Polyphosphate storage in the family *Beggiatoaceae* with a focus on the species *Beggiatoa alba*

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Abbreviations

ADP	adenosine diphosphate
AK	adenylat kinase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
°C	grad celsius
Ca.	*Candidatus*
DAPI	4´,6´-diamidino-2-phenylindole
Da	dalton
DNA	deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures)
EDTA	ethylendiamintetraacetat
EDXA	energy dispersive X-ray analysis
et al.	et alii
g	gram
Glc	glucose
Glc-6-P	glucose-6-phosphate
HPLC	high performance liquid chromatography
L	liter
$l.mm^{-1}$	lines per millimeter
MilliQ	demineralized water in Millipore Quality
PAP	polyphosphate:AMP phosphotransferase
PHA	polyhydroxyalkanoate
PHB	polyhydroxybutyrate
P	phopshorus
P_i	inorganic phosphate
PIPES	1,4-piperazinediethanesulfonic acid disodium salt
poly-P	polyphosphate
PPGK	polyphosphate glucokinase
ppGpp	guanosine tetraphosphate
PPK	polyphosphate kinase
pppGpp	guanosine pentaphosphate

PPX	exopolyphosphatase
NAD	nicotineamid adenosine dinucleotide phosphate
NDP	nucleoside diphosphate
NTP	nucleoside triphosphate
pH	negative $\log_{10}$ of the proton concentration
RNA	ribonucleic acid
rpm	rounds per minute
SEM	scanning electron microscopy
sp.	species
spp.	species (plural)
tRNA	transfer RNA
UV	ultraviolet
V	volt
W	watt
w/v	weight per volume

Summary

Sulfur bacteria of the family *Beggiatoaceae* are of special interest with respect to the phosphorus cycle, because they can store large amounts of polyphosphate and are proposed to influence phosphorus sequestration in marine sediments (e.g. Schulz and Schulz, 2005). The aim of this thesis was to study different aspects of polyphosphate storage in members of the family *Beggiatoaceae* on a physiological and genomic level with a special focus on the heterotrophic freshwater strain *Beggiatoa alba* B15LD. In addition, polyphosphate-related enzymes, which are encoded in different members of the *Beggiatoaceae,* were identified and possible pathways of polyphosphate utilization were discussed, including its exploitation as an energy source.

In the second part, the structure and elemental composition of polyphosphate inclusions in *Beggiatoa alba* were analyzed. Studies using various techniques revealed that these inclusions are not acidic and are associated with sodium cations. This is the first time that a co-occurrence of polyphosphate with Na^+ was observed in *Beggiatoaceae* and among bacteria in general.

In addition, the factors controlling the storage and degradation of polyphosphate in *Beggiatoa alba* were studied under laboratory conditions. Comparison of the triggers for polyphosphate synthesis and degradation to those effective in other members of the family *Beggiatoaceae*, which are lithoautotrophic and originate from freshwater, marine, and hypersaline environments, revealed that only *Beggiatoa alba* stored polyphosphate at nitrogen limitation. Under these conditions, polyphosphate was possibly stored as an energy reserve, since growth was inhibited and excess energy was available through the oxidation of acetate. In *E. coli*, it was shown that polyphosphate was stored at nitrogen limitation to induce the degradation of ribosomal proteins to use them as an intracellular amino acid pool. The produced guanosine tetraphosphate (ppGpp) serves as an important signaling molecule during this process. Elevated ppGpp concentrations were also measured in nitrogen-

limited cultures of *Beggiatoa alba*, suggesting that this mechanism is also present in this species.

Polyphosphate degradation in *Beggiatoa alba* was induced by different stress factors, such as high and low pH (Havemeyer, 2010), elevated temperatures, and high ammonium concentrations, which did not affect polyphosphate storage in the marine strain *Beggiatoa* sp. 35Flor. In contrast, the marine strain degraded polyphosphate in response to high sulfide concentrations and anoxia (Brock and Schulz-Vogt, 2011). While sulfide concentrations in freshwater sediments are much lower than in marine sediments, pH changes are more likely to occur, since freshwater is, in contrast to seawater, not carbonate buffered. Hence, polyphosphate degradation in *Beggiatoaceae* seems to be related to habitat-specific environmental factors.

Finally, polyphosphate storage in environmental samples of filamentous *Beggiatoaceae* from Lake Grevelingen, The Netherlands, and Aarhus Bay, Denmark, were investigated. Fluctuations in redox conditions together with high sulfide concentrations are a prerequisite for polyphosphate degradation and phosphate release in a marine *Beggiatoa* strain (Brock and Schulz-Vogt, 2011). Although both sampling sites have fluctuations in redox conditions, no polyphosphate storage was observed and therefore a potential influence on benthic phosphorus cycling cannot be assumed. The facts that sulfide concentrations were not very high and fluctuations of redox conditions occurred seasonally and not more frequently might explain the difference. Hence, sulfur bacteria do not in general influence benthic phosphorus cycling. Instead, it seems that only special conditions, as found at sites of recent phosphorus sequestration, have the potential to induce massive accumulation of polyphosphate and rapid phosphate release by sulfur bacteria.

Zusammenfassung

Schwefelbakterien der Familie *Beggiatoaceae* sind von besonderem Interesse im Hinblick auf den Phosphorkreislauf, da sie große Mengen an Polyphosphat speichern können und man davon ausgeht, dass sie einen Einfluss auf die Ablagerung von Phosphor in marinen Sedimenten haben (z.B. Schulz and Schulz, 2005). In dieser Doktorarbeit wurden verschiedene Aspekte der Polyphosphatspeicherung in Mitgliedern der Familie *Beggiatoaceae* auf physiologischer und genomischer Ebene studiert, wobei der Fokus auf den heterotrophen Süßwasser-Stamm *Beggiatoa alba* B15LD lag. Zunächst wurde nach Polyphosphat-bezogenen Enzymen gesucht, die in verschiedenen Mitgliedern der Familie *Beggiatoaceae* kodiert werden und Möglichkeiten der Polyphosphatnutzung einschließlich der Verwertung als Energiequelle aufgezeigt.

Im zweiten Teil wurde die Struktur und elementare Zusammensetzung von Polyphosphateinschlüssen in *Beggiatoa alba* analysiert. Studien mit Hilfe verschiedener Methoden haben ergeben, dass diese Einschlüsse nicht sauer und mit Natriumkationen assoziiert sind. Dies ist das erste Mal, dass ein gemeinsames Auftreten von Polyphosphat und Natrium in der Familie *Beggiatoaceae* und generell in Bakterien beobachtet wurde.

Außerdem wurden unter Laborbedingungen die Faktoren untersucht, die Polyphosphatspeicherung und -degradierung kontrollieren. Die Auslöser für Polyphosphatsynthese und -degradierung wurden mit denen anderer litho-autotropher Mitglieder der Familie *Beggiatoaceae* aus Süßwasser, marinen und hypersalinen Habitaten verglichen. Stickstofflimitierung führte nur in *Beggiatoa alba* zu Polyphosphatspeicherung, während Polyphosphat-speicherung der weiteren untersuchten Schwefelbakterien von anderen Faktoren abhing. Unter diesen Bedingungen wurde Polyphosphat möglicherweise als Energiequelle gespeichert, da das Wachstum inhibiert wurde und ein Überschuss an Energie durch die Oxidation von Acetat zur Verfügung stand. In *E. coli* wurde außerdem gezeigt, dass Polyphosphat unter Stickstofflimitierung gespeichert wird, um die Degradierung von ribosomalen

Proteinen zu induzieren und diese als intrazelluläre Aminosäurequelle zu nutzen. Hierbei wird das Signalmolekül Guanosin Tetraphosphate (ppGpp) produziert. Erhöhte ppGpp Konzentrationen konnten ebenfalls in Stickstoff-limitierten *Beggiatoa alba* Kulturen gemessen werden, was darauf hinweist, dass dieser Mechanismus auch in dieser Spezies vorhanden ist.

Polyphosphatdegradierung in *Beggiatoa alba* wurde durch verschiedene Stressfaktoren induziert, wie zum Beispiel hoher und niedriger pH-Wert (Havemeyer, 2010), erhöhte Temperaturen und hohe Ammoniumkonzentrationen. Diese Faktoren hatten hingegen keinen Einfluss auf die Polyphosphatspeicherung im marinen Stamm *Beggiatoa* sp. 35Flor. Im Gegensatz dazu degradiert der marine Stamm Polyphosphat bei hohen Sulfidkonzentrationen unter anoxischen Bedingungen (Brock and Schulz-Vogt, 2011). Während Sulfidkonzentrationen in Süßwassersedimenten viel geringer sind als in marinen Sedimenten, kommen pH-Veränderungen hier eher zum tragen, da Süßwasser im Gegensatz zu Meerwasser nicht Karbonat-gepuffert ist. Folglich scheint Polyphosphatdegradierung in *Beggiatoaceae* eine Antwort auf Habitat-spezifische Umweltfaktoren zu sein.

Zusätzlich wurde Polyphosphatspeicherung in Umweltproben von filamentösen *Beggiatoaceae* von Lake Grevelingen, Niederlande und Aarhus Bay, Dänemark untersucht. Fluktuierende Redox-Verhältnisse zusammen mit hohen Sulfidkonzentrationen sind eine Voraussetzung für Polyphosphatdegradierung in einem marinen *Beggiatoa* Stamm (Brock and Schulz-Vogt, 2011). Obwohl beide Probenahmeorte fluktuierende Redox-Verhältnisse aufweisen, konnte keine Polyphosphatspeicherung festgestellt und damit kein potentieller Einfluss auf den benthischen Phosphorkreislauf prognostiziert werden. Die Tatsache, dass die Sulfidkonzentrationen nicht sehr hoch waren und Fluktuationen der Redox-Verhältnisse saisonal und nicht in kürzeren Zeitintervallen auftraten, könnte die Unterschiede erklären. Demzufolge haben Schwefelbakterien keinen generellen Einfluss auf den benthischen Phosphorkreislauf. Stattdessen scheinen nur spezielle Bedingungen wie an Orten rezenter Phosphorablagerung das Potential zu haben, massive Polyphosphatakkumulierung und schnelle Phosphatabgabe bei Schwefelbakterien zu induzieren.

1. Introduction

1.1 The phosphorus cycle

Within the global element cycles, phosphorus is the only element, which can be considered the ultimate limiting nutrient. Phosphorus and nitrogen control primary production, thus affecting the carbon cycle and thereby indirectly also the climate on earth (Tyrrell, 1999). In contrast to the nitrogen cycle with its abundant atmospheric pool, the phosphorus cycle is mostly abiotically controlled and phosphorus is deposited in phosphorus-rich rocks called phosphorites. On a global scale, the largest phosphorus pools are marine sediments that act both as sinks and sources of phosphorus. Thus, the biological availability of phosphorus is strongly restricted by slow abiotic processes that occur on geological time scales (Föllmi 1996). Therefore, it is crucial to understand the phosphorus cycle and to identify sources and sinks of biologically available phosphate.

Phosphorus is essential for all forms of life. It provides for instance the phosphate-ester backbones of nucleic acids, is a constituent of phospholipids, coenzymes, and energy-rich phosphoanhydride bonds present for example in adenosine triphosphate (ATP) (Paytan and McLaughlin, 2007). Energy-rich phosphoanhydride bonds are very important for the energy metabolism of cells and are further present in the polymer polyphosphate (Kornberg, 1995). Another group of phosphorus-containing molecules are phosphonates, which contain a very stable bond between phosphorus and carbon (Quinn et al., 2007).

Phosphorus can occur in four oxidation states, phosphine (PH_3, oxidation state -3), phosphorus (P_4, oxidation state 0), phosphite (PO_3^{3-}, oxidation state +3), and phosphate (PO_4^{3-}, oxidation state +5), the latter being the most prominent form of phosphorus in nature (White and Metcalf, 2007). Phosphorus in the water column exists in both dissolved and particulate forms. The dissolved

fraction includes inorganic phosphorus (generally orthophosphate), organic phosphorus compounds, and macromolecular colloidal phosphorus. Particulate phosphorus consists of living and dead organisms, precipitates of phosphorus minerals, phosphorus adsorbed to particles, and amorphous phosphorus phases. In each fraction, dissolved and particulate, phosphorus can be in the form of inorganic (orthophosphate, pyrophosphate, polyphosphate, and phosphate-containing minerals) or organic (phosphate-esters, phosphate-diesters, and phosphonates) compounds (Paytan and McLaughlin, 2007). All of these organic and inorganic particulate and dissolved forms of phosphorus undergo continuous transformations. Dissolved inorganic phosphorus, usually at the state of orthophosphate, is the preferred phosphorus source for microbial growth (Karl, 2000) and enters the cycle mainly due to continental weathering of rocks and soils by river runoff (Benitez-Nelson, 2000). Additionally, phosphorus from atmospheric aerosols can contribute to phosphorus loading in the oceans (Migon et al., 2001). Phosphate is assimilated especially by phytoplankton and thereby altered to organic phosphorus compounds (Cotner and Wetzel, 1992). Therefore, the phosphate concentrations in the surface water is usually low (< 1 µmol L^{-1}) (Paytan and McLaughlin, 2007). The organic phosphorus in turn can be remineralized by bacteria and subsequently assimilated again (Cotner and Biddanda, 2002). The remineralization of organic phosphorus leads to increasing phosphate concentrations of approximately 3 µmol L^{-1} in deeper ocean waters (Paytan and McLaughlin, 2007).

Phosphate is continuously removed from the pool of biological available phosphorus by burial into the sediment, called phosphogenesis. Phosphorus in dead biomass sinks towards the seafloor. On its way down, most of the phosphorus gets remineralized, nevertheless a substantial amount of organic biomass reaches the seafloor and gets buried in the ocean sediment (Figure 1.1) (Delaney, 1998). Especially upwelling areas with nutrient-rich waters and consequently high water-surface biomass production in combination with shallow water depths are hotspots of sedimental phosphogenesis (Föllmi, 1996). Here, a significant part of the produced biomass reaches the sediment and half of the global oceanic phosphorus burial occurs in these areas,

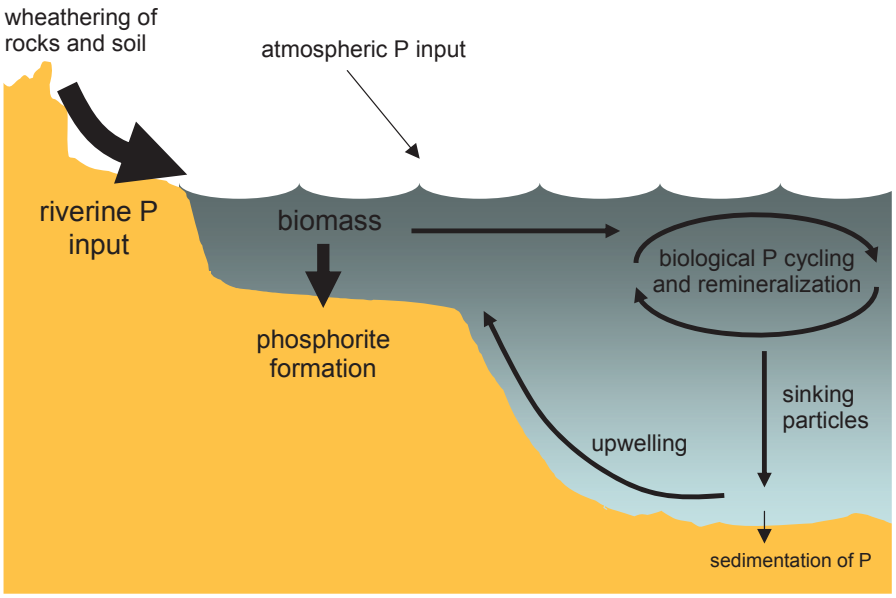

Figure 1.1: The marine phosphorus cycle (modified after Paytan and MacLaughlin (2007)). Weathering of terrestrial rocks and soils delivers phosphorus (P) through river runoff into the marine system. Most of the entered P gets buried in phosphorites in estuarine sediments. The remaining P, which enters the open ocean, is mainly consumed in the upper water column by phytoplankton. Some P sinks down in particulate organic matter and gets remineralized. However, a small part also reaches the seafloor and is buried in the sediment.

although they represent only a small fraction of the total oceanic surface. Phosphogenesis is the result of an oversaturation of phosphate in sediment pore water with respect to the phosphorus mineral francolite (Föllmi, 1996), which leads to the spontaneous precipitation of phosphorus-rich minerals, such as apatite ($Ca_5(PO_4)_3(F,Cl,OH)$). In all apatite minerals, phosphate oxyanions linked by Ca^{2+} cations form a hexagonal framework, but they differ in the elemental composition at the corners of the hexagonal cell (McClellan and Lehr, 1969). The details of phosphogenesis are still not fully understood. As mentioned above, a prerequisite for phosphogenesis to occur is an oversaturation of phosphate in the pore water, which can be induced by dissolution of fish debris (Suess, 1981) or phosphate release due to microbial degradation of organic matter. Another possible reason for high phosphate concentrations is dissolution of phosphates from iron hydroxides at anoxic conditions (Einsele, 1936). The adsorption of phosphate to iron hydroxides at oxic conditions is a well-known abiotic process of phosphate removal (Boström et al.,

1988). Anoxic conditions favor nitrate and sulfate reduction. In case of sulfate reduction, sulfide is produced, which even enhances the dissolution of iron-hydroxides and the release of adsorbed phosphate. However, marine sediments have a rather low iron content (Blomqvist et al., 2004) and therefore phosphate dissolution from iron hydroxides alone is not sufficient to explain the high phosphate concentrations in some marine sediments (Boström et al., 1988). It is very likely that bacteria contribute to increasing phosphate concentrations in the sediment, as it is known that some bacteria can take up phosphate and store it intracellularly as polyphosphate. Upon phosphate release from these storages and locally oversaturating the pore waters with phosphate, these bacteria might play a significant role in apatite formation in ocean sediments (Reimers et al., 1990).

1.1.1 Role of bacteria in the phosphorus cycle

In contrast to the re-entrance of phosphorus from phosphorus-rich rocks, which is strongly restricted by slow abiotic processes (Föllmi, 1996), phosphorus removal by phosphogenesis is probably under abiotic and biotic control (Krajewski et al., 1994). Numerous previous studies discussed a microbial impact on phosphorus sequestration. For example, it was proposed that bacteria, such as *Pseudomonas* and *Acinetobacter*, which also occur in waste water treatments plants and effect phosphorus removal, act in a similar way in natural habitats and actively enhance the genesis of phosphorites (Nathan et al., 1993).

Several recent studies proposed that also the group of large colorless sulfur bacteria play a role in phosphorite formation. Modern sites of phosphogenesis are mainly found in nutrient-rich upwelling areas off the coasts of Peru, Chile, and Namibia (Föllmi, 1996). These are the sites that are densely populated by the large colorless sulfur bacteria, indicating a tight correlation of microbial presence and local phosphogenesis. For instance, chain-forming *Thiomargarita* dominate sediments beneath the Benguela upwelling area off Namibia (Schulz et al., 1999), while bundle-forming filaments, such as "*Ca.* Mari-

hioploca" (former name *Thioploca*, revised according to Salman et al. (2011)), dominate sediments off the South American west coast (Gallardo, 1977) and in the Arabian Sea (Schmaljohann et al., 2001). Free-living filamentous sulfur bacteria like "*Ca.* Maribeggiatoa" dominate vast microbial mats found at the west coast of North America, in the Gulf of Mexcio and the Guaymas basin (Kalanetra et al., 2004; MacGregor et al., 2013). Schulz and Schulz (2005) showed a correlation between *Thiomargarita* abundance, pore water phosphate concentrations and apatite precipitation in modern phosphogenic sediments of Namibia, suggesting sulfur bacteria-driven phosphogenesis. These bacteria are able to store high amounts of phosphate intracellularly as polyphosphate, which they can degrade and subsequently release into the sediment in the form of phosphate. Tracer experiments confirmed that phosphate cycled through these polyphosphate-storing colorless sulfur bacteria is rapidly incorporated into apatite (Goldhammer et al., 2010). A further study revealed that a closely related marine *Beggiatoa* strain has a similar potential to induce phosphogenesis (Brock and Schulz-Vogt, 2011). The authors showed that the marine *Beggiatoa* strain released high amounts of phosphate from internally stored polyphosphate in laboratory experiments in response to high sulfide concentrations and shifts from oxic to anoxic conditions. These are conditions that characterize, for instance, the habitat of colorless sulfur bacteria in upwelling areas. High primary production leads on the one hand to high sulfate reduction rates resulting in high sulfide concentrations and on the other hand to anoxic bottom water masses. Due to changes in the intensity of upwelling, the anoxic bottom water masses frequently change their position on the shelf sediments and concomitantly these are the areas where phosphorites are mainly formed (Figure 1.2) (Burnett et al., 1983).

There is even evidence for an ancient impact of polyphosphate-storing colorless sulfur bacteria on phosphorite formation. Sulfur-containing filamentous microfossils preserved in ancient phosphorites from two major phosphogenic episodes in the geologic record (the Miocene Monterey Formation and the Neoproterozoic Doushantuo Formation) morphologically resemble modern sulfide-oxidizing bacteria (Bailey et al., 2013). These findings suggest that

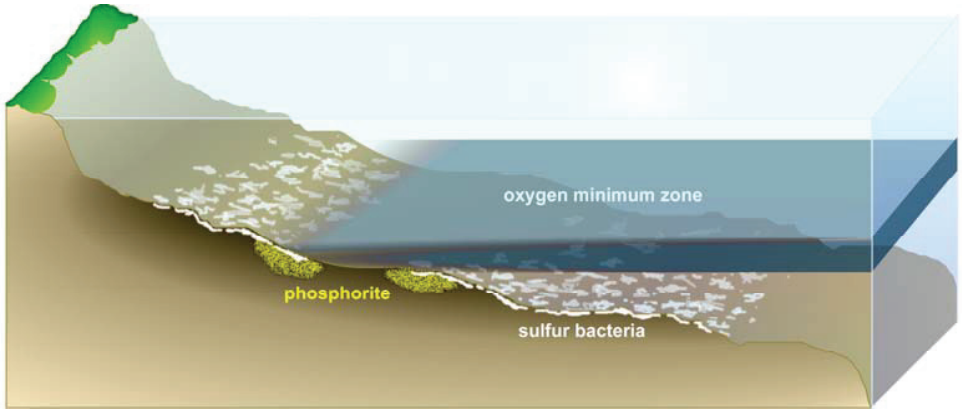

Figure 1.2: Scheme of a coastal upwelling area, where recent phosphorite formation occurs at the border of anoxic bottom water masses due to activity of colorless sulfur bacteria. These areas are characterized by high sulfide concentrations and frequently changing redox conditions (courtesy of H.N. Schulz-Vogt).

colorless sulfur bacteria, which play an important role in modern phosphorite formation, might have also been present in phosphogenic sediments for at least the last 600 million years. If this is true, the requirements of colorless sulfur bacteria for oxygen could explain the temporal correlation between the first appearance of globally distributed marine phosphorites and increasing oxygenation of Neoproterozoic oceans (Bailey et al., 2013). The findings presented in this section lead to the conclusion that the high capacity of colorless sulfur bacteria to store phosphate as polyphosphate is an important prerequisite for an influence on biogeochemical phosphorus cycling.

1.2 Polyphosphate storage in bacteria

Storage of inorganic phosphate in the form of polyphosphate occurs ubiquitously and was found in various organisms from prokaryotes to higher eukaryotes (Kornberg, 1995). However, microorganisms are able to accumulate the highest biomass-specific amount of polyphosphate (Kulaev and Kulakovskaya, 2000). Polyphosphate is an inorganic, linear polymer consisting of three up to hundreds of orthophosphate residues that are linked via energy-rich phosphoanhydride bonds, such as in ATP (Figure 1.3) (Kornberg et al., 1999). Polyphosphate can occur in different forms. It can be dissolved, accumulated in granules, or bound to cytoplasmic compounds, such as ribonucleic acids (Kulaev et al., 2004). In prokaryotes, long-chain polyphosphate are often

called volutin and are accumulated in granules along or near the chromosome (Keasling, 1997), in the cytoplasm membrane, in the cell wall or other organelle membranes, while short-chain polyphosphate can also occur freely dissolved in the cytoplasm (Kulaev et al., 2004).

Diverse functions have been proposed for polyphosphate storage, e.g. a phosphorus reservoir, which can also be used as an energy source due to its energy-rich phosphoanhydrid bonds, or chelator of toxic metals or a regulator of stress and survival (Kornberg, 1995; Brown and Kornberg, 2004). Furthermore, the polymer might buffer intracellular pH (Pick and Weiss, 1991), can have a role in cation storage (Docampo et al., 2010), is necessary in competence for bacterial transformation (Huang and Reusch, 1995), and was shown to be important in physiologic adjustments to growth, development, and deprivation (Kornberg, 1995). Additionally, it was suggested that polyphosphate can regulate gene expression and enzyme activities (Kulaev and Kulakovskaya, 2000). Many of the diverse possible functions are not entirely proven so far and there may be even more undetected functions of polyphosphate among bacteria. Thus, despite the ubiquitous occurrence of polyphosphate in living organisms, its metabolism remains poorly understood and unclear in most cases.

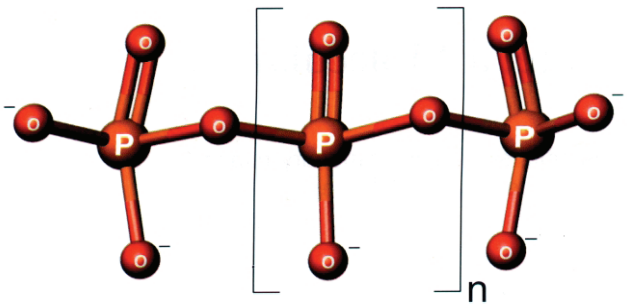

Figure 1.3: A linear polymer of inorganic polyphosphate (Kornberg and Fraley, 2000).

Polyphosphate metabolism in bacteria was mostly studied in microbial communities from wastewater treatment plants, where microorganisms are used for biological phosphorus removal. It was observed that mixed bacterial communities in activated sludge degraded polyphosphate in response to acetate

addition at anoxic conditions followed by subsequent phosphate release (Comeau et al., 1986). Based on these findings, the authors proposed a biochemical model for enhanced biological phosphorus removal (Figure 1.4). In this model, polyphosphate-accumulating bacteria use the energy gained from the breakdown of polyphosphate under anaerobic conditions for the uptake of acetate or other volatile fatty acids, which are then stored as a carbon source in the form of polyhydroxyalkanoates (PHA), such as polyhydroxybutyrate (PHB). The anaerobic phase is then followed by an aerobic phase, in which the bacteria perform a 'luxury uptake' of phosphate and store it as polyphosphate. Simultaneously, stored PHA is degraded to provide the required energy. Hence, the bacterial 'luxury uptake' of phosphate during the aerobic phase is used to remove dissolved phosphate from wastewater.

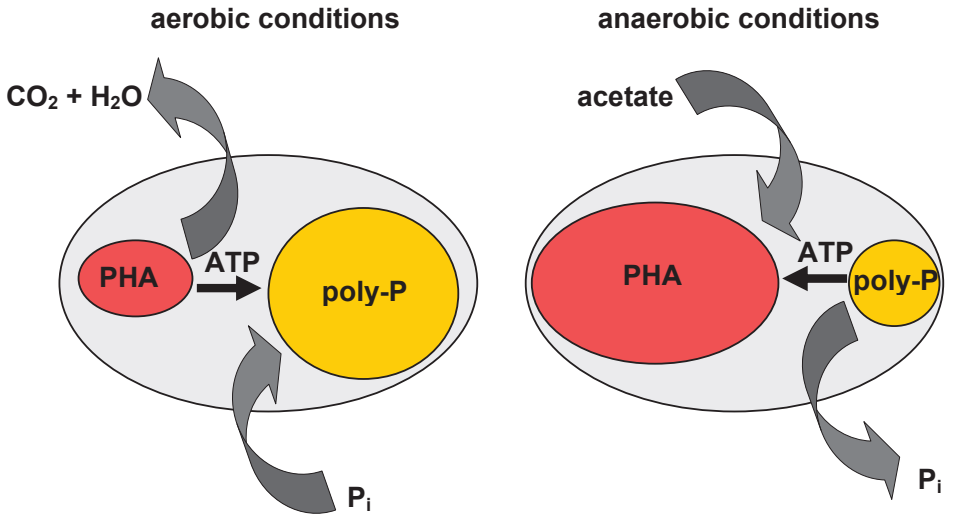

Figure 1.4: Scheme of the proposed biochemical model for polyphosphate storage in bacteria at aerobic and anaerobic conditions in wastewater treatment plants (modified after Forbes et al. (2009)). Under aerobic conditions, the PHA is degraded, the generated energy is used to take up phosphate and to synthesize polyphosphate, while at anaerobic conditions polyphosphate is degraded, phosphate is released and the generated energy is used for the uptake of acetate and the storage of PHA. Abbreviations: ATP, adenosine triphosphate; P_i, inorganic phosphate; PHA, polyhydroxyalkanoates; poly-P, polyphosphate.

Furthermore, Comeau et al. (1986) report phosphate release upon the addition of 2,4-dinitrophenol, H_2S gas, CO_2 gas, and at high pH. However, these observations were not included in the proposed biochemical model for en-

hanced biological phosphorus removal. Although this model derived from mixed bacterial communities and could not be confirmed on pure cultures (Seviour and McIlroy, 2008), it is still generally accepted.

Polyphosphate is synthesized by polyphosphate kinases (PPKs). In bacteria, two different PKKs are known, PPK1 and PPK2. Long polyphosphate chains are synthesized in *E. coli* mainly by the enzyme PKK1 (EC number 2.7.4.1) (Kornberg et al., 1956; Zhang et al., 2002). PPK1 is a membrane-bound homotetramer with a molecular mass of 80 kDa (Akiyama et al., 1992), which is highly conserved (Tzeng and Kornberg, 1998). It requires the presence of divalent cations for activation, especially Mg^{2+} (Murata et al., 1988). The enzyme catalyzes the transfer of the terminal phosphate from ATP to polyphosphate ($[polyphosphate]_n$ + ATP ⇆ $[polyphosphate]_{n+1}$ + ADP). As an intermediate stage, phosphate is presumably linked to a histidine residue of the enzyme via a N-P bond (Ahn and Kornberg, 1990). The reaction is reversible, but polyphosphate synthesis is favored 4-fold over polyphosphate breakdown (Ishige et al., 2002). When polyphosphate is synthesized, PPK1 uses ATP, when polyphosphate is utilized, the enzyme can use both, ADP and GDP, but has a > 30-fold preference for ADP (Ishige et al., 2002).

PPK2 (EC number 2.7.4.1) has a molecular mass of 44 kDa (Ishige et al., 2002). In contrast to PPK1, PPK2 requires Mn^{2+} cations for activation (Zhang et al., 2002). The enzyme has a polyphosphate-driven nucleoside diphosphate kinase activity and uses polyphosphate to generate GTP from GDP ($[polyphosphate]_n$ + GDP ⇆ $[polyphosphate]_{n-1}$ + GTP). This reaction is reversible, but the rate of GTP production is 75-fold greater than that of polyphosphate synthesis from GTP (Zhang et al., 2002). For polyphosphate synthesis, GTP and ATP are equally efficient as phosphate donors, but when polyphosphate is degraded, PPK2 has preference for GDP over ADP. Thus, PPK2 seems to be adapted for the synthesis of GTP from polyphosphate, while PPK1 favors the synthesis of polyphosphate from ATP. In *Pseudomonas aeruginosa* the enzyme appears during stationary phase (Ishige et al., 2002) and most bacteria have both, PPK1 and PPK2 (Rao et al., 2009).

Polyphosphate glucokinase (PPGK, EC number 2.7.1.63) catalyzes the transfer of a terminal phosphate from polyphosphate to glucose forming glucose-6-phosphate, an important substance in cellular metabolism ($[polyphosphate]_n$ + glucose → $[polyphosphate]_{n-1}$ + glucose-6-phosphate) (Szymona and Ostrowski, 1964). PPGK is a bifunctional enzyme, which has a polyphosphate glucokinase activity and an ATP glucokinase activity. However, polyphosphate and ATP have separate binding sites with different regulatory properties (Hsieh et al., 1993; Phillips et al., 1993).

Polyphosphate:AMP phosphotransferase (PAP, EC number 2.7.4.B2) uses polyphosphate to catalyze the phosphorylation of AMP to ADP ($[polyphosphate]_n$ + AMP → $[polyphosphate]_{n-1}$ + ADP). Once bound, the polyphosphate chain does not dissociate from PAP until it has been degraded completely (Bonting et al., 1992). The produced ADP can then be used by the enzyme adenylate kinase (AK) to form ATP (2 ADP → ATP + AMP) or by PPK1 in the reverse reaction ($[polyphosphate]_n$ + ADP → $[polyphosphate]_{n-1}$ + ATP).

