

ORIGINAL ARTICLE

Physiology and behaviour of marine *Thioploca*

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Among prokaryotes, the large vacuolated marine sulphur bacteria are unique in their ability to store, transport and metabolize significant quantities of sulphur, nitrogen, phosphorus and carbon compounds. In this study, unresolved questions of metabolism, storage management and behaviour were addressed in laboratory experiments with *Thioploca* species collected on the continental shelf off Chile. The *Thioploca* cells had an aerobic metabolism with a potential oxygen uptake rate of 1760 $\mu\text{mol O}_2$ per dm^3 biovolume per h, equivalent to 4.4 nmol O_2 per min per mg protein. When high ambient sulphide concentrations ($\sim 200 \mu\text{M}$) were present, a sulphide uptake of $6220 \pm 2230 \mu\text{mol H}_2\text{S}$ per dm^3 per h, (mean \pm s.e.m., $n = 4$) was measured. This sulphide uptake rate was six times higher than the oxidation rate of elemental sulphur by oxygen or nitrate, thus indicating a rapid sulphur accumulation by *Thioploca*. *Thioploca* reduce nitrate to ammonium and we found that dinitrogen was not produced, neither through denitrification nor through anammox activity. Unexpectedly, polyphosphate storage was not detectable by microautoradiography in physiological assays or by staining and microscopy. Carbon dioxide fixation increased when nitrate and nitrite were externally available and when organic carbon was added to incubations. Sulphide addition did not increase carbon dioxide fixation, indicating that *Thioploca* use excess of sulphide to rapidly accumulate sulphur rather than to accelerate growth. This is interpreted as an adaptation to infrequent high sulphate reduction rates in the seabed. The physiology and behaviour of *Thioploca* are summarized and the adaptations to an environment, dominated by infrequent oxygen availability and periods of high sulphide abundance, are discussed.

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Introduction

Extensive areas of the continental shelf along the coast of Peru and Chile (Gallardo, 1963, 1977a, b; Schulz *et al.*, 2000) and also other oxygen minimum zones (OMZs; Schmaljohann *et al.*, 2001; Bernhard and Buck, 2004) are inhabited by so-called ‘macro-bacteria’ (Gallardo and Espinoza, 2007a, b). Among those, we find dense populations of giant filamentous sulphide-oxidizing bacteria belonging to the

genus *Thioploca*. The marine *Thioploca* community is dominated by two species, *T. chileae* and *T. araucae* with average trichome (filament) diameters of 16 and 32 μm , respectively (Maier and Gallardo, 1984a). The bacterial trichomes generally live in bundles surrounded by a common sheath, which can be several centimetres long (Schulz *et al.*, 2000), but occasionally they may also be found as free-living trichomes and thereby resemble *Beggiatoa* (VA Gallardo, personal observation). *Thioploca* accumulate nitrate in concentrations up to 0.5 M (Fossing *et al.*, 1995). The trichomes take up nitrate from seawater at the sediment surface and transport it in intracellular vacuoles down into the sediment, where it is used to oxidize sulphide to intracellular sulphur (Huettel *et al.*, 1996). The simultaneous

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storage of nitrate and sulphur enables the trichomes to sustain a continuous energy metabolism as they migrate up and down in an environment where their electron donor and acceptor do not coexist.

Thioploca show close phylogenetic affiliation to marine *Beggiatoa* and *Thiomargarita* (Teske *et al.*, 1996; Jørgensen *et al.*, 2005; Ahmad *et al.*, 2006). Several studies have shown that all these giant sulphur bacteria employ similar metabolic strategies. These include nitrate and sulphur accumulation, mixotrophic assimilation of CO₂ and organic substrates such as acetate, and the ability to perform dissimilatory nitrate reduction to ammonium (DNRA; Fossing *et al.*, 1995; McHatton *et al.*, 1996; Otte *et al.*, 1999; Schulz *et al.*, 1999; Sayama *et al.*, 2005). Recent genomic studies of *Beggiatoa* and physiological studies of *Thiomargarita* (Schulz and de Beer 2002; Schulz and Schulz 2005; Mussman *et al.*, 2007) indicate that the giant sulphur bacteria have diverse pathways of energy metabolism, which includes both DNRA and denitrification, oxygen respiration and an auxiliary energy reservoir of polyphosphate. The question arises whether these properties also apply to *Thioploca*.

Thiomargarita accumulate and reduce nitrate and also have an aerobic metabolism (Schulz and de Beer, 2002). Aerobic sulphide oxidation is both documented experimentally (Jørgensen and Revsbech, 1983; Nelson *et al.*, 1986b; Sayama *et al.*, 2005) and indicated genetically (Mussmann *et al.*, 2007) in *Beggiatoa*. Similar to *Beggiatoa*, *Thioploca* shows phobic response to oxygen concentrations exceeding 10–20 µM (Huettel *et al.*, 1996). Low oxygen concentrations do not inhibit carbon dioxide assimilation (Otte *et al.*, 1999). However, oxygen uptake by *Thioploca* has not been studied.

Sulphide oxidation by the large marine sulphur bacteria affects mineral cycling in many organic-rich surface sediments (for example, Ferdelman *et al.*, 1997; Mussmann *et al.*, 2003; de Beer *et al.*, 2006; Preisler *et al.*, 2007). Only one study has addressed the biomass-specific sulphide oxidation rate of *Thioploca* (Otte *et al.*, 1999). By living in bundles, *Thioploca* are even more exposed to diffusion limitation than *Beggiatoa* trichomes (Schulz and Jørgensen, 2001). The extent to which this affects sulphide uptake rates, remains to be determined by direct measurements of the biomass-specific sulphide uptake.

