



Species-specific effects of two bioturbating polychaetes on sediment chemoautotrophic bacteria

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ABSTRACT: Bioturbation has major impacts on sediment biogeochemistry, which can be linked to the functional traits of the macrofauna involved. *Nereis (Hediste) diversicolor* and *Marenzelleria viridis* are 2 functionally different bioturbating polychaetes that strongly affect the ecology and biogeochemistry of coastal sediments. However, the different effects of these polychaetes on the activity and composition of microbial communities and on chemoautotrophic bacteria have not been extensively studied. We performed experiments with sediment aquaria that contained each species separately as well as a non-bioturbated control. Bacterial communities in different sediment zones (surface, burrow, subsurface) were characterized by phospholipid-derived fatty acid analysis combined with stable isotope labeling (¹³C bicarbonate) to quantify the dark CO₂ fixation by chemoautotrophic bacteria. Pore water chemistry (ΣH₂S and dissolved inorganic carbon) was additionally assessed in each treatment. The strong ventilation but low bioirrigation capacity in the open-ended burrows of *N. diversicolor* resulted in enhanced aerobic chemoautotrophic activity, potentially by sulfur oxidizing and nitrifying bacteria along the burrow. In contrast, slower ventilation and higher irrigation by *M. viridis* induced an advective mode of pore water transport. This promotes anaerobic chemoautotrophy around the blind-ended burrow and within the subsurface sediment. Sulfate-reducing bacteria were the dominant anaerobic chemoautotrophs that probably disproportionate sulfur. In conclusion, our analysis shows that bioturbating fauna influence the microbial community and chemoautotrophic activity in sediments, but that the effect strongly depends on the structure of the burrow and on species-specific ventilation behavior and irrigation capacity.

KEY WORDS: Dark carbon fixation · Fatty acids · Stable isotope probing · PLFA · Microbes · *Nereis (Hediste) diversicolor* · *Marenzelleria viridis*

INTRODUCTION

Ideally, marine sediments are characterized by a predictable vertical zonation of electron donors and acceptors, but in the presence of benthic fauna this typical 1-dimensional redox zonation is disturbed through particle reworking and solute transport induced by burrow formation and ventilation, a process known as bioturbation (Aller 1988, Meysman et al.

2006, Kristensen et al. 2012). Bioturbation creates steep chemical gradients along burrows, which may modify the spatial distribution of various microbial metabolic pathways such as sulfate reduction, sulfide oxidation, denitrification and nitrification (Aller 1988, Kristensen & Kostka 2005, Bertics & Ziebis 2010). The manner in which chemical gradients and, subsequently, microbial assemblages vary in the sediment has been linked to the functional traits of the biotur-

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bating fauna such as burrow properties (e.g. shape, depth and residence time), ventilation behavior, feeding habits and/or sediment mixing intensity (Marinelli et al. 2002, Papaspyrou et al. 2006, Bertics & Ziebis 2009, Laverock et al. 2010, Gilbertson et al. 2012). Bioturbation therefore has a major impact on sediment biogeochemistry, which differs depending on the ecology and behavior of the macrofaunal organisms involved (Braeckman et al. 2010, Mermilod-Blondin 2011, Kristensen et al. 2014).

Nereis (Hediste) diversicolor and *Marenzelleria viridis* are 2 burrow-dwelling polychaetes that have contrasting effects on the ecosystem functioning of coastal areas in the Baltic Sea (Kristensen et al. 2014). Both species are surface deposit feeders, but *N. diversicolor* is also a suspension feeder (Christensen et al. 2000). In regard to bioturbation activities, both *N. diversicolor* and *M. viridis* promote a limited amount of particle reworking, which consists of a random downward transport of sediment particles over short distances, known as gallery biodiffusion (Kristensen et al. 2012). However, the burrow morphology and ventilation mode (i.e. flushing of the burrow with oxygenated water) differ strongly between the 2 polychaetes, inducing distinct modes of bioirrigation (i.e. biologically enhanced transport of pore water and solutes) that create divergent geochemical conditions. *N. diversicolor* inhabits a mucus-lined open-ended burrow (typically U- or Y-shaped, up to 20 cm deep) that is regularly flushed by undulatory body movements (Kristensen & Kostka 2005). This intense burrow ventilation results in diffusion-based radial solute transport into the surrounding sediment pore water, but creates only limited advective pore water movement in the surrounding sediment (Pischedda et al. 2012, Kristensen et al. 2014). *N. diversicolor* has more than twice as much oxygenated sediment surrounding its burrows as *M. viridis* (5 cm³ versus 1.8 cm³, respectively), creating more oxidized conditions in deeper sediments (Kristensen et al. 2011, Jovanovic et al. 2014) and heterogeneous conditions which appear to promote high microbial diversity (Pischedda et al. 2011). In contrast, *M. viridis* creates deep burrows (up to 30 cm deep) that are typically straight down and blind-ended. *M. viridis* ventilates its burrow slowly through ciliary and muscular movements that promote advective flow of anoxic pore water in the surrounding sediment (Kristensen et al. 2011, Quintana et al. 2011, Jovanovic et al. 2014). *M. viridis* activity therefore simultaneously maintains reduced conditions in the deeper sediment that stimulate sulfate reduction, while potentially enhancing sulfide oxidation in sur-

face sediments, given the upward flow of solutes (Quintana et al. 2007, Kristensen et al. 2011, 2014).

The distinct impacts of *N. diversicolor* and *M. viridis* on the sediment geochemistry suggest substantial differences in the bacterial community composition and function. One microbial metabolism considered to be affected by bioturbation is chemoautotrophy, given the steep chemical gradients and the potential overlap of electron acceptors and donors along the burrow structure (Aller 1988, Kristensen & Kostka 2005). Burrow walls are ideal environments for chemoautotrophic bacteria that fix inorganic carbon by obtaining energy from the oxidation of reduced compounds (e.g. sulfide, ammonium) produced in the sediment. The specific effects of bioturbation on the microbial community composition have occasionally been studied (Steward et al. 1996, Papaspyrou et al. 2006, Cuny et al. 2007, Kunihiro et al. 2011, Yazdani Foshtomi et al. 2015), including only 1 study that quantified the impact on the growth of chemoautotrophic bacteria (Reichardt 1988). The latter study found a substantial increase of dark CO₂ fixation along the burrow of the lugworm *Arenicola marina* when compared to the surface sediment. Therefore, we expect enhanced chemoautotrophic activity along the burrows of *N. diversicolor* and *M. viridis* compared to the adjacent sediment. However, given the contrasting functional traits of these 2 polychaete species, we hypothesize that the bacterial community in these burrow environments could be species-specific. To test this hypothesis, we used phospholipid-derived fatty acid analysis combined with stable isotope probing (PLFA-SIP). This approach allows simultaneous characterization of the total bacterial and chemoautotrophic community composition as well as the quantification of dark CO₂ fixation rates (Boschker et al. 2014). We also determined the effects of polychaetes on sediment pore water chemistry. The impact on chemoautotrophic activity and differences in microbial composition were then linked to the functional traits of *N. diversicolor* and *M. viridis*.

