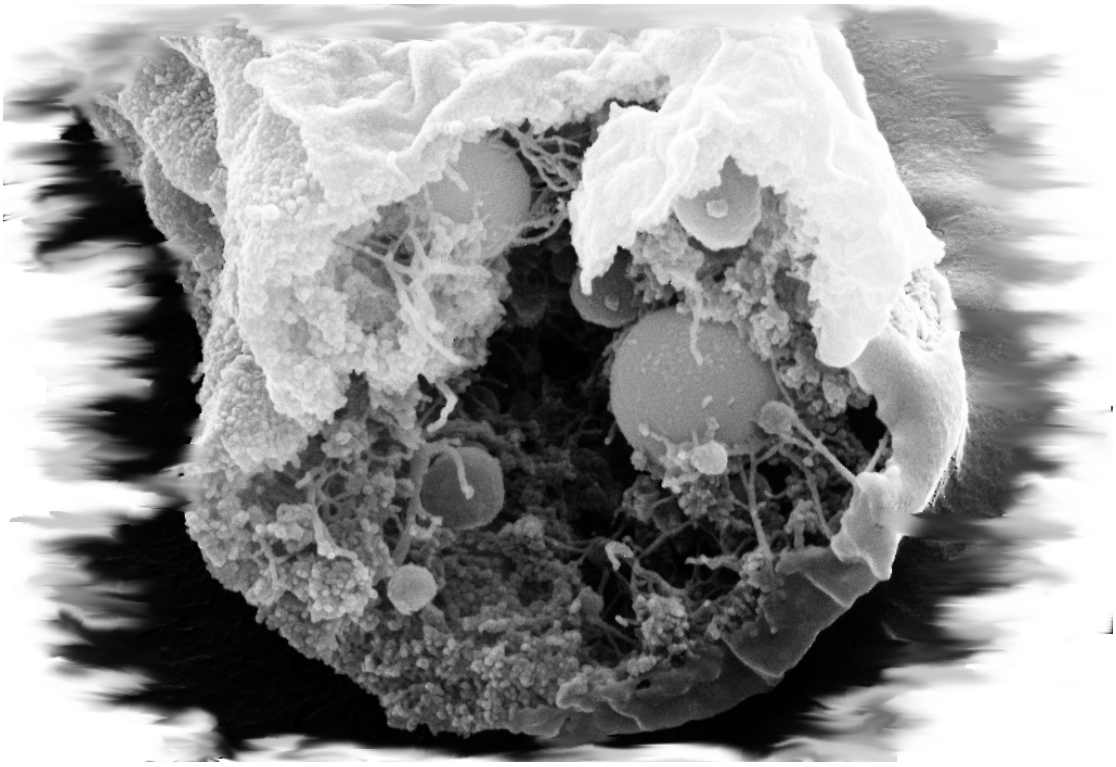
Impact of sulfide-oxidizing bacteria on the phosphorus cycle in marine sediments

Jörg Brock



Scanning electron micrograph of a broken filament of *Beggiatoa* strain 35Flor .

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Dissertation

zur Erlangung des Grades eines

Doktor der Naturwissenschaften

(Dr. rer. nat.)

Dem Fachbereich Biologie/Chemie

der Universität Bremen

vorgelegt von

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Bremen, Januar 2011

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Tag der Promotion: 28.02.2011

Table of contents

Summary

Phosphate is an essential nutrient for living organisms, and its availability controls productivity in the oceans. The main process of phosphate burial, the formation of phosphorus-rich minerals (phosphorites) in organic-rich sediments of coastal upwelling regions, is assumed to be mediated by sulfide-oxidizing bacteria of the family *Beggiatoaceae*.

In the first part of this thesis I studied which factors control the uptake, storage and release of phosphate in the sulfide-oxidizing *Beggiatoa* strain 35Flor, under laboratory conditions. By using a newly developed gradient sampling column for cultivation in combination with microrhizones we obtained phosphate profiles of high resolution. Phosphate profiles of cultures grown under oxic conditions showed, that part of the energy generated by sulfide oxidation was used for the intracellular accumulation of phosphate in the form of polyphosphate. A switch to anoxia in the presence of a high sulfide flux mediated the breakdown of polyphosphate and the rapid release of phosphate, resulting in a significant increase of the phosphate concentration in the medium. This physiological reaction is a so far unknown mechanism of polyphosphate usage in bacteria. It provides an explanation for the co-occurrence of sulfide-oxidizing bacteria and high phosphate concentrations in sediments, which is a prerequisite for the precipitation of phosphorus-rich minerals.

The second part of this thesis focused on the polyphosphate inclusions of the *Beggiatoa* strain 35Flor. With an unusual diameter of more than 3 µm these polyphosphate inclusions are the largest detected so far. By the use of confocal laser scanning microscopy we were able to show that the polyphosphate inclusions are surrounded by a lipid layer. In contrast to many polyphosphate inclusions in other organisms they are not notably acidic. Energy dispersive x-ray analysis revealed enrichment of calcium and to a minor extent enrichment of magnesium in the polyphosphate inclusions. 16S rRNA-based phylogenetic reconstruction showed an affiliation of *Beggiatoa* 35Flor to narrow, mostly non-vacuolated *Beggiatoa* species at the phylogenetic root of the *Beggiatoaceae*. We assume that the storage of large amounts of polyphosphate constitutes an adaptation of narrow

sulfide-oxidizing bacteria to their habitat whereas large species of *Beggiatoaceae* are adapted mainly by the storage of nitrate in a central vacuole.

The third part of the thesis is a study of the element distribution in Namibian sediment and its comparison to changes of the elemental composition of seawater in a *Beggiatoa* 35Flor culture. In the Namibian sediment several elements such as calcium, strontium and uranium were enriched together with phosphorus in a sediment layer where also a high number of *Thiomargarita* spp. occured. These elements were strongly correlated to phosphorus in the whole sediment core. An experiment with the polyphosphate storing *Beggiatoa* strain 35Flor revealed that the uptake and the release of calcium and strontium were not correlated to phosphate. Instead, decrease and increase in the concentrations of calcium and strontium seem to be redox-dependent. In contrast, uranium uptake and release were stoichiometric correlated to phosphate with a U/P ratio of 10^{-4}, which is in the range of ratios observed in natural phosphorites. Thus, we suppose that uranium is associated with polyphosphate. As a consequence of concomitant polyphosphate breakdown and release of phosphate together with uranium, the so formed apatite may be especially enriched in uranium.

Zusammenfassung

Phosphat ist ein essentieller Nährstoff, dessen Verfügbarkeit die Produktivität in den Ozeanen reguliert. Der bedeutendste Prozess der Phosphatablagerung ist die Bildung von phosphathaltigen Mineralien (Phosphoriten) in organisch angereicherten Sedimenten von Auftriebsgebieten. Es wird vermutet, dass Sulfid oxidierende Bakterien der Familie *Beggiatoaceae* diesen Prozess regulieren.

Im ersten Abschnitt dieser Dissertation untersuchte ich unter Laborbedingungen, welche Faktoren die Aufnahme, Speicherung und Abgabe von Phosphat in dem Sulfid oxidierenden Bakterienstamm *Beggiatoa* 35Flor kontrollieren. Durch die Verwendung einer neu entwickelten Gradienten-Beprobungs-Säule zur Kultivierung in Kombination mit Mikrorhizonen erhielten wir Phosphatprofile von hoher Auflösung. In Phosphatprofilen unter oxischen Wachstumsbedingungen ist zu erkennen, dass ein Teil der Energie, die durch die Oxidation von Schwefelwasserstoff gewonnen wurde, zur Speicherung von Phosphat als Polyphosphat verwendet wurde. Ein Wechsel zu Sauerstoffmangel bei einem gleichzeitig hohen Sulfidflux bewirkte einen Abbau von Polyphosphat und eine schnelle Abgabe von Phosphat, wodurch die Phosphatkonzentration im Medium signifikant anstieg. Diese physiologische Reaktion ist ein bislang unbekannter Mechanismus der Verwendung von Polyphosphat in Bakterien. Dieser Mechanismus liefert eine Erklärung für das gleichzeitige Vorkommen von Sulfid oxidierenden Bakterien und hohen Phosphatkonzentrationen in Sedimenten, die eine Voraussetzung für die Ausfällung von phosphorhaltigen Mineralien sind.

Im zweiten Abschnitt dieser Dissertation wurde der Schwerpunkt auf die Polyphosphateinschlüsse des Stammes *Beggiatoa* 35Flor gelegt. Mit einem Durchmesser von 3 µm sind diese Polyphosphateinschlüsse die größten bisher entdeckten. Mittels konfokaler Laser-Scanning-Mikroskopie konnten wir zeigen, dass die Polyphosphateinschlüsse von einer Lipidschicht umgeben sind. Im Gegensatz zu den meisten Polyphosphateinschlüssen in anderen Organismen ist der pH Wert nicht bemerkenswert niedrig. Energiedispersive Röntgenspektroskopie zeigte eine Anreicherung von Kalzium und in geringerem

Maße von Magnesium in den Polyphosphateinschlüssen. 16S rRNA-basierte phylogenetische Rekonstruktion zeigte eine Zuordnung von *Beggiatoa* 35Flor zu vornehmlich nicht vakuolierten schmalen *Beggiatoa* Arten am phylogenetischen Ursprung der *Beggiatoaceae*. Wir vermuten, dass die Speicherung von großen Polyphosphatmengen eine Anpassung von schmalen Sulfid oxidierenden Bakterien an ihren Lebensraum darstellt, während große Arten der *Beggiatoaceae* vor allem durch die vakuoläre Speicherung von Nitrat angepasst sind.

Der dritte Teil der Dissertation ist eine vergleichende Studie zur Elementverteilung in Namibischem Sediment im Vergleich zu Veränderungen der elementaren Zusammensetzung von Meerwasser in einer *Beggiatoa* 35Flor Kultur. Im Namibischen Sediment waren verschieden Elemente, wie zum Beispiel Kalzium, Strontium und Uran zusammen mit Phosphor in einer Sedimentschicht mit einer großen Anzahl an *Thiomargarita* spp. angereichert. Diese Elemente waren über das gesamte Profil mit Phosphor korreliert. In einem Experiment mit dem Polyphosphat speichernden *Beggiatoa* Stamm 35Flor waren die Aufnahme und Abgabe von Kalzium und Strontium nicht mit Phosphat korreliert. Stattdessen scheinen Zunahme und Abnahme der jeweiligen Konzentrationen vom Redoxpotential abhängig zu sein. Im Gegensatz dazu war die Aufnahme und Abgabe von Uran stöchiometrisch mit Phosphat korreliert, wobei das U/P Verhältnis von 10^{-4} im Bereich natürlicher Phosphorite liegt. Wir vermuten daher, dass Uran mit Polyphosphat assoziiert ist. Polyphosphatabbau und Phosphatabgabe zusammen mit der Abgabe von Uran können folglich zur Bildung von stark uranangereicherten Apatiten führen.

Chapter 1

General Introduction

1.1. The marine phosphorus cycle and the involvement of bacteria

In nature, phosphorus occurs mainly fully oxidized as phosphate (PO_4^{3-}, redox state +5), but minor amounts of more reduced species such as phosphite (PO_3^{3-}, +3), phosphonate (C-PO_3^{3-}, +3) and phosphine (PH_3, -3) are also present (White and Metcalf, 2007). The cycling and transformation processes of phosphorus within the oceanic water column are complex and highly variable on spatial and temporal scales. Whereas burial of phosphorus in the sediment occurs on geological time scales, biological uptake, recycling and release of phosphate in the euphotic zone are subject to daily to annual cycles. The concentrations of phosphate range from below 1 µmol l^{-1} in the euphotic zone to around 3 µmol l^{-1} in deep ocean waters (Paytan and McLaughlin, 2007).

Phosphorus is an essential nutrient for all living organisms and accounts for approximately 3% of their dry weight. Phosphate esters are an integral part of nucleic acids and are further found in phospholipids of biological membranes. Energy rich phosphoanhydride bonds are present in adenosine triphosphate (ATP) and therefore of high importance for cellular energy metabolism (Madigan *et al.*, 2009). As phosphorus is a limiting macronutrient for primary production phosphorus cycling is linked to the global carbon cycle and thus influences climate over geological time scales (Tyrrell, 1999). Therefore, it is of great interest to gain a deeper understanding of the phosphorus cycle in view of the anthropogenically generated climate change.

1.1.1. The marine phosphorus cycle

Release of phosphorus in the form of phosphate occurs through weathering of terrestrial rocks and soils (Benitez-Nelson, 2000). Phosphate then enters the marine system through river runoff with the major part of particulate phosphorus

being buried in sediments of estuaries (Compton *et al.*, 2000). Approximately one third of the released phosphorus reaches the open ocean, where it is readily assimilated in the surface zone mostly by primary producers such as algae and photoautotrophic bacteria. Bound in dead biomass phosphate sinks to deeper layers of the water column. On the way to the seafloor, most of the phosphorus is recycled within the water column, but a small fraction is buried in marine sediments of the deep sea and continental margins (Delaney, 1998). Coastal upwelling regions are hotspots of phosphogenesis (Föllmi, 1996), since half of the total oceanic phosphorus burial occurs in theses areas, which represent only 0.1% of the ocean surface. The upwelling of cold and nutrient-rich water generates a high primary production (Figure 1), giving these coastal region a special ecological and economical importance. With shallow water depth of usually less than 130 m (Pinet, 2009) a significant part of the produced biomass reaches the sediment on continental shelves. Phosphorus-rich minerals, such as apatite ($Ca_5(PO_4)_3(F,Cl,OH)$), then arise from the buried phosphorus compounds through the process of phosphogenesis (Figure 1). Thereby, phosphorites with 10-12% of phosphorus (Baturin, 2003) in the form of coated grains and phosphatic laminites can be formed (Arning *et al.*, 2009b).

The complete process of phosphogenesis is very complex and still not resolved in detail. A known prerequisite for phosphogenesis is a supersaturation of phosphate in respect to the phosphorus mineral francolite in the pore water (Föllmi, 1996 and references therein). Possible reasons for high phosphate concentrations in pore water are: (i) Dissolution of fish debris (Suess, 1981), (ii) phosphate release due to microbial degradation of organic matter and (iii) dissolution of phosphates from ironhydroxides. The latter process was first proposed by Einsele (1936), who found a correlation of phosphate increase in the bottom water of freshwater lakes with an increase of ferrous iron under anoxic conditions. The main reason for oxygen depletion is the aerobic respiration of bacteria degrading organic matter. Anoxic conditions then give rise to the reduction of nitrate, oxidized forms of manganese and iron as well as sulfate. Sulfate reduction leads to the production and enrichment of sulfide, which in turn stimulates the dissolution of iron-hydroxides and the release of adsorbed phosphate.

However, pure physico-chemical processes are insufficient to explain the high concentrations of phosphate in marine sediments (Boström *et al.*, 1988), which feature a lower iron content (Blomqvist *et al.*, 2004). It is thought that bacteria, which take up phosphate for growth and are known to store it in high amounts as polyphosphate, play a key role in apatite formation (Krajewski *et al.*, 1994; Reimers *et al.*, 1990). Additionally, the conversion of diatom-derived polyphosphate to apatite may contribute to phosphogenesis (Diaz *et al.*, 2008).

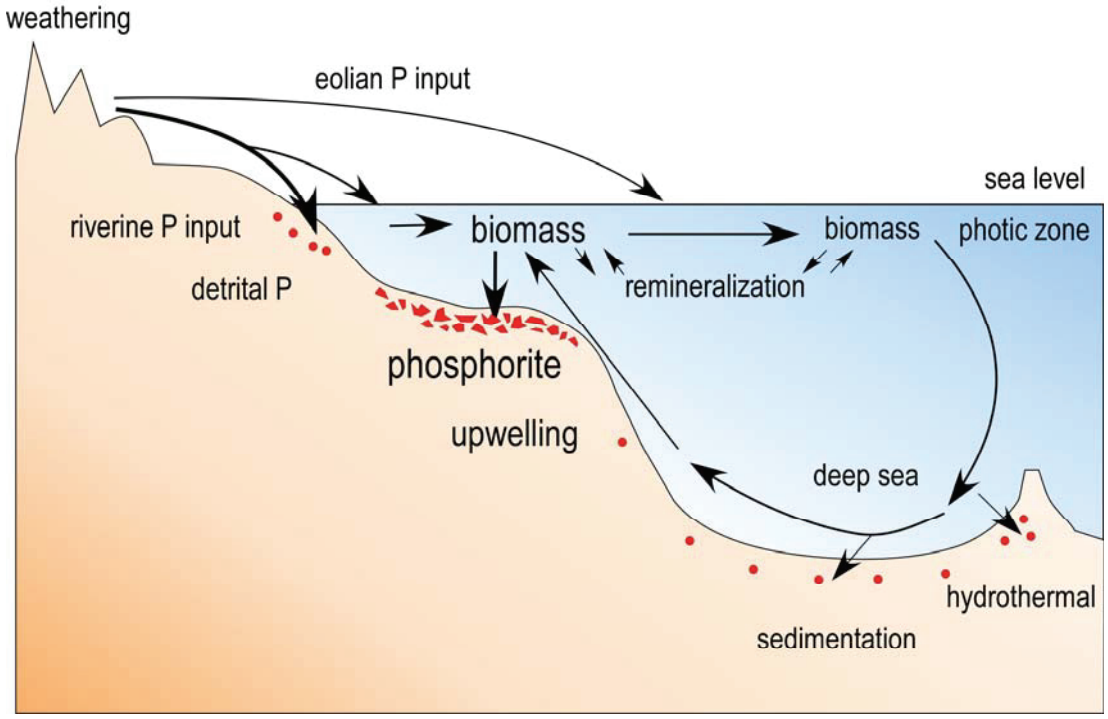

Figure 1 The marine phosphorus cycle. Weathering of rocks and soils delivers phosphorus via rivers into the marine system, where two thirds are buried within estuarine sediments. In the ocean, phosphorus is used mainly by photoautotrophic organisms for growth. Bound in dead biomass phosphorus sinks to the sea floor. On the way a large part is recycled. Burial of phosphorus occurs mostly within sediments of upwelling areas on the continental shelf and upper continental slope by formation of phosphorite. Adapted from Föllmi (1996) and Paytan and MacLaughlin (2007).

1.1.2. Bacteria in modern and ancient phosphorites

Several studies discussed the role of bacteria in the formation of phosphorus-rich minerals. O´Brien *et al.* (1981) suggested that the *post mortem* alteration of phosphorus-rich bacterial cells gave rise to the formation of phosphatic nodules found on the East Australian continental margin. Based on the finding of bacterial fossils in phosphorites from the Middle East (Israel) and studies from the Namibian

upwelling zone, which is an area of modern phosphogenesis, Nathan *et al.* (1993) argued, that Deltaproteobacteria are responsible for elevated phosphate concentrations in the water column.

More recent studies, however, proposed that large, colorless sulfide-oxidizing Gammaproteobacteria are responsible for peaks of phosphate in the pore water of sediments. Sulfide-oxidizing bacteria such as *Thioploca* (Gallardo, 1977; revised *Marithioploca* according to Salman *et al.*,in press), *Beggiatoa* (Arning *et al.*, 2008; revised *Iso- and Parabeggiatoa*) and *Thiomargarita* (Schulz *et al.*, 1999) occur in particularly high biomasses in sediments of modern areas of phosphogenesis. Moreover, ancient phosphorites contain fossils resembling in size and shape *Beggiatoa*-like filaments (Reimers *et al.*, 1990; Williams and Reimers, 1983) and spherical *Thiomargarita*-like cells (Bailey *et al.*, 2007) (Figure 2). Schulz and Schulz (2005) have shown that the giant sulfide-oxidizing bacterium *Thiomargarita namibiensis*, which stores polyphosphate in high amounts, is probably responsible for the observed high peaks of phosphate in the pore water and in the solid phase of sulfidic sediments along the coast of Namibia. This result was recently confirmed by Goldhammer *et al.* (2010), who found that the rate of phosphate-to-apatite conversion in anoxic sediments inhabited by a high number of *Beggiatoa* and *Thiomargarita*, exceeds the rate of phosphorus release during organic matter mineralization. These findings are furthermore in agreement with a study of Arning *et al*. (2008), who observed that the distribution of lipid biomarkers typical for sulfate-reducing bacteria and the distribution of sulfide-oxidizing bacteria are closely associated in sediments off Namibia, Peru and Chile, which feature high concentrations of phosphate in the pore water. The importance of this association in the formation of phosphorite is indicated by a higher abundance of lipid biomarkers of sulfate-reducing bacteria in autochthonous phosphatic laminite compared to other allochthonous facies in Peruvian sediment (Arning *et al.*, 2009a).

Even though an involvement of bacteria in the processes of phosphogenesis is indicated by several studies, the mechanisms by which this mediation is realized are still unknown. In general, bacteria were shown to regulate the flux of phosphorus across the sediment/water interface by phosphate release due to decomposition of organic matter and phosphate uptake for biomass production and

storage as polyphosphate. Thereby, The amount of accumulated polyphosphate varies with changes in the redox-conditions within the sediment (Gächter *et al.*, 1988). For *Thiomargarita namibiensis* it was suggested that the rapid release of phosphate from the breakdown of polyphosphate is triggered by anoxia and uptake of acetate (Schulz and Schulz, 2005) as proposed for polyphosphate-storing bacteria from wastewater treatment plants (Comeau *et al.*, 1986).

