RESEARCH ARTICLE

Acetate-utilizing bacteria at an oxic–anoxic interface in the Baltic Sea

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Abstract

Pelagic redoxclines represent chemical gradients of elevated microbial activities. While chemolithoautotrophic microorganisms in these systems are well known as catalysts of major biogeochemical cycles, comparable knowledge on heterotrophic organisms is scarce. Thus, in this study, identity and biogeochemical involvement of active heterotrophs were investigated in stimulation experiments and activity measurements based on samples collected from pelagic redoxclines of the central Baltic Sea in 2005 and 2009. In the 2009 samples, ^{13}C-acetate 16S rRNA stable isotope probing (16S rRNA-SIP) identified gammaproteobacteria affiliated with *Colwellia* sp. and *Neptunomonas* sp. in addition to epsilonproteobacteria related to *Arcobacter* spp. as active heterotrophs at the oxic–anoxic interface layer. Incubations from sulfidic waters were dominated by two phylogenetic subgroups of *Arcobacter*. In the 2005 samples, organics, manganese(IV), and iron(III) were added to the sulfidic waters, followed by the determination of metal reduction and identification of the stimulated organisms. Here, the same *Arcobacter* and *Colwellia* subgroups were stimulated as in 2009, with *Arcobacter* predominating in samples, in which manganese(IV) reduction was highest. Our results offer new insights into the heterotrophic bacterial assemblage of Baltic Sea pelagic redoxclines and suggest *Arcobacter* spp. as a heterotroph with presumed relevance also for manganese cycling.

Introduction

In the deep basins of the central Baltic Sea, pelagic redoxclines are chemical transition zones between oxygenated and anoxic, sulfidic water masses encompassed by the close proximity of alternating electron acceptors and donors. In addition to redoxclines in sediments or biofilms, pelagic redox gradients typically cover several meters having significant impact on biogeochemical cycles. The energetic exploitation of these gradients by chemotrophs results in an elevated microbial abundance and activity. In the Gotland Deep and Landsort Deep, the specific redox conditions of the suboxic zone, the oxic–anoxic interface layer, and the sulfidic zone support assemblages of key organisms linked to autotrophy. Among these are the thaumarchaeotal subcluster GD2 (Labrenz *et al.*, 2010), Gammaproteobacteria related to the SUP05 group (Glaubitz *et al.*, 2013), and the epsilonproteobacterial *Sulfurimonas* subgroup GD17 (Grote *et al.*, 2007), each of which can make up to one-third of the total cell counts in their respective zones. Moreover, the fixation of inorganic carbon in pelagic redoxclines of, for example, the Cariaco Basin (Taylor *et al.*, 2001), the Baltic Sea (Labrenz *et al.*, 2005), Black Sea (Pimenov & Neretin, 2006; Grote *et al.*, 2008), or in fjords (Zopfi *et al.*, 2001) can contribute significantly to the input of new organic carbon fueling secondary microbial food webs (Taylor *et al.*, 2001; Glaubitz *et al.*, 2009) in these systems. In addition to downward fluxes of particulate organic carbon, both significant grazing by protists (Orsi *et al.*, 2011) and viral lysis (Taylor *et al.*, 2003, Anderson *et al.*, 2012) contribute to the supply of dissolved organic matter, thereby providing valuable sources of energy and carbon to heterotrophs in such habitats.

The uptake of acetate into biomass can be used as an indicator of organic matter utilization, and studies in the

Cariaco Basin have shown an increase in acetate uptake at the oxic–anoxic interface (Taylor *et al.*, 2001; Ho *et al.*, 2002). When coupled to energy conservation via oxidation, besides aerobic respiration, the highest energy yields are provided by denitrification and manganese reduction (Ho *et al.*, 2004). In the pelagic redoxclines of the Baltic Sea, particulate manganese oxides often occur in association with an organic matrix (Neretin *et al.*, 2003), thus serving as a potential source of carbon and as a suitable terminal electron acceptor. Therefore, the utilization of organic compounds is potentially driven by exploitation of inorganic electron acceptors. As Neretin *et al.* (2003) emphasized, manganese(IV) reduction is likely to be coupled to the microbial oxidation of organic compounds that are either present on the particles themselves or supplied by microbial degradation processes or grazing. Manganese reduction by members of *Arcobacter* was reported already in manganese oxide–rich sediments, for example, in the Skagerrak (Vandieken *et al.*, 2012) and the Black Sea (Thamdrup *et al.*, 2000). In contrast, for Baltic Sea pelagic redoxclines, the role and identity of heterotrophic microorganisms and their role in biogeochemical cycles are unknown although presumably relevant in this habitat.

To identify accordant organisms, we sampled Baltic Sea redoxclines and performed incubation experiments, activity measurements, and molecular analyses. Addition of a mixture of organic compounds to Gotland Deep water promoted activity of heterotrophs, while manganese(IV) or iron(III) supplementation served to elucidate potential alternative electron acceptors. From the Landsort Deep redoxcline, active bacteria were identified via ^{13}C-acetate 16S rRNA stable isotope probing complemented by acetate uptake measurements. Phylogenetic analysis of sequences from both sites facilitated identification of heterotrophic bacteria with a potential role in the cycling of manganese.

Materials and methods

Study sites and sampling procedures

Samples were collected from redoxclines in the anoxic basins of the central Baltic Sea, specifically, from the Landsort Deep in 2009 and the Gotland Deep in 2005. Water was retrieved via free-flow bottles attached to a conductivity–temperature–depth (CTD) probe that also recorded turbidity. The Landsort Deep pelagic redoxcline (station 284: 57°19.2′N, 20°03.0′E) was sampled in three characteristic depths with distinct redox conditions: the suboxic zone (78 m), the oxic–anoxic interface layer (86 m), and the sulfidic zone (110 m). The sulfidic zone of the Gotland Deep redoxcline (station 271: 58°34.88′N, 18°14.11′E) was sampled at a depth of 160 m. Concentrations of oxygen, nitrate, nitrite, ammonium, and hydrogen sulfide were analyzed according to Grasshoff *et al.* (1983) on board the research vessel directly after sampling.