It has recently been suggested that giant sulphur bacteria interact with phosphorus cycling in sediments where they are found in high densities (Schulz and Schulz, 2005; Arning *et al.*, 2008). *Thiomargarita* have the ability to store polyphosphate as an energy reserve. The alternating storage and release of phosphate appears to enhance the formation of large phosphorus deposits in the sea bed in the same coastal OMZs where the large sulphur bacteria predominate (Schulz and Schulz 2005). The genetic potential for such a metabolism is also indicated in *Beggiatoa* (Mussmann *et al.*, 2007).

If *Thioploca* would have similar properties, their massive presence in the Peruvian and Chilean sediments might impact on phosphate cycling here. However, experimental evidence of phosphate accumulation and subsequent release is not yet available for *Thioploca*.

DNRA is well documented in both *Beggiatoa* and *Thioploca* (Otte *et al.*, 1999; Sayama, 2001; Sayama *et al.*, 2005; Preisler *et al.*, 2007). In addition to this, it seems that at least some marine and freshwater *Beggiatoa* have the capacity to denitrify (Sweerts *et al.*, 1990; Mussmann *et al.*, 2007). A study of nitrogen metabolism in *Thioploca* left the question about denitrification in this genus open (Otte *et al.*, 1999). *Thioploca* sheaths are a potentially favourable niche for the anammox bacteria, because both nitrite and ammonium could be scavenged from DNRA in *Thioploca* (Prokopenko *et al.*, 2006). This possible association needs molecular and biochemical evidence, and the potential of *Thioploca* to denitrify or perform the anammox reaction requires further testing.

In this study, we wanted to clarify the unresolved aspects of *Thioploca* physiology described above by studying oxygen respiration, sulphide oxidation, polyphosphate storage, denitrification and anammox activity associated with *Thioploca*. We also examine substrate uptake in cleaned bundles of *Thioploca*, analyse carbon incorporation by quantitative microautoradiography and follow trichome movements and behaviour to understand the *Thioploca* way of life.

Materials and methods

Sampling

Sediment cores (9 cm inner diameter) containing *Thioploca* were collected with a multiple corer during January 2006 about 30 km northwest of Concepción, Chile, at Station 7 (36 m water depth, 36° 36'S, 73° 00'W) and Station 18 (93 m water depth, 36° 31'S, 73° 08'W). *Thioploca* used in physiological assays were collected with a GOMEX Box corer (Gilbert Rowe, Texas A&M University, Galveston, TX, USA). All samples were kept at *in situ* temperature (11 °C) until processing at the Dichato Marine Biological Station of the University of Concepción. Samples were stored with 100 µM nitrate in the overlying water phase and were processed within 8 days of sampling.

Microgradients

Gradients of oxygen, nitrous oxide, sulphide and pH were analysed around bundles of *Thioploca araucae* with microsensors. A schematic illustration of the experimental setup is shown in Figure 1. The applied oxygen, nitrous oxide, sulphide and pH microsensors (Revsbech and Jørgensen, 1986; Revsbech, 1989; Kühl *et al.*, 1998; Andersen *et al.*,

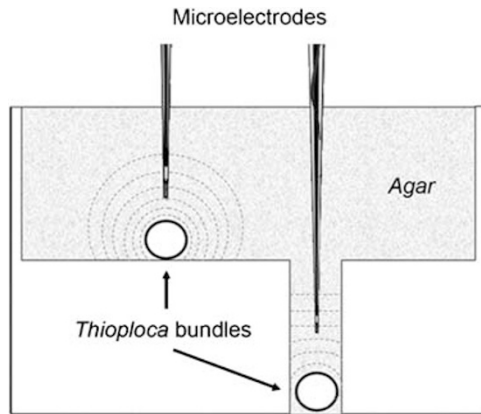


Figure 1 Cross-section of setup with bundle placed in the groove of the glass aquarium. Dotted lines represent concentration isopleths. Not drawn to scale.

2001) had detection limits of $<0.5 \mu\text{M}$, $<1 \mu\text{M}$, $<1 \mu\text{M}$ and <0.05 pH units, respectively. The *Thioploca* bundles were placed either in a 2.5 mm deep and 0.57 mm wide groove (for oxygen, nitrous oxide and pH measurements) or on a flat surface. The use of a groove resulted in steeper gradients and enabled more accurate diffusion flux calculations. However, excessively high sulphide concentrations would have been necessary to saturate the uptake, and these experiments were, therefore, conducted while the trichomes were lying on a flat glass surface and supplied with sulphide by half-cylindrical diffusion. For oxygen measurements one bundle was placed in the groove. For nitrous oxide determinations five bundles with diameters of 0.4–0.5 mm were aggregated to lower the detection limit. To examine possible interference from attached bacteria, the sheaths were carefully peeled off in one end of the bundles, making measurements on free trichomes possible. Control measurements of substrate uptake were also performed on removed sheath material. Bundles were covered by a 1–1.6 cm thick layer of agar-stabilized seawater medium, which prevented advection and allowed for analysis at defined sulphide or low oxygen concentrations.

