visible with the light microscope (Fig. 3B to C).

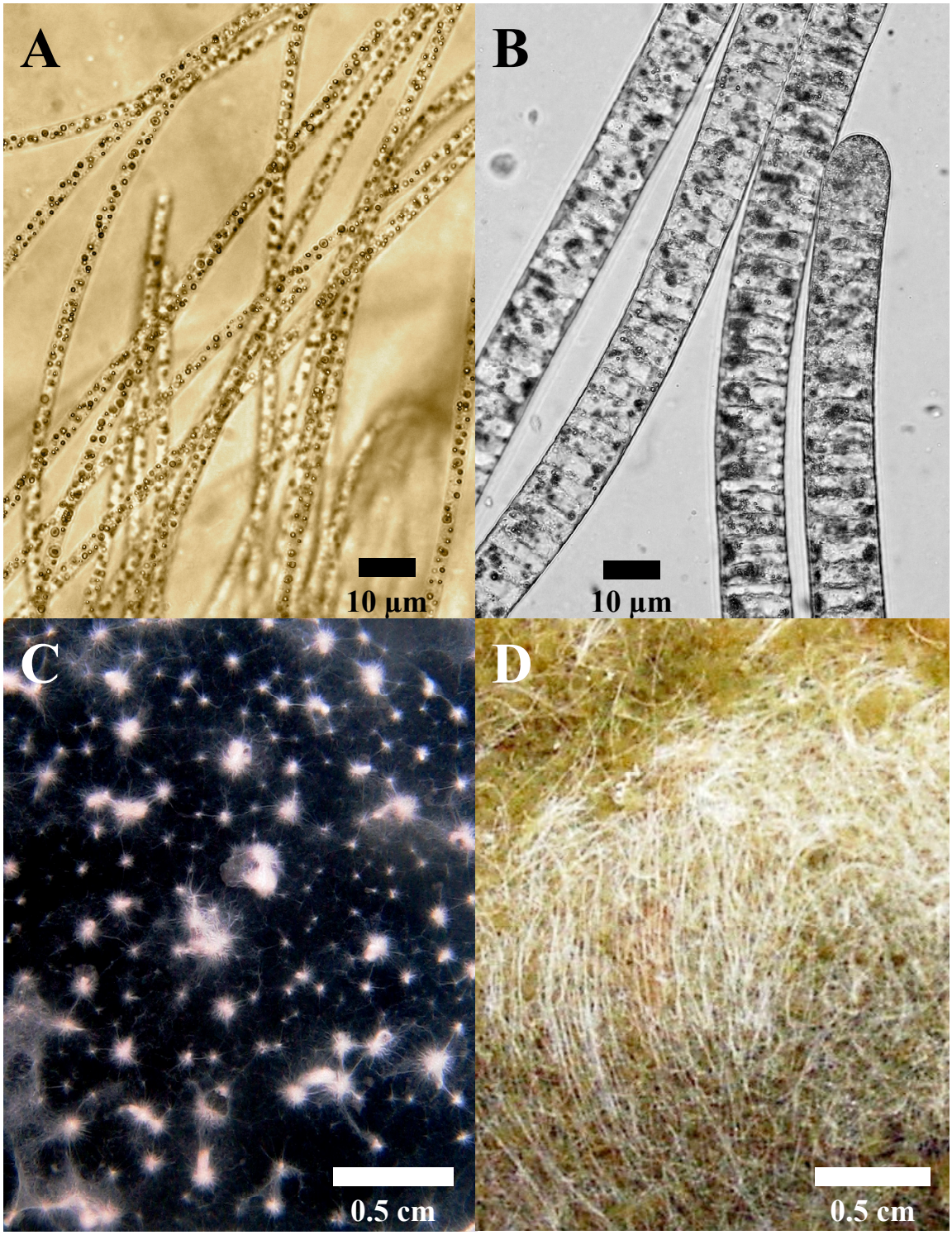

FIG. 1. *Beggiatoa* spp. (A) Light micrograph of a freshwater *Beggiatoa* sp. from a stream in Aarhus, DK, (B) Light micrograph of a marine *Beggiatoa* from harbour sediments in Aarhus. Individual disk-shaped cells are visible. Refractive structures are intracellular sulphur globules (A and B). (C) Tufts of freshwater *Beggiatoa* spp. on mud of sludge deposits around primary treated sewage outlets, Aarhus. Tuft formation protects the filaments from high ambient oxygen concentrations. (D) Large marine *Beggiatoa* spp. on a sediment core from the Namibian shelf, Africa. Single filaments are able to grow to lengths of >1 cm.

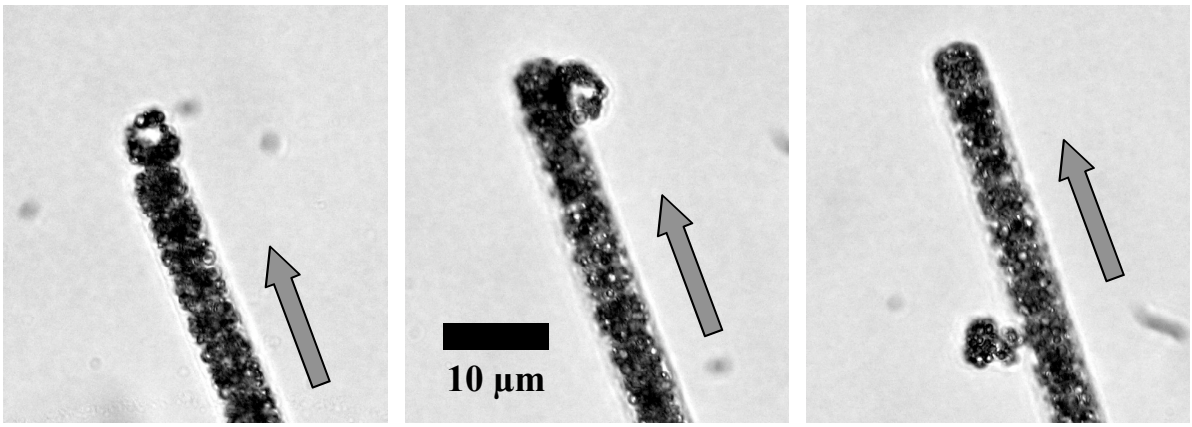

FIG. 2. Series of single light micrographs as an example of the separation of an end cell by a marine *Beggiatoa* that originated from a microbial consortium associated with the black-band disease of scleractinian corals, Florida Keys, USA. Arrows indicate direction of filament gliding.

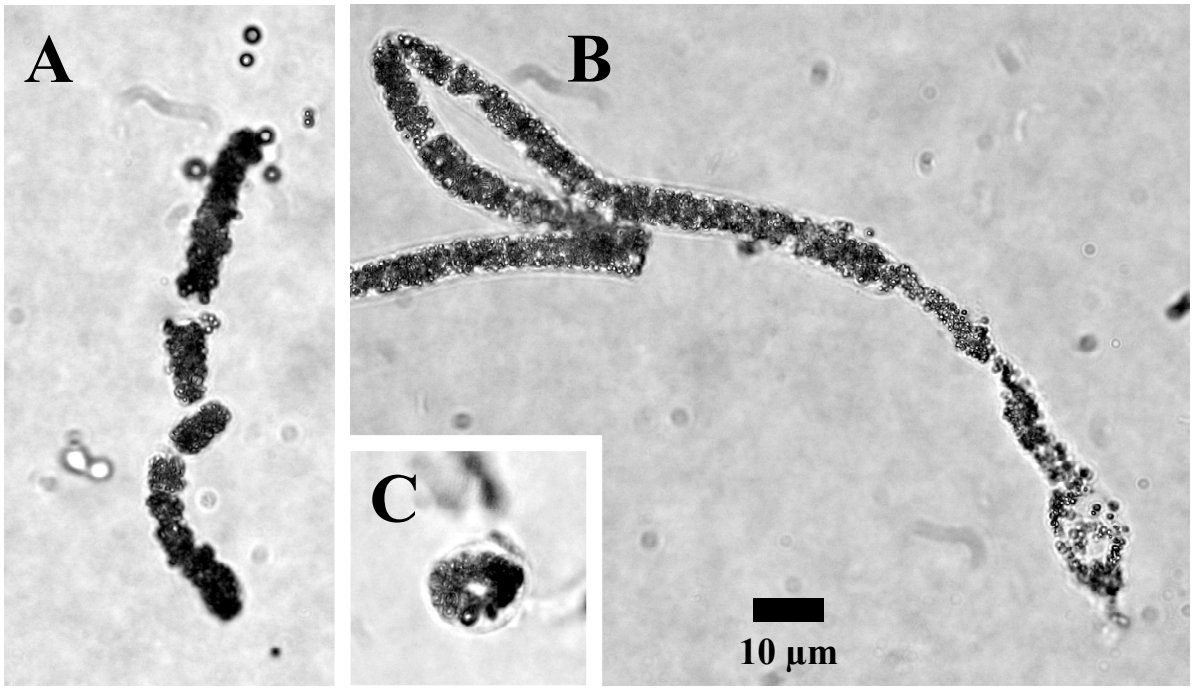

FIG. 3. Light micrographs of marine *Beggiatoa* that originated from a microbial consortium associated with the black-band disease of scleractinian corals, Florida Keys, USA. (A) Dead, fragmented filament. (B) Filament with a massive, terminal extension. (C) Round part of a filament. Refractive structures are intracellular sulphur globules (A to C).

Habitats of *Beggiatoa*

Beggiatoa filaments grow in many aquatic habitats, e.g. at the surface and in the suboxic zone of sulphide-rich freshwater, brackish and marine sediments (Jørgensen, 1977; Sweerts *et al.*, 1990; Sayama, 2001), in hypersaline ponds (Garcia-Pichel *et al.*, 1994), at hydrothermal vents (Jannasch, 1989; Gundersen *et al.*, 1992), at cold seeps (McHatton *et al.*, 1996), and on scleractinian corals infected by the black-band disease (Richardson, 1996). They can reach high biomasses and can form dense, white layers up to 1-3 cm thick on the sediment surface (Jannasch *et al.*, 1989; Gundersen *et al.*, 1992). At a hydrothermal vent site *Beggiatoa* layers of up to 60 cm were found between stands of vestimentiferan tube worms (Nelson *et al.*, 1989). Generally, *Beggiatoa* prefer a microoxic habitat at the interface of a source of reduced sulphur, typically sulphide, and oxygen or nitrate as electron acceptors (Teske and Nelson, 2006). There may be also a gap between the electron donator and acceptor (Mußmann *et al.*, 2003; Preisler *et al.*, 2007). In that case, *Beggiatoa* occur in sediment layers between detectable concentrations of sulphide that diffuses from deeper sediment layers upwards, and the electron acceptors that typically diffuses from the surface water and/or the upper sediment layers downwards.

Gliding motility of *Beggiatoa*

Beggiatoa filaments use gliding motility to precisely track the interface of oxygen and sulphide, to overcome spatial distances between their electron donator and acceptor, and for oxygen-dependant migrations, e.g. as diel migrations in photosynthetically active microbial mats (Garcia-Pichel *et al.*, 1994; Teske and Nelson, 2006; Preisler *et al.*, 2007). *Beggiatoa* gliding velocity is in the range of ca. 1-3 µm s^{-1} (Nelson *et al.*, 1989; Kamp *et al.*, chapter 2 of this thesis). Thus, *Beggiatoa* can glide over a maximum linear path of ca. 26 cm d^{-1}. If *Beggiatoa* are in their preferred microhabitat, the filaments glide in random directions, with a negative chemotactic response to oxygen and sulphide, which is often accompanied by formations of loops and sharp bends (Møller *et al.*, 1985; Preisler *et al.*, 2007). Video supported analysis of *Beggiatoa* that glide in transparent agar shows also that filaments often glide back and forth exactly at the same position, for many times (Kamp *et al.*, unpublished data). This gives the impression that the filaments glide inside their own sheath. Like filamentous cyanobacteria, *Beggiatoa* filaments usually rotate as they move by gliding. It was suggested that pores, which have been observed in parallel rows on the surface of the cells of *Beggiatoa* and *Oscillatoria*, were involved in the gliding movement by excreting slime

(Larkin and Henk, 1996; Hoiczyk and Baumeister, 1998). The slime surrounds the entire filament and becomes a sheath, from which gliding filaments can also be displaced or pressed out (Fig. 4; Møller *et al.*, 1985).

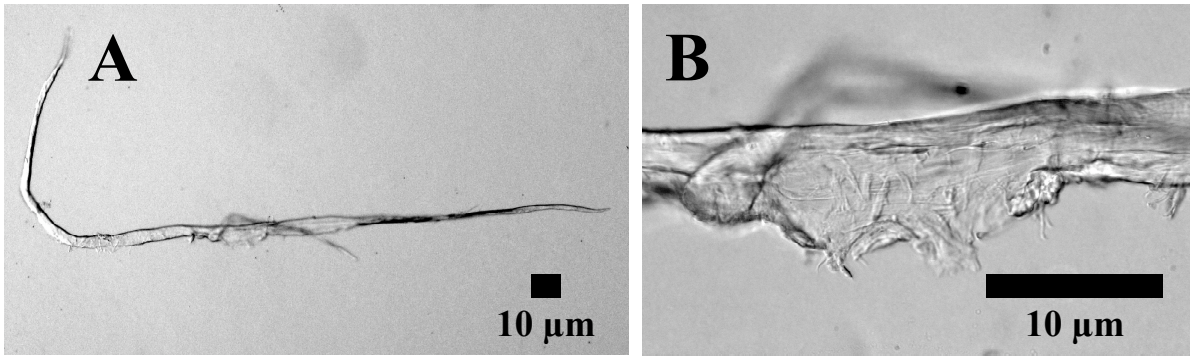

FIG. 4. Light micrographs of an empty sheath of a marine *Beggiatoa* sp. that originated from a microbial consortium associated with the black-band disease of scleractinian corals, Florida Keys, USA (A) Entire sheath. (B) Middle part of the sheath, from which a filament was most probably pressed out.

Cultivation of *Beggiatoa*

Beggiatoa can be enriched from their natural habitat in oxygen-sulphide gradient tubes (Nelson and Jannasch, 1983; Kamp *et al.*, 2006) and sustained for several years. In these gradient tubes, *Beggiatoa* grow under conditions, which mimic essential aspects of the ecophysiological niche of this bacterium. These aspects include opposed gradients of oxygen and sulphide, and a physical substrate that allows gliding of filaments to their preferred microenvironment. The oxygen-sulphide gradient tubes contain two layers of agar, a layer of dense bottom agar containing a high sulphide concentration overlaid by a layer of softer top agar without sulphide, which lead to opposing gradients of sulphide and oxygen in the top agar (Fig. 5A). After *Beggiatoa* are inoculated below the agar surface, the filaments start growing and a mat of *Beggiatoa* filaments is formed (Fig. 5B). The mat occurs usually slightly above the oxygen-sulphide interface (Kamp *et al.*, chapter 3 of this thesis) and moves upwards with time because biological sulphide oxidation by *Beggiatoa* renders the sulphide and oxygen gradients steeper. Subsequently, the *Beggiatoa* filaments have to move upwards to remain at the oxygen-sulphide interface. Thus, there is an interrelation of the metabolism of *Beggiatoa*, the changing chemical gradients, and the gliding behaviour of *Beggiatoa*.

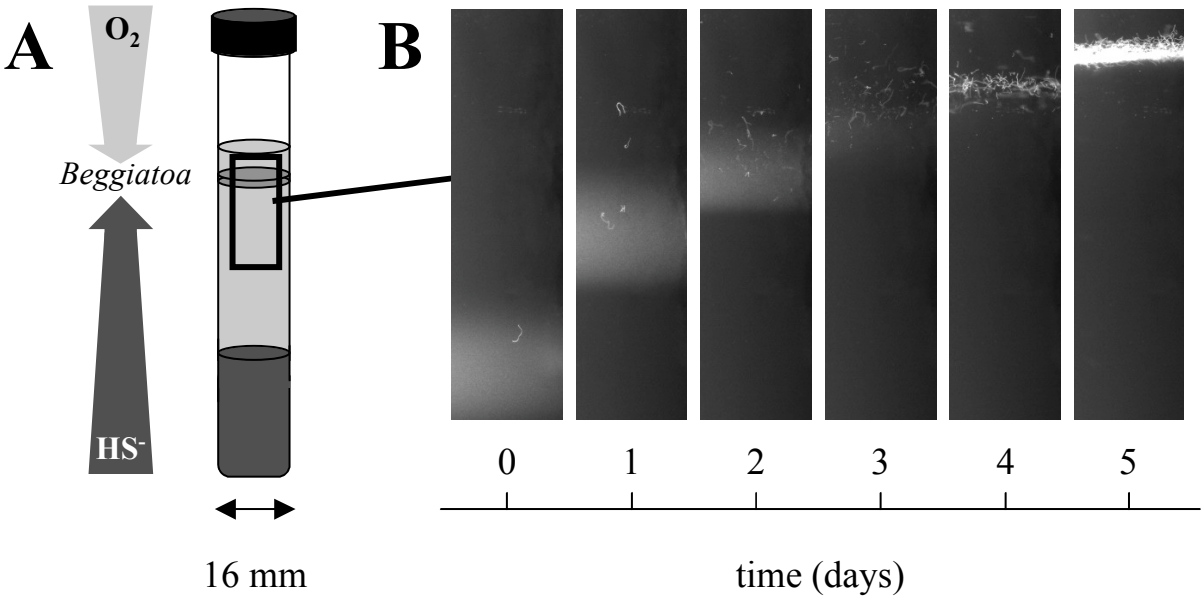

FIG. 5. Oxygen-sulphide gradient tubes. (A) Arrows indicate that oxygen diffuses from the air downwards in the top agar (light grey), and sulphide diffuses from the bottom agar (dark grey) upwards. The *Beggiatoa* mat is right above the interface of the chemical gradients of oxygen and sulphide. (B) Pictures taken from a time-lapse movie demonstrate the formation and the upward movement of the *Beggiatoa* mat in the gradient tube after inoculation with a single marine *Beggiatoa* filament. The filament originated from a microbial consortium associated with the black-band disease of scleractinian corals, Florida Keys, USA. The grey veil below the *Beggiatoa* mat is a sign of chemical sulphide oxidation to elemental sulphur that becomes less dominant the more biological sulphide oxidation proceeds.