MATERIALS AND METHODS

Experimental set-up

Sediment incubations were performed consisting of the following 3 treatments: with addition of *Nereis (Hediste) diversicolor* (Nd); with addition of *Marenzelleria viridis* (Mv); and without addition of polychaetes (Control). Sediment (top 25 cm), polychaetes

and overlying water for incubations were collected at Bregner Bay, Denmark (55.481° N, 10.610° E), in September 2012 (water depth: 50 cm, water temperature: 18°C, salinity: 20). The sediment at the sampling location is described as organic-poor median sand with a porosity of 0.40 ± 0.03 (Quintana et al. 2007, Jovanovic et al. 2014). Sediment was gently sieved at the location through a 1 mm mesh and later homogenized in the laboratory. Active individuals of *M. viridis* and *N. diversicolor* were collected for experiments during sieving, and were kept in buckets with aerated seawater (15°C) and a thin layer of sieved sediment.

For the sediment incubations, we used flat aquaria (height: 35 cm, length: 20 cm, width: 1 cm) with 1 removable side panel which was adapted with injection ports distributed on a 2-dimensional grid (distance of 2 cm between ports). Before assembling the flat aquaria, plastic foil was placed against the inside wall of the removable side panel. This decreased the disturbance of the sediment when the aquarium was opened. A total of 10 aquaria (Nd: 3, Mv: 3 and Control: 4) were filled with homogenized sediment to obtain a ~28 cm sediment depth layer. Aquaria were wrapped in aluminum foil so that only the sediment surface was exposed to light, limiting the development of photosynthetic communities along the walls of the aquaria during incubation. Aquaria were submerged into 90 l water tanks filled with *in situ* seawater at $16 \pm 2^\circ\text{C}$ and exposed to 12 h light:12 h dark cycles for 7 d. Water circulation in the flat aquaria was ensured by aerating each aquarium separately with air pumps and using tubing adapted with needles.

Polychaetes were weighed and transferred to the aquaria after a 24 h settling period of the sediment. Only motile, visibly undamaged individuals were used in the experiments. Average wet weight was 0.11 ± 0.02 g for *N. diversicolor* ($n = 3$) and 0.37 ± 0.03 g for *M. viridis* ($n = 3$). Only 1 individual was added to each flat aquarium to facilitate the identification and collection of burrow sediment for further analysis. The resulting faunal densities (500 ind. m^{-2}) were within the range of 14 to 3013 ind. m^{-2} for *N. diversicolor* and 0 to 1174 ind. m^{-2} for *M. viridis* observed in Odense Fjord by Delefosse et al. (2012).

Biomarker analysis

Six days after the addition of individual polychaetes to the flat aquaria we evaluated the effect of bioturbation on the microbial composition and

chemoautotrophic activity by analyzing the PLFA-SIP (Boschker et al. 1998). First, we employed PLFA fingerprinting, which allows the study of the composition and abundance of the total bacterial community. Second, the composition of the chemoautotrophic community and its activity (i.e. dark CO_2 fixation rate) was characterized by the incorporation of ^{13}C -labeled dissolved inorganic carbon (DIC) into bacterial PLFA. A stock solution of ^{13}C sodium bicarbonate of 60 mM (99% ^{13}C ; Cambridge Isotope Laboratories) was prepared in artificial seawater free of Ca^{2+} and Mg^{2+} to prevent carbonate precipitation. Shortly before use, the bicarbonate solution was bubbled with N_2 in order to remove the O_2 . The line injection method (Jørgensen 1978) was used to introduce ^{13}C -labeled substrate into the sediment. The substrate was injected through the ports in the side wall of the aquaria in 20 μl aliquots from the bottom to the top. Finally, 180 μl substrate was added to the ~2 cm-thick layer of overlying water. The top of the aquaria was covered with Parafilm to prevent evaporation and then returned to the water tanks ($16 \pm 2^\circ\text{C}$). The aquaria were kept elevated above the water line to prevent mixing of overlying water with tank water. Overlying water was bubbled with ^{13}C - CO_2 saturated air to avoid stripping of the label from the overlying water during incubations, as described by Vasquez-Cardenas et al. (2015). Incubations were performed in the dark to exclude ^{13}C incorporation by photosynthetic microorganisms. Of the 4 Control aquaria, 2 did not receive ^{13}C substrate and were used to measure the natural abundance $\delta^{13}\text{C}$.

Stable isotope labeling was terminated after 45 h. Overlying water was removed with a syringe and the sediment surface was examined to locate burrow openings. Aquaria were then placed on their side and the lateral panel was removed. For Nd treatments, more than one burrow was present, so in those cases only the burrow containing the polychaete was considered. The coloration of the sediment surrounding the burrow, burrow diameter and length were recorded. The burrow sediment was defined as the 3 mm-thick layer surrounding the burrow wall. In the Nd cases, the burrow sediment also included the mucoid burrow lining. The sediment from the active burrows was collected with a sterile spatula. Organisms were carefully extracted from their burrows using pincers. The remaining sediment matrix in the aquaria was then sectioned in horizontal layers of 0.5 cm intervals in the top 1 cm, followed by 1 cm intervals to 4 cm depth, and 2 cm intervals to 18 cm depth. The following 4 sediment zones were selected to compare the effects of the burrowing activity on

the PLFA fingerprint and ^{13}C -labeling of the microbial community: burrow (sampled as explained above), surface (0 to 0.5 cm), shallow subsurface (2 to 4 cm) and deep subsurface (12 to 14 cm) (Fig. 1). Sediment was collected in pre-weighed 50 ml double centrifuge tubes and placed on ice until further processing. The bottom of the inner tube was equipped with a GF/C filter to facilitate the collection of pore water. Tubes with sediment were centrifuged at $500 \times g$ (1500 rpm) for 15 min and pore water was collected from the outer tube, while the sediment in the inner tube was frozen overnight and subsequently lyophilized.

PLFA extractions were performed on dry sediment according to Guckert et al. (1985) and Boschker et al. (1998), and analyzed by gas chromatography with isotope ratio mass spectrometry (GC-IRMS; Thermo) on an apolar analytical column (ZB5-MS Phenomenex). The precision of the GC-IRMS is $\sim 0.3\%$ $\delta^{13}\text{C}$, which corresponds to a variation in ^{13}C content of $\sim 0.0003\%$ (Boschker & Middelburg 2002). PLFA concentrations are expressed in carbon units ($\text{nmol C-PLFA [g dry wt]}^{-1}$). Total bacterial biomass was determined as the sum of the concentrations of all bacterial fatty acids (C12:0 to C20:0) converted to biomass, assuming PLFAs constitute 5.5% of the carbon present in the total bacterial carbon biomass (Middelburg et al. 2000). Sediment dry wt was converted to bulk sediment volume using a dry sediment density of $2.55 \text{ g dry wt cm}^{-3}$ and the measured sediment porosity (see 'Pore water analysis'). Characterization of the chemoautotrophic community was based on ^{13}C incorporation into PLFA. Briefly, PLFA ^{13}C -label incorporation per unit of bulk sediment ($\text{pmol } ^{13}\text{C cm}^{-3}$) is calculated as the product of each

bacterial PLFA concentration and its ^{13}C fraction after labeling (corrected for natural ^{13}C fraction of unlabeled samples). Dark CO_2 fixation rates ($\text{nmol C cm}^{-3} \text{ d}^{-1}$) were calculated by summation of the PLFA incorporation data for each sediment sample, which was then converted to total biomass incorporation and divided by the length of the incubation period. Dark CO_2 fixation rates were corrected for $\delta^{13}\text{C}$ DIC values in pore water relative to natural $\delta^{13}\text{C}$ DIC. A detailed description of the PLFA-SIP calculations can be found in Boschker & Middelburg (2002).