Landsort Deep redoxcline 2009: ^{13}C-acetate incorporation and 16S rRNA SIP analyses

To identify acetate-incorporating prokaryotes present in pelagic redoxclines of the Landsort Deep in 2009, a ^{13}C incorporation assay was carried out as described in Glaubitz *et al.* (2009). Water retrieved from the suboxic zone, the oxic–anoxic interface layer, and the sulfidic zone was carefully filled into 1-L glass bottles with overflow, closed gas-tight with polytetrafluoroethylene (PTFE) septum stoppers, and then supplemented with ^{12}C- or ^{13}C-acetate (1,2-^{13}C2-sodium acetate, 99%, Eurisotop, France) to a final concentration of 100 μM. The bottles were incubated for 72 h in the dark at the *in situ* temperature. Cells were harvested via filtration onto 0.2-μm polycarbonate filters (47 mm) (GE Water & Process Technologies). The filters were shock-frozen at −196 °C and stored at −80 °C until further processing. Subsampling for flow cytometric enumeration of total prokaryotic cell numbers was conducted as described by Gasol *et al.* (2004) and Labrenz *et al.* (2007).

RNA was extracted with an acidic extraction protocol as described by Glaubitz *et al.* (2009). Residual DNA was removed with DNase I (Ambion) and the RNA was purified using phenol/chloroform. A maximum of 500 ng RNA was loaded in a cesium trifluoroacetate density gradient (illustra CsTFA, GE Healthcare Lifesciences) and then subjected to isopycnic centrifugation for at least 68 h under vacuum at 111 544 rcf in a Beckman Coulter Ultima L-100 Xp centrifuge with a VTi 65.2 vertical rotor. The RNA was thereby separated according to its buoyant density, in turn a function of the amount of ^{13}C labeling. The resulting gradient was collected in 14 fractions of equal volumes, and the RNA within each fraction was purified.

Bacterial or archaeal 16S rRNA copy numbers within the gradient fractions were quantified in a one-step reverse transcriptional quantitative polymerase chain reaction (RT-qPCR) using the Access One-Step-RT-PCR kit (Promega). ^{13}C incorporation into 16S rRNA during the incubation was assessed by comparing the buoyant densities of the ^{12}C and ^{13}C gradients yielding the maximum 16S rRNA copy numbers.

The PCR assay mixture contained 1 mmol $MgSO_4$ L^{-1}, 0.1 mmol of each dNTP L^{-1}, 0.2 μg BSA μL^{-1}, 0.1× SybrGreen™, 100 μmol fluorescein L^{-1}, 0.26 μmol of each primer L^{-1} (*Bacteria*: Ba519f/Ba907r, Stubner (2002); *Archaea*: Ar109f/Ar912rt, Lueders & Friedrich

(2003), see Supporting Information, Table S1), and 1.5 units of both AMV reverse transcriptase and *Tfl* DNA polymerase. Thermal cycling conditions were as follows: 30 min at 45 °C, 5 min at 95 °C, and 35 cycles of 95 °C for 30 s, 52 °C for 30 s, and 84 °C for 10 s fluorescence measurement, with a final elongation at 68 °C for 5 min. After amplification, a melting curve to exclude unspecific amplicons from the analysis was generated as follows: 1 min at 95 °C, 30 s at 50 °C, and 10 s at 50 °C with +0.5 °C intervals of increasing temperature for 84 repeats.

For single-strand conformation polymorphism (SSCP) fingerprinting analysis, RNA of the gradient fractions was reverse-transcribed and amplified using the same procedure in 50-μL volumes but with a phosphorylated reverse primer; thermal cycling conditions were 45 °C for 30 min, 95 °C for 5 min, and 35 cycles of 95 °C for 1 min, 50 °C for 1 min, and 68 °C for 1 min with a 5-min final elongation at 68 °C. SSCP electrophoresis was carried out according to Schwieger & Tebbe (1998), and the gel was silver-stained as described in Lee *et al.* (1996). Gels were digitalized and relative band intensities quantified based on densitometric curves using Applied Maths GelCompar v 4.5. Selected bands were excised and reamplified according to Labrenz *et al.* (2005). PCR products were purified using the NucleoSpin Extract II kit (Macherey-Nagel) and sequenced by Qiagen (Hilden, Germany) with forward and reverse primers. The excised and eluted bands of interest were reamplified via touch-down PCR (Don *et al.*, 1991) using the primers com1f and com2r (Schwieger & Tebbe, 1998) and the following thermal cycling conditions were as follows: 95 °C for 3 min, 25 cycles of 94 °C for 1 min, initially at 53 °C for 1 min and 72 °C for 1:30 min but then lowering the annealing temperature by 0.1 °C with each cycle; final elongation was carried out at 72 °C for 5 min.