Phylogeny of *Beggiatoa*

The genus *Beggiatoa* belongs to the gamma proteobacteria that include numerous free-living and symbiotic types of sulphur bacteria (Teske and Nelson, 2006). Phylogenetic reconstructions of the 16S rDNA demonstrate that large marine *Beggiatoa* with filament width >9 µm form a well-defined branch together with marine and freshwater strains of the filamentous *Thioploca*, the recently described White Point filamentous bacterium, and the unicellular *Thiomargarita* (Schulz *et al*., 1999; Mußmann *et al*., 2003; Kalanetra *et al.*, 2004; Kalanetra *et al*., 2005; Teske and Nelson, 2006; Ahmad *et al*., 2006; Kamp *et al*., chapter 4 of this thesis). Noticeably, this branch is separated from smaller marine *Beggiatoa* with filament widths of ca. 2-5 µm as well as from freshwater *Beggiatoa*, and it was discussed that the 16S rDNA data may be important for a future taxonomic restructuring of these sulphur bacteria (Strohl, 2005; Teske and Nelson, 2006). In particular, the large, marine, vacuolated *Beggiatoa*

with a filament width >20 µm are more closely related to the large, marine *Thioploca* than to other *Beggiatoa*, and it was questioned whether these strains indeed form separate well-defined genera (Ahmad *et al*., 1999). Optically, a single *Beggiatoa* filament cannot be unequivocally differentiated from a single *Thioploca* filament. The genera can only be distinguished by the observation that several *Thioploca* filaments occur within a common mucus sheath (Jørgensen and Gallardo, 1999; Teske and Nelson, 2006) that may be formed only in particular environments (Ahmad *et al*., 1999).

ECOPHYSIOLOGY OF *BEGGIATOA*

Influence of *Beggiatoa* on the benthic sulphur cycle

As sulphide-oxidizing bacteria, *Beggiatoa* have an important influence on the benthic sulphur cycle. However, this influence is complex, as sulphide oxidation proceeds biologically as well as chemically, in several oxidation steps, with different electron acceptors, and at the sediment surface as well as in the suboxic zone. Biological sulphide oxidation occurs in two steps. First, sulphide is oxidised to elemental sulphur that can be stored as intracellular globules, and subsequently sulphur is oxidised to sulphate (Table 1; Winogradsky, 1887; Strohl, 2005; Teske and Nelson, 2006). For the biological sulphide oxidation, oxygen and nitrate serve as electron acceptors (Teske and Nelson, 2006; Kamp *et al*., chapter 3 and 4 of this thesis). If oxygen and sulphide overlap at the sediment surface, aerobic sulphide oxidation in *Beggiatoa* mats prevents toxic sulphide from diffusing into the water column, because biological sulphide oxidation is much more rapid and efficient than chemical sulphide oxidation (Nelson *et al*., 1986a). However, the anaerobic sulphide oxidation by *Beggiatoa* is discussed controversially. In marine sediments, *Beggiatoa* can be most abundant in the suboxic zone (Jørgensen, 1977; Mußmann *et al*., 2003; Preisler *et al*., 2007), where neither oxygen nor sulphide is detectable. For this zone, it was found that the oxidation of sulphide by *Beggiatoa* with nitrate proceeds so efficiently that it leads to sulphide depletion in the suboxic zone (Sayama *et al*., 2005). In contrast, a recent study on a *Beggiatoa*-inhabited sediment discussed that most of the sulphide that diffused into the suboxic zone was removed by chemical processes, mainly by precipitation and oxidation with iron (Preisler *et al*., 2007). However, further investigations are needed to reveal the factors that determine whether biological or chemical sulphide removal processes are more effective

in the suboxic zone of marine sediments. Perhaps, the dominance of either process is just a question of *Beggiatoa* biomass and the peculiar chemical structure of different sediment types. Little is known about the anaerobic sulphide oxidation by freshwater *Beggiatoa* and their distribution in anoxic zones (Sweerts *et al*., 1990; Kamp *et al*., chapters 3 and 4 of this thesis), but there might by strong differences to *Beggiatoa* in marine sediments. In the latter, sulphide is produced in high amounts by sulphate reduction, which can be the quantitatively most important anaerobic respiration process that promotes remineralization of organic matter (Jørgensen, 1982). In freshwater sediments, sulphide originates mostly from the degradation of organic matter. Therefore, the amount of sulphide produced in freshwater ecosystems is highly dependent on the degree of eutrophication and may also be very patchy, e.g. because of the presence of local sewage outlets. Further, not all freshwater *Beggiatoa* strains have been shown to be chemolithotrophic (Strohl and Larkin, 1978; Nelson *et al*., 1986b; Strohl, 2005). Many strains are chemoorganotrophic, using acetate as their sole energy source. Thus, especially the chemoorganotrophic freshwater *Beggiatoa* may be of minor importance for the sulphide oxidation in freshwater sediments. However, as sulphide is toxic, a sulphide-depleted sediment is essential for the survival of benthic fauna and thus, sulphide removal processes are of major ecological importance.

TABLE 1. Equations for the sulphide oxidation via oxygen reduction (oxic), the dissimilatory nitrate reduction to ammonia (DNRA) and denitrification. Given are the first and second oxidation steps as well as the summarised equation, each

Pathway of sulphide oxidation	Equation
Oxic	$2HS^- + 2H^+ + O_2 \rightarrow 2S^o + 2H_2O$
	$2S^o + 2H_2O + 3O_2 \rightarrow 2SO_4^{2-} + 4H^+$
	$2HS^- + 4O_2 \rightarrow 2SO_4^{2-} + 2H^+$
DNRA	$4HS^- + NO_3^- + 6H^+ \rightarrow 4S^o + NH_4^+ + 3H_2O$
	$4S^o + 7H_2O + 3NO_3^- \rightarrow 4SO_4^{2-} + 3NH_4^+ + 2H^+$
	$4HS^- + 4NO_3^- + 4H_2O + 4H^+ \rightarrow 4SO_4^{2-} + 4NH_4^+$
Denitrification	$5HS^- + 2NO_3^- + 7H^+ \rightarrow 5S^o + N_2 + 6H_2O$
	$5S^o + 2H_2O + 6NO_3^- \rightarrow 5SO_4^{2-} + 3N_2 + 4H^+$
	$5HS^- + 8NO_3^- + 3H^+ \rightarrow 5SO_4^{2-} + 4N_2 + 4H_2O$

Influence of *Beggiatoa* on the benthic nitrogen cycle

Anaerobic sulphide oxidation with nitrate by *Beggiatoa* links the benthic sulphur and nitrogen cycles. Both, elemental sulphur as the electron donator and nitrate as the electron acceptor can be internally stored in concentrations of up to ca. 300-400 mmol l^{-1} (Mußmann *et al.*, 2003; Preisler *et al.*, 2007). In large, marine *Beggiatoa*, nitrate is most probably stored in liquid vacuoles (McHatton *et al.*, 1996). A recent study on freshwater *Beggiatoa* shows that nitrate storage can also be independent of a liquid vacuole (Kamp *et al.*, chapter 4 of this thesis). Nitrate storage enables *Beggiatoa* to occur in the suboxic zone. The gliding filaments shuttle between the sediment surface and the lower part of the suboxic zone where sulphide diffuses upwards from deeper sediment layers. At the sediment surface, the filaments can use oxygen and simultaneously take up and store nitrate; in the upper layer of the suboxic zone they are able to oxidise sulphide and store elemental sulphur (Sayama *et al.*, 2005; Preisler *et al.*, 2007). The anaerobic sulphide oxidation with nitrate by *Beggiatoa* proceeds most probably via DNRA (dissimilatory nitrate reduction to ammonium; Fig. 6 and Table 1; Sayama *et al.*, 2005; Preisler *et al.*, 2007; Kamp *et al.*, chapter 4 of this thesis). For freshwater *Beggiatoa*, denitrification was also discussed (Fig. 6 and Table 1; Sweerts *et al.*, 1990). Energetically, both metabolic pathways are high energy yielding respiration systems in anoxic environments (Strohm *et al.*, 2007). Ecologically, the impact of the one or the other pathway on the environment is complex. If DNRA proceeds, fixed nitrogen may stay in the ecosystem, because ammonium can be reoxidised to nitrate via nitrification. However, nitrification is an oxic process, and since DNRA proceeds in the anoxic zone, the ammonium has to be transferred into the oxic zone for nitrification, e.g., due to sediment resuspension, advective transport processes and possibly by bioturbation/bioirrigation of macrofauna. In anoxic environments, ammonium can also be reoxidised to nitrogen gas via anammox (anaerobic ammonium oxidation). The first indication for anammox was obtained in bioreactors only twelve years ago (Mulder *et al.*, 1995). Ongoing, intensive research on this newly discovered metabolic pathway shows that anammox proceeds in many aquatic environments and can contribute to up to ca. 50% of the total nitrogen loss (Francis *et al.*, 2007). Nitrogen will leave the ecosystem also when denitrification proceeds, because the final product of complete denitrification is gaseous nitrogen. On the other hand, several *Beggiatoa* strains have been shown to fix gaseous nitrogen for assimilatory purposes (Nelson *et al.*, 1982), which eventually will return the organically bound nitrogen back into the nitrogen cycle after filament death and degradation. However, excessive nitrogen concentrations present a water-

quality problem of growing concern (Burgin and Hamilton, 2007), especially in freshwater and near shore environments, where the anthropogenic influence is most noticeable. Thus, the impact of *Beggiatoa* on the benthic nitrogen cycle is of great ecological interest and deserves further attention and research.

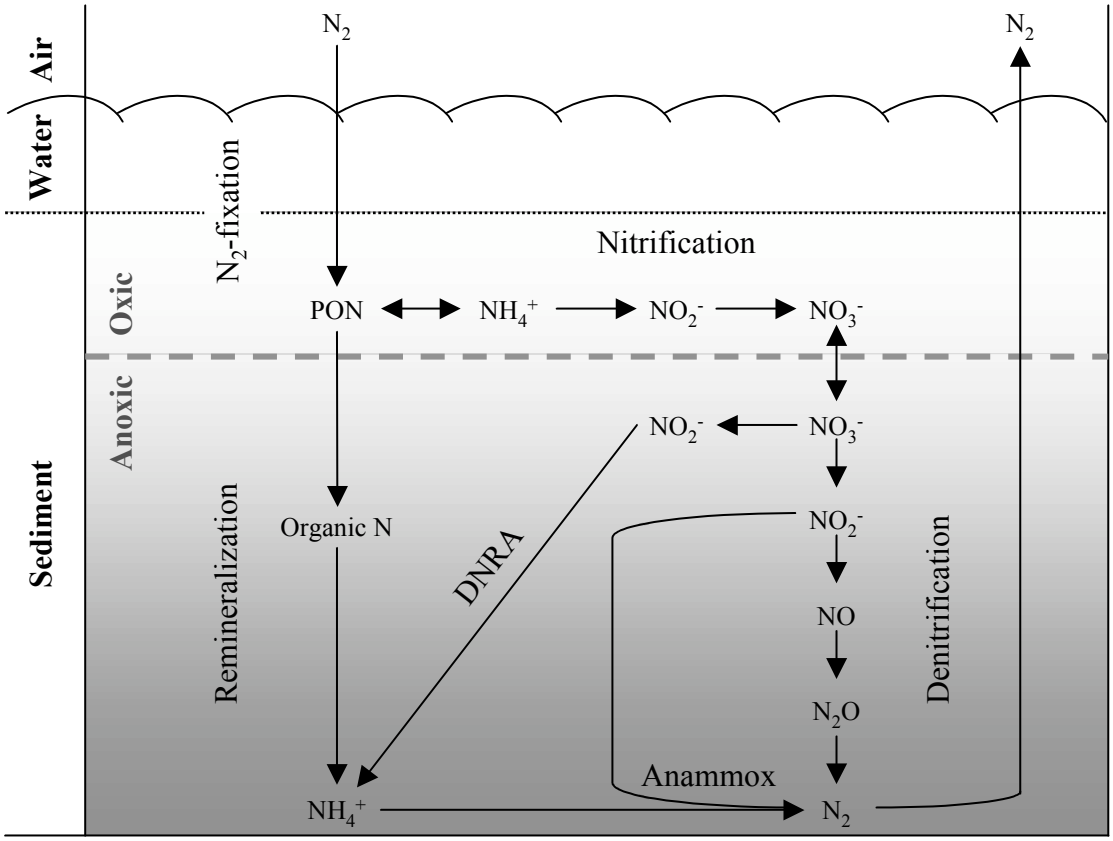

FIG. 6. Aquatic microbial nitrogen cycle. Transformations above, below and across an oxic-anoxic interface of the sediment. PON, particulate organic nitrogen; DNRA, dissimilatory nitrate reduction to ammonium. Modified after Arrigo, 2005; Francis *et al*., 2007.

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OBJECTIVES AND OUTLINE OF THIS THESIS

The main objective of this thesis was to get a better understanding of the influence of the big, filamentous sulphur bacteria *Beggiatoa* on the benthic nitrogen cycle. *Beggiatoa* can be abundant in many aquatic environments, and large *Beggiatoa* from marine environments were found to store nitrate in high concentrations. These large, marine *Beggiatoa* were assumed to use internally stored nitrate to perform DNRA (dissimilatory nitrate reduction to ammonium). In contrast to this, the smaller *Beggiatoa* from freshwater habitats were not known to store nitrate, and little was known about their capability to perform dissimilatory nitrate reduction. Therefore, the use of nitrate by freshwater *Beggiatoa* was of special interest during this thesis.

The experiments were carried out in oxygen-sulphide agar gradient tubes. A freshwater strain, taken from mud of sludge deposits around primary treated sewage outlets in Aarhus (DK), was already in stable gradient cultivation. An original objective was to obtain a pure culture of this strain to perform experiments, in which metabolic pathways could be clearly attributed to the *Beggiatoa*. Unfortunately, a pure culture was not obtained (Conclusions and outlook of this thesis). However, even the study of *Beggiatoa* filaments in enrichment cultures has major advantages. (i) In contrast to the majority of bacteria, the multicellular, large *Beggiatoa* filaments can be easily observed in the transparent agar, and (ii) the gradient tubes can be adjusted to chemically controlled conditions. Thus, a well targeted combination of the cultivation technique with other experimental approaches allowed attributing metabolic pathways to *Beggiatoa* without having a pure culture.

In addition to the freshwater strain, a marine *Beggiatoa* strain that originated from a microbial consortium associated with the black-band disease of scleractinian corals, Florida Keys (USA) was already in stable gradient cultivation. A pure culture of this strain was also not obtained. However, the enrichment cultivation of this strain was straight forward, because even single filaments that were inoculated into the gradient tubes started growing fast. Therefore, this strain stimulated for making time-lapse movies of filaments growing in the transparent agar. The aim was to acquire more information on the growth patterns of the filamentous *Beggiatoa*. In particular, the time course of reproduction of single *Beggiatoa* filaments, and the movement directions of filaments were investigated. This study supplemented basic knowledge on the growth mode of *Beggiatoa*, and is thus addressed in the first manuscript of this thesis (Kamp *et al*., chapter 2 of this thesis).

The second manuscript of this thesis (Kamp *et al*., chapter 3 of this thesis) focuses on the possibility of anaerobic sulphide oxidation with nitrate by a freshwater *Beggiatoa* strain. The oxygen-sulphide agar gradient tubes were supplied with different nitrate and sulphide concentrations. The filaments responded to changed chemical conditions by forming a mat in different depths, which was optically correlated with high-spatial-resolution measurements of nitrate, oxygen, sulphide, and pH using microsensors. The interpretation of the chemical gradients altered by *Beggiatoa*, gave fundamental information on the use of nitrate as an alternative electron acceptor instead of oxygen.

The third manuscript of this thesis (Kamp *et al*., chapter 4 of this thesis) concentrates on the question of the final product of anaerobic sulphide oxidation with nitrate by a freshwater *Beggiatoa* strain. An important finding was, that the investigated *Beggiatoa* strain was capable to store nitrate intracellularly. This ability was used in ^{15}N-labelling experiments to investigate the metabolic fate of nitrate stored within filaments. The gradient tubes were supplied with ^{15}N-labelled nitrate. After the *Beggiatoa* had stored the labelled nitrate, they were transferred into nitrate-free medium and were adjusted to anoxic conditions. After the anoxic incubation, ^{15}N-labelled nitrogen gas and ^{15}N-labelled ammonium, the two possible products of the dissimilatory nitrate reduction pathways DNRA and denitrification, were detected by mass spectrometry.

CONTRIBUTIONS TO PUBLICATIONS

This thesis includes the complete versions of three manuscripts that have been published or will be published as full-length research articles in high quality, peer-reviewed international journals (Kamp *et al*., chapter 2 to 4 of this thesis).

Chapter 2. Anja Kamp, Hans Røy, and Heide N. Schulz-Vogt

Video-supported analysis of *Beggiatoa* filament growth, breakage, and movement

This study was initiated by all co-authors. H.N.S.-V. started the cultivation of the *Beggiatoa* strain. A.K. conducted the further cultivation, performed the experiments and analysed the data. H.R. attended to the hard- and software for the video analyses. A.K. wrote the manuscript with editorial input from all co-authors. The manuscript is in revision for publication in the journal *Microbial Ecology.*

Chapter 3. Anja Kamp, Peter Stief, and Heide N. Schulz-Vogt

Anaerobic sulfide oxidation with nitrate by a freshwater *Beggiatoa* enrichment culture

This study was initiated by all co-authors. H.N.S.-V. started the cultivation of the *Beggiatoa* strain. A.K. conducted the further cultivation and performed the experiments. The O_2, H_2S and pH microgradients were measured by A.K. and P.S. The LIX-type NO_3^- microgradients were measured and analysed by P.S. A.K. analysed all other data and wrote the manuscript with editorial input from all co-authors. The manuscript has been published in the journal *Applied and Environmental Microbiology* (2006) 72: 4755-4760.