Label transfer

To determine the possible grazing on chemoautotrophic bacteria, we measured the ^{13}C -labeling of the polychaetes recovered from the aquaria. Polychaetes were collected at the end of the incubation period, weighed, frozen and then lyophilized. Dried individuals were subsequently pulverized and analyzed by an elemental analyzer-isotope ratio mass spectrometer (EA-IRMS, Finnigan Delta S). Precision of the technique is of the order of $\pm 0.1\%$ $\delta^{13}\text{C}$ for carbon isotopic values. The natural carbon isotopic composition was determined for 2 individuals of each species taken directly from the sampling site and analyzed as described in 'Experimental set-up'.

Pore water analysis

Pore water collected from flat aquaria at the end of the incubation was analyzed for DIC and total free

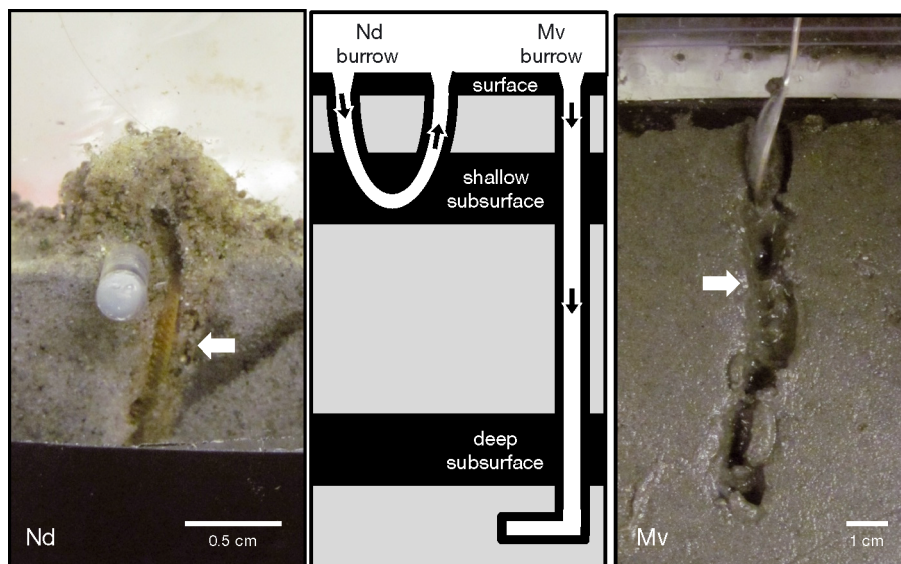


Fig. 1. Left: *Nereis (Hediste) diversicolor* (Nd) and its burrow surrounded by an oxidized halo seen against the side of the flat aquarium. Right: sampled burrow area of *Marenzelleria viridis* (Mv). White arrows indicate the position of the burrow structure. Centre: schematic representation of the burrow geometry, burrow water flow (black arrows) and the 4 sediment zones sampled in black (burrow; surface 0 to 0.5 cm; shallow subsurface 2 to 4 cm; deep subsurface 12 to 14 cm)

sulfide ($\Sigma\text{H}_2\text{S} = [\text{H}_2\text{S}] + [\text{HS}^-] + [\text{S}^{2-}]$). DIC samples (0.5 ml) were collected in gas-tight vials and preserved with 30 μl of a saturated HgCl_2 solution. Total DIC and $\delta^{13}\text{C}$ DIC were measured by the headspace technique using an EA-IRMS equipped with a gas injection port (Boschker et al. 2014). Background carbon isotopic values were analyzed on pore water in parallel samples from the 2 Control aquaria without ^{13}C substrate addition. $\Sigma\text{H}_2\text{S}$ samples (0.5 ml) were fixed with 50 μl of a 1 M zinc acetate solution and analyzed following Cline (1969) (precision of $\pm 2\%$ and sensitivity at pH 0.35 of $29.5 \times 10^3 \text{ l mol}^{-1} \text{ cm}^{-1}$). Weight loss of wet sediment subsamples after drying at 60°C for 48 h was used for porosity determination.

Statistical analysis

All statistical analyses were performed using the extension package CRAN: stats in the open-source software R. Normal distribution (Shapiro-Wilks test) and homogeneity of variance (Bartlett's test) were tested for each data set, and data were log-transformed when necessary to fulfill the assumptions of ANOVA. A 2-way ANOVA was used to test the effect of polychaete treatments and sediment depths on bacterial biomass and chemoautotrophic activity. Depth-integrated pore water DIC and $\Sigma\text{H}_2\text{S}$ were tested with 1-way ANOVA for treatment effects. Significant differences were further analyzed using a Tukey's multiple comparisons of means (Tukey's HSD). Both PLFA concentrations and ^{13}C incorporation values were expressed as a fraction of the total per sediment sample. Relative PLFA values were log-transformed as $[\log(x + 1)]$ and analyzed with principal component analysis (PCA) to determine differences in the microbial assemblage and within the chemoautotrophic community among layers and treatments. Only PLFAs contributing more than 0.1% to the total sediment activity were included in the PCA analysis.

RESULTS

Sediment appearance

The surface sediment in all treatments had a similar appearance after 6 d of acclimatization, and consisted of a light brown surface sediment layer indicative of oxidized conditions, while the sediment below showed a dark grey coloration suggesting reduced conditions. The oxidized surface layer was ~ 4 mm

deep in the Control, 3 to 4 mm in Nd and only ~ 1.5 mm in the Mv sediments. However, on closer inspection, Nd and Mv sediments exhibited several physical differences. The surface sediment in Nd aquaria was loose and fluffy (indicating surface reworking) and detritus accumulations were present around burrow openings, whereas surface sediment in Mv aquaria appeared more compact (Fig. 1). Sediment surrounding the burrow in the Nd aquaria had a brownish coloration similar to the oxidized surface layer (Fig. 1). Nd burrows were typically U-shaped and reached between 2 and 4 cm into the sediment. One aquarium contained 1 burrow, whereas the other 2 aquaria had several burrows; however, only the active and inhabited burrow was sampled for analysis. Each Mv aquarium had only 1 burrow, which was straight, penetrating up to 18 cm deep, and had a similar grey coloration to the subsurface sediment (Fig. 1). One of the 3 Mv aquaria was discarded because the worm was found outside the aquarium at the end of the incubation. The burrows of *Nereis (Hediste) diversicolor* and *Marenzelleria viridis* both had an average burrow diameter of 3 mm.

The porosity ranged from 0.31 to 0.47 between treatments and depths, with an overall mean depth-integrated value of 0.39 ± 0.05 , which was in agreement with previous analyses of the same sediment (Quintana et al. 2007, Jovanovic et al. 2014).