Exopolyphosphatase (PPX, EC number 3.6.1.11) hydrolyses the terminal phosphate residues from long-chain polyphosphate to form orthophosphate ($[polyphosphate]_n + H_2O \rightarrow [polyphosphate]_{n-1} + P_i$; Akiyama et al., 1993). The PPX of *E. coli* is a cytoplasmic membrane-bound dimer with a subunit molecular mass of 58 kDa. The enzyme has a high affinity to long chains of polyphosphate and a high requirement for Mg^{2+} and K^+ (Akiyama et al., 1993).

1.2.1 Polyphosphate storage in colorless sulfur bacteria

Polyphosphate storage has been reported for filamentous and non-filamentous large colorless sulfur bacteria, such as members of the genera *Beggiatoa* and *Thiomargarita*. Concerning the filamentous types, methylene blue staining of narrow heterotrophic freshwater *Beggiatoaceae* revealed the storage of many metachromatic granules of various sizes, which are presumably polyphosphate (Scotten and Stokes, 1962). Maier and Murray (1965) state that polyphosphate was more often found in heterotrophic filaments than

in autotrophic ones. However, the largest polyphosphate inclusions found in bacteria so far are stored by a marine autotrophic *Beggiatoa* strain (Brock et al., 2012). Until now, polyphosphate storage was described for free-living filamentous *Beggiatoaceae* from freshwater (Scotten and Stokes, 1962; Maier and Murray, 1965; Strohl and Larkin, 1978), marine (Brock and Schulz-Vogt, 2011), and hypersaline environments (de Albuquerque et al., 2010). Furthermore, Mußmann et al. (2007) provided genetic evidence for polyphosphate storage in "*Ca.* Isobeggiatoa" and "*Ca.* Parabeggiatoa" (names revised by Salman et al. (2011)). In the genus *Thiomargarita*, the species *Thiomargarita namibiensis,* which is involved in recent phosphorite formation (Section 1.1.1), was shown to store polyphosphate (Schulz and Schulz, 2005).

Among other filamentous relatives of the *Beggiatoceae* the capability to store polyphosphate has not yet been tested or detected, such as the bundle-forming genera *Thioploca* and "*Ca.* Marithioploca" or the attached morphotypes of "*Ca.* Marithrix" (Maier and Murray, 1965; Høgslund et al., 2009). However, "*Ca.* Marithioploca" contains electron-dense inclusions (Maier et al., 1990), which are typical appearance of polyphosphate. Despite the important environmental effect that polyphosphate-storing colorless sulfur bacteria have on phosphorus cycling (Section 1.1.1), the polyphosphate metabolism of these bacteria has not been intensively studied so far. Polyphosphate utilization in *Thiomargarita namibiensis* was proposed to be similar to bacterial communities in waste water treatment plants (Figure 1.4), since in laboratory experiments the cells released phosphate after applying anoxic conditions and acetate addition (Schulz and Schulz, 2005), although their main energy source is the oxidation of sulfide with oxygen or nitrate as the electron acceptor (Schulz et al., 1999). *Thiomargarita namibiensis* cells are not motile and depend on passive transport by externally triggered resuspension of loose sulfidic sediment into the water column, where nitrate and oxygen are available (Schulz and Schulz, 2005). Since *Thiomargarita namibiensis*, in contrast to most of its filamentous relatives, is not motile, the cells need a strategy to overcome anoxia. Therefore, they store nitrate intracellularly as an alternative electron acceptor. Under oxic conditions, *Thiomargarita namibiensis* gains energy by the oxidation of sulfide or intracellular stored sulfur, depending on

sulfide availability. The gained energy can be invested to accumulate both polyphosphate and nitrate, while nitrate is stored in a central vacuole. Under anoxic conditions and an insufficient supply of a suitable external electron acceptor, internally stored nitrate is used to oxidize sulfide or intracellular sulfur globules to gain energy and also polyphosphate could be degraded at this point. Acetate is possibly stored as glycogen instead of PHA (Schulz and Schulz, 2005).

Another sulfur bacterium, which has been studied with respect to polyphosphate utilization, is a marine *Beggiatoa* strain (Brock and Schulz-Vogt, 2011). While *Thiomargarita namibiensis* does not grow in culture, the marine *Beggiatoa* strain was studied under defined laboratory conditions in a growing culture. In contrast to the biochemical model of polyphosphate degradation, which seemed to be valid for *Thiomargarita namibiensis*, it could be shown that the degradation of polyphosphate and release of phosphate in the *Beggiatoa* strain was not triggered by anoxic and acetate-rich conditions. Instead, high sulfide concentrations together with a change from oxic to anoxic conditions triggered polyphosphate degradation and consequently phosphate release, being conditions that are an indirect consequence of acetate introduction into an anoxic, sulfate reducing system. The authors argued that polyphosphate at these conditions might be used to provide energy to overcome sulfide exposure in the absence of an electron donor and enables survival under unfavorable conditions. Comeau et al. (1986), who proposed that bacterial communities in wastewater treatment plants degrade polyphosphate in response to acetate addition and anaerobic conditions, already mentioned that sulfide addition stimulated phosphate release. However, the authors did not further investigate the effect of sulfide on polyphosphate storage. This observation might also have implications for polyphosphate usage in *Thiomargarita namibiensis*, considering that they occur in sediments, which are frequently exposed to high sulfide concentrations and to oxic-anoxic shifts (Figure 1.2). Regarding the findings that *Thiomargarita namibiensis* released phosphate when acetate was added under anoxic conditions (Schulz and Schulz, 2005), it is still possible that the addition of acetate primarily enhanced sulfate reduction rates of associated bacteria present in high numbers in the mucus sheath

around the *Thiomargarita namibiensis* cells. As consequence, this results in higher sulfide concentrations and thus triggers polyphosphate accumulation.

1.3 The family *Beggiatoaceae*

The family *Beggiatoaceae* is a very heterogeneous group of conspicuous bacteria, which share the ability to oxidize fully or partially reduced inorganic sulfur compounds. Recently, the phylogenetic classification of colorless, large sulfur bacteria was revised as it was traditionally based on morphological characteristics and did not reflect genetic heredity (Salman et al., 2011). In addition to the already described genera *Beggiatoa*, *Thioploca*, and *Thiomargarita*, Salman et al. (2011) proposed to add seven new *Candidatus* genera, of which the 16S rRNA gene sequences form distinct monophyletic clusters within the family. According to the reclassification, several genera of large sulfur bacteria that are not monophyletic with the family *Beggiatoaceae*, such as *Thiothrix* and *Leucothrix*, are excluded and form their own distinct families.

Members of the family *Beggiatoaceae* are found worldwide at diverse sulfidic settings and at wide salinity ranges. The bacteria are ubiquitous in freshwater (Strohl and Larkin, 1978), brackish (Jørgensen, 1977), marine (Nelson et al., 1982), and hypersaline sediments (de Albuquerque et al., 2010). They can occur in very dense populations in coastal upwelling areas (Schulz et al., 1999), at hydrothermal vents (Jannasch et al., 1989), at cold seeps (Barry et al., 1996), and in phototrophic microbial mats (Hinck et al., 2007).

Due to their shape and size, representatives of the family *Beggiatoaceae* belong to the largest and most striking bacteria known so far (Schulz and Jørgensen, 2001). Their morphology is diverse, ranging from up to several hundred disc- or cylindrical-shaped cells that are lined up in filaments, over bundles of filaments surrounded by a thick mucous sheath, to enormously large single cells of different shapes and arrangements (Figure 1.5). Filaments have a length between a few micrometers and a few centimeters and diameters between less than one and more than 100 µm. Single cells of *Thiomargarita*

spp. can be up to 750 µm in diameter and occasionally form chains. They can also be arranged in different ways and can be found in asymmetric mucus-surrounded aggregates, in dead diatom frustules, or be simply unicellular (Figure 1.5). Relatives in the candidate genera “Thiopilula” and “Thiophysa” also contain the latter morphological types. Some individuals of these non-filamentous types show a slow rolling motility, others are immobile or even sessile (Salman et al., 2013).

In general, chemolithoautotrophic species gain their energy by the oxidation of reduced sulfur compounds like sulfide (Nelson and Jannasch, 1983) or thiosulfate (Grabovich et al., 2001), and elemental sulfur is stored intracellularly in granules as an intermediate, which can be further oxidized to sulfate (Nelson and Castenholz, 1981). *Beggiatoaceae* filaments are motile by gliding motion and can orient themselves between opposing gradients of oxygen and sulfide (Teske and Nelson, 2006), thereby forming distinct dense mats in the transition zone of the gradients (Jørgensen, 1977). At the transition of the two gradients, the bacteria oxidize the upwards diffusing sulfide with oxygen to elemental sulfur, which is stored intracellularly in granules and leads to a whitish appearance of the cells, and finally to sulfate when sulfide is depleted (Winogradsky, 1887; Nelson and Castenholz, 1981). Due to aerobic sulfide oxidation, the sulfur bacteria steepen the gradients of oxygen and sulfide and narrow the transition zone to a few micrometers (Nelson et al., 1986b).

In the absence of oxygen, nitrate can be used as an alternative electron acceptor, which can be stored in central vacuoles in larger cells of the family *Beggiatoaceae* (McHatton et al., 1996). The ability to store nitrate intracellularly is an adaption to temporary anoxic environmental conditions. For instance, marine free-living filaments (McHatton et al., 1996) as well as bundle-forming types (Otte et al., 1999) are able to store nitrate and oxidize sulfide with it. Also, large single cells of *Thiomargarita namibiensis* accumulate nitrate in their vacuoles, which can account for up to 98% of the cell volume, and nitrate concentrations can reach up to 800 mmol L^{-1} (Schulz et al., 1999). Vacuoles probably allow for wide cell diameters because the diffusion limitation can be prevented by keeping the thickness of the cytoplasmic layer in the

range of conventional bacterial cell sizes (Schulz and Jørgensen, 2001). Sulfide oxidation with nitrate can probably be coupled to dissimilatory nitrate reduction to ammonium (Sayama et al., 2005) or denitrification (Sweerts et al., 1990). In narrow species with cell diameters below 5 µm, no vacuoles were observed. At anoxic conditions, some species can reduce the stored sulfur to sulfide using intracellular stored carbon (Schmidt et al., 1987; Schwedt et al., 2012). Certain strains were shown to fix carbon dioxide (Nelson and Castenholz, 1981) and nitrogen (Nelson et al., 1982) for biomass generation.

Within the family *Beggiatoaceae* different metabolic pathways are known. Besides the chemolithoautotrophic strains, also chemoorganoheterotrophic strains were isolated from freshwater habitats, which are only able to oxidize sulfide in the presence of organic compounds, but are also able to grow solely on organics (Strohl and Larkin, 1978). *Beggiatoa alba,* the type strain of the genus *Beggiatoa,* described in more detail in the next section, is an example for a chemoorganoheterotrophic species, but can also grow mixotrophically when reduced sulfur compounds are available (Güde et al., 1981), thus reflecting the metabolic diversity in the family *Beggiatoaceae*.

Overall, different morphological and physiological features enable the large, colorless sulfur bacteria to inhabit diverse ecological niches that are present in different sulfidic habitats. In these habitats, sulfide-oxidizing bacteria of the family *Beggiatoaceae* can have significant impacts on ecosystems and biogeochemical cycles. They can populate sediments in very high cell densities and thus influence element cycling (Gray and Head, 2005). They affect the phosphorus cycle (Section 1.1.1), as well as the sulfur, nitrogen and carbon cycle.

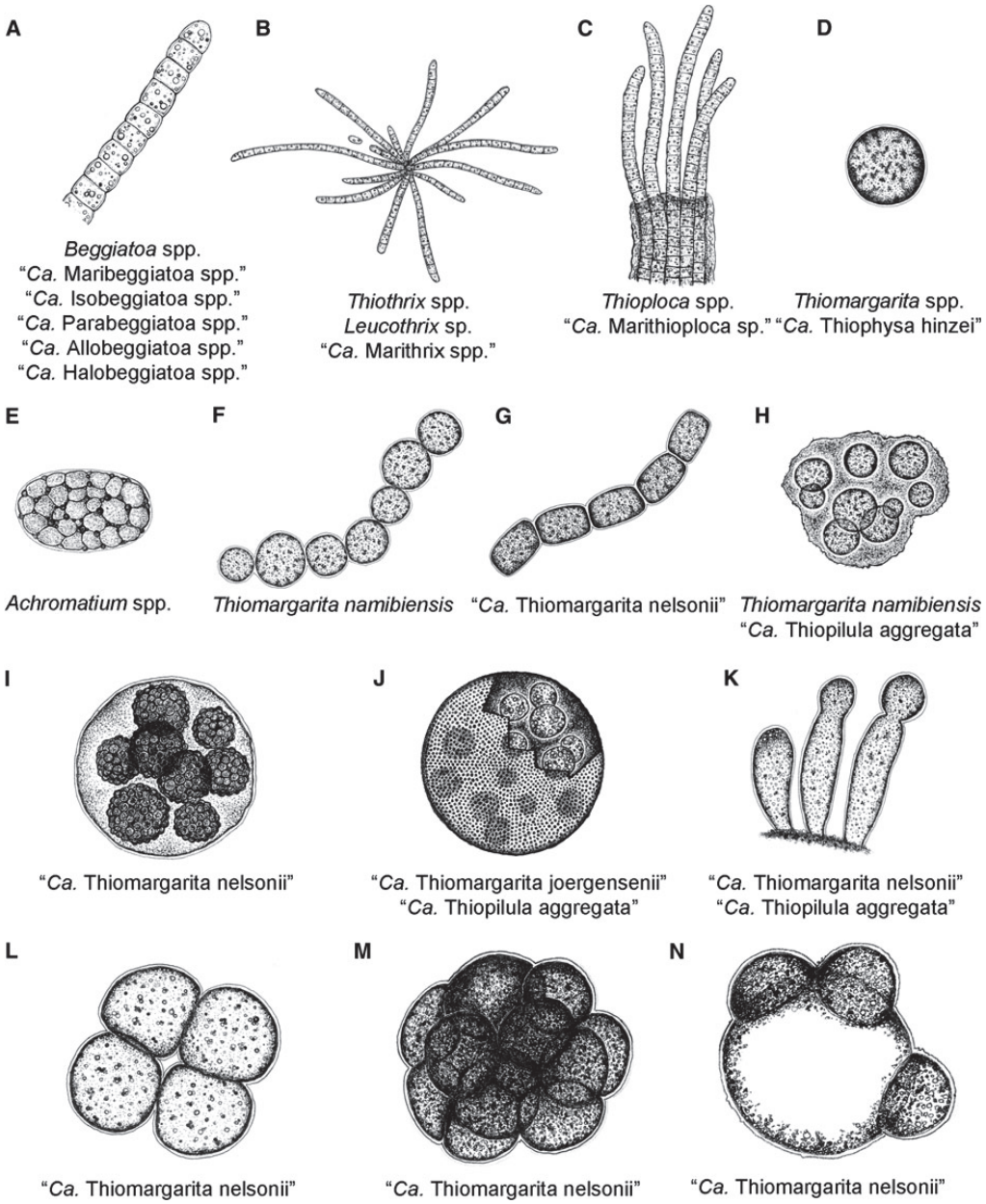

Figure 1.5: Illustration of all currently known morphotypes of large sulfur bacteria with reference to their proposed phylogenetic classification according to Salman et al. (2013). Note that the size proportions are not to-scale. (A) Single, free-living filament; (B) sessile filaments forming buds at the apical end; (C) filaments forming bundles in a common mucus sheath; (D) single, free-living cell; (E) single, free-living cell with calcium carbonate inclusions in addition to elemental sulfur; (F) spherical cells forming chains; (G) cylindrical cells forming chains; (H) cells in a mucous aggregate; (I) cells inside an envelope; (J) cells inside an empty diatom frustule; (K) elongated sessile cells forming buds at the apical end; (L) cell dividing in multiple planes, forming a tetrad; (M) cells dividing in multiple planes forming a large aggregate of several tens of tightly attached cells; (N) putative budding of free-living, extremely large spherical cell.

1.3.1 The species *Beggiatoa alba*

Beggiatoa alba was the first discovered sulfur bacterium in the beginning of the 19th century (Vaucher, 1803). However, at that time the bacteria were assumed to belong to the Cyanobacteria, since the bacteria share a similar morphology; they were initially classified as *Oscillatoria alba*. Later, Trevisan (1842) described them as conspicuous organisms, which were visible to the naked eye and formed long multicellular filaments with numerous sulfur inclusions. He renamed them *Beggiatoa alba,* which is still valid. In 1887, *Beggiatoa alba* was identified of being a sulfur bacterium by Winogradsky, who was the first to propose a lithotrophic lifestyle (Winogradsky, 1887). However, since bacteria at that time were only classified according to their morphology, it can be assumed that the bacteria belonged to filamentous *Beggiatoaceae*, but it could not be proven, that they were indeed representatives of the species *Beggiatoa alba*.

Beggiatoa alba belongs to the genus *Beggiatoa*. According to the recent reclassification (Salman et al., 2011), the genus *Beggiatoa* contains free-living filamentous bacteria with a diameter of 1−9 µm and up to several centimeters length. They are aerobic or microaerophilic with freshwater *Beggiatoa* usually being more oxygen tolerant. Although they depend on oxygen, high concentrations are toxic, since members of the genus *Beggiatoa* lack the enzyme catalase (Larkin and Strohl, 1983), and are therefore sensitive to reactive oxygen species originating from respiration. *Beggiatoa* filaments are motile and form distinct mats at the transition zone of opposing oxygen and sulfide gradients (Kamp et al., 2008). *Beggiatoa* spp. oxidize the sulfide with oxygen to elemental sulfur, which they store intracellularly in granules (Schwedt et al., 2012). In the absence of sulfide, this sulfur can be further oxidized to sulfate. Different storage compounds have been shown in members of the genus *Beggiatoa*, including sulfur globules, PHA (Schwedt et al., 2012), and polyphosphate inclusions (Section 1.2.1).

The freshwater strain *Beggiatoa alba* B15LD was used in the present study and had originally been isolated by Strohl and Larkin (1978) from freshwater

sediments. So far, *Beggiatoa alba* is the only representative of the genus *Beggiatoa* growing in pure culture. Two strains, *Beggiatoa alba* strain B15LD (Strohl and Larkin, 1978) and strain B18LD (Strohl et al., 1981b) are available in type culture collections, and strain B18LD is the type strain of the species (Skerman et al., 1980).

The filament diameter of *Beggiatoa alba* varies from 3−5 µm, the average length of the filament is about 60−120 µm and the trichomes have rounded ends. The bacteria are motile by gliding and disperse by production of necridia and hormogonia (Mezzino et al., 1984). They do not possess vacuoles as typical for narrow *Beggiatoa*, but contain numerous inclusions (Figure 1.6).

Figure 1.6: Differential interference contrast microscopy of a *Beggiatoa alba* filament containing numerous inclusions. Scale bar represents 5 µm.

Beggiatoa alba prefers microaerophilic conditions, since they lack the enzyme catalase (Mezzino et al., 1984). In liquid medium, *Beggiatoa alba* filaments aggregate in tufts to prevent themselves from too high oxygen concentrations (Sweerts et al., 1990). In the presence of oxygen, acetate is oxidized to carbon dioxide and acetate can be used as sole carbon and energy source (Schmidt et al., 1987). Nitrate can also serve as an alternative electron acceptor in addition to oxygen for acetate oxidation (Vargas and Strohl, 1985). When grown heterotrophically on acetate, the cells store PHB (Güde et al., 1981). However, acetate oxidation is slightly suppressed by sulfide oxidation and both reactions might compete for oxygen (Schmidt et al., 1987).

Sulfide is oxidized to sulfur in the presence of oxygen (Schmidt et al., 1986) and may supply energy and reductive potential for mixotrophic growth on acetate (Güde et al., 1981; Schmidt et al., 1987). *Beggiatoa alba* stores sulfur in inclusions enclosed by multiple membranes (Strohl et al., 1981a). These granules are surrounded by the cytoplasmic membrane and are located in the periplasm (Strohl et al., 1982). In contrast to the lithotrophic marine relatives, *Beggiatoa alba* cannot further oxidize sulfur to sulfate (Schmidt et al., 1987). Instead, the stored sulfur can be reduced to sulfide under short-term anoxic conditions with PHB or hydrogen (Schmidt et al., 1987). Therefore, *Beggiatoa alba* is able to survive at least short periods of anoxia (Schmidt et al., 1987). Furthermore, nitrate can be used as an alternative electron acceptor in addition to oxygen for sulfide oxidation (Sweerts et al., 1990). Although *Beggiatoa alba* B18LD was shown to fix nitrogen (Nelson et al., 1982), they did not fix any nitrogen when nitrate or ammonium were added.

Besides sulfur and PHB, *Beggiatoa alba* also stores polyphosphate (Strohl and Larkin, 1978). Although the bacteria belong to the family *Beggiatoacea,* which affects phosphorus cycling (Section 1.1.1), and filamentous freshwater *Beggiatoa*ceae also occur in wastewater treatment plants, where phosphate gets removed by polyphosphate-storing bacteria (Farquhar and Boyle, 1971), their polyphosphate metabolism has not been investigated so far.

1.4 Aims of the thesis

The aim of this thesis was to gain knowledge about polyphosphate storage in sulfur bacteria belonging to the family *Beggiatoaceae*, since they are important in benthic phosphorus cycling in aquatic sediments. Therefore, first the theoretical potential of several large, colorless sulfur bacteria from different genera to synthesize and metabolize polyphosphate was investigated. Then, the studies were focused on the species *Beggiatoa alba*, which is in contrast to the previously investigated relatives *Thiomargarita namibiensis* and *Beggiatoa* sp. 35Flor, an organoheterotroph and inhabits freshwater sediments. The structure and elemental composition of polyphosphate inclusion stored in

Beggiatoa alba were analyzed using various techniques and compared to the polyphosphate inclusions in *Beggiatoa* sp. 35Flor. Furthermore, the conditions under which this strain accumulates and degrades polyphosphate were investigated. Some of these conditions were tested on other lithoautotrophic freshwater, marine and hypersaline *Beggiatoaceae* to see if triggers for polyphosphate storage and degradation can be identified, which are dependent on the habitat or the physiology of the different *Beggiatoaceae* spp.. Finally, polyphosphate storage was investigated in natural environmental samples of free-living filamentous *Beggiatoaceae* to get an understanding under which conditions polyphosphate is stored or degraded in the field.

2. Materials and methods

2.1 Genomic comparison of polyphosphate-related genes in colorless sulfur bacteria

To examine polyphosphate storage in different large, colorless sulfur bacteria in the laboratory, enzymes related to polyphosphate were investigated in a first step by searching in the available genomes of the family *Beggiatoaceae*. Genes coding for predicted polyphosphate-metabolizing enzymes, in partial genomes of six members of the family *Beggiatoaceae* were analyzed using the software tool JCoast, version 1.6 (Richter et al., 2008). The data set included partial genomes of representatives of five currently recognized genera of the family *Beggiatoaceae* (Salman et al., 2011)*; Beggiatoa alba* B18LD (NCBI project ID 62137), "*Ca.* Isobeggiatoa" (Mußmann et al., 2007), "*Ca.* Parabeggiatoa" (Mußmann et al., 2007), "*Ca.* Thiomargarita nelsonii", and an orange *Beggiatoa* filament from Guyamas Basin with an unclear phylogenetic affiliation (MacGregor et al., 2013). The genes of the main polyphosphate synthesizing and degrading enzymes were in focus, such as polyphosphate kinase 1 (PPK1), polyphosphate kinase 2 (PPK2), polyphosphate:AMP phosphotransferase (PAP), polyphosphate glucokinase (PPGK), exopolyphosphatase (PPX), and adenylate kinase (AK). Genes which were not tentatively annotated as encoding for polyphosphate-metabolizing enzymes in JCoast were manually checked for characteristic conserved domains using the NCBI Conserved Domain Database search (Marchler-Bauer et al., 2011).

2.2 Microbiological methods

2.2.1 Microorganisms

2.2.1.1 *Beggiatoaceae* from freshwater habitats

This study mainly investigated the organoheterotrophic freshwater *Beggiatoa alba* (Section 1.3.1), which can also grow "mixotrophically" (chemolithoheterotrophically) with thiosulfate or sulfide as additional energy source. Most laboratory experiments were performed with the strain B15LD, which was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and which had originally been isolated from freshwater sediments by Strohl and Larkin (1978). Strain B18LD was obtained from the American Type Culture Collection (ATCC, Manassas, USA).

Some experiments were also performed with two enrichment cultures of free-living, filamentous, freshwater *Beggiatoacea* spp., which are narrow in diameter and non-vacuolated. Both enrichment cultures grow chemolithoautotrophically and can fix nitrogen. One culture was enriched from a freshwater pond in Hannover, Germany in 2007 and has an average filament diameter of 3.7 µm (Bondarev, 2007). Another culture was enriched from the stream Giber Aa, south of Aarhus, Denmark in 2003, where mats of *Beggiatoaceae* were found on the mud around outlets for primary treated sewage. This strain had about 3 µm wide filaments (Kamp et al., 2006). Pure cultures of these two *Beggiatoaceae* strains could not be obtained so far.

2.2.1.2 *Beggiatoa* sp. strain 35Flor from a marine habitat

The marine chemolithoautotrophic *Beggiatoa* sp. strain 35Flor is able to fix nitrogen and was originally enriched from a black band disease of scleractinian corals from the coast of Florida. This *Beggiatoa* strain is cultivated in a co-culture with the accompanying strain *Pseudovibrio* sp. FO-BEG1, which seems to be required for growth (Schwedt et al., 2012). Filaments have an average diameter of 6 µm (Kamp et al., 2008) and the cells contain sulfur inclusions, polyphosphate inclusions (Brock and Schulz-Vogt, 2011), polyhy-

droxyalkanoates (PHA) (Schwedt et al., 2012), and a central vacuole, probably for polyphosphate storage (Brock et al., 2012).

2.2.1.3 "*Candidatus* Allobeggiatoa spp." from hypersaline habitats

A few experiments were performed on enrichment cultures of wide, chemolithoautotrophic, hypersaline filaments belonging to the genus "*Ca.* Allobeggiatoa" (Hinck et al., 2011). These bacteria are able to fix nitrogen and were enriched from photosynthetic microbial mats in Spain. One culture was obtained in September 2004 from the natural inland Lake Chiprana in northeastern Spain (Hinck et al., 2007) with an average salinity of about 80‰ (Jonkers et al., 2005). The most dominant morphotype had nitrate-containing vacuoles, sulfur inclusions, a filament diameter of 6−8 µm, and a length varying from 1 to > 10 mm (Hinck et al., 2007). Another two cultures were enriched from a saltern pond of the Balearic island Ibiza, Spain at the end of October 2005 (salinity: 60‰ and 150‰, respectively). The filaments had a diameter of 7−10 µm containing a central vacuole for nitrate storage and sulfur inclusions (Hinck, 2008). These hypersaline bacteria are also not yet isolated into pure culture.

2.2.2 Media and cultivation

2.2.2.1 Liquid media for freshwater *Beggiatoa alba* and cultivation

For heterotrophic growth, *Beggiatoa alba* B15LD was cultivated in 250 mL Erlenmeyer flasks containing 100 mL medium on a rotary shaker at 120 rounds per minute (rpm) at 28°C in the dark. The Erlenmeyer flasks were closed with aluminum lids that provided an air atmosphere. Two different heterotrophic media were used, which were both adjusted to pH 7.5 before autoclaving. The first medium was an undefined medium according to Strohl and Larkin (1978) (Table 2.1) without catalase and containing the trace element solution described by Kowallik and Pringsheim (1966). The nutrient broth was purchased from Becton, Dickinson and company, Sparks, USA. The second

medium (Table 2.2) was a defined heterotrophic medium (Schmidt et al., 1987) with a microelement solution described by Vargas and Strohl (1985).

Table 2.1: Composition of liquid medium for *Beggiatoa alba* according to Strohl and Larkin (1978) without catalase.

Medium or solution	Amount per liter
Medium	20 mL saturated $CaSO_4$ solution, 0.45 mg NH_4Cl, 0.2 mg $MgSO_4 \cdot 7\ H_2O$, 1 mg K_2HPO_4, 5 mL trace element solution, 0.82 g $Na(CH_3COO) \cdot 3\ H_2O$, 0.5 g nutrient broth
Trace element solution	0.2 g Na-EDTA, 0.7 g $FeSO_4 \cdot 7\ H_2O$, 0.01 g $ZnSO_4 \cdot 7\ H_2O$, 2 mg $MnSO_4 \cdot 4\ H_2O$, 5 µg $CuSO_4 \cdot 5\ H_2O$, 0.01 g H_3BO_3, 1 mg $Co(NO_3)_2$, 1 mg $Na_2MoO_4 \cdot 2\ H_2O$

Table 2.2: Composition of liquid medium for *Beggiatoa alba* according to Schmidt et al. (1987).

Medium or solution	Amount per liter
Medium	10 mL basal salt solution, 10 mL 1 mol L^{-1} $Na(CH_3COO) \cdot 3\ H_2O$ solution, 5 mL micronutrient solution
Basal salt solution	25.14 g NH_4Cl, 14.7 g $CaCl_2 \cdot 2\ H_2O$, 1 g KH_2PO_4, 0.99 g $MgSO_4 \cdot 7\ H_2O$
Micronutrient solution	11 mL H_2SO_4 (> 98%), 0.2 g Na-EDTA, 0.7 g $FeSO_4 \cdot 7\ H_2O$, 0.01 g $ZnSO_4 \cdot 7\ H_2O$, 0.2 g $MnSO_4 \cdot H_2O$, 0.007 mg $CuSO_4$, 10 mg H_3BO_3, 0.95 mg $CoCl_2 \cdot 6\ H_2O$, 1.21 mg Na_2MoO_4

To test the effect of different components of the medium on polyphosphate storage, the defined medium (Schmidt et al., 1987) was used with different modifications. The following modifications were made to test the effect of nitrogen and phosphate availability on polyphosphate storage: supply of $NaNO_3$ or NH_4Cl as nitrogen source at final concentrations of 0.05, 0.1, 0.5, 1, or 10 mmol L^{-1}. KH_2PO_4 concentrations were 73 µmol L^{-1} or 1 mmol L^{-1}. Initial acetate concentrations were always 10 mmol L^{-1}. To test the effect of carbon supply on polyphosphate storage a medium containing 1 mmol L^{-1} $NaNO_3$ and

1 mmol L^{-1} KH_2PO_4 together with acetate in concentrations of 0.1, 0.5, 1, and 10 mmol L^{-1} was used.

To investigate if polyphosphate is degraded in response to temperature stress, *Beggiatoa alba* B15LD was exposed to high temperatures. For experiments at different temperatures, aliquots of 1 mL culture, which were grown 3 days in medium according to Strohl and Larkin (1978), were filled in 2 mL Eppendorf cups and placed in a water bath at temperatures of 40−50°C for 1 hour.

2.2.2.2 Semi-solid gradient media for *Beggiatoaceae* and cultivation

Semi-solid gradient media mimic natural sediments in which opposing gradients of oxygen and sulfide in the sediment favor growth of colorless sulfur bacteria. In these habitats sulfide is formed by sulfate reduction or has its origin in hydrothermal vent fluids and diffuses upwards, thereby forming a gradient. Often the upper layer of the sediment is oxygenated and forms a gradient in the other direction. The used medium in glass tubes contained a sulfidic bottom agar plug (1.5% w/v Bacto Agar ,BD , NJ, USA) and a sulfide-free, semisolid top agar layer (0.25% w/v Bacto Agar ,BD , NJ, USA), which allows movement of the *Beggiatoaceae* filaments (Figure 2.1).

Semi-solid gradient medium was used to cultivate freshwater, marine and hypersaline filamentous sulfur bacteria of the family *Beggiatoaceae*. The principle was always the same as described above. The details about the composition of the different media are given in the next sections. In general, the agar in the medium washed was three times with demineralized water in Millipore Quality (MilliQ water) before usage. The different components of the medium in top agar and bottom agar were combined after autoclaving them first separately. Afterwards, 4 mL bottom agar were filled in each glass tube (length 150 mm, inside diameter 14 mm). The glass tubes were closed tightly with screw caps to not loose gaseous sulfide. When the bottom agar cooled down and consequently solidified, 8 mL of the top agar (stored at 60°C until use) was filled on top and the tubes were closed loosely to permit exchange of the

headspace gas with the atmosphere. After 1 day, when opposing gradients of oxygen and sulfide were established, the medium was inoculated ca. 1 cm below the air-agar interface with 100 µL of a *Beggiatoa* culture. All cultures growing in semi-solid gradient medium were incubated at room temperature (~ 20°C) in the dark. At the interface of the two opposing gradients of sulfide and oxygen a *Beggiatoa* mat formed, which oxidized sulfide with oxygen (Figure 2.1 right).