Chapter 4. Anja Kamp, Peter Stief, Lars Peter Nielsen, and Heide N. Schulz-Vogt

Dissimilatory nitrate reduction to ammonium by a freshwater *Beggiatoa* strain

This study was initiated by all co-authors. L.P.N. and A.K. carried out the sampling of the *Beggiatoa*. A.K. conducted the cultivation of the *Beggiatoa* strain, made the (internal) NO_3^- measurements and performed the experiments. P.S. acquired the 16S rRNA sequence, made the phylogenetic analysis, and performed the N_2O measurements. The ^{15}N-NH_4^+ and ^{15}N-N_2 samples were measured professionally at the National Environmental Research Institute, Silkeborg, DK. A.K., P.S., and L.P.N. analysed the data. A.K. wrote the manuscript with editorial input from all co-authors. The manuscript has been submitted to the journal *Environmental Microbiology.*

Chapter 2

Video-supported analysis of *Beggiatoa* filament growth, breakage, and movement

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The manuscript is in revision for publication in the journal *Microbial Ecology*

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ABSTRACT

A marine *Beggiatoa* strain was cultured in semi-solid agar with opposing oxygen-sulphide gradients. Growth pattern, breakage of filaments for multiplication, and movement directions of *Beggiatoa* filaments in the transparent agar were investigated by time-lapse video recording. The initial doubling time of cells was 15.7 ±1.3 h (mean ±SD) at room temperature. Filaments grew up to an average length of 1.7 ±0.2 mm, but filaments of up to ca. 6 mm were also present. First breakages of filaments occurred ca. 19 h after inoculation, and time-lapse movies illustrated that a parent filament could break into several daughter filaments within a few hours. In many cases filament breakage occurred at the same point where the tip of a loop of the filament was observed before. As filament breakage is accomplished by the formation of a sacrificial cell, we hypothesise that sacrificial cells interrupt the communication between two parts of one filament. As a consequence, the two parts of one filament can move towards each other forming the tip of a loop at the sacrificial cell.

INTRODUCTION

The filamentous, colourless sulphur bacteria of the genus *Beggiatoa* (Thiotrichales) were named after the Italian medic and botanist F. S. Beggiato [27]. Originally they had been described as a colourless cyanobacterium named "*Oscillatoria alba*" nearly 40 years earlier [28]. Apart from the morphological resemblance of the filamentous genera *Beggiatoa* and *Oscillatoria*, similarities in life cycle and gliding motility were found [6, 7, 12, 13, 16, 24]. The filaments often rotate as they move by gliding, and it was discussed that pores that have been found in parallel rows on the surface of the cells of *Beggiatoa* and *Oscillatoria* were involved in the rotating gliding motility by excretion of slime [8, 13]. The slime surrounds the entire filament and becomes a sheath, from which gliding filaments can also be displaced or pressed out [8, 15].

Gliding motility is used by *Beggiatoa* to track their chemical microenvironment, the interface between oxygen and sulphide [26]. They grow in many aquatic habitats, e.g. at the surface and in the suboxic zone of sulphide-rich marine and freshwater sediments [10, 21, 25], in hypersaline ponds [4], at hydrothermal vents [5, 9], at cold seeps [14], and on scleractinian corals infected by the black-band disease [20]. *Beggiatoa* can be enriched from their natural habitat in oxygen-sulphide gradient tubes [17] and sustained for several years.

In this study, cultivation in transparent agar was used in combination with time-lapse video recording to investigate the growth and multiplication of *Beggiatoa* under conditions, which mimicked essential aspects of the niche of this bacterium. These aspects include opposed gradients of oxygen and sulphide, and a physical substrate that allows gliding of filaments to their preferred microenvironment. The experimental approach was used to address the following questions. (i) What is the growth pattern of *Beggiatoa* filaments? (ii) What is the time course of filament breakage for multiplication? (iii) What are the movement directions of filaments?

METHODS

Strain and cultivation. The 6.3 µm wide *Beggiatoa* strain used for this study originated from a microbial consortium associated with the black-band disease of scleractinian corals (Florida Keys). It was enriched and has been kept in stable gradient cultivation for more than six years.

The *Beggiatoa* filaments were cultured in agar gradient cultures, modified after Nelson & Jannasch [17]. These gradient cultures contained two layers of media, a layer of bottom agar medium (0.75% Bacto Agar [Difco Laboratories]) containing a high sulphide concentration overlaid by a layer of softer top agar medium (0.25%) without sulphide, which led to opposing gradients of sulphide and oxygen in the top agar. The compositions of the artificial seawater, nutrient solutions, vitamin solutions and agar media are shown in Table 1A and B. The pH of the top agar was adjusted to approximately 8 with KOH. The autoclaved bottom agar medium was adjusted to 4 mmol/L Na_2S. The autoclaved top agar medium was adjusted to 4 mmol/L $NaHCO_3$, and 0.04% sterile vitamin solution was added. The gradients were prepared in screw-cap tubes (length, 150 mm; inside diameter, 14 mm). The tubes were filled with 4 ml of final bottom agar medium and 8 ml of top agar medium. The screw caps were left loose to permit exchange of the headspace gas with the atmosphere. To allow gradient development, the agar was aged for at least 2 days before inoculation. For the different experiments, *Beggiatoa* filaments were inoculated approximately 5 mm below the agar surface with a sterile Pasteur pipette. Cultures used for determination of protein content and filament length were grown at room temperature in the dark. Cultures used for time-lapse movies were kept in monochromatic red light (see below).

TABLE 1A. Composition of artificial seawater, mineral solution, trace solution, and vitamin solution

Artificial seawater or solution	Composition
Artificial seawater	27.5 g NaCl, 10.7 g $MgCl_2 \cdot 6\ H_2O$, 4.1 g $MgSO_4 \cdot 7\ H_2O$, 0.66 g $CaCl_2 \cdot 2\ H_2O$, 1.02 g KCl, 1,000 ml distilled water
Mineral solution	555 mg K_2HPO_4, 50 mg Na_2MoO_4, 750 mg $Na_2S_2O_5$, 29 mg $FeCl_3 \cdot 6\ H_2O$, 1,000 ml distilled water
Trace element solution	5.2 g EDTA, 1.5 g $FeCl_2 \cdot 4\ H_2O$, 0.07 g $ZnCl_2$, 0.1 g $MnCl_2 \cdot 4\ H_2O$, 0.062 g H_3BO_4, 0.19 g $CoCl_2 \cdot 6\ H_2O$, 0.017 g $CuCl_2 \cdot 2\ H_2O$, 0.024 g $NiCl_2 \cdot 6\ H_2O$, 0.036 g $Na_2MoO_4 \cdot 2\ H_2O$, 1,000 ml distilled water
Vitamin stock solution[a]	1 mg B12, 1 mg Inositol, 1 mg Biotin, 1 mg Folic Acid, 10 mg PABA, 100 mg Nicotinic Acid, 100 mg d-Pantothenate, 200 mg Thiamine (each vitamin was dissolved in 10 ml distilled water)

[a]For the final vitamin solution 1 ml of each vitamin stock solution was added to 100 ml (final volume) of distilled water.

Protein determination. Protein was quantified by the Coomassie Brilliant Blue assay (Bio-Rad, Munich, Germany) of Bradford [2]. The inoculum for replicate cultures was harvested from 23 culture tubes, pooled, thoroughly mixed, and identical subsamples (100 µL) were used for inoculation of new gradient tubes. Sets of 6 cultures were taken after 1, 2, 3, 4, 6, 8, 10, 12, and 14 days, and the top 1.5 - 2.5 ml agar, which contained the *Beggiatoa* filaments, was harvested. Prior to protein measurement, the samples were incubated with 10% (w/v) trichloracetic acid at 90°C for 20 min [18], then cooled to 4°C overnight and centrifuged at 20800 g for 10 min at 4°C. After the supernatant was removed, 1 ml 0.1 mol/L NaOH was added to the pellet and heated to 55°C for 20 min. Equivalent volumes of agar from uninoculated gradient tubes were processed likewise and served as blanks; bovine plasma albumin was used as the standard (Bio-Rad). The final assay mix contained 0.4 ml standard (in 0.1 mol/L NaOH) or supernatant of samples, 0.4 ml 0.15 mol/L HCl, and 0.2 ml

dye reagent concentrate. OD_{595} of standards and samples was measured after 15 min against the blank.

TABLE 1B. Composition of agar media

Agar media[a]	Composition
Bottom agar medium	180 ml artificial seawater, 1.35 g agar
Top agar medium[b]	1) 240 ml artificial seawater, 4.32 g NaCl
	2) 96 ml distilled water, 0.9 g agar
	3) 24 ml mineral solution, 0.36 ml trace solution

[a]Each medium was autoclaved.

[b]The three components of the top agar medium were combined after they were autoclaved.

Camera system. The gradient cultures were imaged from the side every 10 to 20 sec with a Sony XCD-X710 digital monochrome camera (1024 x 768 pixels). Illumination was provided with a red 1W Luxeon™ Star LED with collimating optics. The LED emits light in a narrow band around 625 nm that does not affect the movement of *Beggiatoa* [16]. Cultures were filmed several days and the images were collected to time-lapse movies using the computer program VideoMach (Gromada Multimedia, Cologne, Germany).

Doubling time of cells. The doubling time of cells was determined with images from three independent time-lapse movies. Single *Beggiatoa* filaments were inoculated into new gradient tubes. The time-lapse movies were screened for images on which the *Beggiatoa* filaments glided parallel to the image plane of the camera. *Beggiatoa* filament lengths were measured on the chosen images (movie 1: n = 8, movie 2: n = 29, movie 3: n = 19) with the free computer program UTHSCSA Image Tool (**IT**; University of Texas Health Science Center at San Antonio, USA) during the first day after inoculation. Filament lengths were converted into a number of standard cells (average cell length = 2.7 μm), which was plotted against the time elapsed between the individual images. The function of the exponential plot was used to calculate the doubling time.

Filament length. Filament lengths were measured from *Beggiatoa* light micrographs with **IT**. Incubation start and inoculum were the same as for the protein determinations (see

above), but samples were taken in daily intervals. The *Beggiatoa* filaments were harvested from one gradient culture per incubation time with a Pasteur pipette. Using a stereomicroscope, care was taken to avoid damaging filaments during the harvest. Harvested filaments were put on an objective slide for taking the light micrographs of randomly chosen *Beggiatoa* filaments at 100-fold magnification (n = 42).

FISH/DAPI. FISH (Fluorescence In Situ Hybridization) was performed by use of published methods [1, 22] modified as follows. *Beggiatoa* filaments were taken from gradient cultures two days after inoculation. The filaments were fixed in 1 ml freshly prepared paraformaldehyde (4%) for 4 h and washed 2 times in 1xPBS (Phosphate-buffered Saline) before storage in 0.5 ml 1xPBS and 0.5 ml 96% (v/v) ethanol. Prepared filaments were spotted on hybridization wells of non-coated microscopic slides and dried at 48°C before dehydration in a 50-80-96% (v/v) ethanol series. To each well, 9 µl hybridization buffer (35% formamide) and 1 µl Cy3-labelled EUB338-III probe [3] was added. Hybridization was carried out at 46°C for 90 min in a hybridization oven. After hybridization, the slides were rinsed and incubated with preheated washing buffer (48°C) for 15 min. Finally, the slides were carefully washed with H_2O_{dist} and air dried. Approximately 20 min before microscopic analysis, the slides were mounted in Citifluor-DAPI (4',6',-diamidino-2-phenylindole). Fluorescence micrographs were recorded with a Zeiss Axiovert 200M microscope with apotome (545 nm excitation wavelength for FISH and 375 nm for DAPI).

Filament gliding speed. Measurements of gliding speed of *Beggiatoa* filaments were obtained from the same time-lapse movies that were used for the determination of the growth rate of cells. The time-lapse movies were screened for sequences in which the filament or at least tips of filaments glided parallel to the image plane of the camera. Single images (n = 757) of the chosen movie sequences (n = 61) were analysed by marking the position of the filament tip image by image and saving its x/y coordinates with the computer program **IT**. Distances between the x/y coordinates from one to another image were calculated, summed-up and divided by the total duration of each sequence.

Filament breakage and movement. Time-lapse movies were screened for sequences in which filament breakage was well visible. Filament lengths before and after their first 3 - 4 breakages were measured in three independent movies as described above. Further, 64 breakages were observed in seven independent time-lapse movies, and the direction of movement of the dividing filaments was determined.

RESULTS

Protein determination and doubling time of cells. The average total protein content of the *Beggiatoa* consortium was 0.9 ±0.3 µg for the inoculum (standard error [SE]) and increased linearly to 8.8 ±0.7 µg during the incubation time of two weeks (Fig. 1A). The initial doubling time of cells was 15.7 ±1.3 h. However, a minimum doubling time of cells was calculated with 5 h in movie 2 during the first 300 min.

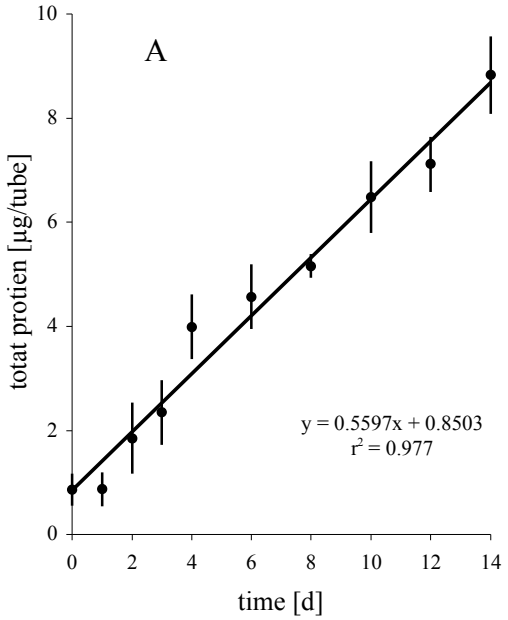

FIG. 1. Average total protein content of the *Beggiatoa* consortium (µg/tube) over time (d). Day 0 = inoculum. Error bars indicate SE (n = 6).

Filament length determination. The average filament length in the inoculum was 0.7 ±0.1 mm, which increased to 1.7 ±0.2 mm on day seven, and remained constant thereafter (Fig. 2A). The frequency distribution of classes of filament lengths (Fig. 2B and C) revealed that filaments of up to 2 mm dominated the gradient culture numerically both during the early and the later incubation period (day 1-5 and 6-14, respectively, Fig. 2B and C). However, filaments of 2-6 mm in length occurred also during the whole experiment.

FISH/DAPI and light microscopy. An overlay of FISH and DAPI images shows regions along the filament in which neither ribosomes nor DNA occurred (Fig. 3A). Such "empty" regions were also visible in ordinary light micrographs.

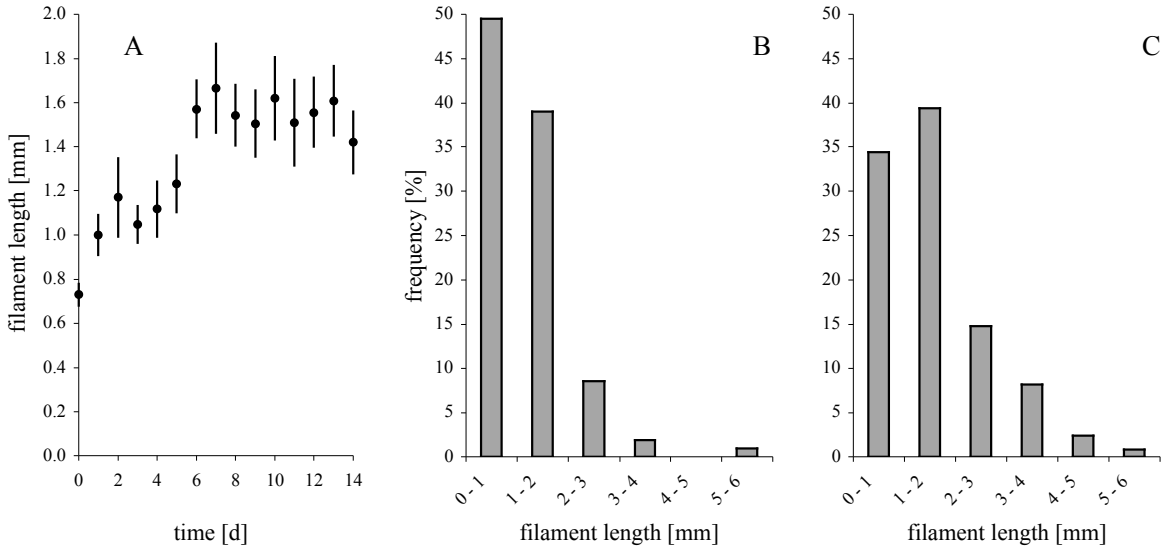

FIG. 2. (A) Average *Beggiatoa* filament length (mm) over time (d). Day 0 = inoculum. Error bars indicate SE (n = 42). (B, C) Frequency (%) of *Beggiatoa* filament lengths in classes of 1 mm, averaged for day 1 – 5 (n = 210; B) and for day 6 – 14 (n = 378; C).

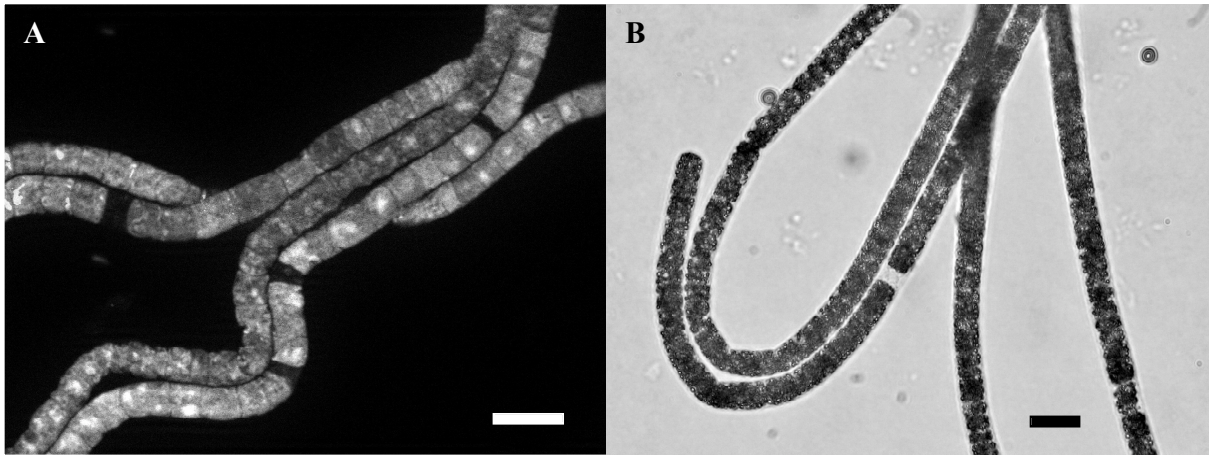

FIG. 3. (A) *Beggiatoa* filaments hybridized with the universal *Bacteria* probe EUB338-III (FISH) and counterstained with DAPI, showing regions along the filament that neither hybridized to the FISH probe, nor were stained with DAPI. (B) Light micrograph of *Beggiatoa* filaments, showing an "empty" region where a cell lysed. Bars, 10 µm.