Alongside with the ^{13}C incubations, the incorporation rates of ^{14}C-labeled sodium bicarbonate or ^{3}H-labeled acetate were determined for the same depths in separate individual vials following the methods of Jost *et al.* (2008) and Jost & Pollehne (1998). From the CTD, water was transferred directly into 10-mL test tubes with glass stoppers (OMNILAB). Labeled sodium bicarbonate (40–60 μL) or acetate (25 μL; final concentration 28.5 nM) was added with a gas-tight syringe (Hamilton) from an anoxic stock solution (9.25 MBq mL^{-1} for sodium bicarbonate, 8.51 MBq mL^{-1} for acetate). The samples were incubated at the *in situ* temperature (6–10 °C) in the dark: the bicarbonate incubations for 24 h, while the acetate incubations varied between 22 h and 29 h. Negative controls were stopped with 100 μL formaldehyde (37%) 10 min prior to substrate addition and subsequently treated as described above. The incubation period was terminated by adding 100 μL formaldehyde. To determine the total activity added, 50 μL was withdrawn prior to filtration and merged with 50 μL of ethanolamine and 5 mL of scintillation cocktail (UltimaGold XR). The remaining volume was filtered onto 0.2-μm cellulose acetate membrane filters (25 mm), which were exposed to HCl fumes for 30 min (sodium bicarbonate incubations only) and subsequently mixed with scintillation cocktail for counting in a scintillation counter (TriCarb 2560 Tr/X). For acetate, incorporation rates are expressed as percentage of the recovered radioactivity in biomass compared with the added radioactivity because the *in situ* concentrations of acetate were unknown. CO_2 incorporation was calculated based on a total dissolved inorganic carbon concentration of 2 mM, which is the concentration typically found for Baltic Sea pelagic redoxclines (Grote *et al.*, 2008).

Gotland Deep redoxcline 2005: growth on organic carbon and metal oxides as electron acceptors

For the sulfidic zone samples of the Gotland Deep, obtained in 2005, heterotrophic and metal-oxide-reducing bacteria were stimulated with a mixture of organic substrates (Table S2), manganese(IV) (Merck), or iron (III) (AppliChem). These were added in different combinations to the sample water to final concentrations of 4 μM (each organic substrate) and 100 μM (metal oxides). The samples and controls (no additions) were incubated at the *in situ* temperature in the dark for 48 and 96 h until subsampling and harvesting of the cells as described above.

Manganese concentrations were determined as described in Labrenz *et al.* (2005) using the formaldoxime method (Brewer & Spencer 1971). Dissolved iron(II) concentrations were determined photometrically using ferrozine according to the method described by Stookey (1970). For rinsing the filter, 10-mL sample were filtered through a 0.4-μm polycarbonate filter and the filtrate was then discarded. Afterward, 20 mL of sample water was filtered, followed by the addition of ferrozine, incubation in the dark, and the measurement of absorbance at 562 nm against a ddH_2O blank. $FeCl_2$ standards between 0 and 50 μM were prepared anoxically in preboiled ddH_2O, which was purged with N_2 while the solution cooled to room temperature. Particulate iron(III) concentrations were calculated by subtracting the concentrations of the filtered from the unfiltered fraction.

RNA and DNA were extracted according to Weinbauer *et al.* (2002), followed by the digestion of co-precipitated DNA with DNAse I (Ambion). Then, cDNA was synthesized from RNA using the iScript cDNA synthesis kit (Bio-Rad) as described in Labrenz *et al.* (2005) and the universal primer 1492r (Lane,

1991). The reaction started at 25 °C for 5 min, followed by reverse transcription at 42 °C for 30 min and terminal elongation at 85 °C for 5 min. Amplification of the cDNA with the primers com1f/com2rpH (Schwieger & Tebbe, 1998) and gel electrophoresis were carried out as described above.

Phylogenetic analysis

Sequencing data were quality-revised with DNAStar SeqMan II v5.06, and forward and reverse sequences were assembled into a combined contig. Sequences were aligned using the ARB 5.1 software package (Ludwig *et al.*, 2004), and phylogenetic trees were reconstructed based on the PHYML maximum-likelihood (ML), neighbor joining (NJ), and maximum parsimony (MP) algorithms with a specific filter. 16S rRNA partial sequences of the excised fingerprinting bands were integrated into the constructed tree using the quick add marked function in ARB.

Results

Chemical characterization of the Landsort Deep and Gotland Deep redoxclines

In 2009, the Landsort Deep redoxcline was located between 70 and 100 m depth (Fig. 1). The three sampling depths covered the suboxic zone, the oxic–anoxic interface layer, and the sulfidic zone. The turbidity maximum was found as expected (Neretin *et al.*, 2003), at the oxic–anoxic interface layer. Oxygen was not measurable at depths below 82 m, whereas hydrogen sulfide first appeared at 78 m. Thus, these chemical compounds slightly overlapped at the oxic–anoxic transition zone. Nitrate peaked at 70 m in the upper suboxic zone and decreased to the detection limit at 80 m. Nitrite also peaked within the suboxic zone, but below the nitrate maximum. Ammonium overlapped with oxygen in the suboxic zone and further increased with depth. Dark CO_2 fixation rates (Table 1) increased with depth, reaching the highest rate of 1 μmol C L^{-1} day^{-1} in the sulfidic zone.

The sulfidic zone of the Gotland Deep redoxcline in 2005 was located below 150 m, where sulfide was already detected together with low concentrations of oxygen (Fig. 2). Due to a major inflow of North Sea water into the Baltic Sea from 2002 to 2003 (Hannig *et al.*, 2007), oxygen did not disappear until depths between 150 and 165 m. The detection limits for O_2 and H_2S are 2 and 0.2 μM, respectively.

