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Chemosynthetic bacterial signatures in Frenulata tubeworm *Oligobrachia* sp. in an active mud volcano of the Canadian Beaufort Sea

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ABSTRACT: We performed bulk and compound-specific stable carbon isotope analyses to constrain specific carbon sources utilized for the chemosynthetic metabolisms of bacterial communities inhabiting the tube and worm of *Oligobrachia* sp. Together with bulk carbon isotopic compositions (–57.1 ± 1.2‰, mean ± SD) observed in the worm, the most depleted ^{13}C values of predominant fatty acids (FAs) (i.e. C16:1ω7 [–71.4 ± 2.9‰] and C18:1ω7 [–76.7 ± 4.3‰]) indicated that sulfur-oxidizing symbionts were preferentially utilizing anaerobic oxidation of methane-derived dissolved inorganic carbon (–31.6 ± 4.2‰), rather than methane (–59.5 ± 3.9‰), as a carbon source. In contrast, the isotopic signatures of FAs of the tube sections indicated that both autotrophic and heterotrophic bacterial communities utilized dissolved inorganic carbon supplied from ambient bottom seawater and sediment porewater. In this regard, the metabolisms of chemosynthetic bacterial communities inhabiting the tube may be regarded as potentially supporting tubeworm nutrition. Given that the tubeworm host incorporates locally adapted microbial communities, the isotopic signatures suggest that different micro-niches identified from the tube and the worm, particularly in relation to complex metabolic interactions, may be correlated with *in situ* microbial processes in sediment and bottom seawater.

KEY WORDS: Mud volcano · Siboglinid tubeworm · Sherlock microbial identification system · MIDI · Fatty acids · Carbon isotopic composition · $\delta^{13}C$

1. INTRODUCTION

Siboglinids are tube-dwelling marine annelids that lack a digestive track and thrive in obligatory association with endosymbionts housed in a particular organ, such as the trophosome (Felbeck 1981, Southward et al. 1986, 2005 and references therein). As traditionally recognized, the family Siboglinidae comprises 4 main lineages: (1) Vestimentifera, (2) Frenulata, (3) Monilifera, and (4) *Osedax* (Rouse 2001, Rouse et al. 2004, Halanych 2005). Among them, Frenulata, which represents 75% of all known siboglinids, has been the least studied lineage when it comes to chemosymbiotic associations, as they are typically diminutive (usually no more than a few mm in diameter and 10–20 cm in length) and often poorly preserved (Webb 1971, Southward et al. 2005, Hilário et al. 2011). Only a few studies have addressed Frenulata endosymbiont diversity, i.e. *Oligobrachia mashikoi* (Kimura et al. 2003, Kubota et al. 2007), *Oligobrachia* sp. JT-1 (Naganuma et al. 1997), *Siboglinum fiordicum* (Thornhill et al. 2008), *O. haakonmosbiensis* (Lösekann et al. 2008), and specimens of the genera *Lamellisabella*, *Polybrachia*, *Spirobrachia,* and *Bobmarleya* (Rodrigues et al. 2011).

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Endosymbionts can synthesize organic molecules from reduced compounds that are delivered to the host (including the trophosome) through the host's vascular and coelomic systems (Felbeck 1981, Cavanaugh 1985, Fisher 1990). In most cases, Frenulata harbors sulfur-oxidizing endosymbionts belonging to *Gammaproteobacteria* (Southward et al. 1986, Fisher 1990, Thornhill et al. 2008), but the presence of methane- and methyl-oxidizing endosymbionts has also been reported in several regions (e.g. the Skagerrak and the Gulf of Cadiz) (Schmaljohann & Flügel 1987, Rodrigues et al. 2011). The habitat-specific endosymbionts appear to have an ecologically flexible strategy that allows hosts to incorporate locally adapted microbial species (Won et al. 2003, Rodrigues et al. 2011). The tubes (including roots) provide structural support, allowing tubeworms to maintain their positions at the oxic–anoxic interface. Thus, they offer suitable habitats for the growth of microbial communities in accordance with environmental changes at the interface between water and sediment (Wahl et al. 2012). Previous studies reported the distinct microbial niche of chemosynthetic communities (e.g. *Riftia pachyptila* and *Lamellibrachia* sp.) associated with the tube, emphasizing the occurrence of both aerobic and anaerobic lineages in interior and exterior surfaces of the tube (López García et al. 2002, Duperron et al. 2009). Accordingly, metabolisms of bacterial groups (e.g. *Epsilon-* and *Gammaproteobacteria*) that colonize tubes may support chemosymbiotic associations between the host and its associated endosymbionts over a long period (Fisher et al. 1997, Duperron et al. 2009).