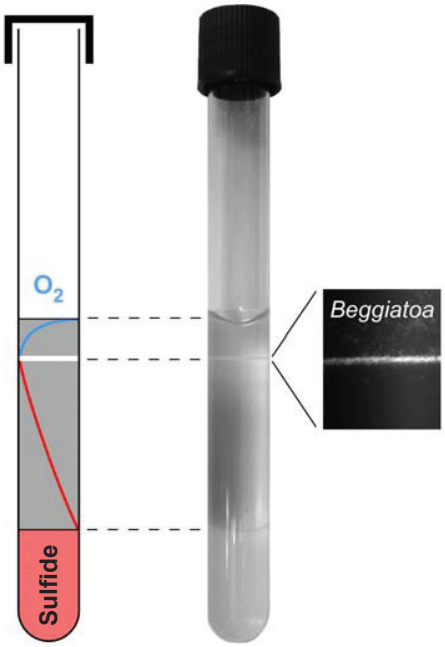

Figure 2.1: Schematic view of a *Beggiatoa* culture in a glass tube containing semi-solid gradient medium (left), picture of a *Beggiatoa* culture in a glass tube containing semi-solid gradient medium (middle), and an enlarged picture of a *Beggiatoa* mat (right). The bottom agar (red) contains a sulfide reservoir, the top agar (grey) is semi-solid and allows *Beggiatoa* filaments to move. At the interface of opposing gradients of sulfide from below and oxygen from above *Beggiatoa* filaments form a mat (right). The tubes were closed loosely to permit exchange of the headspace gas with the atmosphere (courtesy of A.-C. Kreutzmann).

Semi-solid gradient media for freshwater Beggiatoaceae and cultivation

Semi-solid gradient medium for mixotrophic growth of freshwater *Beggiatoa alba* contained modified medium according to Schmidt et al. (1987) (Table 2.2). The top agar was additionally buffered at pH 7.5 with 20 mmol L^{-1} 1,4-piperazine-diethanesulfonic acid disodium salt (PIPES). The ammonium concentration was 0.1 mmol L^{-1}. Sulfide concentrations in the bottom agar ranged from 0−4 mmol L^{-1}. For experiments at anoxic conditions, the glass

tubes were closed with butyl rubber stoppers and flushed with dinitrogen gas for 5 minutes.

The composition of the freshwater medium for lithoautotrophic growth of freshwater enrichment cultures of *Beggiatoaceae* (Table 2.3) was described by Kamp et al. (2006). The top agar was additionally buffered with 20 mmol L^{-1} PIPES to maintain a pH of 7.5. The different components of the medium in the bottom and top agar (Table 2.4) were combined after separate autoclaving. The sulfide concentration in the bottom agar was 2−4 mmol L^{-1}.

In experiments testing the effect of an additional nitrogen source on polyphosphate storage, $NaNO_3$ and NH_4Cl was added to the top agar in concentrations of 1 mmol L^{-1}. In experiments testing the effect of nitrogen limitation on polyphosphate storage, nitrogen fixation was inhibited by acetylene addition. Therefore, the tubes were closed with butyl rubber stoppers and 10% of the gas phase was exchanged by acetylene gas.

Table 2.3: Solutions for semi-solid gradient medium for freshwater *Beggiatoaceae* enrichment cultures according to Kamp et al. (2006).

Medium or solution	**Amount per L MilliQ water**
Medium	0.01 g Na-EDTA, 0.12 g $CaSO_4 \cdot 2\ H_2O$, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 0.016 g NaCl, 0.14 g Na_2HPO_4*, 0.138 g NaH_2PO_4*, 0.264 g $CaCl_2 \cdot 2\ H_2O$, 2 mL $FeCl_3$ solution (0.29 g L^{-1}), 1 mL micronutrient solution
Micronutrient solution	0.5 mL H_2SO_4 (> 98%), 2.28 g $MnSO_4 \cdot H_2O$, 0.5 g $ZnSO_4 \cdot 7H_2O$, 0.5 g H_3BO_3, 0.025 g $CuSO_4 \cdot 5\ H_2O$, 0.025 g $Na_2MoO_4 \cdot 2\ H_2O$, 0.045 g $CoCl_2 \cdot 6\ H_2O$
Vitamin stock solution**	0.1 g cyanocobalamine, 0.1 g inositol, 0.1 g biotin, 0.1 g folic acid, 1.0 g *p*-aminobenzoic acid, 10 g nicotinic acid, 10 g *d*-pantothenate, 20 g thiamine

* Was separately dissolved to avoid precipitation.
** All vitamins were dissolved separately, then combined in a final stock solution (1 mL of each vitamin solution in a final volume of 100 mL distilled water) and filter-sterilized twice.

Table 2.4: Composition of semi-solid gradient medium for freshwater *Beggiatoaceae* enrichment cultures modified after Kamp et al. (2006).

Final medium	Component*	Composition
Bottom agar	A	90 mL medium, 90 mL MilliQ water, 2.7 g agar**, 180 µL Phenol Red, 1 drop 1 mol L^{-1} KOH
	B	0.375 mL 1 mol L^{-1} Na_2S solution (final concentration of 2 mmol L^{-1}) or 0.75 mL 1 mol L^{-1} Na_2S solution (final concentration of 4 mmol L^{-1})
Top agar	A	180 mL medium, 2.18 g PIPES, 360 µL Phenol Red
	B	180 mL MilliQ water, 0.9 g agar**
	C	0.36 mL $FeCl_3$ solution (0.29 g L^{-1})
	D	0.72 mL 1 mol L^{-1} $NaHCO_3$ solution
	E	150 µL vitamin solution

* All components were sterilized separately.
** The agar was washed three times in MilliQ water before usage.

Semi-solid gradient media for marine Beggiatoa and cultivation

For lithoautotrophic growth of *Beggiatoa* sp. strain 35Flor, tubes with semi-solid gradient medium modified after Nelson and Jannasch (1983) using artificial seawater (Kamp et al., 2008) (Table 2.5) were prepared and the top agar was buffered with 20 mmol L^{-1} PIPES to maintain a pH of 7.0. The sulfide concentration in the bottom agar was 4 mmol L^{-1}. The different components of the bottom and top agar (Table 2.6) were combined after separate autoclaving. In experiments testing the effect of an additional nitrogen source on polyphosphate storage, $NaNO_3$ and NH_4Cl was added to the top agar in concentrations of 1−10 mmol L^{-1}. In experiments testing the effect of nitrogen limitation on polyphosphate storage, nitrogen fixation was inhibited by acetylene addition. Therefore, the tubes were closed with butyl rubber stoppers and 10% of the gas phase was exchanged by acetylene gas.

Table 2.5: Solutions for semi-solid gradient medium modified after Nelson and Jannasch (1983) for marine *Beggiatoa.*

Solution	Amount per L MilliQ water
Artificial seawater	27.5 g NaCl, 5 g $MgCl_2 \cdot 6\ H_2O$, 4.1 g $MgSO_4 \cdot 7\ H_2O$, 0.66 g $CaCl_2 \cdot 2\ H_2O$, 1.02 g KCl
Mineral solution	555 mg K_2HPO_4, 28.72 mg Na_2MoO_4, 750 mg $Na_2S_2O_5$, 29 mg $FeCl_3 \cdot 6\ H_2O$
Trace element solution*	5.2 g Na-EDTA, 1.5 g $FeCl_2 \cdot 4\ H_2O$, 70 mg $ZnCl_2$, 100 mg $MnCl_2 \cdot 4H_2O$, 62 mg H_3BO_4, 190 g $CoCl_2 \cdot 6\ H_2O$, 17 mg $CuCl_2 \cdot 2\ H_2O$, 24 mg $NiCl_2 \cdot 6\ H_2O$, 36 mg $Na_2MoO_4 \cdot 2\ H_2O$
Vitamin solution**	0.1 g cyanocobalamine, 0.1 g inositol, 0.1 g biotin, 0.1 g folic acid, 1.0 g *p*-aminobenzoic acid, 10 g nicotinic acid, 10 g *d*-pantothenate, 20 g thiamine

* pH adjusted to 6.5.
** All vitamins were dissolved separately, then combined in a final stock solution (1 mL of each vitamin solution in a final volume of 100 mL distilled water) and filter-sterilized twice.

Table 2.6: Composition of semi-solid gradient medium modified after Nelson and Jannasch (1983) for marine *Beggiatoa.*

Medium	Component*	Composition
Bottom agar	A	100 mL artificial seawater, 2.9 g NaCl, 1 drop 1 mol L^{-1} KOH
	B	80 mL MilliQ water, 2.7 g agar**
	C	0.75 mL 1 mol L^{-1} Na_2S solution
Top agar	A	240 mL artificial seawater, 4.32 g NaCl, 2.18 g PIPES
	B	96 mL MilliQ water, 0.9 g agar**
	C	24 mL mineral solution, 0.36 ml trace element solution, 7 drops 1 mol L^{-1} KOH
	D	0.72 mL 1 mol L^{-1} $NaHCO_3$ solution
	E	150 µL vitamin solution

* All components were sterilized separately.
** The agar was washed three times in MilliQ water before usage.

The influence of different stress factors on polyphosphate storage in *Beggiatoa* sp. strain 35Flor, such as pH and temperature, was also tested. For experiments testing pH effects, the pH in the top agar was adjusted to pH values of 6.0, 6.5, 7.0, 7.5, and 8.5. Concerning temperature stress, culture tubes of *Beggiatoa* sp. strain 35Flor were put for 1 hour in a water bath at temperatures of 40−80°C.

Semi-solid gradient media for hypersaline "Candidatus Allobeggiatoa spp." and cultivation

For lithoautotrophic growth of hypersaline enrichment cultures of "*Ca.* Allobeggiatoa spp.", a semi-solid gradient medium modified after Nelson and Jannasch (1983) using artificial seawater (Kamp et al., 2008) with increased salinity (Table 2.7) was used. The pH was adjusted to approximately 7.0 with KOH, as indicated by the addition of the pH indicator phenol red. Sulfide concentration in the bottom agar plug was 4 mmol L^{-1}. The different components of the bottom and top agar (Table 2.8) were combined after separate autoclaving. As the enrichment culture contained agar-degrading microorganisms, the agar concentration in the top agar was increased to 1 g.

In experiments testing the effect of nitrogen limitation on polyphosphate storage, nitrogen fixation was inhibited by acetylene addition. Therefore, the tubes were closed with butyl rubber stoppers and 10% of the gas phase was exchanged by acetylene gas.

Table 2.7: Solutions for semi-solid gradient medium modified after Nelson and Jannasch (1983) with increased salinities for hypersaline enrichment cultures of "*Ca.* Allobeggiatoa spp.".

Solution	Amount per L MilliQ water
Artificial seawater	43 g NaCl, 5 g $MgCl_2 \cdot 6\ H_2O$, 4.1 g $MgSO_4 \cdot 7\ H_2O$, 0.66 g $CaCl_2 \cdot 2\ H_2O$, 1.02 g KCl
Mineral solution	555 mg K_2HPO_4, 28.72 mg Na_2MoO_4, 750 mg $Na_2S_2O_5$, 29 mg $FeCl_3 \cdot 6\ H_2O$
Trace element solution*	5.2 g Na-EDTA, 1.5 g $FeCl_2 \cdot 4\ H_2O$, 70 mg $ZnCl_2$, 100 mg $MnCl_2 \cdot 4H_2O$, 62 mg H_3BO_4, 190 g $CoCl_2 \cdot 6\ H_2O$, 17 mg $CuCl_2 \cdot 2\ H_2O$, 24 mg $NiCl_2 \cdot 6\ H_2O$, 36 mg $Na_2MoO_4 \cdot 2\ H_2O$
Vitamin solution**	0.1 g cyanocobalamine, 0.1 g inositol, 0.1 g biotin, 0.1 g folic acid, 1.0 g *p*-aminobenzoic acid, 10 g nicotinic acid, 10 g *d*-pantothenate, 20 g thiamine

* pH adjusted to 6.5.
** All vitamins were dissolved separately, then combined in a final stock solution (1 mL of each vitamin solution in a final volume of 100 mL distilled water) and filter-sterilized twice.

Table 2.8: Composition of semi-solid gradient medium modified after Nelson and Jannasch (1983) with increased salinities for hypersaline enrichment cultures of "*Ca.* Allobeggiatoa spp.".

Medium	Component*	Composition
Bottom agar	A	180 mL artificial seawater, 2.7 g agar**, 180 µL Phenol Red, 1 drop 1 mol L^{-1} KOH
	B	0.75 mL 1 mol L^{-1} Na_2S solution
Top agar	A	240 mL artificial seawater, 8 g NaCl (= 80 ‰) or 6 g (= 60 ‰) or 15 g (= 150 ‰)
	B	96 mL MilliQ water, 1 g agar**
	C	24 mL mineral solution, 360 µL trace element solution, 360 µL Phenol Red, 4 drops 1 mol L^{-1} KOH
	D	0.72 mL 1 mol L^{-1} $NaHCO_3$ solution
	E	150 µL vitamin solution

* All components were sterilized separately.
** The agar was washed three times in MilliQ water before usage.

2.2.3 Studies with sediment cores from different habitats to detect polyphosphate storage in environmental *Beggiatoaceae*

To investigate, if natural populations of filamentous, colorless sulfur bacteria store polyphosphate under environmental conditions and to compare, whether these findings are in agreement with observations made in cultures in the laboratory, sediment cores containing *Beggiatoaceae* filaments were taken. The sediment cores were sampled at two different sites: Aarhus Bay, Denmark, and Lake Grevelingen, the Netherlands.

2.2.3.1 Sediment core from Aarhus Bay, Denmark

The first sampling site was Aarhus harbor at the Aarhus Bay, Denmark. Aarhus Bay is a shallow semi-enclosed embayment in the northwestern part of the Baltic Sea, characterized by seasonal variations of temperature and elevated primary production during the summer months (Glud et al., 2003). The sediment core was taken in February 2012 using a corer connected to a 2 m-long stick and stored at 4°C in the dark. Microsensor measurements were performed by A.-C. Kreutzmann for pH, oxygen, and total sulfide. A mat of *Beggiatoa*ceae filaments was present approximately 2 mm below the sediment surface. Sulfide was consumed within the bacterial mat. The oxygen concentration in the water column was around 70 µmol L^{-1}. Oxygen was consumed within the sediment and the sediment on top of the bacterial mat was depleted in oxygen. The pH in the upper sediment layer was around 7.12 and increased with depth. In the *Beggiatoa* mat the pH was around 7.25 (personal communication A.-C. Kreutzmann).

2.2.3.2 Sediment cores from Lake Grevelingen, The Netherlands

The second sampling site was the marine Lake Grevelingen, the Netherlands, which is an artificially enclosed estuary that is relatively shallow and has limited seawater exchange with the North Sea. The lake is known to be subject to seasonal hypoxia; in summer the lake is anoxic, in winter oxic. Sediment cores were taken in August and September 2012 in 23.1 m depth using a

hand-held corer. Oxygen, nitrate, ammonium, total sulfide, and total phosphorus were determined by F. Sulu-Gambari. Oxygen concentration in the bottom water was 0 in August and about 200 µmol L^{-1} in September. No nitrate was measured in the water overlaying the sediment in either month. The total phosphorus dropped from 4.5 in August to 0.1 µmol L^{-1} in September, ammonium from 55−13 µmol L^{-1} and sulfide was only measurable in the bottom water in the most anoxic month August at 1.2 µmol L^{-1} (personal communication F. Sulu-Gambari).

2.3 Analytical methods

2.3.1 Detection and characterization of polyphosphate inclusions

The presence of polyphosphate inclusions in *Beggiatoa* samples were investigated by applying various techniques. Structure and properties of polyphosphate inclusions were then compared to the marine lithotrophic *Beggiatoa* strain 35Flor (Brock et al., 2012).

2.3.1.1 Staining with 4´,6´-diamidino-2-phenylindole

4',6-diamidino-2-phenylindole (DAPI) was used to stain polyphosphate inclusions in *Beggiatoa* cells. DAPI is a fluorescent cationic dye commonly used for staining of DNA with a maximum emission wavelength around 460 nm upon UV excitation (Kapuściński and Skoczylas, 1978) leading to a blue signal. When used at high concentrations, DAPI also stains negatively charged polyphosphate inclusions, thereby shifting its maximum emission wavelength upon UV excitation to about 525 nm, resulting in a green-yellow signal (Tijssen et al., 1982). Tjissen et al. (1982) also tested small, negatively charged molecules like orthophosphate but found no effect on DAPI fluorescence. Ruiz et al. (2001) furthermore excluded an effect of pyrophosphate or other anions on the emission shift of DAPI when binding to polyphosphate. Tjissen et al. (1982) discussed that the special behavior of polyphosphate might be due to the extraordinary high density of negative charges. DAPI bound to lipids also show a

yellow fluorescence, but the lipid fluorescence signal is weak and fades within a few seconds (Streichan et al., 1990).

For polyphosphate staining, 10 µL of DAPI solution (1 mg mL^{-1} in MilliQ water) was added to 90 µl of a *Beggiatoa* sample or sediment and incubated 30 minutes at room temperature. Polyphosphate inclusions inside the *Beggiatoa* filaments were visualized either with a fluorescence microscope or with a confocal laser scanning microscope (CLSM). The fluorescence microscope was 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). The CLSM (LSM 510; Zeiss, Oberkochen, Germany) was equipped with a 100-fold Zeiss Apochromat NA 1.4 oil immersion objective lens. The excitation wavelength was 351 and 364 nm, DNA fluorescence was observed through a band pass 385-470 nm filter and polyphosphate fluorescence was observed through a band pass 505-550 nm filter. Overlays of the DNA and polyphosphate images were performed using the LSM Browser Rel. 4.2 software.

2.3.1.2 Staining with Toluidine Blue

Toluidine Blue is a metacromatic dye, which reveals blue red inclusions when binding to polyphosphate (Kulaev et al., 2004). For staining, a small volume of culture was spotted onto a glass slide and air-dried. The sample was heat-fixed by passing the slide several times through a Bunsen burner flame. After cooling, the sample was rinsed with MilliQ water and dried with a paper tissue. Afterwards the specimen was covered with a Toluidine Blue solution (0.3% in 0.5% acetic acid) for 30–45 seconds and then washed with 0.5% acetic acid and finally with MilliQ water. Upon air-drying the sample was examined by bright field microscopy using an Axioplan universal microscope (Zeiss, Oberkochen, Germany).

2.3.1.3 Staining with Nile Red

Nile Red is a lipophilic fluorescent dye, which binds to membrane lipids (Greenspan and Fowler, 1985) and PHA. In this study it was tested, if polyphosphate inclusions of *Beggiatoa alba* are surrounded by a membrane, as observed in a marine *Beggiatoa* strain (Brock et al., 2012). For staining, 90 µL sample of a liquid *Beggiatoa alba* culture was incubated for 5 minutes with 10 µL Nile Red (25 mg L^{-1} in dimethyl sulfoxide; Sigma-Aldrich, Steinheim, Germany). Fluorescence of Nile Red was excited with an argon ion laser at 488 nm and 546 nm and emission was recorded through a long pass 585 nm filter (Zeiss LP 585).

2.3.1.4 Staining with Acridin Orange

The acidophilic dye Acridin Orange was used to verify, whether there is a pronounced pH difference between cytoplasm and polyphosphate inclusions as observed in polyphosphate-containing inclusions in eukaryotes (Ramos et al., 2010). Acridine Orange is a cationic fluorescent dye used for staining of nucleic acids, which in principle binds to any kind of polyanion (Bradley and Wolf, 1959). Intercalation into DNA double helices results in a green fluorescence, which is detected at 530 nm (Lerman, 1963), whereas interaction with single stranded nucleic acids like RNA results in a red fluorescence detectable at 640 nm (Bradley and Wolf, 1959). Furthermore Acridine Orange is a pH-sensitive dye that accumulates in acidic cell compartments in dependence of the pH difference and shows a shift in emission at higher concentrations from green to red because of aggregate formation (Han and Burgess, 2010). Acridine Orange (Sigma Aldrich, Steinheim, Germany) was applied to the *Beggiatoa alba* samples in a final concentration of 6 µmol L^{-1} and incubated for 5 hours in the dark. The filaments were examined with a CLSM (LSM 510; Zeiss, Oberkochen, Germany). Fluorescence of Acridine Orange was excited with an argon ion laser at 488 nm and emission was recorded above 505 nm using a long pass 505 nm filter (Zeiss LP 585).

2.3.1.5 Staining with Rhodamin 123

To check for the presence of an electric potential (inside positive) over membranes of inclusions, *Beggiatoa alba* and *Beggiatoa* sp. strain 35For filaments were stained with the fluorescent lipophilic cationic dye Rhodamine 123. Rhodamin 123 is commonly used in eukaryotic cells to stain mitochondria, in which it accumulates because of the membrane potential and shows a green fluorescence upon excitation at 485 nm (Johnson et al., 1980). Rhodamin 123 (Invitrogen, Karlsruhe, Germany) was added to the *Beggiatoa* samples at a final concentration of 200 µmol L^{-1} and incubated for 1 hour in the dark. The stained filaments were investigated with a CLSM (LSM 510; Zeiss, Oberkochen, Germany). Fluorescence of Rhodamine 123 was excited with an argon ion laser at 488 nm and emission was observed above 505 nm using a long pass 505 nm filter (Zeiss LP 585).

2.3.1.6 Raman micro-spectroscopy

Raman microscopy is useful to identify polyphosphate in bacteria (Majed et al., 2009). Raman micro-spectroscopy was used to identify and visualize polyphosphate in *Beggiatoa* filaments. This method combines Raman spectroscopy with confocal microscopy and allows for mapping of polyphosphate on a microscopic scale, based on analysis of Raman scattering spectra. Sodium phosphate glass type 45 was used as polyphosphate standard (Sigma-Aldrich, Steinheim, Germany). Filaments from *Beggiatoa* sp. strain 35Flor cultivated in semi-solid gradient medium were rinsed in artificial seawater to remove agar and were then transferred onto round glass cover slips (1 mm thickness) coated with poly-L-lysine. *Beggiatoa alba* cells were directly pipetted from liquid cultures onto the glass cover slips. Subsequently, the medium was removed and the adhered cells were air-dried. The samples were analyzed with a confocal Raman micro-spectroscope NTEGRA Spectra (NT-MDT, Eindhoven, the Netherlands) coupled to an inverted Olympus IX71 microscope (Olympus, Japan). All measurements were performed with a 532 nm solid-state laser and a 100x oil-immersion objective lens (NA 1.30, Olympus UPlanFL N, Hamburg, Germany). The confocal pinhole was set to 100 µm

corresponding to a spatial resolution of approximately 300 nm. The Raman analyses were performed without a neutral density filter, which resulted in an illumination power at the sample of ca. 40 µW. Exposure times were in the range of several seconds for point spectra and 2 seconds per pixel during Raman mapping. A grating of 150 l.mm^{-1} was used to disperse the Raman-scattered light and the signal was collected by an electron multiplying charge coupled device (EMCCD) camera (Andor Technology, Belfast, Northern Ireland) cooled to -70°C. The Raman spectra were recorded between 0 and approximately 4500 cm^{-1} with a spectral resolution of 0.2 cm^{-1}. Spectral data were processed using Nova Px 3.1.0 software.

2.3.1.7 Scanning electron microscopy and energy dispersive X-ray analysis

Scanning electron microscopy (SEM) in combination with energy dispersive X-ray analysis (EDXA) was performed to visualize polyphosphate inclusions and to detect elements associated to them. *Beggiatoa alba* samples from liquid cultures were washed with water and placed on 10 · 10 mm silicon wafer supports (Plano, Wetzlar, Germany) on which they dried. The silicon wafer supports were attached to aluminum stubs. SEM pictures were obtained with a Quanta 250 FEG SEM (FEI, Eindhoven, the Netherlands) operating at 10 kV. The specimens were investigated at high vacuum mode using an Everhart-Thornley detector (ETD). EDXA measurements of whole filaments or selected spots and areas were performed using a Bruker Double Detector system equipped with two XFlash 6/30 (Bruker nano, Berlin, Germany).

2.3.2 Protein determination

The protein content was determined with a modified method according to Bradford (1976). 1 mL of culture was centrifuged at 20°C for 5 minutes at 13,000 rpm (centrifuge 5417 R, Eppendorf, Hamburg, Germany). The cell pellet was washed with 100 mmol L^{-1} Tris-HCl buffer pH 7.5 containing 5 mmol L^{-1} $MgCl_2$ and then resuspended in 0.5 mL of the same buffer. 0.5 mL

1 mol L^{-1} NaOH was added and the sample was incubated for 10 minutes at 95°C and 300 rpm (thermomixer comfort, Eppendorf, Hamburg, Germany). 0.2 mL sample was supplied with 0.6 mL distilled water and 0.2 mL Bradford reagent (Bio-Rad Laboratories, München, Germany). After 30 minutes incubation in the dark, the absorption at 595 nm was measured using a SpectroDirect Spectrophotometer (Aqualytic, Dortmund, Germany). For calibration bovine serum albumin (Bio-Rad Laboratories, München, Germany) was used as standard. Three technical replicates of each of the three biological replicates were measured.

2.3.3 Polyphosphate determination

Polyphosphate was determined with the DAPI-based method described by Kulakova et al. (2011) by taking three technical samples from each biological replicate. The cells were broken by snap-freezing in liquid nitrogen. Sodium phosphate glass type 45 was used as polyphosphate standard (Sigma-Aldrich, Steinheim, Germany). The fluorescence of the samples was measured with a luminescence spectrometer LS-50B (Perkin-Elmer, Überlingen, Germany). Three technical replicates of each of the three biological replicates were measured.

2.3.4 Phosphate determination

Phosphate was determined colorimetrically by the ascorbic acid method modified after Hansen and Koroleff (1999). 10 µL of an ascorbic acid solution (containing 0.1 g ascorbic acid dissolved in 5 mL MilliQ water and 5 mL 4.5 mol L^{-1} H_2SO_4) were added to 500 µL sample. The reaction was started by addition of 10 µL heptamolybdate solution (containing 4 g $(NH_4)_6\ Mo_7O_{24} \cdot 4\ H_2O$ in 30 mL MilliQ water, 90 mL 4.5 mol L^{-1} H_2SO_4, and 5 mL tartrat solution (2.5 g $C_4H_4KO_7Sb \cdot 0.5\ H_2O$ in 100 mL MilliQ water)). After 30 minutes the absorption was measured at a wavelength of 695 nm using a SpectroDirect Spectrophotometer (Aqualytic, Dortmund, Germany). Calibration was performed with phosphate standards prepared from a titrisol stock

solution (Merck, Darmstadt, Germany). Two technical replicates of each of the three biological replicates were measured.

2.3.5 Acetate determination

Acetate was determined with a HPLC system (Sykam GmbH, Eresing, Germany) equipped with an anion neutral pre-column (4 · 20 mm; Sykam GmbH, Eresing, Germany) and an Aminex HPX-87H separation column (300 · 7.8 mm; Biorad, München, Germany) at a temperature of 60°C. The eluent consisted of 5 mol L^{-1} H_2SO_4 in HPLC-grade water with a flow rate of 0.6 mL $minute^{-1}$. Acetate was quantified with a 7515A RI detector (ERC, Riemerling, Germany). Two technical replicates of each of the three biological replicates were measured.

2.3.6 Nitrate determination

Nitrate was determined with a NO_X analyzer (CLD 86, Eco Physics, Rösrath, Germany) according to the vanadium chloride reduction method (Braman and Hendrix, 1989). At 90°C, the acidified 0.1 mol L^{-1} vanadium chloride in the reaction chamber reduces both nitrate and nitrite. The nitrate concentration was calculated by subtracting the nitrite concentration determined separately by a standard colorimetric method. Two technical replicates of each of the three biological replicates were measured.

2.3.7 Ammonium determination

Ammonium was determined using the flow injection analysis system as described by Hall and Aller (1992). 30 mmol L^{-1} NaOH with 0.2 mol L^{-1} sodium citrate tribasic dehydrate and 50 µmol L^{-1} HCl were used as carrier and receiver phase, respectively. Two technical replicates of each of the three biological replicates were measured.

2.3.8 Guanosine tetraphosphate determination

Guanosine tetrahosphate (ppGpp) was extracted from *Beggiatoa alba* cells grown for 3 days at nitrogen surplus or limiting conditions in liquid cultures in modified media according to Schmidt et al. (1987) (Section 2.2.2.1). The nucleotides were extracted as described by Traxler et al. (2008), except that for each sample cells of 100 mL cultures were collected and the cell pellet was resuspended in 2.5 mol L^{-1} ice-cold formic acid. The ppGpp content was determined by anion exchange chromatography on Mono Q 5/50 GL column (GE Healthcare, Dassel, Germany) based on a procedure published by Traxler et al. (2008). The ppGpp standard was purchased from TriLink Bio Technologies, San Diego, USA. Two technical replicates of each of the three biological replicates were measured.

2.3.9 Measurements with different microsensors

To measure microsensor profiles through *Beggiatoa alba* tufts, they first had to be embedded in agar-containing medium (1% w/v Bacto Agar ,BD , NJ, USA). Tufts grown in medium with 10 mmol L^{-1} NH_4Cl were picked with glass needles and transferred to 45°C warm medium containing agar, which solidified upon cooling. After this procedure *Beggiatoa alba* tufts were still alive and respired oxygen as previously shown (personal communication A.-C. Kreutzmann). In case of ammonium measurements, the agar-containing medium was prepared without KH_2PO_4 and $Na(CH_3COO) \cdot 3\ H_2O$, because the microsensors for ammonium measurements are sensitive to sodium and potassium.

The pH microsensor (PH-10, Unisense A/S, Aarhus, Denmark) was used together with an external self-made reference electrode. Buffer solutions with a pH of 4.01, 7.00, and 9.21 (Mettler-Toledo, Giessen, Germany) were used for calibration. For ammonium measurements, self-made microsensors with liquid ion-exchanging (LIX) membranes (de Beer and van den Heuvel, 1988) and self-made external reference electrodes were used.

The used micosensors were calibrated directly before and after the measurements to correct for a possible drift. The measurements started above the tuft in the agar and were performed through the tuft by vertical profiling in 50 µm steps. For this profiling, the microsensors were mounted on a motorized linear positioner (VT-80, Pollux motor, Micos, Eschbach, Germany) controlled by a computer using a software for automated microsensor measurements (µ-Profiler, L. Polerecky, http://www.microsen-wiki.net).

3. Results

3.1 Genes encoding polyphosphate-related enzymes in colorless sulfur bacteria

The enzymes related to polyphosphate metabolism in the available genomes of colorless sulfur bacteria were investigated. The investigated partial genomes are from six different chemotrophic sulfur bacteria of the family *Beggiatoaceae* (Section 2.1). The polyphosphate-building polyphosphate kinase 1 (PPK1) and the polyphosphate-degrading polyphosphate:AMP phosphotransferase (PAP) were identified in all analyzed genomes except for the "*Ca.* Parabeggiatoa sp." genome (Table 3.1). In contrast, polyphosphate kinase 2 (PPK2) could not be identified in any of the tested genomes. The polyphosphate-utilizing polyphosphate glucokinase (PPGK) was only found in the genomes of "*Ca.* Thiomargarita nelsonii", "*Ca.* Isobeggiatoa sp." and in the genome of the Guaymas filament (Table 3.1). The polyphosphate-degrading exopolyphosphatase (PPX) was identified in all genomes except for "*Ca.* Thiomargarita nelsonii". In *Beggiatoa alba* B18LD and *Beggiatoa* sp. strain 35Flor two PPX genes were found. Adenylate kinase (AK) was found in genomes of *Beggiatoa alba* strain B18LD, "*Ca.* Thiomargarita nelsonii", the Guaymas filament, and *Beggiatoa* sp. strain 35Flor (Table 3.1). No regulators were found for the identified polyphosphate-related genes.

Table 3.1: Genes detected in the genomes of sulfur bacteria of the family *Beggiatoaceae* coding for enzymes putatively involved in polyphosphate metabolism. Genes tentatively not identified in the not closed partial genomes are indicated with '?'. Incomplete gene sequences are written in brackets.

Product	Gene	EC number	Sulfur bacterium	Locus
Polyphosphate kinase 1	*ppk1*	2.7.4.1	*Beggiatoa alba* B18LD	orf27_glimmer3
			"*Ca.* T. nelsonii"	(THI45_0)
			"*Ca.* Parabeggiatoa sp."	?
			"*Ca.* Isobeggiatoa sp."	(BGP_5434)
			Guaymas filament	BOGUAY_0085
			Beggiatoa sp. 35Flor	FLOR_02687
Polyphosphate kinase 2	*ppk2*	2.7.4.1	*Beggiatoa alba* B18LD	?
			"*Ca.* T. nelsonii"	?
			"*Ca.* Parabeggiatoa sp."	?
			"*Ca.* Isobeggiatoa sp."	?
			Guaymas filament	?
			Beggiatoa sp. 35Flor	?
Polyphosphate glucokinase	*ppgK*	2.7.1.63	*Beggiatoa alba* B18LD	?
			"*Ca.* T. nelsonii"	(THI112_0)
			"*Ca.* Parabeggiatoa sp."	?
			"*Ca.* Isobeggiatoa sp."	(BGP_0205)
			Guaymas filament	BOGUAY_0012
			Beggiatoa sp. 35Flor	?
Polyphosphate:AMP phosphotransferase	*pap*	2.7.4.B2	*Beggiatoa alba* B18LD	BA10_37
			"*Ca.* T. nelsonii"	(THI191911041837)
			"*Ca.* Parabeggiatoa sp."	?
			"*Ca.* Isobeggiatoa sp."	BGP_4237
			Guaymas filament	BOGUAY_0604
			Beggiatoa sp. 35Flor	FLOR_02303
Exopolyphosphatase	*ppx*	3.6.1.11	*Beggiatoa alba* B18LD	BA10_67 BA12_96
			"*Ca.* T. nelsonii"	?
			"*Ca.* Parabeggiatoa sp."	(BGS_0277)
			"*Ca.* Isobeggiatoa sp."	BGP_0779
			Guaymas filament	BOGUAY_0388
			Beggiatoa sp. 35Flor	FLOR_02379 FLOR_02608
Adenylate kinase	*ak*	2.7.4.3	*Beggiatoa alba* B18LD	BA02_295
			"*Ca.* T. nelsonii"	THI811_0
			"*Ca.* Parabeggiatoa sp."	?
			"*Ca.* Isobeggiatoa sp."	?
			Guaymas filament	BOGUAY_2262
			Beggiatoa sp. 35Flor	FLOR_00305

3.2 Detection and characterization of polyphosphate inclusions in *Beggiatoa alba*

The structure and properties of polyphosphate inclusions in the heterotrophic freshwater strain *Beggiatoa alba* B15LD were studied with different methods and the inclusions were compared with the ones in the marine lithotrophic strain *Beggiatoa* sp. 35Flor (Brock et al., 2012).