Landsort Deep redoxcline 2009: ^{13}C-acetate-incorporating heterotrophs

For all Landsort Deep incubations with acetate, total cell numbers increased within 72 h (Table 1), with the incubation originating from the sulfidic zone yielding the highest cell numbers among the three depths. Radiolabeled acetate was incorporated by the bulk microbial community at all three sampled depths (Table 1), whereas uptake was maximal at the oxic–anoxic interface layer followed by the sulfidic zone. Comparatively low uptake was determined in water from the suboxic zone. Additionally, RNA-SIP analysis confirmed the microbial incorporation of acetate into biomass via 16S rRNA RT-qPCR, using the primers com1f/com2r for all three depths. This was indicated by a shift in the peak of the copy numbers toward fractions of higher buoyant density obtained from the 16S rRNA extracted from the ^{13}C-acetate incubations (Fig. S1). For the suboxic zone incubation, incorporation into archaeal 16S rRNA was also assessed using the primers Ar109f and Ar912rt, but a shift was not detected. Notably, copy numbers were significantly lower for archaea than for bacteria (Table S3). Subsequent bacterial 16S rRNA based fingerprint gels revealed that the composition of the microbial assemblages changed strongly during the 72-h incubations with acetate, compared with the control before amendment and incubation (Fig. 3). This

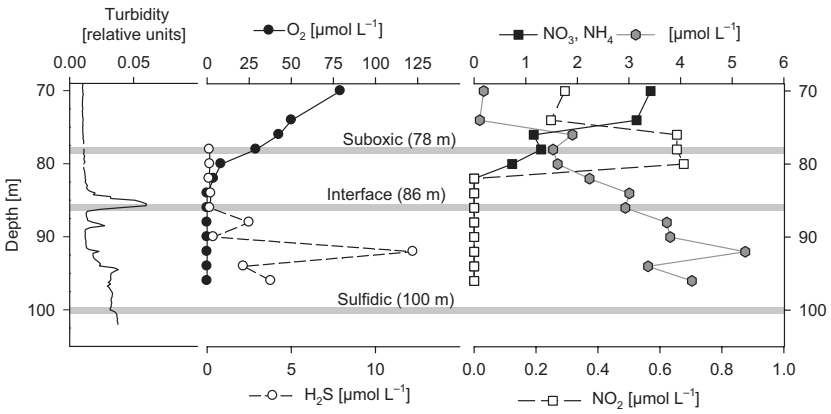

Fig. 1. Physicochemical structure of the Landsort Deep redoxcline in October 2009, showing turbidity (left panel), oxygen and hydrogen sulfide (middle), and ammonium, nitrate, and nitrite concentrations (right). The three sampled depths used for the RNA-SIP incubation experiment are marked by gray lines.

Table 1. Total ^{14}C bicarbonate and ^{3}H acetate incorporation rates and total cell numbers before (0 h) and after (72 h) incubation with ^{12}C- and ^{13}C-acetate in water retrieved from the suboxic zone, the oxic–anoxic interface layer, and the sulfidic zone of the Landsort Deep in 2009. For the 72 h ^{12}C- and ^{13}C-incubations, duplicate subsamples were analyzed; in parentheses, the average of pooled ^{12}C- and ^{13}C-values is given with standard deviation (SD)

	CO_2-fixation (μM day^{-1})	Acetate uptake (% of added)	Total cell numbers (cells mL^{-1})		
			0 h (± SD)	72 h ^{12}C (average ± SD)	72 h ^{13}C
Suboxic zone	0.1 ± 0.0	0.8 ± 0.1	0.44×10^6 ($\pm 3.02 \times 10^4$)	1.33×10^6 1.52×10^6 ($1.33 \times 10^6 \pm 1.39 \times 10^5$)	1.19×10^6 1.28×10^6
Interface layer	0.5 ± 0.1	14.2 ± 1.2	1.13×10^6 ($\pm 9.17 \times 10^4$)	1.67×10^6 1.24×10^6 ($1.35 \times 10^6 \pm 2.17 \times 10^5$)	1.25×10^6 1.22×10^6
Sulfidic zone	1.0 ± 0.5	4.7 ± 3.3	1.12×10^6 ($\pm 5.01 \times 10^4$)	2.50×10^6 2.67×10^6 ($2.58 \times 10^6 \pm 1.10 \times 10^5$)	2.47×10^6 2.68×10^6

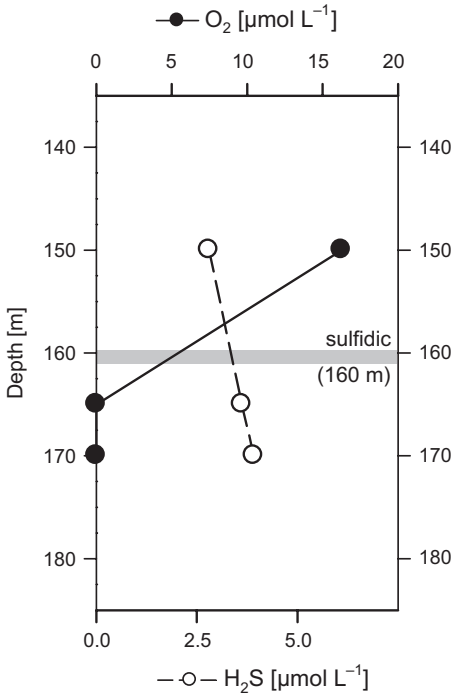

Fig. 2. Depth profile for oxygen and hydrogen sulfide around the redoxcline of the Gotland Deep in 2005. The gray line indicates the depth used for both sampling and subsequent stimulation experiments with organic compounds, manganese(IV), and iron(III).

feature was characteristic for all three depths; however, ^{13}C-enriched bands were detected only in the incubations from the oxic–anoxic interface layer and the sulfidic zone. Specifically, bands 6–13 showed enrichment in the ^{13}C-gel compared with the ^{12}C-gel based on both visual comparison and densitometric curves (Fig. S2).