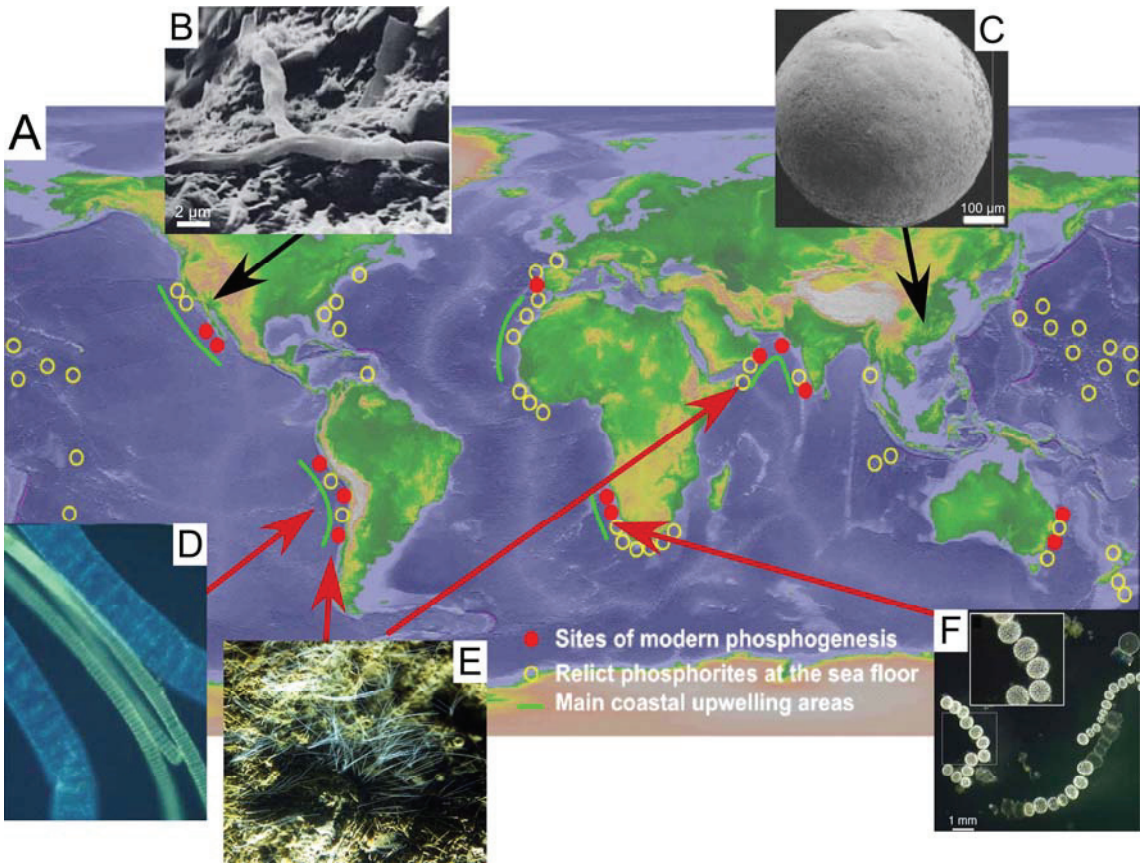

Figure 2 Sites of phosphogenesis and distribution of large sulfide-oxidizing bacteria. Modern phosphogenesis occurs mainly in regions of intensive coastal upwelling (A), which are prominent habitats of large sulfide-oxidizing bacteria of the genera *Beggiatoa* (D, revised *Maribeggiatoa*), *Thioploca (*E, revised *Marithioploca*) and *Thiomargarita* (F). Relict phosphorites are found in regions of ancient and modern upwelling as well as at sea mounts (A). Fossils which resemble filamentous *Beggiatoa*-like bacteria (B) or *Thiomargarita* spp. (C) have been discovered in ancient continental phosphorite deposits. (A (Hensen *et al.*, 2006) modified after (Baturin, 1982; Föllmi, 1996), B (Reimers *et al.*, 1990), C (Bailey *et al.*, 2007), D and E (Teske and Nelson, 2006), F (Schulz, 2006))

1.2. Sulfide-oxidizing bacteria

In this section sulfide-oxidizing bacteria of the family *Beggiatoaceae* are described in detail owing to their proposed importance in the formation of phosphorite.

1.2.1. Beggiatoaceae

Sulfide-oxidizing bacteria of the family *Beggiatoaceae* are ubiquitous in freshwater and marine habitats, such as lake sediments (Lauterborn, 1907), hydrothermal vents and springs (Jannasch *et al.*, 1989), eutrophic coastal sediments in areas of coastal upwelling (Gallardo, 1977; Schulz *et al.*, 1999) and also occur associated with the black band disease of corals (Richardson, 1996). Due to their shape and size sulfide-oxidizing bacteria of the genera *Beggiatoa*, *Thioploca* (for revision see Salman *et al.*,in press) and *Thiomargarita* belong to the most conspicuous bacteria known so far (Schulz and Jørgensen, 2001). Filamentous representatives of the family *Beggiatoaceae* such as *Marithioploca* spp., *Maribeggiatoa* spp., *Thioploca* spp. and *Beggiatoa* spp. are highly motile bacteria with filament lengths reaching from a few micrometers up to a few centimeters and diameters ranging from only one to more than hundred micrometers (Jørgensen, 1977; Nelson *et al.*, 1989; Pringsheim, 1964; Strohl and Larkin, 1978). *Thiomargarita* spp. occur as single cells or in chains of either barrel-shaped or spherical cells, whereas *Thiopilula* spp. form mucus-covered aggregates (Salman *et al.*, in press; Schulz, 2006).

Within the family *Beggiatoaceae* both organoheterotrophic and lithoautotrophic species are known. Winogradsky's (1887) pioneering work on the discovery of chemolithoautotrophy was based on his studies of an enrichment culture of *Beggiatoa*. Chemolithoautotrophic species of sulfide-oxidizing bacteria gain their energy solely by the oxidation of the reduced sulfur compounds like sulfide (Nelson and Jannasch, 1983) and thiosulfate (Grabovich *et al.*, 2001) with oxygen or nitrate. In the presence of sulfide, elemental sulfur is stored as an intermediate in form of globules enclosed by multiple membranes (Strohl *et al.*, 1981). For the wider marine species of the *Beggiatoaceae* storage of nitrate in concentrations of up to 800 mmol l^{-1} was shown within a central vacuole that can account for up to 98 % of the cell volume (Fossing *et al.*, 1995; McHatton *et al.*, 1996; Schulz *et al.*,

1999). For smaller species of the genus *Beggiatoa* that feature a diameter of less than 7 µm vacuolar nitrate storage has not yet been shown.

1.2.2. The marine Beggiatoa strain studied in this thesis

The examined marine *Beggiatoa* strain 35Flor originates from the microbial community associated with the black band disease of scleractinian corals from the Florida Keys. In contrast to large sulfide oxidizing bacteria such as *Thiomargarita* spp., *Marithioploca* spp. and *Maribeggiatoa* this strain can be cultivated under defined laboratory conditions. Presently, the culture is accompanied by a *Pseudovibrio denitrificans* strain (Anne Schwedt, personal communication), which seems to be essential for the growth of the *Beggiatoa* strain. The strain grows chemolithoautotrophically from the oxidation of sulfide with oxygen. Within its natural habitat (Richardson, 1996) and in artificial mineral gradient medium the filaments accumulate in the transition zone of oxygen and sulfide, where a distinct bacterial mat is formed. The studied *Beggiatoa* strain 35Flor shows physiological similarities to the large nitrate-storing members of the family *Beggiatoaceae* (Salman *et al.*, in press), as the cells possess sulfur globules, a central vacuolar structure (Figure 3) and accumulate polyphosphate. However, the filaments of this strain are only 6 µm in diameter and nitrate storage was not detected.

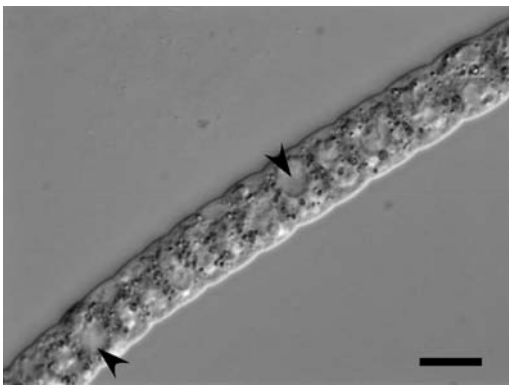

Figure 3 Differential interference contrast (DIC) image of a filament from the studied marine *Beggiatoa* strain 35Flor. In the center of some cells a large vacuolar structure (arrowheads) is visible. Sulfur globules are visible as dark spots. Scale bar 5 µm

1.3. Polyphosphate and its storage by sulfide-oxidizing bacteria

Many organisms from bacteria to higher eukaryotes are known to store phosphate in the form of polyphosphate (Docampo and Moreno, 2001; Harold, 1966; Kornberg *et al.*, 1999; Kulaev *et al.*, 2004). Polyphosphate can be found within cell walls and membranes, in granules and also in the nuclei of eukaryotes (Kulaev *et al.*, 2004). Structurally, polyphosphate consists of three to over hundred phosphate monomers that are linked to each other via energy-rich phosphoanhydride bonds (Kornberg *et al.*, 1999).

1.3.1. Metabolism and function of polyphosphate

The formation of polyphosphate via the enzyme polyphosphate-kinase (PPK) requires ATP. Polyphosphate decomposition can be catalyzed by different enzymes. Endo-polyphosphatase (PPN) cleave polyphosphate intramolecularly and thus produce short-chain polyphosphates whereas polyphosphate-exokinase (PPX), reverse PPK, polyphosphate-AMP-phosphotransferase (PAP), polyphosphate-glucose-phosphotransferase and further enzymes cleave off single, terminal phosphate residues (Figure 4). Some bacterial enzymes like NAD kinase are both ATP- and polyphosphate-dependent. The breakdown of an energy-rich phosphoanhydride bond in polyphosphate releases about as much energy as delivered by the breakdown of a phosphoanhydride bond in ATP (about 38 kJ mol^{-1} at pH 5; Kulaev *et al.*, 2004).

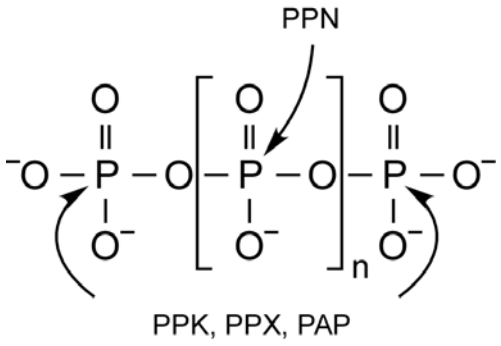

Figure 4 General structure of polyphosphate and important enzymes involved in its decomposition. Up to a few hundred phosphate groups are linked by energy-rich anhydride bonds. Terminal phosphate groups can be cleaved by polyphosphate-kinase (PPK), polyphosphate-exokinase (PPX) and polyphosphate-AMP-phosphotransferase (PAP). Endo-polyphosphatase (PPN) can divide the polyphosphate internally into smaller units. Adapted from Kornberg *et al.* (1999).

The function of polyphosphate is not yet clear. It is assumed to serve as an ATP substitute, phosphorus storage, a chelator of metal ions and to play an important role in adaptation to various forms of stress such as pH imbalance (Ault-Riche *et al.*, 1998; Jahid *et al.*, 2006; Seufferheld *et al.*, 2008). Pick and Weiss (1991) suggested that the hydrolysis of polyphosphate counterbalances amine-induced alkaline stress. Interestingly, many polyphosphate-dependent enzymes are multifunctional and can catalyze reactions with both, polyphosphate and nucleotide triphosphates. Therefore, an important role of polyphosphate in the regulation of basal cellular functions can be assumed (Kulaev *et al.*, 2004).

It was shown that polyphosphate inclusions in some bacterial species have properties similar to acidocalcisomes of eukaryotes (Seufferheld *et al.*, 2003). These electron-dense acidic organelles are enclosed by a membrane, which is associated with vacuolar-ATPases, vacuolar-PPases and different ion transporters. The enzymes PPK and PPX were shown to be located within the matrix of acidocalcisomes in trypanosomes (Moreno and Docampo, 2009).

1.3.2. Polyphosphate accumulation by sulfide-oxidizing bacteria

Several studies have shown polyphosphate accumulation in different strains of the sulfide-oxidizing genus *Beggiatoa* by staining with methylene blue or transmission electron microscopy (TEM) (de Albuquerque *et al.*, 2010; Maier and Murray, 1965; Strohl and Larkin, 1978) (Figure 5). For *Thiomargarita namibiensis*, which was found in Namibian sediment with high phosphate concentrations in the pore water and a high phosphorus content in the solid phase, it was shown by toluidine blue staining that polyphosphate is stored in large inclusions (Schulz and Schulz, 2005) (Figure 5). In contrast to the genera *Beggiatoa* and *Thiomargarita* there is no direct evidence for polyphosphate storage in the genera *Thioploca* and *Marithioploca* so far (Høgslund *et al.*, 2009; Maier and Murray, 1965). However, in a comparative TEM study with ultrathin sections of the marine species *Thioploca araucaeae* and *Thioploca chileae* (both revised *Marithioploca*) Maier *et al.* (1990) observed in *Thioploca chileae* electron dense bodies, but the elemental composition was not tested.

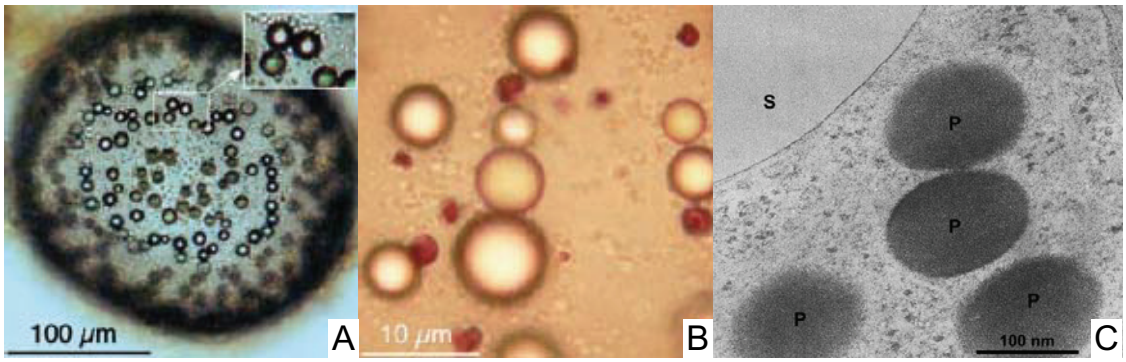

Figure 5 Polyphosphate inclusions in sulfide-oxidizing bacteria. (A) A single *Thiomargarita* sp. cell with large sulfur globules and many smaller inclusions as visible in the magnification (inset). (B) Polyphosphate inclusions within *Thiomargarita* sp. are stained red by toluidine blue. (C) Transmission electron microscopy reveals electron dense bodies (P) next to a large sulfur globule (S) within a narrow marine *Beggiatoa* strain (A and B (Schulz and Schulz, 2005), C (de Albuquerque *et al.*, 2010)).

1.4. Aim of the thesis

The overall aim of this thesis was to understand how sulfide-oxidizing bacteria contribute to phosphogenesis. For this reason, we first studied the polyphosphate accumulation capacity of the marine *Beggiatoa* strain 35Flor. Then, we searched for the main stimulus inducing polyphosphate breakdown and rapid release of phosphate that leads to increased phosphate concentrations in the culture medium (Chapter 2). To gain a deeper understanding of polyphosphate storage we studied the structure and elemental composition of the polyphosphate inclusions (Chapter 3). Based on these results we investigated changes in the elemental composition of natural seawater medium that were caused by growth of *Beggiatoa* 35Flor. The results of this experiment were compared to the elemental composition of sediment from the Namibian upwelling zone, in which a high number of *Thiomargarita* spp. occurs at the same depth as sediment enriched in apatite (Chapter 4).

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Chapter 2

Sulfide induces phosphate release from polyphosphate in cultures of a marine *Beggiatoa* strain

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The ISME Journal advance online publication, 9 September 2010; doi:10.1038/ismej.2010.135 Subject Category: geomicrobiology and microbial contributions to geochemical cycles

Abstract

Sulfur bacteria such as *Beggiatoa* or *Thiomargarita* have a particularly high capacity for storage because of their large size. In addition to sulfur and nitrate, these bacteria also store phosphorus in the form of polyphosphate. *Thiomargarita namibiensis* has been shown to release phosphate from internally stored polyphosphate in pulses creating steep peaks of phosphate in the sediment and thereby inducing the precipitation of phosphorus-rich minerals. Large sulfur bacteria populate sediments at the sites of recent phosphorite formation and are found as fossils in ancient phosphorite deposits. Therefore, it can be assumed that this physiology contributes to the removal of bioavailable phosphorus from the marine system and thus is important for the global phosphorus cycle. We investigated under defined laboratory conditions which parameters stimulate the decomposition of polyphosphate and the release of phosphate in a marine *Beggiatoa* strain. Initially, we tested phosphate release in response to anoxia and high concentrations of acetate, because acetate is described as the relevant stimulus for phosphate release in activated sludge. To our surprise, the *Beggiatoa* strain did not release phosphate in response to this treatment. Instead, we could clearly show that increasing sulfide concentrations and anoxia resulted in a decomposition of polyphosphate. This physiological reaction is a yet unknown mode of bacterial polyphosphate usage and provides a new explanation for high phosphate concentrations in sulfidic marine sediments.

Keywords: sulfur bacteria, marine sediment, polyphosphate, phosphorus cycle

Introduction

Phosphorus is considered as the ultimate limiting nutrient, because over geological time scales the amount of biologically available phosphorus determines, how much carbon is incorporated into living biomass. In contrast to this, nitrogen often limits growth over shorter time scales, but is ultimately available in large amounts from the atmosphere via the activity of nitrogen fixing microorganisms (Tyrrell, 1999).

Therefore, it is crucial to know the sources and sinks of phosphorus in order to understand the global carbon cycle and thus the climate on Earth. Phosphorus enters the biosphere in the form of phosphate by the weathering of phosphorus-rich rocks on land and is removed by phosphogenesis, which refers to the formation of phosphorus-rich minerals on the seafloor (Föllmi, 1996). While weathering is mostly a physicochemical process, phosphogenesis is initiated by the spontaneous precipitation of phosphorus-rich minerals due to oversaturation in the pore water with respect to apatite, which can be induced by the activity of microorganisms. Depending on the initial degree of oversaturation, different mechanisms of precipitation reactions may prevail, resulting in a slow formation of dispersed apatite or a fast formation of phosphatic bodies (Krajewski *et al.,* 1994). In addition, calcium-associated polyphosphate of biological origin can reduce the kinetic nucleation barrier to the precipitation of calcium phosphate minerals, and diagenetic transformation into fine-grained, geologically stable authigenic apatite particles can thereby occur (Diaz *et al.,* 2008). In today's oceans, active phosphogenesis is mainly found beneath the nutrient rich upwelling areas off the coasts of Peru, Chile and Namibia (Föllmi, 1996).

Large sulfur bacteria of the genera *Beggiatoa*, *Thioploca* and *Thiomargarita* are suspected to be involved in phosphogenesis, because they occur in remarkably high biomasses precisely in the areas of the most active modern phosphorite formation (Fossing *et al.,* 1995; Schulz *et al.,* 1999) and they are found as fossils in phosphorite rocks (Bailey *et al.*, 2007; Reimers *et al.*, 1990; Williams and Reimers, 1983). Recently, *Thiomargarita namibiensis* was found to accumulate polyphosphate, which can be released as phosphate under certain conditions producing steep peaks of phosphate in the pore water. The generated oversaturation then results in the formation of phosphorus-rich minerals in sediments off the coast of Namibia (Schulz and Schulz, 2005). Like other large sulfur bacteria, *Thiomargarita namibiensis* gains energy by the oxidation of sulfide with oxygen or internally stored nitrate (Schulz and Jorgensen, 2001). Polyphosphate is a storage compound, which is accumulated at the expense of energy during favorable growth conditions and then can again be used to gain energy (Kornberg, 1995). As the accumulation and decomposition of polyphosphate is not part of the main energy-gaining metabolism, recurrent

phosphate release must be initiated by an environmental stimulus. The identification of this stimulus is important for both the general understanding of polyphosphate in the microbial metabolism and the identification of the environmental conditions inducing phosphate release and phosphogenesis.

Most studies on bacterial polyphosphate accumulation and release were conducted on microbial communities from wastewater treatment plants, in which polyphosphate-accumulating bacteria are used for biological phosphorus removal. More than three decades ago, a correlation between the phosphate-removal capacity of activated sludge and the acetate concentration during the anaerobic treatment was observed (Fuhs and Chen, 1975). In later studies, Comeau *et al.* (1986) proved that addition of acetate to anoxic activated sludge triggered phosphate release, and based on this observation the authors postulated a biochemical model for enhanced biological phosphorus removal. According to this model, polyphosphate-accumulating bacteria store acetate in form of poly-β-hydroxybutyrate during the anaerobic treatment phase using the energy provided by the decomposition of polyphosphate. In the following oxic treatment phase, the same bacteria exhibit a “luxury uptake” of phosphate along with accumulation of polyphosphate. This concept is generally accepted, although the identification and cultivation of the relevant polyphosphate-accumulating organisms has proven to be problematic (Seviour and McIlroy, 2008). Moreover, Comeau *et al.* (1986) report that sulfide addition stimulated phosphate release, but this result was not included into the concept of polyphosphate usage and, to our knowledge, has not further been investigated. However, sulfate reduction accompanied by the growth of sulfur-oxidizing bacteria is known to occur in anaerobic activated sludge (Okabe *et al.,* 1999) and a higher phosphate release was detected simultaneously with an increase in sulfate reduction rates (Baetens, 2000).