3.2.1 Staining with 4´,6´-diamidino-2-phenylindole

Polyphosphate inclusions in *Beggiatoa alba* were visualized by 4´,6´-diamidino-2-phenylindole (DAPI) staining. Filaments were stained after 3 days of growth in a modified liquid medium according to Strohl and Larkin (1978) (Section 2.2.2.1) resulting in a blue fluorescent DNA signal and a yellow fluorescent signal for polyphosphate inclusions. Figure 3.1 shows that *Beggiatoa alba* stored polyphosphate in large inclusions.

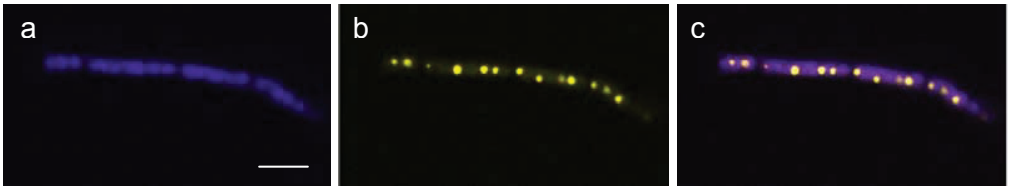

Figure 3.1: Confocal laser scanning microscopy (CLSM) images of a *Beggiatoa alba* filament stained with DAPI after 3 days of growth in a modified medium according to Strohl and Larkin (1978). (a) The blue fluorescent signal is due to DNA staining, (b) polyphosphate staining results in a yellow fluorescent signal and (c) an overlay of both signals. The scale bar represents 5 µm.

3.2.2 Staining with Toluidine Blue

Polyphosphate in *Beggiatoa alba* was also stained with Toluidine Blue. Using this dye, polyphosphate inclusions appeared blue red under the light microscope. As shown in Figure 3.2, *Beggiatoa alba* accumulated polyphosphate internally in high amounts when grown with medium according to Strohl and Larkin (1978).

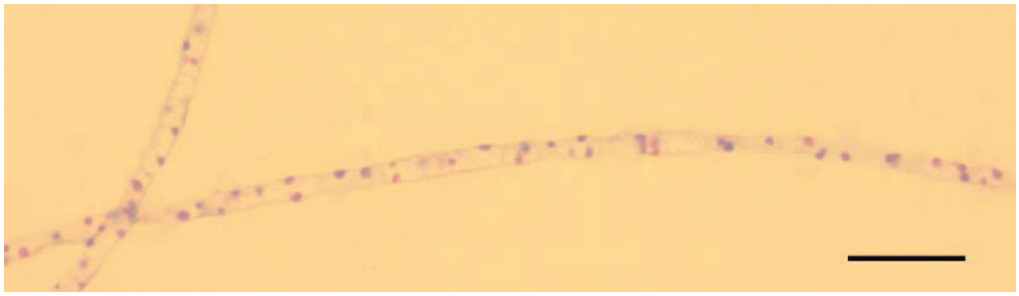

Figure 3.2: Toluidine Blue staining of polyphosphate inclusions in *Beggiatoa alba* filaments after 3 days of growth in a modified medium according to Strohl and Larkin (1978). Polyphosphate inclusions are visible as blue red dots within the filaments. The scale bar represents 10 µm.

3.2.3 Staining with Nile Red

Nile Red was used to visualize polyhydroxyalkanoates (PHA) and to investigate if polyphosphate inclusions of *Beggiatoa alba* are surrounded by a membrane. Therefore, *Beggiatoa alba* filaments grown for 3 days in modified medium according to Strohl and Larkin (1978) were simultaneously stained with Nile Red and DAPI. The Nile Red staining revealed a high number of red fluorescent inclusions (Figure 3.3) that resulted in very strong signals, prohibiting the identification and localization of potentially stained membranes (Figure 3.3).

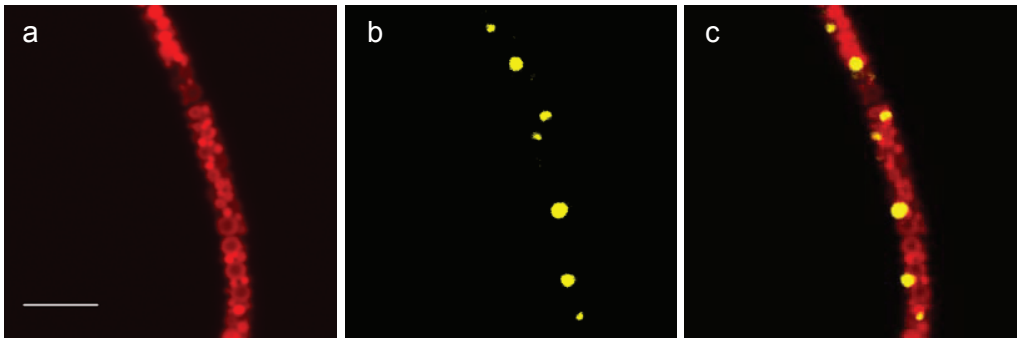

Figure 3.3: CLSM images of a *Beggiatoa alba* filament stained with Nile Red and DAPI after 3 days of growth in a modified medium according to Strohl and Larkin (1978). (a) Nile Red staining results in a red fluorescence of membranes and PHA inclusions, (b) DAPI staining reveals polyphosphate inclusions by a yellow fluorescence and (c) the overlay of both signals. The scale bar represents 10 µm.

3.2.4 Staining with Acridine Orange

Beggiatoa alba was stained with the acidophilic dye Acridine Orange for the investigation of acidic inclusion inside the cells. For this reason, filaments of a culture grown for 3 days in medium according to Strohl and Larkin (1978), in which the strain was shown to store polyphosphate (Figure 3.1), were used. Figure 3.4 shows that the fluorescent orange signal of the dye is visible in the cytoplasm along the whole filament, except in larger inclusions. No obvious accumulation in inclusions was visible.

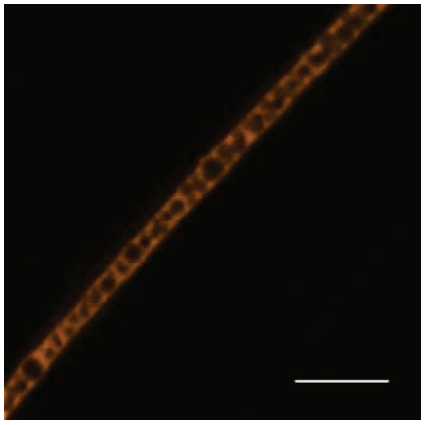

Figure 3.4: CLSM image of a *Beggiatoa alba* filament stained with Acridine Orange after 3 days of growth in a modified medium according to Strohl and Larkin (2007). The scale bar represents 10 µm.

3.2.5 Staining with Rhodamin 123

By staining with Rhodamin 123, it was investigated if an electrical potential over the membrane of polyphosphate inclusions (inside positive) exists in *Beggiatoa alba*. Staining was performed on a culture grown for 3 days in medium according to Strohl and Larkin (1978) in which they store polyphosphate as shown above. Figure 3.5 shows a green fluorescent signal in large and small inclusions, while the cytoplasm seemed to be unstained.

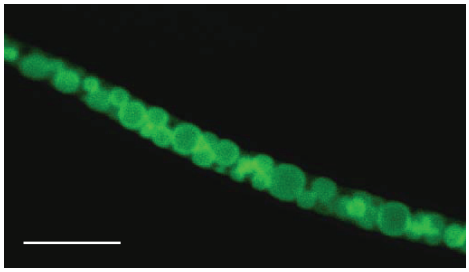

Figure 3.5: CLSM image of a *Beggiatoa alba* filament stained with Rhodamin 123 after 3 days of growth in a modified medium according to Strohl and Larkin (1978). The scale bar represents 10 µm.

Also marine *Beggiatoa* sp. 35Flor were stained with Rhodamin 123 after 7 days of growth in semi-solid gradient medium with 4 mmol L^{-1} sulfide in the bottom agar (Section 2.2.2.2). Additionally, polyphosphate inclusions were stained by DAPI. In contrast to *Beggiatoa alba*, Rhodamin 123 stained the cytoplasm (Figure 3.6 a) and accumulated in small inclusions (arrows in Figure 3.6 a) but not in big inclusions containing polyphosphate (Figure 3.6 b, c).

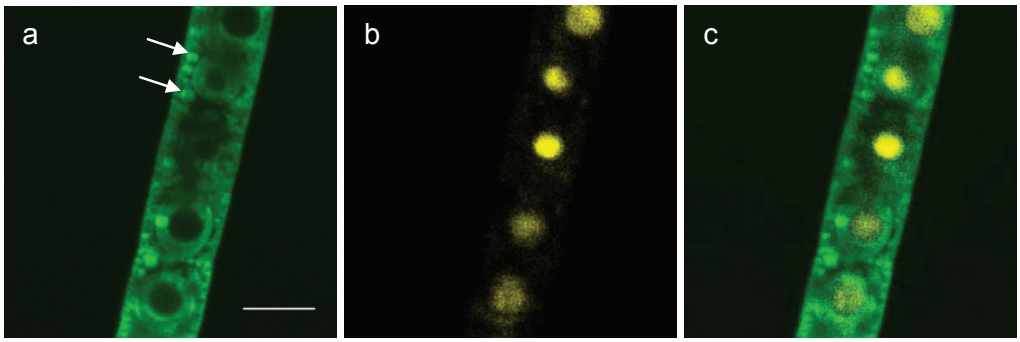

Figure 3.6: CLSM image of a *Beggiatoa* sp. 35Flor filament stained with Rhodamin 123 and DAPI after 7 days of growth in a modified medium according to Nelson and Jannasch (1983) with 4 mmol L^{-1} sulfide in the bottom agar, top agar buffered at pH 7.0 with 20 mmol L^{-1} 1,4-piperazinediethanesulfonic acid disodium salt (PIPES). (a) Green fluorescent signal of Rhodamin 123, (b) yellow fluorescent signal of polyphosphate after DAPI staining and (c) overlay of both signals. The scale bar represents 5 µm.

3.2.6 Raman micro-spectroscopy

Raman micro-spectroscopy was used to identify and visualize polyphosphate in *Beggiatoa* sp. 35Flor and *Beggiatoa alba* filaments due to its Raman scattering spectra. *Beggiatoa* sp. 35Flor was cultivated for 7 days in semi-solid gradient medium with 4 mmol L^{-1} sulfide in the bottom agar (Section 2.2.2.2). Raman spectra from polyphosphate inclusions of *Beggiatoa* sp. 35Flor contained two strong peaks at 1187 and 710 cm^{-1} (Figure 3.7 b) close to the polyphosphate standard having peaks at 1167 cm^{-1} and at 690 cm^{-1} (Figure 3.7 c). These two peaks are absent in Raman spectra of the cytoplasm around the polyphosphate (Figure 3.7 a).

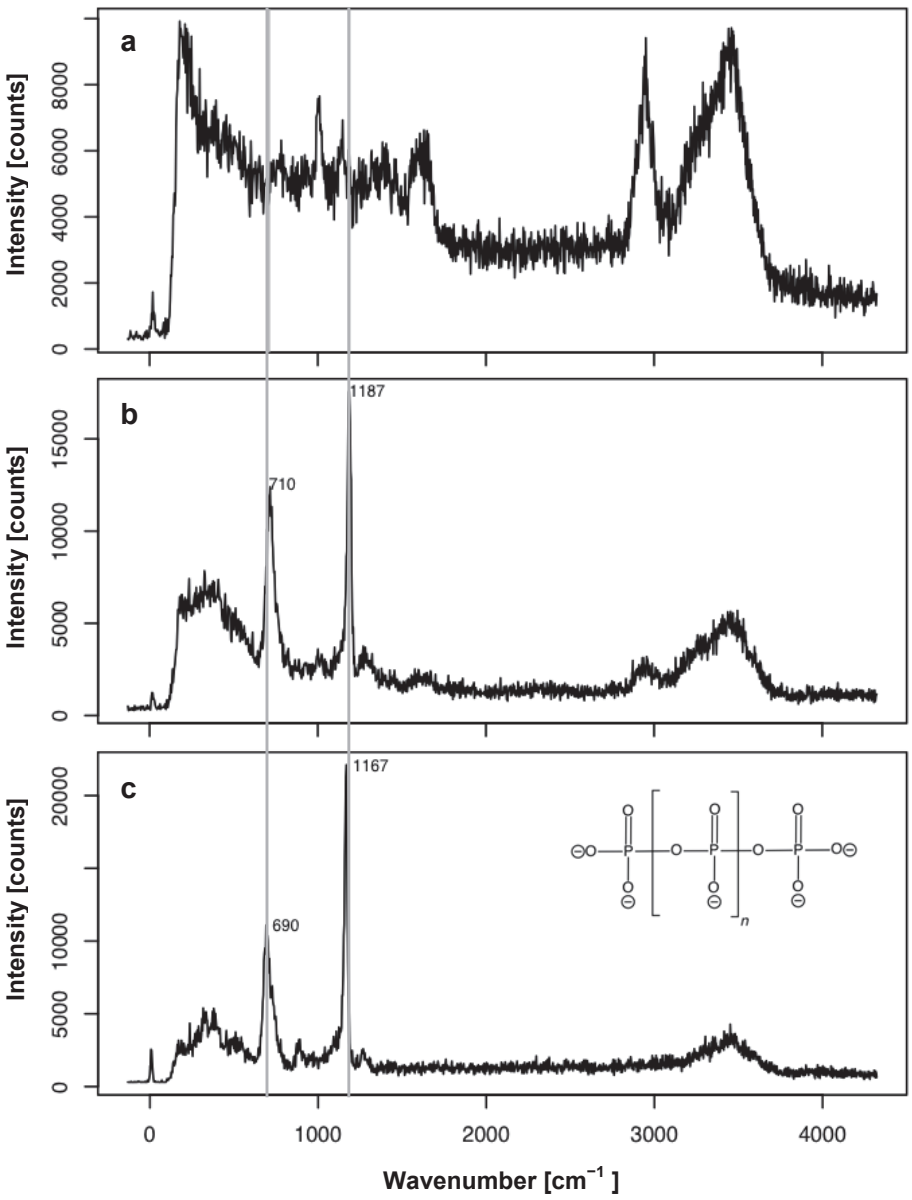

Figure 3.7: Raman spectra from a marine *Beggiatoa* sp. 35Flor filament (a) in the cytoplasm, (b) in the region of a polyphosphate inclusion, and (c) the sodium phosphate glass type 45 as polyphosphate standard. Vertical lines in grey show the positions of the polyphosphate peaks missing in the spectrum of the cytoplasm (courtesy of Jana Milucka).

Spectral mapping of the Raman scattering revealed that the filament exhibited polyphosphate inclusions of different sizes (Figure 3.8 b). In the regions of polyphosphate inclusions, the density of organic material decreased (Figure 3.8 c,d).

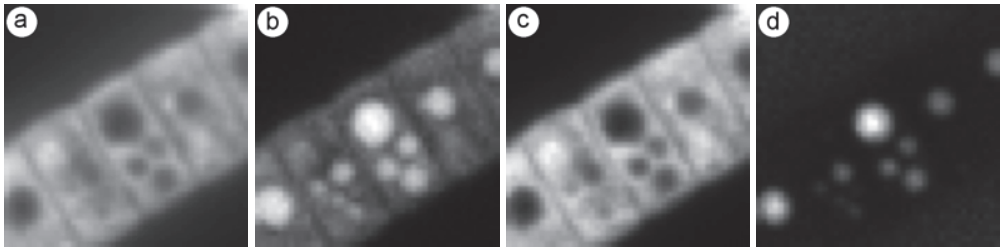

Figure 3.8: Raman spectral maps of a marine *Beggiatoa* sp. 35Flor filament showing polyphosphate signals in inclusions of different sizes. (a) Overall Raman scatter intensity, (b) intensity in region 1170-1190 cm^{-1} (polyphosphate), (c) intensity in region 2900-3000 cm^{-1} (organic matter), (d) ratio of intensity in region 1170-1190 to 2900-3000 cm^{-1}. Map dimension is 10.2 · 10.2 µm, 44 · 44 pixels (courtesy of Jana Milucka).

Beggiatoa alba samples for Raman micro-spectroscopy were taken from liquid cultures grown for 3 days in medium according to Strohl and Larkin (1978), which were already shown to store polyphosphate by staining with DAPI and Toluidine Blue (Figure 3.1 and 3.2). These polyphosphate inclusions are much smaller than the ones observed in the marine *Beggiatoa* sp. 35Flor (Brock et al., 2012). Spectral mapping revealed regions with polyphosphate signals in the filaments (Figure 3.9 a), which have a lower density of organic material (Figure 3.9 b). Raman spectra of these regions of potential polyphosphate inclusions showed peaks at 1175 and 700 cm^{-1} (Figure 3.10 b). Raman spectra in the cytoplasm had no peaks representing polyphosphate (Figure 3.10 a), instead peaks at 845, 905, 1065, 1465, 1750 cm^{-1} dominated the spectra.

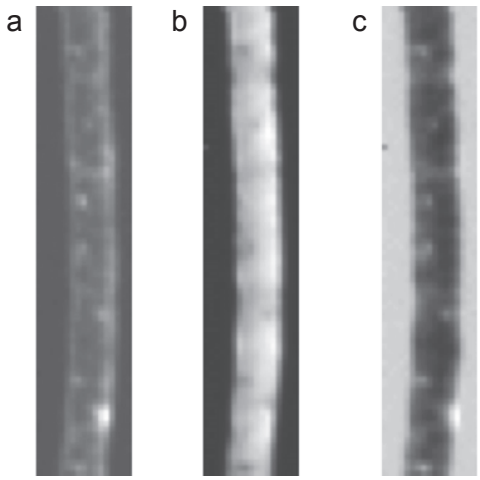

Figure 3.9: Raman spectral maps of a *Beggiatoa alba* filament. (a) Intensity in region 1170-1190 cm^{-1} (polyphosphate), (b) intensity in region 2900-3000 cm^{-1} (organic matter), (c) ratio of intensity in region 1170-1190 to 2900-3000 cm^{-1}. Map dimension is 4.0 · 19.8 µm, 17 · 85 pixels (courtesy of Jana Milucka).

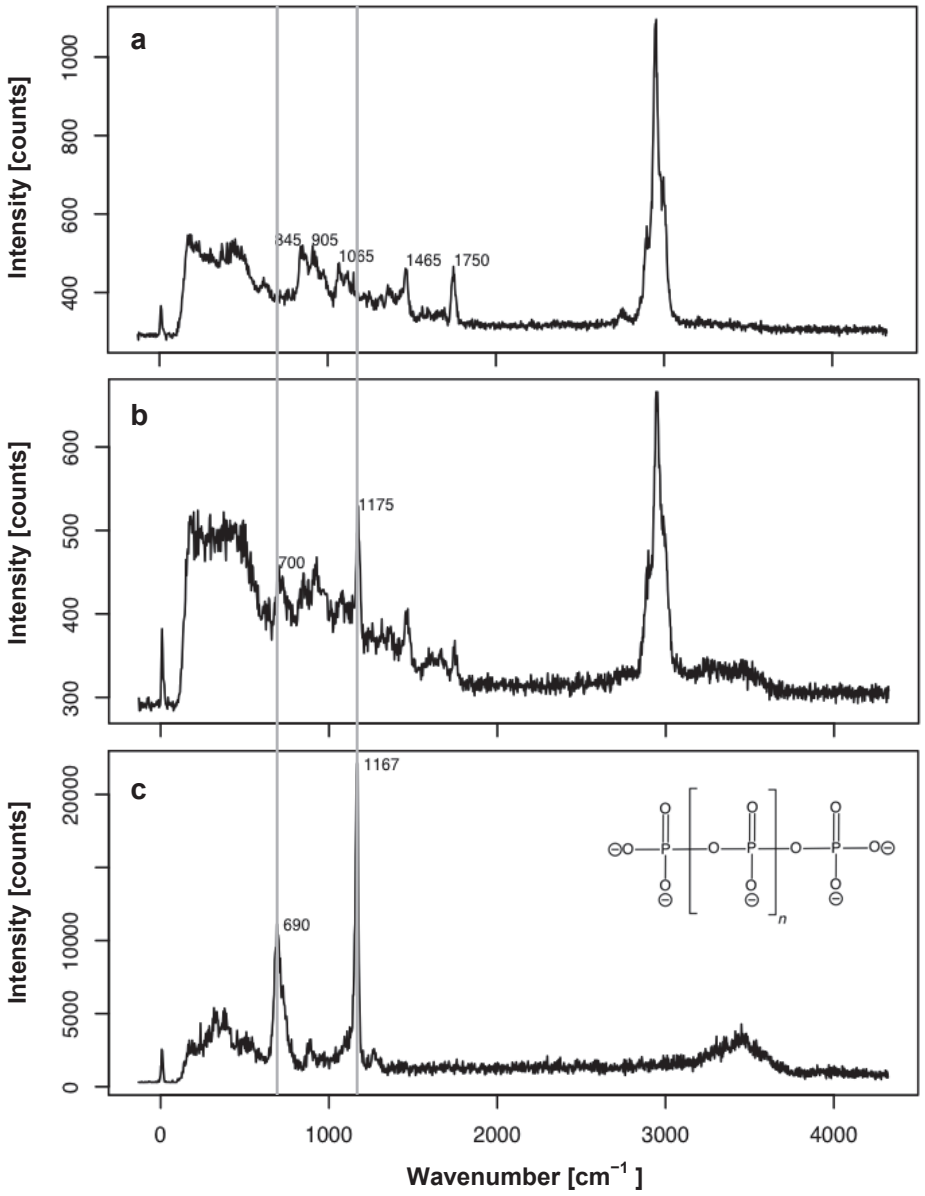

Figure 3.10: Raman spectra from a *Beggiatoa alba* filament (a) in the cytoplasm, (b) in the region of a polyphosphate inclusion, and (c) the sodium phosphate glass type 45 as polyphosphate standard. Vertical lines in grey show the positions of the polyphosphate peaks missing in the spectrum of the cytoplasm (courtesy of Jana Milucka).

3.2.7 Scanning electron microscopy and energy dispersive X-ray analysis

Scanning electron microscopy and energy dispersive X-ray analysis (SEM and EDXA) was performed to detect elements in the *Beggiatoa alba* filaments and to investigate, if there is a co-occurrence of phosphorus with other elements. The electron micrograph obtained by back-scattered electrons showed small light grey spots in the *Beggiatoa alba* filament, indicating regions of higher concentrations of heavier elements (Figure 3.11 a, d). EDXA spectra revealed significantly high concentrations of phosphorus, oxygen and sodium in these regions (Figure 3.11 b), whereas no phosphorus and less oxygen and potassium was detected in darker areas (Figure 3.11 c).

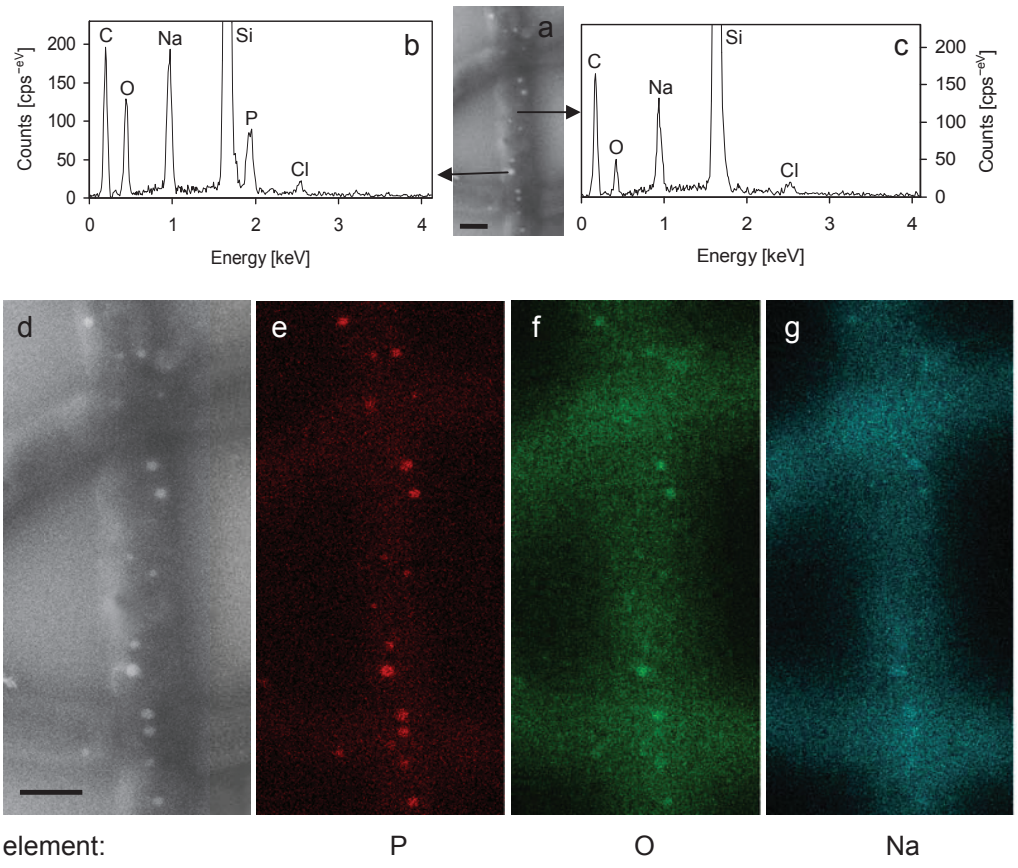

Figure 3.11: SEM and EDXA of *Beggiatoa alba* filaments. (a, d) Back-scattered electron micrograph, (b) EDXA spectra of light grey spot of the back-scattered electron micrograph in the filament, which means there are heavier elements at higher concentrations, (c) EDXA spectra of darker area of the back-scattered electron micrograph in the filament. (e-g) Elemental mapping of *Beggiatoa alba* filaments. The scale bars represent 2 µm.

The high silica peak in Figure 3.11 is due to the silicon wafer support used for the analysis. Elemental mapping of the filament revealed a co-occurrence of phosphorus and oxygen (Figure 3.11 f, g). Areas of heavier elements and the distribution of phosphorus and oxygen were smaller than polyphosphate inclusions stained by DAPI. Furthermore, an association of sodium with phosphorus and oxygen was observed. Although the cytoplasm contains significantly high amounts of sodium, the amount was higher in areas where phosphorus and oxygen dominated.

3.3 Polyphosphate storage in *Beggiatoaceae* in dependence of different culture conditions and in natural, environmental samples

General remarks:
The focus of these studies was to investigate polyphosphate storage in organoheterotrophic freshwater *Beggiatoa alba*. The effect of different nitrogen, phosphorus, and carbon concentrations as well as the effect of sulfide and potential stress factors were tested on polyphosphate storage. The effect of some of these factors on polyphosphate storage were also tested on other lithoautotrophic freshwater, marine, and hypersaline representatives of the family *Beggiatoaceae* to see if habitat- or metabolism-dependent triggers for polyphosphate storage can be identified. Finally, natural, environmental *Beggiatoaceae* samples were analyzed for polyphosphate storage to verify, if the findings can be explained by results obtained in the laboratory.

3.3.1 Polyphosphate storage in organoheterotrophic freshwater *Beggiatoa alba*

Beggiatoa alba stored polyphosphate in medium according to Strohl and Larkin (1978) but not in medium according to Schmidt et al. (1987) (Figure 3.12).

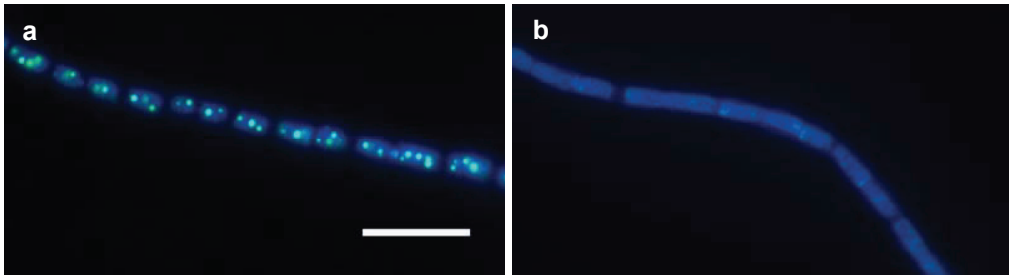

Figure 3.12: DAPI stained polyphosphate in *Beggiatoa alba* after 3 days of growth in (a) a modified medium according to (Strohl and Larkin, 1978) and (b) a modified medium according to (Schmidt et al., 1987). Polyphosphate inclusions are visible as greenish inclusions, while DNA is leading to a blue signal. The scale bar represents 10 µm.

The main differences between the two media are the presence of broth in the undefined medium (Strohl and Larkin, 1978) and the high ammonium concentration (4.7 mmol L^{-1} vs. 0.84 mmol L^{-1}) in the defined medium (Schmidt et al., 1987). It could be excluded that broth is responsible for presence or absence of polyphosphate storage, because *Beggiatoa alba* still stored polyphosphate in the undefined medium without broth (then being a defined medium) and did not store polyphosphate in the defined medium supplemented with broth (data not shown). Therefore, it was tested whether nitrogen availability has an effect on polyphosphate storage.

Effect of different nitrogen and phosphate concentrations on polyphosphate storage in Beggiatoa alba

Beggiatoa alba was cultivated in the defined medium according to Schmidt et al. (1987) containing nitrate or ammonium as nitrogen source in concentrations of 0.05, 0.1, 0.5, 1, and 10 mmol L^{-1}. The acetate concentration was 10 mmol L^{-1} and the phosphate concentration was 73 µmol L^{-1}, as described in the original recipe (Section 2.2.2.1). After 3 days of growth, filaments grown with 0.1, 1, and 10 mmol L^{-1} nitrogen were stained with DAPI for polyphosphate (Figure 3.13). Many polyphosphate inclusions were visible when grown with 0.1 mmol L^{-1} ammonium or nitrate (Figure 3.13 a, d). Much less or no polyphosphate was stored when grown at higher nitrogen concentrations (Figure 3.13 b, c, e, f).

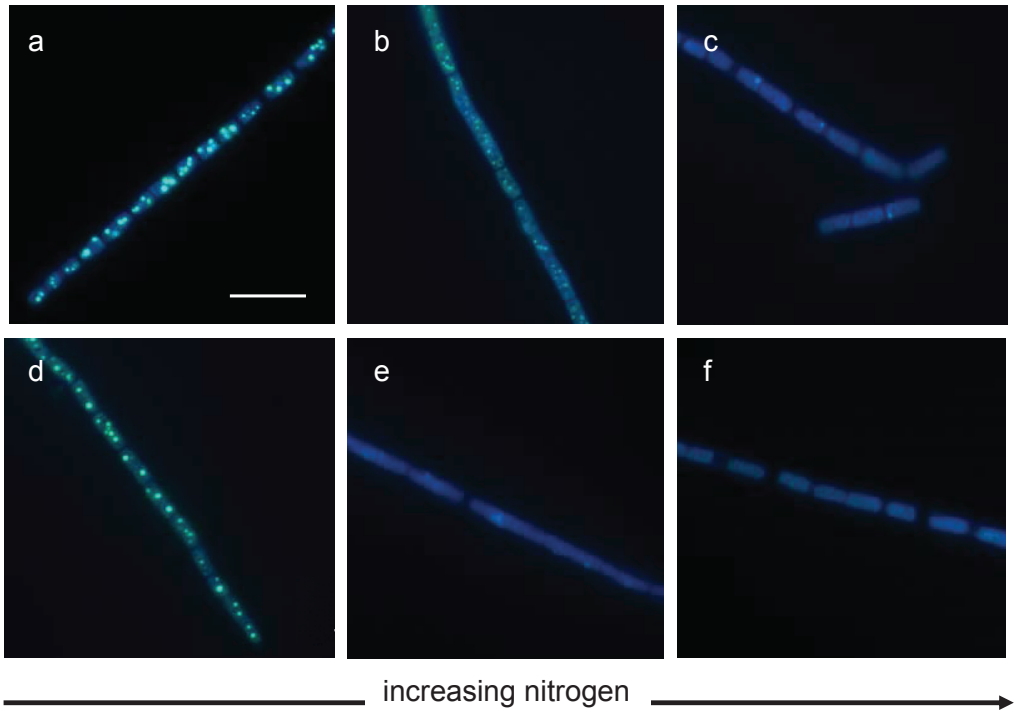

Figure 3.13: DAPI stained polyphosphate in *Beggiatoa alba* after 3 days growth in a modified medium according to (Schmidt et al., 1987) with 73 µmol L^{-1} phosphate and nitrate in concentrations of (a) 0.1 mmol L^{-1}, (b) 1 mmol L^{-1}, and (c) 10 mmol L^{-1} or with ammonium in concentrations of (d) 0.1 mmol L^{-1}, (e) 1 mmol L^{-1}, and (f) 10 mmol L^{-1}. Polyphosphate inclusions are visible as greenish inclusions, while DNA is leading to a blue signal. The scale bar represents 10 µm.