Sequencing of the respective bands revealed members affiliated with the *Gamma*- and *Epsilonproteobacteria* as actively acetate-incorporating bacteria (Table 2; Fig. 4). Acetate incorporation into 16S rRNA by both of these groups was restricted to the incubation from the oxic–anoxic interface layer, whereas in the incubation from the sulfidic zone, only members affiliated with the epsilonproteobacterial *Arcobacter* sp. were identified as acetate utilizers. Although the suboxic zone incubation was dominated by members affiliated with *Arcobacter* sp., incorporation of ^{13}C label was not detectable for the respective bands (Fig. 3).

Gotland Deep sulfidic zone 2005: growth on organic carbon and metal oxides as electron acceptors

During the incubation period of 48 and 96 h, total cell numbers increased in all four setups containing substrate additions (Table 3). The most pronounced increase was detected in those amended solely with organic substrates or organic substrates and manganese(IV). In contrast, total cell numbers decreased within 48 h in the setups amended with organic substrates and iron(III) or organic substrates and iron(III) and manganese(IV).

A similar trend was reflected in the amounts of reduced manganese(IV) and iron(III) (Table 3). Within 48 and 96 h of incubation in the presence of organic substrates, 17.3 and 22.3 μM manganese(IV) were reduced, respectively. If iron(III) was added as well, within 48 h, manganese(IV) reduction was not detectable or, within 96 h, it was much less pronounced (with 6.9 μM). In general, iron(III) was reduced to a much lesser extent than manganese(IV).

During the incubations, members of the *Gamma*- and *Epsilonproteobacteria* were stimulated, based on the results of 16S rRNA based SSCP fingerprinting analysis (Fig. 5). As expected, the chemoautotrophic epsilonproteobacterial

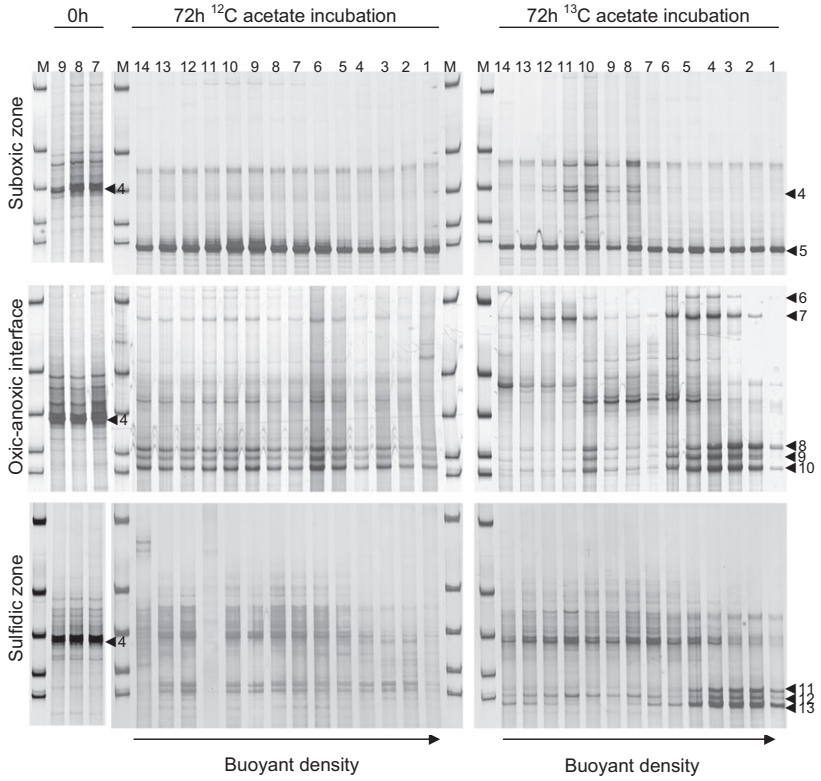

Fig. 3. Bacterial 16S rRNA based SSCP fingerprints from the three depths of the Landsort Deep redoxcline in 2009 as determined based on full CsTFA density-resolved gradients (fractions 1–14) after 72 h of incubation with ^{12}C- and ^{13}C-labeled acetate. For each depth, central gradient fractions (7–9) in which the 16S rRNA copy maximum was located are shown before incubation (0 h). Excised and sequenced bands are indicated by an arrow. M = marker (1-kb Ladder, GeneCraft). For closest relatives and phylogenetic affiliations, see Table 2 and Fig. 5, respectively.

Table 2. Overview of sequences from SSCP bands identified (○) in which ^{13}C label (★) was incorporated after 72 h of incubation in ^{13}C-acetate-amended water originating from different layers of the Landsort Deep redoxcline sampled in 2009

SSCP band	Closest relative, accession number (% 16S rRNA gene similarity)	Suboxic	Interface	Sulfidic
Arcobacter subgroup ArcBaltic1				
5	Uncultured *Arcobacter* sp. clone SVB_Fis_pl34b07, JF837809 (100%)	○		
10	Uncultured *Arcobacter* sp. clone ATB-KS-13875, JQ845782 (99%)		★	
12	Uncultured *Arcobacter* sp. clone SVB_Fis_pl34b07, JF837809 (100%)			★
13	Uncultured *Arcobacter* sp. clone ATB-KS-13875, JQ845782 (97%)			★
Arcobacter subgroup ArcBaltic2				
9	Uncultured bacterium, SSCP band OS-PT70, FR714989 (97%)		★	
8	Uncultured bacterium, SSCP band OS-PT70, FR714989 (99%)		★	
11	Uncultured bacterium, SSCP band OS-PT70, FR714989 (99%)			★
Neptunomonas sp.				
6	Bacterium endosymbiont of *Osedax*, clone Omu 16 c5881, FN773262 (99%)		★	
Colwellia subgroup ColwBaltic1				
7	Uncultured bacterium clone Nat2-24, JN092254 (99%)		★	
Sulfurimonas subgroup GD17				
4	Uncultured epsilon proteobacterium isolate SSCP gel band D, EU673344 (100%)	○	○	○

Sulfurimonas subgroup GD17 (band 4) was abundant within the unamended controls but it was not stimulated by the supplementation of organics (Fig. 5). Instead, GD17 bands were less pronounced in the incubations with organics and metal oxides.

