RESEARCH ARTICLE

Distribution, ecology and molecular identification of *Thioploca* from Danish brackish water sediments

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Abstract

The distribution of *Thioploca* populations was investigated in Danish fjords, brackish lakes and coastal waters. *Thioploca* was found in three geographically distinct populations, where biomasses reached 33.8 ± 14.3 g wet weight m^{-2} (mean $\pm$ SD). Mats or lawns were not formed at the sediment surfaces and *Thioploca* biomasses peaked 4–7 cm into the sediment and extended down to 18 cm depth. Morphology and 16S rRNA gene sequences classified all populations as *Thioploca ingrica*. A sequence divergence of 1.7–2.2% indicated that *T. ingrica* comprise at least two genotypes. Physiological analysis showed that *T. ingrica* accumulate nitrate in concentrations of approximately 3 mM and that bicarbonate and acetate are used as a carbon source. The presence of oxygen promoted carbon incorporation, but *T. ingrica* could survive up to 3 months without an external supply of nitrate or oxygen. *Thioploca ingrica* populations were exclusively found close to river outlets in a bioturbated sediment with separate sulphidic spots and worm burrow walls containing nitrate and oxygen. It is hypothesized that the subsurface *T. ingrica* have a special advantage in this heterogeneous environment using their sheath surrounding the bacterial trichomes when navigating between electron donor and acceptor.

Introduction

The aim of microbial ecology has been defined as . . . *finding principles which explain the structure and function of microbial communities* (Fenchel, 1992). Focusing on easily detectable members of a defined microbial community has been a valuable strategy in microbial ecological research (Ward, 2006). An example of this is the studies of the large, shiny white sulphur bacteria *Achromatium*. These studies have demonstrated how niche differentiation promotes diversity, and it has also been possible to experimentally test the importance of natural selection in structuring microbial communities (Gray & Head, 1999; Gray *et al.*, 2004, 2007). Their size and remarkable morphology make these bacteria and other large sulphur bacteria suitable for population studies, because abundance and distributional patterns are assessable by simple light microscopy or even the naked eye. These large bacteria allow experimental manipulation under *in situ* conditions and are therefore useful models to establish links between physiology and natural occurrence (Gray & Head, 2005). This also applies to *Thioploca*, a filamentous sulphur bacterium, which is easy to recognize, as the trichomes (filaments) live in centimetre-long bundles surrounded by a common sheath. The marine *Thioploca* have trichome diameters up to 43 μm and store nitrate in concentrations around 300 mM within the cell (Fossing *et al.*, 1995; Schulz *et al.*, 2000). This nitrate is used in oxidation of sulphide, which is accumulated as elemental sulphur. *Thioploca* trichomes move back and forth between the nitrate source at the sediment surface and the sulphide within the sediment and this commuting is thought to be governed by the sheath (Jørgensen & Gallardo, 1999). The freshwater species *Thioploca ingrica* morphologically resembles the well-characterized marine *Thioploca*, but has a smaller trichome diameter ranging from 2 to 4.5 μm (Wislouch, 1912). Less attention has been paid to this freshwater species and its ecology is poorly studied. It was first found in the Neva river, Russia (Wislouch, 1912), and later in scattered populations in European freshwater habitats (Koppe, 1922; Kolkwitz, 1955; Maier & Preissner, 1979)

and in Lake Erie (Maier & Murray, 1965). More recently, populations in Lake Biwa and Lake Ogawara, Japan (Nishino *et al.*, 1998; Kojima *et al.*, 2007), Lake Ontario, North America (Dermott & Legner, 2002), and Lake Baikal, Russia (Zemskaya *et al.*, 2001, 2009), have been reported.

In surveys of Danish aquatic environments in 1982, we observed *T. ingrica* within one area of 4 km^2 located in the Randers fjord estuary (unpublished data). During the past 27 years, this population has been followed and was molecularly identified in 1996 by Teske *et al.* (1996). The persistence and restricted occurrence of this *T. ingrica* population stimulated an interest in the distinct factors supporting the population, i.e. the parameters defining the ecological niche of *T. ingrica*. Many bacterial population surveys are confined to predefined environmental gradients such as salinity or light, because of the need for time-demanding molecular identification and quantification. *Thioploca*, however, can be mapped over large areas using a stereomicroscope or by simply looking into the sediment. Knowing where this bacterium is found and not found, we can then start searching for environmental parameters defining the bacterial niche.

Here, we describe the distribution of *T. ingrica* in Danish waters and sequence 16S rRNA genes from *T. ingrica* found at tree different locations. We examine the ability of *T. ingrica* to accumulate nitrate and phosphate. Carbon source and electron acceptor utilization are studied by microautoradiography and in anaerobic survival experiments. Cell-bound nitrate is measured in sediment cores with dense populations of *T. ingrica*.

Materials and methods

Sampling and site description

Thioploca ingrica distribution was investigated in fjords, brackish lakes and coastal waters throughout Denmark (Fig. 1). Thirteen sites were visited in October 2003 and 23 sites had been routinely visited during the past few years. Detailed mapping of distributional patterns was carried out from March to September 2003 in Randers fjord and from April to July 2004 in Hjarbæk fjord. Sediment samples for biomass quantification were obtained by push- and kajak-corers, which ensured an undisturbed vertical stratification of the sediment. Sediment for vertical biomass distribution and nitrate measurements was sampled in Hjarbæk fjord in May 2004 at 20 cm water depth next to the rhizosphere of the reed zone. All cores were maintained at *in situ* temperature in aerated water until processing. Samples were processed within 4 days.