Total bacterial community

Surface, burrow and 2 subsurface depths (shallow and deep) were examined using PLFA analysis. A total of 39 PLFAs were identified, of which 29 were considered as bacterial biomarkers. The PLFAs not used in our data analysis were polyunsaturated fatty acids (PUFAs), which are considered to be eukaryote biomarkers (Vestal & White 1989). These PUFAs were mostly present in the surface samples and accounted for 6% of the total PLFAs. A subset of 14 out of 29 bacterial fatty acids represented more than 90% of the total bacterial biomass per sample (Fig. 2). Major bacterial PLFAs were 16:1 ω 7c ($25 \pm 0.3\%$), 18:1 ω 7c ($22 \pm 0.9\%$), 16:0 ($19 \pm 0.6\%$), 18:1 ω 9c ($5 \pm 0.2\%$), 14:0 ($5 \pm 0.3\%$), ai15:0 ($4 \pm 0.3\%$), 16:1 ω 7t ($2 \pm 0.9\%$), i15:0 ($2 \pm 0.1\%$) and 18:0 ($2 \pm 0.1\%$), whereas minor fatty acids (1 to 2%) included 15:0, 16:1 ω 5, 17:1 ω 8c, 17:1 ω 6c and 17:0.

The 2-way ANOVA (Shapiro-Wilks $p = 0.39$; Bartlett $p = 0.56$) on log-transformed bacterial biomass data showed a statistically significant effect of sediment depth ($F_{3,24} = 10.95$, $p = 0.0001$) rather than

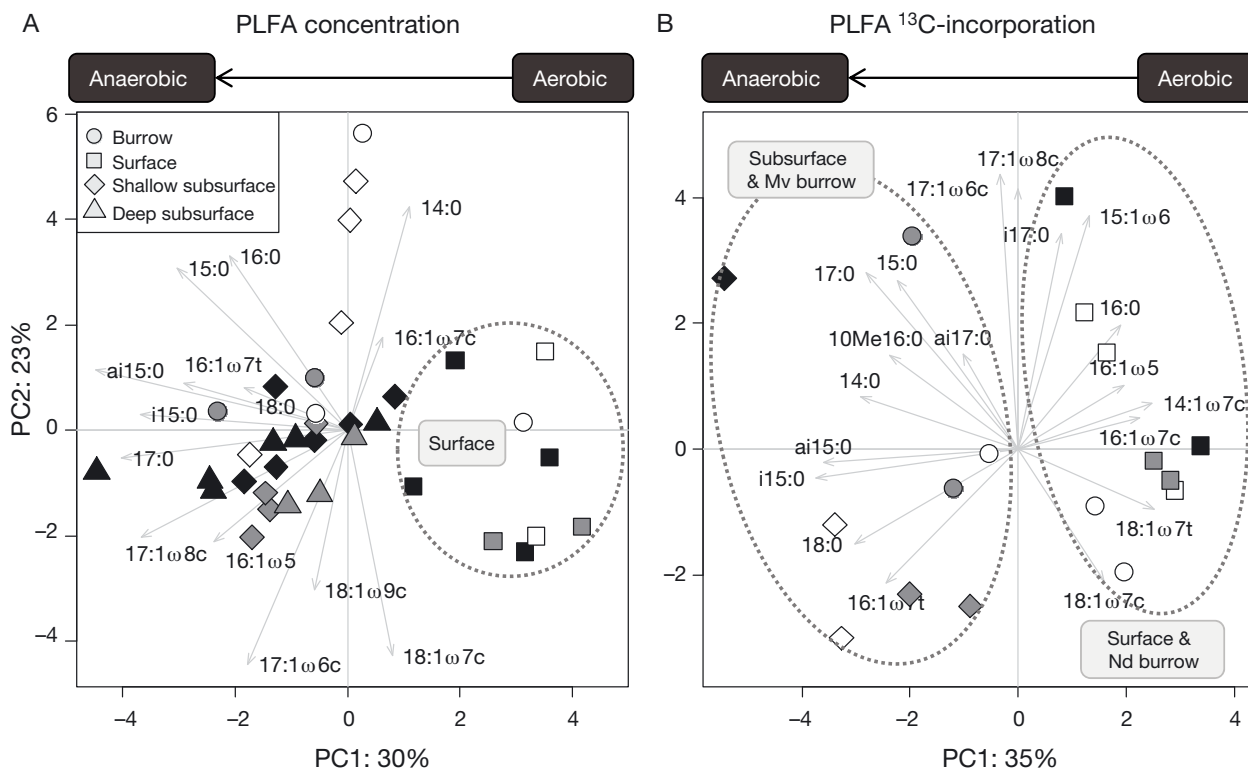


Fig. 2. PCA of (A) PLFA concentrations ($\text{nmol C-PLFA [g dry wt]}^{-1}$) and (B) ^{13}C incorporation ($\text{pmol } ^{13}\text{C [g dry wt]}^{-1}$) for each sediment depth. Values were log-transformed prior to analysis. The percent of variation amongst samples explained by each of the first 2 principal components (PC) is given on the corresponding axis. Grey arrows represent the proportion of total variance explained by each PLFA. Black: Control (without bioturbation); white: *Nereis (Hediste) diversicolor* (Nd); grey: *Marenzelleria viridis* (Mv). Sediment depths are represented by different symbols as noted in legend (burrow; surface 0 to 0.5 cm; shallow subsurface 2 to 4 cm; deep subsurface 12 to 14 cm)

treatment ($F_{2,24} = 00.189$, $p = 0.83$). Tukey's HSD showed that differences occurred mainly between the higher bacterial abundance in surface sediments compared to the lower values in both subsurface sediments (shallow subsurface $p = 0.0009$, deep subsurface $p = 0.00009$). In general, subsurface bacterial biomass represented 55 to 65% of the surface biomass (Table 1). The 1-way ANOVA for the bacterial biomass in the Control showed statistical differences between layers ($F_{2,11} = 4.421$, $p = 0.039$), with the bac-

terial biomass in surface sediment not significantly different from that in the shallow subsurface (Tukey's HSD $p = 0.52$) but significantly higher than in the deep subsurface (Tukey's HSD $p = 0.05$). In the Nd treatment, 1-way ANOVA ($F_{2,6} = 6.961$, $p = 0.027$) showed that the bacterial biomass in the burrow was not statistically different from that in the surface sediment (Tukey's HSD $p = 0.53$), whereas for the Mv treatment 1-way ANOVA ($F_{3,7} = 7.923$, $p = 0.012$), the bacterial biomass in the burrow was lower than that

Table 1. Bacterial biomass and chemoautotrophy rates (mean \pm SD) for the different treatments and sediment depths studied. Number of replicates is stated in parentheses next to the treatment code. Control: no bioturbation; Nd: *Nereis (Hediste) diversicolor*; Mv: *Marenzelleria viridis*; na: not applicable; nd: not detected; surface: 0 to 0.5 cm; shallow subsurface: 2 to 4 cm; deep subsurface: 12 to 14 cm. Ranges are stated when there were only 2 replicates

	Bacterial biomass ($\mu\text{mol C cm}^{-3}$)			Chemoautotrophy rate ($\text{nmol C cm}^{-3} \text{d}^{-1}$)		
	Control (4)	Nd (3)	Mv (2)	Control (2)	Nd (3)	Mv (2)
Surface	20 ± 8	15 ± 2	13–16	43–145	49 ± 9	52–76
Burrow	na	13 ± 4	9–12	na	59 ± 68	10–11
Shallow subsurface	11 ± 2	9 ± 1	9–11	1.6–1.9	2 ± 0.3	3.6–4.0
Deep subsurface	9 ± 2	na	8–10	<1	na	nd

in the surface (Tukey's HSD $p = 0.07$), but not statistically different from subsurface biomass (Tukey's HSD $p > 0.6$).