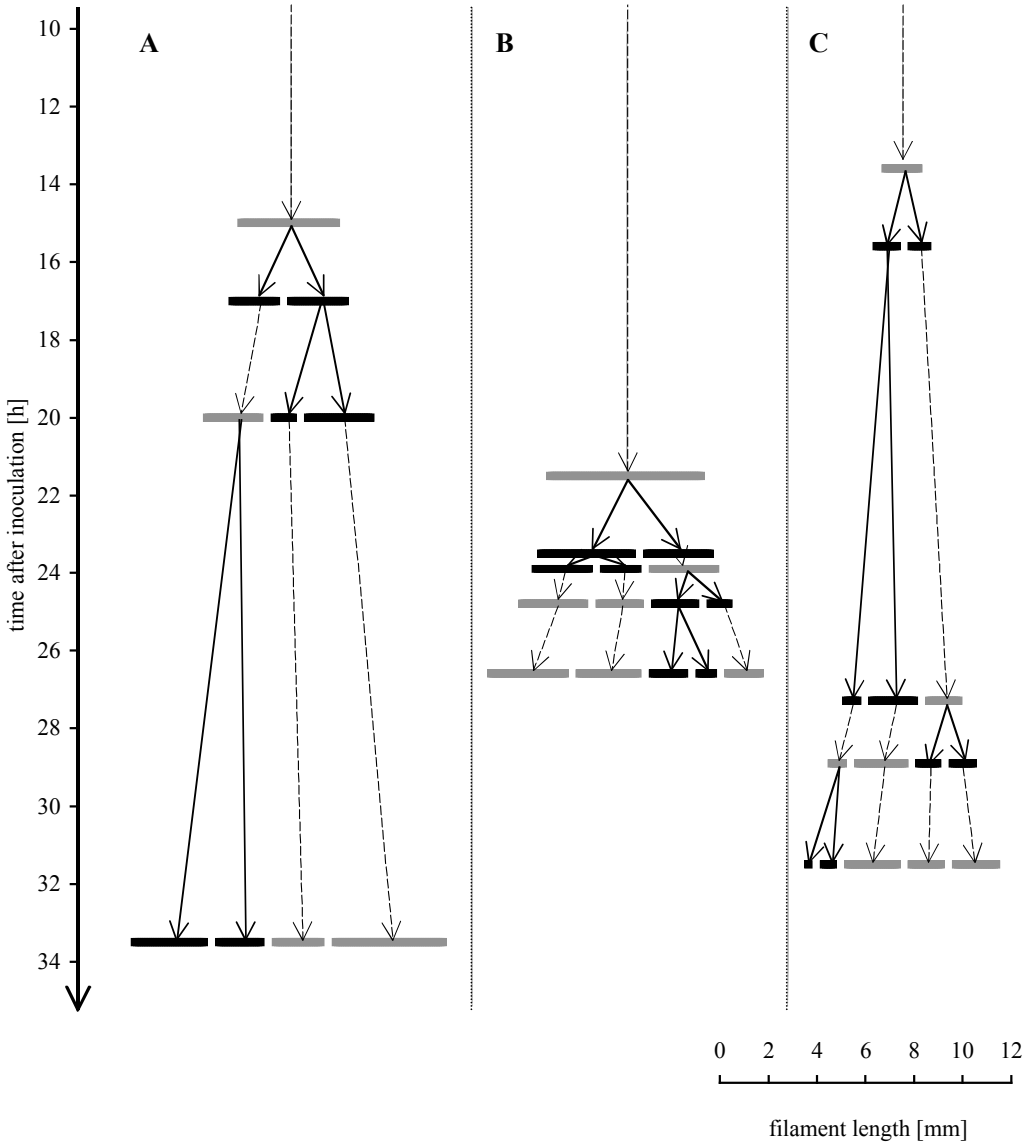

FIG. 4. First filament breakages observed after inoculation. Time after inoculation (h) and filament length (mm) were taken from movies 1(A), 2 (B), and 3 (C). Arrows with continuous lines show filament breakage (black filaments); arrows with dashed lines show filament growth (grey filaments).

Filament gliding speed, breakage, and movement. The average gliding speed of the *Beggiatoa* filaments was 3.2 ±0.1 µm/sec. Variation of the gliding speed over time was not observed, and the gliding speed did not depend on the position of the filaments in the gradient cultures.

Single filaments that were inoculated into new gradient tubes, started to break after 17.0 (movie 1), 23.5 (movie 2), and 15.6 h (movie 3; Fig. 4). The next 3-4 filament breaks occurred during the following 3-16 h of incubation. Filament breakage can be roughly

grouped into three types. (i) Breakage of a stretched filament, (ii) breakage at the tip of a filament loop, and (iii) breakage at the position where formerly the tip of a loop was located (Fig. 5A to C). Furthermore, some of the breakages occur via separation of one or a few end cells, where a hormogonium may have been produced, and sometimes it was observed that two parts of formerly one filament continued to share their slime sheath for ca. 1 min before separation.

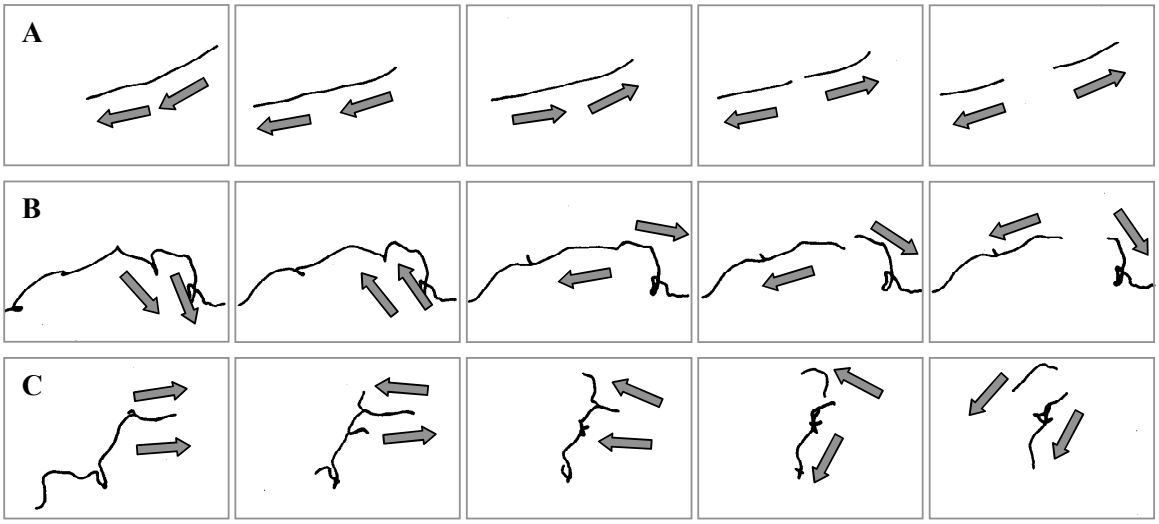

FIG. 5. Series of single picture as examples of filament breakage types. (A) Breakage in the middle of a stretched filament. (B) Breakage at the tip of a filament loop. (C) Breakage at the position where formerly a tip of a loop was located. Arrows indicate direction of filament movement. Filaments were redrawn manually from movie images.

DISCUSSION

The increase of the total protein content of the *Beggiatoa* consortium was linear for as long as 14 days (Fig. 1A). However, cell doubling within single *Beggiatoa* filaments that were inoculated in freshly prepared gradient tubes was exponential. This indicates that the initial exponential growth of *Beggiatoa* could not be resolved by measuring the total protein content. Nelson et al. [18] also initiated the growth of marine *Beggiatoa* by inoculation of replicate gradient culture tubes to investigate the increase of biovolume and protein content. Similar to our results, *Beggiatoa* grew linearly throughout much of their incubation time of ca. 11 days, and linear growth occurred only after an initial period of exponential biomass growth (doubling time of 11 h). The linear increase of *Beggiatoa* biomass in gradient cultures

was accompanied by an essentially constant sulphide flux, which suggests that sulphide availability limited growth [18].

The average filament length in the inoculum was remarkably short (Fig. 2A) and was most likely the result of mechanical disruption of *Beggiatoa* filaments during the thorough mixing of the inoculum prior to inoculation. This procedure was essential to obtain identical subsamples for the inoculation of replicate culture tubes. However, filament disruption through mixing did not impair the overall viability of the *Beggiatoa* filaments, as indicated by the immediate growth of the cultures after inoculation. The video analysis clearly documented that the *Beggiatoa* filaments grew up to an average length that remained constant (ca. 1.7 mm). Further, the frequency distribution of classes of filament lengths (Fig. 2B and C) revealed that filaments of up to 2 mm in length were equally common during the early and the later incubation period, and that filaments of 2-6 mm in length occurred with low frequency during the whole experiment. The growth of the *Beggiatoa* culture was thus not achieved by ever growing filaments, but by alternating filament elongation and breakage that controlled the average filament length. The constant average filament length could possibly be an invariable trait of the investigated *Beggiatoa* strain, or it could be influenced by environmental conditions, e.g. by the availability of sulphide, or the physical structure of the habitat. Field studies in the sediments of a brackish fjord (Limfjorden, DK) revealed that the average filament length of *Beggiatoa* spp. was positively correlated with the filament width [10]. In this sediment, *Beggiatoa* spp. with filament widths that were comparable to the strain investigated by us, had an average filament length of 1.0 mm and were thus considerably shorter than the average filament length of 1.7 mm we determined. According to Jørgensen [10], the grain size of sediments has an impact on the distribution of *Beggiatoa*. Perhaps, it is also possible that growth of filament length in soft agar was enhanced compared to more compact sediments in which the interstitials could to be space limiting.

The “empty regions” along the filament that were detected with a combination of FISH and DAPI staining as well as with light micrographs (Fig. 3) showed regions where one or a few cells have lysed. It is known that *Beggiatoa* filament breakage is accomplished by formation of sacrificial cells (necridia) at various points within the filament. Upon dying, the sacrificial cells lyse, dividing the filament into daughter filaments [24]. This process is comparable with filament breakage in the filamentous cyanobacterium *Oscillatoria* [12]. The mechanism that controls the formation of necridia in *Beggiatoa* is still unclear. However, time-lapse movies provided examples of the time course of filament breakage and the position at which the filaments break (Fig. 4). It took ca. 19 h after inoculation before first filament

breakages occurred. The paradox that optimal growth conditions leads to cell death is plausible because the breakage into daughter filaments facilitates multiplication. Further, the time-lapse movies illustrate that a filament may not only break once into two daughter filaments, but a couple of times within a short time period so that several daughter filaments can occur within a few hours. An explanation for this could be the simultaneous induction of the death of several sacrificial cells inducing the multiple breakage of the filament.

The time-lapse movies allowed also the observation of gliding directions in breaking filaments. The following patterns were observed. (i) If the filament breakage occurred in a stretched filament, the two parts of one filament usually glided in the same direction before the breakage and away from each other after the breakage (Fig. 5A). (ii) If the filament breakage occurred at the tip of a loop, or at the position where formerly the tip of a loop was located, the two parts of one filament moved towards each other before the breakage, whereby the loop was formed, and away from each other after the breakage (Fig. 5B and C). Before the breakage it was not possible to detect the position of sacrificial cells in the filament. Therefore, it was also not possible to analyse whether sacrificial cells were present at the positions at which the tips of loops were formed. However, in more than 20% of the observed filament breakages the filament loops were formed at the breakage point immediately before breakage so that a correlation between loop formation and the position of the sacrificial cell was shown retrospectively. A filament of an average length of 1.7 mm has ca. 630 cells. Assuming that the filament has one sacrificial cell, the probability for each position along the filament to be the breakage point would be 0.16%. The probability for a cell to be the tip of a loop is not given, but, if there would be no correlation between loop formation and the position of the sacrificial cell, the probability for a cell to be both would be even smaller (< 0.16%). Thus, a casual co-occurrence of loops and sacrificial cells is unlikely.

The gliding speed of the *Beggiatoa* strain was ca. 3 μm/s and thus in the range of other strains from various habitats (i.e., 1-8 μm/s [23]). Gliding motility of *Beggiatoa* is often accompanied by flexing, bending, or rotation of the filaments [23]. *Beggiatoa* filaments can reverse the direction of movement in response to increasing oxygen concentrations [15]. This phobic response to oxygen is observed whenever either a filament tip or a filament loop glides into a stable microgradient of increasing oxygen concentration. In many cases, only a part of the filament responds, which leads to the formation of sharp bends or loops within the filament. According to Møller et al. [15], this partial response facilitates the positioning of filaments at the oxygen-sulphide interface, the microhabitat of *Beggiatoa*. The biochemical nature of the cellular response that controls the direction of movement of filaments is not

known until now. Further, a probable mechanism of communication between cells, which could mediate the biochemical control of loop formation, has not been identified. We propose that the tip of the loop occurs at the position of a sacrificial cell, which could explain the correlation between loop formation and filament breakage. Further, we hypothesise that a sacrificial cell interrupts the communication between two parts of one filament. In this case, two parts of one filament, which share one common slime sheath, can act independent of each other, which increases the probability of opposite directions of movement. If the movement direction is towards each other a loop will be formed. After loop formation, filament breakage can occur when the two independently acting parts of one filament, both change their gliding direction, and move away from each other.

Video-supported analysis of *Beggiatoa* in combination with culture techniques in transparent agar represents a powerful method for side view observations of filament growth, breakage, and movement. This method has the advantage that the *Beggiatoa* can be observed exactly in their typical habitat of opposite gradients of oxygen from above and sulphide from below, which is an addition to the top view images of *Beggiatoa* that were possible in the sediment [Dunker R (2005) Microsensor studies on a *Beggiatoa* mat under changing oxygen concentrations. MSc thesis, University of Bremen, Germany]. Further studies should combine the video-supported analysis with microsensor measurements in gradient cultures [11, 19]. The correlation of the exact position of the filaments to the vertical gradients of oxygen and sulphide could lead to further insight into the mechanism that enables the filaments to respond to the chemical parameters of their microenvironment.

ACKNOWLEDGMENTS

S. Viehmann collected the *Beggiatoa* strain that was enriched in gradient culture by H. N. Schulz-Vogt in the laboratory of D. C. Nelson. P. Stief is gratefully acknowledged for the initial idea of making these movies and the critical reading of the manuscript. L. P. Nielsen and B. B. Jørgensen are thanked very much for fruitful discussions. M. B. Lund gave valuable help with FISH. M. Schubert and P. Stolle are thanked for technical support. This study was funded by the grant SCHU1416/2-1 from the Deutsche Forschungsgemeinschaft (German Research Foundation) and by the Max Planck Society, Germany.

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Chapter 3

Anaerobic sulfide oxidation with nitrate by a freshwater *Beggiatoa* enrichment culture

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The manuscript has been published in the journal *Applied and Environmental Microbiology* (2006) 72: 4755-4760

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ABSTRACT

A lithotrophic freshwater *Beggiatoa* strain was enriched in O_2-H_2S gradient tubes to investigate its ability to oxidize sulfide with NO_3^- as an alternative electron acceptor. The gradient tubes contained different NO_3^- concentrations, and the chemotactic response of the *Beggiatoa* mats was observed. The effects of the *Beggiatoa* sp. on vertical gradients of O_2, H_2S, pH, and NO_3^- were determined with microsensors. The more NO_3^- that was added to the agar, the deeper the *Beggiatoa* filaments glided into anoxic agar layers, suggesting that the *Beggiatoa* sp. used NO_3^- to oxidize sulfide at depths below the depth that O_2 penetrated. In the presence of NO_3^- *Beggiatoa* formed thick mats (>8 mm), compared to the thin mats (ca. 0.4 mm) that were formed when no NO_3^- was added. These thick mats spatially separated O_2 and sulfide but not NO_3^- and sulfide, and therefore NO_3^- must have served as the electron acceptor for sulfide oxidation. This interpretation is consistent with a fourfold-lower O_2 flux and a twofold-higher sulfide flux into the NO_3^--exposed mats compared to the fluxes for controls without NO_3^-. Additionally, a pronounced pH maximum was observed within the *Beggiatoa* mat; such a pH maximum is known to occur when sulfide is oxidized to S^0 with NO_3^- as the electron acceptor.

INTRODUCTION

Beggiatoa spp. are gliding, filamentous, colorless sulfur bacteria (22). These multicellular bacteria can occur in dense mats at the surface of sulfide-rich sediments in many freshwater and marine habitats (2, 10, 11, 21). The filaments of bigger marine species of *Beggiatoa* can be more than 120 μm wide (2) and >1 cm long, are white, and are visible with the naked eye; even single filaments of narrow freshwater *Beggiatoa* species whose filaments are ca. 3 μm wide (14, 21) can be observed with a stereomicroscope. *Beggiatoa* spp. are sulfide-oxidizing bacteria that have an important effect on the benthic sulfur cycle (4, 6). The presence of *Beggiatoa* mats at the sediment surface prevents toxic sulfide from diffusing into the water column, because biological sulfide oxidation is much more rapid and efficient than chemical sulfide oxidation (13).