To investigate whether the cultures were growth-limited at 0.1 mmol L^{-1} nitrogen concentrations, the protein content was determined as a proxy for growth. Growth curves of *Beggiatoa alba* grown at different nitrogen concentrations are shown in Figure 3.14 and 3.15. The standard deviations are relatively high due to the formation of tufts by filaments in liquid culture, which complicated the retrieval of homogenous samples. At concentrations of 1 and 10 mmol L^{-1} nitrate or ammonium, the cultures reached the stationary phase after approximately 3 days. Only when grown at low nitrogen concentrations of 0.1 mmol L^{-1} the cultures did not reach the stationary phase and the biomass was low. To reveal whether this is caused by nitrogen limitation, nitrogen as well as phosphate concentrations in the media were monitored together with the protein content (Figure 3.16).

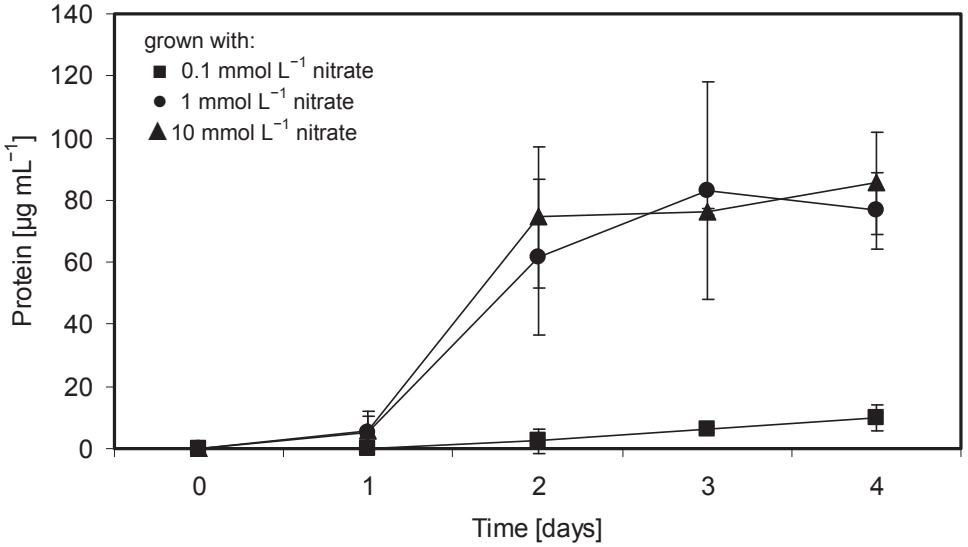

Figure 3.14: Growth curve based on protein content of *Beggiatoa alba* grown in modified medium according to (Schmidt et al., 1987) with 73 µmol L^{-1} phosphate, 10 mmol L^{-1} acetate, and nitrate as nitrogen source. Nitrate concentrations were (■) 0.1 mmol L^{-1}, (●) 1 mmol L^{-1}, and (▲) 10 mmol L^{-1}. Error bars represent the standard deviation of biological replicates.

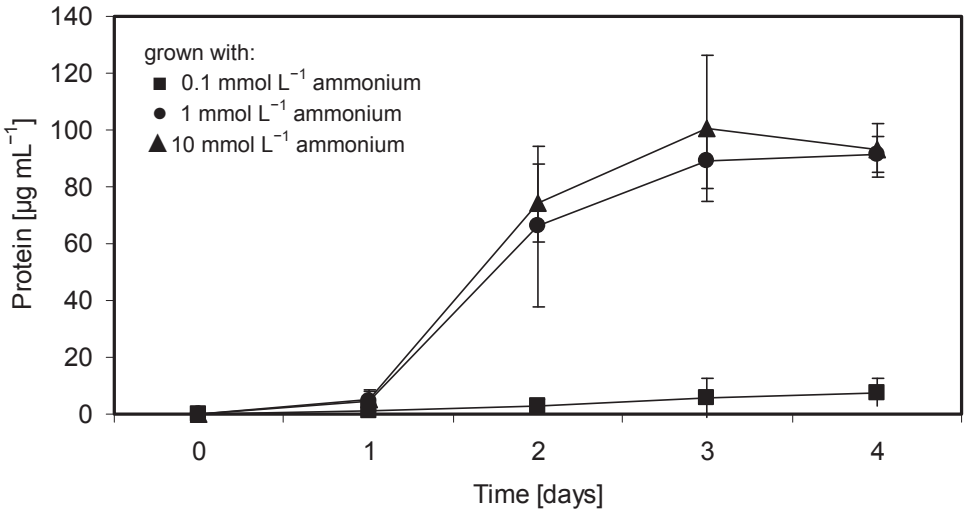

Figure 3.15: Growth curve based on protein content of *Beggiatoa alba* grown in modified medium according to (Schmidt et al., 1987) with 73 µmol L^{-1} phosphate, 10 mmol L^{-1} acetate, and ammonium as nitrogen source. Ammonium concentrations were (▲) 0.1 mmol L^{-1}, (●) 1 mmol L^{-1}, and (■) 10 mmol L^{-1}. Error bars represent the standard deviation of biological replicates.

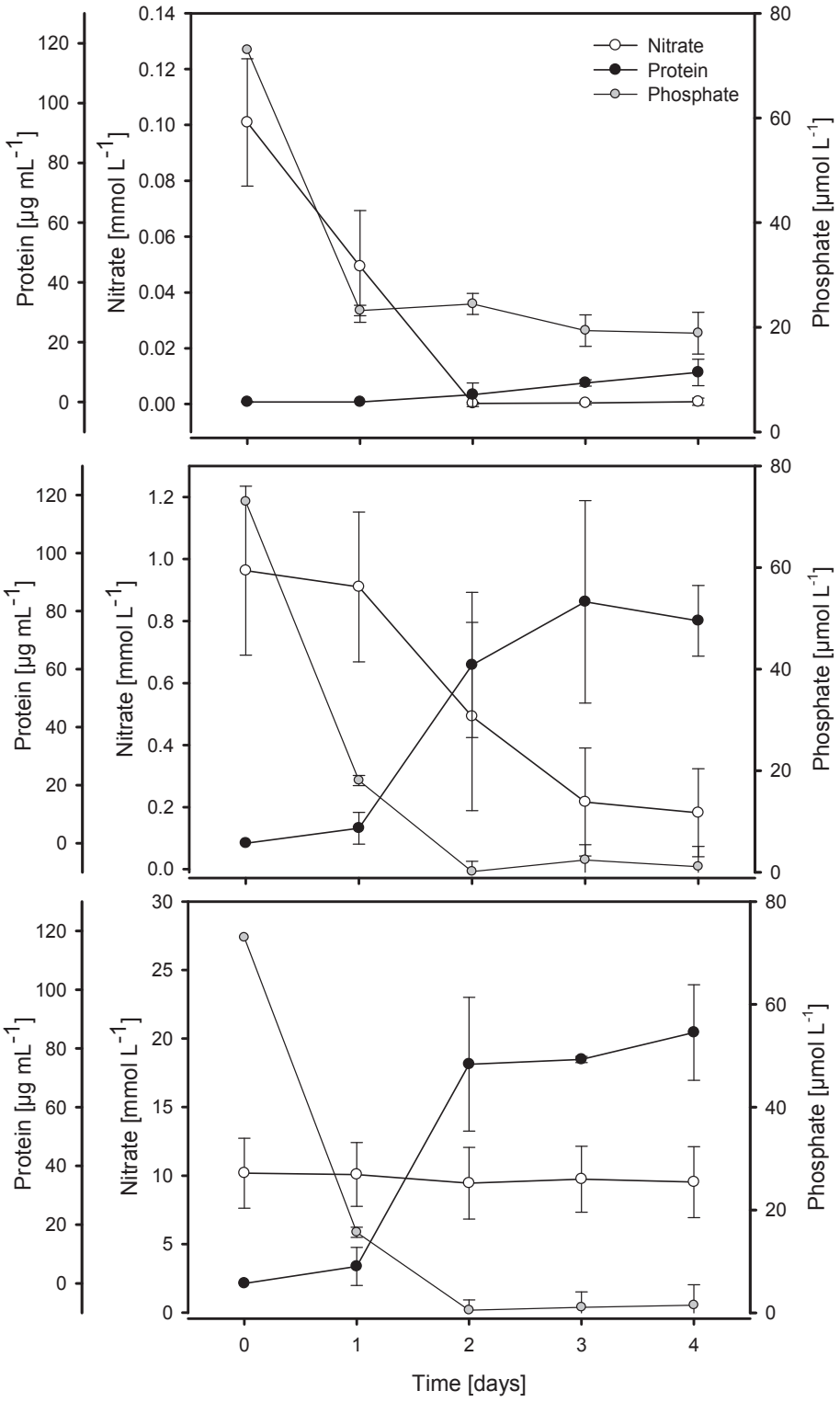

Figure 3.16: (●) Protein, (○) nitrate, and (●) phosphate concentrations over time in *Beggiatoa alba* cultures that grew with initially 10 mmol L^{-1} acetate, 73 µmol L^{-1} phosphate, and (upper panel) 0.1 mmol L^{-1} nitrate, (middle panel) 1 mmol L^{-1} nitrate or (lower panel) 10 mmol L^{-1} nitrate. Error bars represent the standard deviation of biological replicates.

In Figure 3.16, growth is shown as protein content together with nitrate and phosphate concentrations over time in *Beggiatoa alba* cultures grown with 0.1, 1, and 10 mmol L^{-1} nitrate together with 10 mmol L^{-1} acetate and 73 µmol L^{-1} phosphate. At 0.1 mmol L^{-1} nitrate, when the cells stored high amounts of polyphosphate (Figure 3.13 a), all nitrate was used up within 2 days. Under these conditions almost no growth occurred and phosphate was still available in the medium. In contrast, when grown with 1 and 10 mmol L^{-1} nitrate, phosphate was used up after 2 days and not all nitrate was consumed during growth. At these conditions much less polyphosphate was stored (Figure 3.13 b, c). In summary, *Beggiatoa alba* stored higher amounts of polyphosphate at low nitrogen concentrations and stored less polyphosphate when the nitrogen concentrations were higher and phosphate was consumed.

To test the hypothesis that *Beggiatoa alba* stored less polyphosphate when growing at high nitrogen concentrations due to phosphate limitation, they were cultivated in the same medium as before but with a surplus of phosphate in concentrations of 1 mmol L^{-1} and compared it to the polyphosphate content, which was build up in medium with only 73 µmol L^{-1} phosphate (Figure 3.17).

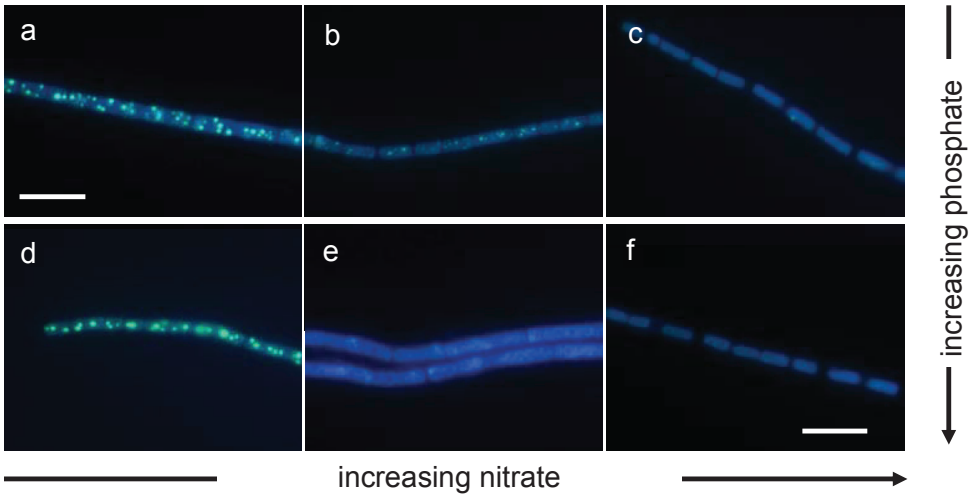

Figure 3.17: DAPI stained polyphosphate in *Beggiatoa alba* after 3 days of growth in a modified medium according to Schmidt et al. (1987) containing 73 µmol L^{-1} phosphate (upper panels) or 1 mmol L^{-1} phosphate (lower panels), and (a, d) 0.1 mmol L^{-1} , (b, e) 1 mmol L^{-1}, and (c, f) 10 mmol L^{-1} nitrate. Polyphosphate inclusions are visible as greenish inclusions, while DNA is leading to a blue signal. The scale bar represents 10 µm.

Figure 3.17 shows that with increasing nitrate concentrations *Beggiatoa alba* stored less polyphosphate independent of the phosphate supply. With increasing nitrate concentrations the cells contained less polyphosphate inclusions in the cultures grown with 73 µmol L^{-1} as well as with 1 mmol L^{-1} phosphate concentration.

Polyphosphate was also quantified in *Beggiatoa alba* cultures after growth at different nitrogen concentrations and 1 mmol L^{-1} phosphate (Figure 3.18). At higher nitrogen concentrations the polyphosphate content decreased as already observed in the microscopic pictures (Figure 3.13 and 3.17). When *Beggiatoa alba* grew at 0.05 mmol L^{-1} nitrate, the polyphosphate content was about 87 µg mg^{-1} protein (Figure 3.18). At 10 mmol L^{-1} nitrate *Beggiatoa alba* contained around 40 µg mg^{-1} protein.

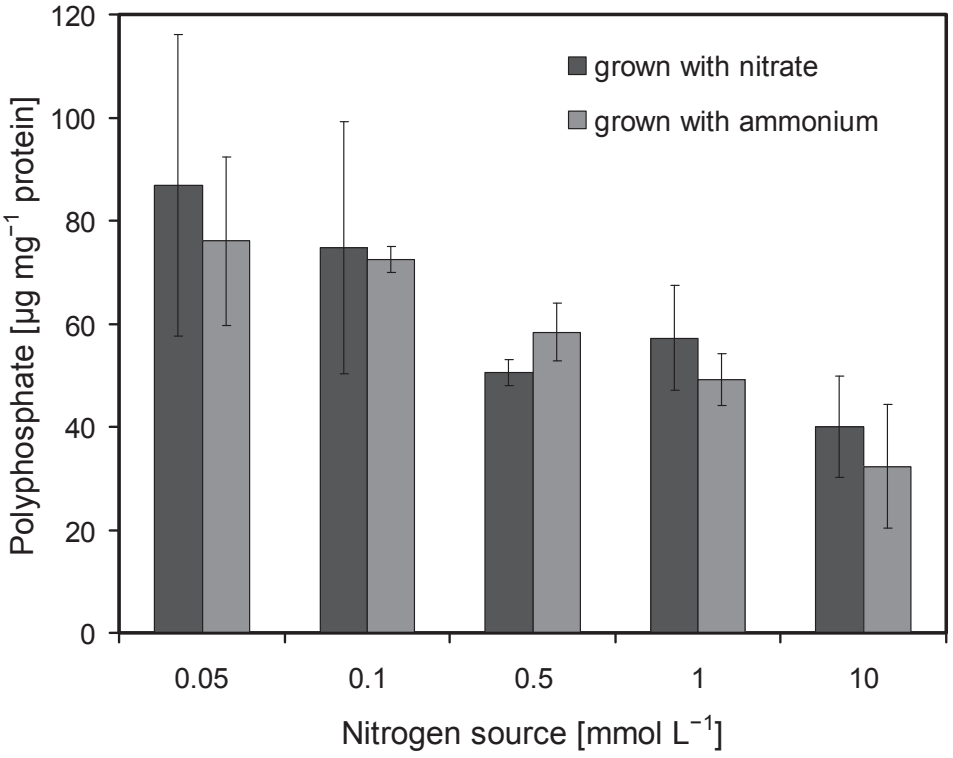

Figure 3.18: Effect of different nitrate and ammonium concentrations on the polyphosphate content of *Beggiatioa alba* filaments after 3 days of growth in a modified medium according to Schmidt et al. (1987) containing different (■) nitrate and (■) ammonium concentrations ranging from 0.05 mmol L^{-1} to 10 mmol L^{-1}. Acetate concentrations were 10 mmol L^{-1} and phosphate concentrations were 1 mmol L^{-1}. Error bars represent the standard deviation of biological replicates.

The polyphosphate content decreased also at higher ammonium concentrations. After growth at 0.05 mmol L^{-1} ammonium, the polyphosphate content in *Beggiatoa alba* cells was about 76 µg mg^{-1} protein and about 32 µg mg^{-1} protein when grown at 10 mmol L^{-1} ammonium (Figure 3.18). Consequently, polyphosphate was preferably stored at low nitrogen concentrations and was not dependent on phosphate concentrations in the medium.

In cultures originally grown with low nitrogen concentrations and then supplemented with high nitrogen concentrations filaments lost their polyphosphate inclusions after 1 day of incubation (Figure 3.19).

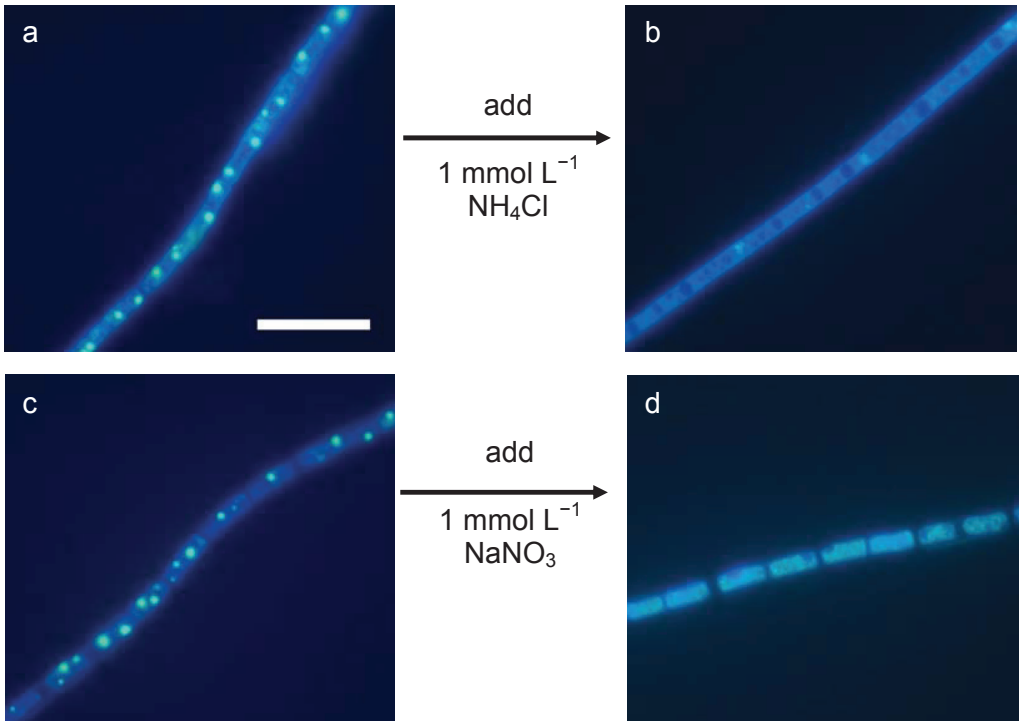

Figure 3.19: DAPI stained polyphosphate in *Beggiatoa alba* after 3 days of growth in a modified medium according to (Schmidt et al., 1987) with (a) 0.1 mmol L^{-1} ammonium or (c) 0.1 mmol L^{-1} nitrate. 1 mmol L^{-1} ammonium or nitrate was added and after 1 day the filaments were again stained for polyphosphate (c, d). Polyphosphate inclusions are visible as greenish inclusions, while DNA is leading to a blue signal. The scale bar represents 10 µm.

Guanosin tetraphosphate production by Beggiatoa alba

Since nitrogen limitation results in amino acid limitation and in *Escherichia coli* guanosin tetraphosphate (ppGpp) is known to induce polyphosphate storage under amino acid limiting conditions (Kuroda et al., 1997), it was investigated whether ppGpp is also produced by *Beggiatoa alba* under nitrogen limiting growth conditions. High pressure liquid chromatography (HPLC) analysis of *Beggiatoa alba* extracts from nitrogen-limited and not limited cultures revealed that at nitrogen limitation ppGpp was produced in concentrations of around 25 pmol L^{-1} mg $^{-1}$ protein, while cultures, which were not nitrogen-limited, produced around 5 pmol L^{-1} mg $^{-1}$ protein (Figure 3.20).

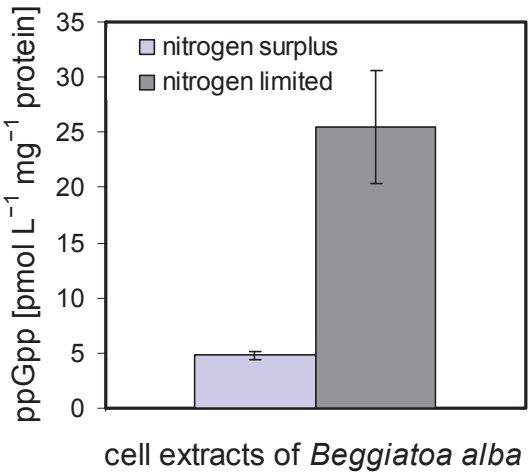

Figure 3.20: Effect of different nitrate concentrations on the ppGpp content of *Beggiatoa alba* cultures after 3 days of growth in a modified medium according to Schmidt et al. (1978) containing (□) 1 mmol L^{-1} nitrate and (■) 0.1 mmol L^{-1} nitrate. Acetate concentrations were 10 mmol L^{-1} and phosphate concentrations were 1 mmol L^{-1}. Error bars represent the standard deviation of biological replicates.

Tuft formation of Beggiatoa alba filaments grown at high ammonium concentrations

Cultures of *Beggiatoa alba* growing at high ammonium concentrations looked different compared to cultures growing at high nitrate concentrations. Filaments growing in medium with ammonium were attached to each other and formed big tufts (Figure 3.21). At 10 mmol L^{-1} ammonium the culture died already after 4 days. Compared to these observations, cultures grown at 1 or 10 mmol L^{-1} nitrate did not form many or large tufts and survived longer. Microsensor measurements through tufts were performed to test whether the

conditions differed compared to the surrounding medium. pH and ammonium measurements revealed no changes within the tufts (Figure 3.22).

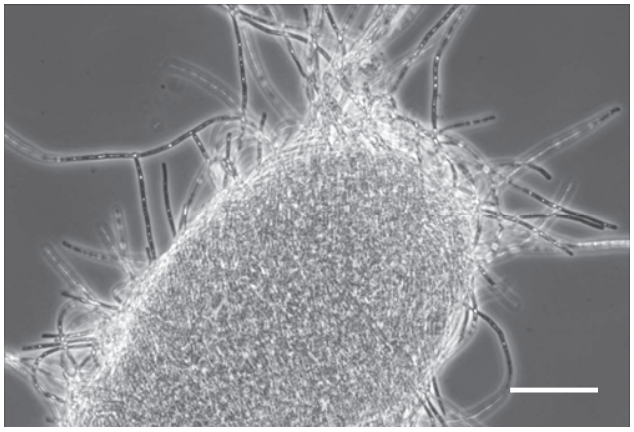

Figure 3.21: Microscopic phase contrast image of *Beggiatoa alba* filaments forming a tuft after 3 days of growth in a modified medium according to (1987) with 1 mmol L^{-1} ammonium and 1 mmol L^{-1} phosphate. The scale bar represents 20 µm.

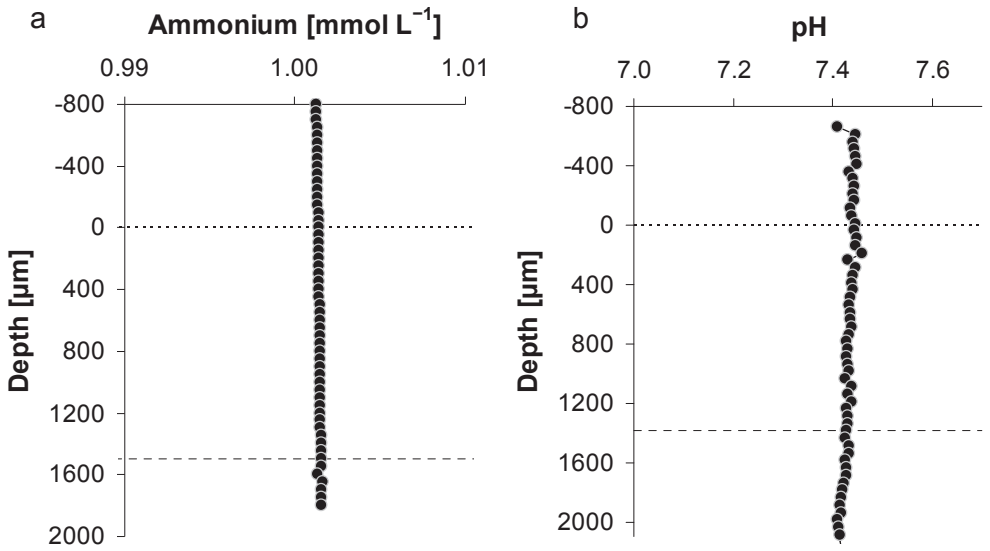

Figure 3.22: Microsensor profiles of a *Beggiatoa alba* tuft embedded in agar. (a) Ammonium profile and (b) pH profile. Horizontal lines indicate the beginning and end of the tuft.

Effect of different carbon concentrations on polyphosphate storage in Beggiatoa alba

In addition to phosphate and nitrogen limitation, the effect of carbon limitation on polyphosphate storage was tested in *Beggiatoa alba.* Therefore the amount of acetate in the cultures was reduced from 10 to 1, 0.5, and, 0.1 mmol L^{-1} and a surplus of phosphate and nitrate was supplied in concentrations of 1 mmol L^{-1}. In cultures with 0.1 mmol L^{-1} acetate the growth was too low to measure the protein and polyphosphate content. The cultures grown with acetate concentrations of 0.5 mmol L^{-1} and 1 mmol L^{-1} used up all acetate within 3 days (data not shown) and only the cultures grown with 10 mmol L^{-1} acetate did not use up all acetate after 3 days. In cultures grown with 0.5 mmol L^{-1} acetate, the polyphosphate content was lower than in cultures grown with 1 or 10 mmol L^{-1} acetate (Figure 3.23). When cultivated under different acetate concentrations, a tendency towards less polyphosphate storage with less acetate is obvious. This result is different to nitrogen limitation. While lower nitrogen concentrations led to increased polyphosphate storage, lower acetate concentrations led to decreased polyphosphate storage.

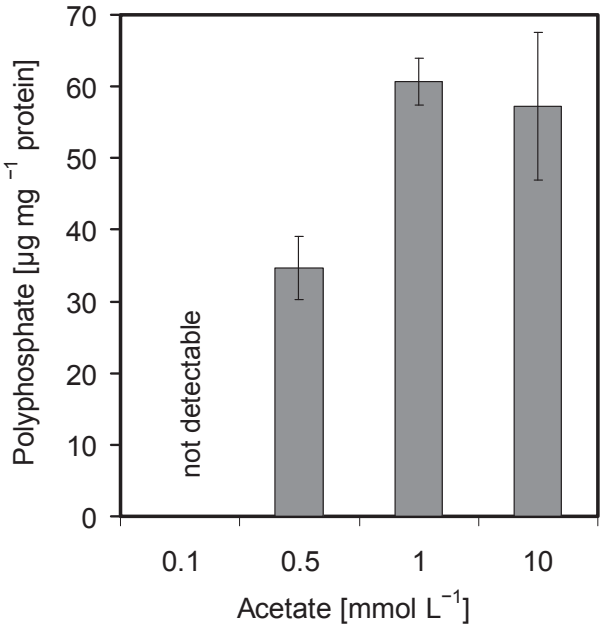

Figure 3.23: Effect of different acetate concentrations on the polyphosphate content in *Beggiatoa alba* after 3 days of growth in a medium according to Schmidt et al. (Schmidt et al., 1987) at different acetate concentrations ranging from 0.1 to 10 mmol L^{-1}. Nitrate and phosphate concentrations were 1 mmol L^{-1}. Error bars represent the standard deviation of biological replicates.

Effect of sulfide and anoxia on polyphosphate storage in Beggiatoa alba

The polyphosphate storage in *Beggiatoa alba* during mixotrophic growth in semi-solid gradient medium containing sulfide (Section 2.2.2.2) was studied. To investigate the effect of sulfide on polyphosphate storage in freshwater *Beggiatoa alba*, the strains were cultivated at different sulfide concentrations and anoxic conditions were applied. After 4 days of mixotrophic growth, *Beggiatoa alba* filaments were examined for sulfur inclusion by phase contrast microscopy. When imaged with phase contrast microscopy sulfur inclusions are easily visible as birefringent white spheres. Strain B18LD contained intracellular sulfur inclusion, while strain B15LD had no sulfur inclusions in the cells, instead sulfur globules outside of the filaments were visible in the growth medium (Figure 3.24). Furthermore, only strain B18LD seemed to have additional large inclusions in the cells (Figure 3.24 b).

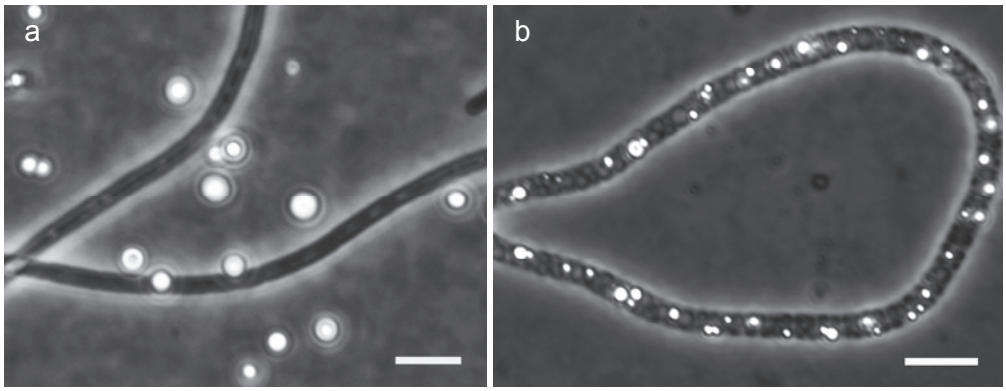

Figure 3.24: *Beggiatoa alba* examined by phase contrast microscopy after 4 days of mixotrophic growth in a modified semi-solid gradient medium according to Nelson and Jannasch (1983) using the medium composition described by Schmidt et al. (1987) with 0.1 mmol L^{-1} ammonium, 10 mmol L^{-1} acetate, and 2 mmol L^{-1} sulfide in the bottom agar. The top agar was buffered at pH 7.5 with 20 mmol L^{-1} PIPES. (a) Strain B15LD contained no intracellular sulfur inclusion, while (b) strain B18LD contained intracellular sulfur inclusions visible as white globules in the filament. The scale bars represent 5 µm.

Potential polyphosphate storage in strain B15LD was detected by DAPI staining (Figure 3.25), which revealed that the strain cultivated in semi-solid gradient medium stored less polyphosphate with and without the addition of sulfide compared to cultures grown in the same medium, but in liquid cultures without sulfide (Figure 3.13 d). Upon 5 days of oxic growth in the gradient media, the

cultures were exposed to 1 day anoxia. After this treatment, the cells seemed to store less polyphosphate than before, but the difference was not pronounced (Figure 3.25 c).