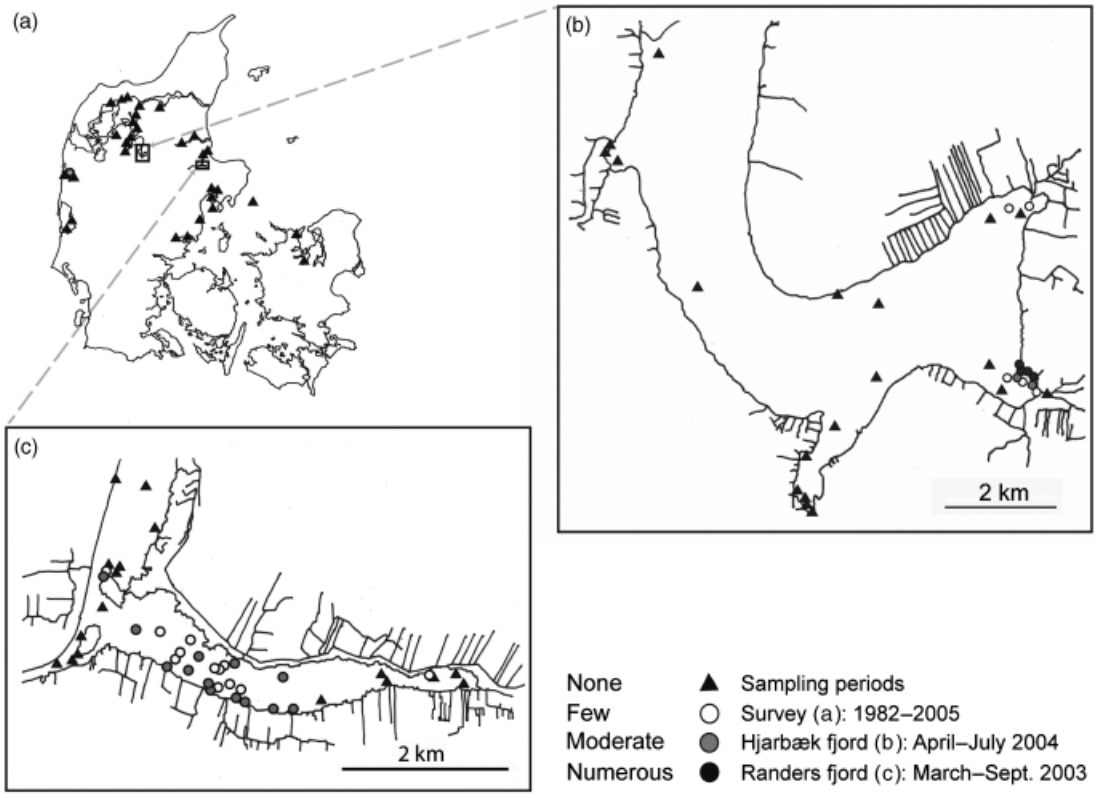

Fig. 1. (a) Locations searched for *Thioploca*. Enlarged sections show *Thioploca ingrica* distribution in Hjarbæk fjord (b) and Randers fjord (c).

PCR amplification, cloning, sequencing and phylogenetic reconstruction

Thioploca bundles for molecular identification were picked from Hjarbæk fjord, Randers fjord and Nissum fjord sediment and rinsed as described previously (Teske *et al.*, 1996). Rinsed bundles were left in sterile water on a microscope slide for 20 min, during which *Thioploca* trichomes would protrude from their sheath. Trichomes from each sampling location were subsequently transferred by sterile glass needles to individual PCR tubes containing 33.5 μL dH_2O. A PCR mixture consisting of 1 μM of the primers 26 F (Hicks *et al.*, 1992) and 1492 R (Lane *et al.*, 1992) (MWG AG Biotech), 2.5 U of Taq DNA polymerase (Sigma-Aldrich Denmark A/S), 12.5 μM of each dNTP (AH Diagnostics), 1 × DNA buffer and 1.5 mM $MgCl_2$ was added to the PCR tubes, forming a total reaction volume of 50 μL. Thermal cycling conditions for PCR amplification were as follows: initial denaturation of 5 min at 94 °C, 34 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 1 min and elongation at 72 °C for 45 s adding 1 s per cycle. The final elongation was 7 min at 72 °C. PCR products from the three locations were purified (QIAquick PCR purification kit; Qiagen), and cloned using the Topo XL cloning kit (Invitrogen). Three clones were picked from each of the three clone libraries and purified using the QIAprep spin miniprep kit (Qiagen) for sequencing with the M13 F vector primer. The nine partial 16S rRNA gene sequences were compared with the closest relative in the RDP database by BLAST searches (Altschul *et al.*, 1990) at the NCBI server (http://www.ncbi.nlm.nih.gov/). All except one sequence, which was chimeric, affiliated with *Thioploca ingrica*. One *Thioploca* sequence was picked from each library for further sequencing using the vector primer M13 R and the universal and eubacterial primers 518 F, 518 R (Muyzer *et al.*, 1993), 907 F, 907 R (Lane *et al.*, 1992), yielding sequences with overlapping sequence information at all positions. All sequencing was carried out using the DYEnamic ET terminator cycle sequence kit (Amersham Biosciences) and reactions were run on an ABI 3100 genetic analyzer sequencer. The 16S rRNA gene sequences were compiled and aligned to previously sequenced large vacuolated sulphur bacteria using the ARB program package (Ludwig *et al.*, 2004). Alignments were refined by manual inspection.