The bacterial community composition characterized through PLFA fingerprinting showed differences between depth layers along the first principal component axis, with a total of 53% of the variation between samples explained by the first 2 principal components (Fig. 2A). Surface samples for all treatments showed a clear differentiation from deeper layers, with a higher contribution of 16:1 ω 7c, 18:1 ω 7c/9c and 14:0. Of the 3 Nd burrow samples, 2 also clustered with the surface samples, whereas Mv burrows resembled PLFA patterns of the subsurface. Deeper sediments increased by ~15% in the relative contribution of ai15:0 compared to surface samples, and 16:1 ω 7t was only present in deeper layers.

Chemoautotrophic community

Combining the PLFA fingerprint technique with SIP allows for a targeted study of the active chemoautotrophic bacterial community. The incorporation of ^{13}C -label was determined for 20 bacterial fatty acids. The following 3 PLFAs consistently displayed the highest ^{13}C assimilation: 16:1 ω 7c ($34 \pm 4\%$), 18:1 ω 7c ($26 \pm 5\%$) and 16:0 ($17 \pm 2\%$). However, PCA showed a clear divergence in the composition of the chemoautotrophic bacterial community between surface and subsurface samples (Fig. 2B). Surface samples were characterized by more label uptake into even-numbered mono-unsaturated fatty acids, while synthesis of branched and saturated fatty acids prevailed in subsurface samples. Burrow sediments exhibited a specific PLFA fingerprint for each polychaete treatment (Fig. 2B). Nd burrow samples showed the highest relative ^{13}C incorporation into 16:1 ω 7c ($36 \pm 2\%$) and 18:1 ω 7c ($30 \pm 5\%$), which was similar to that observed for surface samples. Nonetheless, 1 replicate Nd burrow had less 18:1 ω 7c (25%) and thus clustered towards the subsurface PLFA fingerprint in the PCA (Fig. 2B). Mv burrows showed at least 3 times higher ^{13}C incorporation into ai15:0 (8%) and i15:0 (2.5%) than surface sediments, therefore closely resembling subsurface PLFA patterns. Moreover, mono-unsaturated fatty acid 16:1 ω 7t was prevalent in subsurface sediment but differed between treatments, with an average incorporation of 10% for Nd and 4% for Mv and the Control treatment.

Overall, the highest chemoautotrophy rates were found in the surface and the lowest in the subsurface

sediments (Table 1). Very low chemoautotrophic activities were detected within the deep subsurface layer of all treatments (12 to 14 cm deep) and, therefore, these data were not considered for further analysis. The 2-way ANOVA (Shapiro-Wilks $p = 0.053$; Bartlett $p = 0.55$) performed on log-transformed (volumetric) chemoautotrophy rates showed significant differences between all sediment zones ($F_{2,11} = 47.432$, $p = 3.9 \times 10^{-6}$). However, Nd burrow and surface rates were the only pair that were not significantly different (Tukey's HSD $p = 0.857$) and both were on average 20 times higher than subsurface rates. In contrast, the mean dark CO_2 fixation rate of Mv burrows was only 15% of the surface rate and 2.5 times higher than subsurface activity. Subsurface chemoautotrophic activity was 2-fold higher in Nd and 4-fold higher in Mv when compared to the subsurface activity in the Control treatment.

Trophic interactions

Isotope results from field-collected specimens revealed a natural $\delta^{13}\text{C}$ signal of -15 to -14% for *N. diversicolor* and -14 to -13% for *M. viridis*. All polychaetes retrieved from ^{13}C -labeled incubations were alive and motile at the end of the incubation period, and were used to determine possible ^{13}C enrichment. All polychaetes were enriched in ^{13}C , and $\delta^{13}\text{C}$ values were more pronounced for *M. viridis* (-1 to -2%) than for *N. diversicolor* (-11 to -7%), suggesting a higher grazing of chemoautotrophic bacteria by *M. viridis* than by *N. diversicolor*.

Pore water chemistry

Free sulfide ($\Sigma\text{H}_2\text{S}$) concentrations in the pore water of the Control and Nd treatment were low at the surface (~ 0.1 mM), increasing to 2.5 mM at 5 cm depth, and remaining fairly constant deeper down (Fig. 3A). The increase in $\Sigma\text{H}_2\text{S}$ for Mv was less pronounced, only reaching 1 mM at 5 cm depth and then gradually increasing to 3 mM at 17 cm depth. However, 1-way ANOVA (Shapiro-Wilks $p = 0.74$; Bartlett $p = 0.94$) showed that the pore water $\Sigma\text{H}_2\text{S}$ inventories (depth-integrated $\Sigma\text{H}_2\text{S}$ up to 20 cm) were not statistically different ($F_{2,6} = 0.592$, $p = 0.582$) between treatments (Nd: 82 ± 38 ; Mv: 29 to 69; Control: 73 ± 32 mmol m^{-2}). DIC showed a pattern similar to that of $\Sigma\text{H}_2\text{S}$, with a rapid increase in the Control and Nd treatment from overlying water values (~ 2 mM) at the sediment surface to ~ 9 mM at 5 cm depth