In addition, *Beggiatoa* spp. can have a great effect on the aquatic nitrogen cycle when they use NO_3^- anaerobically as an alternative electron acceptor in place of O_2. The ability of freshwater and marine *Beggiatoa* spp. to oxidize sulfide anaerobically with NO_3^- has been studied for some time (11, 19, 20, 21), especially because large marine species contain a

vacuole in which NO_3^- can be stored at concentrations up to 160 mmol/liter (11). This enables the filaments to penetrate into anoxic sediment layers and perform anaerobic sulfide oxidation. However, anaerobic sulfide oxidation by freshwater *Beggiatoa* species has not been unequivocally documented, and the impact of freshwater *Beggiatoa* species on the nitrogen cycle is unclear (5, 11). Therefore, there is significant interest in obtaining more information about possible anaerobic sulfide oxidation with NO_3^- by freshwater *Beggiatoa* species.

The freshwater *Beggiatoa* strain that was used in this study was sustained for more than 2 years in highly enriched O_2-H_2S gradient tubes (12). Using microsensors to measure changes in the O_2 contents, H_2S contents, pH, and NO_3^- contents in these gradient tubes, the position of the *Beggiatoa* filaments in the transparent agar could be optically related to high-resolution chemical gradients. This experimental approach was used to address the following questions. (i) Does the freshwater *Beggiatoa* sp. exhibit a chemotactic response to the presence of different NO_3^- and H_2S concentrations? (ii) Does a *Beggiatoa* mat use NO_3^- as an alternative electron acceptor in place of O_2? (iii) Do the *Beggiatoa* filaments alter the vertical O_2, H_2S, and pH gradients differently when they are exposed to NO_3^- in addition to O_2?

MATERIALS AND METHODS

Sampling site and cultivation. Samples of *Beggiatoa* sp. with a filament width of 3 μm were collected in 2003 from the NO_3^--rich stream Giber Aa, south of Aarhus, DK. Here, mats of *Beggiatoa* were found on the mud around outlets for primary treated sewage.

The *Beggiatoa* filaments were enriched in lithotrophic agar gradient tubes, modified as described by Nelson and Jannasch (12). These gradient tubes contained two layers of agar, a layer of dense bottom agar (1.5% Bacto Agar [Difco Laboratories]) containing a high $\sum H_2S$ concentration ($[\sum H_2S] = [H_2S] + [HS^-] + [S_2^-]$) overlaid by a layer of softer top agar (0.25%) without $\sum H_2S$, which led to opposing gradients of $\sum H_2S$ and O_2 in the top agar. The composition of the medium is shown in Table 1. The pH was adjusted to approximately 7.0 with NaOH. The gradients were prepared in screw-cap tubes (length, 150 mm; inside diameter, 14 mm). The tubes were filled with 4 ml of autoclaved bottom agar and 8 ml of top agar. Unless indicated otherwise, the bottom agar was prepared with 4 mmol/liter Na_2S. The top agar also contained 150 μl of a sterile vitamin solution (Table 1), 4 mmol/liter $NaHCO_3$, and, unless indicated otherwise, 50 μmol/liter $NaNO_3$, 50 μmol/liter NH_4Cl, and 50 μmol/liter sodium acetate. The screw caps on the tubes were left loose to permit exchange of the headspace gas with the atmosphere. To allow gradient development, the agar was aged for at

least 2 days before inoculation. For the different experiments, *Beggiatoa* filaments were taken from existing gradient tubes, pooled, and mixed, and identical subsamples of enriched *Beggiatoa* biomass were inoculated approximately 5 mm below the agar surface. All cultures were grown at room temperature in the dark.

Vertical position of the *Beggiatoa* mats. For determination of the NO_3^-- and $\sum H_2S$ -dependent vertical positions of the *Beggiatoa* mats, the agar was prepared with 0, 100, 200, 400, and 600 μmol/liter $NaNO_3$ and with 4 and 8 mmol/liter Na_2S, respectively (n = 3). The mat positions within the gradient system were determined using the tip of a microsensor dummy as a pointer. The dummy was mounted vertically on a micromanipulator, which was attached to a heavy stand. Via its motor drive, the micromanipulator allowed slow, small-scale insertion of the microsensor dummy into the agar down to the *Beggiatoa* mat, while the tip was viewed through the side of the gradient tube with a stereomicroscope (magnification, x10 to x20). The meniscus of the agar surface was defined as a depth of 0 μm, from which the position of the clearly visible upper boundary of the *Beggiatoa* mat was measured. The mat position was determined 1 to 6 days after inoculation.

Chemical microgradients. The O_2 concentrations, H_2S concentrations, pH values, and NO_3^- concentrations in the gradient tubes were measured with microsensors. Agar was prepared with 0 and 600 μmol/liter $NaNO_3$, and profiles were determined 2 and 4 days after inoculation; profiles in uninoculated tubes that were the same age were also determined.

The microsensors were either purchased from Unisense A/S (Aarhus, DK) or manufactured at the Max Planck Institute for Marine Microbiology (Bremen, Germany). The O_2 microsensors with a guard cathode (17) had tip diameters of 10 to 15 μm and 90% response times of <5 s. They were calibrated with air- and N_2-flushed medium used for agar preparation (100 and 0% air saturation, respectively). The glass-type pH microsensors (18) had tip diameters of <12 μm and 90% response times of <20 s and were calibrated with commercial buffer solutions (pH 4.0, 7.0, and 9.2; Mettler-Toledo, Switzerland). The pH microsensors were used together with homemade reference electrodes, which consisted of a chlorinated Ag wire (length, 30 mm; diameter, 0.5 mm) that was inserted into one end of a glass capillary. The capillaries (length, 100 mm; inside diameter, 1 mm) were filled with 1% agar prepared in 3-mol/liter KCl and thus served as a salt bridge. The H_2S microsensors (3) had tip diameters of 10 μm and 90% response times of <10 s. They were calibrated with deoxygenated PO_4 buffer (200 mmol/liter K_2HPO_4/KH_2PO_4, pH 7.5) to which Na_2S was added stepwise to obtain final concentrations of approximately 0 to 400 μmol/liter (9). The precise $\sum H_2S$ concentration of each calibration solution was determined

spectrophotometrically by the method of Pachmeyer (16). The concentrations of free H_2S in the calibration solutions were calculated as follows:

$$[H_2S] = [\sum H_2S]/(1 + (10^{pH}/10^{pK1})] \quad (1)$$

where $pK_1 = 7.027$ is the negative logarithm of K_1, the first dissociation constant of the sulfide equilibrium system (pK_2 can be neglected at pH <9). From these data, the calibration curve for the H_2S microsensor was plotted. $\sum H_2S$ gradients in the tubes were calculated as follows:

$$[\sum H_2S] = [H_2S] \times (1 + (10^{pH}/10^{pK1})] \quad (2)$$

using the $[H_2S]$ and the pH gradients measured with microsensors.

LIX-type NO_3^- microsensors (1) with tip diameters of 5 to 10 μm and 90% response times of <30 s were prepared on the day before use to improve the signal stability. NO_3^- microsensors were used together with homemade reference electrodes (see above). Calibration was performed using uninoculated gradient tubes in which the $NaNO_3$ concentration was adjusted to 0, 15, 30, 60, 150, 300, or 600 μM. All sensors were calibrated before and after measurement at room temperature. One microsensor at a time was mounted on a motorized micromanipulator that was operated by the software *Profix* (Unisense A/S, Aarhus, DK). The microsensor was positioned in the center of the tube cross section and then lowered toward the agar surface (depth, 0 μm [see above]). Starting at this depth, vertical profiles were recorded at increments of 100, 200, or 400 μm down to 30 mm. The O_2, pH, H_2S, and NO_3^- profiles were determined at the same spot of the same tube whenever possible and were related to the position and thickness of the *Beggiatoa* mat in the inoculated enrichment culture (for mat position designations see above). The lower boundary of the mat was defined as the position where filaments were present more than just sporadically.

Flux calculations. The amounts of O_2 and $\sum H_2S$ that flowed across a unit of area per unit of time (flux) were determined for uninoculated controls as well as for the tubes that were inoculated with the *Beggiatoa* enrichment. Assuming steady-state conditions, Fick's first law of diffusion was used:

$$J = -D\,(\delta C/\delta x) \quad (3)$$

where J is the flux (in nmol cm^{-2} s^{-1}), D is the diffusion coefficient (in cm^2 s^{-1}), C is the concentration (in nmol cm^{-3}), and x is the depth (in cm). The diffusion coefficients for O_2 and

$\sum H_2S$ (in agar at room temperature) were 2.03 x 10^{-5} and 1.57 x 10^{-5} cm^2 s^{-1}, respectively (13). For the uninoculated controls, the linear regions of the concentration gradients above and below the O_2-$\sum H_2S$ overlap zone were used for $\delta C/\delta x$ (13); for the *Beggiatoa*-containing gradient tubes, the linear regions above and below the *Beggiatoa* mat were used.

TABLE 1. Compositions of medium, micronutrient solution, and vitamin solution

Medium	Micronutrient solution	Vitamin stock solution[a]
0.01 g EDTA	0.5 ml H_2SO_4 (>98%)	1 mg vitamin B_{12}
0.12 g $CaSO_4 \cdot 2H_2O$	2.28 g $MnSO_4 \cdot H_2O$	1 mg inositol
0.2 g $MgSO_4 \cdot 7H_2O$	0.5 g $ZnSO_4 \cdot 7H_2O$	1 mg biotin
0.016 g NaCl	0.5 g H_3BO_3	1 mg folic acid
0.14 g Na_2HPO_4	0.025 g $CuSO_4 \cdot 5H_2O$	10 mg *p*-aminobenzoic acid
0.138 g NaH_2PO_4	0.025 g $Na_2MoO_4 \cdot 2H_2O$	100 mg nicotinic acid
0.264 g $CaCl_2 \cdot 2H_2O$	0.045 g $CoCl_2 \cdot 6H_2O$	100 mg *d*-pantothenate
2 ml $FeCl_3$ solution (0.29 g/l)	1,000 ml distilled water	200 mg thiamine
1 ml micronutrient solution		
1,000 ml distilled water		(each vitamin was dissolved in 10 ml distilled water)

[a]For the final vitamin solution 1 ml of each vitamin stock solution was added to 100 ml (final volume) of distilled water.

RESULTS

Mat position experiments. The experiments showed that the mat position depended on three factors: the concentrations of NO_3^- and $\sum H_2S$ and the length of incubation (Fig. 1). Generally, the mat position was deeper when the NO_3^- concentration was higher. This effect was less pronounced when 8 mmol/liter Na_2S was used instead of 4 mmol/liter Na_2S. In all treatments *Beggiatoa* mats moved upward with time (12). Three-way analysis of variance with NO_3^- and $\sum H_2S$ concentrations as between-subject factors and with time as a within-subject factor revealed that the dependence of the mat position on all three factors (for NO_3^-, $F_{4,19} = 478$ and $P < 0.001$; for $\sum H_2S$, $F_{1,19} = 529$ and $P < 0.001$; and for time, $F = 1{,}229$, $df = 5$, and $P < 0.001$) was highly significant.

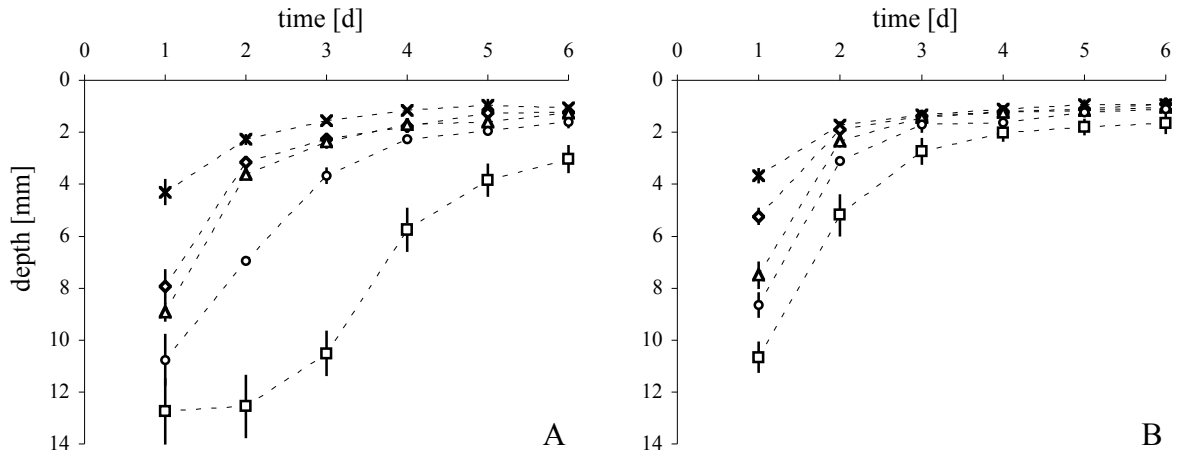

FIG. 1. Mean depth (in mm) of the upper boundary of the *Beggiatoa* mat, depending on the NO_3^- and $\sum H_2S$ concentrations in the gradient tubes over time (days [d]). (A) Bottom agar prepared with 4 mmol/liter Na_2S. (B) Bottom agar prepared with 8 mmol/liter Na_2S. Symbols: cross, no NO_3^-; diamond, 100 μmol/liter NO_3^-; triangle, 200 μmol/liter NO_3^-; circle, 400 μmol/liter NO_3^-; square, 600 μmol/liter NO_3^-. Some of the error bars, which indicate standard deviations (n = 3), are smaller than the symbols.

O_2 and $\sum H_2S$ microgradients. Without NO_3^- addition, the vertical O_2 and $\sum H_2S$ gradients were steeper in the *Beggiatoa* gradient tubes than they were in the uninoculated controls (Fig. 2A to D). Correspondingly, the O_2 and $\sum H_2S$ fluxes into the *Beggiatoa* mats were greater than those into the O_2-$\sum H_2S$ overlap zone (Table 2). Furthermore, the O_2 and $\sum H_2S$ gradients became steeper with time, which resulted in upward movement of both the O_2-$\sum H_2S$ overlap zone (uninoculated controls) and the *Beggiatoa* mat (Fig. 2A to D; cf. Fig. 1). The *Beggiatoa* mat in the experiment without added NO_3^- was approximately 0.4 mm thick and was slightly above the O_2-$\sum H_2S$ overlap zone. NO_3^- addition to *Beggiatoa* tubes had a strong effect on the O_2 and $\sum H_2S$ microgradients, on the mat position, and on the thickness of the mat, which increased to >8 mm (Fig. 2E and F). The NO_3^- effect was most pronounced 2 days after inoculation. An approximately 4-mm gap appeared between the O_2 and $\sum H_2S$ profiles (Fig. 2E). Additionally, the corresponding O_2 microgradient was considerably less steep, resulting in a flux of 3.6 pmol cm^{-2} s^{-1}, which was only one-half the value obtained for the uninoculated control and less than one-fourth the value obtained for the treatment without NO_3^- (Table 2). In contrast, the $\sum H_2S$ flux was about twofold higher than that in the *Beggiatoa* gradient tube without NO_3^- and about fourfold higher than that in the uninoculated control (Table 2). The NO_3^- effect was less pronounced after 4 days; the O_2 profile in the

NO_3^--containing *Beggiatoa* enrichment culture became steeper, and the $\sum H_2S$ profile became less steep (Fig. 2F).

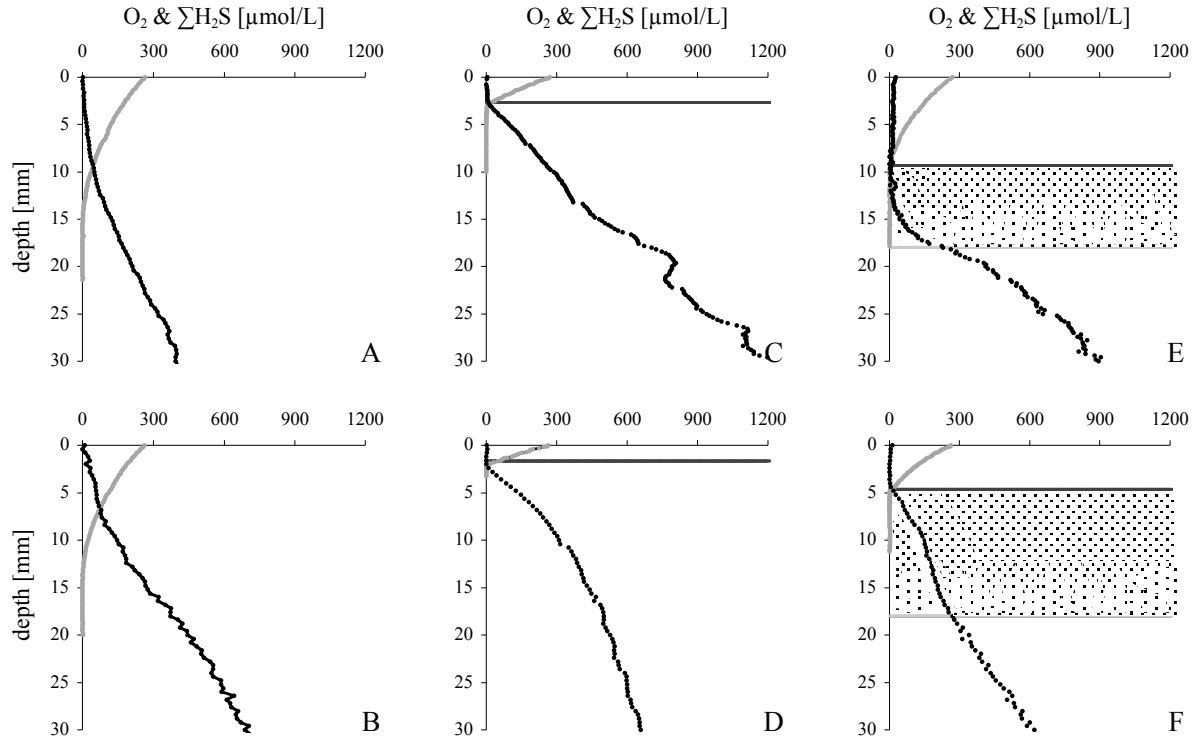

FIG. 2. Microprofiles of O_2 (gray circles) and $\sum H_2S$ (black circles) and positions of the upper (dark gray lines) and, where applicable, lower (light gray lines) boundaries of the *Beggiatoa* mats. (A and B) Uninoculated gradient tubes. (C and D) *Beggiatoa* gradient tubes without NO_3^-. (E and F) *Beggiatoa* gradient tubes with an initial NO_3^- concentration of 600 µmol/liter. The incubation times were 2 days (A, C, and E) and 4 days (B, D, and F) after inoculation. The shaded areas within the boundaries of the *Beggiatoa* mats (E and F) indicate that filaments were more abundant in the upper mat regions. Gray and black circles overlap in some panels.