A 50% conservation filter was calculated for all in-group sequences and subsequently manually edited. Evolutionary trees were inferred by neighbour-joining and maximum likelihood algorithms in the ARB program. Bootstrap values were generated by 1000 replicate samplings using the SEQBOOT program. Neighbour-joining distances were calculated using Jukes–Cantor corrections. Bayesian phylogeny was inferred from 16S rRNA gene sequences >1300 nucleotides using MRBAYES v. 3.1.2 (Huelsenbeck & Ronquist, 2001). Partial sequences were subsequently added to this tree using the parsimony interactive tool of the ARB program. All trees showed the same topology of the *T. ingrica* clade and the neighbour-joining tree was chosen for presentation. Out-group sequences were removed after tree construction to improve the clarity of the tree.

The nucleotide sequences have been deposited in GenBank under the accession nos. EU718069, EU718070 and EU718071.

Biomass quantification

Three procedures were used for quantifying *Thioploca*: Split-core inspection, dissection microscopy and microscopic counting, allowing different resolutions and speeds of sample processing.

Split-core inspection was used for the detection and coarse quantification of *Thioploca* in Hjarbæk fjord and in the search for *Thioploca* locations throughout Danish aquatic environments. The amount of white, elastic bundles appearing when the cores were slowly split was observed (Fig. 2) and a none - few < moderate < numerous scale was developed for biomass estimation. Split-core estimates were calibrated by comparison with biomasses determined by dissection microscopy as described below ($n = 31$). Split-core estimates corresponded to

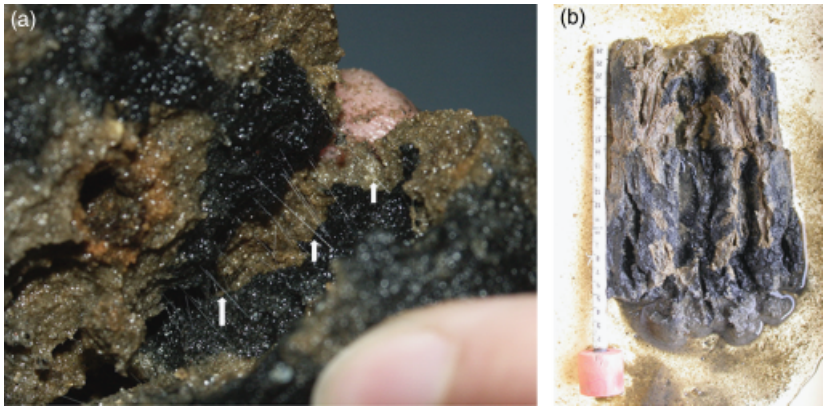

Fig. 2. (a) Split-core inspection, *Thioploca ingrica* visible as white bundles (arrows). (b) Mosaic sediment from a *T. ingrica* location with reduced (black) and oxidized (brown) spots.

the following biomasses: none: 0–0.5 g m^{-2}, few: 0.2–5 g m^{-2}, moderate: 2.5–13 g m^{-2} and numerous: 24–50 g m^{-2}. A sampling point was classified as '*Thioploca* negative' if a thorough split-core examination of at least four sediment cores was negative; this yielded a sensitivity that could detect a population with a density $< 1\%$ of the maximum population densities.

Dissection microscopy was used for horizontal mapping of *Thioploca* abundance in Randers fjord. Sediment from 3 to 6 cm depth was mixed, and a 1 g sediment subsample was transferred to a Petridish, suspended in 2 mL water, distributed evenly over the bottom of the dish and *Thioploca* bundles were counted by dissection microscopy ($\times$ 12 magnification). Biomasses were estimated by multiplying the number of *Thioploca* bundles with the average bundle volume measured by light microscopy (bundle diameter 20 μm, bundle length 0.5 cm). A cell density of 1 g cm^{-3} was used (Jørgensen, 1977), and extrapolation to biomass per area was performed by anticipating that 60% of the population occurred in the 3–6 cm fraction. This assumption was based on visual inspection of intact cores and confirmed by counting of *Thioploca* in 2 cm horizons from 0 to 12 cm depth in five sediment cores.

Light microscopy enumeration was used for detailed vertical biomass profiles. It is difficult to count and measure the length of individual *Thioploca* trichomes that are packed inside a sheath, and the line-intercept method (Newman, 1966; Nedoma *et al.*, 2001) was therefore used excluding trichome length measurements. Three cores were sliced at 1 cm intervals from 1 to 19 cm depth. Each fraction was mixed with 1 mL 24.5% formaldehyde. A subsample of 20–30 mg sediment was placed on a microscope slide and weighed. It was then spread over an area corresponding to a cover slip (1.8 $\times$ 1.8 cm) by applying a few water droplets to the microscope slide. The preparation was examined by light microscopy ($\times$ 100 magnification), going systematically through 10 transects counting intersects with trichomes. If entangled bundles were encountered, $\times$ 250 magnification was used to resolve the number of trichomes crossed by the transects. Biomass per cm^{-3} was calculated using the formula $(((I/2) \times L \times \pi^2 \times r^2)/m) \times \delta$, where I is the number of intersects along the transect, L is the transect length, r is the radius of the trichomes, m is the sample weight and δ is the sediment density (adapted from Newman, 1966).

The average *Thioploca* trichome width was determined by light microscopy ($\times$ 1000 magnification) on 25–27 randomly picked trichomes from each fjord. Macrofauna abundance was determined by sieving sediment cores ($D = 5.4$ cm, $h = 14$ cm) through a 1-mm mesh, and counting the fauna ($n = 3$).