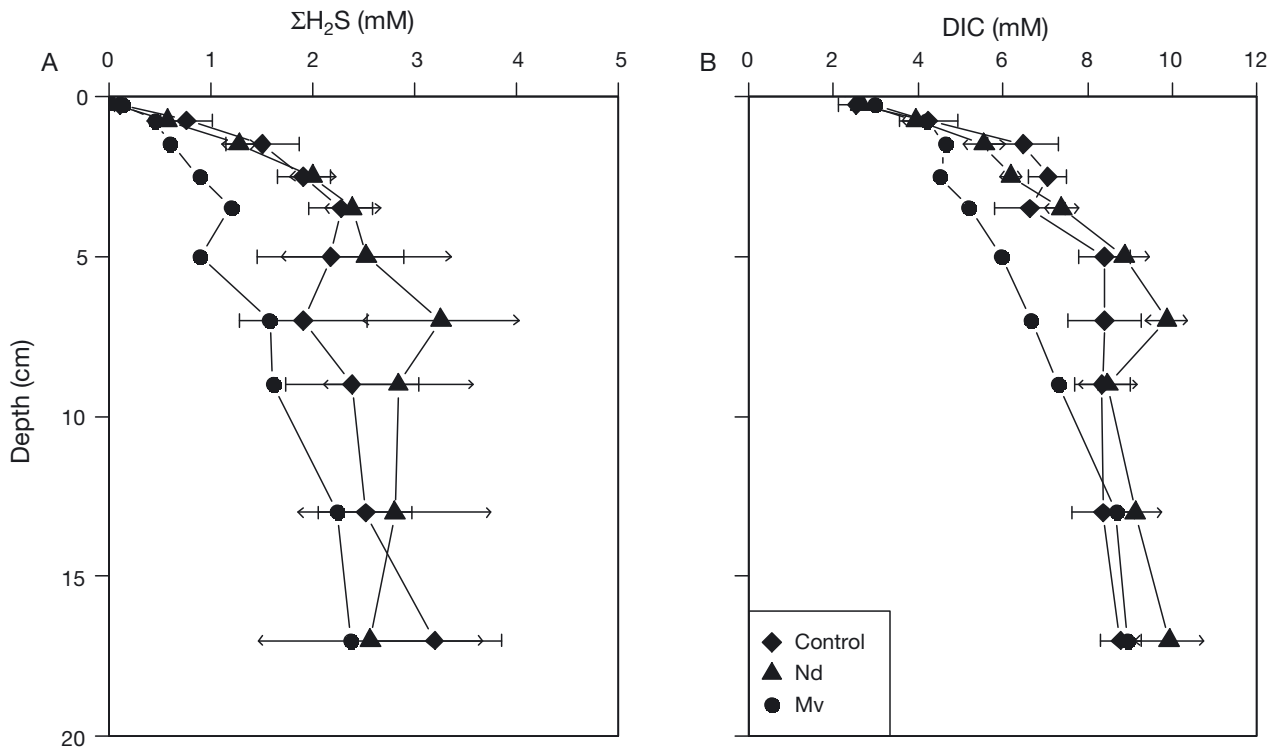


Fig. 3. Sediment pore water profiles for (A) ΣH_2S and (B) dissolved inorganic carbon (DIC) for the different treatments (Control: no bioturbation; Nd: *Nereis (Hediste) diversicolor*; Mv: *Marenzelleria viridis*). Error bars show SE (Control: n = 4; Nd: n = 3; Mv: n = 2)

(Fig. 3B). The 1-way ANOVA (Shapiro-Wilks $p = 0.65$; Bartlett $p = 0.19$) showed that depth-integrated DIC was statistically different between treatments ($F_{2,6} = 6.839$, $p = 0.028$), with a DIC inventory of 223 to 224 mmol m^{-2} for Mv, $276 \pm 15 \text{ mmol m}^{-2}$ for Nd and $260 \pm 19 \text{ mmol m}^{-2}$ for the Control treatment. The Mv treatment showed a more gradual increase in DIC, which was significantly different from Nd (Tukey's HSD $p = 0.02$) and to a lesser degree from Control (Tukey's HSD $p = 0.08$).

DISCUSSION

Bacterial community

Sediment bioturbated by *Nereis (Hediste) diversicolor* and *Nereis virens* has shown 4 to 5 times higher bacterial abundance at the surface and burrow when compared to subsurface sediment (Papaspyrou et al. 2006). Likewise, Dobbs & Guckert (1988) observed an enrichment of bacterial biomass associated with the burrow lining of deep-burrowing shrimp *Callinassa trilobata*, while sediment from the burrow wall had a similar bacterial biomass to that of subsurface sediment. PLFA analysis of sediment biotur-

bated by 3 polychaete species (*Notomastus lobatus*, *Branchioasychus americana* and *Balanoglossus aurantiacus*) has shown a 20 to 75% species-dependent decrease in bacterial biomass from the surface to the burrow (Steward et al. 1996). Our Mv and Nd treatments followed a similar trend, with ~36% higher bacterial biomass in the surface sediment than in the subsurface, while bacterial biomass in Mv and Nd burrows was only 13 and 26%, respectively, lower than surface bacterial abundances.

The biomarker patterns revealed distinct differences in microbial communities between surface and subsurface sediments. Furthermore, PLFA patterns in burrow and surface sediment were alike for Nd, whereas in the Mv treatment, the biomarker fingerprint of the burrow resembled that of the subsurface sediment. Nd burrow and surface sediment contained higher quantities of 16:1 ω 7c and 18:1 ω 7c, and although these compounds are ubiquitous amongst bacteria, they are typically more abundant in aerobic environments (Vestal & White 1989). The dominance of an aerobic microbial community in the Nd burrow is in line with our observation of oxidized conditions (light brown halo around the burrow lining) and the intense ventilation (i.e. pumping of burrow with oxygenated waters) known to be performed by *N. diversicolor*.

color (Pischedda et al. 2012). In contrast, the burrow of *Marenzelleria viridis* and subsurface sediments showed increased concentrations of ai15:0 and i15:0, which are compounds that indicate a high proportion of anaerobic metabolism (Findlay et al. 1990, Bühring et al. 2005). The enhancement of anaerobic activity in burrow sediments of *M. viridis* rather than *N. diversicolor* is consistent with the known impact of *M. viridis* bioturbation on sediment geochemistry, which boosts anaerobic processes such as sulfate reduction (Kristensen et al. 2011, Quintana et al. 2013).

A decrease in the concentration of 16:1 ω 7c combined with an increase in its trans form (16:1 ω 7t) observed in the subsurface of all treatments may be linked to ambient stress on the bacteria (Findlay et al. 1989, Vestal & White 1989). Nevertheless, sieving compared to freezing or asphyxiation has been found to be the least disruptive method in regard to the magnitude of the effect and recovery time, both of the microbial community and the geochemical conditions (Porter et al. 2006, Stocum & Plante 2006). Manipulated sediments can, however, take between 5 d and 2 wk to reach a steady state or resemble natural biogeochemical conditions (Findlay et al. 1990, Kristensen 2001, Porter et al. 2006, Stocum & Plante 2006), which suggests that after a short acclimatization period, opportunistic, fast-growing bacteria may be favored in sediments. It is possible that the week-long acclimatization of the microbial community in our incubations may not have allowed for the complete recovery of the slow-growing microbial community after the initial disturbance of the sediment (i.e. sieving and homogenization). However, chemoautotrophic bacteria, generally exhibiting slow growth, have been shown to develop rapidly (~1 wk) in homogenized cohesive, sulfide-rich sediments (Vasquez-Cardenas et al. 2015). Hence, both the sediment manipulation technique and the incubation time may strongly determine the microbial community in the sediment and should be considered during the experimental design phase in similar studies.

Chemoautotrophic community

PLFA-SIP analysis showed that the chemoautotrophic bacteria associated with the Nd burrow sediment grouped with those of surface sediments, whereas chemoautotrophs from the Mv burrow clustered with those of subsurface samples. Prevalence of aerobic or anaerobic bacterial communities in burrow environments has previously been linked to the functional traits of the macrofauna (Dobbs & Guckert

1988, Steward et al. 1996, Papaspyrou et al. 2006, Laverock et al. 2010, Gilbertson et al. 2012), and our results confirm this species-specific bioturbation effect.