NO_3^- microgradients. The NO_3^- microsensor measurements for the uninoculated control (Fig. 3A) and the *Beggiatoa* enrichment culture after 2 and 4 days (Fig. 3B and C) illustrate that the NO_3^- concentrations decreased in the presence of *Beggiatoa* sp. during incubation. The mean NO_3^- concentration in the upper 30-mm agar layer decreased from the initial concentration (600 µmol/liter) to 86 µmol/liter after 2 days and to 54 µmol/liter after 4 days. Furthermore, the profiles show that all of the NO_3^- diffused from the small upper agar volume into the mat, whereas some NO_3^- was still diffusing upward from the much larger

volume of agar below the mat that also contained a larger total amount of NO_3^-. In contrast to O_2 and $\sum H_2S$, which were spatially separated after 2 days in the NO_3^--containing treatment, NO_3^- and $\sum H_2S$ overlapped in the *Beggiatoa* mat (Fig. 2E and 3B).

TABLE 2. O_2 and $\sum H_2S$ fluxes in uninoculated controls and in *Beggiatoa*-enriched gradient tubes without NO_3^- and with an initial NO_3^- concentration of 600 μmol/liter[a]

Time (d)	Flux (pmol cm^{-2} s^{-1})					
	Controls		*Beggiatoa* enrichments without NO_3^-		*Beggiatoa* enrichments with 600 μmol/liter NO_3^-	
	O_2	$\sum H_2S$	O_2	$\sum H_2S$	O_2	$\sum H_2S$
2	7.2	2.8	16.7	5.9	3.6	11.9
4	7.8	3.9	23.4	7.4	7.7	4.3[b]

[a]data correspond to profiles of Fig. 2.

[b]flux may have been underestimated because there were no long-term steady-state conditions for $\sum H_2S$

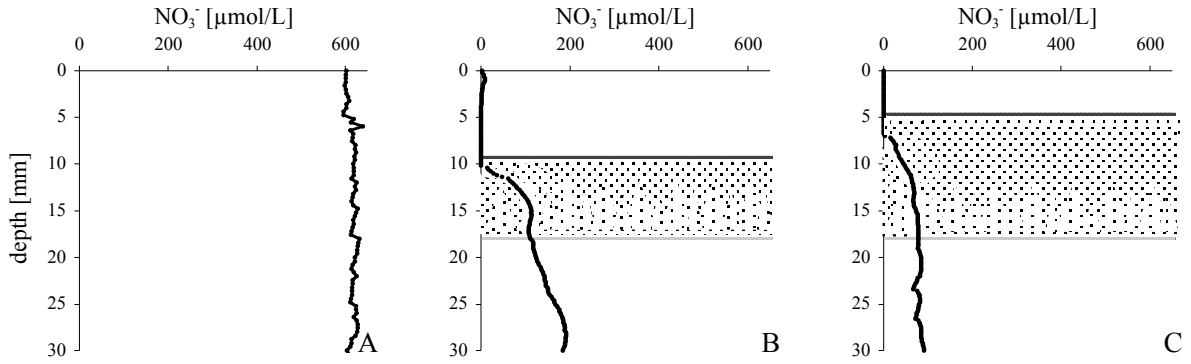

FIG. 3. Microprofiles of NO_3^- (circles) and positions of the upper (dark gray lines) and lower (light gray lines) boundaries of the *Beggiatoa* mats. (A) Uninoculated gradient tube. (B and C) *Beggiatoa* gradient tubes 2 days (B) and 4 days (C) after inoculation. The initial NO_3^- concentration was 600 μmol/liter. The shaded areas within the boundaries of the *Beggiatoa* mats (B and C) indicate that filaments were more abundant in the upper mat regions. Circles overlap in some panels.

pH microgradients. In the uninoculated control, the pH was 7.8 at the agar surface and increased to 8.3 at a depth of 30 mm due to the increasing $\sum H_2S$ concentration (Fig. 4A). In the *Beggiatoa* enrichment culture without NO_3^-, the pH profile showed that the minimum pH was close to the *Beggiatoa* mat (Fig. 4B). In contrast, in the *Beggiatoa* enrichment culture with NO_3^- the pH profile had a completely different shape and there was a pronounced maximum pH in the *Beggiatoa* mat (Fig. 4C).

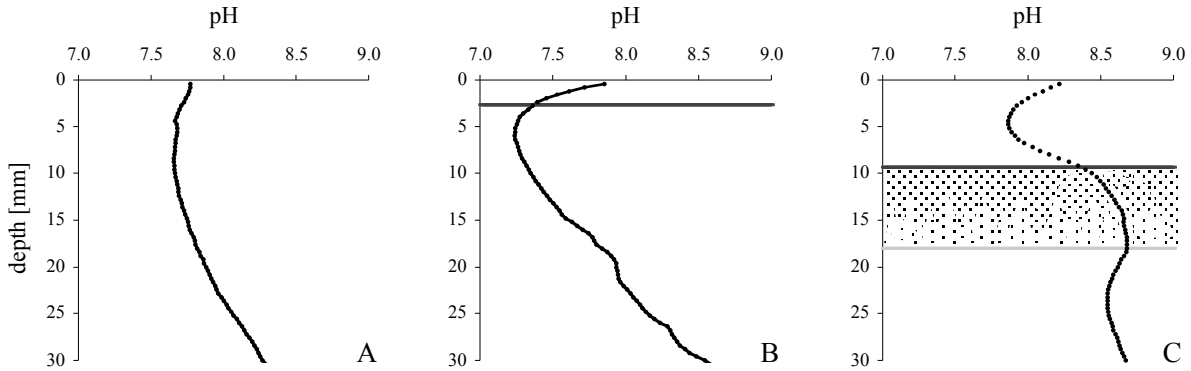

FIG. 4. Microprofiles of pH (circles) and positions of the upper (dark gray line) and, where applicable, lower (light gray line) boundaries of the *Beggiatoa* mats. (A) Uninoculated gradient tube. (B and C) *Beggiatoa* gradient tubes without NO_3^- (B) and with an initial NO_3^- concentration of 600 μmol/liter (C). The incubation time was 2 days. The shaded area within the boundaries of the *Beggiatoa* mat (C) indicates that filaments were more abundant in the upper mat regions. Circles overlap in some panels.

DISCUSSION

The hypothesis that the freshwater *Beggiatoa* strain investigated is able to oxidize $\sum H_2S$ anaerobically with the alternative electron acceptor NO_3^- originated from observations made during the mat position experiments; at higher NO_3^- concentrations the *Beggiatoa* mats moved deeper into the agar toward the electron donor $\sum H_2S$ (Fig. 1). This hypothesis was supported by microsensor profiles and flux calculations, which demonstrated that the *Beggiatoa* filaments indeed moved into anoxic, NO_3^--rich agar layers and could oxidize even more $\sum H_2S$ if NO_3^- was available (Fig. 2C to F and Table 2). Furthermore, the O_2 flux into the *Beggiatoa* mat exposed to NO_3^- was much lower than the O_2 fluxes in the tubes without NO_3^- and the uninoculated control tubes after 2 days (Table 2). This finding can be explained by the missing O_2-$\sum H_2S$ overlap zone in the NO_3^--amended *Beggiatoa* tubes (Fig. 2E).

Because of the spatial separation of O_2 and $\sum H_2S$, neither chemical nor biological $\sum H_2S$ oxidation with O_2 could take place. The effect of the initial NO_3^- concentration on *Beggiatoa* sp. became less pronounced over time (Fig. 1 and 2C to F), which is explained by the finding that NO_3^- limitation occurred as incubation progressed (Fig. 3). It is likely that not all NO_3^- was immediately used for anaerobic $\sum H_2S$ oxidation and that an unknown fraction of NO_3^- was assimilated or stored intracellularly (11, 23). Vacuoles in freshwater *Beggiatoa* have not been detected so far (22), but cytoplasmic storage of NO_3^- is another possibility. This could explain the finding that more NO_3^- was taken up during the first 2 days of incubation than during the second 2 days (Fig. 3).

Beggiatoa oxidizes $\sum H_2S$ first to S^0, which can be stored as intracellular globules, and subsequently to SO_4^{2-} (22, 24). When O_2 is used as the electron acceptor, the oxidation of H_2S to S^0 is pH neutral (if HS^- is used as the electron donor, its oxidation to S^0 is moderately alkaline; S^{2-} can be neglected at pH <9), whereas the oxidation of S^0 to SO_4^{2-} is acidogenic. In total, the aerobic oxidation of $\sum H_2S$ to SO_4^{2-} is acidogenic, which explains the pH profile found in the *Beggiatoa* enrichment culture without NO_3^-, in which the minimum pH largely coincided with the position of the *Beggiatoa* mat (Fig. 4B) (7, 13). When NO_3^- is used as the electron acceptor, the oxidation of $\sum H_2S$ to S^0 increases the pH, while the oxidation of S^0 to SO_4^{2-} decreases the pH (20). This was visible in the pH profiles that were determined for the NO_3^--containing treatments; after 2 days of incubation, the maximum pH was 8.7 in the lower region of the *Beggiatoa* mat (Fig. 4C), which must have resulted from the oxidation of $\sum H_2S$ to S^0 with NO_3^-. Toward the upper region of the *Beggiatoa* mat, where less $\sum H_2S$ was available, the pH decreased. However, the pH in this layer did not decrease to values lower than those in the uninoculated control (Fig. 4A and C). Therefore, there was no indication that oxidation of S^0 to SO_4^{2-} took place in the upper region of the *Beggiatoa* mat. However, if oxidation of S^0 to SO_4^{2-} occurred at all, NO_3^- rather than O_2 must have been used as the electron acceptor, because the O_2 flux into the *Beggiatoa* mat was extremely low. The measured pH profiles are consistent with the results of a recent study of Sayama et al. (20), in which these authors found similar pH profiles in marine sediment colonized with *Beggiatoa* spp. It was hypothesized that the oxidation of H_2S to S^0 occurred with NO_3^- and was not necessarily spatially coupled to the oxidation of S^0 to SO_4^{2-}.

Furthermore, Sayama et al. (20) demonstrated that the marine *Beggiatoa* spp. investigated reduce NO_3^- to NH_4^+ under anoxic conditions (dissimilatory nitrate reduction to ammonium). This metabolic pathway was also hypothesized to occur in other marine sulfur bacteria (19) and is known to occur in large marine *Thioploca* spp. (15) that are close relatives

of large marine *Beggiatoa* spp. (22). Another possibility for anaerobic $\sum H_2S$ oxidation with NO_3^- is denitrification, which was discussed by Sweerts at al. (21) for freshwater *Beggiatoa* spp. To date, this study is the only study in which anaerobic $\sum H_2S$ oxidation with NO_3^- was postulated for freshwater *Beggiatoa* spp., but questions about contamination of the *Beggiatoa* filaments with unicellular denitrifying bacteria have been raised by other authors (5, 11). The *Beggiatoa* enrichment culture used in our study also contained unicellular bacteria. Despite numerous trials, a pure culture could not be obtained, suggesting that this *Beggiatoa* strain is not able to grow without associated bacteria, which is a well-known phenomenon for other bacteria (8). However, the visibility of the *Beggiatoa* filaments in the transparent agar can be used. Using a stereomicroscope, it was observed that NO_3^- had an effect on the filaments because the *Beggiatoa* mat position and thus the chemotactic response of the filaments to O_2 and $\sum H_2S$ were indeed changed. Alternatively, the movement of the *Beggiatoa* filaments may have resulted from an intimate association with unicellular NO_3^- reducers, which were directly responsible for the $\sum H_2S$ oxidation, and because of an absolute dependence of the *Beggiatoa* sp. on these reducers, the *Beggiatoa* sp. followed the movement of the NO_3^- reducers in the gradient tubes. However, this seems unlikely because in this case the *Beggiatoa* sp. would have had to disassociate from the energetically favorable electron acceptor O_2. Hence, the changed chemotactic response of the *Beggiatoa* sp. strongly suggests that the freshwater *Beggiatoa* filaments themselves were chiefly responsible for the anaerobic $\sum H_2S$ oxidation with NO_3^-.

ACKNOWLEDGMENTS

L. P. Nielsen is gratefully acknowledged for providing the *Beggiatoa* sp. from his sewage outlet, as well as for fruitful discussions. A.-T. Henze and H. Plattner are thanked very much for valuable help. G. Eickert and M. Schubert provided technical support.

This study was funded by grant SCHU1416/2-1 from the Deutsche Forschungsgemeinschaft (German Research Foundation) and by the Max Planck Society, Germany.

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Chapter 4

Dissimilatory nitrate reduction to ammonium by a freshwater *Beggiatoa* strain

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The manuscript has been submitted to the journal *Environmental Microbiology*

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SUMMARY

Filamentous colourless sulphur bacteria from a nitrogen-polluted stream in Denmark were phylogenetically analysed and physiologically studied with respect to the capability of intracellular NO_3^- storage and the dissimilatory use of NO_3^-. Filaments were sampled from mats formed on the surface of the natural sediment and highly enriched in oxygen-sulphide gradient tubes in which experiments with ^{15}N-labelled NO_3^- were performed. The 16S rDNA sequence retrieved from the filaments revealed the affiliation with the genus *Beggiatoa* and with the confined cluster of freshwater strains. Intracellular NO_3^- storage was found with concentrations of 35 ±32 mmol L^{-1} (mean ±SD), which was ca. 230-fold higher than the ambient concentration. The ^{15}N-labelling experiment showed that the freshwater *Beggiatoa* used the intracellular NO_3^- for the dissimilatory nitrate reduction to ammonium (DNRA), whereas denitrification activity was not detected. This study reveals for the first time that a freshwater *Beggiatoa* strain is capable of intracellular accumulation of NO_3^-, which is used for DNRA.

INTRODUCTION

Beggiatoa are multicellular, filamentous colourless sulphur bacteria of the order Thiotrichales (gamma proteobacteria) that deposit elemental sulphur globules within each cell (Strohl, 2005; Teske and Nelson, 2006). *Beggiatoa* filaments show gliding motility, can grow up to several mm in length (Jørgensen, 1977; Gundersen *et al.*, 1992; Larkin and Henk, 1996; Kamp *et al.*, unpublished), and reach high biomass in many aquatic habitats, e.g. in and on freshwater and marine sediments (Jørgensen, 1977; Sweerts *et al.*, 1990; Gundersen *et al.*, 1992; Sayama, 2001; Sayama *et al.*, 2005). Beside their important influence on the benthic sulphur cycle (Jørgensen, 1977; Jørgensen and Nelson, 2004), marine *Beggiatoa* and the phylogenetically closely related *Thioploca* have also been recognised to link the benthic sulphur and nitrogen cycles (Fossing *et al.*, 1995; Otte *et al.*, 1999; Sayama *et al.*, 2005). They are capable to store the electron acceptor NO_3^- in addition to elemental sulphur as the electron donator (Fossing *et al.*, 1995; McHatton *et al.*, 1996; Jørgensen and Gallardo, 1999; Otte *et al.*, 1999; Sayama, 2001; Mußmann *et al.*, 2003). This enables *Beggiatoa* and *Thioploca* to survive anoxic conditions and, in combination with their gliding motility, to take advantage of steep chemical gradients in sediment. At the sediment surface, they can use O_2 and simultaneously take up and store NO_3^-, whereas in the sediment they are able to oxidise

sulphide and store elemental sulphur (Fossing *et al.*, 1995; Jørgensen and Gallardo, 1999; Sayama *et al.*, 2005). In contrast to marine *Beggiatoa* and *Thioploca*, findings on the dissimilatory use of NO_3^- by freshwater *Beggiatoa* and *Thioploca* are rare (Sweerts *et al.*, 1990; Kamp *et al.*, 2006). However, the physiological capabilities of these bacteria are of great ecological interest because excessive nitrogen concentrations present a water-quality problem of growing concern (Burgin and Hamilton, 2007). If NO_3^- is reduced to NH_4^+ under anoxic conditions (DNRA, dissimilatory nitrate reduction to ammonium), the produced NH_4^+ stays in the ecosystem and can be reoxidised to NO_3^- by nitrifying bacteria. In contrast, the final products of denitrification are gaseous nitrogen-compounds (mostly N_2) and thus nitrogen will leave the ecosystem.

The freshwater *Beggiatoa* strain investigated in this study originated from a nitrogen-polluted stream in Denmark. Filaments that were kept in an aquarium with natural sediment were used to address the following questions. (1) What is the phylogenetic position of the freshwater *Beggiatoa* strain? (2) Is this freshwater *Beggiatoa* strain capable of intracellular NO_3^- storage? (3) What is the final product of the dissimilatory use of NO_3^- by the freshwater *Beggiatoa* strain? To answer the final question, *Beggiatoa* filaments were highly enriched in oxygen-sulphide gradient tubes. The culture technique was combined with the application of ^{15}N-labelled NO_3^-. This approach allowed adjusting the *Beggiatoa* filaments to chemically controlled conditions and follow the pathway of dissimilatory reduction of NO_3^-.