Intracellular nitrate and polyphosphate

Intracellular nitrate was determined on individual *Thioploca* bundles picked from 3 to 17 cm depths in the sediment. Briefly, the bundles were cleaned in nitrate-free artificial seawater (7‰ NaCl) with 300 μM of the nitrification inhibitor thiourea and left to dry on a glass needle. This procedure ruptured the cells and released cell-bound nitrate. There was no need for fixatives or other liquids, which would interfere with the biosensor and dilute released nitrate. The nitrate content was determined using either a NO_x-biosensor with flowcell (Unisense, Aarhus, Denmark) or chemiluminescence detection (Høgslund *et al.*, 2008). Glass needles with sediment aggregates were used as contamination controls.

Staining for polyphosphate was carried out on *Thioploca* bundles picked from 5 and 8 cm depth in Hjarbæk fjord sediment sampled in January 2008. Cells were stained with toluidine blue as described by Schulz & Schulz (2005). Marine *Beggiatoa* from Århus harbour, Denmark, were used as positive controls.

Microautoradiography

To examine autotrophic activity under *in situ* conditions, we incubated a sediment core populated with *T. ingrica* with ^{14}C-bicarbonate-amended pore water. Pore water was extracted anoxically from another sediment core using rhizon soil moisture samplers (Rhizosphere Research Products, Wageningen, the Netherlands) and amended with 8 μCi of ^{14}C-bicarbonate mL^{-1}. To exchange pore water, a grid was inserted at the bottom of the *T. ingrica* sediment core and the ^{14}C-amended pore water was added anoxically to the top of the core. The core was placed in a small water-filled container, allowing the elevation of the water surface determine the hydrostatic pulling force, dragging ^{14}C-amended pore water through the *T. ingrica* sediment core. Incubations were stopped after 11 days by exchanging the pore water with 4% PFA-amended water. *Thioploca* bundles were picked from the cores, washed and left to dry on glass slides.

For incubations with various electron acceptors, *Thioploca* bundles were picked from Hjarbæk fjord sediment held in anaerobic *in situ* water. Approximately 100 bundles were transferred to each serum bottle containing 3 mL anaerobic pore water. Bundles were preincubated for 5 days with either nitrate (1 mM), nitrite (0.5 mM), oxygen (atmospheric concentration) or no external electron acceptor. Experiments were started by adding either 20 μCi of ^{14}C-bicarbonate plus 0.05 mM bicarbonate or 20 μCi ^{14}C-acetate and unlabelled acetate to a final concentration of 1 mM to the bottles. Incubations were stopped by adding PFA to a final concentration of 4% after 2 days of incubation at 10 °C. Application of a film emulsion and developing was carried out as described by Nielsen & Nielsen (2005).

Anaerobic incubations

To test survival without the supply of nitrate and oxygen, 25 mL serum bottles were filled with sediment slurries

containing *Thioploca* from Randers fjord and sealed with rubber stoppers. Nine bottles were incubated at 20 °C in the dark for 1, 2, 5, 12, 20, 29, 54, 81 and 209 days, respectively. When the bottles were opened, 10–14 bundles were randomly picked and inspected by light microscopy.

Sediment nitrate

The depth distribution of pore water nitrate and total nitrate was measured in four parallel sediment cores (Ø = 5.4 cm). Five hours before analysis, water was removed above the sediment surface. This reduced the pore water nitrate concentration, because nitrate was metabolized in the sediment. Cores were sliced into 1 cm horizons to 10 cm depth and 2 cm horizons from 10 to 18 cm depth. Fractions were immediately transferred to centrifuge tubes and centrifuged at 2000 ***g*** for 10 min. From each fraction, 500 μL supernatant was removed and frozen. Centrifuge tubes were then heated to 80 °C to lyse cells, shaken to dissolve released nitrate and centrifuged again. The supernatant was removed and frozen until analysis. Nitrate concentrations were determined using a NO_x-biosensor with flowcell adaptation (Unisense, Denmark).

Results

Taxonomic identification

Trichome diameters ranged from 3 to 4.1, 3 to 4.5 and 2.3 to 4 μm in Randers fjord, Nissum fjord and Hjarbæk fjord populations, respectively. This classifies the *Thioploca* as *Thioploca ingrica* (Wislouch, 1912). The mean trichome diameters were 3.6 ± 0.07 μm ($n = 25$) for Randers fjord, 3.7 ± 0.08 μm ($n = 27$) for Nissum fjord and 3.4 ± 0.08 μm ($n = 26$) (mean ± SEM) for Hjarbæk fjord populations. Trichomes from Nissum fjord were significantly larger than trichomes from Hjarbæk fjord ($P = 0.01$, Student's *t*-test). Divergence between populations was also seen in the molecular phylogeny. Almost full-length 16S rRNA gene sequences with overlapping sequencing at all sites were obtained from the populations in Randers fjord, Nissum fjord and Hjarbæk fjord. All three sequences affiliated with *T. ingrica* within the group of large vacuolated sulphur-oxidizing bacteria (Fig. 3). The sequences obtained from Randers fjord and Nissum fjord populations formed a cluster (99.9% similarity), which was set apart from previously sequenced freshwater *Thioploca* and the sequence obtained from Hjarbæk fjord (distance 2.2–1.7%).