The agar cover for oxygen uptake experiments was made from *in situ* bottom seawater with 10% of atmospheric oxygen saturation obtained by nitrogen bubbling. It was subsequently heated to 45 °C in serum bottles with 10% atmospheric oxygen tension in the headspace before 10% (liquid vol/vol) 2% agar in 3.5% NaCl solution was injected. Equilibrium between headspace and agar was approached by leaving the bottles in a horizontal position at 11 °C until the next day. The medium for nitrous oxide and sulphide measurements was made in a similar manner but without oxygen. Acetylene blocks the last step in the denitrification pathway, and denitrification can, therefore, be quantified by measuring nitrous oxide production (Smith *et al.*, 1978). For the nitrous oxide detection experiment, nitrate was added to a concentration of 250 μM and a

headspace of 20% acetylene and 80% argon was applied in the serum bottles. Medium for sulphide measurements was buffered with 10 mM morpholinethanesulphonic acid buffer. Sulphide was added to final concentrations of 100 and 200 μM . The pH in the final solutions varied from 7.00 in the 100 μM solution to 7.07 in the 200 μM solution. All manipulations of *Thioploca* and measurements were conducted at 11 °C.

For bundles placed in the groove, calculation of diffusion fluxes per length of bundle (J) from the concentration (C) change with depth (x) were performed by Fick's first law of diffusion:

$$J = wD\delta C/\delta x$$

where D is the diffusion coefficient and w is the width of the groove.

When bundles were placed at the flat glass surface, the flux could be calculated from the concentration gradient $\delta C/\delta x$ measured at some distance from the periphery of the bundle anticipating a half cylindrical diffusion geometry (one half cylinder being blocked by the glass; Figure 1). Cylindrical diffusion fluxes can be calculated from:

$$J = 2\pi RD\delta C/\delta x$$

where R is the radial distance from the centre of the bundle to the point of determination of $\delta C/\delta x$ (Crank, 1983). The diffusion coefficient for oxygen in seawater at 11 °C is $1.51 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ (tables at www.unisense.com), and the diffusion coefficient for sulphide was estimated to be $1.11 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ (Nelson *et al.*, 1986a). The sum of H_2S and HS^- (S_{tot}) was calculated from pH and sulphide data (Hershey *et al.*, 1988). From fluxes and bundle diameters, metabolic rates could be expressed in units of biovolume. In converting these rates to activity per biomass protein a conversion factor of 7 mg protein per cm^3 was used based on our direct measurements of average volume and protein, as well as on measurements of other vacuolated sulphur bacteria of similar size (Kalanetra *et al.*, 2004).

Activity assays

The upper 1–2 cm of sediment was removed from the box corer and placed on ice in a nitrogen-filled glove bag (Sigma-Aldrich, Zwijndrecht, The Netherlands). For each bottle, 100 to 200 *Thioploca* bundles were picked from the sediment. The bundles were washed twice in the incubation medium before anaerobic transfer to 6.1 ml vials equipped with rubber septa (Exetainer; Labco, High Wycombe, United Kingdom), or 30 ml rubber-stoppered bottles filled with anaerobic medium. The incubation medium was made from seawater, which was made anoxic by bubbling with nitrogen and supplied with 1 mg ml^{-1} catalase. Subsequently, substrates were injected through the rubber septum under anoxic conditions. Bottles were incubated at 12 °C and the medium was changed twice during

incubation by decanting under a nitrogen atmosphere. At specific time intervals, samples were taken and analysed for ammonium, nitrite, sulphide, thiosulphate and phosphate. Ammonium was determined colorimetrically according to Grasshoff *et al.* (1999). Dissolved sulphide was determined spectrophotometrically at 670 nm on Zn-preserved pore water by the methylene blue method (Cline, 1969). The concentration of phosphate was determined with the molybdenum blue method as described by Murphy and Riley (1962). Protein content of batches of *Thioploca* bundles was assayed by the microbiuret method, which is insensitive to sulphide and elemental sulphur (Otte *et al.*, 1999). For control experiments, *Thioploca* bundles were disintegrated mechanically by a Potter–Elvehjem homogenizer (Fisher Scientific, Zoetermeer, The Netherlands). This treatment disrupts *Thioploca* cells but leaves bacteria attached to the sheath intact (Otte *et al.*, 1999). The control was necessary to check for activity of epibiontic bacteria observed by fluorescence microscopy (A Teske, personal communication) and autoradiography (Otte *et al.*, 1999).

For the detection of polyphosphate inclusions under the light microscope, intact *Thioploca* trichomes were randomly collected from Stations 7 and 18 and immersed for 30–60 s in a solution containing 0.3 % toluidine blue in 0.5 % acetic acid, then washed and inspected under the light microscope in 0.5 % acetic acid (Schulz and Schulz, 2005).

Quantitative microautoradiography

Incubations for microautoradiography (MAR) were carried out on *Thioploca* bundles incubated in serum bottles. Individual *Thioploca* bundles were collected anaerobically in the same way as bundles used in physiological assays, and five bundles were placed in each 9 ml serum bottle containing 2 ml anaerobic pore water. The bundles were preincubated anaerobically for 0.5 h to remove all traces of oxygen.

Experiments were started by adding 20 μCi of ^{14}C -bicarbonate and unlabelled bicarbonate to a final concentration of 100 μM . Combinations of phosphate (50 μM), sulphide (50 μM), nitrate (50 μM), nitrite (10 μM), oxygen (10%) and organic substrates (100 μM) were added anaerobically to individual bottles (see Table 1).

Incubations with 20 μCi $^{33}\text{PO}_4^{3-}$ for detection of phosphate accumulation were prepared as described for bicarbonate and acetate, except for the addition of 10 μM PO_4^{3-} , 100 μM bicarbonate and combinations of sulphide (50 μM), nitrate (50 μM), oxygen (10%) and organic substrates (100 μM). Bottles were incubated at *in situ* temperature in the dark for 4 or 16 h. Incubations were stopped by adding paraformaldehyde to a final concentration of 4%. All microautoradiographic procedures were carried out as described by Nielsen and Nielsen

Table 1 Carbon fixation by *Thioploca* with various substrates available.