RESULTS AND DISCUSSION

The phylogenetic analysis of the nearly full-length 16S rDNA sequence confirmed the affiliation of the investigated filamentous bacteria with the genus *Beggiatoa* as well as the relation to the genus *Thioploca* and the recently described White Point filamentous bacterium [Fig. 1; (Kalanetra *et al.*, 2004)]. Therefore, the filamentous bacteria investigated here are further referred to as *Beggiatoa* sp. LPN. The strain clusters with all other known freshwater *Beggiatoa* spp. with sequence similarities of 89-99%. The phylogenetic reconstruction demonstrated that the closest relative is a freshwater *Beggiatoa* strain occurring in sulphidic caves [99% similarity; (Macalady *et al.*, 2006)]. However, different filament widths suggest different species (Mußmann *et al.*, 2003). The filament width of *Beggiatoa* sp. LPN was ca. 3 µm, and the filament width of the *Beggiatoa* sp. from the sulphidic caves was 5-7 µm. In contrast, *Beggiatoa* sp. LPN is less closely related to the type species *Beggiatoa alba* (89%

similarity) that exhibit also a filament width of ca. 3 µm (Mezzino *et al.*, 1984; Teske *et al.*, 1995; Ahmad *et al.*, 2006).

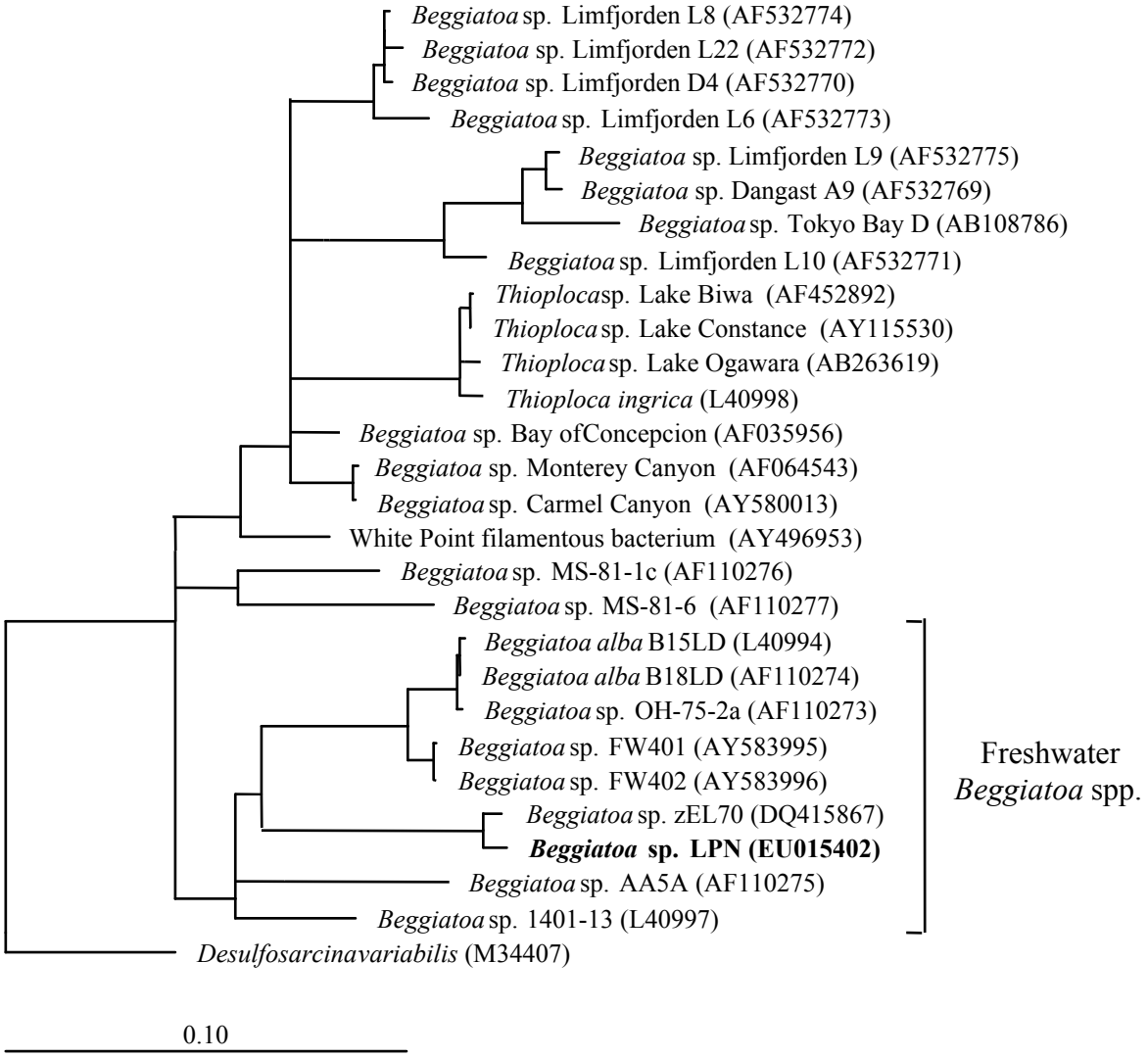

FIG. 1. Phylogenetic tree of the investigated freshwater *Beggiatoa* strain (*Beggiatoa* sp. LPN) and diverse filamentous sulphur-oxidising gamma proteobacteria based on almost full-length 16S rDNA sequences. *D. variabilis* (delta proteobacteria) is included as the outgroup. The scale bar corresponds to 10% estimated sequence divergence. GenBank accession numbers are shown in parentheses.

NO_3^- measurements of lysed filaments revealed that the freshwater *Beggiatoa* sp. LPN was capable of NO_3^- storage in concentrations of 35 ±32 mmol L^{-1} (mean ±SD, n=11), representing a ca. 230-fold increase over the ambient NO_3^- concentration. This is the first report of intracellular NO_3^- accumulation by freshwater *Beggiatoa*. So far NO_3^- storage has only been documented for marine *Beggiatoa*, as well as for marine and freshwater *Thioploca*.

(McHatton *et al.*, 1996; Otte *et al.*, 1999; Sayama, 2001; Zemskaya *et al.*, 2001; Mußmann *et al.*, 2003, Preisler *et al.* 2007). In these bacteria, the NO_3^- was probably stored in liquid vacuoles, with highest concentrations of up to 500 mmol L^{-1}, as documented for large, marine *Thioploca* (Fossing *et al.*, 1995; Otte *et al.*, 1999). A vacuolate-dependent NO_3^- storage was also demonstrated for the large sulphur bacteria *Thiomargarita namibiensis* and the newly described *Thiomargarita*-like bacteria, at concentrations of up to 800 and 460 mmol L^{-1} NO_3^-, respectively (Schulz *et al.*, 1999; Kalanetra *et al.*, 2005). Since vacuoles were not detected in freshwater *Beggiatoa* (Maier and Murray, 1965; Strohl *et al.*, 1982), the NO_3^- storage in the freshwater *Beggiatoa* sp. LPN was most probably cytoplasmic.

The detected ability of intracellular NO_3^- storage by the freshwater *Beggiatoa* sp. LPN was used in ^{15}N-labelling experiments on the metabolic fate of intracellular NO_3^-. These experiments showed that the freshwater *Beggiatoa* sp. LPN was capable of DNRA. To arrive at this finding, the oxygen-sulphide gradient culture technique (Nelson and Jannasch, 1983; Kamp et al., 2006) was used to pre-incubate the *Beggiatoa* enrichment with ^{15}N-labelled NO_3^- that was internally stored during the incubation time of 3 days. After $^{15}NO_3^-$ was stored, the *Beggiatoa* enrichment was transferred into fresh gradient tubes. Here, the freshwater *Beggiatoa* were exposed to anoxic conditions to induce the use of NO_3^- as the alternative electron acceptor instead of O_2. Indeed, 1.5 ±0.5 and 1.4 ±0.3 nmol $tube^{-1}$ $^{15}NH_4^+$ (mean ±SD, n=4) were produced in two independent treatments after 4 days of anoxic incubation, whereas no $^{15}NH_4^+$ production was detected in the non-labelled control (Fig. 2). In contrast, $^{29}N_2$ and $^{30}N_2$ were not detected and hence there was no indication that the investigated freshwater *Beggiatoa* sp. LPN exhibited denitrification activity (data not shown). All of the ^{15}N in the produced NH_4^+ must have originated from the $^{15}NO_3^-$ that was stored intracellularly, as this was the only ^{15}N-labelled NO_3^- source during the final anoxic incubation. This experimental approach allows to attribute the dissimilatory reduction of $^{15}NO_3^-$ specifically to the *Beggiatoa* sp. LPN rather than to unicellular bacteria that accompanied the *Beggiatoa* enrichment. It can be ruled out that those accompanying bacteria contributed significantly to the production of $^{15}NH_4^+$ because, even if they had the capability to store NO_3^- intracellularly, they were outnumbered by far (>>100-fold in terms of biovolume) by *Beggiatoa* filaments.

The biovolume of the *Beggiatoa* filaments was 0.03 µl in each tube. Thus, the $^{15}NH_4^+$ production was equivalent to 49.8 ±16.1 and 46.5 ±9.8 nmol μl^{-1} of *Beggiatoa* biovolume for the two independent $^{15}NO_3^-$ treatments (Fig. 2). Assuming that the entire pool of $^{15}NO_3^-$ stored in the *Beggiatoa* filaments was reduced to $^{15}NH_4^+$, the biovolume-specific production of $^{15}NH_4^+$ represents also the amount of $^{15}NO_3^-$ that was formerly stored intracellularly. Hence,

the intracellular $^{15}NO_3^-$ concentrations were 49.8 ±16.1 and 46.5 ±9.8 mmol L^{-1} for the two independent $^{15}NO_3^-$ treatments, which is within the range of the NO_3^- storage capacity found in this study (35 ±32 mmol L^{-1}). Vacuolate marine *Beggiatoa* were found to use up ca. 14 mmol L^{-1} of stored NO_3^- per day for survival under anoxic conditions and in the absence of an additional NO_3^- source (Preisler *et al.*, 2007). Assuming similar consumption rates for *Beggiatoa* sp. LPN, the given amount of NO_3^- stored intracellularly would enable this freshwater strain to survive ca. 3 days with no access to extracellular electron acceptors.

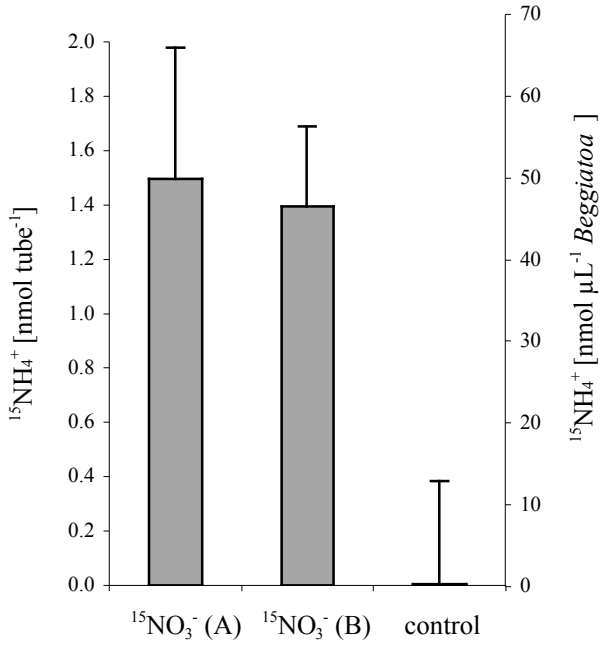

FIG. 2. $^{15}NH_4^+$ produced by *Beggiatoa* sp. LPN after four days of anoxic incubation in gradient tubes (given in nmol $tube^{-1}$ and nmol μl^{-1} *Beggiatoa*). The final, anoxic incubation tubes were inoculated with *Beggiatoa* enrichments that were pre-incubated with $^{15}NO_3^-$ (A and B, see Fig. 3) as well as with a *Beggiatoa* enrichment that was not pre-incubated with $^{15}NO_3^-$ (control). Before the pre-incubation with $^{15}NO_3^-$, the *Beggiatoa* enrichments were kept in cultures with (A) or without $^{14}NO_3^-$ (B and control). Means +SD (n=4) are given.

The two independent $^{15}NO_3^-$ treatments (A and B, Fig. 2) differed in the supply of $^{14}NO_3^-$ to the *Beggiatoa* before the pre-incubation with $^{15}NO_3^-$ (100 vs. 0 µmol L^{-1} $^{14}NO_3^-$ in A vs. B, respectively). Nevertheless, the production of $^{15}NH_4^+$ was identical in both treatments. Obviously, the NO_3^- storage capacity of the freshwater *Beggiatoa* sp. LPN was

not fully reached in the $^{14}NO_3^-$ cultures (before the pre-incubation with $^{15}NO_3^-$) because the same amount of ^{15}N-label was stored intracellularly in both treatments.

TABLE 1. Denitrification activity by the *Beggiatoa* enrichment incubated with different NO_3^- concentrations and 10% acetylene in the headspace. Mean values ±SD (n=4) are shown

NO_3^- added (nmol tube^{-1})	N_2O produced (nmol tube^{-1})	N_2O produced (% of NO_3^- added) [a]
0	0.1 ±0.1	-
100	0.3 ±0.5	0.6 ±1.0
200	1.8 ±0.4	1.8 ±0.9
300	2.0 ±0.8	1.3 ±0.5
400	4.5 ±0.9	2.3 ±0.5

[a]Considering that 2 moles NO_3^- are needed to produce 1 mole N_2O and that NO_3^- in the tube was entirely depleted within one week of incubation.

In addition to testing *Beggiatoa* sp. LPN with an internal NO_3^- pool, it was also investigated whether the *Beggiatoa* enrichment as a whole (*Beggiatoa* plus the accompanying unicellular bacteria) was capable of denitrification. In tubes in which all bacteria of the enrichment had access to extracellular NO_3^-, small amounts of N_2O were produced after treatment with acetylene gas to inhibit the reduction of N_2O to N_2 (Table 1). This denitrification activity must be attributed to the accompanying bacteria of the *Beggiatoa* enrichment because for *Beggiatoa* sp. LPN itself denitrification activity could not be detected in the labelling experiment. The NO_3^- added to the tubes of the latter experiment was completely taken up and/or used during the incubation time (data not shown). Thus, the whole enrichment used as little as 1.5% (mean across all different NO_3^- concentrations) of the added NO_3^- for denitrification. The remaining NO_3^- must have been used for DNRA by the freshwater *Beggiatoa* sp. LPN (and possibly by some accompanying unicellular bacteria of the enrichment) or used for assimilatory processes that incorporate nitrogen into biomass.

This study reveals for the first time that a freshwater *Beggiatoa* strain is able to perform DNRA as the metabolic pathway for dissimilatory reduction of intracellularly stored NO_3^-. This finding agrees well with those of most studies on marine *Beggiatoa* and *Thioploca*, which come to the conclusion that intracellularly stored NO_3^- is reduced to NH_4^+ (Otte *et al.*, 1999; Zopfi *et al.*, 2001; Sayama *et al.*, 2005). Evidence on the possible

dissimilatory use of NO_3^- by freshwater *Thioploca* is missing so far, whereas for freshwater *Beggiatoa*, denitrification was formerly discussed to be the pathway of dissimilatory NO_3^- reduction (Sweerts *et al.*, 1990). Denitrification was initially also suggested for marine *Thioploca* (Fossing *et al.*, 1995), and was not entirely ruled out in a later investigation (Otte *et al.*, 1999). Given these heterogeneous findings, one could assume that the realisation of a certain metabolic pathway is a strain-specific trait. A more general observation in benthic aquatic ecosystems is that the presence of high sulphide concentrations favours the occurrence of DNRA rather than denitrification because sulphide inhibits the final two reduction steps of denitrification (Brunet and Garcia-Gil, 1996; Burgin and Hamilton, 2007). Consequently, further research should address the spatial distribution of sulphur bacteria that perform different pathways of dissimilatory NO_3^- reduction relative to ambient sulphide concentrations.

EXPERIMENTAL PROCEDURES

Sampling site. Sediment samples that contained *Beggiatoa* sp. with filament widths of ca. 3 µm and maximum lengths of >2500 µm (ca. 400 cells) were collected in 2006 from the NO_3^- rich stream Giber Aa, south of Aarhus, DK. Here, dense mats of *Beggiatoa* occur on sludge deposits around primary treated sewage outlets.

16S rDNA Sequencing. The *Beggiatoa* strain was phylogenetically classified by sequence analysis of its 16S rRNA gene as amplified with the universal bacterial primers 26F (Hicks *et al.*, 1992) and 1492R (Lane, 1991). Fifty filaments were rinsed with sterile culture medium and transferred to a PCR tube containing 50 µl of PCR mixture (Kjeldsen *et al.*, 2005), except for Taq polymerase. The PCR tube was frozen at –20°C for cell lysis (≥4 h) and 0.5 µl of Taq polymerase (5 U μl^{-1}) was added after thawing. Thermal cycling followed the protocol of Kjeldsen *et al.* (2005). Purified PCR products were ligated and then transformed into *Escherichia coli* JM109 using the pGEM-T-Easy vector kit (Promega, Madison, WI). Twelve clones were selected for sequencing with vector primers on a model ABI 3730XL sequencer (Applied Biosystems). For the sequences retrieved, BLAST searches were performed against the nucleotide database at the National Center for Biotechnology Information's website (www.ncbi.nlm.nih.gov). One of the clone sequences, appearing at a frequency of 58%, was closely affiliated with freshwater *Beggiatoa* strains. This sequence was deposited in GenBank under the Accession No. EU015402 and further analysed with the ARB software package (www.arb-home.de). Phylogenetic trees of nearly full-length

sequences of filamentous sulphur bacteria were calculated by neighbour-joining, maximum-likelihood, and maximum parsimony analysis of an alignment of positions 126-1417 (*E. coli* numbering). In the consensus tree, those branching orders that were not supported by all methods are shown as multifurcations.

Intracellular NO_3^-. To measure intracellular NO_3^-, the *Beggiatoa* sp. and natural sediment were incubated in an aquarium with tap water (15°C). NO_3^- concentration was adjusted to ca. 150 µmol L^{-1}, which corresponded to the in situ concentration. For each sample (n=11), 100 filaments were picked from the aquarium, rinsed, and transferred into a 1.5 ml tube with 1 ml filtered tap water. The sample was centrifuged (20 min, 55 × *g*), and the supernatant was removed carefully except for a small volume of ca. 12 µl containing the filaments (exact volume was calculated via weighing). To extract intracellular NO_3^- from the *Beggiatoa* filaments, the sample was frozen and heated three times (≥4 h at -20°C; 10 min at 90°C). NO_3^- content was measured using the VCl_3 reduction method (Braman and Hendrix, 1989) with a chemiluminescence detector (Model CLD 86; Eco Physics). Filtered tap water samples without *Beggiatoa* sp. were processed likewise and served as blanks.