Distribution of *T. ingrica*

Thioploca ingrica populations were found in three separate Danish fjords: Nissum fjord, Hjarbæk fjord and Randers fjord (Fig. 1a). Nissum fjord and Hjarbæk fjord populations were new findings, whereas the Randers fjord population has been known since 1982. The populations were not visible at the sediment surface, but had maximum biomasses 4–7 cm down in the sediment. *Thioploca ingrica* were in all three cases found close to river outlets in sediments having a characteristic mosaic pattern of reduced and oxidized zones throughout the sediment profile (Fig. 2). The salinity was < 9‰ in the fjords, and the rivers adjacent to *T. ingrica* populations contained nitrate all year round, with average concentrations above 100 μM (Danish national environmental database). Texture ranged from gravel (Nissum fjord), fine/medium-sized silt with a high organic content (Randers fjord) to peat and sand with a high organic content (Hjarbæk fjord). No *Beggiatoa* were found at the Hjarbæk

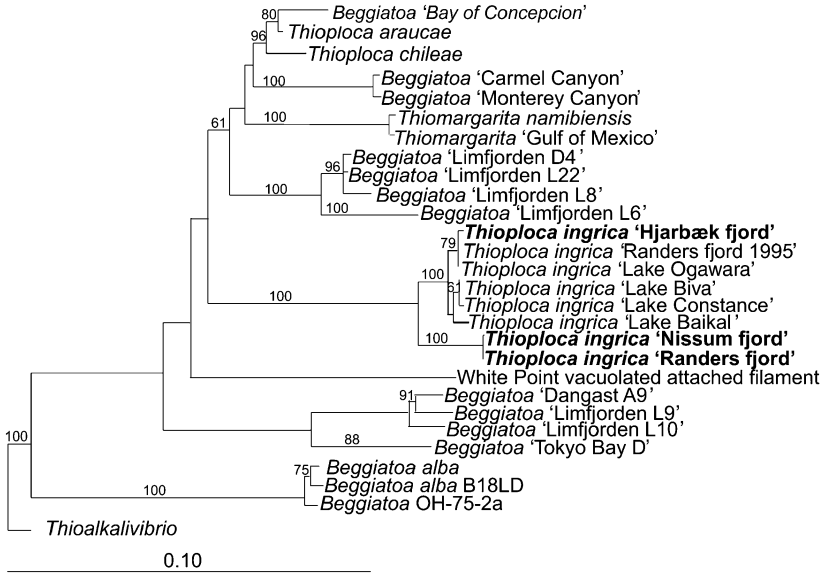

Fig. 3. 16S rRNA gene phylogeny of large vacuolated sulphur bacteria and freshwater *Beggiatoa* inferred by neighbour joining. Scale bar corresponds to 10% sequence divergence. Bootstrap values above 60% are shown on the branches.

fjord and Nissum fjord locations. In Randers fjord, scattered small *Beggiatoa* specimens (trichome diameter $< 3\,\mu m$) were observed.

Rivers, lakes and marine environments away from river outlets did not support *Thioploca* and it was also absent from many fjord locations. A more detailed mapping of *T. ingrica* was carried out in two of the tree fjords: Randers fjord and Hjarbæk fjord. *Thioploca ingrica* in Randers fjord showed a well-defined occurrence within an area of 4×1 km (Fig. 1c). In this area, population densities varied an order of magnitude within 1 m. Maximum biomasses of $13\,g\,m^{-2}$ were found close to reed belts, but outside the rhizosphere. A similar pattern was seen in Hjarbæk fjord by the split-core inspection (Fig. 2b). The Hjarbæk fjord population was located within an area of 1×1 km at the mouth of the southern river outlet. Microscopic enumeration at this site yielded a total biomass of $33.8 \pm 14.3\,g$ wet weight m^{-2} (mean $\pm$ SD) and showed peak biomasses 5–7 cm down in the sediment, with the population extending down to 18 cm depth (Fig. 4). This high-density *T. ingrica* site in Hjarbæk fjord was heavily bioturbated by the polychaete *Hediste diversicolor*, which was present at a density of 2183 ± 504 individuals m^{-2} ($n = 3$) (mean $\pm$ SEM).

Physiological analysis

Out of 35 *T. ingrica* bundles, 25 contained nitrate and had an average nitrate content of 5 ± 8 pmol per bundle (average $\pm$ SD). Bundles picked from the upper centimetres of the sediment column contained the same amount of nitrate as bundles picked from the 15–17-cm-deep horizon. Contamination controls contained 1 ± 0.4 pmol nitrate (average $\pm$ SEM, $n = 5$). This amount was subtracted from all measurements. Using average dimensions of 5-mm-long bundles with five trichomes beside each other, the internal nitrate concentrations were about 3 mM.

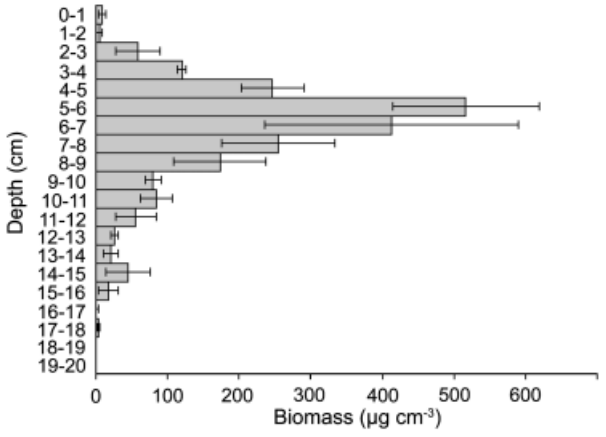

Fig. 4. Vertical *Thioploca ingrica* biomass profile from Hjarbæk fjord. Error bars show SEM ($n = 3$).