N. diversicolor intermittently ventilates its U-shaped burrow in regular cycles of ~10 min ventilation and ~5 min resting periods (Pischedda et al. 2012). The final result is a strong oxygenation of the burrow, with a mean oxygen concentration of ~70% saturation in the burrow. The O₂ concentration in the inhalant opening matches that of the overlying water and decreases towards the exhalant opening, with the lowest O₂ values being encountered at the bottom of the burrow (Pischedda et al. 2012). From the oxygenated burrow, oxygen diffuses into the sediment, sustaining oxidized conditions (over 0.5 to 3 mm) and generating steep chemical gradients radially from the center of the burrow towards the surrounding sediment. This radial transport of oxygen stimulates the aerobic re-oxidation of reduced compounds by bacteria within the burrow (Banta et al. 1999). Strong aerobic activity is also inferred from the high chemoautotrophic labeling of even-numbered mono-unsaturated 16- and 18-carbon fatty acids in the Nd burrow sediment, similar to the aerobic PLFA pattern noted in the surface sediment of all treatments. These mono-unsaturated PLFAs are also highly abundant in sulfur-, nitrite- and ammonium-oxidizing chemoautotrophic bacteria (Blumer et al. 1969, Lipski et al. 2001, Knief et al. 2003, Inagaki et al. 2004, Labrenz et al. 2013, Boschker et al. 2014). The nitrification potential of *Nereis* spp. burrows substantially exceeds that of surface sediments (Kristensen & Kostka 2005); yet, here chemoautotrophy rates in the burrow and surface sediment of the Nd treatment were similar. Nitrifying bacteria have generally lower growth yields than sulfur-oxidizing bacteria (Boschker et al. 2014) and thus their activity may be underrepresented by the PLFA-SIP technique. Nonetheless, biomarker analysis of Nd burrows and surface sediments points to an aerobic chemoautotrophic community composed of sulfur-oxidizing and nitrifying bacteria.

Chemoautotrophy rates in Nd burrows were variable, with 1 replicate exceeding the other 2 by more than 80%, possibly due to the distinct burrowing behavior in the replicate aquaria. Several burrow openings were observed in the aquaria with the lowest chemoautotrophy rates, while only 1 burrow was found in the most active aquarium. In the aquaria with more than 1 burrow, Nd individuals apparently rebuilt and changed burrow over the course of the experiment. PCA analysis further showed that the less active Nd burrow had a larger contribution of anaero-

bic bacterial biomarkers (ai15:0 and i15:0), suggesting that the burrow was occupied only shortly before sampling. In fact, the residence time of burrows is an important factor determining the succession and maturation of the microbial community (Marinelli et al. 2002). Therefore, the higher aerobic chemoautotrophy found in the aquarium with 1 Nd burrow suggests that stable burrows with steady ventilation strongly affect the chemoautotrophic bacterial community.

The effects of burrowing by *M. viridis* on the geochemistry and microbial community are opposite to those of *N. diversicolor*. *M. viridis* ventilates its blind-ended burrow 10 times less than *N. diversicolor*, which results in 75% air saturation at the burrow opening, which quickly decreases with depth along the burrow axis until anoxic conditions are obtained, interrupted by sporadic bursts of faint oxygenation (~8% air saturation) at the burrow end (Quintana et al. 2011, Jovanovic et al. 2014). Nonetheless, *M. viridis* has a stronger irrigation capacity due to deep burrowing and the percolation of return water through the sediment, which tends to dilute the pore water chemistry, as seen in the DIC and $\Sigma\text{H}_2\text{S}$ depth profiles over the top 10 cm (Fig. 3). Pore water is injected from the burrow into the surrounding sediment and is pushed upwards to the sediment–water interface through advective transport, which increases the efflux of pore water solutes (Quintana et al. 2007, Jovanovic et al. 2014). This transport induced by *M. viridis* translates into a 10-fold increase of the chemoautotrophic activity in the burrow compared with the Control subsurface sediment, but the chemoautotrophic activity still remains lower than that in the sediment surface. In agreement with the overall anaerobic conditions in the Mv burrow, the burrow sediment was characterized by biomarkers typical for sulfate reduction, such as i15:0, ai15:0, i17:1, 10Me16:0, 17:1 and 16:1 (Taylor & Parkes 1983, Edlund et al. 1985, Boschker & Middelburg 2002, Webster et al. 2006). A chemoautotrophy study by Miyatake (2011) found similar PLFA-SIP patterns to those in our Mv sediment, which were attributed to sulfate-reducing bacteria from the *Deltaproteobacteria* performing sulfur disproportionation. The enhanced chemoautotrophic anaerobic activity in the presence of *M. viridis* was, therefore, attributed to such *Deltaproteobacteria*.

Chemoautotrophy rates

A long-held notion is that burrows are interfaces between oxic and anoxic sediments, and serve as hot

spots of re-oxidation processes and hence of chemoautotrophic activity (Aller 1988, Kristensen & Kostka 2005). Apart from our study, only Reichardt (1988) directly quantified chemoautotrophic carbon fixation in the burrow of the lugworm *Arenicola marina* (~500 nmol C cm⁻³ d⁻¹). These chemoautotrophy rates are 8 times higher than the rates from Nd burrows and 48 times higher than the Mv treatment of our study. *A. marina* is a strong bioturbator, both in regards to particle reworking and burrow ventilation (Meysman et al. 2005), thus higher re-oxidation rates would be expected in burrows of lugworms rather than in Mv and Nd burrows (Fig. 4).

Aerobic chemoautotrophy rates obtained in our study (40 to 145 nmol C cm⁻³ d⁻¹) are in the lower range reported for coastal sediments characterized by non-mat-forming sulfur oxidizers (100 to 200 nmol C cm⁻³ d⁻¹ from 0 to 2 cm deep; Thomsen & Kristensen 1997, Lenk et al. 2011, Boschker et al. 2014). In organic-poor, permeable sediments, ~70% of the total dark CO₂ fixation can be attributed to sulfur-oxidizing *Gammaproteobacteria* not related to filamentous morphotypes (Lenk et al. 2011). However, much higher *in situ* chemoautotrophy rates (900 to 7000 nmol C cm⁻³ d⁻¹) have been found in surface sediments colonized by white mats of filamentous sulfur oxidizers (Boschker et al. 2014). White bacterial mats have been observed at Mv burrow openings in several studies (Kristensen et al. 2011, Quintana et al. 2013, Renz & Forster 2014), and white patches appeared on the surface of some of our incubations (Fig. 4). It is plausible that the upward flow of sulfide- and ammonium-rich pore water to the sediment–water interface, induced by the advective mode of pore water irrigation by *M. viridis*, favors the development of these microbial mats of filamentous sulfur oxidizers at the surface.