In this study we tested whether acetate or sulfide is responsible for the release of phosphate by a marine *Beggiatoa* strain through the decomposition of polyphosphates. Members of the genus *Beggiatoa* are filamentous, highly motile sulfur bacteria, which occur abundantly in sulfidic sediments all over the world (Teske and Nelson, 2006) and are also encountered as fossils in phosphorite deposits (Reimers *et al.*, 1990; Williams and Reimers, 1983). Typically, the long

filaments populate organic matter-rich sediments, in which they form a mat in the narrow zone where oxygen and sulfide overlap, close to the sediment surface.

Materials and Methods

Strain and cultivation

The investigated marine *Beggiatoa* strain grows in presence of a single accompanying bacterium. This *Pseudovibrio* strain occurs in low cell numbers but could not be removed during purification and therefore seems to be required for the growth of the culture. The *Beggiatoa* filaments have an average diameter of 6 µm; the cells possess sulfur inclusions, a central vacuole, probably for nitrate storage, and polyphosphate inclusions of different sizes.

For cultivation, a mineral medium with opposing gradients of oxygen and sulfide modified after Nelson and Jannasch (1983) was strongly buffered with 1,4-Piperazinediethanesulfonic acid disodium salt (20 mmol l^{-1}) to maintain a pH of 7 and thus avoid phosphate precipitation. The initial phosphate concentration of the medium was lowered to 20 µmol l^{-1}. The gradient medium was prepared in a Plexiglas tube of 20 mm inner diameter and 12 cm length, closed at the bottom and filled with 7.5 ml sulfidic bottom agar and 15 ml sulfide-free top agar. On the wall of the tube 2-mm-wide holes were drilled in 1-mm-depth intervals and closed with autoclavable tape (Figure 1).

Distribution of *Beggiatoa* filaments

Due to highly refractive internal sulfur globules, filaments of *Beggiatoa* appear white and can easily be seen in a transparent agar medium. The distribution of the filaments in the top part of the gradient medium was recorded by a sensicam CCD camera (PCO, Kelheim, Germany) while the tubes were exposed to an amber Luxeon LED (Philips, Amsterdam, The Netherlands).

Experiments

In order to test the influence of different sulfide fluxes on the release of phosphate, gradient media with 8, 16, 24 and 36 mmol l^{-1} Na_2S in the bottom agar were prepared. Na_2S was added to the bottom agar after autoclaving, and afterward the pH of the bottom agar was adjusted to about 7 by addition of 1 mol l^{-1} HCl. After solidification of the bottom agar at 4 °C the top agar was added. If necessary the pH was adjusted to 7 after autoclaving by addition of 1 mol l^{-1} HCl. The tubes were closed by aluminum foil, allowing exchange of headspace gas with the atmosphere. After aging for one day, a sulfide gradient had established in the top agar and the experiment was started by inoculation with 80 µl of a *Beggiatoa* culture, which contained 8 mmol l^{-1} Na_2S in the bottom agar and the same concentration of phosphate and buffer as noted above. Sampling of 3 parallel cultures was carried out after 7 days of oxic growth or 7 days of oxic growth followed by 24 hours of anoxic incubation. Anoxic conditions were obtained by closing the tubes with rubber stopper and flushing with nitrogen for 5 minutes.

The effect of volatile fatty acids in combination with anoxia on phosphate release was tested by injecting acetate or propionate (32 µl of a 1 mol l^{-1} solution) directly into the bacterial mat in 3 parallel cultures (7 days old, 8 mmol l^{-1} Na_2S in the bottom agar) followed by 24 hours of anoxic incubation. As a control, three parallel cultures (6 days old) were left oxic for 24 hours after the injection.

Additionally, it was tested whether phosphate release is an active process of living cells or occurs as a consequence of cell death. Thick mats of *Beggiatoa* were harvested and immediately inactivated by exposure to 60 °C for 15 minutes. A 1-mm-thick layer of dead filaments was placed on top of a sterile and phosphate-free gradient medium that contained 36 mmol l^{-1} sulfide in the bottom agar. Phosphate profiles were measured in three parallels after 3 days of oxic conditions or 2 days of oxic conditions followed by 24 h of anoxic incubation.

Phosphate profiles

For phosphate determination, samples of 80 µl were taken from subsequent depth layers through the holes in the wall of the tubes using microrhizons (Rhizosphere Research products, Wageningen, The Netherlands). After addition of 420 µl H_2O phosphate concentrations were determined colorimetrically by the ascorbic acid method modified after Hansen and Koroleff (1999) using a Beckman DU 640 Spectrophotometer (Beckman Coulter, Fullerton, CA, USA) or a SpectroDirect Spectrophotometer (Aqualytic, Dortmund, Germany).

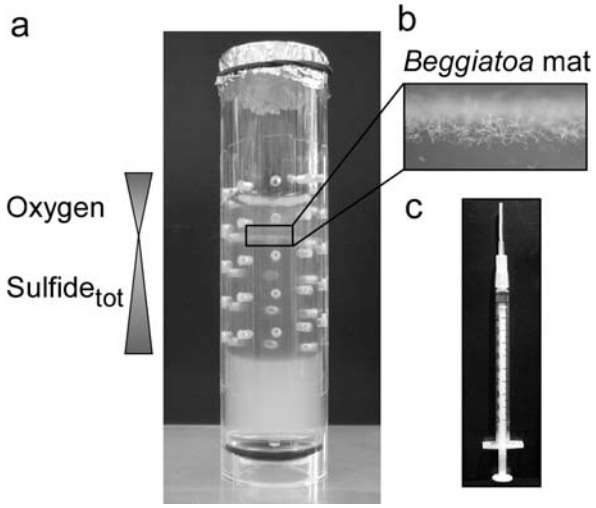

Figure 1 Gradient sampling tube with an oxygen/sulfide gradient medium. **(a)** The tube consists of polymethyl methacrylate (Plexiglas) and features spherical drilled holes of 2 mm diameter separated by a vertical distance of 1 mm for high-resolution sampling. The holes are sealed with an autoclavable adhesive tape. **(b)** A *Beggiatoa* mat forms at the interface of oxygen and sulfide. **(c)** A microrhizone attached to a 1-ml syringe is used for sampling of liquid from the agar

Sulfide profiles

Sulfide profiles were determined with commercially available microelectrodes (Unisense, Aarhus, Denmark). Total dissolved sulfide concentrations were calculated using a simultaneously measured pH profile according to Kühl *et al.* (1998). The flux of total sulfide was calculated as the sum of the H_2S and the HS^- fluxes according to Fick´s first law of diffusion:

$$J = -D^0 \times \partial C/\partial x$$

The used diffusion coefficients (D^0) were 1.49×10^{-9} for HS^- and 1.75×10^{-9} m^2 s^{-1} for H_2S (Schulz, 2006).

Microscopy and staining of polyphosphate inclusions

Filaments of *Beggiatoa* were imaged by differential interference contrast (DIC) microscopy to visualize internal cell structures such as sulfur globules, which appear as dark refractive spots. 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) is a common stain for DNA with a blue signal and a maximum emission wavelength of 460 nm when bound to DNA. DAPI as well binds to polyphosphate, but then the dye's maximum emission wavelength is shifted to 525 nm, resulting in a yellow signal (Tijssen *et al.,* 1982). For polyphosphate staining 5 µl of a DAPI solution (1 g l^{-1} in H_2O) was added to 50 µl of a fresh *Beggiatoa* sample and incubated over night at room temperature. Polyphosphate inclusions were observed by fluorescence microscopy using an Axioplan universal microscope (Zeiss, Oberkochen, Germany) with a HBO 50 mercury lamp (Osram, München, Germany) for UV light and a UV-G 365 filter set (G 365 exciter filter, FT 395 chromatic beam splitter and an LP 420 barrier filter, Zeiss, Oberkochen, Germany).

Results

Culture growth

All cultures showed the formation of a distinct *Beggiatoa* mat 1-2 days after inoculation. Higher sulfide concentrations in the bottom agar resulted in higher growth rates and higher positions of the *Beggiatoa* mats in the gradient medium (Figure 2). After one week the mat positions varied from 5.0 mm depth in the cultures with the lowest sulfide flux to 0.5 mm depth in the cultures with the highest flux (Figures 3a and 4, hatched lines). The addition of acetate or propionate followed by oxic incubation induced an upward movement of the mat from 3 to 1 and 5 to 2 mm depth, respectively, within 24 h (Figure 3, hatched lines). Under anoxic incubation the *Beggiatoa* mat dispersed in all cultures and the *Beggiatoa* filaments moved to the top of the gradient medium within 24 h (Figures 2d-f, 3 and 4, gray lines).

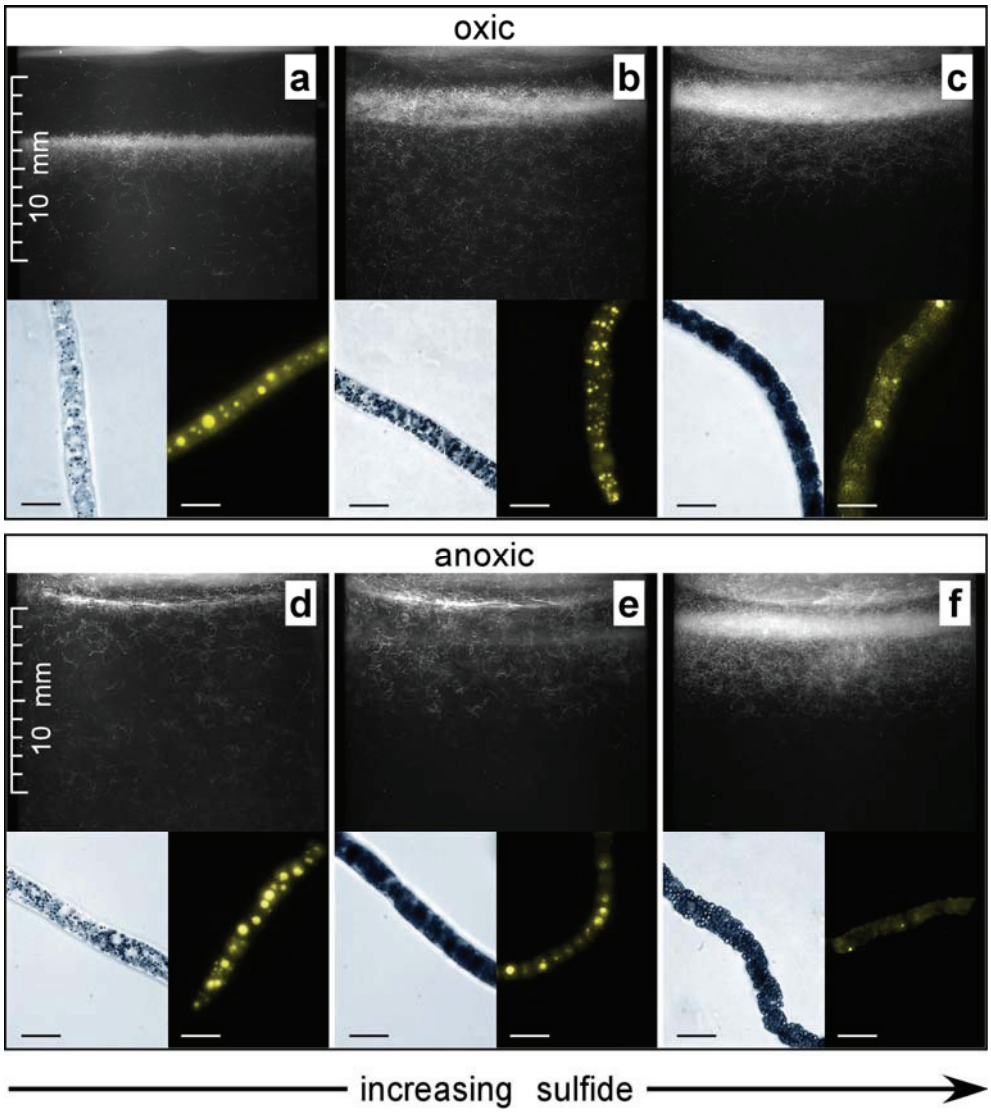

Figure 2 Response of a marine *Beggiatoa* strain to increasing sulfide fluxes and anoxia. (**a-c**) After 7 days of oxic conditions *Beggiatoa* filaments have established a distinct mat at the interface of oxygen and sulfide. With increasing sulfide fluxes the mats are situated at higher positions. (**d-f**) The same cultures after 24 h of anoxic incubation. The mats have dispersed and the filaments have migrated to the top. Insets show micrographs of filaments from the respective cultures. In the left images sulfur globules are visualized as dark spots by DIC microscopy. In the right images polyphosphate inclusions stained with DAPI show a yellow fluorescence. Filaments contain more sulfur granules with increasing sulfide fluxes. At higher sulfide concentrations, anoxic conditions cause the decomposition of polyphosphate inclusions. Scale bars are 10 µm. (Pictures **a-f** by courtesy of Anne Bachmann)

Phosphate uptake and release in response to addition of fatty acids

Under oxic conditions the profiles of dissolved phosphate (Figure 3, triangles) showed a decrease in concentration from 20 to nearly 0 µmol l^{-1} at the top of the gradient media where the *Beggiatoa* mats were situated, owing to bacterial growth and accumulation of polyphosphate (Figure 3, triangles). Below the *Beggiatoa* mats, the phosphate concentration increased with ongoing depth. Because *Beggiatoa* filaments are hardly found in these deeper layers, the phosphate concentration was regulated by diffusion. In sterile controls the initial phosphate concentration of about 20 µmol l^{-1} was found throughout the depth of the medium (Figure 3, crosses). No significant difference in phosphate profiles was evident when acetate or propionate (Figures 3b and c) was added to cultures without volatile fatty acids (Figure 3a). After 24 h of anoxic incubation no change in the phosphate profiles could be observed (Figure 3, filled circles). However, under oxic conditions the *Beggiatoa* mat responded to addition of acetate or propionate with an upward movement from 3 mm to 1 mm or 5 mm to 2 mm depth, respectively, within 24 h (Figure 3, hatched lines).

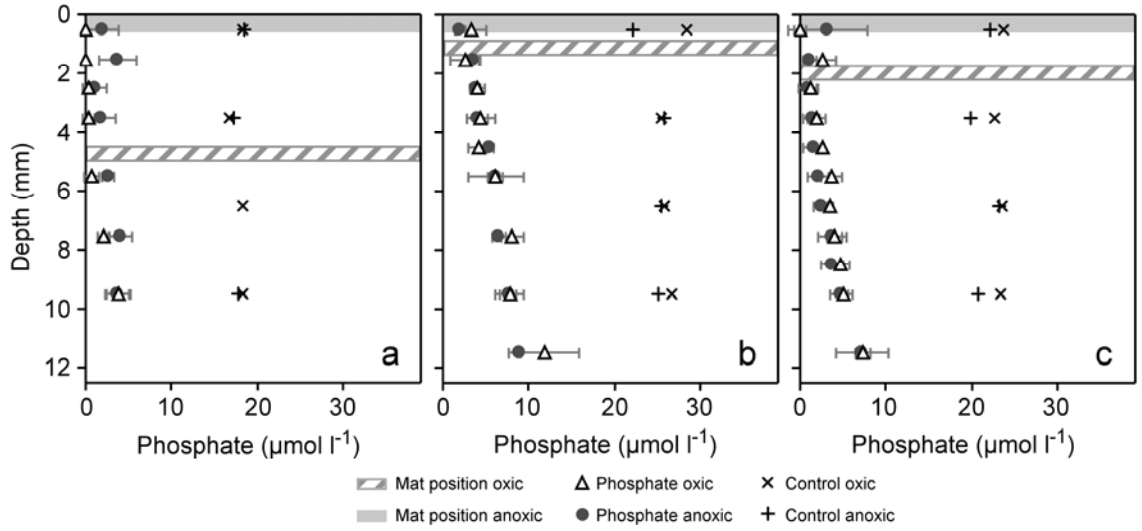

Figure 3 Influence of volatile fatty acids on phosphate release. Mean phosphate concentrations and standard deviations of three parallels after 6 days of oxic growth followed by the injection of a volatile fatty acid into the mat and 24 h of oxic incubation (Δ), or 24 h of anoxic incubation (●). The hatched horizontal lines indicate mat positions after oxic condition and the gray lines after anoxic incubation. (**a**) Profiles without addition of volatile fatty acids, (**b**) after addition of acetate and (**c**) after addition of propionate to cultures with 8 mmol l^{-1} sulfide in the bottom agar. Under oxic conditions phosphate is rapidly taken up, but after switching to anoxic conditions phosphate is not released in the absence or in the presence of acetate or propionate.

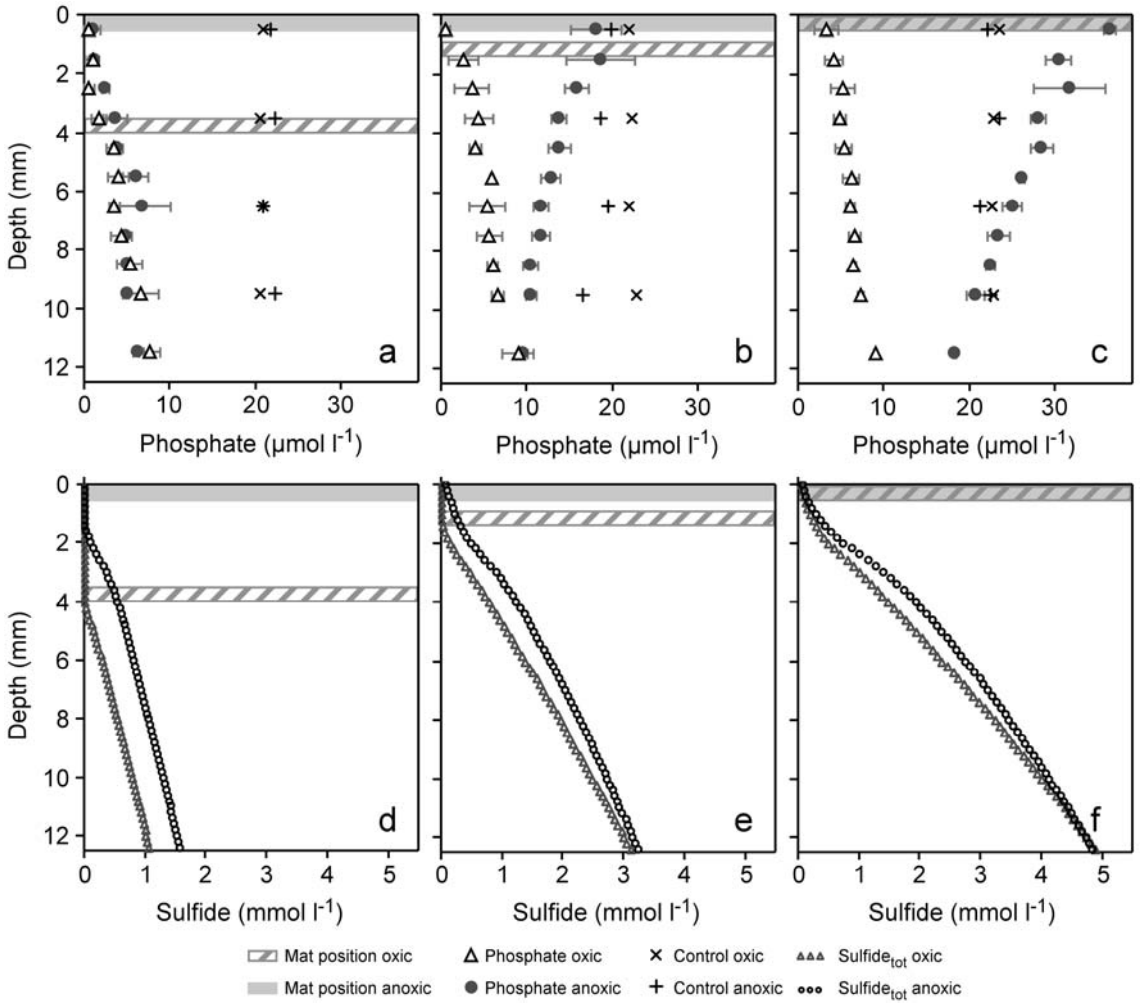

Figure 4 Influence of different sulfide fluxes on phosphate release. Cultures with (**a, d**) 16 mmol l^{-1} (**b, e**) 24 mmol l^{-1} and (**c, f**) 36 mmol l^{-1} sulfide in the bottom agar are shown. (**a-c**) Mean phosphate concentrations and standard deviation of three parallels after 7 days of oxic growth (∆) and after 7 days of oxic growth followed by 24 h of anoxic incubation (●). (**d-f**) Concentrations of total sulfide under oxic (∆) and after switching to anoxic conditions (**O**). The hatched horizontal lines indicate mat positions after oxic condition and the gray lines after anoxic incubation. Under oxic conditions phosphate is rapidly taken up. With increasing sulfide more biovolume is produced and a mat is established at a higher position. During the following anoxic incubation steeper sulfide gradients stimulate an increasing release of phosphate.