Product of dissimilatory NO_3^- reduction. To analyse the product of dissimilatory NO_3^- reduction by *Beggiatoa* sp., the filaments were purified and cultivated at room temperature in oxygen-sulphide gradient tubes as described in Kamp *et al.* (2006), except that the *Beggiatoa* enrichment was cultured with 100 µmol L^{-1} $^{14}NO_3^-$ or without $^{14}NO_3^-$. From these cultures, *Beggiatoa* were transferred to newly prepared tubes for a pre-incubation with 200 µmol L^{-1} ^{15}N-labelled NO_3^- (Fig. 3, 98 atom%; Cambridge Isotope Laboratories). As a control, *Beggiatoa* were pre-incubated in tubes without $^{15}NO_3^-$. After three days, $^{15}NO_3^-$ was completely taken up, which was confirmed by measuring NO_3^- concentrations with the VCl_3 reduction method (<1 µmol L^{-1} NO_3^-). Identical biovolumes of pre-incubated *Beggiatoa* sp. were taken for inoculation of fresh tubes (n=4). After inoculation, the fresh tubes were supplied with 1 ml sterile culture medium that contained 100 µmol L^{-1} $^{14}NO_3^-$, and were made anoxic by flushing the headspace with helium. After four days, production of $^{29}N_2$ and $^{30}N_2$ by denitrification was measured as described by Risgaard-Petersen and Rysgaard (1995). In the same tubes, the production of ^{15}N-labelled NH_4^+ by DNRA was measured with the combined microdiffusion-hypobromite oxidation method (Risgaard-Petersen *et al.*, 1995). For the calculation of the biovolume of *Beggiatoa* sp., subsamples of the inocula were put on an objective slide for taking light micrographs at 100-fold magnification. Images were taken cross-like over the cover slip (n=40). Length of all imaged filaments or parts of filaments was

measured with the computer program Image Tool (University of Texas Health Science Center), summed up and converted to biovolume using the filament diameter of 3 µm.

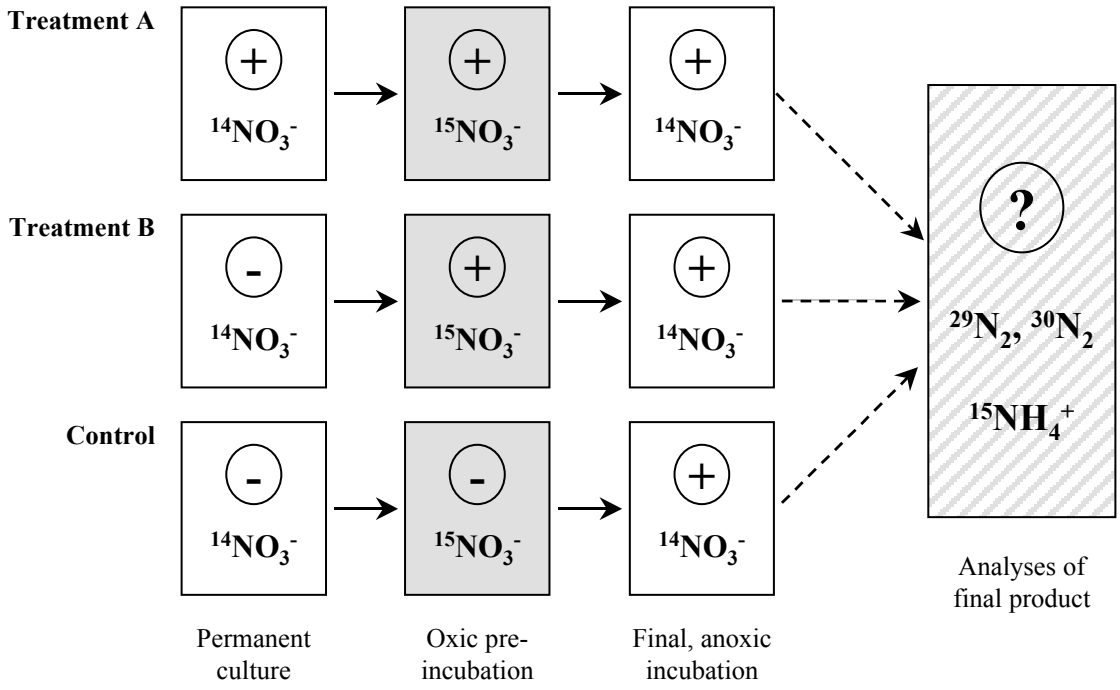

FIG. 3. Procedure of the ^{15}N-labelling experiment: *Beggiatoa* enrichments that were (treatment A) and were not habituated to NO_3^- (treatment B and control) were taken from permanent culture tubes. The harvested biomass was transferred to newly prepared tubes for the oxic pre-incubation with and without $^{15}NO_3^-$ (gray squares). During this stage, $^{15}NO_3^-$ could be stored intracellularly. After the $^{15}NO_3^-$ was completely taken up, the biomass was transferred into fresh tubes for the final, anoxic incubation, during which the stored $^{15}NO_3^-$ could be used for dissimilatory NO_3^- reduction. The headspace and the agar of the final incubation tubes were analysed for $^{29}N_2$, $^{30}N_2$, and $^{15}NH_4^+$, respectively, as the possible final products of dissimilatory NO_3^- reduction.

Acetylene inhibition. Denitrification was also investigated via measuring the N_2O production of the *Beggiatoa* enrichment in the presence of acetylene, which inhibits the reduction of N_2O to N_2 (Balderston *et al.*, 1976; Yoshinari and Knowles, 1976). Newly inoculated tubes were made anoxic by flushing the headspace with N_2 before adding 1 ml sterile, anoxic culture medium with 0, 100, 200, 300 or 400 µmol L^{-1} NO_3^- and replacing 10% of the headspace with acetylene (n=4). After one week of incubation, the N_2O production was measured with a gas chromatograph (GC-8A; Shimadzu Corporation).

ACKNOWLEDGMENTS

Andreas Schramm, Hans Brix, and Nils Risgaard-Petersen are gratefully acknowledged for their help and advice. The practical work of this study was mostly done in the Department of Biological Sciences, Microbiology, of the University of Aarhus, Denmark. All members of the Microbiology Group are thanked very much for the remarkably good working atmosphere.

This study was funded by the grant SCHU1416/2-1 from the Deutsche Forschungsgemeinschaft (German Research Foundation) and by the Aarhus University Research Foundation. P.S. was supported by the European Commission with grant no. 515536 within the Marie Curie Mobility Actions.

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Chapter 5

Concluding remarks and outlook

CONCLUDING REMARKS AND OUTLOOK

The main objectives of this thesis were (i) to acquire more information on the growth patterns of the filamentous, colourless sulphur bacteria *Beggiatoa*, and (ii) to investigate the dissimilatory nitrate reduction pathways possibly utilised by freshwater *Beggiatoa*.

The majority of experiments were carried out in oxygen-sulphide agar gradient cultures of a marine and a freshwater *Beggiatoa* strain. An original objective was to obtain pure cultures of these strains (Objectives and outline of this thesis). Despite numerous trials, pure cultures could not be obtained, suggesting that not all *Beggiatoa* strains were able to grow without accompanying unicellular bacteria. This hypothesis is supported by numerous unsuccessful isolation trials with a marine *Beggiatoa* strain. Single filaments of this strain were carefully picked with a sterile needle and rinsed up to 20 times in a series of petri dishes filled with sterile seawater. After inoculation of the rinsed filaments into new oxygen-sulphide gradient tubes, approximately half of the washed filaments did not show growth. The other half that did start growing had accompanying unicellular bacteria with them. The better the filaments resumed growing, the more accompanying bacteria were detected via phase contrast microscopy. The freshwater *Beggiatoa* never started growing after an isolation trial. In addition, the pure culture isolation technique on agar plates (Nelson and Castenholz, 1981) was intensively tried by A.-T. Henze for both strains investigated during this thesis, but unfortunately with no success (A.-T. Henze, personal communication). It is a well-known phenomenon that not all *Beggiatoa* strains can be isolated in pure culture, probably because of the dependence on (not identified) metabolic products of the accompanying bacteria (Winogradsky, 1887). However, even the experimental addition of these compounds may not lead to successful pure culture isolation. In more recent literature, it is hypothesised that some bacteria require specific signals originating from their “neighbours” that indicate the presence of a “familiar” environment (Kaeberlein *et al.*, 2002). These researchers bypassed the isolation of bacteria in pure culture with a diffusion chamber method. Certain bacteria that resisted traditional isolation were successfully separated from the accompanying bacteria via a membrane that allowed exchange of chemicals and probably specific signals. This experimental design may also be successful for *Beggiatoa*.

The video-supported analyses of *Beggiatoa* filament growth, breakage, and movement supplemented the basic knowledge on the life history of *Beggiatoa* (Kamp *et al*., chapter 2 of this thesis). In particular, the time course of reproduction of single *Beggiatoa* filaments, and the movement directions of filaments enhanced the current information about *Beggiatoa*. In

addition, the movies of *Beggiatoa* led to intriguing observations, which have not been addressed in the manuscripts included in this thesis, but support the recently published hypothesis that the steepness of the sulphide gradient is an important factor for the orientation of *Beggiatoa* (Preisler *et al.*, 2007). It was repeatedly observed that *Beggiatoa* filaments suddenly moved into deeper sulphide-rich agar layers for no obvious reason. In these deeper agar layers, the sulphide gradient is less steep than usually favoured by *Beggiatoa*. According to the above hypothesis, the majority of filaments were not able to move back into their natural microenvironment, which is slightly above the steepest zone of the sulphide gradient. In further studies, the video-supported analysis should be combined with microsensor measurements in gradient cultures. The correlation of the exact position of the filaments to the vertical gradients of sulphide, pH, and oxygen could reveal the mechanism that enables the filaments to respond to the chemical parameters of their microenvironment.

In parallel to the studies of this thesis it was tried to cultivate large marine *Beggiatoa* with filaments widths of ca. 40 µm from the Namibian Shelf, Africa. So far, stable oxygen-sulphide agar gradient cultures of large marine *Beggiatoa* were never obtained, which is also the case for the *Beggiatoa* sampled from the Namibian Shelf. After any inoculation effort, the filaments loose orientation in deep agar layers, in which the sulphide gradient is less steep, but where the sulphide concentrations are high. Perhaps, the steepness of the sulphide gradient is also a much more important factor for the cultivation of large marine *Beggiatoa* than previously assumed.

The study on the possibility of freshwater *Beggiatoa* to perform anaerobic sulphide oxidation with nitrate, strongly suggested that the freshwater *Beggiatoa* were able to use nitrate instead of oxygen as an alternative electron acceptor (Kamp *et al.*, chapter 3 of this thesis), which was confirmed in later investigations (Kamp *et al.*, chapter 4 of this thesis). In addition, it was revealed that the investigated freshwater *Beggiatoa* strain was capable of nitrate storage in concentrations of 35 ±32 mmol L^{-1} (Kamp *et al.*, chapter 4 of this thesis). Since vacuoles were not detected in freshwater *Beggiatoa* (Maier and Murray, 1965; Strohl *et al.*, 1982), the nitrate storage was most probably cytoplasmic. However, freshwater *Thioploca*, that are close relatives to freshwater *Beggiatoa* and have comparable filament widths, contain several very small vacuoles (Maier and Murray, 1965). Therefore, it might be that some freshwater strains also contain so far overlooked small compartments in which nitrate can be stored. Such compartments could be detected via combining labelling experiments, in which *Beggiatoa* are supplied with ^{15}N-labelled nitrate, with the recently introduced multi-isotope imaging mass spectrometry (MIMS) or nanoSIMS (Lechene *et al.*,

2006; Kuypers, 2007; Kuypers and Jørgensen, 2007; Lechene *et al*., 2007). This method allows high-resolution imaging of stable isotopes within single cells, and could thus visualise the location of nitrate storage.

The study on the metabolic pathway for dissimilatory reduction of intracellularly stored nitrate revealed that the investigated freshwater *Beggiatoa* strain was able to perform DNRA (dissimilatory nitrate reduction to ammonium; Kamp *et al*., chapter 4 of this thesis). Most studies on marine *Beggiatoa* and their close relatives *Thioploca* found DNRA to be the pathway of dissimilatory nitrate reduction (Otte *et al.*, 1999; Zopfi *et al.*, 2001; Sayama *et al.*, 2005; Preisler *et al*., 2007). By contrast, freshwater *Beggiatoa* were previously discussed to perform denitrification (Sweerts *et al.*, 1990). Denitrification was formerly also suggested for marine *Thioploca* (Fossing *et al.*, 1995), and was not entirely ruled out in a later investigation (Otte *et al.*, 1999). Given these non-consistent findings, one could consider that not all *Beggiatoa* and *Thioploca* strains perform the same metabolic pathway. Generally, it is well known that high sulphide concentrations favour the occurrence of DNRA rather than denitrification because sulphide inhibits the final two reduction steps of denitrification (Brunet and Garcia-Gil, 1996; Burgin and Hamilton, 2007). Thus, the spatial distribution of *Beggiatoa* strains that perform the one or the other pathway of dissimilatory nitrate reductions may depend on the ambient sulphide concentration. One could also hypothesise that some *Beggiatoa* strains switch between DNRA and denitrification. An experimental design to address this question could be to supply a *Beggiatoa* strain with different sulphide concentrations. It is conceivable that the performance of the one or the other pathway depends on the ambient sulphide concentration. In addition to physiological studies on *Beggiatoa*, molecular techniques could be applied to study the presence and expression of functional genes for dissimilatory reduction of nitrate.

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LIST OF PUBLICATIONS

Publications presented this thesis

Kamp, A., Stief, P., Nielsen, L.P., and Schulz-Vogt, H.N. (submitted) Dissimilatory nitrate reduction to ammonium by a freshwater *Beggiatoa* strain. *Environ. Microbiol.*

Kamp, A., Røy, H., and Schulz-Vogt, H.N. (in revision) Video-supported analysis of *Beggiatoa* filament growth, breakage, and movement. *Micob. Ecol.*

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Further publications

Papenbrock, J., Riemenschneider, A., **Kamp, A.**, Schulz-Vogt, H.N., and Schmidt, A. (2007) Characterization of cysteine-degrading and H_2S-releasing enzymes of higher plants - From the field to the test tube and back. *Plant. Biol.* **9**:582-588

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Poster presentations

Kamp, A., Stief, P., and Schulz-Vogt, H.N. (2006) Anaerobic sulfide oxidation with nitrate by a freshwater *Beggiatoa* enrichment culture. ISME – 11th International Symposium on Microbial Ecology, Vienna, Austria

Kamp, A., Stief, P., and Schulz, H.N. (2005) Microscale gradients of oxygen, sulfide, and nitrogen compounds in a lithotrophic *Beggiatoa* mat grown in enrichment culture. ASLO – Aquatic Science Meeting, Salt Lake City, USA

Kamp, A., and Witte, U. (2004) The importance of macrofauna for carbon processing in a fine-grained sandy shelf sediment (North Sea): Experiments with ^{13}C labelled phytoplankton. 1st Nereis Park Conference, Carry-Le-Rouet, France

DANKSAGUNG

Zunächst möchte ich mich bei Juniorprof. Dr. Heide Schulz-Vogt und bei Prof. Dr. Bo Barker Jørgensen für die Begutachtung meiner Dissertation bedanken. Beiden danke ich, neben Juniorprofessor Dr. Stefan Könemann, ebenfalls dafür, dass sie Prüfer bei meiner Disputation waren. PD Dr. Stefan Irniger danke ich für den Prüfungsvorsitz.

Mein besonders großer Dank gilt Juniorprof. Dr. Heide Schulz-Vogt dafür, dass sie mich als Doktorandin betreut und so großes Vertrauen in mich gesetzt hat. Sie hat in mir die Faszination an *Beggiatoa* geweckt und mich stets darin unterstützt mich als Wissenschaftlerin zu entwickeln.

Ein großes Dankschön geht auch an Prof. Bo Barker Jørgensen dafür, dass er sich immer wieder Zeit für mich genommen und sich für meine Forschung interessiert hat.

Mein ganz besonders herzlicher Dank geht an Dr. Peter Stief und Associate Prof. Dr. Lars Peter Nielsen. Beide haben sich als Kooperations- und Diskussionspartner stets Zeit für mich genommen und meine Doktorarbeit dadurch sehr bereichert. Ein besonderer Dank für sehr gute Zusammenarbeit geht auch an Dr. Hans Røy.

Den früheren und jetzigen Mitgliedern der Arbeitsgruppe um Juniorprof. Dr. Heide Schulz-Vogt danke ich für die gute Stimmung und Hilfsbereitschaft. Stellvertretend möchte ich hier vor allem Anne-Theres Henze, Jörg Brock, Anne Bachmann und Jutta Graue nennen.

Den MitarbeiterInnen des Instituts für Mikrobiologie and der Leibniz Universität Hannover danke ich für die vielseitige Unterstützung. Insbesondere Dr. Hans Plattner und Michael Schubert haben wertvolle Hilfe geleistet.

Teile meiner Doktorarbeit habe ich im Department of Biological Sciences, Microbiology, University of Aarhus (Denmark) durchgeführt. Ein Riesendank geht an alle Mitglieder der Mikrobiologie für die hervorragende Arbeitsatmosphäre.

Meinen Eltern danke ich sehr herzlich für alles was sie mir mit auf den Weg gegeben haben und immer noch geben.

Mein tiefster Dank gilt Peter! Er hat mich während meiner Doktorandenzeit stets mit sehr viel Liebe, Verstand und Tatkraft unterstützt. Die Zuversicht, die er dadurch immer wieder in mir mobilisieren konnte, war und ist von ganz besonderem Wert.

ERKLÄRUNG

Hierdurch erkläre ich, dass die Dissertation zum Thema „Growth and nitrate reduction of *Beggiatoa* filaments studied in enrichment cultures“ selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden. Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, den 08.10.2007