Staining with toluidine blue did not indicate any accumulation of polyphosphate granules in *T. ingrica*, whereas stained polyphosphate was clearly seen in control trichomes of marine *Beggiatoa*.

Microautoradiographic incubations in intact sediment cores showed fixation of bicarbonate by *T. ingrica* (Fig. 5) and incubations in serum bottles showed that acetate was also taken up and incorporated into the biomass. Incubations with nitrate and oxygen yielded positive microautoradiography signals, whereas incubations with nitrite and without an external electron acceptor were negative (Table 1).

Thioploca ingrica survived sediment incubations without oxygen or other externally supplied electron acceptors for more than 81 days. A decrease in stored sulphur was not noted until after 54 days. Sulphur storage was clearly reduced after 81 days, but trichomes were still moving. After 209 days, the *T. ingrica* had died and only empty sheaths were present.

Sediment nitrate

Heat treatment of sediment from the high-density *T. ingrica* site in Hjarbæk fjord caused a marked increase in the pore water nitrate concentration (Fig. 6). The increase was most pronounced in the surface sediment, where the nitrate concentration increased from 2 to 98 µM. Pore water nitrate in the heat-treated sediment varied around 15 µM to a depth of 18 cm, whereas nitrate in the untreated sediment disappeared at 3 cm depth.

Discussion

Phylogenetic diversity of *T. ingrica*

The Nissum fjord and the Hjarbæk fjord populations differed both in trichome diameters and in 16S rRNA gene sequences. Trichome diameter, however, does not map onto the molecular phylogeny within the *T. ingrica* cluster. Large *T. ingrica* (i.e. diameter 4.2 µm: Kojima *et al.*, 2003 and 7.4 µm: Zemskaya *et al.*, 2009) affiliate with the small Hjarbæk fjord sequence.

The divergence between the sequence obtained from Randers fjord in this study and the sequence obtained from the same location in 1995 (Teske *et al.*, 1996) suggests a population shift in Randers fjord or the coexistence of different *T. ingrica* genotypes.

The genetic divergence found in this study could theoretically be caused by different 16S rRNA gene operons in the same individuals. Denaturing gradient gel electrophoresis (DGGE) analysis of *T. ingrica* from Lake Biwa and Lake Constance with *Thioploca*-specific primers produced only single DGGE bands (Kojima *et al.*, 2003). This implies that only one type of 16S rRNA gene sequence is found in the

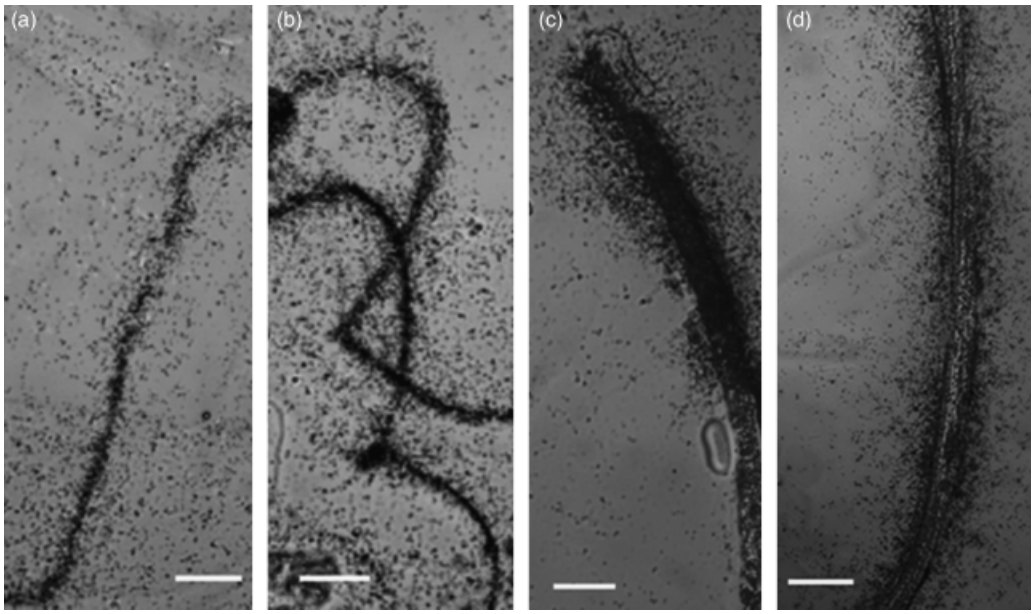

Fig. 5. Microautoradiography signal of *Thioploca ingrica* bundles. (a) Incubation in intact sediment core with ^{14}C-bicarbonate. (b–d) Incubation in serum bottles with ^{14}C-bicarbonate and oxygen, ^{14}C-acetate and oxygen, ^{14}C-acetate and nitrate, respectively.

Table 1. Microautoradiography signals by *Thioploca ingrica* incubated with various electron acceptors and carbon sources

	Acetate	Bicarbonate
Nitrate	+	–
Oxygen	+	+
Nitrite	–	–
None	–	–

T. ingrica genome, and that the divergence observed is due to different *T. ingrica* genotypes. The genetic distance within the cosmopolitan *T. ingrica* clade does not show a geographical pattern and geographic separation seems not to drive diversification within the species.