A previous study of the Håkon Mosby mud volcano showed that *O. haakonmosbiensis* harbors chemoautotrophic sulfur oxidizers rather than methane oxidizers as endosymbionts within the trophosome (Lösekann et al. 2008). However, the symbionts inhabiting *O. haakonmosbiensis,* and the metabolic interactions between the worm and its hosting tube, remain unknown. Recently, high Frenulata populations, with high similarity in the mitochondrial cytochrome c oxidase subunit I gene with *O. haakonmosbiensis* (~97%), were discovered in an active mud volcano of the Canadian Beaufort Sea (Paull et al. 2015). However, the carbon isotopic composition ($\delta^{13}C$) values (ca. −66.7‰) of the bulk tissue *Oligobrachia* sp. were not indicative of specific carbon sources (e.g. methane or dissolved inorganic carbon) for bacterial communities. In this study, we combined bulk stable carbon isotope analysis with compound-specific stable carbon isotope analysis of fatty acids (FAs) to constrain specific carbon sources utilized for chemosynthetic metabolisms of bacterial communities inhabiting the top, middle, and bottom of the tube and the worm of *Oligobrachia* sp.

2. MATERIALS AND METHODS

2.1. Site description and sample information

The active mud volcano investigated is located on the Canadian Beaufort continental slope at a depth of 420 m (Paull et al. 2011, 2015). Since its discovery in 2009 (see www.omg.unb.ca/Projects/Arctic/google for the data catalog), the presence of benthic chemosynthetic communities (e.g. tubeworms and bacterial mats) has been shown using a remotely operated vehicle (ROV) operated by the Monterey Bay Aquarium Research Institute (Paull et al. 2011, 2015).

For this study, a push core (ARA08C-DIVE104-10) was collected near the center of the mud volcano (70° 47′ 30″ N, 135° 33′ 21″ W, 420 m water depth) during the ARA08C expedition in 2017 with the RV 'Araon' using an ROV (Fig. 1). On board, *Oligobrachia* sp. specimens were roughly separated from ambient sediments by washing with distilled water. The specimens were then thoroughly washed in filtered seawater and stored at −80°C. The visual color of the collected *Oligobrachia* sp. clearly showed 3 transitions (black–red–grayish white) from top to bottom (Fig. 1). In the laboratory, ca. 10 individuals of these specimens were sectioned into 3 parts (i.e. top, middle, and bottom) along color transitions. Moreover, worms were separated from the trophosome positioned at the middle sections by dissection under a microscope (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m628p095_supp.pdf) and then washed 3 times in autoclaved water to prevent potential contamination with tube or sediment microbes. Finally, 3 parts (top, middle, and bottom) of the pooled tubes (n = 3 for each part) as well as the pooled worms (n = 3) were used for bulk and compound-specific isotope analyses.

2.2. Methane analysis

Upon sediment core recovery, the sediments were subsampled at 2 to 4 cm intervals, and then put into 20 ml serum vials for methane analyses. Serum vials were sealed with butyl rubber stoppers immediately after subsampling to prevent gas exchange and stored at 4°C. In the laboratory, these vials were shaken thoroughly to equilibrate porewater methane