Phosphate uptake and release in response to increasing sulfide concentrations

The shape of the phosphate profile under oxic conditions did not change significantly with increasing sulfide concentrations in the bottom agar (Figure 4, triangles). Only in the culture with the highest sulfide flux did phosphate concentrations not reach 0 µmol l^{-1} at the position of the mat but remained around 3

µmol l^{-1}. Sulfide profiles measured with microelectrodes showed that under these conditions sulfide diffused up to the top of the medium. Accordingly, *Beggiatoa* filaments in these cultures were permanently exposed to higher sulfide concentrations compared with cultures in which a lower sulfide flux allows a complete oxidation of sulfide within the mat (Figure 4 f; Table 1).

Table 1 Concentration of phosphate and total dissolved sulfide at the top of the culture as well as total sulfide fluxes into the *Beggiatoa* mat at different sulfide concentrations in the bottom agar

c (Na_2S) (mmol l^{-1})[a]	c(PO_4^{3-}) (µmol l^{-1})[b]		c ($sulfide_{tot}$) (µmol l^{-1})[b]		$sulfide_{tot}$ flux (µmol m^{-2} s^{-1})[c]	
	Oxic	Anoxic	Oxic	Anoxic	Oxic	Anoxic
8	0	0-4	0	0	0.14	0.11
16	0-1	0-2	0	0	0.19	0.19
24	0-1	16-23	0	70	0.43	0.36
36	2-5	30-32	45	63	0.61	0.53

The values were determined after 7 days of oxic growth or after 7 days of growth followed by 24 h of anoxic incubation. A release of phosphate after anoxic incubation coincides with the presence of sulfide at the top of the culture when *Beggiatoa* filaments are exposed to sulfide.
[a]) Initial concentration in bottom agar.
[b]) Concentration at the top.
[c]) Sum of HS^- and H_2S flux

The *Beggiatoa* filaments migrated to the top of the gradient medium during anoxic incubation at all sulfide fluxes tested (Figures 2d-f; Figure 4, gray lines). Anoxic conditions for 24 h resulted in increasing phosphate release when the *Beggiatoa* strain was cultivated in presence of steeper sulfide gradients (Figures 4b and c, filled circles; Table 1). Sulfide microprofiles showed that filaments in these cultures were exposed to elevated sulfide concentrations during anoxic conditions (Figure 4e and f; Table 1). At 16 mmol l^{-1} Na_2S in the bottom agar the flux of total sulfide was 0.19 µmol m^{-2} s^{-1} under oxic and anoxic conditions. At a sulfide concentration of 36 µmol l^{-1} Na_2S in the bottom agar the flux of total sulfide decreased from 0.61 µmol m^{-2} s^{-1} under oxic conditions to 0.53 µmol m^{-2} s^{-1} after anoxic incubation, indicating a decrease in sulfide uptake.

In the control experiment in which the dead filaments were placed on top of a phosphate-free medium, no significant release of phosphate could be detected

under oxic or anoxic conditions (Figure 5). The *Beggiatoa* filaments, which originally contained polyphosphate in similar amounts as in the experiments, were mostly fractured, and possessed after the treatment a lower amount of polyphosphate inclusions compared with untreated filaments. It is likely that the majority of polyphosphate was dispersed in the medium and was therefore not detectable by DAPI staining.

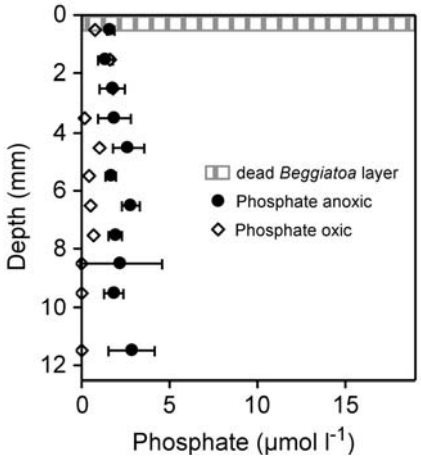

Figure 5 Control experiment with dead *Beggiatoa* filaments. A thick *Beggiatoa* mat was harvested and inactivated by exposure to 60°C for 15 min. A 1-mm-thick layer of dead filaments was placed on top of a sterile, phosphate-free medium with 36 mmol l^{-1} sulfide in the bottom agar. Phosphate profiles after 3 days of oxic conditions (◇) and 2 days of oxic followed by 24 h of anoxic conditions (●) showed no significant phosphate release.

Accumulation of Polyphosphate

When incubated oxically, the *Beggiatoa* strain accumulated polyphosphate in high amounts within the cells as visualized by DAPI staining (Figure 2a-c). At low sulfide concentrations big round polyphosphate inclusions with a diameter up to 3 µm were found in the center of the cells in addition to a few smaller inclusions. With increasing sulfide concentrations the size of the polyphosphate inclusions decreased and at very high concentrations also their number.

At low sulfide concentrations no change in size and number of polyphosphate inclusions was visible after 24 h of anoxic incubation (Figures 2a and d), whereas the amount of the inclusions decreased at medium sulfide concentrations (Figures 2b and e). At high sulfide concentrations, inclusions were hardly found after anoxic incubation (Figure 2f). In addition to the decrease in size and number of

polyphosphate inclusions the fluorescence signal weakened, which could be an effect of the increasing amount of sulfur globules diffracting the fluorescence signal.

Sulfur storage

Under oxic conditions the number of sulfur globules, which were visible as dark spots by differential interference contrast microscopy, increased along with sulfide concentrations in the bottom agar (Figures 2a-c). At low sulfide concentrations only some sulfur globules were found, whereas at high sulfide concentrations the cells were filled with sulfur globules and the filaments appeared completely dark. After 24 h of anoxic incubation the amount of sulfur globules had increased considerably for the respective sulfide concentrations (Figures 2d-f). The sulfur globules did not increase only in number but also in size (Figures 2c and f).

Discussion

Our study demonstrates a very high phosphate accumulation capacity of the investigated marine *Beggiatoa* strain under oxic conditions. The excessive energy, which can be gained through sulfide oxidation and does not need to be invested in overall metabolism and cell growth, is spent for a “luxury uptake” of phosphate, which is stored in form of polyphosphate as demonstrated by DAPI staining (Figure 2). If sulfide exceeds concentrations at which the supply of oxygen is not sufficient for a complete oxidation of the sulfide, the capacity to build up polyphosphate decreases (Figure 2c and 4c). In contrast to the results obtained in studies on activated sludge from wastewater treatment plants (Comeau *et al.*, 1986; Fuhs and Chen, 1975), we never observed phosphate release in direct response to the addition of fatty acids under anoxic conditions (Figure 3). However, the upward movement of the *Beggiatoa* filaments under oxic conditions indicates the usage of these compounds. Therefore, it can be concluded that polyphosphate is not decomposed to provide energy for the uptake of fatty acids and the synthesis of polyhydroxyalkanoates. Instead, our data reveal that exposure to high sulfide

concentrations under anoxic conditions is the direct stimulus for polyphosphate decomposition (Figure 2) and phosphate release in the studied *Beggiatoa* strain (Figure 4 and 6). This release is due to the metabolism of living filaments, because it did not occur in controls with heat-inactivated filaments (Figure 5).

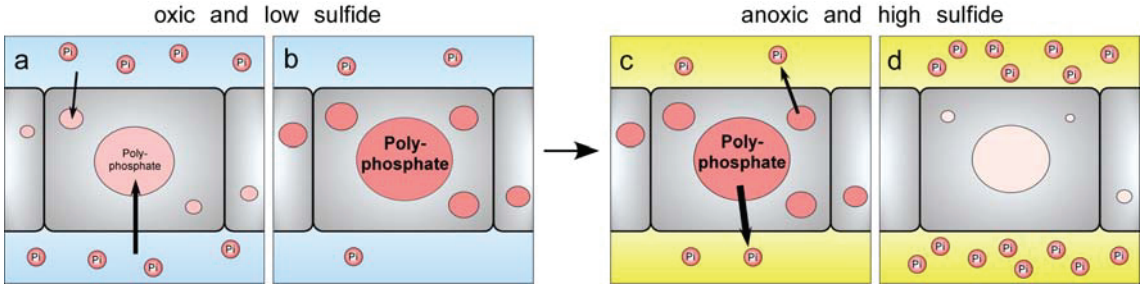

Figure 6 Proposed phosphate uptake and release by *Beggiatoa*. (**a, b**) Under oxic conditions and exposure to low sulfide concentrations phosphate is taken up by *Beggiatoa* and accumulated as polyphosphate. The phosphate concentration in the medium decreases. (**c, d**) When the conditions change to anoxia and exposure to sulfide increases, the *Beggiatoa* decompose polyphosphate and release phosphate. This leads to an increase of phosphate in the medium.

The most common source of high sulfide concentrations in nature is the anaerobic oxidation of organic carbon, including acetate (Widdel and Pfennig, 1981), by sulfate reducing bacteria. Earlier studies on the giant sulfur bacterium *T. namibiensis* showed that phosphate was released under anoxic conditions in response to acetate addition (10 mmol l^{-1} final concentration) (Schulz and Schulz, 2005). In this experiment, *Thiomargarita* cells were taken directly from their original, sulfidic sediment, because no culture of this bacterium is available. In view of the new findings reported here, we suspect that acetate was used by sulfate-reducing bacteria, which derived from the mucus sheaths of *Thiomargarita*, leading to sulfide formation in the medium, which in turn induced the decomposition of polyphosphate. This explanation is in agreement with the observation that phosphate release in anoxic sediments is enhanced in lakes with increased sulfate concentrations (Caraco *et al.,* 1993). As one possible mechanism, the authors suggest an increased phosphate release from microbial polyphosphate pools.

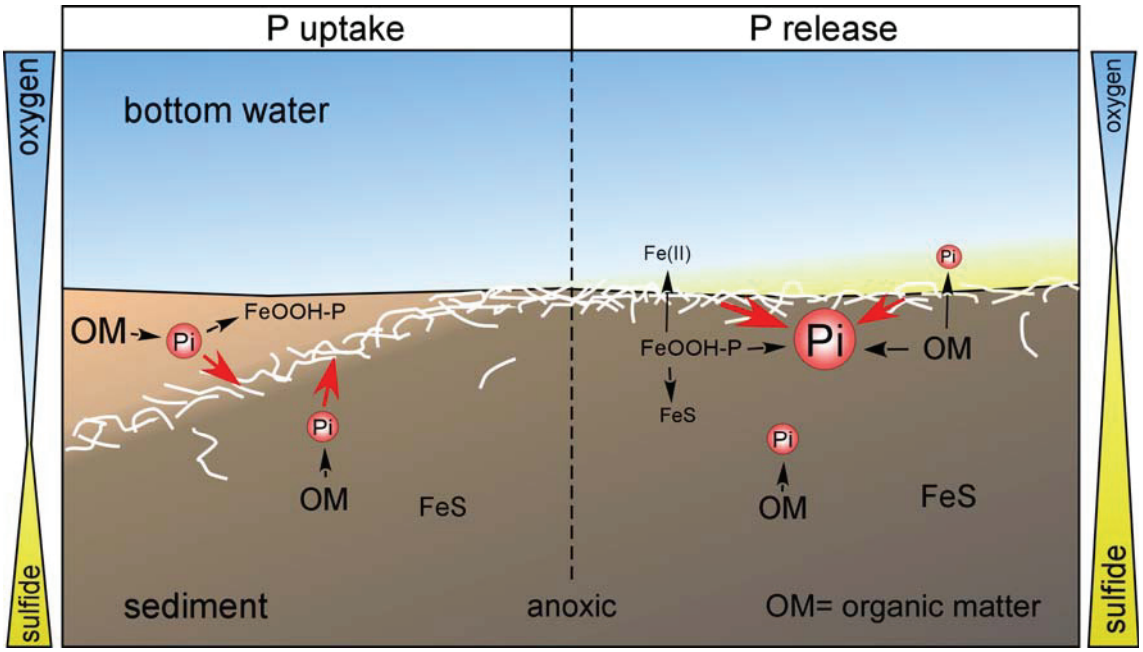

Figure 7 Postulated impact of sulfur bacteria on the phosphorus cycle in coastal marine sediments. Owing to bacterial degradation of organic matter, phosphate is released into the pore water. Under oxic conditions phosphate then is taken up by sulfur bacteria and accumulated internally as polyphosphate (red arrows, left side). Another part of the released phosphate is adsorbed to iron hydroxides. With increasing sulfide concentrations, the sediment becomes completely anoxic and phosphate is released due to dissolution of iron hydroxides. The sulfur bacteria decompose polyphosphate once they have no access to oxygen and are exposed to sulfide. The phosphate is released (red arrows, right side) and the concentration of phosphate in the pore water increases drastically. Part of the released phosphate diffuses into the bottom water.

In addition to the active microbial release of phosphate from polyphosphate, purely chemical processes, collectively referred to as reductive dissolution, could explain the increased phosphate concentrations recorded in some anoxic sediments. According to the classical model established by Einsele (1936), phosphate is retained in oxic lake sediments by coprecipitation on iron hydroxides. This phosphate is released from anoxic sediments when iron is reduced, thus dissolving iron hydroxides and releasing the associated phosphate, as shown in Figure 7. Einsele also showed experimentally that sulfide reduces iron hydroxides and thereby induces the release of phosphate. In spite of the plausibility of this model, Einsele and later authors (Boström *et al.*, 1988; Hupfer and Lewandowski, 2008) realized that it does not always sufficiently explain the observed phosphate fluxes in lake sediments. In marine environments, reactive iron concentrations are lower, as are iron-associated phosphate concentrations (Blomqvist *et al.,* 2004). Nevertheless, high sulfide concentrations in anoxic marine bottom water often co-

occur with increased phosphate concentrations, which is interpreted as a concurrent release of sulfide and phosphate by the bacterial degradation of organic matter (Shen *et al.,* 2002). In sulfide incubation experiments with sediments from eutrophic lagoons Heijs *et al.* (2000) detected a 10-fold higher phosphate release related to the initial ironbound amount of phosphate. Interestingly, these sediments showed a high biological sulfide oxidation potential, which the authors attributed to the presence of colorless sulfur bacteria.

In addition to the two classical models, biological phosphate release in response to acetate and chemical release of iron-bound phosphate, we suggest a third alternative mechanism by which a switch to anoxia may induce increased phosphate concentrations in marine sediments: Phosphate is being trapped by sulfur bacteria through “luxury uptake” under oxic conditions. In response to elevated sulfide concentrations and anoxia, enhanced decomposition of bacterial polyphosphate leads to strong phosphate release. We assume that this mode of phosphate release is the dominant mechanism in coastal sediments with a high input of phosphorus bound in organic matter and dense populations of sulfur bacteria (Figure 7).

Even though the effect of sulfide on phosphate release by the studied *Beggiatoa* strain is obvious, it still remains unclear why anoxic exposure to sulfide has this physiological effect. Among the many functions of polyphosphate, such as an ATP substitute, this compound is important in the physiological adjustment to stress, such as pH changes and nutrient limitation (Kornberg *et al.,* 1999). In the present case, an explanation could be, that the energy provided by the decomposition of polyphosphate is needed to endure sulfide exposure in the absence of a suitable electron acceptor like oxygen or nitrate, which is known to be used by sulfur bacteria for sulfide oxidation (Teske and Nelson, 2006). As changes in the redox conditions are frequent in sulfidic sediments populated by *Beggiatoa*, *Thioploca* and *Thiomargarita*, this so far unknown usage of polyphosphate could act as a kind of “safety system”, which enables survival under unfavorable conditions of low redox potential. On a stoichiometric level it is a good example of how key resources such as energy and mineral nutrients lead to a high variability in the elemental composition of autotrophs (Sterner and Elser, 2002). The C:P ratio of

sulfide-oxidizing bacteria due to a change from oxic to anoxic and sulfidic condition is highly dynamic and is of general ecological importance. It remains to be shown wether other bacteria from habitats with comparably fluctuating conditions like sulfidic hot springs, hydrothermal vents or sulfidic cyanobacterial mats show similar responses.

Impact on precipitation of phosphorus-rich minerals

The active release of phosphate by polyphosphate accumulating sulfur bacteria may lead directly to the precipitation of phosphorous-rich minerals as observed off the coast of Namibia (Schulz and Schulz, 2005), or it could mainly enhance the flux of phosphate from the sediment into the bottom water. The process prevails depends on the rate of phosphate release and the sediment depth at which the release occurs. In general, it seems reasonable to assume that a frequent change from oxic to anoxic conditions in a sulfidic environment, inducing pulses of bacterial phosphate release, will enhance the chances for phosphorite formation on average.

One important difference between freshwater and seawater is that sulfate concentrations are considerably higher in seawater. Consequently, bacterial sulfate reduction is a much more important process in marine sediments compared with freshwater environments. Assuming that phosphogenesis is stimulated by phosphate release of polyphosphate accumulating bacteria and that the key stimulus for bacterial phosphate release is a change from oxic to anoxic conditions in the presence of high sulfide concentrations, we would expect that large accumulations of phosphorites occur more in marine than in freshwater environments, which is certainly the case (Föllmi, 1996). Furthermore, phosphogenesis should occur at locations with very high sulfate reduction rates and changing redox conditions. Indeed, pronounced phosphogenesis is today found in areas of local upwelling, where sulfate reduction rates are exceptionally high (Thamdrup and Canfield, 1996). Within these upwelling areas, phosphorites are preferentially formed in shelf sediments that are located at the boarder of oxygen-depleted water masses (Burnett *et al.,* 1983), where frequent changes between oxic and anoxic conditions in the bottom water prevail. In addition, active

phosphate release in sediments populated by large sulfur bacteria is mostly found in the same depth as a peak of lipid biomarkers indicative of sulfate-reducing bacteria (Arning *et al.,* 2008). Recently, Arning *et al.* (2009) showed that Pleistocenic phosphorite crusts off Peru (ca. 1 mill years old) formed as a result of closely coupled bacterial sulfate reduction and sulfide oxidation.

Massive phosphorite deposits from the geological past usually formed in organic matter-rich sediments such as black shales (Piper and Codispoti, 1975) and are often associated with oceanic anoxic events (Handoh and Lenton, 2003). This supports our assumption that phosphorites formed preferentially in sediments with high sulfate reduction rates. On the other hand, phosphorites are typically enriched in uranium, which indicates a change from oxic conditions, wherein uranium is soluble, to anoxic conditions, wherein uranium precipitates (Baturin and Dubinchuk, 2005). All this is principally in agreement with our observations, which suggest an increased release of bacterial-bound phosphate, inducing the precipitation of phosphorus-rich minerals, in response to a switch from oxic to anoxic conditions under exceptionally high sulfide fluxes. Other factors that seem to have an important role in this process are pH, alkalinity and calcite within the sediment (Reimers *et al.,* 1990; Tribovillard *et al.,* 2010). Consequently, if the massive phosphorite deposits of the past where formed at the boarders of expanding anoxic water bodies, the gradually increasing burial of phosphorus into the sediment would have counteracted further eutrophication. As a result, the rate of oxygen-consuming organic matter degradation would have been decreased. Concurrently with a flux of oxygen from the prior oxygen-enriched atmosphere, oceanic anoxia would have been reversed by this negative feedback mechanism (Handoh and Lenton, 2003).

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (through MARUM Center for Marine Environmental Sciences) and the Max Planck Society.

We thank B Barker Jørgensen, SB Joye, J Peckmann and F Widdel for their helpful remarks on the manuscript, A Bachmann and M Meyer for technical help and M Schubert for construction of the cultivation tubes.

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Chapter 3

Unusual polyphosphate inclusions observed in a marine *Beggiatoa* strain

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In preparation for Journal of Bacteriology

Abstract

Sulfide-oxidizing bacteria of the genus *Beggiatoa* are known to accumulate phosphate as polyphosphate in intracellular inclusions, but little is known about the structure and properties of these inclusions. By the application of different staining techniques we show the presence of unusually large polyphosphate inclusions in the marine *Beggiatoa* strain 35Flor. These inclusions revealed a co-occurrence of polyphosphate, calcium and magnesium when analyzed by scanning electron microscopy and energy dispersive X-ray analysis. Similar to polyphosphate enriched acidocalcisomes of prokaryotes and eukaryotes, the polyphosphate inclusions are enclosed by a lipid layer and store cations. However, they are not notably acidic. 16S rRNA-based phylogenetic reconstruction showed an affiliation of the *Beggiatoa* strain 35Flor to a monophyletic branch, comprising other narrow vacuolated and non-vacuolated *Beggiatoa* species. Concluding from our results, the polyphosphate inclusions represent a new type of storage compartment within the genus *Beggiatoa* aside of mostly nitrate-storing vacuoles known from other marine sulfide-oxidizing bacteria of the family *Beggiatoaceae*.

Introduction

Filamentous *Beggiatoaceae* are ubiquitous in freshwater (Kojima *et al.*, 2003; Strohl and Larkin, 1978), marine (Jørgensen, 1977; Mußmann *et al.*, 2003; Rosenberg and Diaz, 1993) and hypersaline sediments (Hinck *et al.,* 2007). Depending on the sulfide level in the environment they occur as single filaments in the interstitial space of sediments (Mußmann *et al.,* 2003) or as dense mats at hydrothermal vents (Kalanetra *et al.,* 2004; Nelson *et al.,* 1989) and on the sediment surface along coasts with local upwelling (Schmaljohann *et al.,* 2001; Schulz *et al.,* 2000). Filamentous *Beggiatoaceae* consist of up to several hundreds of cylindrical cells and can reach a length of more than 1 cm. The filament widths range in marine strains from 1 to 200 µm, whereas freshwater strains mostly possess widths less than 5 µm (Strohl, 2005).