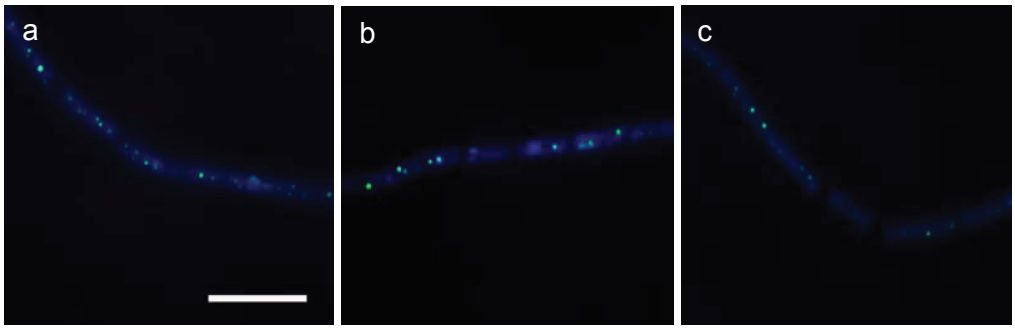

Figure 3.25: DAPI stained polyphosphate in *Beggiatoa alba* after 5 days of mixotrophic growth in a modified semi-solid gradient medium according to Nelson and Jannasch (1983) using the medium composition described by Schmidt et al. (1987) with 10 mmol L^{-1} acetate and 0.1 mmol L^{-1} ammonium. The top agar was buffered at pH 7.5 with 20 mmol L^{-1} PIPES. The bottom agar contained (a) no sulfide, (b) 2 mmol L^{-1} sulfide. (c) After 5 days of growth cultures with 2 mmol L^{-1} sulfide in the bottom agar were exposed to 1 day anoxia. Polyphosphate inclusions are visible as yellow inclusions, while the DNA signal is blue. The scale bar represents 10 µm.

3.3.2 Effect of different nitrogen concentrations on polyphosphate storage in lithoautotrophic freshwater *Beggiatoaceae*

Based on the observation that polyphosphate storage in the organoheterotrophic freshwater *Beggiatoa alba* depended on nitrogen availability, it was tested whether nitrogen availability also has an effect on polyphosphate storage in lithoautotrophic freshwater *Beggiatoaceae*. For this purpose, the enrichment culture from Aarhus, Denmark, and the enrichment culture from Hannover, Germany (Figure 3.26) were used (Section 2.2.1.1). They were cultivated in modified semi-solid gradient medium described by Kamp et al. (2006) with a top agar buffered at pH 7.5 with 20 mmol L^{-1} PIPES. The medium was buffered according to Brock and Schulz-Vogt (2011) because the filaments of freshwater enrichments stored more polyphosphate in buffered medium. Instead of buffering at pH 7.0 the medium was buffered at pH 7.5, because this is the pH optimum for freshwater *Beggiatoa alba*. The medium was prepared with and without the addition of 1 mmol L^{-1} nitrate or ammonium and filaments were stained for polyphosphate after 7 days of growth.

When grown with the addition of ammonium, the filaments did not store more or less polyphosphate compared to cultures cultivated without additional nitrogen source (Figure 3.27). The same was observed for the addition of nitrate (data not shown).

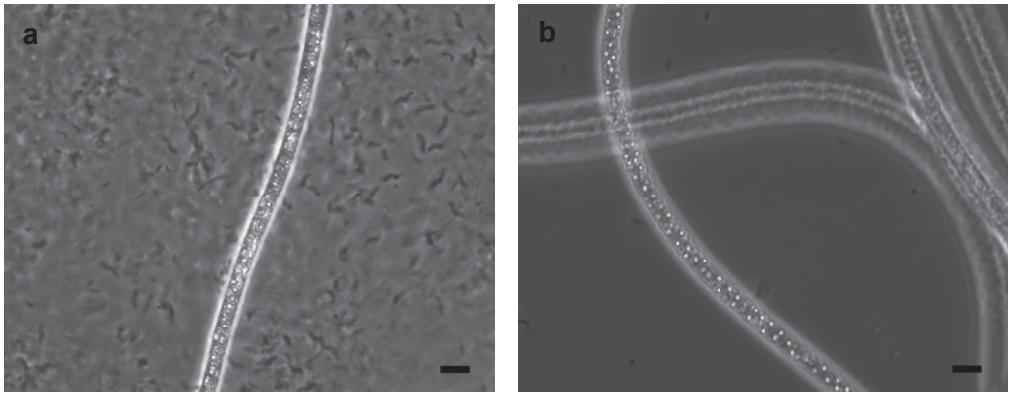

Figure 3.26: Freshwater *Beggiatoaceae* enrichment cultures (a) from Hannover, Germany, and (b) Aarhus, Denmark examined by phase contrast microscopy after 4 days of lithotrophic growth in a modified semi-solid gradient medium according to Kamp et al. (2006) with 4 mmol L^{-1} sulfide in the bottom agar and the top agar buffered at pH 7.5 with 20 mmol L^{-1} PIPES. Scale bars represent 5 µm.

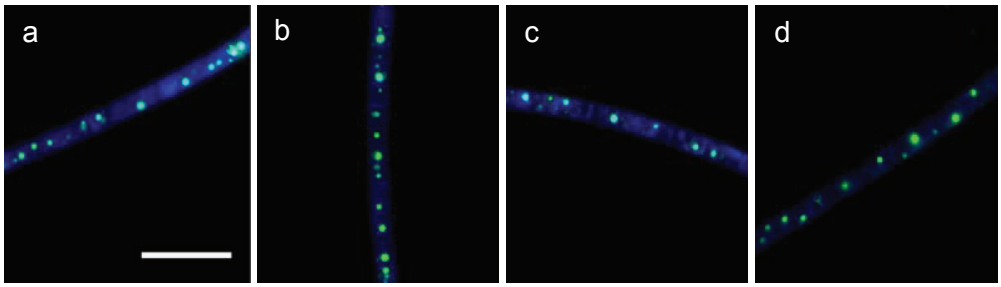

Figure 3.27: DAPI stained freshwater *Beggiatoaceae* enrichment cultures (a, b) from Aarhus, Denmark and (c, d) Hannover, Germany. *Beggiatoaceae* were cultivated for 7 days in a semi-solid gradient medium according to Kamp et al. (2006) with 2 mmol L^{-1} sulfide in the bottom agar and the top agar buffered at pH 7.5 with 20 mmol L^{-1} PIPES. The cultures grew (a, c) without any additional nitrogen source or (b, d) with 1 mmol L^{-1} ammonium. Polyphosphate inclusions are visible as greenish inclusions, while DNA led to a blue fluorescent signal. The scale bar represents 10 µm.

Furthermore, it was investigated whether the inhibition of nitrogen fixation by acetylene, which should lead to nitrogen limitation, has an effect on polyphosphate storage in lithotrophic freshwater *Beggiatoaceae*. For this purpose, acetylene was added to growing cultures and filaments were stained before and after acetylene addition for polyphosphate. As shown in Figure 3.28, the

Beggiatoaceae of both enrichment cultures contained numerous polyphosphate inclusions after 4 days of growth in semi-solid gradient medium without the addition of a nitrogen source. After 1 of day incubation with acetylene, the *Beggiatoaceae* of both enrichment cultures looked the same as before with respect to polyphosphate content (Figure 3.28). There was no obvious tendency of enhanced or reduced polyphosphate storage.

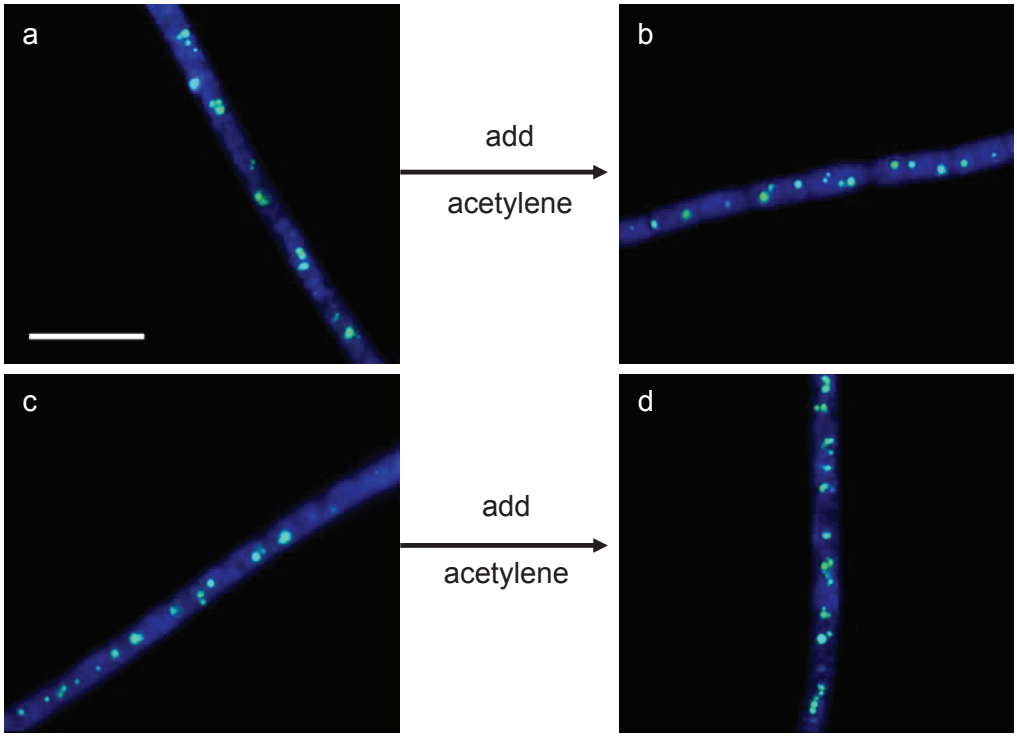

Figure 3.28: DAPI stained freshwater *Beggiatoaceae* enrichment cultures from (a, b) Aarhus, Denmark and (c, d) Hannover, Germany. *Beggiatoaceae* were cultivated in a modified semi-solid gradient medium according to Kamp et al. (2006) without any additional nitrogen source, 2 mmol L^{-1} sulfide in the bottom agar and the top agar buffered at pH 7.5 with 20 mmol L^{-1} PIPES. (a, c) After 4 days of growth and (b, d) after 4 days growth and 1 day incubation with acetylene. Polyphosphate inclusions are visible as greenish inclusions, while DNA led to a blue fluorescent signal. The scale bar represents 10 µm.

3.3.3 Effect of different nitrogen concentrations on polyphosphate storage in the lithoautotrophic marine *Beggiatoa* strain 35Flor

Also the marine *Beggiatoa* sp. 35Flor was investigated for its potential to store less or no polyphosphate in the presence of high nitrogen concentrations, as observed for *Beggiatoa alba*. This strain was cultivated in semi-solid gradient medium (Section 2.2.2.2) with additional nitrate or ammonium. In contrast to *Beggiatoa alba*, the additional nitrogen sources did not lead to reduced polyphosphate storage (Figure 3.29). Also very high ammonium and nitrate concentrations of 10 mmol L^{-1} had no effect (data not shown).

Furthermore, the effect of acetylene addition on polyphosphate storage in growing cultures of marine *Beggiatoa* sp. 35Flor was tested. The strain was cultivated without any additional nitrogen source in the medium and a substantial storage of polyphosphate was observed after 4 days of growth (Figure 3.30 a). When incubated for 1 day with acetylene, no obvious change of the polyphosphate content in the cells could be detected (Figure 3.30 b).

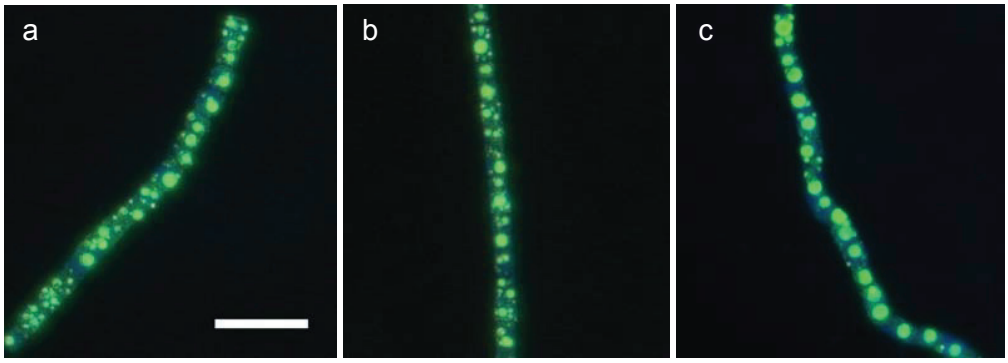

Figure 3.29: DAPI stained polyphosphate in *Beggiatoa* sp. 35Flor. The cultures grew for 5 days in a modified semi-solid gradient medium according to Nelson and Jannasch (1983) with 4 mmol L^{-1} sulfide in the bottom agar, top agar buffered at pH 7.0 with 20 mmol L^{-1} PIPES, and (a) no addition of nitrogen in the medium, (b) 2 mmol L^{-1} nitrate, and (c) 2 mmol L^{-1} ammonium. Polyphosphate inclusions are visible as greenish inclusions, while DNA led to a blue fluorescent signal. The scale bar represents 20 µm.

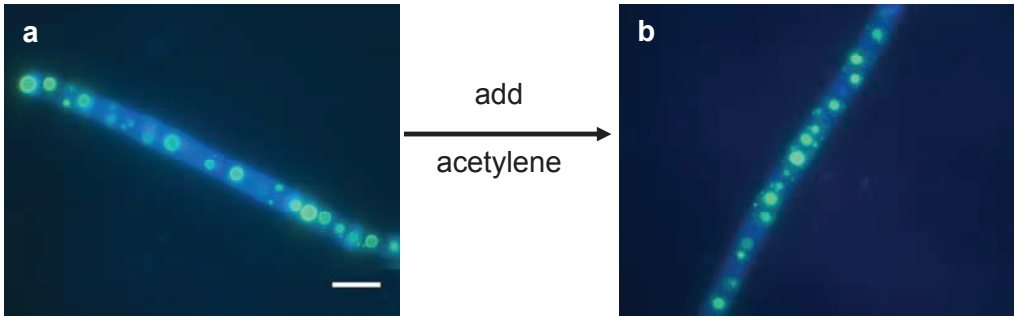

Figure 3.30: DAPI stained polyphosphate in *Beggiatoa* sp. 35Flor. Filaments cultivated in a modified semi-solid gradient medium according to Nelson and Jannasch (1983) without any additional nitrogen source, 4 mmol L^{-1} sulfide in the bottom agar and the top agar buffered at pH 7.5 with 20 mmol L^{-1} PIPES. (a) After 4 days growth and (b) after 4 days growth and 1 day incubation with acetylene. Polyphosphate inclusions are visible as greenish inclusions, while DNA led to a blue fluorescent signal. The scale bar represents 10 µm.

3.3.4 Effect of different nitrogen concentrations on polyphosphate storage in lithoautotrophic hypersaline "*Candidatus* Allobeggiatoa spp."

Also hypersaline enrichment cultures of "*Ca.* Allobeggiatoa spp." from Ibiza, Spain, and Lake Chiprana, Spain (Section 2.2.1.3) were tested for their response to acetylene addition with respect to polyphosphate storage. They were cultivated for 7 days in modified medium according to Nelson and Jannasch (1983) with increased salinities (Section 2.2.2.2). Figure 3.31 clearly shows for all three enrichment cultures that polyphosphate was not stored, neither with nor without the addition of acetylene. When the medium was buffered at pH 7.0 with 20 mmol L^{-1} PIPES, as in marine *Beggiatoa* cultures, the hypersaline "*Ca.* Allobeggiatoa spp." did not store any polyphosphate (data not shown).

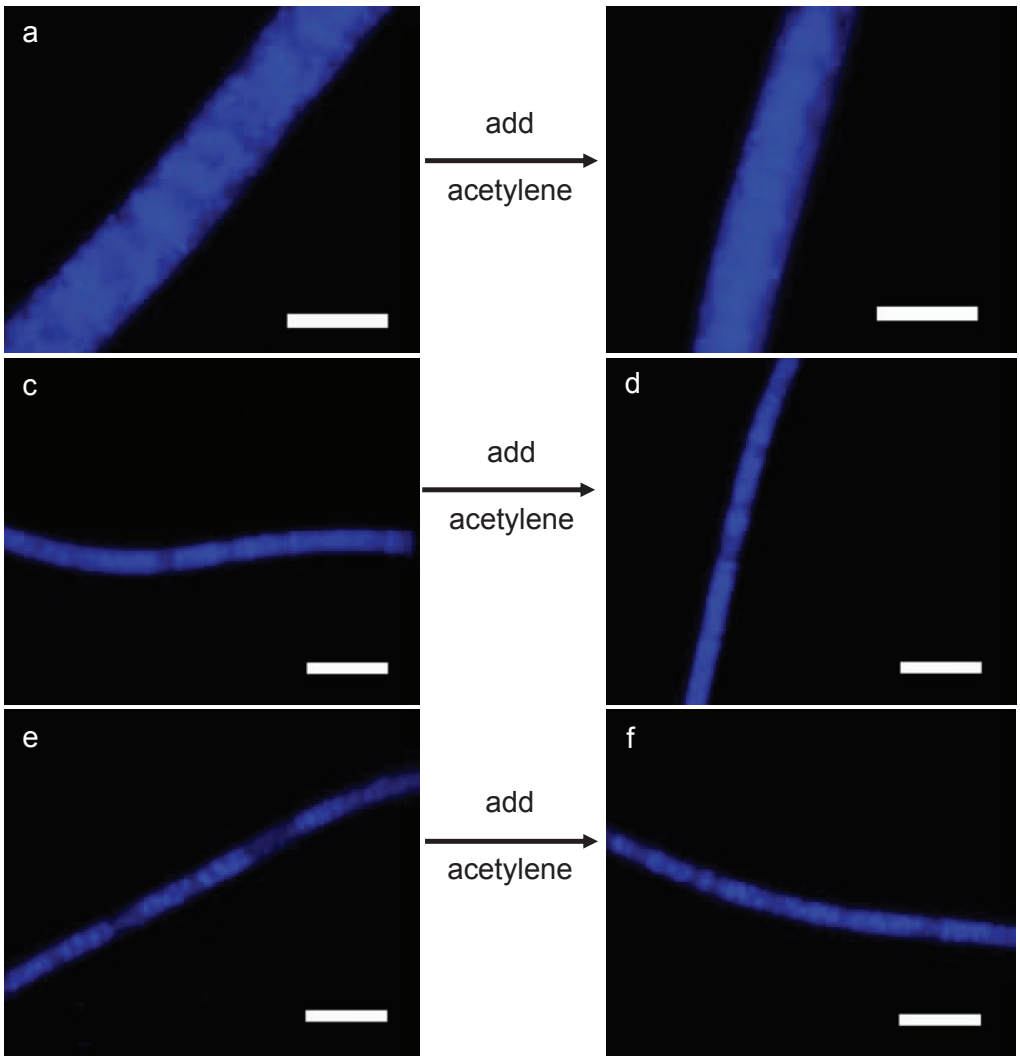

Figure 3.31: DAPI stained hypersaline enrichment cultures of "*Ca.* Allobeggiatoa spp." from (a-d) Ibiza, Spain and (e-f) Lake Chiprana, Spain. The "*Ca.* Allobeggiatoa spp." were cultivated in a modified semi-solid gradient medium according to Nelson and Jannasch (1983) with 4 mmol L^{-1} sulfide in the bottom agar, top agar buffered at pH 7.0 with 20 mmol L^{-1} PIPES, and a salinity of (a, b) 150‰, (c, d) 60‰, and (e, f) 80‰. Cultures grew (a, c, e) for 7 days in medium without additional nitrogen source and (b, d, f) 7 days and 1 day incubation with acetylene. Polyphosphate inclusions would have led to a greenish signal, while DNA led to a blue fluorescent signal. Scale bars represent (a, b) 20 µm and (c-f) 10 µm.

3.3.5 Effect of various pH values and temperature ranges on polyphosphate storage in *Beggiatoaceae*

Since it was already observed that freshwater heterotrophic *Beggiatoa alba* degrade their polyphosphate in response to high and low pH (Havemeyer,

2010), the marine lithotrophic *Beggiatoa* sp. 35Flor was studied with respect to this behavior. Therefore, *Beggiatoa* sp. 35Flor was cultivated at pH 6.0, 6.5, 7.0, 7.5, and 8.5. At pH 6.0 there was no growth. At pH from 6.5 to 8.5 the bacteria grew and stored similar amounts of polyphosphate (Figure 3.32).

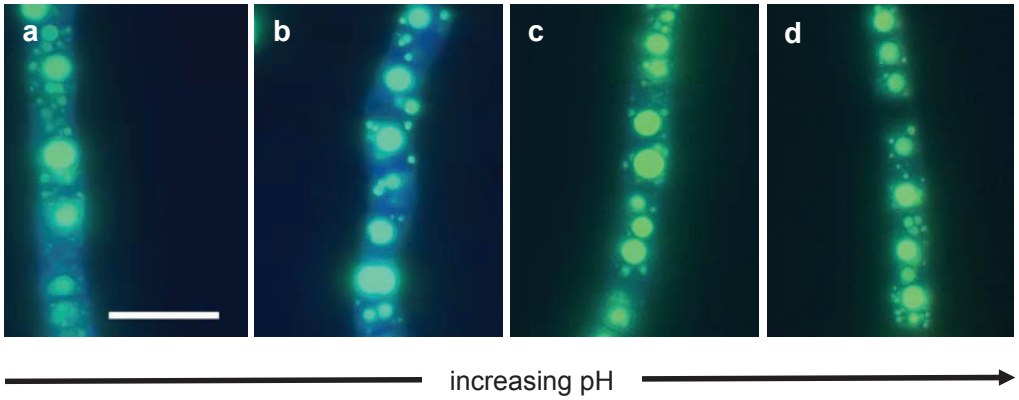

Figure 3.32: DAPI stained *Beggiatoa* sp. 35Flor cultivated at different pH values. The cultures grew 7 days in a modified semi-solid gradient medium according to Nelson and Jannasch (1983) with 4 mmol L^{-1} sulfide in the bottom agar, top agar buffered at (a) pH 6.5, (b) 7.0, (c) 7.5, and (d) 8.5. Polyphosphate inclusions are visible as greenish inclusions, while DNA led to a blue fluorescent signal. Scale bar represents 10 µm.

Stress was reported to be one regulating factor for polyphosphate storage in bacteria (Kulaev and Kulakovskaya, 2000) and as *Beggiatoa alba* degrades polyphosphate in response to pH changes (Havemeyer, 2010), temperature was also tested as another potential stress factor on this strain as well as on the marine lithotrophic *Beggiatoa* sp. 35Flor. Liquid *Beggiatoa alba* cultures, which grew 3 days in medium according to Strohl and Larkin (1978) and *Beggiatoa* sp. 35Flor, which grew 7 days in modified semi-solid gradient medium according to Nelson and Jannasch (1983) with 4 mmol L^{-1} sulfide in the bottom agar and top agar buffered at pH 7.0 with 20 mmol L^{-1} PIPES, were incubated for 1 hour at different temperatures. With increasing temperatures the polyphosphate inclusions became smaller in both *Beggiatoa* strains compared to the controls, but the temperature inducing this reaction differed. Figure 3.33 shows that *Beggiatoa alba* degraded polyphosphate when incubated at higher temperatures than 45°C, whereas strain 35Flor degraded polyphosphate at 70°C and higher temperatures (Figure 3.35). In case of *Beggiatoa alba* the filaments formed tufts and were obviously damaged at 50°C (Figure 3.34 b, c).

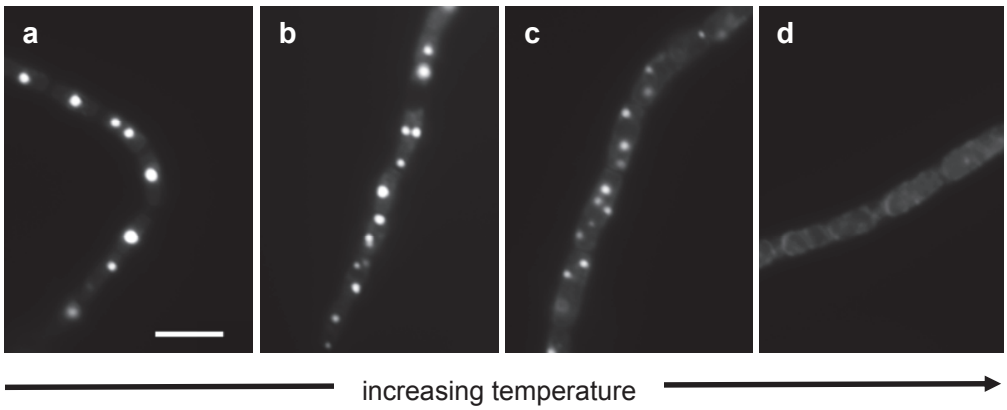

Figure 3.33: Black-white image of DAPI stained *Beggiatoa alba* after 1 hour heat stress. The cultures grew 3 days in modified medium according to Strohl and Larkin (1978) without catalase and were afterwards exposed to different temperatures. (a) Control at 28°C, (b) at 45°C, (c) at 48°C, and (d) at 50°C. Polyphosphate is visible as white inclusions, while the DNA signal is grey. The scale bar represents 5 µm.

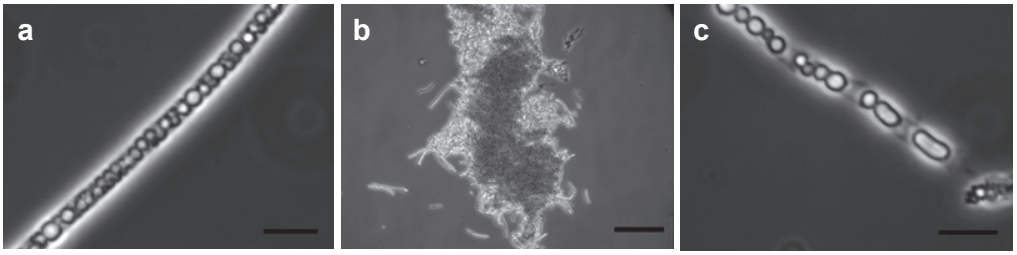

Figure 3.34: Phase contrast microscopy of *Beggiatoa alba* after 1 hour heat stress at (a, b) 48°C and (c) 50°C. The cultures grew 3 days in modified medium according to Strohl and Larkin (1978) and were afterwards exposed to different temperatures. The scale bars represent 5 µm.

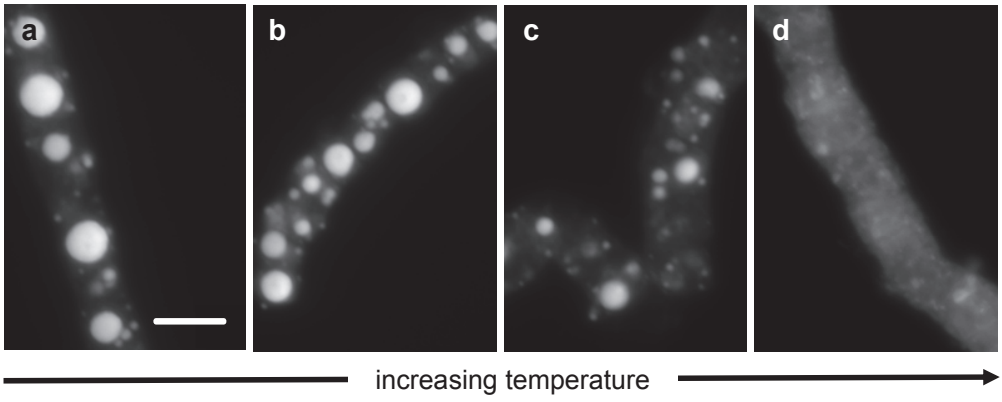

Figure 3.35: Black-white image of DAPI stained *Beggiatoa* sp. 35Flor after 1 hour heat stress. The cultures grew 7 days in modified medium according to Nelson and Jannasch (1983) with 4 mmol L^{-1} sulfide in the bottom agar, top agar buffered at pH 7.0 with 20 mmol L^{-1} PIPES. (a) Control at 20°C, (b) at 50°C, (c) at 70°C, and (d) at 80°C. Polyphosphate is visible as white inclusions, while the DNA signal is grey. The scale bar represents 5 µm.

3.3.6 Polyphosphate storage in natural, environmental samples of *Beggiatoaceae*

Polyphosphate storage in environmental samples of filamentous *Beggiatoaceae* in sediments from Aarhus Bay, Denmark (Section 2.2.3.1), and Lake Grevelingen, the Netherlands (Section 2.2.3.2), was investigated. In the sediment core from Aarhus the *Beggiatoaceae* formed a mat approximately 2 mm below the sediment surface (Figure 3.36), while no distinct *Beggiatoaceae* mat was visible in the sediment core from Aarhus. Staining with DAPI revealed *Beggiatoaceae* filaments of different sizes from both sampling sites, ranging in filament diameters from ~ 1.5 to ~ 45 µm in the core from Aarhus and from ~ 0.6 to ~ 10 µm in the core of Lake Grevelingen. However, in none of these filaments polyphosphate inclusion were stained (Figure 3.37 and 3.38).

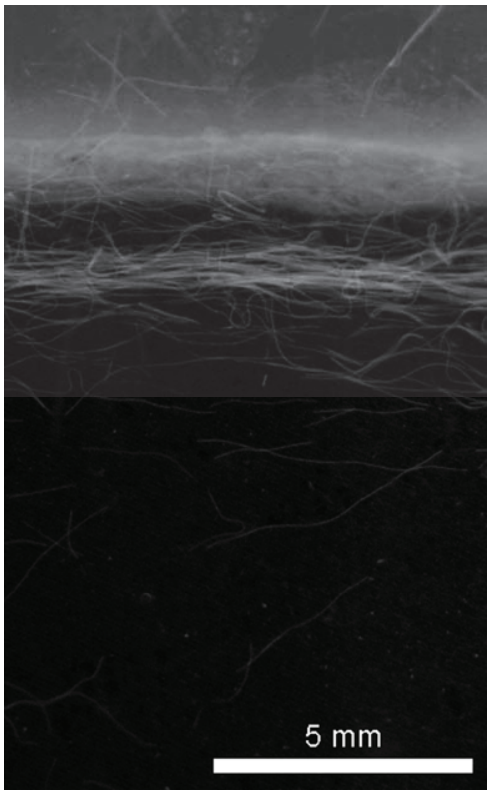

Figure 3.36: Mat of filamentous *Beggiatoaceae* in a sediment core from Aarhus Bay, Denmark, taken in February 2012. The scale bar represents 5 mm (courtesy of A.-C. Kreutzmann).

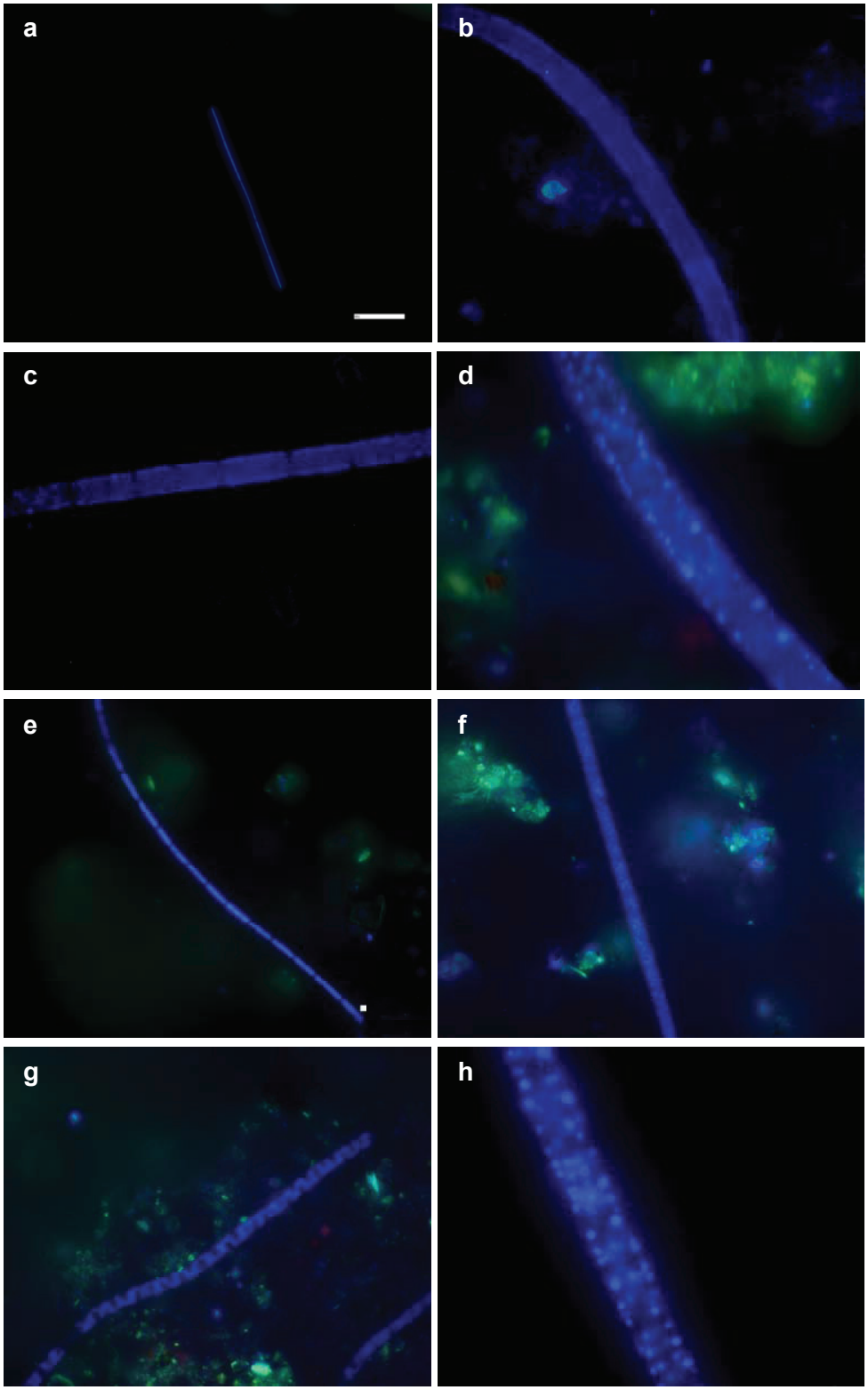

Figure 3.37: DAPI stained *Beggiatoaceae* from a sediment core from Lake Grevelingen, the Netherlands, taken (a-d) in August 2012 and (e-h) in September 2012. Polyphosphate inclusions would have led to a greenish signal, while DNA led to a blue fluorescent signal. The scale bar represents 10 µm.