Alternatively, sulfate reducers within the *Deltaproteobacteria*, capable of sulfur disproportionation or H₂ oxidation, have been proposed as key players of anaerobic chemoautotrophy in subsurface sediments (Thomsen & Kristensen 1997, Miyatake 2011, Boschker et al. 2014). Anaerobic dark carbon fixation (10 to 40 nmol C cm⁻³ d⁻¹) is usually lower than aerobic chemoautotrophy, as seen in intact sediments with limited bioturbation (Enoksson & Samuelsson 1987, Lenk et al. 2011, Boschker et al. 2014) and in laboratory incubations with sieved sediment (Thomsen & Kristensen 1997, Vasquez-Cardenas et al. 2015). Higher chemoautotrophic activity in the subsurface than in surface sediment has been observed in heavily bioturbated sediment by the lugworm *A. marina* (Reichardt 1988), plausibly as a result of

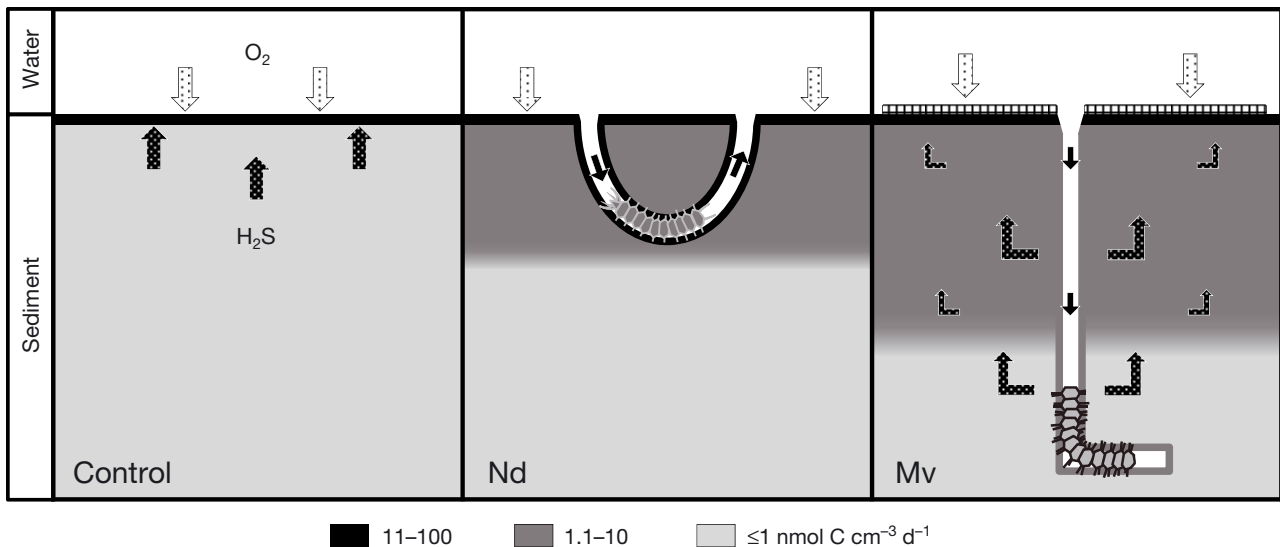


Fig. 4. A conceptual scheme summarizing the distribution of chemoautotrophic activity in the 3 treatments: Control (no bioturbation), *Nereis (Hediste) diversicolor* (Nd) and *Marenzelleria viridis* (Mv). Shading shows 3 ranges of chemoautotrophic activity. Black arrows in the sediment represent the direction of $\Sigma\text{H}_2\text{S}$ flux and white arrows in the overlying water indicate O_2 flux in the Control sediment. Burrows are U-shaped for *N. diversicolor* and L-shaped for *M. viridis*. Polychaetes are represented at the bottom of each burrow. Solid black arrows in burrows indicate the direction of pore water flow. Meshed areas at the burrow opening of *M. viridis* denote sulfur-oxidizing bacterial mat formations

the intense injection of oxic overlying water into the subsurface sediment (Meysman et al. 2005). However, we found limited chemoautotrophic activity in subsurface sediments (1 to 4 $\text{nmol C cm}^{-3} \text{d}^{-1}$), which accounted for less than 1% of the surface activity in non-bioturbated Controls and ~5% in bioturbated sediments. *N. diversicolor* and *M. viridis* bioirrigate to a much lesser degree than *A. marina* (Kristensen et al. 2014) and thus only slightly stimulate subsurface chemoautotrophic activity compared to non-bioturbated subsurface sediments (Fig. 4).

Trophic interaction

Hylleberg (1975) first introduced the concept of bacterial gardening in lugworms, where bacterial biomass is enhanced for feeding purposes by the lugworms as a result of particle mixing, excretion of fecal pellet, breakdown of particles through grazing or transport of solutes by ventilation (Aller & Yingst 1985). A study of the potential carbon sources of the head-down deposit feeder *Heteromastus filiformis* indicated a large dietary contribution by heterotrophic rather than chemoautotrophic bacteria (Clough & Lopez 1993). Alternatively, an increased survival of the polychaete *Capitella* sp. 1 has been reported in organic-rich, sulfidic coastal sediments, which was linked to the ingestion of chemoautotrophic

bacteria (Tsutsumi et al. 2001). Our stable isotope analysis also suggests that both *M. viridis* and *N. diversicolor* graze on chemoautotrophic bacteria in the sediment, given the observed ^{13}C enrichment. Comparison of excess ^{13}C fraction of the polychaetes with the corresponding chemoautotrophic activity showed that *M. viridis* can assimilate $0.017 \pm 0.003 \text{ d}^{-1}$ of the chemoautotrophic production, while *N. diversicolor* had a lower uptake rate of $0.010 \pm 0.002 \text{ d}^{-1}$. These results suggest a stronger grazing capacity of *M. viridis* on chemoautotrophic bacteria than *N. diversicolor*. Additionally, growth rates of *N. diversicolor* (0.020 to 0.035 d^{-1} ; Kristensen 1984, Christensen et al. 2000) suggest that 30 to 50% of its diet could be supplemented by chemoautotrophs. However, *N. diversicolor* can switch from filter to deposit feeding when phytoplankton concentrations are low in overlying water (Christensen et al. 2000), therefore the high grazing of benthic chemoautotrophic bacteria observed in our incubations may be a consequence of depleted phytoplankton stocks in the water reservoir. Conversion of our uptake rates to biomass increment resulted in $2.8 \pm 0.9 \text{ mg C ind.}^{-1} \text{ d}^{-1}$ for *M. viridis* and 6 times less for *N. diversicolor* ($0.5 \pm 0.1 \text{ mg C ind.}^{-1} \text{ d}^{-1}$). *M. viridis* has been shown to have turnover rates twice as high as *N. diversicolor*, which is in agreement with the opportunistic behavior exhibited by *Marenzelleria* spp. (Sarda et al. 1995).

CONCLUSIONS

In the absence of bioturbating fauna, chemoautotrophic activity and re-oxidation processes such as sulfur oxidation were primarily limited to the surface oxic–anoxic interface, given the depletion of oxidants in anoxic sediments (Fig. 4). In contrast, the 2 bioturbating polychaetes studied induced complex O₂ and H₂S dynamics in the sediment, modulating the composition of the microbial community and the associated metabolic activity. Strong ventilation by *Nereis (Hediste) diversicolor* extended the oxic microenvironments into the sediment, where aerobic dark carbon fixation through sulfide oxidation and nitrification were mainly promoted along the burrow whereas the slower pumping behavior combined with deeper burrows of *Marenzelleria viridis* enhanced anaerobic microbial metabolism (i.e. sulfate reduction and sulfur disproportionation) in subsurface sediment. Concomitantly, the upward pumping of sulfidic and nutrient-rich pore water by *M. viridis* may potentially promote the development of sulfur-oxidizing microbial mats on the surface, as seen in field observations (Kristensen et al. 2011, Quintana et al. 2013, Renz & Forster 2014). More targeted food web studies are necessary to determine the potential gardening of these microbial mats by *M. viridis*. In conclusion, the spatial distribution and magnitude of microbial processes, such as chemoautotrophy, can be strongly regulated by the species-specific functional traits of macrofauna, and the extent of these effects should be examined in intact coastal sediments.

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