The different filamentous *Beggiatoaceae* investigated so far show a broad spectrum of metabolic capacities and are heterotrophic or autotrophic (Mußmann *et al.*, 2007; Teske and Nelson, 2006). Autotrophic representatives are able to gain their energy exclusively by the oxidation of the reduced sulfur compounds sulfide (Nelson and Jannasch, 1983) or thiosulfate (Grabovich *et al.,* 2001). As electron acceptor, oxygen (Nelson *et al.,* 1986) and alternatively nitrate (Kamp *et al.,* 2006; Sweerts *et al.,* 1990) are used. In the larger marine representatives, nitrate was shown to be stored in a vacuole, which makes up most of the cell volume (Fossing *et al.,* 1995; McHatton *et al.,* 1996). Elemental sulfur is stored as an intermediate compound within the periplasma in form of spherical inclusions, which are enclosed by invaginations of the cytoplasmic membrane (Strohl *et al.,* 1981). Short-chain fatty acids are stored as polyhydroxyalkanoates (PHA) (Strohl and Larkin, 1978). Other genera of sulfide-oxidizing bacteria of the family *Beggiatoaceae* show similar characteristics (Salman *et al.*, in press). However, *Thiomargarita namibiensis* stores glycogen instead of PHA (Schulz and Schulz, 2005) and species of the genus *Marithrix* (originally described as vacuolated-attached filaments) do not store nitrate in their vacuole (Kalanetra *et al.,* 2004).

Some *Beggiatoa* strains accumulate phosphate as polyphosphate. This was shown by staining with methylene blue (Strohl and Larkin, 1978) or 4',6-Diamidino-2-phenylindole dihydrochlorid (DAPI) (Brock and Schulz-Vogt, 2010), by transmission electron microscopy (TEM) of thin sections (Maier and Murray, 1965) and of whole filaments of *Beggiatoa* (de Albuquerque *et al.,* 2010). Polyphosphate is a polymer of many tens or hundreds of orthophosphate residues linked by high-energy phosphoanhydride bonds (Kornberg, 1995). It is assumed to be a molecule of many functions such as ATP substitute, phosphate reservoir, chelator of metals and may play an important role in the survival and fitness of bacterial cells in general (Ault-Riche *et al.,* 1998; Kornberg *et al.,* 1999; Seufferheld *et al.,* 2008). In a recent study, we could show that the breakdown of internally accumulated polyphosphate within the marine *Beggiatoa* strain 35Flor and the following release of phosphate are mediated by a change from oxic to anoxic cultivation conditions at high sulfide concentrations (Brock and Schulz-Vogt, 2010).

In the present study, we investigated the polyphosphate inclusions in filaments of the same *Beggiatoa* strain in more detail. We stained filaments with DAPI for the detection of polyphosphate inclusions simultaneously with dyes specific for staining lipid layers and acidic cell compartments. By means of scanning electron microscopy (SEM) in combination with energy dispersive X-ray analysis (EDXA) we studied the elemental composition of the polyphosphate inclusions. Further, we investigated the phylogenetic affiliation of the *Beggiatoa* strain 35Flor based on its 16S rRNA gene sequence, enabling the comparison of intracellular structures with closely related strains of the same genus.

Materials and Methods

Cultivation of *Beggiatoa* 35Flor

We used a culture of the marine *Beggiatoa* strain 35Flor, which has been maintained in modified gradient medium according to Nelson and Jannasch (1983) since nine years. Originally, it was collected from a microbial consortium of a black band disease at scleractinian corals from Florida Keys. Beside the *Beggiatoa* strain the culture contains a *Pseudovibrio* sp., which seems to be required for the growth of the *Beggiatoa* strain. To simulate natural growth conditions, the *Beggiatoa* strain was cultivated in sterile aged natural seawater obtained from the North Sea near the island of Helgoland, Germany. A sulfide oxygen gradient (Nelson and Jannasch, 1983) was generated as follows: 4 ml of a solid bottom agar (1.5% Bacto Agar ,BD , NJ, USA), containing 2 mmol l^{-1} Na_2S, 1 mmol l^{-1} NH_4^+ and 40 µmol l^{-1} K_2HPO_4, were overlaid with 8 ml of a semi solid top agar (0.25% Bacto Agar), containing vitamins, 1 mmol l^{-1} NH_4^+, 2 mmol l^{-1} $NaHCO_3$ and 40 µmol l^{-1} K_2HPO_4. The pH of both agars was adjusted to around 8 using HCl. To allow gas exchange with ambient air, the caps of the cultivation tubes were loosely closed. The sulfide gradient in the culture tubes was allowed to establish for one day, before inoculation with the *Beggiatoa* filaments.

Staining of polyphosphate

Granules of polyphosphate were detected by staining with toluidine blue and DAPI. Toluidine blue reveals red inclusions due to a metachromatic effect when binding to polyphosphate (Kulaev *et al.,* 2004). One part of the *Beggiatoa* culture was mixed with two parts of 96% ethanol to dissolve stored sulfur globules. After two days at room temperature, the *Beggiatoa* filaments were heat fixated on a glass slide, washed with H_2O, stained for 30 seconds with toluidine blue (0.3% in 0.5% acetic acid), washed with 0.5% acetic acid and finally with H_2O. The filaments were observed by bright field microscopy using an Axioplan universal microscope (Zeiss, Oberkochen, Germany) equipped with a 100-fold Zeiss NA 1.3 oil immersion objective lens.

DAPI is a common stain for DNA and shows a shift from a blue DNA signal with an emission wavelength around 460 nm to a yellow emission around 525 nm when binding to polyphosphate (Tijssen *et al.,* 1982). A stock solution containing 2.8 mmol l^{-1} DAPI was added to a sample of the *Beggiatoa* culture to a final concentration of 140 µmol l^{-1}. After incubation for 6 hours at room temperature the *Beggiatoa* filaments were inspected for polyphosphate inclusions using a confocal laser scanning microscope (CLSM) LSM 510 (Zeiss, Oberkochen, Germany) equipped with a 100-fold Zeiss Apochromat NA 1.4 oil immersion objective lens. Excitation wavelengths of 351 nm and 364 nm were used and emission detected between 505 − 550 nm. In parallel, a transmission image was collected on the in-build photodiode at a wavelength of 630 nm to check for cell compartments such as vacuoles and sulfur globules. Due to light refraction, sulfur globules appear as dark spots or spheres.

Staining of cell membranes and acidic cell compartments

To study the structure of the polyphosphate inclusions we used several fluorochromes, which are specific for different cell constituents. Imaging was done with the CLSM (see section above). Lipids were stained by the lipophilic dye Nile Red (8 µmol l^{-1}, 10% DMSO, Sigma Aldrich, St Louis, USA; CLSM excitation: 488 nm and 543 nm, CLSM emission: Zeiss LP 585), which is suitable for lipid layer

detection at an emission wavelength above 590 nm (Greenspan and Fowler, 1985), and the yeast vacuole membrane marker MDY-64 (100 µmol l^{-1}, 1% DMSO, Invitrogen, Carlsbad, USA; CLSM excitation: 458 nm, CLSM emission: Zeiss 475 − 525) (Hinck *et al.,* 2007). Acidic cell compartments were visualized by pH-sensitive dyes. Acridine Orange (6 µmol l^{-1}, Sigma Aldrich; CLSM excitation: 488 nm, CLSM emission: Zeiss LP 505) accumulates in acidic cell compartments in dependence of the pH difference. At higher concentrations Acridine Orange shows a shift in emission from green to red because of aggregate formation (Han and Burgess, 2010). As it also binds to DNA and RNA the interpretation of images can be difficult. LysoSensor Green DND-189 (2 µmol l^{-1}, 0.2% DMSO, Invitrogen; CLSM excitation: 488 nm, CLSM emission: 505 − 530 nm) has an emission maximum around 510 nm at low pH values of 4 − 5 (Lin *et al.,* 2001). Each dye was added to a DAPI treated *Beggiatoa* sample and incubated for at least 30 min at room temperature.

Scanning electron microscopy and energy dispersive X-ray analysis

To detect the main elements of which the polyphosphate inclusions are composed of, *Beggiatoa* filaments were observed by SEM in combination with EDXA. After 7 days of growth, *Beggiatoa* filaments were fixed by addition of glutaraldehyde to the growth medium to a final concentration of 2.5% for at least 3 hours. Fixed or unfixed filaments were picked out of their growth medium by means of self-made glass needles. Filaments were washed in H_2O and placed onto 10 × 10 mm silicon wafer supports (Plano, Wetzlar, Germany) that were attached to aluminum stubs and subsequently dried in an exsiccator. SEM was performed in a Hitachi S3200N scanning electron microscope (Hitachi, Tokyo, Japan) operating at 20 kV. The uncoated specimens were investigated either immediately in the low vacuum mode at 5 Pa or after desiccation in the high vacuum mode using a Robinson BSE detector. Digitized pictures were recorded using either the DISS and DIPS software packages (point electronic GmbH, Halle, Germany) or the INCA software package (Oxford Instruments, Wiesbaden, Germany). EDXA measurements of whole filaments or selected spots and areas were performed using an Oxford INCA system (PentaFET Precision INCA X-act detector, INCA software package).

16S rRNA gene sequence amplification and phylogenetic analysis

From the *Beggiatoa* culture tube, 20 µl of the mat were removed with a pipette and collected in 10 ml sterile artificial seawater. With a self-made glass needle, a single *Beggiatoa* filament was removed and washed by dragging it through fresh artificial seawater. Subsequently, the filament was directly used for polymerase chain reaction (PCR). The reaction mix in a final volume of 25 µl contained 0.2 mmol l^{-1} dNTPs, 1 µmol l^{-1} each of forward and reverse primer (GM3F and GM4R) (Muyzer *et al.,* 1995), 1× Fermentas (Fermentas, St. Leon-Rot, Germany), High Fidelity PCR enzyme mix reaction buffer with 1.5 mmol l^{-1} $MgCl_2$ and 1.25 U Fermentas High Fidelity enzyme mix. The filament was disrupted inside the reaction vial using a sterile needle before applying the PCR program with the following steps: initial denaturation at 94°C for 2 min, followed by 25 cycles of 94°C for 30 seconds, 48°C for 30 seconds and 72°C for 2 min. The program was terminated with a final elongation for 7 minutes at 72°C. The PCR product was separated on a 1% agarose gel, cut out and extracted with a kit (Quiagen, Hilden, Germany). The amplicon was directly sequenced using the Big Dye Cycle Sequencing Kit (Applied Biosystems, Carlsbad, USA) and further analyzed on an ABI Genetic Analyzer 3130x (Applied Biosystems). The sequence was deposited in the GenBank/EMBL/DDBJ databases under accession number FR717278.

The phylogenetic affiliation of the sequence was inferred with the ARB software package (Ludwig *et al.,* 2004) based on release 102 of the SILVA SSURef database (Pruesse *et al.,* 2007). For tree reconstruction, 229 nearly full-length 16S rRNA gene sequences and nucleotide positions between 252-1436 (according to *E. coli* numbering) were included. Calculation was based on neighbor joining, maximum parsimony and maximum likelihood methods applying 10, 30 and 50% positional conservatory filters. After comparison of the retrieved trees, a multifurcation tree based on the 10% neighbor joining tree was manually constructed according to the Standard Operating Procedure for Phylogenetic Inference (SOPPI) (Peplies *et al.,* 2008). A distance matrix with similarity correction of the obtained sequence and its closest relatives was calculated with neighbor joining criteria in the ARB program.

Results

Polyphosphate inclusions

Staining of *Beggiatoa* filaments with toluidine blue and DAPI revealed the existence of several polyphosphate inclusions within *Beggiatoa* cells (Figure 1). The width of the *Beggiatoa* filaments was around 6 µm. The size of the polyphosphate bodies was highly variable and ranging from barely visible in the light microscope to inclusions of more than 3 µm (Figure 1 B). The polyphosphate inclusions were irregularly distributed in the cells. Within a single cell, large inclusions were prevalently found in the center whereas several smaller inclusions were found all over the cell volume. Some cells either lacked polyphosphate inclusions completely or possessed only few small ones. In the transmission mode of the CLSM, different cell compartments such as sulfur globules, which were clearly visible as dark spots or small circles, were detected. Large spherical areas in the cell showed congruence with large polyphosphate inclusions stained with DAPI (Figure 1 B-D). Some cell compartments could neither be related to sulfur globules nor to polyphosphate inclusions.

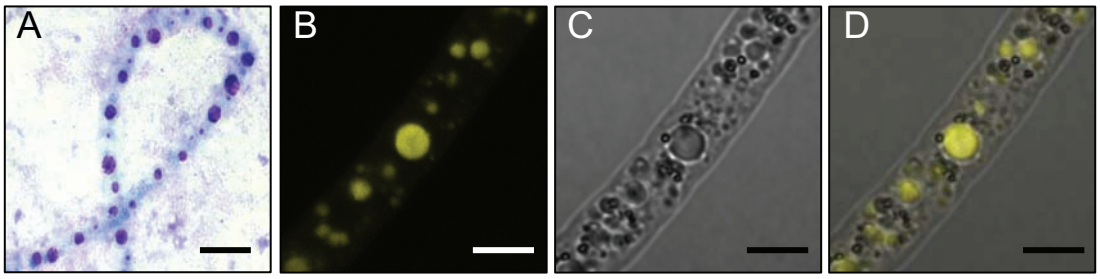

Figure 1 Polyphosphate inclusions within the marine *Beggiatoa* strain 35Flor after 7 days of growth. **(A)** Bright field image of a toluidine blue stained *Beggiatoa* filament. Due to metachromasy, violet polyphosphate inclusions of different sizes are visible. **(B-D)** CLSM images of a DAPI-stained *Beggiatoa* filament. **(B)** Staining with DAPI reveals polyphosphate inclusions of different sizes by a yellow fluorescence signal. **(C)** In the transmission mode of the CLSM, many spherical cell compartments of different sizes are visible. **(D)** The overlay of (B) and (C) reveals congruence of polyphosphate inclusions with the large and with some of the smaller cell compartments. Small dark spots probably represent sulfur granules. Scale bar 5 µm

Membrane and pH staining

Both lipid staining dyes Nile Red and MDY-64 revealed several lipid layer enclosed cell areas and some small completely stained inclusions (Figure 2). The areas enclosed by a lipid layer were of different sizes and mostly of spherical shape. Dual staining with DAPI and Nile Red or MDY-64 revealed that polyphosphate inclusions were enclosed by a lipid layer, probably a membrane. This was less obvious for smaller polyphosphate inclusions as their size was close to the resolution limit of the laser scanning microscope.

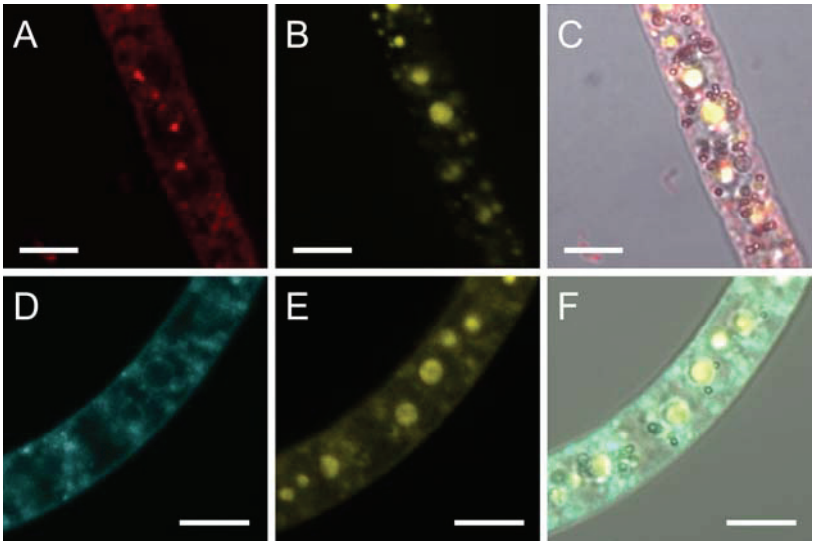

Figure 2 Fluorescence of *Beggiatoa* strain 35Flor filaments obtained by dual staining with DAPI for polyphosphate and Nile Red or MDY-64 for lipid detection. **(A)** Lipid layers of spherical structure and different sizes are visible by staining with Nile Red. **(B)** In the same filament, stained with DAPI, polyphosphate inclusions of different sizes are visible by a yellow fluorescence signal. **(C)** An overlay of the Nile Red and DAPI fluorescence show the existence of lipid layers for most of the large and some of the small polyphosphate inclusions. **(D)** Staining with MDY-64 reveals the same pattern of lipid layers as for Nile Red staining. **(E)** Polyphosphate inclusions of different sizes **(F)** The overlay of MDY-64 and DAPI fluorescence reveals that most polyphosphate inclusions are enclosed by a lipid layer indicating a membrane. Note: The detected internal lipid layers do not exclusively surround polyphosphate inclusions. Scale bar 5 µm

By staining with LysoSensor Green DND-189, some spots with an intensive fluorescent signal were visible (Figure 3 A). Furthermore, spherical areas, which were mostly distributed within the cytoplasmic cell periphery, were detected. The cytoplasm itself showed a weak fluorescent signal. No enhanced fluorescence signal for LysoSensor Green DND-189 indicating a notably acidic milieu was revealed for cell areas with polyphosphate inclusions, which were detected by

DAPI staining (Figure 3 A – C). A similar result was obtained with the pH-sensitive dye Acridine Orange, as this fluorochrome did not accumulate within the large central area of the cell (Figure 3 D – F), which consisted of polyphosphate inclusions, as shown by DAPI staining. In contrast, it highlighted numerous unknown inclusions, which were neither polyphosphate nor sulfur globules. These inclusions showed an intensive fluorescence and burst within seconds under laser illumination.

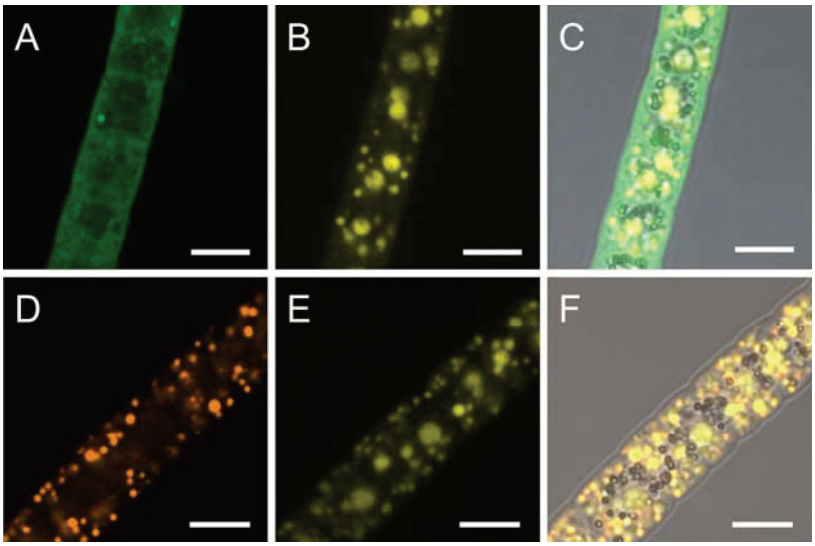

Figure 3 Fluorescence of *Beggiatoa* strain 35Flor filaments obtained by dual staining with DAPI for polyphosphate and the pH-sensitive dyes LysoSensor Green DND-189 or Acridine Orange. **(A)** LysoSensor Green DND-189 shows fluorescence mostly in the cell periphery, indicating a low pH. **(B)** DAPI reveals polyphosphate inclusions of different sizes in the same filament. **(C)** The overlay of (D) and (E) shows no fluorescence for LysoSensor Green DND-189 within polyphosphate inclusions, indicating a not notably acidic pH. **(D)** Acridine Orange reveals several spheres of acidic pH in the cell periphery. **(E)** DAPI reveals polyphosphate inclusions of different sizes in the same filament. **(F)** The overlay of (D) and (E) reveals no fluorescence for Acridine Orange in polyphosphate inclusions, indicating a not notably acidic pH in the inclusions. Scale bar 5 µm

SEM and EDXA

The electron micrograph obtained by back-scattered electrons showed a heterogeneous distribution of elements in the *Beggiatoa* cells (Figure 4 A). Areas with higher concentrations of heavier elements are indicated by a lighter grey value (Figure 4 A). These areas were either detected as a single large spot in the center of a cell or as many small spots in the whole cell. By applying EDXA, significantly higher peaks of phosphorus and oxygen compared to carbon were measured in

these areas. An association with calcium and magnesium was also shown by peaks of these elements at the respective sites within the filament (Figure 4 B). The cytoplasm surrounding these inclusions, as indicated by darker grey values in Figure 4 A, showed much smaller peaks for phosphorus and oxygen than for carbon and no peaks for calcium and magnesium were detected (Figure 4 C). In accordance to the EDXA spectra, element mapping revealed higher amounts of phosphorus, oxygen and calcium for the lighter areas of the electron micrograph as compared to the ambient cytoplasm (Figure 4 D – H). Magnesium, which showed a distinct peak by EDXA revealed a very weak signal by element mapping. The pattern of the cell areas with higher concentrations of heavier elements and the distribution of phosphorus, oxygen and calcium was similar to the distribution of polyphosphate inclusions observed in DAPI stained cells.