Organic C	Substrates					MAR index	
	PO_4^{3-}	H_2S	NO_3^-	NO_2^-	O_2	4 h	16 h
Acetate	+	+	+			100	100
Propionate	+	+	+			91	92
None		+	+			59	65
None	+	+	+			63	59
None	+		+			63	55
None	+	+		+		61	52
None	+	+			+	37	29
None	+	+				18	26

Microautoradiography index is normalized to the incubation showing the highest activity.

(2005). Length of exposure was optimized to avoid saturation of silver grains in any of the incubations. Quantification of silver grain density on *Thioploca* cells was done by image analysis, using the Image J software (Papadopoulos *et al.*, 2007).

^{15}N -stable isotope experiments

Unlabelled (^{14}N) and ^{15}N -labelled ammonium, nitrite and nitrate were used to assay denitrification and anammox associated with *Thioploca*. Whole *Thioploca* bundles, disrupted *Thioploca* bundles or sediment surrounding *Thioploca* bundles were transferred to separate airtight 6.1 ml vials equipped with rubber septa (Exetainer; Labco) under anaerobic conditions. The vials were filled with anaerobic medium (see above) leaving no headspace, and left overnight at 12 °C for temperature equilibration and consumption of residual nitrate, nitrite and ammonium. Labelled and unlabelled ammonium, nitrite, nitrate and sulphide were added in concentrations of 50–100 μM at the start of the experiments, after which the samples were incubated for 24 h at 12 °C. Then, 1 ml of medium was replaced with 1 ml of argon at atmospheric pressure and the reaction was stopped with 50 μl of saturated ZnCl_2 . The samples were stored upside down to minimize the amount of gas exchange through the septum. In some samples, the 1 ml of removed medium was pasteurized for 15 min at 70 °C, and used for nitrate and nitrite analysis. After arrival in Denmark, samples were analysed in a Tracermass stable isotope ratio mass spectrometer with triple collector.

Observations on trichome movements

To examine the behaviour of individual trichomes in relation to the common sheaths, a 1.8 cm bundle was placed in a Petri dish with oxic seawater and cut into six pieces of different length with initially no trichomes extending out. The movement of the trichomes was subsequently monitored by photography (Ricoh Caplio RR30).

Results

Microprofiling

Oxygen microgradients above *Thioploca* bundles, placed in the groove, showed that *Thioploca* took up oxygen. The oxygen uptake by individual *Thioploca* bundles with diameters of 0.4–0.5 mm varied from 0.3 to 1.8 nmol cm⁻¹ h⁻¹, that is, per cm of bundle length. Knowing the diameter of the individual trichome bundles and assuming complete packing, this corresponds to a biovolume-specific uptake rate of 210–1040 μmol dm⁻³ h⁻¹ (corresponding to 0.5–2.5 nmol min⁻¹ per mg protein). Six different locations along a 3 cm long bundle showed an average oxygen uptake of 340 ± 90 μmol dm⁻³ h⁻¹ (mean ± s.d.) when incubated with low oxygen (~30 μM) in the agar above the incubation groove (data not shown). The inside of the bundle remained anoxic at low ambient oxygen concentrations, but at oxygen concentrations of 100 μM, the inside of the bundle became oxic and a maximum oxygen uptake of 1760 μmol dm⁻³ h⁻¹ was measured (Figure 2a), which is equivalent to 4.4 nmol min⁻¹ per mg protein. Eight hours later, the *Thioploca* trichomes exposed to 100 μM oxygen were dead. The oxygen uptake measured in these experiments was not due to contaminants on the sheath, because no oxygen uptake could be detected above empty sheath material and because biovolume-specific respiration rates were similar above intact bundles and trichomes protruding from the sheath.

Sulphide microgradients were measured above *Thioploca* bundles placed on the chamber bottom, and half-cylindrical diffusion geometry around the bundle was used to calculate sulphide uptake rates. *Thioploca* achieved internal zero sulphide concentrations when incubated on the flat chamber bottom in anoxic 100 μM sulphide agar and, thus, the uptake was diffusion limited (Figure 2b). When the sulphide concentration in the bulk agar was raised to 200 μM, the inside of the *Thioploca* bundle became sulphidic and maximum uptake rates were calculated (6220 ± 2230 μmol dm⁻³ h⁻¹, average ± s.e.m.; Figure 2c) equivalent to 15 ± 5.3 nmol min⁻¹ per mg protein. Knowing the biovolume-specific uptake, the minimum sulphide concentration at the bundle surface, which saturates this uptake, can be calculated using a cylindrical diffusion model with mass consumption.

$$C_b = \frac{1}{4} * \frac{u}{D} * b^2$$

Where C_b is the concentration at the bundle surface, u is the uptake rate, D is the diffusion coefficient and b is the bundle radius (derived from Sten-Knudsen, 2002). Hence, a bundle with a diameter of 200 μm would need a sulphide concentration of 3.9 μM at the surface to reach the maximum sulphide uptake. Measurements with pH microsensors during the sulphide uptake experiment showed no pH gradients (<0.05 pH unit) around the *Thioploca* bundles, due to the buffer in the medium.

No nitrous oxide microgradients were detectable above *Thioploca* bundles placed in the groove with acetylene and nitrate. Given the sensor detection limit of 1 μM nitrous oxide, this means that denitrification rates were below 8 μmol dm⁻³ h⁻¹ (0.02 nmol min⁻¹ per mg protein), corresponding to only about 0.2% of the sulphide uptake rates. Denitrification by *Thioploca* or associated bacteria was therefore insignificant, if present at all.