Thioploca ingrica belong to a phylogenetically cohesive cluster of large vacuolated sulphur bacteria containing large marine *Beggiatoa*, *Thiomargarita*, a *Thiotrix*-like filamentous bacterium and *Thioploca* (Fig. 3). *Thioploca ingrica* occupies a special position within this cluster. It is the only representative from brackish and freshwater environments and some, like the one in this study, lack the characteristic large vacuole in the cell. It has been suggested that the vacuole was lost in the course of adaptation to life outside the marine realm (Ahmad *et al.*, 2006), but the finding of vacuolated *T. ingrica*, which has a 16S rRNA gene sequence similarity of 99.1% to nonvacuolated *T. ingrica*, contradicts this theory and the vacuole may be a more ductile taxonomic trait than previously thought.

Physiological characteristics

Nitrate accumulation by *T. ingrica* from the Danish fjords was shown by measurements on single bundles. The concentrations were similar to those found in freshwater *Thioploca* in Lake Biwa (Kojima *et al.*, 2007), but an order of magnitude lower than in freshwater populations from Lake Baikal (Zemskaya *et al.*, 2001) and in other nitrate-accumulating sulphur-oxidizing bacteria (Fossing *et al.*, 1995; Mchatton *et al.*, 1996; Schulz *et al.*, 1999). Ultrastructural descriptions of *T. ingrica* show small separate or no vacuoles (Maier & Murray, 1965; Kojima *et al.*, 2003), and the *T. ingrica* from the Danish fjords resemble these by lacking a large central vacuole. High ($>$ 100 mM) intracellular nitrate concentrations are therefore only found in the vacuolated sulphur oxidizers such as *T. ingrica* from Lake Baikal (Zemskaya *et al.*, 2009) and the closely related marine *Thioploca*, *Beggiatoa* and *Thiomargarita* (Gallardo, 1977; McHatton *et al.*, 1996; Schulz *et al.*, 1999). It is therefore tempting to speculate that the ability to store nitrate in large quantities is linked to vacuoles having higher nitrate concentrations than the surrounding cytoplasm. Other physiological functions may also be facilitated by the vacuole because the related vacuolated *Thiotrix*-like filaments lack the ability to store nitrate (Kalanetra *et al.*, 2004).

The nitrate storage by *T. ingrica* could only explain about 5% of the intracellular nitrate found throughout sediment columns from Hjarbæk fjord, and the distribution of nitrate

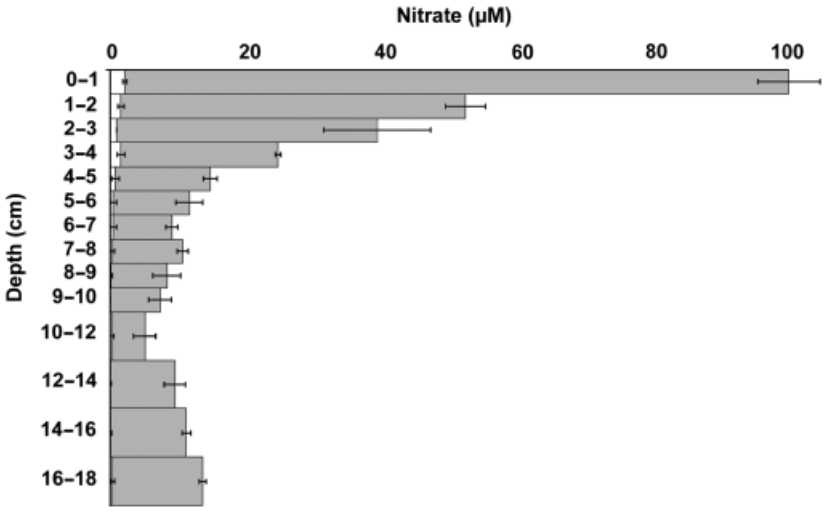

Fig. 6. Depth distribution of nitrate pools in *Thioploca ingrica*-populated sediment. White and grey bars show the pore water nitrate concentration before and after the extraction of cellular nitrate.

in the sediment did not correspond to the *T. ingrica* biomass distribution (Figs 4 and 6). It has been known for a while that microalgae accumulate nitrate (Lomstein *et al.*, 1990) and also benthic foraminifera are capable of nitrate storage (Risgaard-Petersen *et al.*, 2006). Buried microalgae may perhaps account for most of the cell-bound nitrate because living diatoms can be recovered from half a metre below sediment surfaces (Jewson *et al.*, 2006) and only a few living foraminiferal individuals could be found at the site.

Anticipating a biomass-specific nitrate reduction rate similar to the marine *Thioploca* (Otte *et al.*, 1999), *T. ingrica* could deplete the nitrate storage in 1 day. It therefore seems likely that stored nitrate did not support the two and a half months of survival by *T. ingrica* in anoxic slurry incubations. Oxidation of organic matter with the stored elemental sulphur might be a possibility. This is known to occur in *Beggiatoa* (Nelson & Castenholz, 1981), and the observed reduction in stored sulphur over time in *T. ingrica* could indicate sulphur reduction.

Microautoradiography on *T. ingrica* incubated with ^{14}C-bicarbonate in intact sediment cores showed, contrary to recent findings (Kojima *et al.*, 2007), that *T. ingrica* is autotrophic like marine *Thioploca* species (Otte *et al.*, 1999). Both bicarbonate and acetate incorporations were seen in serum bottle incubations with oxygen, showing that *T. ingrica* live mixotrophically and have an aerobic metabolism. This has been confirmed by measuring the oxygen uptake by *T. ingrica* using microelectrodes (L. P. Nielsen, unpublished data). *Thioploca ingrica* also took up acetate in anoxic incubations with nitrate. Thus, *T. ingrica* incorporate carbon under oxic conditions as well as under anoxic conditions when having access to nitrate like the marine *Thioploca* (Høgslund *et al.*, 2009).