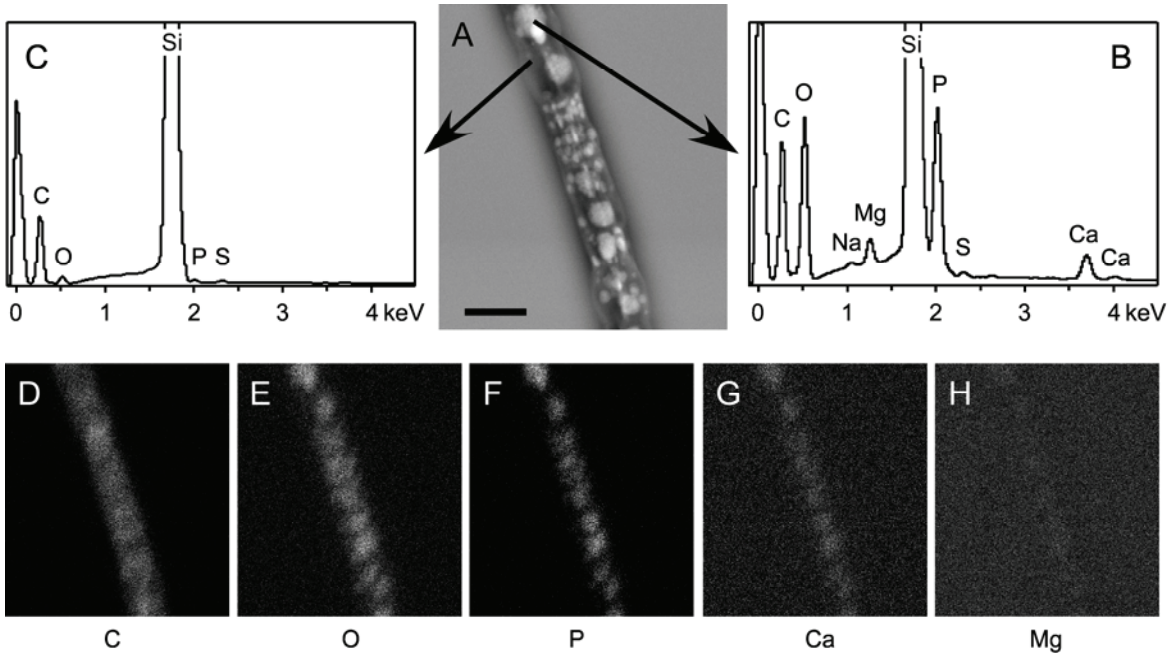

Figure 4 Back-scattered electron micrograph, EDXA spectra and elemental mapping of a *Beggiatoa* strain 35 Flor filament. **(A)** Light areas in the back-scattered electron micrograph of an intact, fixed and air-dried *Beggiatoa* filament indicate high concentrations of elements with higher atomic weight. **(B)** EDXA spectra of these areas reveal large peaks for phosphorus and oxygen and small peaks for calcium and magnesium. **(C)** In adjacent areas of the filament neither calcium nor magnesium peaks are detectable. Very large peaks of silica are resulting from the usage of silica wafer. **(E, F and G)** A clear spatial correlation of oxygen, phosphorus and calcium is obtained by elemental mapping of the filament. **(H)** The signal for magnesium is much weaker than observed for the other elements. Scale bar 5 µm.

16S rRNA-based Phylogeny

Phylogenetic analysis based on the nearly full-length 16S rRNA gene sequence obtained from *Beggiatoa* strain 35Flor confirmed its close affiliation to a monophyletic cluster including other narrow vacuolated and non-vacuolated *Beggiatoa* spp. within the family *Beggiatoaceae* (Figure 5). Within this monophyletic cluster, the obtained sequence was affiliated to a distinct sub-cluster and was closest related to the sequence of the marine strain *Beggiatoa* sp. MS-81-6 (Ahmad *et al.,* 2006), sharing 98.1% sequence identity. The latter strain was isolated from a salt march (Nelson *et al.,* 1982) and exhibited a similar diameter as the strain investigated in this study (4−5 µm). The identified sub-cluster contained sequences of other narrow *Beggiatoa* spp. originating from marine and hypersaline habitats. Interestingly, *Beggiatoa* strain 35Flor was the only one within this monophyletic sub-cluster featuring intracellular vacuole-like structures. Within the other sub-cluster of the genus *Beggiatoa*, only for two other strains, *Beggiatoa* sp. 1124 and *Beggiatoa* sp. “Chiprana” (6−8 µm diameter) internal vacuoles were reported. However, in these bacteria the vacuoles were proposed to contain nitrate as high internal nitrate concentrations were detected (Hinck *et al.,* 2007).

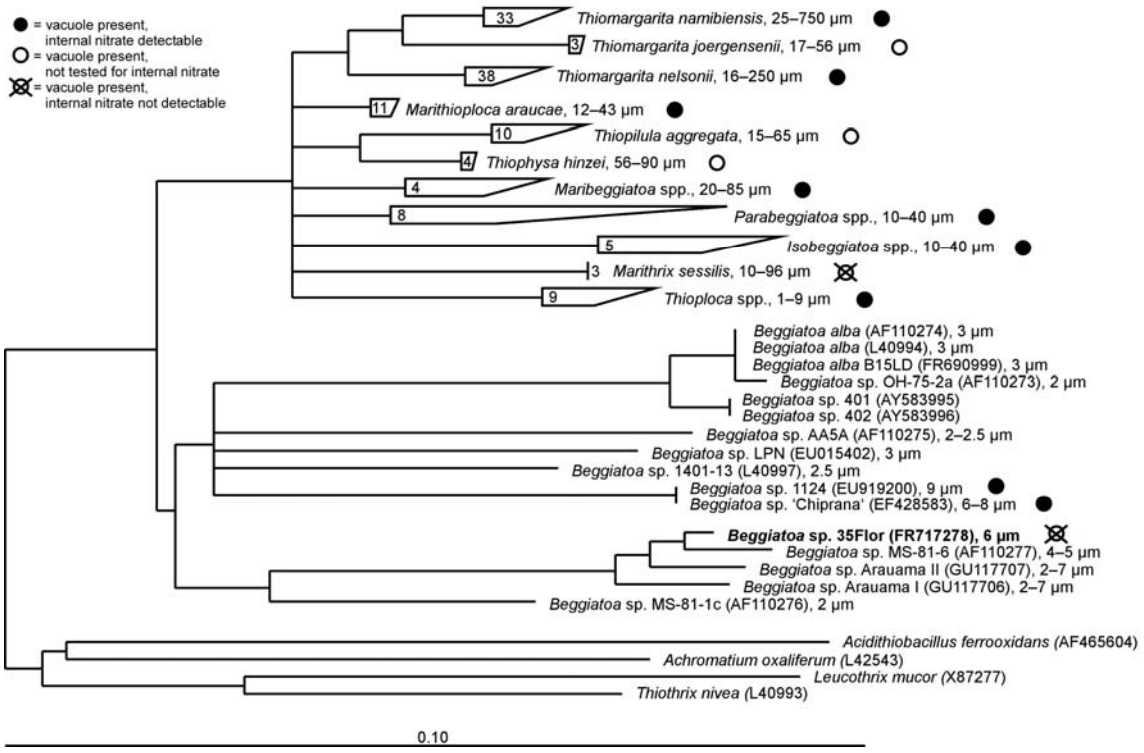

Figure 5 Phylogenetic tree based on 16S rRNA gene sequences showing the affiliation of the investigated *Beggiatoa* strain 35Flor to the genus *Beggiatoa*. The closest relative is the *Beggiatoa* strain MS-81-6. *Beggiatoa* strain 35Flor is the only known species within this sub-cluster which contains intracellular vacuoles. Vacuolation is a common characteristic within the family of *Beggiatoaceae*. Only within the genera *Beggiatoa* and *Thioploca* narrow non-vacuolated representatives have been found so far. For most vacuolated representatives, storage of nitrate within the vacuole is proposed (filled circles) whereas the here described strain is the only one so far having central vacuoles filled with polyphosphate. The nomenclature of sulfide-oxidizing bacteria presented here follows a recent revision of the family *Beggitoaceae* suggested by Salman *et al.* (in press).

Discussion

By staining filaments of the marine *Beggiatoa* strain 35Flor with DAPI, we observed small polyphosphate inclusions and large polyphosphate inclusions of up to 3 µm in diameter (Figure 1). Compared to previous studies on polyphosphate inclusions in bacteria and acidocalcisomes in eukaryotic protists, polyphosphate inclusions of such sizes are very unusual. In *Agrobacterium tumefaciens*, polyphosphate inclusions have been reported with diameters around 210 nm (Seufferheld *et al.,* 2003). Larger polyphosphate bodies were found in the cyanobacterium *Synechococcus* sp. (Lawry and Jensen, 1979), which had a maximum size of

400−500 nm. The largest polyphosphate inclusions of a size more than 2 µm have so far been observed in the non-filamentous, giant sulfide-oxidizing bacterium *Thiomargarita namibiensis* (Schulz and Schulz, 2005), a close relative of *Beggiatoa* (Figure 5). In narrow freshwater *Beggiatoa* spp. polyphosphate inclusions of less than 1 µm have been reported by Maier and Murray (1965). In some narrow, hypersaline *Beggiatoa* such inclusions of similar size were just recently reported by de Albuquerque *et al*. (2010). However, both size and number of polyphosphate inclusions greatly depend on the environmental conditions, such as the sulfide and oxygen regime, the bacteria are exposed to (Brock and Schulz-Vogt, 2010). Therefore, it is difficult to compare the storage capabilities of polyphosphate in different organisms which experienced different environmental conditions leading to differences in the physiological state of the cell. In conclusion, we propose that the polyphosphate inclusions detected in the marine *Beggiatoa* strain 35Flor are the largest described so far in the family *Beggiatoaceae* and probably in bacteria in general.

The polyphosphate inclusions analyzed in this study are similar to acidocalcisomes of eukaryotes as they are also enclosed by a lipid layer, likely a membrane (Figure 2), and store cations such as calcium and magnesium within the inclusion (Figure 4). Bacterial organelles similar to acidocalcisomes have already been found in other bacteria like *Agrobacterium tumefaciens* (Seufferheld *et al.,* 2003). Acidocalcisomes are described as acidic polyphosphate enriched cell compartments, which are enclosed by a lipid membrane possessing ATPases, pyrophosphatases and different cation and anion transporters (Docampo *et al.,* 2005). In contrast to the properties of acidocalcisomes, we could not detect significant acidic conditions within the polyphosphate inclusions by dual staining with DAPI and LysoSensor Green DND-189 or Acridine Orange (Figure 3). A specific fluorescence increase between pH 4 and 5 as typical for LysoSensor Green DND-189 could not be observed. Also Acridine Orange, which would be indicative for a pronounced pH difference between cytoplasm and polyphosphate, showed no accumulation in the inclusion (Ramos *et al.,* 2010). Nevertheless, we observed smaller acidic compartments within the cell (Figure 3), which are not associated with polyphosphate, but indicate that a staining for acidic parts was

principally successful. Thus, we conclude that at least under our experimental conditions the large polyphosphate inclusions are not notably acidic.

Among the group of large sulfide-oxidizing bacteria, the investigated polyphosphate inclusions constitute a new type of vacuolar compartments. They are unique intracellular components in addition to nitrate storing vacuoles, which are known from the large marine genera such as *Marithioploca*, *Maribeggiatoa* and *Thiomargarita* and from smaller freshwater *Thioploca* species. In these genera the stored nitrate serves as electron acceptor for sulfide oxidation (McHatton *et al.,* 1996; Otte *et al.,* 1999; Schulz *et al.,* 1999; Zemskaya *et al.,* 2009). For the genus *Marithrix* vacuoles without nitrate have been observed, but the function of these vacuoles is still under debate (Kalanetra and Nelson, 2010). Therefore, vacuolar structures in sulfide-oxidizing bacteria can serve other purposes than nitrate storage. The only genus, in which both a large nitrate storing vacuole and polyphosphate inclusions have been unambiguously detected, is the genus *Thiomargarita*. In contrast to the genera *Beggiatoa* and *Thiomargarita*, polyphosphate inclusions seem to be absent in *Marithioploca* spp. (Holmkvist *et al.,* 2010).

In an earlier study (Brock and Schulz-Vogt, 2010) we could show for *Beggiatoa* strain 35Flor that decomposition of polyphosphate enables the *Beggiatoa* filaments to survive short periods of exposure to sulfide without an electron acceptor such as oxygen or nitrate. Although, the cellular mechanism behind this physiological correlation remains unknown we assume that polyphosphate-filled vacuoles in narrow *Beggiatoa* spp. might have similar functions as nitrate-filled vacuoles in the larger species of the family *Beggiatoaceae.* In the latter, the vacuoles contain nitrate concentrations of up to 800 mmol l^{-1}, which is used for sulfide oxidation under diminished oxygen concentrations (McHatton *et al.,* 1996; Otte *et al.,* 1999; Schulz *et al.,* 1999).

The narrow *Beggiatoa* spp. define the phylogenetic root of the family *Beggiatoaceae* as shown by Ahmad *et al.* (2006) and by our 16S rRNA data (Figure 5). Since the polyphosphate inclusions in *Beggiatoa* 35Flor are enclosed by a lipid layer, probably a vacuolar membrane, it is likely that the polyphosphate

inclusions are related to the nitrate storing vacuoles found in the other genera of the *Beggiatoaceae*. This implies that the ability to form vacuoles is a basic evolutionary feature of the family *Beggiatoaceae* and that the modulation of the vacuolar size and function might reflect adaptations to different environmental conditions. Within the genus *Beggiatoa*, a large central vacuole for nitrate storage has so far only been found in two strains, which are the widest representatives of this genus (6-9 µm). Likewise, the *Beggiatoa* strain 35Flor is among the widest of its phylogenetic group (6 µm) and features a compartmentalization by polyphosphate inclusions. This observation suggests that the overall expression of vacuoles in a filament strongly depends on the filament diameter, as only organisms beyond 5 µm so far contain intracellular vacuoles. This conclusion is in congruence with the absence of diffusion limitation in cell diameters below 5 µm (Schulz and Jørgensen, 2001). However, also narrow filaments (around 4.4 µm) possess small polyphosphate inclusions (around 150 nm) that even compartmentalize these thin filaments (de Albuquerque *et al.*, 2010).

In conclusion, the large polyphosphate inclusions of *Beggiatoa* strain 35Flor share similarities with acidocalcisomes and vacuoles in general. They possibly constitute an adaptation of narrow sulfide-oxidizing bacteria to their habitat and to their increasing cell size comparable to the nitrate storing vacuoles known from other genera of *Beggiatoaceae*. The habitats of *Beggiatoa* spp. and its relatives include organic rich sediments, vent systems and cyanobacterial mats, which are characterized by frequently changing gradients of sulfide, oxygen and nitrate. The storage of metabolites such as energy reserves is therefore a crucial life-strategy of gradient organisms and now includes the here identified large polyphosphate inclusions as one more extraordinary feature among these unusual bacteria.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (through MARUM Center for Marine Environmental Sciences) and the Max Planck Society. We thank M. Meyer for technical help.

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Chapter 4

Large sulfide-oxidizing bacteria can accumulate and release uranium concurrently with phosphate

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In preparation for Geobiology

Abstract

The phosphorus-rich mineral apatite, which is formed in sediments of coastal upwelling areas, is enriched in major and minor elements. Release from organic matter during degradation and abiotic redox processes are proposed to be responsible for this enrichment. In this study we compared metal enrichment in an apatite bearing Namibian sediment populated by a high number of *Thiomargarita* spp. and uptake and release of metals by a culture of the phylogenetic related *Beggiatoa* strain 35Flor in a laboratory experiment. Energy dispersive x-ray fluorescence analysis revealed an enrichment and correlation of phosphorus with strontium, uranium, aluminum and yttrium within a sediment layer of newly formed apatite. In the laboratory experiment with *Beggiatoa* 35Flor we measured by inductivity coupled plasma analysis a decrease in concentration of metals, such as calcium, strontium and uranium according to growth of the culture at oxic conditions and an increase under following anoxic conditions. Uranium was the only metal which was taken up and released in correlation to phosphate, when polyphosphate was build up and later degraded. From these results we conclude that uranium is stored together with polyphosphate by the bacteria and released concurrently with phosphate. Thereby, high pore water concentrations of uranium are generated directly by the bacteria when phosphate is released, which can enhance the enrichment of uranium in precipitating phosphorous rich minerals.

Introduction

Areas of coastal upwelling are hot spots of modern phosphogenesis, the formation of phosphorus-rich minerals (Föllmi, 1996), which is assumed to be mediated by bacterial activity (Krajewski *et al.,* 1994; Nathan *et al.,* 1993). The organic-rich sediments beneath areas of coastal upwelling, such as the shelf of Namibia, Chile, Peru and the northeastern Arabian Sea, are densely populated by large sulfide-oxidizing bacteria of the family *Beggiatoaceae* including the genera *Beggiatoa*, *Thiomargarita* and *Thioploca* (*Marithioploca* according to revision by Salman *et al.*, (in press)) (Gallardo, 1977; Schmaljohann *et al.*, 2001; Schulz *et al.*, 1999). Further

more, fossils, which resemble in size and shape the large sulfide-oxidizing bacteria *Thiomargarita* spp. (Bailey *et al.,* 2007) or filamentous *Beggiatoa* spp. (Reimers *et al.,* 1990) often occur in ancient formations rich in phosphorite. Thus, sulfide oxidizing-bacteria have been suggested as one possible trigger for phosphorite formation. This assumption was confirmed by Schulz and Schulz (2005), who could show that the large sulfide-oxidizing bacterium *Thiomargarita namibiensis* can mediate the formation of phosphorite by the release of phosphate from internally accumulated polyphosphate.

Recently, we identified the stimulus for the decomposition of polyphosphate and fast release of phosphate in the marine *Beggiatoa* strain 35Flor. Phosphate release was stimulated by a change from oxic to anoxic conditions in the presence of high sulfide concentrations (Brock and Schulz-Vogt, 2010). This finding is supported by the results of Arning *et al.* (2008; 2009a) who reported a close spatial association of sulfate-reducing and sulfide-oxidizing bacteria in both organic-rich sediments which feature very high phosphate concentrations in the pore water and in authigenic apatite, from the upwelling areas off Peru, Chile and Namibia. More recently, Goldhammer *et al.* (2010) could show by incubation experiments with radioactively labeled phosphate that *Thiomargarita* spp. as well as *Beggiatoa* spp. are responsible for authigenic apatite formation in Namibian sediments. Furthermore, they reported a rate of phosphate-to-apatite conversion under anoxic bottom waters that exceeded the rate of phosphate release during organic matter mineralization.

In this study we investigated which elements are enriched in apatite formed by the phosphate release of *Thiomargarita* spp. in Namibian sediments (Schulz and Schulz, 2005). To evaluate, whether the accumulation of the identified elements can be mediated by the activity of large sulfide-oxidizing bacteria, we performed a laboratory study with a polyphosphate accumulating marine *Beggiatoa* strain, grown under defined conditions in natural seawater. As we can manipulate this strain to accumulate and release polyphosphate (Brock and Schulz-Vogt, 2010) we could observe which elements are stored and released concurrently with phosphate.

Methods

Sediment sampling and processing

The investigated sediment was sampled during a Meteor cruise in March 2003 by means of a multicorer on the Namibian shelf at 22°10´S; 14°03´E at a water depth of 70 m. For element determination of the solid phase the core was cut in intervals of 1 cm for the upper 10 cm and 2 cm or 5 cm layers for deeper parts. The sediment was dried in a laboratory oven and ground by hand to obtain a homogenous material. Elements were determined on board by energy dispersive x-ray fluorescence analysis (EDXRF) using a Spectro Xepos (Spectro, Kleve, Germany).

Cultivation

We used the marine *Beggiatoa* strain 35Flor as model for the impact of sulfide-oxidizing bacteria of the family *Beggiatoacea* on pore water chemistry. The *Beggiatoa* strain was isolated from a black band disease of scleractinian corals from Florida Keys, and has been grown in mineral gradient medium since 9 years. A *Pseudovibrio* strain is associated with the *Beggiatoa* strain. In a previous study the *Beggiatoa* strain was shown to accumulate phosphate as polyphosphate in high amounts in internal inclusions (Brock and Schulz-Vogt, 2010). Cultivation was carried out in a modified sulfide oxygen gradient medium (Nelson and Jannasch, 1983) using buffered aged natural seawater from the North Sea near Helgoland, Germany. In the culture tubes 15 ml of a solid agar containing 1.5% bactoagar (BD, NJ, USA), 24 mmol l^{-1} Na_2S, 10 mmol l^{-1} HEPES buffer, 0.5 mmol l^{-1} $(NH_4)_2SO_4$ and 40 µmol l^{-1} K_2HPO_4 in natural seawater were overlaid with 30 ml of a semi solid agar containing seawater with 0.75% bactoagar, 10 mmol l^{-1} HEPES buffer, 0.5 mmol l^{-1} $(NH_4)_2SO_4$, 2 mmol l^{-1} $NaHCO_3$, 40 µmol l^{-1} K_2HPO_4 and vitamins. After autoclaving the pH of both components was adjusted to 7.5. The medium was prepared in polycarbonate tubes of 125 mm heights, 26 mm inner diameter and with 2 mm wide holes drilled in 1 mm intervals into the wall. The holes were sealed

with autoclavable tape and the top closed with autoclaved aluminium foil allowing gas exchange with ambient air.