One member of *Colwellia* sp. (band 1) and two different *Arcobacter* spp. (bands 2 + 3) were identified, of which *Arcobacter* subgroup ArcBaltic2 dominated the active bacteria in the incubations amended with organic substrates and manganese(IV) or iron(III). Activity of *Colwellia* sp. was not stimulated within 48 h but was strongly stimulated within 96 h (band 1) in incubations amended solely with the organic mixture. Additional manganese(IV) or iron(III) availability reduced the intensity of the bands affiliated with *Colwellia* sp. but did not change the time required for band enrichment. In

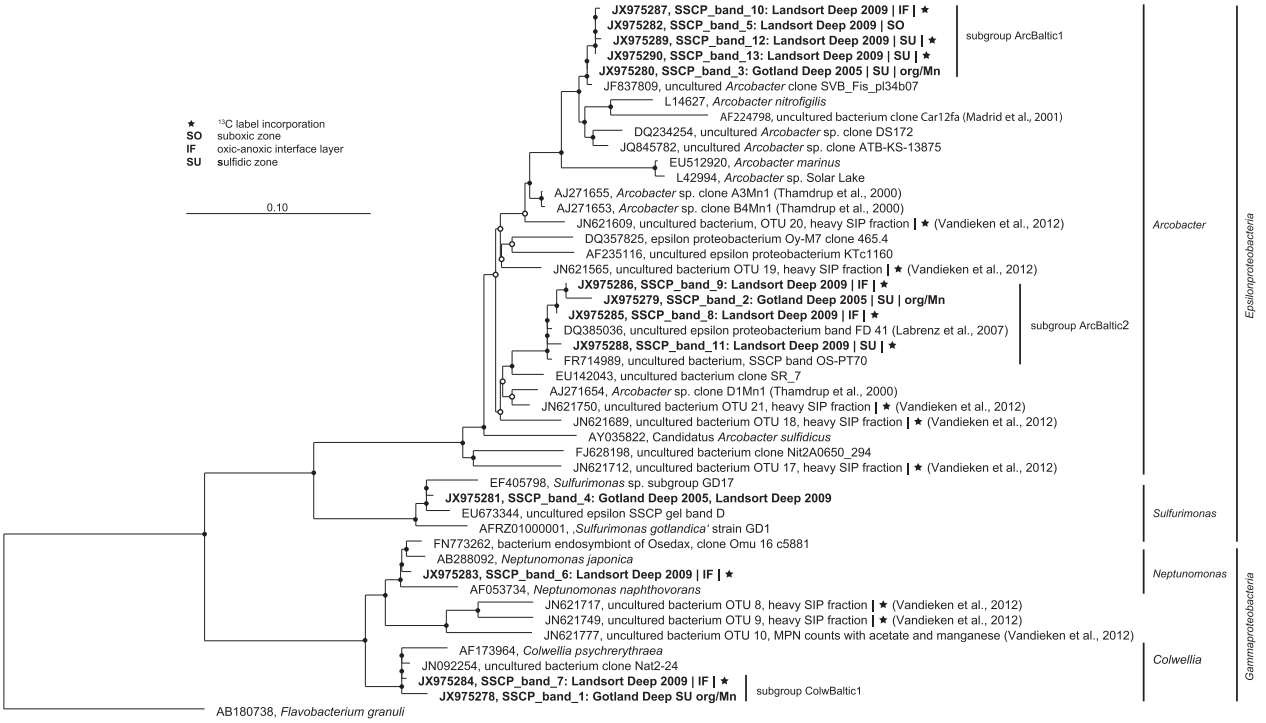

Fig. 4. ML phylogenetic tree showing affiliations of the 16S rRNA partial sequences (~400 bp) of members of *Gamma-* and *Epsilonproteobacteria* recovered in this study (shown in bold). The tree was generated using PHYML and the ARB software package. Branching points supported by ML, NJ, and MP algorithms are indicated by a filled circle; branching points supported by two algorithms are indicated by an open circle. A star indicates sequences for which ^{13}C label incorporation was confirmed, including sequences from Thamdrup *et al.* (2000), Madrid *et al.* (2001) and Vandieken *et al.* (2012).

Table 3. Total cell numbers and reduction of metal oxides in water from the sulfidic zone of the Gotland Deep sampled in 2005 and incubated with different combinations of organic substrate, iron(III), and manganese(IV) for 48 and 96 h

	Total cell number (cells mL^{-1})			Fe(III)/Mn(IV) reduction (μM)			
				48 h		96 h	
Substrate	0 h	48 h	96 h	Fe(III)	Mn(IV)	Fe(III)	Mn(IV)
Organic	2.39×10^6	2.79×10^6	7.08×10^6	0	0	0	0
Organic + Fe^{3+}		1.48×10^6	N/A	0	0	4.3	0
Organic + Mn^{4+}		3.60×10^6	8.83×10^6	0	17.3	0	22.3
Organic + Fe^{3+} + Mn^{4+}		1.69×10^6	3.54×10^6	0	0	1.1	6.9

contrast, the combined availability of the organic mixture, manganese(IV), and iron(III) resulted in an enrichment of these bands already within 48 h. However, their intensity was lower than that in the solely organic mixture setup and was not further increased in the 96-h incubation.

Bands related to *Arcobacter* sp. were pronouncedly enriched (Fig. 5, band 2) or slightly present (band 3) within 96 h of incubation in all setups with amendments. Notably, the additional availability of manganese(IV) reduced the time required for the stimulation of *Arcobacter* (band 2) from 96 to 48 h.