Activity assays and microautoradiography

Thioploca protein content was 310 ± 56 μg ($n = 10$) per 100 bundles. The ammonium production rates and nitrate consumption rates measured in activity

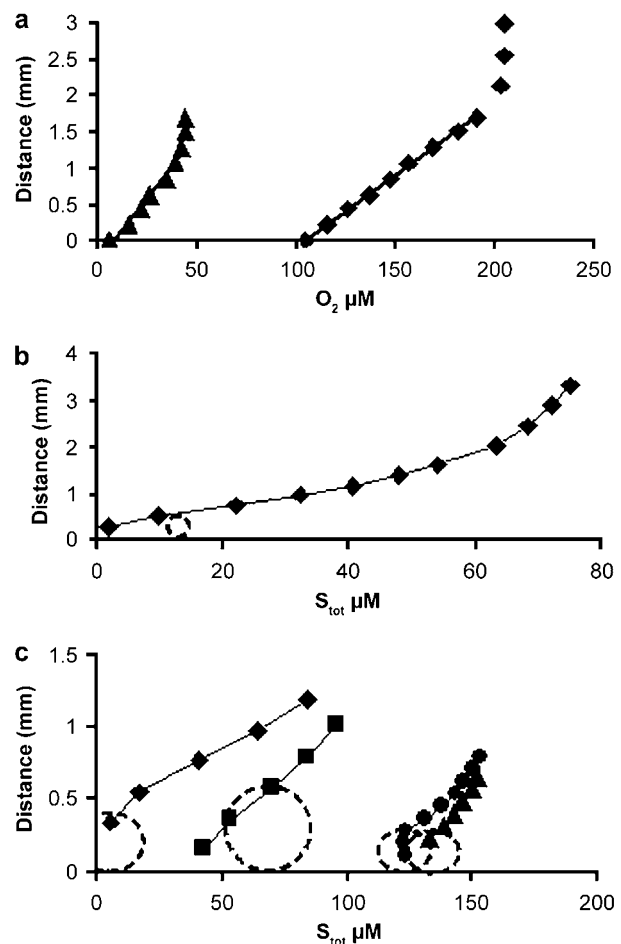


Figure 2 (a) Oxygen profiles above a *Thioploca* bundle (diameter 0.34 mm) that had been incubated in the groove for 15 min (▲) and for 5.5 h (◆). The position of the sheath periphery is defined as zero depth. (b) Sulphide concentrations around a 0.5 mm *Thioploca* bundle placed on the surface of a glass container. Depth zero is defined by the glass surface. The position of the bundle periphery is illustrated with a broken line. (c) Sulphide gradients around bundles incubated with 200 μM sulphide in the bulk agar. Diameters and biovolume-specific respiration rates of the four bundles: ◆, 0.37 mm, 12630 μmol dm⁻³ h⁻¹; ■, 0.55 mm, 2890 μmol dm⁻³ h⁻¹; ●, 0.28 mm, 5820 μmol dm⁻³ h⁻¹ and ▲, 0.32 mm, 3570 μmol dm⁻³ h⁻¹. Depth zero is defined by the glass surface, the position of the bundle periphery is illustrated with a broken line. No pH gradient could be detected around the bundles in this buffered medium.

assays were 1–2 nmol per mg protein per min and 5–6.5 nmol mg protein per min, respectively. After 25 h of incubation some *Thioploca* cells started to lyse irrespective of incubation conditions.

No uptake of phosphate could be detected in three independent activity experiments where *Thioploca* bundles were incubated with 50–100 μM phosphate, with or without sulphide and/or with or without nitrate over a period of 24 h. Two additional activity experiments showed that release of phosphate could not be induced by adding acetate in concentrations of 50–100 μM at different time intervals. Intracellular polyphosphate granules were not observed microscopically on staining with toluidine blue, and MAR incubations with $^{33}\text{PO}_4^{3-}$ did not show phosphate uptake (results not shown).

Results from ^{14}C -bicarbonate incubations and MAR are presented in Table 1. The number of silver grains per bacterial area was calculated by dividing the counted number of silver grains by the length of the trichome. Variation between bacterial cells in the same trichome was <5% whereas variation between trichomes from different bundles was up to 50%. Counts per area were averaged for each incubation and scaled relative to the incubation showing the highest density of silver grains, set to 100. This resulted in a MAR index, from which it is possible to compare carbon fixation between different incubations. Phosphate addition did not affect carbon fixation, neither did the presence of oxygen or sulphide. Addition of nitrate and nitrite enhanced $^{14}\text{CO}_2$ fixation by 100% to 250%, and presence of organic substrates also caused increased CO_2 fixation, indicating potential for mixotrophy, which supports earlier findings of Maier and Gallardo (1984b). The same trends were observed in bottles incubated for four hours and in bottles incubated for 16 h.