Beggiatoa 35Flor experiment

The medium was inoculated after one day to allow for the development of oxygen and sulfide gradients. After 8 days of growth, when a dense *Beggiatoa* mat had established, about 1.15 ml of the medium was sampled from three replicates without bacteria and from three replicates with the *Beggiatoa* culture. At the same time 15 tubes with *Beggiatoa* and 3 tubes without bacteria were closed with butyl rubber stoppers and the headspace was flushed with nitrogen for 5 minutes to establish anoxic conditions. The tubes were placed in an anaerobic jar, which was flushed two times with nitrogen for 10 minutes at an interval of 30 minutes. Three anoxic *Beggiatoa* cultures were sampled each after 12, 24, 36, 48 and 72 hours. At the last time point three anoxic tubes without bacteria were sampled. Sampling was carried out using microrhizones (Rhizosphere Research Products, Wageningen, The Netherlands) with a length of 20 mm, a diameter of 1 mm and a pore size of 0.2 µm through a hole of the tube 3 mm below the surface of the semi solid agar.

Microscopy and Staining

From one of three replicates a sample of 95 µl was taken from the zone of high abundance of *Beggiatoa* filaments. 4',6-Diamidino-2-phenylindole dihydrochlorid (DAPI) was added from a stock solution of 2.8 mmol l^{-1} to a final concentration of 140 µmol l^{-1}. DAPI binds to polyphosphate and emits fluorescence with a maximum at 525 nm when excited by UV-light (Tijssen et al., 1982). The *Beggiatoa* filaments were observed by fluorescence microscopy using an Axiophot universal microscope (Zeiss, Oberkochen, Germany) equipped with a 100-fold Zeiss Neofluar NA 1.3 oil immersion objective lens. UV-light was generated using a HBO 50 mercury lamp (Osram, München, Germany). Emission was detected using a UV-G 365 filter set (G 365 exciter filter, FT 395 chromatic beam splitter and an LP 420 barrier filter, Zeiss). Images were taken by an AxioCam MRm monochrome CCD camera and processed by zeiss axiovision 4.3 software (Zeiss).

Phosphate measurement

Phosphate was determined colorimetrically by the ascorbic acid method modified after Hansen and Koroleff (Hansen and Koroleff, 1999). To avoid possible interference of hydrogen sulfide a subsample of 100 µl was filled into 2 ml glass tubes and gently bubbled with dinitrogen gas for one minute. Subsequently, the sample was diluted with 400 µl H_2O. 10 µl of an ascorbic acid solution containing 0.1 g ascorbic acid dissolved in 5 ml H_2O and 5 ml 4.5 mol l^{-1} H_2SO_4 were added. The reaction was started by addition of 10 µl heptamolybdate solution containing 4 g $(NH_4)_6$ Mo_7O_{24} x 4 H_2O in 30 ml H_2O, 90 ml 4.5 mol l^{-1} H_2SO_4 and 5 ml tartrat solution (2.5 g $C_4H_4KO_7Sb$ x 0.5 H_2O in 100 ml H_2O). After 30 min the absorption was measured at a wavelength of 695 nm using a SpectroDirect Spectrophotometer (Aqualytic, Dortmund, Germany). Calibration was performed with standards of 0, 6.3, 12.6, 18.9 and 25.2 µmol l^{-1} phosphate prepared from a titrisol stock solution (Merck, Darmstadt, Germany).

Element determination

A sample volume of 1 ml was acidified with 30 µl of suprapur HNO_3 and stored at 4°C. Before measurement the samples were again filtered using microrhizones to remove colloidal sulfur, which precipitated during storage. Part of the sample was diluted 1:10 with diluted HNO_3 and the following elements were detected using an Inductivity Coupled Plasma - Optical Emission Spectrometer (ICP-OES): aluminum, barium, calcium, iron, potassium, magnesium, manganese, strontium and titanium. Another part of the sample was diluted 1:50 with diluted HNO_3 and the following elements were detected by the use of an Inductivity Coupled Plasma - Quadrupole Mass Spectrometer (ICP-MS): iron, arsenic, cadmium, yttrium, uranium, titanium, chromium and manganese.

Results

Element distribution in Namibian sediment

EDXRF analysis of the investigated sediment from the Namibian shelf showed very high contents of phosphorus in the first 5 cm for all three replicates. Peaks with maximal contents of phosphorus from 36880 to 50750 mg/kg are found at 3 and 4 cm (Figure 1a). Within these layers a high number of *Thiomargarita* cells were observed (Schulz and Schulz, 2005). Layers below 8 cm without cells of *Thiomargarita* show phosphorus contents below 5000 mg/kg. In the same layer, where the phosphorus peak was detected, distinct peaks of calcium, strontium, yttrium, aluminum and uranium occurred (Figure 1 b-f) as well as less pronounced peaks of barium and titanium.

The elements calcium, strontium, yttrium, aluminum and uranium show a strong positive correlation with phosphorus and barium, titan, chromium and potassium a weak positive correlation (Table 1). Other detected elements show neither a peak in the depth of high phosphorus contents nor a correlation to phosphorus in general. Silica is negatively correlated to phosphorus. By averaging the contents of the elements within the phosphorus peak layers from 2 to 4 cm and comparison to their average contents in deeper layers from 10 to 20 cm it becomes apparent that the elements with the strongest positive correlation are also the ones with the highest enrichment. Enrichment factors vary between 4 for uranium and aluminum, 7 for strontium and 15 for phosphorus (Table 1).

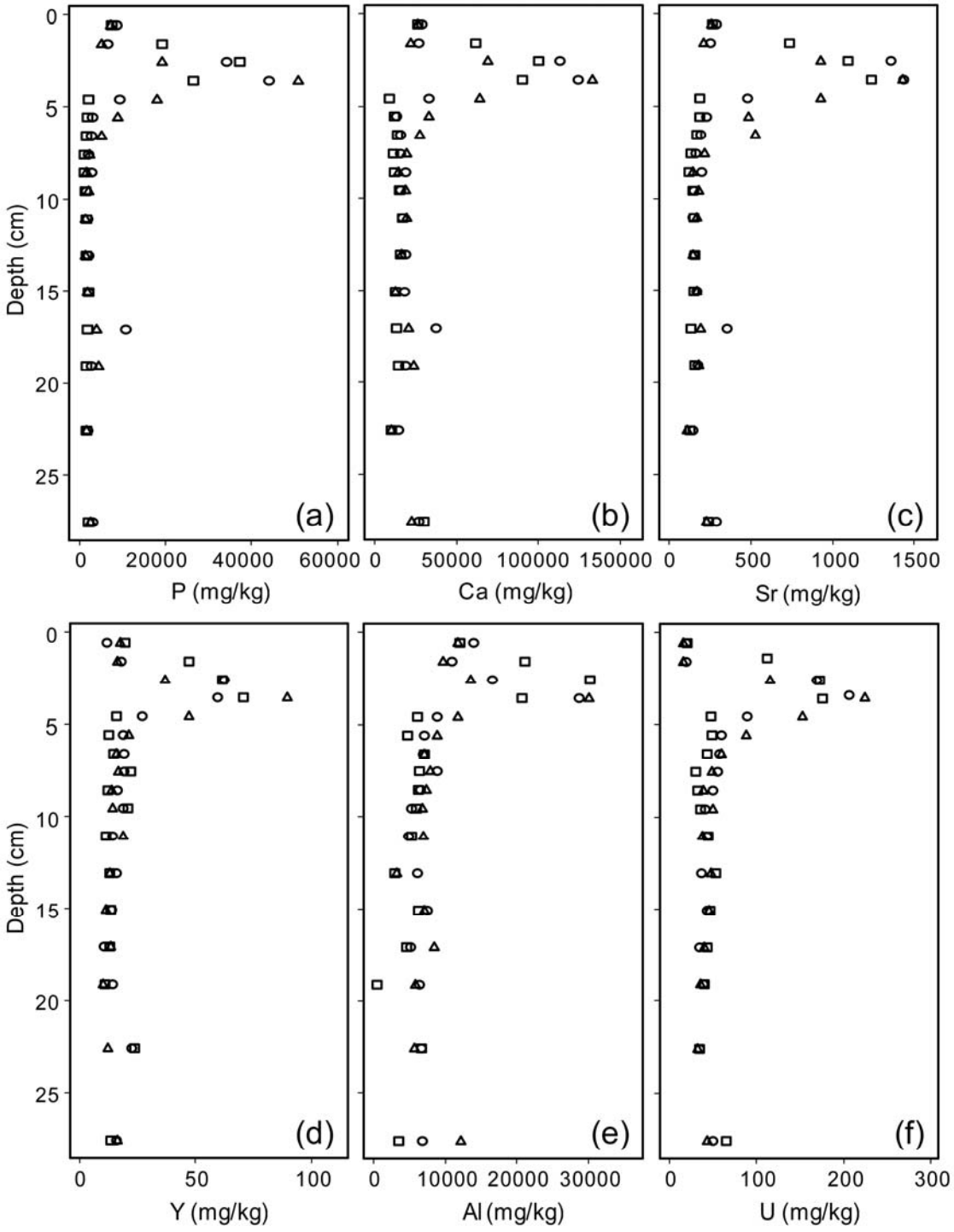

Figure 1 Element distribution in sediment from the Namibian shelf populated by a *Thiomargarita* mat at 1-3 cm depth. Contents of three replicates are shown in mg/kg for (a) phosphorus, (b) calcium, (c) strontium, (d) yttrium, (e) aluminum and (f) uranium.

Table 1 Average contents of elements in sediment from the Namibian shelf with a *Thiomargarita* mat at 1-3 cm depth. Phosphorus-rich layers from 2 to 4 cm and deeper layers with no significant apatite content as background are averaged. The element enrichment and correlation to phosphorus are calculated.

element	In the P peak (mg/kg, 2-4 cm, n=6)	background (mg/kg, 10-20 cm, n=15)	enrichment in the P peak	correlation with P (R^2, n=51)
P	35017	2411	15	
Ca	104322	17759	6	0,95
Sr	1239	168	7	0,93
Y	63	13	5	0,88
Al	23165	5242	4	0,85
U	177	41	4	0,85
Ba	284	109	3	0,71
Ti	2762	1662	2	0,65
Cr	165	94	2	0,65
K	14392	9667	1	0,61
Mn	259	180	1	0,38
Mg	17017	10080	2	0,34
S	13878	10104	1	0,27
Zn	54	47	1	0,21
Cd	33	21	2	0,19
Fe	18217	16422	1	0,13
Rb	55	48	1	0,11
As	46	17	3	0,08
Ga	8	7	1	0,01
V	232	219	1	0,001
Pb	10	11	1	-0,01
Th	9	10	1	-0,03
Br	200	330	1	-0,07
Tl	4	6	1	-0,13
Cl	19532	34767	1	-0,26
Ni	43	63	1	-0,28
Cu	22	33	1	-0,36
Se	10	18	1	-0,4
Si	200150	448820	0,4	-0,64

Beggiatoa experiment

The total phosphate concentration in the *Beggiatoa* culture of about 40 µmol l^{-1}, which was added to the culture medium, decreased to about 0 µmol l^{-1} within 8 days of oxic growth (Figure 2a). The *Beggiatoa* filaments were mostly found within a distinct mat about 1 mm below the agar surface, but a high number of filaments were also found below the mat (data not shown). DAPI staining of filaments sampled from the *Beggiatoa* mat revealed several inclusions of polyphosphate in the filaments (Figure 3a) at this time point. The phosphate concentration increased to around 7 µmol l^{-1} after 48 h and around 21 µmol l^{-1} after 72 h of anoxic and sulfidic conditions (Figure 2a). At this time point most *Beggiatoa* filaments were found at the top of the medium. Concurrently to the release of phosphate, the amount of polyphosphate within the *Beggiatoa* filaments decreased and only a few polyphosphate inclusions could be detected by DAPI staining (Figure 3b).

Together with phosphate uranium was taken up by the *Beggiatoa* culture during oxic growth (Figure 2b). As compared to the sterile control the concentration decreased by about 0.004 µmol l^{-1}. During 72 h of anoxic and sulfidic conditions the uranium concentration increased by 0.001 µmol l^{-1} to around 0.0078 µmol l^{-1}. In the same time, the concentration of uranium in the sterile control decreased by about 0.001 µmol l^{-1}. For the elements calcium and strontium a decrease in concentration was observed during oxic growth, but without pronounced differences in the *Beggiatoa* cultures compared to the sterile controls (Figure 2c and d). With values of about 9900 µmol l^{-1} calcium and 81 µmol l^{-1} strontium in the *Beggiatoa* culture the background concentrations were of several orders higher as for uranium. For both *Beggiatoa* culture and sterile control an increase in concentration could be detected during 72 h of anoxic and sulfidic conditions. Similar results were obtained for magnesium and potassium (data not shown). The concentrations of yttrium and aluminum in the seawater medium were too low to draw conclusions.

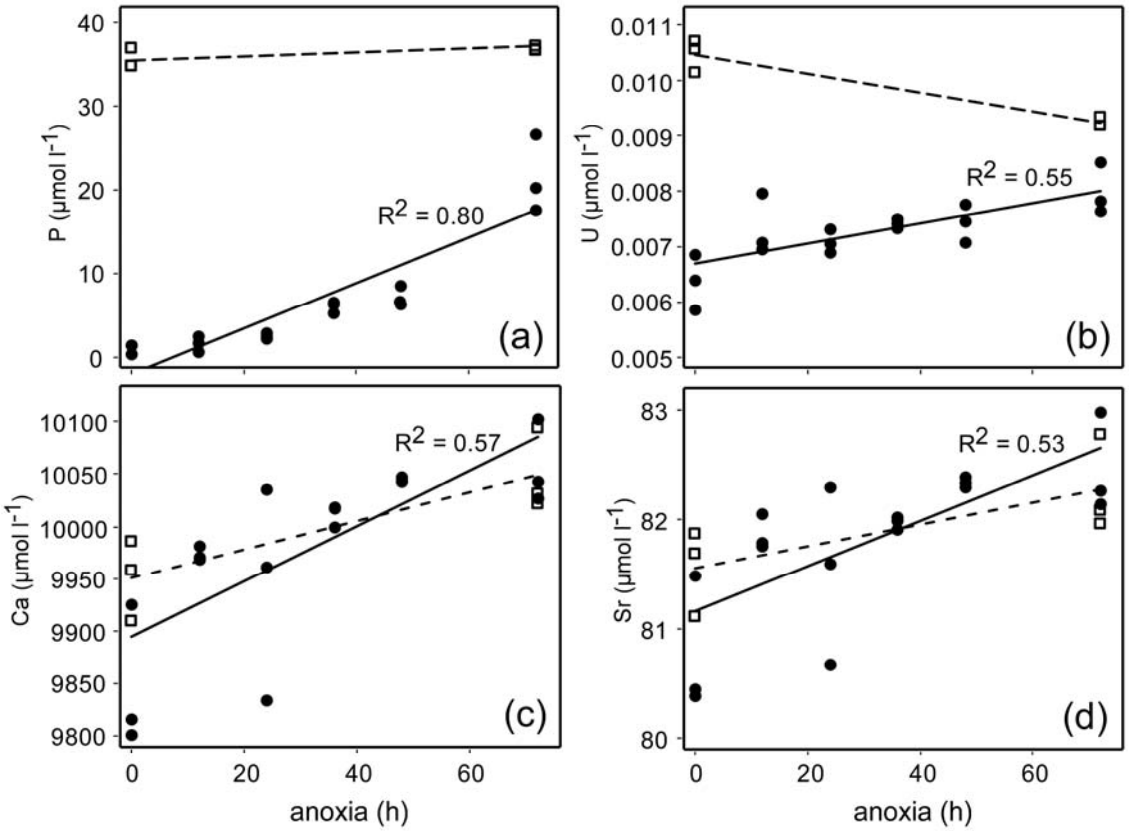

Figure 2 Influence of anoxia on the phosphate and element concentration in a *Beggiatoa* culture. Concentrations in the medium of three parallels are shown in µmol l^{-1} for (a) phosphate as phosphorus, (b) uranium, (c) calcium and (d) strontium during 72 h of anoxic and sulfidic conditions in a *Beggiatoa* culture (●) and a sterile control (□). The solid line shows the trend line for the *Beggiatoa* culture and the dashed line for the sterile control. The experiment was started after 8 days of oxic growth.

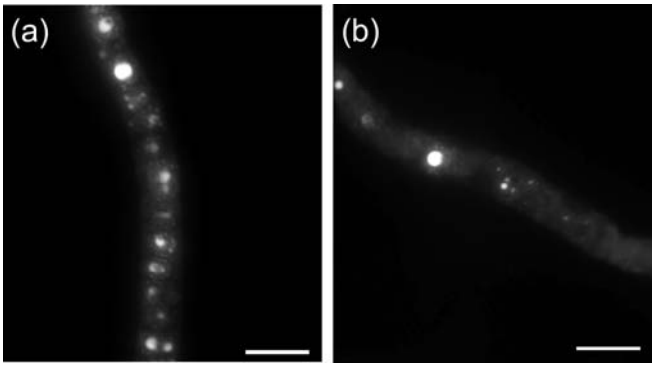

Figure 3 Accumulation of polyphosphate in the *Beggiatoa* strain. (a) DAPI staining reveals several polyphosphate inclusions of different sizes in a *Beggiatoa* filament after 8 days of oxic growth. (b) After 72 h of anoxic and sulfidic conditions less polyphosphate inclusions are detectable. Scale bar 10 µm

Discussion

The depth profiles in the investigated Namibian sediment revealed several elements, which are enriched in the same depth as phosphorus, above all calcium, strontium, yttrium, aluminum and uranium (Figure 1). The high contents of phosphorus and calcium reflect apatite as demonstrated by X-ray diffraction analysis (Schulz and Schulz, 2005). Due to the co-occurrence of a high number of polyphosphate storing sulfide-oxidizing bacteria of the genus *Thiomargarita*, which were shown to release phosphate in an incubation experiment (Schulz and Schulz, 2005), it was suggested that the precipitation of apatite is likely mediated by these bacteria. The correlation of strontium, yttrium and uranium with phosphorus in the apatite layer of the sediment can be explained by substitution of calcium within the apatite matrix (Ames, 1960). Other elements like potassium, magnesium, and cadmium are described as possible calcium substitutes (Jarvis *et al.*, 1994 and references therein), but the distribution of these three elements in the sediment did not show a pronounced correlation with phosphorous (Table 1). The aluminum peak is surprising, because it is not typically associated with phosphorous rich layers. Higher contents of aluminum in marine sediments are generally attributed to clay of terrigenous source, but the overall terrestrial input into sediments of the Namibian Shelf is low (Borchers *et al.*, 2005).

In our laboratory experiment with the *Beggiatoa* strain 35Flor, a close relative of *Thiomargarita namibiensis*, uranium was the only detectable element, that was taken up and released in correlation with the build up and break down of polyphosphate (Figure 2). During oxic growth around 0.004 µmol l^{-1} uranium, one third of the total amount was taken up together with around 40 µmol l^{-1} phosphate. Under the following anoxic and sulfidic conditions 0.001 µmol l^{-1} uranium was released together with 20 µmol l^{-1} phosphate. Due to the change of redox conditions a part of the uranium was probably lost by precipitation (Jarvis *et al.*, 1994). If we assume that this part was similarly high as the decrease of uranium in the sterile control, about 0.002 µmol l^{-1} uranium would have been released together with 20 µmol l^{-1} phosphate. Thus, during both phosphate uptake and release uranium is accumulated and excreted with a factor of 1:10000 which indicates that

uranium is associated with the phosphate metabolism and may be bound to polyphosphate.

The relationship of uranium and phosphate, the U/P ratio for uptake and release is around 1 x 10^{-4}, which is in the range of U/P ratios of natural phosphorites. Calvert and Price (1983) reported in phosphorites from the Namibian shelf U/P ratios around 9 x 10^{-4}. Baturin and Kochenov (2001) calculated from a wide range of ancient and modern phosphorites an average U/P_2O_5 ratio of 3.2 x 10^{-4}. However, the U/P ratio of 5 x 10^{-3} in the investigated Namibian sediment in the zone of the phosphorus peak is higher. This can be explained by the very recent precipitation of apatite, because subsequent catagenic processes, such as oxidation of the insoluble uranium(IV) to soluble uranium(VI), lead to a depletion of uranium in phosphorites (Baturin and Kochenov, 2001). Another reason might be that the amount of uranium taken up by sulfide-oxidizing bacteria probably depends on the concentration of uranium in their surrounding. The *Beggiatoa* in our experiment have accumulated uranium from seawater with a rather low uranium concentration, whereas sediments may contain much higher concentrations (Baturin, 1971). Furthermore, it has to be considered, that the experiment is carried out in a closed system, where the concentration of uranium in the medium is decreasing during uptake in contrast to the natural system, where uranium can be replaced by diffusion from the bottom water (Baturin and Kochenov, 2001; Veeh *et al.*, 1974). Therefore, we assume that the uptake and release of uranium concomitantly with phosphate under natural conditions can be even higher than demonstrated in our laboratory experiment.

The concurrent release of uranium and phosphate can enhance the concentrations of uranium during apatite formation resulting in an enrichment of uranium in the apatite. The assumption, that sulfide-oxidizing bacteria could enrich uranium in apatite, is confirmed by the observation that phosphorites from the Peruvian shelf, where the sediment is often dominated by marine species of *Beggiatoa* (Arning *et al.*, 2008), contain very high concentrations of uranium, which range from about 125 to more than 400 mg/kg (Arning *et al.*, 2009b). Thus, sediments from Peru and Namibia, populated by polyphosphate accumulating sulfide-oxidizing bacteria, show in general higher enrichment factor of trace elements and uranium compared

to sediments from the Chilean shelf (Böning *et al.,* 2009; Borchers *et al.,* 2005), which are mostly dominated by marine *Thioploca* sp. (Arning *et al.*, 2008). In a recent study by Holmkvist *et al.* (2010) it was shown that *Thioploca* spp. likely do not store polyphosphate and do not contribute to authigenic formation of apatite, which was only a minor part of bound phosphate. In contrast authigenic apatite formation was experimentally shown in sediment from the Namibian shelf, which was populated by the polyphosphate storing sulfide oxidizers *Beggiatoa* and *Thiomargarita* (Goldhammer *et al.*, 2010). Therefore, polyphosphate storing sulfide-oxidizing bacteria and recent phosphorite, which is especially enriched in uranium, seem to show a certain co-occurrence.