Staining with toluidine blue supported the findings of Maier & Murray (1965) that phosphate storage is present in *Beggiatoa* cells, but not in *T. ingrica*. Polyphosphate storage was also not detectable in marine *Thioploca* (Høgslund *et al.*, 2009). Hence, the phosphate storage is not a unifying trait of the group of large nitrate-accumulating sulphur bacteria, even though it has also been found in the *Thiomargarita* genus (Schulz & Schulz, 2005).

Distribution and ecological niche of subsurface *T. ingrica* populations

Thioploca ingrica population densities reached 13 g m^{-2} in Randers fjord and 50 g m^{-2} in Hjarbæk fjord. These high biomasses are similar to the dense *Beggiatoa* populations found in Limfjorden, Denmark (Jørgensen, 1977; Mussmann *et al.*, 2003), and even higher in terms of active cytoplasm because marine *Beggiatoa* cells are occupied by large vacuoles.

Thioploca ingrica did not form populations at the sediment surface, but were generally found below a 3-cm-thick light-brown sediment layer with biomass peaks 4–7 cm into the sediment. Recent reports on freshwater *Thioploca* describe surface populations similar to the lawns of marine *Thioploca* off the Chilean coast (Nishino *et al.*, 1998; Zemskaya *et al.*, 2001; Dermott & Legner, 2002), whereas early reports on populations from Europe do not mention these conspicuous lawns (Wislouch, 1912; Koppe, 1922; Kolkwitz, 1955; Perfilev, 1965; Maier & Preissner, 1979). These two distinct distributional patterns are likely controlled by environmental factors because the *T. ingrica* lawns from Japanese lakes and Lake Baikal are genetically similar to the populations having peak biomasses within the sediment (Fig. 3).

All three *T. ingrica* populations were confined to small areas (< 4 km^2) and it therefore seems that these areas had special environmental characteristics that are prerequisites

for the occurrence of *T. ingrica*. Sediment texture is known to shape *Beggiatoa* communities (Jørgensen, 1977), but *T. ingrica* did not show specific texture requirements. It seemed that the river outlets provided the right nutritional inflow with a year-round supply of nitrate and organic matter. The supply of electron donor and acceptor could, however, not explain the distribution alone, because *T. ingrica* was absent from many sediments where sulphide and nitrate are available. The simplest explanation for the observed horizontal and vertical biomass distribution is that *T. ingrica* occupy a niche where nitrate and sulphide are physically separate, allowing *T. ingrica* to take advantage of their sheath, motility and dual storage capacity.

Microelectrode measurements show that nitrate is available in a radius of 2 mm around burrows of *H. diversicolor* in sediment similar to that found at the Hjarbæk fjord site (Nielsen *et al.*, 2004). Because *T. ingrica* was found far from the nitrate source at the sediment surface and because specimens contained nitrate even below 13 cm depth, it seems likely that these *Thioploca* do not rely on migration between horizontal zones of nitrate and sulphide such as *Beggiatoa* (Mussmann *et al.*, 2003), but take up nitrate around worm burrows that frequently extended to a depth of 18 cm (Fig. 2).

The subsurface biomass peaks may correspond to a zone where a sufficient rate of sulphate reduction, combined with low amounts of oxidized metals, created a niche with appropriate amounts of free sulphide. Sulphide produced by sulphate reduction is rapidly immobilized or oxidized in the presence of reactive metals (Jørgensen & Nelson, 2004). Therefore, almost no *T. ingrica* were found in the light brown upper sediment, which was oxidized through sediment resuspension, bioirrigation and bioturbation. The distribution of *T. ingrica* in depth was probably bound by the density of worm burrows supplying nitrate and oxygen. *Thioploca ingrica* is a poor competitor for organic substrates compared with free-living bacteria. The main competence of *T. ingrica* is to store and move an electron acceptor and donor. Their distribution therefore likely follows sulphur and nitrate prevalence.

The distance between hotspots of free H_2S and the nitrate source should be about 1 cm if *T. ingrica* should take advantage of the sheath in directional migration similar to the marine *Thioploca*. Such a heterogeneous distribution of reduced and oxidized zones was evident in sediment populated by *T. ingrica* (Fig. 2), but maintenance of this heterogeneity must occur by an intermediate disturbance level. Frequent relocation would require high investments in rebuilding of sheath connections between sulphide and the electron acceptor, in particular because the first connection cannot be guided by chemical gradients and are likely established by trial and error. *Thioploca ingrica* therefore seems to be dependent on the intensity and type of bioturbation. Respiration with stored sulphur would allow prolongation of motility and continuation of energy metabolism after depletion of nitrate storage. However, being a poor competitor for carbon, the *T. ingrica* most likely do not grow under these conditions.

It is suggested that high populations of *H. diversicolor* create a niche with intermediate disturbance (Kristensen, 2001), supplying nitrate and oxygen to sediment layers with reduced sulphidic spots. Without gradients to guide chemotaxis in this heterogeneous three-dimensional system, a high degree of controlled directional movement is required. The problem seems solved by the sheath, connecting sources of electron donors and acceptors. The free-living *Beggiatoa* trichomes, contrary to *T. ingrica*, occupy a vertically stratified system between the electron acceptor at the sediment surface and sulphide within the sediment. There, random walk is sufficiently effective to trace the chemical gradients (Preisler *et al.*, 2007). This could explain why no *Beggiatoa* are found at the *T. ingrica* sites. The sheath might be a prerequisite for thriving as a nitrate-accumulating sulphide oxidizer within a three-dimensional worm burrow system.

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