^{15}N -stable isotope experiments

Intact *Thioploca*, disrupted *Thioploca* or sediment surrounding *Thioploca* were incubated with ^{15}N labelled NH_4^+ together with unlabelled NO_2^- or NO_3^- to detect potential anammox activity associated with *Thioploca*. $^{29}\text{N}_2$ was produced in concentrations of 0.05 to 0.4 μM in incubations with intact *Thioploca*. This corresponds to a low but significant anammox activity of 0.007 to 0.06 nmol N per min per mg protein. This activity was similar to incubations with disrupted *Thioploca* and to incubations with sediment surrounding the *Thioploca* (Figure 3). Therefore, the intact *Thioploca* did not contribute to the observed anammox activity. Addition of ^{15}N -labelled NO_2^- or NO_3^- to sediment surrounding *Thioploca* cells resulted mainly in the accumulation of 10 μM $^{30}\text{N}_2$, which is at least two orders of magnitude higher than the observed anammox activity. A small production of $^{30}\text{N}_2$ was detectable in the cleaned *Thioploca* preparations and also in control incubations with disrupted *Thioploca*. This

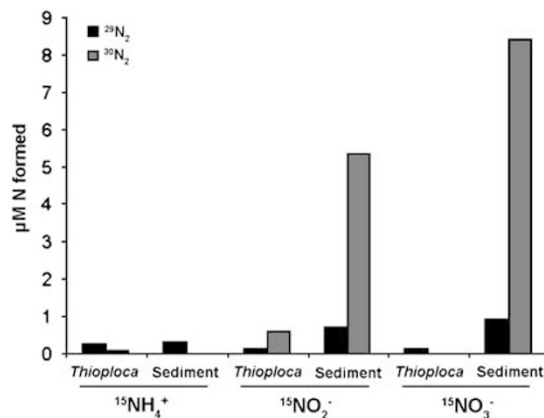


Figure 3 ^{15}N labelled nitrogen gas formation after 24 h of incubation with ^{15}N labelled ammonium, nitrite or nitrate, respectively. $^{29}\text{N}_2$ formation in incubations with labelled ammonium and unlabelled nitrite or nitrate is an indication for anammox activity. $^{30}\text{N}_2$ formation in incubations with labelled nitrate and nitrite (with unlabelled ammonium present in the sediment) is an indication for denitrification. Incubations were made in duplicates except for *Thioploca* incubated with $^{15}\text{NH}_4^+$ ($n = 11$) and sediment incubated with $^{15}\text{NO}_3^-$ ($n = 1$).

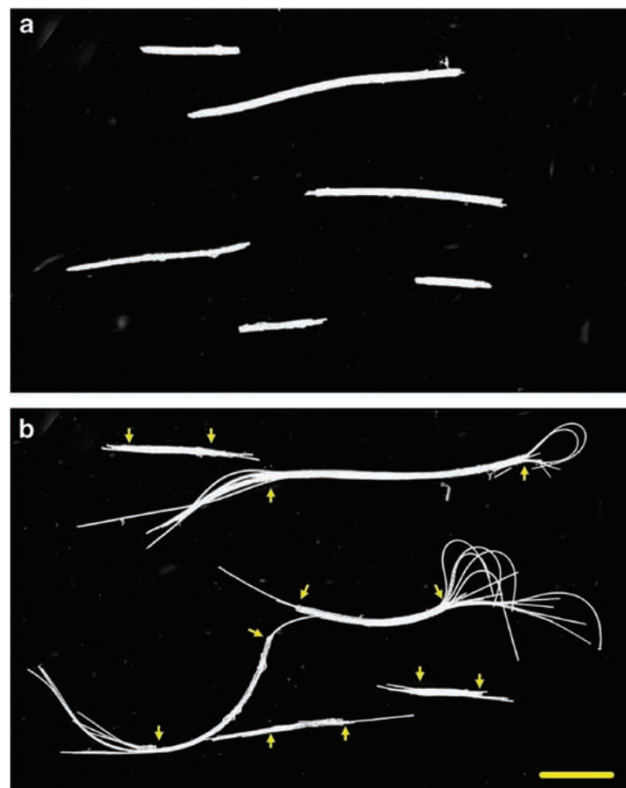


Figure 4 Bundle of *Thioploca* photographed just after being cut into six pieces (a) and 1 h later (b). The arrows indicate the limits of the slime sheaths from where the trichomes have moved out. Scale equals 0.2 cm.

$^{30}\text{N}_2$ could have been produced by contaminating bacteria.

Behavioural observations

Thioploca bundles were divided in pieces (Figure 4a) and placed in oxic seawater. The

individual trichome fragments instantly started to move out of the sheaths and after about 1 h, numerous trichomes had migrated almost completely out (Figure 4b). Interestingly, no trichomes completely abandoned the sheath although they all kept moving back and forth at the same speed. Trichomes had a known maximum length corresponding to the length of the sheath piece, which they occupied. If trichomes from the short pieces had moved as long a net distance as trichomes from the long pieces, most of them would have been free of the slime sheath after one hour, but this did not happen.

Discussion

Oxygen, nitrate, sulphide and phosphorus metabolism

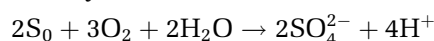
Here we showed that *Thioploca* has an aerobic metabolism with a maximum oxygen uptake rate of $1760 \mu\text{mol dm}^{-3} \text{h}^{-1}$. The maximum uptake rate is similar to the oxygen uptake by *Thiomargarita* (Schulz and de Beer, 2002), but contrary to *Thiomargarita*, *Thioploca* did not sustain this oxygen uptake under high oxygen concentrations for longer periods. This indicates that *Thioploca* is microaerophilic as suggested in motility studies by Maier and Gallardo (1984a) whereas *Thiomargarita* is a facultative aerobe. It is well documented that *Beggiatoa* is microaerophilic (Jørgensen and Revsbech, 1983; Møller *et al.*, 1985; Nelson *et al.*, 1986a), but there are few direct measurements of biovolume-specific oxygen uptake. Oxygen uptake rates measured on marine *Beggiatoa* (trichome diameter $20 \mu\text{m}$) in a setup similar to the one of this study showed a 10 times higher biovolume-specific oxygen consumption ($32000 \mu\text{mol dm}^{-3} \text{h}^{-1}$, LP Nielsen *et al.*, 2006 (unpublished data)). Thus, it seems that *Thioploca* and *Thiomargarita*, which populate OMZs and which rarely experience oxygenated conditions, have a metabolic potential less optimized for the use of oxygen than large marine *Beggiatoa*, which thrive in sediments underlying oxygenated bottom water (for example, Mussman *et al.*, 2003; Preisler *et al.*, 2007; de Beer *et al.*, 2006).