An enrichment of uranium in polyphosphate granules is likely, because the negative charge of the polyphosphate is typically compensated by cations of calcium, magnesium, potassium and other metals (Docampo, 2006 and references therein). In *Acidithiobacillus ferrooxidans*, a sulfide-oxidizing bacterium which is phylogenetically loosely related to the large sulfur bacteria, accumulation of uranium from uranium polluted water within polyphosphate complexes has been shown (Merroun *et al.*, 2002). Interestingly, uranium can be used as alternative electron acceptor by different bacteria, such as sulfate reducing bacteria (Lovley and Phillips, 1992; Merroun and Selenska-Pobell, 2008), which are found in high numbers in the organic rich sediments beneath upwelling areas. Nevertheless, uranium, which is released concomitantly with phosphate after breakdown of polyphosphate, could be incorporated simultaneously with calcium into apatite or a precursor of it (Jarvis *et al.*, 1994).

Although, calcium is bound to polyphosphate in the investigated *Beggiatoa* strain (Brock *et al.*, Chapter 3) it shows a different response to anoxic conditions in the experiment than uranium. Calcium concentrations increase in both *Beggiatoa* culture and the sterile control without a correlation to the phosphate release (Figure 2c). Furthermore, the concentrations of calcium, strontium and magnesium decreased during the oxic growth in an amount which exceeds the amount of phosphate taken up by *Beggiatoa* several times. It is likely, that the positive cations of these elements are bound to negative groups within the cell walls of *Beggiatoa* and to the extracellular polymeric substances (EPS) released by *Beggiatoa* (Larkin

and Henk, 1996; Volesky and Holan, 1995). We assume that a change from oxic to anoxic conditions induces a release of these elements because of the change in redox conditions. An additional release from polyphosphate does not contribute much to the overall concentration in the medium. However, despite the stoichiometry of uranium and phosphate an additional binding of uranium to the cell wall or EPS may also occur, as several studies have shown the binding of uranyl in particular on carboxyl and phosphate groups (Merroun and Selenska-Pobell, 2008 and references therein).

Interestingly, we did not detect a peak of magnesium in the sediment together with phosphorus, even though we know from the analysis of the *Beggiatoa* strain, that magnesium is associated with the polyphosphate, but contrary to seawater in which magnesium concentrations are higher than those of calcium, in a lower amount than calcium (Brock *et al.*, in prep.). This may be important in the formation of apatite as magnesium inhibits the formation of apatite from its precursor (Jarvis *et al.*, 1994). Moreover, the absence of a magnesium peak in sediment maybe an indication, that the release of phosphate simultaneously with magnesium does not lead to precipitation of magnesium-rich apatite or a precursor, such as amorphous calcium magnesium phosphate (Krajewski *et al.*, 1994).

Yttrium and aluminum, which are enriched together with phosphorus, may also be associated to polyphosphate and could be excreted together with phosphate but the concentrations in our experiment with a marine *Beggiatoa* strain were too low to allow an interpretation. An accumulation of aluminum in polyphosphate as well as in the cell wall was shown for *Anabaena cylindrica* under high aluminum concentration (Pettersson *et al.*, 1985). It was proposed that aluminum was detoxified by the build up of polyphosphate, which can bind aluminum. A similar accumulation of aluminum in polyphosphate inclusions of sulfide-oxidizing bacteria could not be shown yet but seems possible.

Conclusions

In Namibian sediment where a steep phosphorous peak was produced by the activity of the large sulfur bacterium *Thiomargarita* we observed a corresponding peak in uranium and a strong correlation between the uranium and the phosphorus content in different sediment depths. In an experiment with a marine polyphosphate storing *Beggiatoa* strain, a close relative of *Thiomargarita*, which can be grown in culture, we revealed a stoichiometric uptake and release of uranium together with phosphate, suggesting that uranium is accumulated inside the polyphosphate granules and released together with phosphate when polyphosphate is degraded. Thereby, high concentrations of uranium in recently formed apatite may be due to the active release of uranium by polyphosphate accumulating sulfide-oxidizing bacteria.

Acknowledgment

This work was supported by the Deutsche Forschungsgemeinschaft (through MARUM Center for Marine Environmental Sciences) and the Max Planck Society. We thank M. Meyer and S. Hessler for technical assistance.

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Chapter 5

Vacuolar respiration of nitrate coupled to energy conservation in *Beggiatoa* sp.

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Running title: Vacuolar respiration of nitrate in *Beggiatoa*

In preparation for Environmental Microbiology

Summary

We show that the nitrate storing vacuole of sulfur oxidising bacteria *Beggiatoa* has an electron transport chain (ETC) that generates a proton motive force (PMF) used for cellular energy conservation. Protons are pumped from the cytoplasm into the vacuole resulting in a low internal pH. Immunostaining by antibodies showed that cytochrome c, an ETC protein, is present in the vacuole. Using pH imaging, we studied the effect of different inhibitors on the vacuolar pH. The inhibition of vacuolar ATPases and pyrophosphatases resulted in a pH decrease, showing that the proton gradient over the vacuolar membrane is used for ATP and pyrophosphate generation. The inhibition of the ETC increased the pH, indicating that the proton gradient is build up by a vacuolar ETC. Furthermore, the addition of nitrate resulted in a decrease of the pH value in the vacuole. Adding chlorate, an inhibitor of nitrate reduction led to increased pH. Both findings suggest, that nitrate respiration drives the high proton concentration within the vacuole (pH 2-3). Further, we detected nitric oxide in vacuoles of cells exposed to nitrate, which also supports intravacuolar reduction of nitrate. Thus, the vacuole acts as an active side of energy conservation, resembling an inverted periplasmatic space.

Chapter 6

Concluding remarks and outlook

The findings in this thesis contribute to both microbiology and geobiology. The major focus of this investigation was laid on the study of intracellular storage of phosphate as polyphosphate in *Beggiatoa*, the regulation of their polyphosphate storage by environmental stimuli and, above all, the impact of sulfide-oxidizing bacteria on the phosphorus cycle in marine sediments. This chapter connects the different findings and gives an outlook for future research.

6.1. Concluding remarks

By cultivation of *Beggiatoa* 35Flor in a newly developed gradient sampling column and the use of modified microrhizones (Rhizoshere Research Products, Wageningen, The Netherlands), it became possible to obtain phosphate profiles of an afore unmatched resolution (Chapter 2). This enabled the study of uptake and release of phosphate within a mineral gradient medium under specific laboratory conditions. In contrast to earlier models of polyphosphate usage by bacteria derived from enhanced biological phosphorus removal (Comeau *et al.*, 1986; Seviour *et al.*, 2003), a polyphosphate breakdown and phosphate release in response to anoxia in the presence of volatile fatty acids, such as acetate was not observed. Instead, experiments with the *Beggiatoa* culture in this thesis revealed that polyphosphate is degraded and phosphate is released in response to a switch from oxic conditions to anoxia at high sulfide concentrations (Chapter 2). This outcome was surprising, as sulfide has not been suggested as a trigger for polyphosphate degradation before. However, it is plausible with regard to the environment of these bacteria, which includes organic-rich sediments featuring fluctuating gradients of sulfide, oxygen and pH (Teske and Nelson, 2006).

The findings in this thesis indicate that narrow sulfide-oxidizing bacteria, such as *Beggiatoa* 35Flor, which are not able to store nitrate, as it is known for

Marithioploca, *Maribeggiatoa* and *Thiomargarita* (Fossing *et al.*, 1995; McHatton *et al.*, 1996; Salman *et al.*, in press; Schulz *et al.*, 1999), withstand temporary depletion of electron acceptors in a highly sulfidic environment by the storage and breakdown of polyphosphate (Chapter 2 and 3). Polyphosphate inclusions have already been described in sulfide-oxidizing bacteria (Schulz and Schulz, 2005; Strohl and Larkin, 1978), but with sizes of up to 3 µm the polyphosphate inclusions in *Beggiatoa* 35Flor are unusually large (Chapter 3).

Since the investigated *Beggiatoa* strain 35Flor is very closely related to other narrow species of marine and freshwater *Beggiatoaceae* (Chapter 3) the observed usage of polyphosphate (Chapter 2) is of great importance for the understanding of the phosphorus cycle in aquatic systems in general, since these bacteria are ubiquitously found in anoxic sediments of freshwater lakes and marine habitats (Teske and Nelson, 2006). So far, it was assumed that polyphosphate storage under oxic conditions and degradation under anoxic conditions are coupled to the storage of polyhydroxyalkanoates (PHA) from labile organic sources such as volatile fatty acids under anoxia (Comeau *et al.*, 1986; Hupfer *et al.*, 2007). In future investigations, it has to be considered that in some bacteria anoxia and exposure to high sulfide concentrations trigger polyphosphate breakdown and rapid phosphate release. Especially for organisms from environments with fluctuating redox conditions such as organic-rich sediments, hydrothermal vents and cold seeps this physiological trait is probably widely distributed.

The investigated *Beggiatoa* strain 35Flor is phylogenetically affiliated to other narrow *Beggiatoa* strains, which form a monophyletic branch at the phylogenetic root of the *Beggiatoaceae* (Chapter 3) (Ahmad *et al.*, 2006). A basic evolutionary feature of the family *Beggiatoaceae* is the ability to form vacuoles. The size and function of these vacuoles might reflect an adaptation to differing conditions in the respective habitats of the organisms, including also the absence of vacuoles in some narrow (2-5 µm) strains (Chapter 3). The polyphosphate inclusions described in this thesis are enclosed by a lipid layer, probably a vacuolar membrane. Thus, it is very likely that the polyphosphate inclusions are evolutionarily related to the nitrate storing vacuoles found in the other taxa of the *Beggiatoaceae*. Furthermore, the overall expression of vacuoles in a filament seems to correlate

with the filament diameter, implying that vacuoles are required in organisms with diameters beyond 5 µm. However, also some narrow filaments (around 4.4 µm) are even compartmentalized by small polyphosphate inclusions (around 0.15 µm) in their cytoplasm (de Albuquerque *et al.*, 2010).

Moreover, the polyphosphate inclusions in *Beggiatoa* 35Flor feature other interesting properties. Beside the surrounding lipid layer, they are enriched in calcium and to a minor extent also in magnesium, as directly demonstrated by energy dispersive X-ray analysis (EDXA) (Chapter 3) and indirectly by the uptake and release of these elements by a *Beggiatoa* 35Flor culture (Chapter 4). These properties are typical for acidocalcisomes, which are polyphosphate-enriched acidic cellular compartments in eukaryotes and some bacteria (Docampo, 2006; Seufferheld *et al.*, 2003). It is very likely that in addition to calcium and magnesium other metal cations are associated with polyphosphate in *Beggiatoa*, such as aluminum and uranium, which was shown to be correlated to phosphate uptake and release (Chapter 4). This finding indicates that sulfide-oxidizing bacteria are crucial in retaining uranium in sediments, which sheds a new light on the question why uranium is highly concentrated in phosphorites (Baturin and Kochenov, 2001). It shows that bacteria not only contribute to apatite precipitation, as assumed so far (Krajewski *et al.*, 1994; Schulz and Schulz, 2005), but also contribute to the chemical structure of the apatite.

Although the cellular processes behind the finding that anoxia and sulfide trigger rapid phosphate release remain unknown, it enhances our knowledge concerning the role of sulfide-oxidizing bacteria in the precipitation of apatite - the first step of phosphogenesis in sediments of coastal upwelling (Arning *et al.*, 2009). Phosphate is liberated into the pore water via degradation of organic matter by sulfate-reducing bacteria resulting in high phosphate concentrations. A substantial part of the phosphate is taken up by sulfide-oxidizing bacteria, when oxygen or nitrate is available, and stored as polyphosphate. When oxygen and nitrate are depleted, the sulfide concentration in the pore water increases drastically, which triggers the rapid release of phosphate by sulfide-oxidizing bacteria. Thus, the phosphate concentration increases in the pore water, which becomes oversaturated in respect to a precursor of apatite, as described by Krajewski *et al.* (1994). The precursor

precipitates and is transformed into apatite. It is likely, that the phosphorus peak in the investigated Namibian sediment was created by this process (Chapter 4). Moreover, it explains the close association of lipid biomarkers indicative for sulfate-reducing bacteria and a high number of sulfide-oxidizing bacteria in phosphogenic sediments (Arning *et al.*, 2008).

As discussed in chapter 2, the findings in this thesis are in agreement with results of several earlier studies from the fields of geology and geobiology. For example, they are an explanation for the observation that phosphorite formation is mainly found at the boundaries of oxygen minimum zones (Burnett *et al.*, 1983; Föllmi, 1996). Within these areas, frequent changes from suboxic to anoxic conditions occur at the sediment-water interface, which are important in the formation of phosphorite (Arning *et al.*, 2009). Such conditions are in agreement with the outcome of chapter 2, reporting that a switch from oxic to anoxic conditions in a sulfidic environment is a trigger for phosphate release by sulfide-oxidizing bacteria. As the formation of phosphorites is the major sink of phosphorus in the oceans (Föllmi, 1996), it shows that sulfide-oxidizing bacteria play an important role in the global phosphorus cycle, which is linked to the global carbon cycle (Tyrrell, 1999).

However, the question remains, if the results of this thesis can be directly transferred to sulfide-oxidizing bacteria of the family *Beggiatoaceae* in general. In contrast to the genera *Thiomargarita* and *Beggiatoa,* species of the genus *Marithioploca* (formerly marine *Thioploca*) do not seem to be involved in phosphorite formation. Despite high phosphate concentrations in pore waters, nearly no authigenically formed hydroxyapatite was found in Chilean sediment bearing a large *Marithioploca* community (Holmkvist *et al.*, 2010). As in earlier studies on the ultrastructure of *Marithioploca* (Maier *et al.*, 1990), the authors did not detect significant polyphosphate storage. Unfortunately, attempts to cultivate *Marithioploca* spp. have failed so far, inhibiting the application of experiments with these organisms as carried out with *Beggiatoa* 35Flor.

Finally, the findings of this thesis are of general importance, since mineable phosphorite deposits will be depleted in the near future, and phosphate recycling from anthropogenic influenced waters, such as urban- and agricultural wastewater,

will become more important to avoid the deficiency of this essential nutrient (Gilbert, 2009). Furthermore, it might be of importance for the improvement of enhanced biological phosphorus removal in wastewater treatment plants or the remediation of radioactive contaminated sediments and aquifers (Merroun and Selenska-Pobell, 2008).

6.2. Outlook

On a microbiological level, it will be a future challenge to describe the polyphosphate inclusions of *Beggiatoa* 35Flor in more detail. The knowledge of functional properties of the polyphosphate inclusions might help to explain the new finding of polyphosphate usage described in chapter 2. More appropriate pH sensitive dyes, such as the ratio-based dye SNARF-1 AM, could be used to test for pH differences, which were not detectable by the so far used dyes (Chapter 3). By the application of cationic lipophilic dyes, such as rhodamine 123 or the ratio-based jc-1, it could be tested if an electric potential exists over the membrane of the polyphosphate inclusions. The membrane should be investigated also with respect to a possible similarity of polyphosphate storing and nitrate storing vacuolar structures found in different genera of sulfide-oxidizing bacteria, as discussed in chapter 3.

Immunofluorescence microscopy could be used to identify certain proteins such as V-ATPases, pyrophosphatases and ion channels, which occur in membranes of acidocalcisomes or vacuoles in general (Docampo, 2006; Seufferheld *et al.*, 2003). Transmission electron microscopy (TEM) and freeze-fracturing may be useful to get information about the structure of the polyphosphate inclusions of sulfide-oxidizing bacteria as it has been in the studies of sulfur globules of these bacteria (Strohl *et al.*, 1981). In combination with EDXA, TEM could be used to study the elemental composition of the polyphosphate inclusions and the cells as a whole (Seufferheld *et al.*, 2003) to prove if uranium, aluminum or other elements, which are enriched in phosphorites, are associated with polyphosphate inclusions, the cell wall or extracellular polymeric substances, as discussed in chapter 4. Since the cultivation in mineral gradient medium is a closed system in contrast to natural

sediment, which is in exchange with overlying bottom water and deeper sediment layers, depletion of elements of interest has to be considered. Therefore, cultivation should be carried out in a medium specifically enriched with the element in question.

Furthermore, it is necessary to study the potential involvement of other sulfide-oxidizing bacteria, in particular those from upwelling areas, where apatite precipitation occurs. Because of the common difficulty to cultivate large sulfide-oxidizing bacteria in pure cultures, genomic analysis of single filaments could resolve this constraint. Using this technique, Mußmann *et al.* (2007) showed that large vacuolated *Parabeggiatoa* (formerly *Beggiatoa*) possess the gene polyphosphate-kinase which is essential for polyphosphate synthesis. In a similar way, other species of the family *Beggiatoaceae* could be tested for genes encoding enzymes involved in polyphosphate metabolism and phosphate transport.

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My own contribution to the manuscripts presented in chapter 2-5 of this thesis.

Chapter 2

Sulfide induces phosphate release from polyphosphate in cultures of a marine *Beggiatoa* strain

Jörg Brock & Heide N. Schulz-Vogt

The concept was developed together with Heide N. Schulz-Vogt. I developed the gradient-sampling-tube and the sampling method as a prerequisite for the study. Moreover, I carried out the cultivation of *Beggiatoa* strain 35Flor, the experiments and the data analysis. The manuscript was written in collaboration with Heide N. Schulz-Vogt.

Chapter 3

Unusual polyphosphate inclusions observed in a marine *Beggiatoa* strain

Jörg Brock, Erhard Rhiel, Martin Beutler, Verena Salman & Heide N. Schulz-Vogt

The concept of the study was developed by me and I performed most of the experiments. Confocal laser scanning microscopy was supported by Dr. Martin Beutler. Scanning electron microscopy and energy dispersive X-ray analysis were done in collaboration with Dr. Erhard Rhiel, University of Oldenburg. 16S rRNA sequencing and phylogenetic analysis were carried out by Verena Salman. I wrote the manuscript with assistance of the co-authors.

Chapter 4

Large sulfide-oxidizing bacteria can accumulate and release uranium concurrently with phosphate

Jörg Brock, Heide N. Schulz-Vogt & Martin Kölling

The study is based on a discussion with Heide N. Schulz-Vogt, who contributed the Namibian sediment data. I performed the laboratory experiments with the *Beggiatoa* strain 35Flor and the data analysis. Inductive coupled plasma analysis

was done in collaboration with Dr. Martin Kölling, University of Bremen. The manuscript was written in collaboration with Heide N. Schulz-Vogt.

Chapter 5

Vacuolar respiration of nitrate coupled to energy conservation in *Beggiatoa* sp.

Martin Beutler, Susanne Borgwardt, Frank Schreiber, Jana Milucka, Jörg Brock, Marc Mussmann, Heide N. Schulz-Vogt and Dirk de Beer

I assisted Dr. Martin Beutler in confocal laser scanning microscopy and supported the writing of the manuscript.

Acknowledgment

This thesis has been made possible by the help, support and input of many people.
In the first place, I thank Dr. Heide N. Schulz-Vogt for her great supervision, support and encouragement over the last years. She helped me to develop the enthusiasm and openness, which are necessary for scientific research.
My deepest thanks go to Prof. Dr. Bo Barker Jørgensen for his willingness to review this thesis and to Prof. Dr. Ulrich Fischer, Prof. Dr. Michael W. Friedrich, Vladimir Bondarev and Viola Krukenberg for joining my thesis committee.
A big thank you to Dr. Martin Beutler for his help with confocal laser scanning microscopy and the discussions about it.
Many thanks to Dr. Erhard Rhiel, Institute for Chemistry and Biology of the Marine Environment, Carl von Ossietzky University Oldenburg, for the very good cooperation in electron microscopy.
Also thanks to Dr. Martin Kölling and the people at the department of geochemistry and Center for Marine Environmental Science (MARUM) at the University of Bremen, who contributed to this thesis.
Sincere thanks to my very nice colleagues in the ecophysiology group: Martina Meyer, Susanne Menger, Anne Schwedt, Verena Salman, Anne-Christin Girnth, Sandra Havemeyer, Stefano Romano and all former group members.
Furthermore, I thank all the people in the department of Microbiology and the Max Planck Institute for Marine Microbiology, Bremen. I had the pleasure to meet and work with many people, in particular with Ulrike Jaekel, Dennis Enning, Jens Harder, Lubos Polerecky, Thomas Vagner, Andreas Krupke, Petra Pop Ristova and Anja Kamp.
Moreover, I thank the people at the Institute of Microbiology, Leibniz University of Hannover, for the very nice first year.
Finally, thanks a lot to my friends, my family and Matylda.

Erklärung gemäß § 6 Abs. 5 der Promotionsordnung der Universität Bremen für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche

Hiermit versichere ich, dass ich die vorliegende Dissertation mit dem Titel **„Impact of sulfide-oxidizing bacteria on the phosphorus cycle in marine sediments“**

1) ohne unerlaubte Hilfe angefertigt habe
2) keine anderen, als die von mir angegebenen Quellen und Hilfsmittel benutzt habe und
3) die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Jörg Brock Bremen, den 07.01.2011