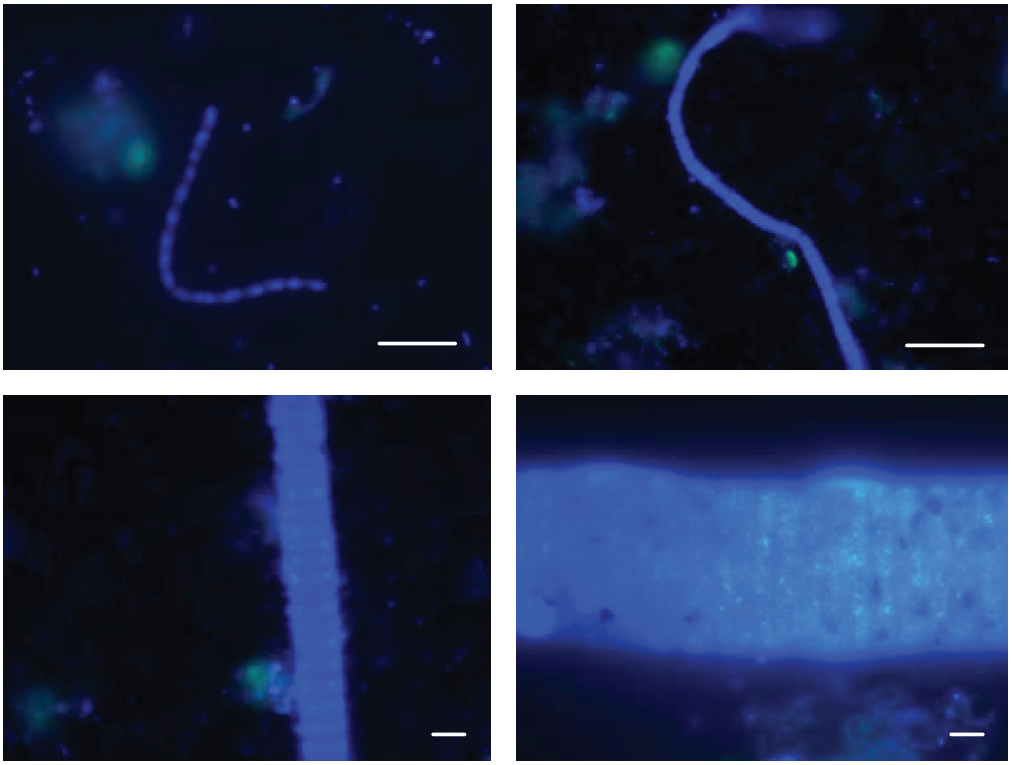

Figure 3.38: DAPI stained *Beggiatoaceae* from a sediment core from Aarhus Bay, Denmark, taken in February 2012. Polyphosphate inclusions would have led to a greenish signal, while DNA led to a blue fluorescent signal. The scale bars represent 10 µm.

4. Discussion

4.1 Genes encoding for polyphosphate-related enzymes in sulfur bacteria

In order to study polyphosphate storage, an understanding of the enzymes involved in polyphosphate metabolism is required. The availability of six draft genomes from *Beggiatoaceae* offered the possibility to get a first insight into the diversity of polyphosphate-metabolizing enzymes encoded by members of the family. However, it has to be noted that the draft-status of the genomes did not allow drawing a comprehensive picture.

Polyphosphate kinase 1 (PPK1) occurred ubiquitously in members of the *Beggiatoaceae*. PPK1 was identified in almost all analyzed genomes except that of *"Ca.* Parabeggiatoa sp." (Table 3.1). Hence, genetic evidence was provided for polyphosphate synthesis ($[polyphosphate]_n$ + ATP ⇆ $[polyphosphate]_{n+1}$ + ADP) in *Beggiatao alba* B18LD, *"Ca.* Thiomargarita nelsonii", *"Ca.* Isobeggiatoa sp.", the Guaymas filament, and *Beggiatoa* sp. 35Flor. Indeed, polyphosphate storage was reported for *Beggiatoa alba* (Strohl and Larkin, 1978), *Thiomargarita namibiensis* (Schulz and Schulz, 2005), and *Beggiatoa* sp. 35Flor (Brock and Schulz-Vogt, 2011). In contrast to PPK1, the preferentially polyphosphate-degrading enzyme polyphosphate kinase 2 (PPK2) was not identified in any of the *Beggiatoaceae* genomes, which means that members of the family seemingly do not couple polyphosphate breakdown to the synthesis of GTP ($[polyphosphate]_n$ + GDP ⇆ $[polyphosphate]_{n-1}$ + GTP). The polyphosphate-utilizing polyphosphate glucokinase (PPGK) was found in genomes of "*Ca.* Thiomargarita nelsonii", "*Ca.* Isobeggiatoa sp.", and the Guaymas filament (Table 3.1), suggesting they are likely able to use polyphosphate as a phosphorus donor instead of ATP to activate glucose ($[polyphosphate]_n$ + glucose → $[polyphosphate]_{n-1}$ + glucose-6-phosphate).

The polyphosphate-degrading enzyme polyphosphate:AMP phosphotransferase (PAP) was identified in all analyzed genomes except that of *"Ca.* Parabeggiatoa sp." (Table 3.1), whereas the transphosphorylating enzyme adenylat kinase (AK) was found in genomes of *Beggiatoa alba* B18LD, "*Ca.* Thiomargarita nelsonii", the Guyamas filament, and *Beggiatoa* sp. 35Flor. The presence of both PAP and AK in *Beggiatoa alba* B18LD, "*Ca.* Thiomargarita nelsonii", the Gyamas filament, and *Beggiatoa* sp. 35Flor suggests that members of the *Beggiatoaceae* can use polyphosphate as an energy source in order to produce ATP (PAP: $[polyphosphate]_n$ + AMP → $[polyphosphate]_{n-1}$ + ADP; AK: 2 ADP → ATP + AMP). For *Thiomargarita namibiensis* and *Beggiatoa* sp. 35Flor it has been suggested that energy provided by the decomposition of polyphosphate is needed to endure conditions, under which no suitable electron acceptor is available for the oxidation of sulfide or internally stored sulfur (Schulz and Schulz, 2005; Brock and Schulz-Vogt, 2011).

Similar to PAP, the exopolyphosphatase (PPX) degrades polyphosphate, however, without generating an energy-rich compound, such as ATP. Instead, PPX releases orthophosphate from polyphosphate ($[polyphosphate]_n + H_2O \rightarrow [polyphosphate]_{n-1} + P_i$). PPX was identified in all genomes except that of "*Ca.* Thiomargarita nelsonii" and two homologues of this enzyme were found in the genomes of *Beggiatoa alba* B18LD and *Beggiatoa* sp. 35Flor. Orthophosphate release upon polyphosphate degradation was previously shown for *Beggiatoa* sp. 35Flor (Brock and Schulz-Vogt, 2011). It was suggested that the breakdown of polyphosphate and release of phosphate in sulfur bacteria could have an effect on benthic phosphorus cycling and can even drive phosphogenesis (Section1.1.1) (Schulz and Schulz, 2005). Since the giant sulfur bacterium *Thiomargarita namibiensis* was proposed to drive phosphogenesis by phosphate release from polyphosphate degradation in Namibian sediments (Schulz and Schulz, 2005), it is surprising that PPX was not found in the related "*Ca.* Thiomargarita nelsonii". However, it has to be considered that the analyzed genomes are not closed and genes, which have not been found, could still be present. Figure 4.1 summarizes the main enzymes involved in polyphosphate metabolism found in the genomes of *Beggiatoaceae*.

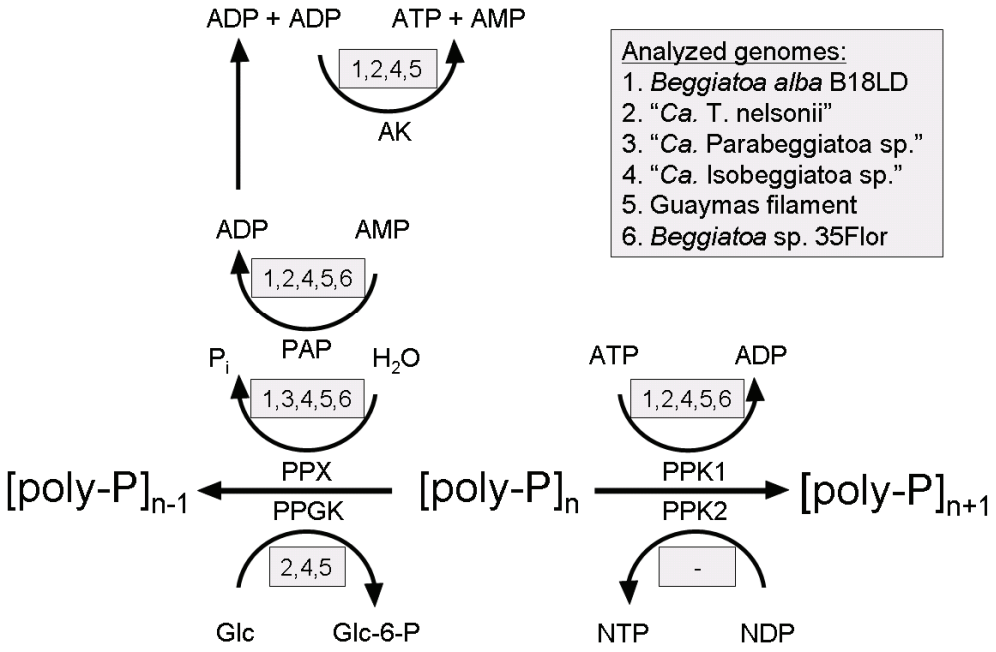

Figure 4.1: Scheme of the main enzymes involved in prokaryotic polyphosphate metabolism found and their presence in members of the family *Beggiatoaceae.* Notably, enzymes not identified so far can be located in the not yet sequenced parts of the draft genomes.
Abbreviations: ADP, adenosine diphosphate; AK, adenylat kinase; AMP, adenosine monophosphate; ATP, adenosine triphosphate; *Ca., Candidatus*; Glc, glucose; Glc-6-P, glucose-6-phosphate; PAP, polyphosphate:AMP phosphotransferase; P_i, inorganic phosphate; poly-P, polyphosphate; PPGK, polyphosphate glucokinase; PPK, polyphosphate kinase; PPX, exopolyphosphatase; GDP, guanosin diphosphate; GTP, guanosin triphosphate.

4.2 Polyphosphate storage and degradation in *Beggiatoaceae*

4.2.1 Polyphosphate inclusions in *Beggiatoa alba*

Size of polyphosphate inclusions

Polyphosphate storage of the freshwater heterotrophic *Beggiatoa alba* B15LD was observed earlier (Strohl and Larkin, 1978), but has not been studied in detail. Here, the polyphosphate inclusion of *Beggiatoa alba* were characterized and compared to already investigated polyphosphate inclusions of the related marine lithotrophic *Beggiatoa* sp. 35Flor (Brock et al., 2012). A high

polyphosphate storage capacity of *Beggiatoa alba* was demonstrated by DAPI and Toluidine Blue staining (Figure 3.1 and 3.2). While the wider marine strain contains polyphosphate inclusions up to 3 µm in diameter (Brock et al., 2012), narrow *Beggiatoa alba* stored polyphosphate in smaller inclusions of up to 1.2 µm in diameter, but mostly below 1 µm in diameter. This is in the range of polyphosphate inclusions observed in other narrow filamentous freshwater *Beggiatoaceae* (Maier and Murray, 1965) and hypersaline *Beggiatoaceae* (de Albuquerque et al., 2010). In contrast, the giant marine lithotrophic *Thiomargarita namibiensis* stores polyphosphate in inclusions of up to 2 µm in diameter (Schulz and Schulz, 2005). Like *Beggiatoa* sp. 35Flor, *Beggiatoa alba* contained polyphosphate inclusions of different sizes and numbers, depending on the cultivation conditions (Section 3.3). For this reason, it is difficult to compare the storage capabilities for polyphosphate between bacteria, which have been exposed to different growth conditions.

pH of polyphosphate inclusions

Apart from confirming polyphosphate storage in *Beggiatoa alba*, the inclusions were further characterized. Since acidocalcisomes of eukaryotes, which are polyphosphate enriched inclusions, are described to be acidic (Docampo et al., 2005), the pH in polyphosphate inclusions was investigated. Acridine Orange is an acidophilic dye, which was used to indicate pronounced pH differences between cytoplasm and polyphosphate inclusions (Ramos et al., 2010). The dye did not accumulate or stain inclusions in the tested strains. Instead, the cytoplasm along the entire filament was stained (Figure 3.4). Probably this signal was due to nucleic acid staining (Bradley and Wolf, 1959). This observation is in agreement with polyphosphate inclusions of *Beggiatoa* sp. 35Flor, which also did not stain with Acridine Orange and are therefore assumed to be not acidic (Brock et al., 2012). However, the marine strain *Beggiatoa* sp. 35Flor contained small acidic inclusions of yet unknown function, which were not observed in *Beggiatoa alba*. Overall, staining with Acridine Orange revealed that the polyphosphate inclusions in *Beggiatoa alba* were not acidic.

Additionally, a potential pH difference between inclusions and the cytoplasm was tested by using the cationic lipophilic dye Rhodamin 123. In contrast to Acridine Orange, this dye does not accumulate in acidic inclusion; it is excluded from acidic inclusions. In a previous study, wide vacuolated marine *Beggiatoaceae* were stained with Rhodamin 123. In that study, only the cytoplasm was stained, not the vacuoles, indicating the presence of an electric potential (inside positive) over this membrane and that the vacuole was acidic (Mußmann et al., 2007). In *Beggiatoa alba*, Rhodamin 123 stained large and small inclusions and no obvious signal was observed in the cytoplasm (Figure 3.5). In contrast, in stained *Beggiatoa* sp. 35Flor filaments, Rhodamin 123 fluorescence was detected in the cytoplasm and accumulated in small inclusion, but not in large polyphosphate inclusions (Figure 3.6). The exclusion of the Rhodamine 123 from these large inclusions indicated that they were acidic. This raises the question whether polyphosphate inclusions in strain 35Flor are actually not acidic as indicated by the negative Acridine Orange staining (Brock et al., 2012). Possibly, they are at least more acidic than the cytoplasm, although they did not stain with Acridine Orange. Concerning *Beggiatoa alba*, many inclusions were stained, which were therefore not acidic. However, so many inclusions were stained that it could not be distinguished, whether they were PHA or polyphosphate inclusions or both.

Compartmentalization of polyphosphate inclusions by a membrane

While the large polyphosphate inclusions in the marine *Beggiatoa* sp. 35Flor were shown to be surrounded by a membrane by staining with Nile Red and MDY-64 (Brock et al., 2012), no lipid layers were observed around polyphosphate in *Beggiatoa alba*. Nile Red staining in *Beggiatoa alba* revealed many red fluorescent inclusions due to PHA staining (Figure 3.3), which were very large and had such strong signals that is was impossible to distinguish if there was a staining around polyphosphate inclusions or not. However, *Beggiatoa* sp. 35Flor has a wider filament diameter than *Beggiatoa alba* and is more likely to be compartmentalized by membrane-bound polyphosphate inclusions. So far, only sulfur bacteria beyond 5 µm contain intracellular membrane-

bound compartments, such as vacuoles. This observation is in congruence with the calculated assumption of an increased diffusion limitation in cells with diameters exceeding a few micrometers. Vacuoles probably allow for wide filament diameters because the diffusion limitation within cells can be prevented by keeping the thickness of the cytoplasmic layer in the range of conventional bacterial cell sizes (Schulz and Jørgensen, 2001). However, we could neither show nor exclude the presence of a membrane around polyphosphate inclusions in *Beggiatoa alba*.

Characterization by Raman scattering due to bond stretching vibration

Raman micro-spectroscopy confirmed polyphosphate storage by *Beggiatoa* sp. 35Flor, as already observed by Brock and Schulz-Vogt (2011) and in *Beggiatoa alba* B15LD. Compared to the application of dyes, Raman spectroscopy is more specific, because Raman spectra can be compared to standards. Furthermore, the method is less invasive, since it does not require chemical fixation or addition of other substances, which possibly change the composition of cell components, as is necessary for staining. Raman spectra of polyphosphate inclusions of *Beggiatoa* sp. 35Flor showed two main peaks at 1187 and 710 cm^{-1} (Figure 3.8). These two peaks were close to the polyphosphate standard with a peak at 1167 cm^{-1}, corresponding to the stretching vibration of the PO_2^- group, and a peak at 690 cm^{-1}, representing the P-O-P bond vibration (de Jager and Heyns, 1998). In areas of polyphosphate inclusions less organic material was measured (Figure 3.7), represented in the Raman spectra by the peak at ca. 2950 cm^{-1} due to the C-H bond stretching vibration (Figure 3.8). In case of *Beggiatoa alba*, Raman spectra at the regions of potential polyphosphate inclusions showed peaks at 1175 and 700 cm^{-1} (Figure 3.10), which were down-shifted by approximately 10 cm^{-1} each, as compared to the peaks of polyphosphate inclusions in *Beggiatoa* sp. 35Flor, but which were still in the range of reported values for polyphosphate in other bacteria (Majed et al., 2009). Raman spectra of *Beggiatoa alba* filaments in regions without polyphosphate often showed peaks at 845, 905, 1065, 1465, and 1750 cm^{-1}, which are similar to the pattern created by PHB (Majed and Gu,

2010). Polyphosphate storage in *Beggiatoa* sp. 35Flor and *Beggiatoa alba* was not only confirmed by Raman micro-spectroscopy, but also differences of the polyphosphate types were observed. The polyphosphate peaks of the two strains had different Raman shift values with respect to each other and to the sodium polyphosphate standard, although their relative heights and separation are similar. Since peak positions of polyphosphate depend for example on associated cations and average chain length (Corbridge and Lowe, 1954), it might explain peak shifts.

Elemental composition of polyphosphate inclusions

Indeed, scanning electron microscopy (SEM) in combination with energy dispersive X-ray analysis (EDXA) revealed different cations associated with polyphosphate in *Beggiatoa* sp. 35Flor and *Beggiatoa alba*. The negative charge of polyphosphate inclusions is typically compensated by associated positively charged, low-molecular-weight compounds like amino acids or divalent cations. The cations most often associated with polyphosphate are Ca^{2+}, K^{+}, and Mg^{2+} (Keasling, 1997), as for example in *Acinetobacter* (Bonting et al., 1993). In contrast to polyphosphate in narrow hypersaline *Beggiatoaeae* and *Beggiatoa* sp. 35Flor, which are associated with Ca^{2+} and Mg^{2+} (de Albuquerque et al., 2010; Brock et al., 2012), polyphosphate in *Beggiatoa alba* was not associated with these elements. Instead, in regions of phosphorus and oxygen storage a co-occurrence with significantly higher Na^{+} concentrations compared to the surrounding cytoplasm was detected (Figure 3.11). So far, Na^{+} was not reported to typically associate with polyphosphate, instead acidocalcsisomes of protoza, which are polyphosphate enriched inclusions, also contain Na^{+} (Docampo et al., 2005). Hence, for the first time an obvious association of sodium with polyphosphate in bacteria was reported.

4.2.2 Polyphosphate storage in *Beggiatoaceae* in dependence of different culture conditions

General remarks:
The focus of these studies was to investigate polyphosphate storage in organoheterotrophic freshwater *Beggiatoa alba*. The culture conditions differed in nitrogen, phosphorus, carbon, and sulfide concentrations as well as in various pH values and temperatures. The possible functions of polyphosphate at different culture conditions will be discussed and compared to observations made on other lithoautotrophic freshwater, marine, and hypersaline representatives of the family *Beggiatoaceae*.

Polyphosphate storage in *Beggiatoa alba* strongly depended on nitrogen availability. Initially, it was observed that *Beggiatoa alba* stored polyphosphate in medium according to Strohl and Larkin (1978) but not in medium according to Schmidt et al. (1987) (Figure 3.12). The component correlating with the presence or absence of polyphosphate was ammonium, which was in low amounts (0.84 mmol L^{-1}) in the medium according to Strohl and Larkin (1978), and in high amounts (4.7 mmol L^{-1}) in medium according to Schmidt et al. (1987). An increased polyphosphate storage in medium according to Schmidt et al. (1987), which generated nitrogen limiting conditions with lowered concentrations of 0.1 mmol L^{-1} in the presence of excess carbon, was observed, whereas less or no polyphosphate was stored when growing in media with higher nitrogen concentrations (Figure 3.13 and 3.18). Similar to our observations, many bacteria accumulate polyphosphate under conditions of nutritional imbalance unfavorable for growth (Kornberg et al., 1999). For instance, *Pseudomonas putida* (Tobin et al., 2007) and *Escherichia coli* (Ault-Riché et al., 1998) store high amounts of polyphosphate during nitrogen limitation. Furthermore, in the yeast *Candida utilis* the polyphosphate content depends on the growth rate and the nitrogen source in the medium (Núñez and Callieri, 1989). Nitrogen limitation was even used in research for waste water treatment plants to select for polyphosphate-accumulating bacteria in a mixed culture that did not previously exhibit enhanced biological phosphorus removal characteristics (Harper et al., 2005).

Polyphosphate as energy source

Nitrogen limitation led to limited growth (Figure 3.14 and 3.15), while excess energy was available through oxidation of acetate. Polyphosphate was reported to be accumulated at the expense of energy (Kornberg, 1995). During nitrogen limitation, there was an imbalance between energy generation and biosynthesis, because more energy could be gained through the oxidation of acetate than it was needed for growth, which was limited by nitrogen. Such an imbalance can lead to polyphosphate storage (Wentzel et al., 1986). According to Wilkinson (1959), an energy reserve is stored if the energy supply is in excess of that required for growth and related processes. For example, the *Acinetobaeter* strain 210A stores high amount of polyphosphate as an energy sink product at low growth rates, when the energy generated from the extracellular energy source is in excess due to nitrogen limitation (van Groenestijn et al., 1989).

It could be hypothesized that polyphosphate was stored as an energy reserve in *Beggiatoa alba,* because there was excess energy available through the oxidation of acetate, but growth was limited by low concentrations of nitrogen. The energy reserve could immediately be used for growth when nitrogen was available again. This might explain, why polyphosphate in *Beggiatoa alba* was degraded after nitrogen addition to a previously nitrogen limited culture (Figure 3.19). An example for such a process to occur is *Klebsiella aerogenes.* A *Klebsiella aerogenes ppx* mutant rapidly degraded polyphosphate when growth is resumed after nutritional downshift, suggesting that PPK consumed polyphosphate by its reverse reaction and provided ATP *in vivo* (Kuroda and Ohtake, 2000).

However, if polyphosphate is assumed to be an energy source, it should be metabolized according to the biochemical model described by Comeau et al. (1986) (Section 1.2). This model implies that polyphosphate is stored during oxic conditions and the energy required for its synthesis is gained by the degradation of PHA. Under anoxic conditions, polyphosphate would serve as an energy source and the energy released by its degradation could be used to

take up acetate and store it as PHA. However, *Beggiatoa alba* does not degrade polyphosphate in response to the combination of acetate addition and anoxic conditions (Havemeyer, 2010), which is in congruence with its relative *Beggiatoa* sp. 35Flor (Brock and Schulz-Vogt, 2011). These two strains accumulate polyphosphate and PHA simultaneously. Therefore, it can be assumed that polyphosphate is not used as an energy source to drive the synthesis of PHA. Furthermore, *Pseudomonas putida* CA-3 accumulates these two biopolymers at the same time and polyphosphate is assumed to not provide energy for the synthesis of PHA (Tobin et al., 2007). Additionally, the rapid turnover rate of ATP in the cell (the entire pool of ATP is typically turned over in 0.2 seconds) argues against polyphosphate simply being an ATP store (Chapman and Atkinson, 1977).

In *Beggiatoa alba*, acetate is used as a sole carbon and energy source when no sulfide is available (Schmidt et al., 1987). Under acetate limitation, the polyphosphate content in *Beggiatoa alba* decreased (Figure 3.23). These findings are not in agreement with other bacteria, for instance *Acinetobacter* strain 210A stores more polyphosphate when carbon is limiting (Bonting et al., 1992) and no effect of carbon supply on polyphosphate storage was observed in *E. coli* (Ault-Riché et al., 1998). During acetate limitation, *Beggiatoa alba* growth was not only limited by the lack of carbon, but also by energy, since acetate is used as a sole carbon and energy source when no sulfide is available (Schmidt et al., 1987). Linton and Stephenson (1978) even argued that growth on limited amounts of acetate means primarily energy limitation and not carbon limitation. Therefore, it could be argued that less polyphosphate was stored because less energy was available. However, the addition of an alternative energy source in *Beggiatoa alba*, namely sulfide (Güde et al., 1981; Schmidt et al., 1987), did not enhance polyphosphate storage (Figure 3.25). Consequently, the ability to store polyphosphate seems to be directly linked to the availability of an organic carbon source in *Beggiatoa alba*. Interestingly, when grown with sulfide, *Beggiatoa alba* strain B15LD did not store sulfur in inclusions as strain B18LD did (Figure 3.24), although both strains were reported to store sulfur inclusions intracellular (Strohl et al., 1981a). Instead, sulfur globules in the medium of B15LD were visible, which were likely

formed by the bacteria, since in the uninoculated control medium less sulfur globules were observed. So far, this is the first time that a colorless sulfur bacterium seems to excrete sulfur into the medium. In cultures of the marine *Beggiatoa* sp. 35Flor sulfide is the only energy source. However, also at very low sulfide concentrations, when growth was limited by the gain of energy, the filaments still stored polyphosphate (Brock and Schulz-Vogt, 2011). This suggests that at least in this marine strain polyphosphate was not stored solely as an energy reserve, but may have important regulatory functions, since the cells even invested energy for its synthesis during energy limiting conditions.

On the one hand, polyphosphate is used up in *Beggiatoa alba* cells after shifting them from nitrogen limitation to nitrogen surplus, which might indicate the usage of polyphosphate as an energy source for growth. Furthermore, acetate limitation led to decreased polyphosphate storage, which argues for polyphosphate being an energy source. On the other hand, polyphosphate in *Beggiatoa alba* is not used according to the biochemical model described by Comeau et al. (1986) (Section 1.2) and an additional energy source like sulfide had no obvious influence on polyphosphate storage, suggesting that polyphosphate is not an energy source. Consequently, it is still under debate, if polyphosphate in *Beggiatoa alba* is used as an energy source.

Polyphosphate triggers protein degradation at nitrogen limitation

As decribed above, high amounts of polyphosphate were stored in *Beggiatoa alba* in response to nitrogen limitation. Polyphosphate storage at nitrogen limitation is best studied in *E. coli*. In *E. coli*, polyphosphate is required for adaptation to amino acid starvation (Kuroda et al., 1999). Nitrogen is particularly necessary to build up amino acids. Consequently, nitrogen limitation results in amino acid starvation, which results in a lack of both, amino acid biosynthetic enzymes and the amino acids required to make these enzymes (Kuroda, 2006). One possibility for bacteria to overcome this condition is to use their own proteins as amino acid pool (Gottesman and Maurizi, 2001).

The mechanism behind the usage of proteins as intracellular amino acid pool is called the stringent response (Kuroda and Ohtake, 2000). The depletion of amino acids results in uncharged transfer RNAs (tRNAs). These uncharged tRNAs can bind to a vacant ribosomal A-site if the corresponding amino acid is not available and thereby block translation. This is the molecular event sensed by the enzyme RelA (Haseltine and Block, 1973). RelA stabilizes an unusual distorted form of the tRNA on the ribosome, with the acceptor arm making contact with RelA and far from its normal location. By this mechanism, RelA is probably able to sense the uncharged tRNA on the ribosome as a result of amino-acid starvation (Agirrezabala et al., 2013). RelA then catalyses the transfer of phosphates from ATP to GTP or GDP, thereby producing the alarmone guanosine pentaphosphate (pppGpp) (Haseltine and Block, 1973). The pppGpp is hydrolyzed to guanosine tetraphosphate (ppGpp) and both compounds are key signaling molecules in the adaptation to amino acid starvation.

ppGpp and pppGpp inhibit the activity of PPX, resulting in up to 1000-fold higher polyphosphate accumulation (Rao and Kornberg, 1996; Kuroda et al., 1997). PPK builds up polyphosphate and PPX degrades polyphosphate (Section 1.2). In *E. coli*, both enzymes are constitutively expressed and inhibition of PPX therefore leads to polyphosphate accumulation. *E. coli* mutants that fail to produce the stringent factors RelA and therefore are not able to generate ppGpp and pppGpp, also fail to accumulate polyphosphate at amino acid starvation (Kuroda and Ohtake, 2000). The accumulated polyphosphate can then form a complex with the ATP-dependent Lon protease, which degrades free ribosomal proteins (Amerik et al., 1991). It is assumed that the polyphosphate-dependent degradation of these proteins will supply the lacking amino acids (Kuroda et al., 2001). Polyphosphate probably first binds to Lon at the central ATPase domain and then stimulates the activity of the protease by interacting with ribosomal proteins (Kuroda et al., 2001). Thus, it seems that polyphosphate directs the degradation of ribosomal proteins (Nomura et al., 2004).

Lon homologues were discovered in many organisms, from prokaryotes to eukaryotes. Lon is an oligomer of identical subunits (87 kDa) that form a ring-shaped hexamer (Kuroda et al., 2001). Each subunit contains three domains: an N terminus, possibly involved in substrate recognition and binding (Tsilibaris et al., 2006), a central ATPase containing a typical ATP-binding motif (Ebel et al., 1999), and a C-terminal proteolytic domain with a catalytically active residue (Fischer and Glockshuber, 1994). Lon activity generates short peptides that are broken down by cytoplasmic peptidases and amino acids become available for the cell (Figure 4.2). With these amino acids, new enzymes can be build to adjust to nutrient-poor conditions and to recover from nutritional downshifts (Kuroda et al., 2001).

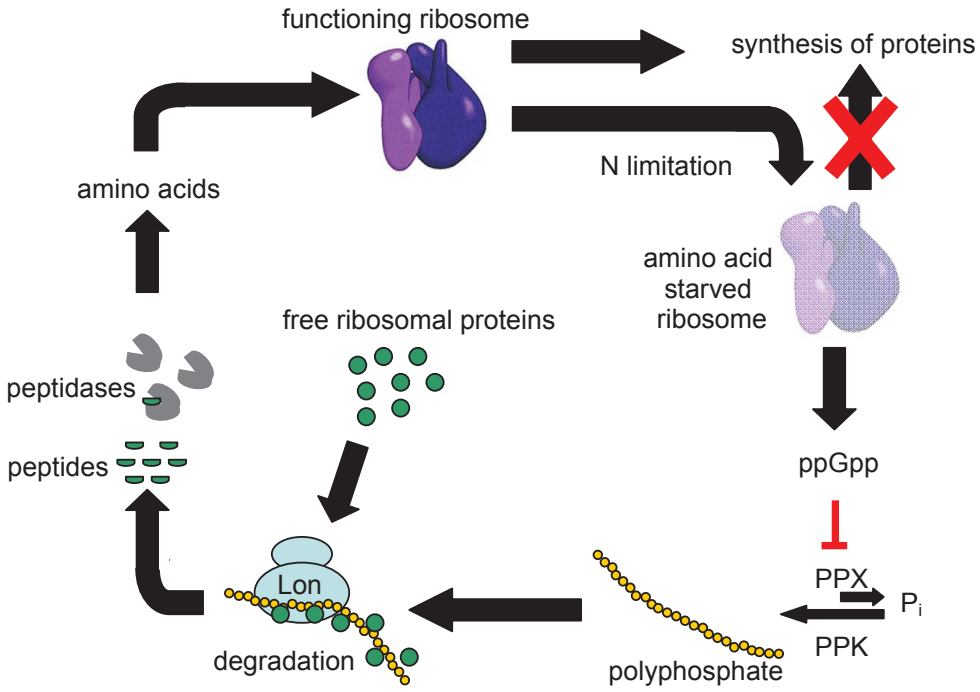

Figure 4.2: Scheme of nitrogen limitation induced protein degradation in *E. coli* (modified after Gottesman and Maurizi (2001)). During amino acid depletion, an increased production of ppGpp leads to accumulation of polyphosphate due to inhibition of PPX. Polyphosphate binds to a subclass of free ribosomal proteins and to the Lon protease, which then degrades ribosomal proteins. Consequently, amino acids are made available to the cell by cytoplasmic peptidases that chop up the short peptides released by Lon. The cell is then able to adjust to these conditions by synthesizing biosynthetic enzymes from the released amino acids.
Abbreviations: Lon, protease Lon; N, nitrogen; P_i, inorganic phosphate; ppGpp, guanosine tetraphosphate; PPK, polyphosphate kinase; PPX, exopolyphosphatase.