Denitrification by *Thioploca* was not detectable, either by microelectrodes (<0.2 % of the sulphide uptake rates) or by ^{15}N experiments. The ^{15}N -stable isotope experiments showed anammox activity associated with the surrounding sheath and sediment, but not with the *Thioploca* cells. Co-occurrence of anammox bacteria and *Thioploca* may explain earlier reports on formation of N_2 with lower ^{15}N labelling percentage than expected from the added ^{15}N nitrate source (Otte *et al.*, 1999). Anammox bacteria would have produced 14–15 dinitrogen gas from the added labelled nitrate and the unlabelled ammonium produced by *Thioploca*. At the time of those experiments, anammox bacteria were not known to be present in marine environments, and the results were taken as an indication of

minor denitrification by epibiotic denitrifiers, although denitrification by *Thioploca* could not completely be ruled out (Otte *et al.*, 1999). Our present evidence shows that these large marine *Thioploca* only use DNRA as a dissimilatory nitrate reduction pathway. The role of nitrite reduction in the metabolism of *Thioploca* was confirmed by increased carbon fixation when nitrite was supplied (Table 1). Positive taxis towards nitrite has been shown by Zopfi *et al.* (2001).

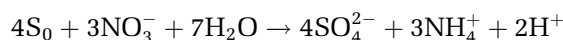
The reduction of nitrate to ammonium rather than to dinitrogen is possibly the more optimal use of the costly nitrate reservoir. More elemental sulphur can be gathered because DNRA transfer eight electrons per nitrate molecule reduced, compared to five by denitrification. The thermodynamic energy gain by DNRA is actually similar to denitrification when expressed per mole nitrate reduced (Jørgensen and Nelson, 2004; Gray and Head, 2005) and in terms of growth yield it may be even higher (Strohm *et al.*, 2007).

To compare the measured rates of sulphide uptake to rates of elemental sulphur oxidation by oxygen or nitrate, it is useful to convert all measured rates into moles of oxidized sulphur. The oxygen uptake was measured in a setup without external supply of sulphide or other electron donors. The consumed oxygen can, therefore, be assumed to be used primarily for the oxidation of stored sulphur:



According to this stoichiometry, the maximum sulphur oxidation by oxygen should be $1170 \mu\text{mol S per dm}^3 \text{ per h}$ (equal to $2.9 \text{ nmol S per min per mg protein}$).

Elemental sulphur is also oxidized by nitrate.



DNRA rates were 1–2 nmol N per min per mg protein when no external sulphide was present. Thus, stored sulphur was oxidized in these experiments and the DNRA rate translates to a sulphur oxidation rate of 1.3–2.6 nmol S per min per mg protein. Therefore, aerobic and anaerobic oxidation of stored sulphur occur at similar rates, which likely reflects the basic metabolic rate in *Thioploca* cells.

The measured potential sulphide uptake rate of $6220 \mu\text{mol S per dm}^3 \text{ per h}$ ($15 \text{ nmol S per min per mg protein}$) was 5–6 times faster than the continuous oxidation rate of stored sulphur. This rate difference shows that, during sulphide oxidation, elemental sulphur accumulates fast compared to the subsequent oxidation of stored elemental sulphur to sulphate. The presence of sulphide in the bulk medium did not induce higher carbon fixation rates in the MAR experiments (Table 1). This indicates that the rapid sulphide uptake is uncoupled from carbon fixation and energy conservation, and displays a mechanism for fast accumulation of elemental sulphur, when high concentrations of sulphide are present.

The use of polyphosphate as a dynamic energy storage, as described for *Thiomargarita* (Schulz and Schulz, 2005), could not be detected in *Thioploca*, neither with toluidine blue staining nor with activity assays with phosphate or the more sensitive $^{33}\text{PO}_4^{3-}$ MAR incubations. For comparison, the latter method would have detected a cellular phosphate content corresponding to 0.1% of the phosphate stored by phosphate accumulating bacteria from wastewater treatment plants (Hesselsøe *et al.*, 2005).

Thioploca physiology: adaptations to ephemeral electron donors and acceptors

Many basic physiological parameters have been quantified in this as well as in other studies (Table 2) and we now see a more complete picture of the *Thioploca* physiology. *Thioploca* is well adapted to a fluctuating environment. This can be seen in the accumulation and transport of nitrate and sulphur, which permit a metabolic strategy independent of the coexistence of electron donor and acceptor (Fossing *et al.*, 1995), and also in the fast rate of sulphur storage.

Thioploca trichomes are usually not observed to protrude from the sheath within the sediment (BB Jørgensen, personal observation), and the uptake of

sulphide occurs while the trichomes are within the sheath. From the maximum sulphide uptake rate it was calculated that an average *Thioploca* bundle needs a minimum of $4\ \mu\text{M}$ sulphide at the bundle surface to saturate the sulphide uptake. This indicates that, in the present environment, *Thioploca* are adapted to long periods with no measurable dissolved sulphide ($<1\ \mu\text{M}$; Thamdrup and Canfield, 1996; Ferdelman *et al.*, 1997) by holding a high capacity to exploit the occasional presence of sulphide concentrations up to $800\ \mu\text{M}$ (Høglund *et al.*, 2008; L Holmkvist *et al.*, in preparation).