Taken together, phylogenetic 16S rRNA analyses of the sequences recovered from the heterotrophic bacteria of the Landsort Deep and Gotland Deep redoxclines (Fig. 4) revealed that one *Colwellia* subgroup (ColwBaltic1) and two *Arcobacter* subgroups (ArcBaltic1 + ArcBaltic2) were recovered from both. The sequences within the respective subgroups were closely related and were recovered in both years and from both sampling sites.

Nucleotide sequence accession numbers

The 16S rRNA partial sequences from this study have been deposited under the GenBank accession numbers JX975278- JX975280 (Gotland Deep) and JX975281–JX975290 (Landsort Deep).

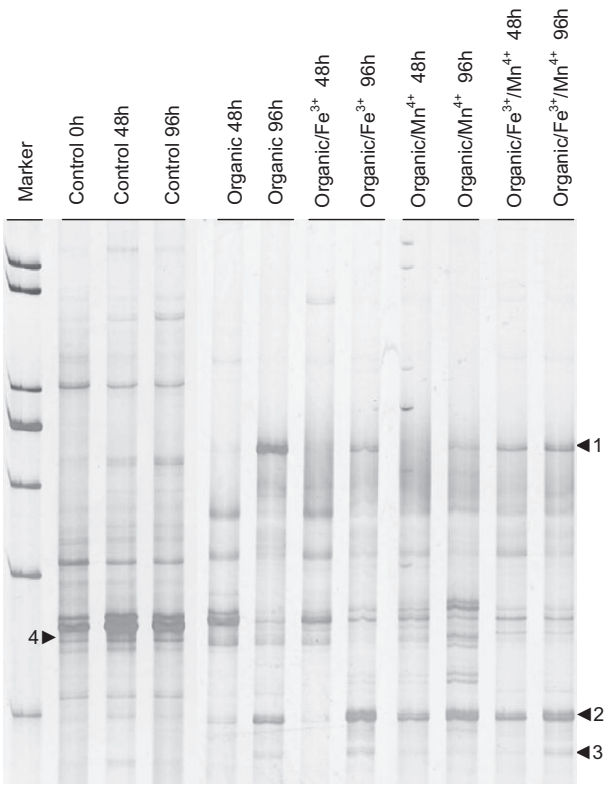

Fig. 5. SSCP fingerprint based on bacterial 16S rRNA from incubations with different combinations of iron(III), manganese(IV), and an organic substrates mixture with water from the sulfidic zone of the Gotland Deep redoxcline 2005 before and after 48 h and 96 h of incubation. Excised and sequenced bands are indicated by an arrow. For phylogenetic affiliations of the bands, see Fig. 5.

Discussion

Heterotrophy in Baltic Sea pelagic redoxclines with low or no oxygen

Our study shows that in central Baltic Sea redoxclines, members of the *Gamma-* and *Epsilonproteobacteria* are able to utilize organic carbon in short-term experiments, predominantly around the oxic–anoxic interface. Experiments in which *Arcobacter* sp. was additionally stimulated by manganese(IV) suggested that Mn oxides are used as electron acceptors for organic carbon degradation and that representatives of this bacterial group participate in manganese cycling across the oxic–anoxic interface of the central Baltic Sea (Neretin *et al.*, 2003). In the Landsort Deep redoxcline, heterotrophy was detected in particular at the oxic–anoxic interface layer and, albeit slightly less pronounced, in the sulfidic zone, as respectively expressed by the elevated uptake of acetate (Table 1). For the redox transition zone of the Cariaco Basin, Taylor *et al.* (2001) also reported increased turnover of acetate, followed by the study by Ho *et al.* (2004) who showed that amendment of water from the oxic–anoxic interface layer with MnO_2 results in an increase in acetate oxidation. Chemoautotrophic activity in our study, as indicated by dark CO_2 fixation, was also evident at the oxic–anoxic interface and, similar to previous work in the Baltic Sea (Grote *et al.*, 2007, 2008) or the Cariaco Basin (Taylor *et al.*, 2001), most pronounced in the upper sulfidic zone (Table 1). This zonation generally implies that acetate, as an important intermediate of anoxic degradation processes, is preferentially utilized at the oxic–anoxic interface layer. This observation was reinforced by our rRNA-SIP results (Fig. 3), which in these water layers identified several active heterotrophs (Table 2), mainly represented by members of *Arcobacter* but also by *Colwellia* and *Neptunomonas*. Previously, members affiliated with *Arcobacter* and *Colwellia* were shown to incorporate acetate and to reduce manganese oxides in sediments (Vandieken *et al.*, 2012) of geographically separated sites (Skagerrak, Gullmar Fjord, Ulleung Basin). We detected members of the same genera actively utilizing acetate in oxygen-deficient or anoxic waters of a Landsort Deep pelagic redoxcline, which suggests that the functional role for these taxa in pelagic redoxclines is similar to that in sediments.

Heterotrophic *Arcobacter* sp. linked to manganese reduction

Extensive diversity is a remarkable feature of the genus *Arcobacter* (Campbell *et al.*, 2006), which comprises many host-associated pathogens (Snelling *et al.*, 2006) but also environmental representatives with a wide range of metabolic capabilities. The latter participate in the ecosystem-relevant biogeochemical cycles of C, S, and N, as is the case for the autotrophic sulfide-oxidizing *Arcobacter sulfidicus* (Wirsen *et al.*, 2002; Sievert *et al.*, 2007), nitrogen-fixing *Arcobacter nitrofigilis* (McClung *et al.*, 1983), and nitrate-reducing *Arcobacter marinus* (Kim *et al.*, 2010), respectively. The recently recognized feature of dissimilatory manganese reduction (Thamdrup *et al.*, 2000; Vandieken *et al.*, 2012) extends the known ecological capabilities of these *Epsilonproteobacteria* but it has so far not been studied extensively in Baltic Sea pelagic redoxclines or in other water columns of hypoxic systems.