As described, polyphosphate is a crucial component in the adaptation to amino acid starvation and triggers the degradation of ribosomal proteins. In *E. coli* mutants lacking PPK1 no proteins were degraded in response to a nutritional downshift (Kuroda and Ohtake, 2000). Since polyphosphate synthesis does not require amino acids, it is suitable as a regulator during nutritional downshifts (Maurizi, 1992).

The ppGpp content in *Beggiatoa alba* was measured to test if the described mechanism might be also present, as it is supported by the finding of the genes coding for RelA in the genome of *Beggiatoa alba* (data not shown). When grown at high nitrogen concentrations, *Beggiatoa alba* did not produce detectable amounts of ppGpp. However, when grown at nitrogen limitation, elevated concentrations of ppGpp in the cell extracts were detected (Figure 3.20). Thus, these results demonstrate massive polyphosphate accumulation and its putative induction by the production of ppGpp. In *Beggiatoa alba* it can therefore be hypothesized that polyphosphate is also stored at nitrogen limitation to regulate enzymes, which degrade proteins, in order to use the generated peptides as intracellular amino acid pool.

Concerning polyphosphate-storing lithotrophic freshwater *Beggiatoaceae* (Figure 3.26 and 3.27) and marine *Beggiatoa* sp. 35Flor, it was not possible to correlate storage of even higher amounts of polyphosphate in response to nitrogen limitation, which in this case was induced by inhibiting nitrogen fixation with acetylene (Figure 3.28 and 3.30). Furthermore, inhibition of nitrogen fixation did not induce polyphosphate storage in enrichment cultures of hypersaline "*Ca.* Allobeggiatoa spp." (Figure 3.31). At the same time, high concentrations of additional nitrogen sources did not lead to decreased polyphosphate storage in *Beggiatoa* enrichment cultures and *Beggiatoa* sp. 35Flor (Figure 3.27 and 3.29). In contrast to the studied lithotrophic freshwater, marine, and hypersaline *Beggiatoaceae*, only heterotrophic freshwater *Beggiatoa alba* stored polyphosphate in response to nitrogen availability and hence, polyphosphate was not used in lithoautotrophs in the adaptation to nitrogen depletion. It might be that the nitrogen-fixing *Beggiatoaceae* did not adapt their polyphosphate metabolism to varying nitrogen concentrations, because

they usually do not experience nitrogen limitation due to the abundant nitrogen pool in the atmosphere.

Polyphosphate as intracellular pH buffer

During the course of the experiments, it was observed that the morphology of *Beggiatoa alba* cultures grown at high ammonium concentrations differed compared to cultures grown at high nitrate concentrations. When cultivated at high ammonium concentrations, the filaments formed large tufts (Figure 3.21) and died already after 4 days, which is earlier than cultures grown with nitrate that died approximately after 7 days. Tuft formation of *Beggiatoa alba* is a common behavior at high oxygen concentrations to diminish direct contact with the oxygenated medium, since *Beggiatoa alba* does not have the enzyme catalase and cannot degrade toxic peroxides (Strohl and Larkin, 1978; Agirrezabala et al., 2013). It could be also observed that *Beggiatoa alba* formed tufts at high and low pH (data not shown) and high temperatures (Figure 3.34). Therefore, tuft formation in *Beggiatoa alba* could be interpreted as a general behavior during stress, indicating that high ammonium concentrations stress the bacteria.

Stress factors in general are reported to induce polyphosphate decomposition (Kulaev and Kulakovskaya, 2000). As mentioned before, *Beggiatoa alba* forms tufts at high oxygen concentrations, creating a microenvironment with lowered oxygen concentration by respiration (Sweerts et al., 1990). Ammonium profiles were measured through the tufts to test whether there is a similar effect compared to oxygen, i.e. a decrease in ammonium concentrations within the tuft. This assumption could not be confirmed. There was no change in the ammonium concentrations inside the tuft in comparison with the surrounding medium (Figure 3.22). With increasing pH, more ammonium speciates to ammonia, which as a gas freely diffuses into cells and can be toxic. To test whether the pH inside the tufts is reduced, which would indirectly protect the cells from the exposure of high ammonia concentrations, pH profiles were measured through tufts, but no pH change was detected (Figure 3.22). Con-

sequently, tuft formation of *Beggiatoa alba* at high ammonium concentrations cannot be explained yet.

In eukaryotes, polyphosphate degradation upon high ammonium concentrations was observed earlier. In the yeast *Saccharomyces cerevisiae*, ammonium addition caused rapid degradation of polyphosphate and in this case, this reaction was also assumed to balance the intracellular pH (Greenfield et al., 1987). In another study with the unicellular algae *Dunaliella salina* it was shown that high ammonium concentrations stressed these organisms and that the polyphosphate content was lower at high ammonium concentrations (Pick et al., 1990). The authors assumed that ammonia diffused into the cell where it got protonated to ammonium, thus leading to cytoplasmic alkalinization. To counteract this intracellular pH change, polyphosphate was degraded since the hydrolysis of anhydride bonds generates protons. The generated protons could serve to stabilize the intracellular pH. Thereby, polyphosphate functioned as an intracellular buffer counterbalancing internal alkaline pH stress (Figure 4.3) (Pick et al., 1990).

A second study on amine-induced alkaline stress in the alga *D. salina* supports the hypothesis of a high-capacity buffering system provided by polyphosphate hydrolysis (Pick and Weiss, 1991). In *Dunaliella salina*, polyphosphate was hydrolyzed by an endopolyphosphatase, which cleaves phosphoanhydride bonds inside the polyphosphate chain with tripolyphosphates as the main products (Pick et al. 1990). This enzyme was so far not found in bacteria, but possibly the same mechanism mediated by PPX is present in bacteria, since PPX also hydrolyzes polyphosphate but in contrast to the endopolyphosphatase it cleaves off terminal phosphate groups. The gene encoding PPX was found in *Beggiatoa alba* (Table 3.1), hence this mechanism might be also active at high ammonium concentrations and could explain, why the cells died early compared to cultures that grew with nitrate. When the polyphosphate was used up and a possible intracellular pH change could not be buffered anymore by polyphosphate hydrolysis, the cells started to die. Since the diffusion of the gas cannot be controlled, the cells could not prevent the toxic effect anymore.

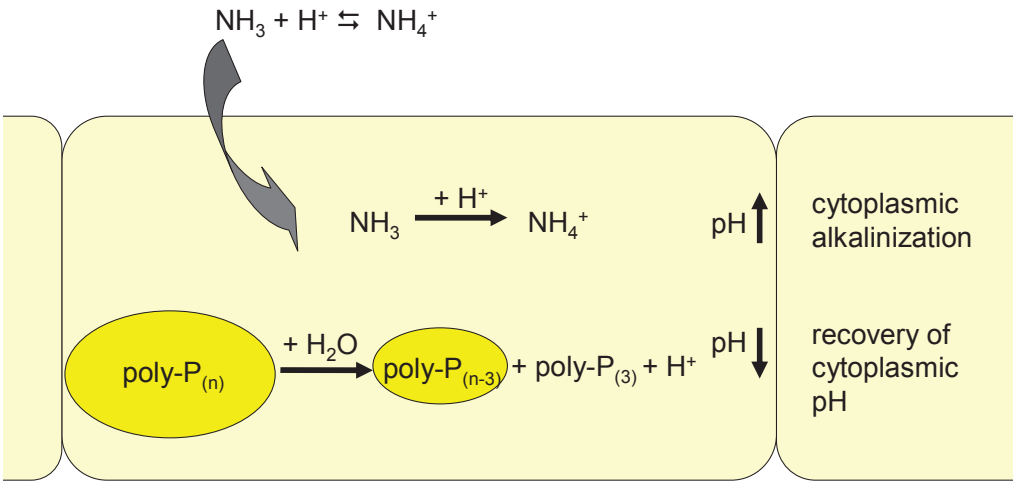

Figure 4.3: Scheme of polyphosphate (poly-P) functioning as an intracellular buffer to counterbalance internal alkaline pH stress in the unicellular algae *Dunaliella salina* (modified according to Pick et al.(1990). Ammonia diffuses into the cells where it gets protonated to ammonium leading to a cytoplasmic alkalinization. Consequently, poly-P is hydrolyzed and tripoly-P and protons are generated. The generated protons lead to recovery the cytoplasmic pH.

A possible detoxification of toxic gas by polyphosphate was also observed in *Beggiatoa* sp. 35Flor (Brock and Schulz-Vogt, 2011). In that study, it was shown that the marine strain degraded polyphosphate in response to high sulfide concentrations and anoxic conditions. Although marine *Beggiatoa* oxidize sulfide, high concentrations are also toxic to them, because it is a strong reducing agent. It might be that polyphosphate in *Beggiatoa* is used to detoxify gases like ammonia or sulfide that are harmful at high concentrations. However, no effect of ammonium on polyphosphate in other freshwater *Beggiatoaceae* or the marine strain *Beggiatoa* sp. 35Flor was observed (Figure 3.27 and 3.29). It still needs to be tested whether polyphosphate can act as an internal pH buffer in *Beggiatoa alba*.

Polyphosphate as phosphorus source

Interestingly, phosphate supply did not influence polyphosphate storage in *Beggiatoa alba*. As mentioned earlier, the filaments did not store high

amounts of polyphosphate at high nitrogen concentrations (Figure 3.13). In these cultures, phosphate was already consumed after 2 days (Figure 3.16). The experiment was repeated with a surplus of 1 mmol L^{-1} phosphate in the medium to exclude that polyphosphate was not stored due to the rapid phosphate depletion in the medium. However, the results remained the same: little or no polyphosphate was stored in *Beggiatoa alba* in response to high nitrogen concentration, independent of the phosphate concentration (Figure 3.17 and 3.18). This result is different compared to *Acinetobacter* strain 210A, which stores polyphosphate in response to a shift from phosphate starved to phosphate surplus conditions (Ault-Riché et al., 1998). Since polyphosphate storage in *Beggiatoa alba* seems to be independent of the phosphate concentration in the medium, it probably does not only serve as phosphorus source, but rather has other functions in the cells. Furthermore, polyphosphate is probably also not a phosphorus source in the related *Beggiatoa* sp. 35Flor, because the strain does not degrade polyphosphate after phosphate depletion in the medium (personal communication J. Brock).

Effect of stress factors on polyphosphate storage in Beggiatoacae

Stress factors in general are reported to induce polyphosphate decomposition (Kulaev and Kulakovskaya, 2000). Different environmental parameters can be considered as stress factors, but the responses of polyphosphate storage in bacteria to these factors differ, even between strains of one species (van Groenestijn et al., 1989). Different stress factors were investigated, namely pH, temperature, and high ammonium concentrations and it was observed that *Beggiatoa alba* and *Beggiatoa* sp. 35Flor responded differently to stress concerning their intracellular polyphosphate pools.

Effect of various pH values on polyphosphate storage

Beggiatoa alba stored polyphosphate at neutral pH, whereas a decrease in size and amount of inclusions was observed at moderately acidic (pH 6.0−6.5)

and at alkaline (pH 8.5−9.5) conditions (Havemeyer, 2010). In addition, the freshwater *Beggiatoaceae* enrichment cultures stored higher amounts of polyphosphate when the medium was buffered at a neutral pH (Figure 3.27). This is in agreement with Filipe et al. (2001), who reported an ideal pH range for polyphosphate-accumulating organisms of 7.3−7.6. In contrast, polyphosphate storage in marine *Beggiatoa* sp. 35Flor was not affected by changing the pH in the wide range of 6.5−8.5 (Figure 3.32). Decreasing the pH to 6 resulted in no growth of strain 35Flor. Also polyphosphate storage in *E. coli* (Ault-Riché et al., 1998) and *Acinetobacter* strain 210A (van Groenestijn et al., 1989) was not affected during changes in pH between 6.5 and 9. However, in some *Shigella* spp. and *Salmonella* spp., *ppk* mutants were less tolerant towards acids, indicating that polyphosphate is necessary to overcome pH stress in these species (Kim et al., 2002). Thus, in contrast to marine *Beggiatoa* and many other bacteria, polyphosphate in freshwater *Beggiatoa alba* is degraded in response to pH stress.

Concerning the different effects of pH on polyphosphate storage in freshwater *Beggiatoa alba* and marine *Beggiatoa* sp. 35Flor, the differences in their natural habitats have to be considered. In comparison, natural freshwater environments are in contrast to marine environments not carbonate buffered and pH changes are probably more drastic. For example, pH changes in the sediments can be caused by high primary production (Boström et al., 1988) or increased sulfate reduction rates (Caraco et al., 1993). The capability of *Beggiatoa alba* to overcome pH changes by using intracellular stored polyphosphate might serve as advantage in the competition with other microorganisms during unfavorable conditions in the environment. The proposed adaptation to use polyphosphate to overcome pH changes evolved presumably only in *Beggiatoa alba*, since pH changes in freshwater are more variable compared to seawater.

Effect of potentially toxic gases on polyphosphate storage

Furthermore, high sulfide concentrations can be considered as unfavorable conditions especially under anoxic conditions in the absence of a suitable electron acceptor like oxygen or nitrate, since sulfide is highly reactive, corrosive, and toxic to microorganisms (Widdel, 1988). In an earlier study, it was assumed that the degradation of polyphosphate in response to high sulfide concentrations and anoxia in *Beggiatoa* sp. 35Flor might be a mechanism to withstand temporary depletion of electron acceptors in a highly sulfidic environment and that the energy provided is needed to endure sulfide exposure in the absence of a suitable electron acceptor (Brock and Schulz-Vogt, 2011). In contrast to their marine relatives, *Beggiatoa alba* did not significantly degrade its polyphosphate in response to sulfide and anoxia (Figure 3.25). Instead, *Beggiatoa alba* degraded polyphosphate at high ammonium concentrations, while *Beggiatoa* sp. 35Flor did not respond to ammonium. Furthermore, ammonium at high concentrations can have toxic effects. Ammonium does not readily diffuse through lipid membranes, but depending on the pH, a portion occurs in the unprotonated form ammonia (Boussiba, 1989), which diffuses across the cell membrane (Kleiner, 1993). Concerning ammonia, some toxic effects were postulated. Ammonia could for example directly inhibit the activity of cytosolic enzymes (Sprott et al., 1984), or ammonia, which accumulated inside cells, might be toxic by its effect on intracellular pH (Sprott et al., 1984; Pick et al., 1990), or the concentration of other cations, such as K^+, is influenced (Sprott and Patel, 1986).

One crucial difference between freshwater and marine sediments is that sulfide concentrations are much higher in marine sediments. Since the main source of sulfide in nature is the anaerobic oxidation of organic carbon by sulfate reducing bacteria (Widdel and Pfennig, 1981), sulfate reduction rates are considerably higher in marine sediments leading to higher sulfide concentration compared to freshwater environments. Thus, freshwater *Beggiatoa alba* most likely did not adapt their polyphosphate metabolism to high variations of sulfide concentrations in their surrounding as the marine relatives did.

Effect of various temperatures on polyphosphate storage

Beggiatoa alba as well as *Beggiatoa* sp. 35Flor degraded their polyphosphate in response to high temperatures, but the temperature inducing this reaction differed. *Beggiatoa alba* degraded polyphosphate when incubated at temperatures higher than 45°C (Figure 3.33), formed tufts, and were obviously damaged at 50°C (Figure 3.34). Contrary, strain 35Flor degraded polyphosphate at 70°C and higher temperatures (Figure 3.35). In comparison, *Acinetobacter* strains accumulated less polyphosphate at 35°C than at room temperature (van Groenestijn et al., 1989) and in *E. coli* no effect of temperatures up to 42°C on polyphosphate storage was observed (Ault-Riché et al., 1998). The effect of heat stress on *E. coli ppk* mutants that were not able to accumulate polyphosphate was tested by exposure of cells to 55°C for 2 minutes. The characteristic of being heat tolerant for this short time period was greatly diminished in these mutants. Only 2% of the mutant cells survived compared to about 90% of the wild-type cells, which still had the ability to accumulate polyphosphate (Rao and Kornberg, 1996). Similar results were obtained for *ppk* mutants of *Shigella* and *Salmonella* spp. (Kim et al., 2002). Furthermore, in *Pseudomonas fluorescens* Pf0-1 polyphosphate was shown to be required for heat tolerance (Silby et al., 2009).

Heat is known to destabilize many proteins and polyphosphate was suggested to stabilize proteins or stimulate degradation of inhibitory denatured protein aggregates in *Salmonella enterica* (Price-Carter et al., 2005). In *E. coli,* exposure to 70°C for 1 hour was used to provoke heat-induced leakage of polyphosphate (Kashihara et al., 2010). The authors described that the heat disrupted the cell wall and polyphosphate leaked out. In activated sludge, heating at 70°C for 1 hour is even used to induce the release of polyphosphates from bacterial cells into the surrounding liquid (Kuroda et al., 2002; Hirota et al., 2010). Although *Beggiatoa* sp. 35Flor filaments exposed to 70°C and higher temperatures did not look damaged when examined under the light microscope, the cells were probably damaged and it cannot be argued that the disappearance of polyphosphate inclusions at these elevated temperatures was a physiological reaction. In a previous study, these bacteria

were even killed when exposed to 60°C for 15 minutes (Brock and Schulz-Vogt, 2011). Therefore, only results for up to 50°C are considered as physiologically relevant. Similar to experiments with potential pH stress, marine *Beggiatoa* sp. 35Flor did not respond to temperature stress regarding polyphosphate storage. In contrast, freshwater *Beggiatoa alba* showed a stress response towards elevated temperatures and degraded polyphosphate.

Concerning different stress factors like pH, temperature, and high ammonium concentrations, there is a clear difference between the studied freshwater *Beggiatoa alba* and the marine *Beggiatoa* sp. 35Flor. In *Beggiatoa alba* polyphosphate is degraded in response to these stress factors, while they did not effect the polyphosphate accumulation in *Beggiatoa* sp. 35Flor. The only so far described conditions, at which *Beggiatoa* sp. 35Flor degrades polyphosphate, is at high sulfide concentration and anoxia (Brock and Schulz-Vogt, 2011). Although this strain inhabits the marine system, high sulfide concentrations are also toxic to them and can be considered as a stress factor. Hence, polyphosphate accumulation in *Beggiatoa* is not a general response to stress, but occurs as a result of specific stresses, which might occur in their habitats, and to which they hence are adapted to.

4.2.3 Polyphosphate storage in *Beggiatoaceae* in natural, environmental samples

Recently, it has been suggested that giant polyphosphate-accumulating sulfur bacteria influence phosphorus cycling in sediments under certain conditions where they are found in high densities (Schulz and Schulz, 2005; Brock and Schulz-Vogt, 2011). Therefore, polyphosphate storage in filamentous *Beggiatoaceae* from environmental samples at natural conditions were investigated at two different sampling sites: 1) Aarhus harbor at the Aarhus Bay, Denmark, and 2) the marine Lake Grevelingen, the Netherlands. Both sediments contained filamentous *Beggiatoaceae*, which did not store polyphosphate inclusions as tested when directly retrieved from the sample (Figure 3.37 and 3.38).

In the sediment of Aarhus, *Beggiatoaceae* filaments formed a mat approximately 2 mm below the sediment surface in the anoxic zone (Figure 3.36). Usually filamentous *Beggiatoaceae* grow at micro-oxic conditions (Nelson et al., 1986a). Sulfide diffusing from below was consumed within the *Beggiatoacea* mat, indicating that the bacteria probably moved downwards into the anoxic zone to oxidize sulfide with nitrate instead of oxygen. Because no nitrate data are available, this explanation is still unproven. Usually, *Beggiatoaceae* oxidize sulfide with oxygen and form a mat at the interface between oxygen and sulfide. Marine vacuolated *Beggiatoaceae* filaments however have been shown to be able to anaerobically oxidize sulfide with nitrate as electron acceptor, which they can store in their vacuoles in concentrations of up to 44 mmol L^{-1} (McHatton et al., 1996). Dissimilatory nitrate reduction enables *Beggiatoaceae* to colonize anoxic environments in deeper layers in sediments (Mußmann et al., 2003). Since the observed morphotypes of *Beggiatoaceae* in the sediment of Aarhus had filament diameters of ~ 1.5 to ~ 45 µm, it is not unlikely that the wider filaments were able to store nitrate in their vacuoles. However, also non-vacuolated narrow *Beggiatoaceae* use nitrate as a terminal electron acceptor for sulfide oxidation (Mußmann et al., 2003). The pH in the tested *Beggiatoaceae* mat was around 7.25, which is in the pH range of polyphosphate storage in *Beggiatoaceae* in laboratory experiments (Section 4.3.2). However, the *Beggiatoaceae* from the sediment samples did not store polyphosphate (Figure 3.38).

Similar results were obtained for polyphosphate storage in filamentous *Beggiatoaceae* from sediment of the second sampling site, the marine Lake Grevelingen, the Netherlands. However, in contrast to the sediment from Aarhus, hardly any *Beggiatoaceae* filaments were found in the sediment of Lake Grevelingen and the ones found did not store polyphosphate inclusions, neither the narrow filaments nor the wider ones (Figure 3.37). During sampling in August 2012 no oxygen and nitrate was detected in the bottom water but it contained sulfide in the range of 1.2 µmol L^{-1}. These anoxic conditions are not favorable for growth of *Beggiatoaceae*, since they lack a terminal electron acceptor for sulfide oxidation. Even if they would have stored nitrate intracellularly, this pool would have probably been used up within one month of anoxia.

This explains, why no distinct sulfur bacterial mats at this site was present and sulfide could diffuse from the sediment into the bottom water. Under these conditions it was also not expected to observe polyphosphate storage in the few surviving *Beggiatoaceae*, since both anoxia and high sulfide concentrations induce polyphosphate degradation in marine a *Beggiatoa* strain (Brock and Schulz-Vogt, 2011).

In the following month after the initial sampling, conditions in the marine Lake Grevelingen had changed. In September 2012, oxygen concentrations in the bottom water were 200 µmol L^{-1} and no nitrate was detected. The total phosphorus concentration dropped from 4.5 µmol L^{-1} in August to 0.1 µmol L^{-1} in September, ammonium concentrations decreased from 55 to 13 µmol L^{-1} and no sulfide was measured in the bottom water. These conditions were more favorable for sulfur bacterial growth, since oxygen was present to oxidize sulfide at the sediment-water interface and the drop of phosphorus and ammonium concentrations in the bottom water might be an indication for biological uptake. Even then it was difficult to find any *Beggiatoaceae* and those did not store polyphosphate. It is possible that the population of sulfur bacteria was just about to start growing again, however, usually with the return of oxic conditions *Beggiatoaceae* mats establish rapidly within days at the sediment-water interface, and phosphate concentrations in the bottom water decrease (personal communication F. Sulu-Gambari). Seasonal fluctuations in oxygen concentrations therefore lead to seasonal changes in *Beggiatoaceae* occurrence (personal communication F. Sulu-Gambari).

As mentioned above, Lake Grevelingen shows seasonal fluctuations in oxygen penetration and redox conditions leading to seasonal changes in *Beggiatoaceae* occurrence. *Thiomargarita namibiensis,* also belonging to the family *Beggiatoaceae* (Salman et al., 2011), was shown to store and degrade polyphosphate under fluctuating conditions. A change in redox conditions from oxic to anoxic induced polyphosphate degradation and consequently phosphate release in *Thiomargarita namibiensis,* which possibly drives phosphogenesis in Namibian sediments (Schulz and Schulz, 2005). Although *Beggiatoaceae* from Lake Grevelingen also do not store polyphosphate at anoxic

conditions, which is similar to the studied marine *Beggiatoa* strain, it cannot be predicted that metabolic activities of *Beggiatoaceae* might influence the benthic phosphorus cycling in Lake Grevelingen, since no polyphosphate storage could be observed under extended oxic conditions, yet. So far, no correlation of *Beggiatoaceae* abundance and phosphate concentration could be revealed.

Assuming that phosphogenesis is stimulated by phosphate release of polyphosphate-accumulating sulfur bacteria in marine systems due to a change from oxic to anoxic conditions in the presence of high sulfide concentrations (Brock and Schulz-Vogt, 2011) it can be hypothesized that such an effect also occurs in Lake Grevelingen and in Aarhus sediments. Both sites have seasonal changes of redox conditions due to primary production during the summer months. However, the investigated Lake Grevelingen and Aarhus sediments are not sites of recent phosphogenesis. Phosphorites are preferentially formed in shelf sediments of upwelling areas that are located at the border of oxygen-saturated and -depleted water masses (Burnett et al., 1983), where frequent changes between oxic and anoxic conditions occur. In comparison, in Lake Grevelingen and Aarhus sediments such frequent sudden changes in redox conditions do not occur, but rather long-lasting periods of oxic and anoxic conditions alternate. This fact, together with comparably lower sulfate reduction rates and consequently lower sulfide concentrations compared to areas of recent phosphogenesis probably explain why *Beggiatoaceae* in Lake Grevelingen and Aarhus Bay do not seem to considerably influence phosphorus cycling.

5. Conclusion

The results presented in this thesis contribute to the understanding of polyphosphate metabolism in sulfur bacteria, which are considered to influence phosphogenesis (Bailey et al., 2013). Genetic evidence for polyphosphate storage was provided for representatives of three genera (*Beggiatoa*, "*Ca*. Thiomargarita", and "*Ca.* Isobeggiatoa") within the family *Beggiatoaceae*. Furthermore, the studied representatives of the genus *Beggiatoa* and "*Ca*. Thiomargarita" contain genes, which suggest that they can use polyphosphate as an energy source.

The characterization of polyphosphate inclusions in *Beggiatoa alba* revealed a new cation-polyphosphate association. In contrast to the already investigated polyphosphate inclusions of *Beggiatoa* sp. 35Flor (Brock et al., 2012) and hypersaline *Beggiatoaceae* (de Albuquerque et al., 2010), which are associated with magnesium and calcium, *Beggiatoa alba* has polyphosphate inclusions associated with sodium, which is a newly identified association for bacterial polyphosphate.

Although organoheterotrophic freshwater *Beggiatoa alba* and lithoautotrophic marine *Beggiatoa* sp. 35Flor contain the same genes for polyphosphate related enzymes, their polyphosphate metabolism differs. *Beggiatoa alba* stored high amounts of polyphosphate at nitrogen limitation. There are indications that *Beggiatoa alba* accumulated polyphosphate under these conditions to activate enzymes, which degrade intracellular ribosomal proteins in order to use them as amino acid pool. In contrast, at carbon limitation, less polyphosphate was stored, which led to the assumption that polyphosphate also functions as an energy reserve. The observation that polyphosphate storage was independent of phosphate availability argues against the storage compound being solely a phosphorus source. Hence, polyphosphate in *Beggiatoa alba* might serve as an energy source, but not as a phosphorus source and probably regulates enzymes at nitrogen limitation. The storage of polyphosphate as a regulator at nitrogen limitation can favor these gradient organisms in habi-

tats with changing nutrient supply and might be a crucial life-strategy when competing with other bacteria.

Polyphosphate degradation in sulfur bacteria seems to be triggered by different environmental factors. *Beggiatoa alba* degraded its polyphosphate in response to stress, such as high and low pH (Havemeyer, 2010), elevated temperatures, and high ammonium concentrations, while sulfide and anoxia did not lead to obvious polyphosphate degradation, contrary to *Beggiatoa* sp. 35Flor (Brock and Schulz-Vogt, 2011). Possibly, in *Beggiatoa* spp. polyphosphate detoxifies freely permeable toxic gases like ammonia and sulfide, but the regulation is dependent on the adaption to the habitat. For example, sulfide concentrations in marine sediments are much higher compared to freshwater sediments, which could mean that marine *Beggiatoa* spp. have adapted their polyphosphate metabolism to this factor. On the other hand, seawater is carbonate buffered and thus more pH stable than freshwater. A drastic pH rise in freshwater could lead to an unfavorable speciation of ammonium to ammonia, which could be compensated with an adaptive pH regulating system, such as polyphosphate storage in freshwater *Beggiatoa alba*. Accordingly, polyphosphate degradation seems to be triggered by different environmental factors in the different investigated strains and polyphosphate accumulation in closely related *Beggiatoa* spp. is not just a general response to stress but is modified in response to specific conditions and potential stresses of their habitats.

Finally, investigation of environmental samples of *Beggiatoaceae* from Lake Grevelingen, the Netherlands, and Aarhus Bay, Denmark, revealed that sulfur bacteria do not in general influence benthic phosphorus cycling. Seasonal instead of frequent fluctuations in redox conditions together with moderate sulfide concentrations found at these sites seem to not induce massive accumulation of polyphosphate and rapid phosphate release. Consequently, at these conditions, an influence of *Beggiatoaceae* on benthic phosphorus cycling cannot be assumed as reported for sites of recent phosphorite formation in upwelling areas.

6. Outlook

Polyphosphate storage in bacteria in general will be an interesting topic to study, since phosphorus is considered to be the ultimate limiting nutrient and mineable phosphorite deposits will be depleted in the near future. Therefore, the role of polyphosphate-storing bacteria on phosphorus cycling is still an interesting topic. Furthermore, due to the predicted phosphorus limitation in the future, phosphate recycling from waste water will become more important and knowledge on polyphosphate-storing bacteria is necessary to use them in an industrial way.

The results of this thesis provide new insights into polyphosphate storage in *Beggiatoaceae*. Nevertheless, the obtained results also raise new questions that need to be addressed in the future. To test if polyphosphate serves as an energy source in *Beggiatoa alba*, experiments in liquid culture using thiosulfate as an additional energy source can be performed, since in semi-solid liquid medium the cultures store less polyphosphate and do not grow homogenously. Furthermore, the agar makes it difficult to measure for example protein content.

To confirm the assumption that in *Beggiatoa alba* polyphosphate is used to trigger the degradation of ribosomal proteins at nitrogen limitation, it needs to be tested if PPX is indeed inhibited by ppGpp and if an increased protein degradation can be detected. Additionally, more freshwater representatives of the family *Beggiatoaceae* should be investigated with respect to nitrogen-dependent polyphosphate storage to clarify, if this mechanism is restricted to representatives, which do not fix nitrogen. This is of special interest, because the nitrogen-fixing freshwater enrichment cultures of filamentous *Beggiatoaceae* did not store polyphosphate in response to nitrogen limitation. The question whether polyphosphate storage depends on nitrogen concentrations is in particular interesting, because further eutrophication in freshwater could have an effect on polyphosphate storage. In case polyphosphate would be degraded in response to high nitrogen concentrations, as observed in cultures

of *Beggiatoa alba*, this would be a positive feedback and not only high nitrogen, but also high phosphate concentration due to a phosphate release by bacteria upon polyphosphate degradation could be expected

As discussed, ammonia might lead to a pH change within the *Beggiatoa alba* cells. This hypothesis can be tested either by applying pH sensitive stains, such as BCECF (Invitrogen, Karlsruhe, Germany) or by in vivo measurements of the intracellular pH using ^{31}P NMR studies. In the last case, the pH can be measured from the shift of the intracellular inorganic orthophosphate peak position. Furthermore, concentrations of phosphorus-containing metabolites can also be readily obtained from the spectra.

Concerning polyphosphate storage in environmental *Beggiatoa*, the investigated sites (Lake Grevelingen and Aarhus bay) should be studied at different time points during the year. Possibly time points are missing, at which the environmental conditions favored polyphosphate storage and therefore underestimated the relevance of *Beggiatoaceae* on phosphorus cycling at these sites. Additionally, more environmental parameters, as for example nitrate concentrations, have to be determined in order to explain presence or absence of polyphosphate. Furthermore, it is necessary to study polyphosphate storage in sulfur bacteria at sites of recent phosphogenesis. Fluorescence *in situ* hybridization using probes for specific polyphosphate-related genes could be used to combine the identification of the phylogenetic affliliation of the bacteria and their function in benthic phosphorus cycling. Additionally, since sulfur bacteria that inhabit recent sites of phosphogenesis do not grow in cultures so far, for instance *Marithioploca* spp. from the coast off Chile, genomic analysis of these bacteria could help to understand their potential role in biogeochemical cycles. This would contribute to both microbiology and geobiology.

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Erklärung

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

Hiermit erkläre ich, dass ich die Arbeit mit dem Titel:

„Polyphosphate storage in the family *Beggiatoaceae* with a focus on the species *Beggiatoa alba* “

selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit eidesstattlich, dass es sich bei den von mir abgegebenen Arbeiten um 3 identische Exemplare handelt.

(Sandra Havemeyer)