Thioploca is a slow-growing mixotrophic bacterium capable of using inorganic carbon as well as acetate and propionate for biomass production (Table 1). This observation is in accordance with previous studies (Maier and Gallardo, 1984b). In the present study MAR also showed that carbon dioxide fixation was enhanced by the presence of external electron acceptors (Table 1), and previous labelling experiments have shown that *Thioploca* cells readily reduce external nitrate without first storing it inside the vacuoles (Otte *et al.*, 1999). This strategy differs from the cellular management of sulphur. We showed that high sulphide concentrations did not increase carbon fixation (Table 1) but induced rapid sulphur storage. This difference in response to

Table 2 Summation of quantitative measures of physiology and behaviour of marine *Thioploca*

	Range	Unit	Reference
Sulphide uptake	0.7–15 ^a	nmol min ⁻¹ per mg protein	Present study; Otte <i>et al.</i> (1999)
Sulphate production	2–3	nmol min ⁻¹ per mg protein	Otte <i>et al.</i> (1999)
Oxygen consumption	0.5 ^a –4.4 ^a	nmol min ⁻¹ per mg protein	Present study
DNRA	1–4	nmol min ⁻¹ per mg protein	Present study; Otte <i>et al.</i> (1999)
Denitrification	<0.02 ^a	nmol min ⁻¹ per mg protein	Present study
Nitrate uptake	5–6.5	nmol min ⁻¹ per mg protein	Present study
Internal nitrate storage	10–500	mM	Otte <i>et al.</i> (1999); Fossing <i>et al.</i> (1995) Zopfi <i>et al.</i> (2001)
<i>Internal sulphur storage</i>			
<i>T. chileae</i>	50–800	mM	
<i>T. araucae</i>	10–355	mM	
SCM	10–800	mM	
Phosphate storage	None		Present study
Doubling time	69–139	days	Otte <i>et al.</i> (1999)
CO ₂ fixation	0.4–1.5	nmol min ⁻¹ per mg protein	Otte <i>et al.</i> (1999); Ferdelman <i>et al.</i> (1997)
Acetate uptake	0.4	nmol min ⁻¹ per mg protein	Otte <i>et al.</i> (1999)
Motility	0.37–14.8	mm h ⁻¹	Huettel <i>et al.</i> (1996)
<i>Negative taxi</i>			
O ₂	Threshold value > 10% air saturation		Huettel <i>et al.</i> (1996)
H ₂ S	> 500 μM		
<i>Positive taxi</i>			
NO ₂	> 4 μM		Zopfi <i>et al.</i> (2001)
NO ₃ ⁻	> 25 μM		Huettel <i>et al.</i> (1996)
H ₂ S	< 100 μM		
Organic substrates	Acetate, amino acids, glucose, glycine, thymidine		Otte <i>et al.</i> (1999); Maier and Gallardo (1984b)

Abbreviations: DNRA, dissimilatory nitrate reduction to ammonium; SCM, short cell morphotype.
^aConversion factor 7 mg protein per cm³.

sulphide and nitrate indicates that sulphide is the limiting substrate for *Thioploca*.

The organization of *Thioploca* trichomes in large bundles enhances the diffusion limitation already imposed by the large cell size. In the case of nitrate, this problem is effectively overcome by stretching the individual trichomes out of the sheaths in the sediment and into the overlying water, thus penetrating the diffusive boundary layer (Huettel *et al.*, 1996). Nitrate uptake can, therefore, occur efficiently even though nitrate is present in low ($\sim 10\ \mu\text{M}$) concentrations. In the case of sulphide uptake, trichomes remain in the sheath and are well adapted to exploit periodically high ($< 200\ \mu\text{M}$) sulphide concentrations following massive organic matter sedimentation. The low substrate affinity of the intact bundle seems to be counterbalanced by the capacity for rapid sulphur storage. This might be their major advantage in the competition with other sulphide consuming organisms and processes in the sediment.

The *Thioploca* may benefit from the sheath in sediments that lack a sulphide gradient to guide trichome movement. Previous studies show that the largely vertical orientated sheaths allow a more directional and, therefore, much more rapid return from the sediment to the nitrate source, than would be possible if the trichomes were moving in a 'random walk' fashion (Schulz *et al.*, 1996). The closely related *Beggiatoa*, which do not have a sheath to give direction, seem to require a sulphide gradient to make the trichomes return from the subsurface sediment to the surface (Preisler *et al.*, 2007).

The behaviour of *Thioploca* is optimized for the periodic availability and spatial separation of electron donor and acceptor. Previous studies have shown that *Thioploca* trichomes have a positive taxis towards nitrate, which has priority over a phobic response to oxygen when nitrate and oxygen are present in the bottom water (Huettel *et al.*, 1996). Interestingly, protruding trichomes reverse the direction of movement just in time to avoid leaving their common sheath (Figure 4). However, the mechanisms that allow the trichomes not to lose contact with the sheath remain to be resolved. Low sulphide concentrations cause a fast positive chemotactic response by *Thioploca* trichomes, but *Thioploca* shows phobic reaction to high sulphide concentrations (Huettel *et al.*, 1996). The return of trichomes from the deep sediment to the surface is not triggered by low vacuolar nitrate concentrations (Zopfi *et al.*, 2001) and it is still unclear whether depletion or saturation of nitrate and sulphur storage control their behaviour.

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