A comparison of the 2009 Landsort Deep sequences with the 2005 Gotland Deep sequences allowed the establishment of a link between heterotrophy and manganese reduction for the identified acetate-utilizing *Arcobacter* relatives. Sequences from both experiments fell into identical subgroups of the genera *Arcobacter* and *Colwellia*, respectively (Fig. 4). In the 2005 experiments, the stimulation of *Arcobacter* subgroup ArcBaltic2 by organic substrates reproducibly demonstrated its heterotrophy,

coinciding with the reduction of manganese(IV). A phylogenetic analysis of *Arcobacter* subgroups ArcBaltic1 and ArcBaltic2 (Fig. 4) evidenced their relation to heterotrophic manganese reducers recovered from Black Sea shelf sediments (Thamdrup *et al.*, 2000). The occurrence of particulate manganese oxides directly at or slightly above the oxic–anoxic interface in redoxclines of, for example, the Cariaco Basin (Taylor *et al.*, 2001; Percy *et al.*, 2008) or the Baltic Sea (Neretin *et al.*, 2003; Dellwig *et al.*, 2010) provides a considerable electron acceptor pool. These findings suggest that bacteria of the *Arcobacter* subgroup ArcBaltic2 not only utilize acetate but also reduce manganese.

The vertical distribution of manganese oxides suggests that microbial manganese reduction takes place mainly in the oxygen-deficient or anoxic parts of redoxclines. A pronounced peak of particulate manganese is usually located at or above the oxic–anoxic interface layer (Dellwig *et al.*, 2010; Jost *et al.*, 2010), while in the subjacent anoxic waters, concentrations are drastically lower but still detectable down to the CO_2 fixation maximum (Jost *et al.*, 2010). Therefore, manganese-rich particles should be considered as potential microhabitats where prokaryotes may reside in high abundances while contributing to the microbiological reduction of manganese oxides. Consistent with this view, Jost *et al.* (2010) reported that these particles are densely colonized by microorganisms although it is as yet unclear whether the colonizers are related to *Arcobacter*. Regarding particulate attachment and terminal electron acceptors, Fedorovich *et al.* (2009) showed that in an acetate-fed microbial fuel cell, *Arcobacter butzleri* strain ED-1 is able to transfer electrons to extracellular insoluble electron acceptors. Physical contact with the electron acceptor was also reported for the manganese-reducing *Alteromonas putrefaciens* strain MR-1 (Myers & Nealson, 1988). This ability of manganese reducers to exploit particulate manganese oxides should also be considered as a means of energy conservation for *Arcobacter* sp. in Baltic Sea pelagic redoxclines.

Our recovery of *Arcobacter* sequences in 2005 and 2009 from the Landsort Deep and Gotland Deep together with the previous detection of a sequence from *Arcobacter* subgroup ArcBaltic2 (Fig. 4) in 2003 by Labrenz *et al.* (2007) suggests that these heterotrophs comprise a stable albeit low-abundance component of Baltic Sea pelagic redoxclines, a habitat to which they are probably metabolically well adapted. Rapid stimulation of *Arcobacter* sp. solely with organics was achieved within 72 h and with organics and manganese within 48 h. This underlines the apparent ability of these bacteria to adapt to the short-term availabilities of organic carbon sources. *In situ* acetate concentrations reported for the Black Sea and Cariaco Basin vary in the range of 1–4 μM (Albert *et al.*, 1995; Ho *et al.*, 2002), which corresponds to the amount of organic substrates added in the Gotland Deep experiment. In fact, our stimulation experiments induced drastic changes in the microbial community resulting in a different diversity compared with the natural one. While on the one hand this was our aim, to primarily stimulate heterotrophs, on the other hand elevated substrate concentrations are required for rRNA-SIP experiments to yield detectable enrichment of ^{13}C in the RNA. It remains to be clarified to what extent *Arcobacter* relatives dominate organic substrate degradation *in situ* in conjunction with manganese oxide reduction.

Conclusions

Thus far, knowledge about the microorganisms associated with the manganese cycle in Baltic Sea pelagic redoxclines is limited. Our results are among the first to provide insight into the heterotrophs of these waters, especially highlighting acetate-utilizing *Arcobacter* relatives as potential manganese reducers. The quantitative impact of these organisms in the Baltic Sea redoxcline should be elucidated by investigating spatial distribution, abundance, and the conditions under which they actively influence the carbon and manganese cycles.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers used in this study.

Table S2. Contents of the 5 mM organic mixture stock solution used in the Gotland Deep 2005 incubation experiment.

Table S3. Buoyant densities (g mL^{-1}) of CsTFA density-resolved gradient fractions, showing maximum 16S rRNA copy numbers.

Table S4. Chemical parameters (μM) and turbidity (relative units) of the environmental water retrieved from the Landsort Deep redoxcline in 2009 and used for RNA-SIP incubation experiments with acetate.

Fig. S1. Quantification of bacterial and archaeal 16S rRNA in CsTFA density-resolved ^{12}C and ^{13}C gradients The samples were incubated for 72 h with ^{12}C- and ^{13}C-acetate added to water retrieved from three different zones of the Landsort Deep redoxcline in 2009.

Fig. S2. Densitometric curves of bands of interest from ^{12}C- and ^{13}C-rRNA gradient fingerprints showing relative band intensities in comparison to total band intensities within an individual gel lane throughout the buoyant density (BD) gradient.