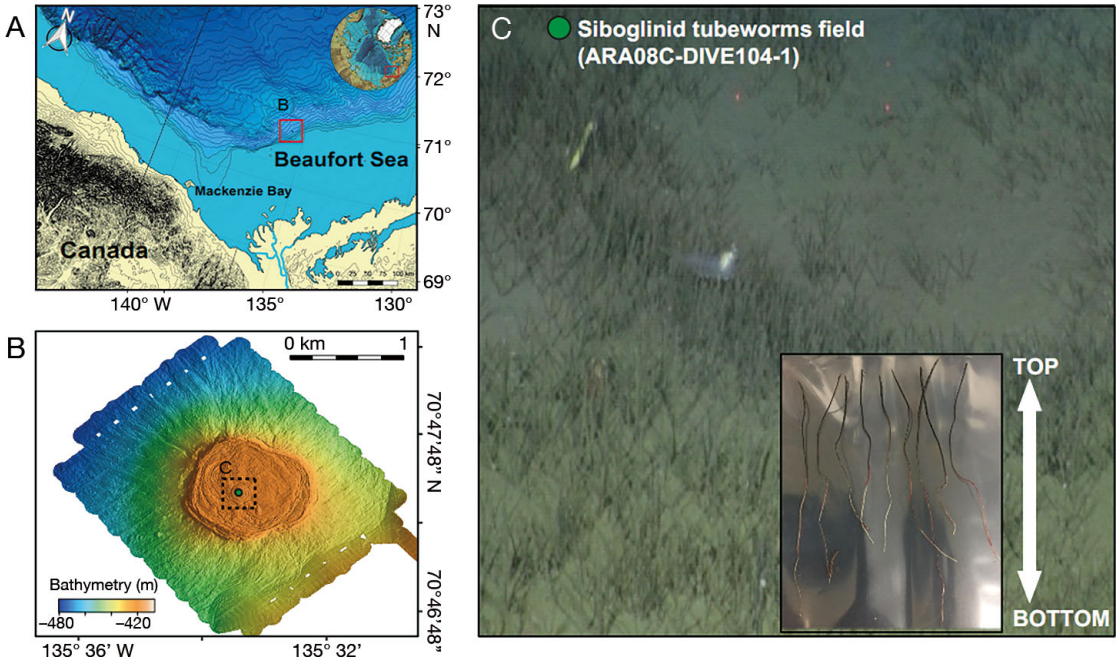

Fig. 1. (A) General bathymetric map of the continental slope of the Canadian Beaufort Sea, (B) mud volcano at a water depth of 420 m with the siboglinid tubeworm sampling site shown by dotted black box, and (C) siboglinid tubeworm field, identified as *Oligobrachia haakonmosbiensis*, and collected specimens

between aqueous and gas phases. To measure the $\delta^{13}C$ values of methane, a 1 ml aliquot of the headspace was injected into an isotope ratio mass spectrometer (Finnigan MAT 252, Thermo Fisher Scientific) with a Combustion III interface (Thermo Fisher Scientific) at Nagoya University. Values were reported as per mille (‰) using δ notation, relative to the standard Vienna Pee Dee Belemnite (VPDB) for carbon. The $\delta^{13}C$ values were calibrated using a methane standard with a $\delta^{13}C$ value of −35.2‰, certified by the US National Institute of Standards and Technology. The precision for $\delta^{13}C$ determinations was better than ±0.2‰.

2.3. Dissolved inorganic carbon analysis

Seawater was filtered through a 0.20 µm disposable polytetrafluorethylene in-line filter. Porewater was extracted from the holes drilled at 2 to 3 cm intervals down the core liner using rhizon samplers connected to 20 ml syringes. Extracted porewater was filtered through a 0.20 µm disposable polytetrafluorethylene in-line filter. The dissolved inorganic carbon (DIC) samples for $\delta^{13}C$ analysis were collected in 2 ml glass vials and preserved with 10 µl of saturated $HgCl_2$ under 4°C. The $\delta^{13}C$ values of DIC were obtained using an isotope ratio mass spectrometer (Finnigan DELTAplusXL, Thermo Fisher Scientific) with a Finnigan GasBench II headspace autosampler at Oregon State University (USA). Values were reported as per mille (‰) using δ notation, relative to the standard VPDB for carbon. The $\delta^{13}C$ values were calibrated using an internal laboratory water standard with a $\delta^{13}C$ value of −0.4‰, certified by Oregon State University. The precision for $\delta^{13}C$ determinations was better than ±0.02‰.

2.4. Bulk stable carbon isotope analysis

All triplicate samples (i.e. tube sections and worms) were freeze-dried, homogenized, and acidified to remove any inorganic carbon. All samples were treated with 200 µl 1M HCl (Junsei Chemical), using silver capsules (10 × 10 mm). These samples were dried at 60°C for 6 h and analyzed for bulk stable carbon isotopic compositions. The $\delta^{13}C$ values of each sample were measured via CO_2 generated by an elemental analyzer (vario PYRO cube, Elementar) coupled with an isotope ratio mass spectrometer (Isoprime 100, Isoprime). Values were reported as per mille (‰) using δ notation, relative to the standard VPDB for carbon. The $\delta^{13}C$ values were calibrated using a CH_3 standard with a $\delta^{13}C$ value of −24.7‰ certified by the International Atomic Energy Agency

(Austria). The precision for $\delta^{13}C$ determinations was better than ±0.1‰.

2.5. Lipid extraction and preparation

For FA analysis, all samples in triplicate were freeze-dried, homogenized, and then ultrasonically extracted (3×) with solvent mixtures (dichloromethane [DCM]: methanol [MeOH], 2:1 v/v). An internal standard (i.e. nonadecanoic acid) of known concentration (10 µg ml^{-1}, 50 µl injection) was added prior to extraction. The total lipid extract was passed over anhydrous Na_2SO_4, and then reacted with 500 µl 14% BF_3 in MeOH at 60°C for 10 min to yield FA methyl esters. After cooling, methylated FAs were extracted 4 times with 1 ml DCM in the presence of 500 µl distilled water. Combined extracts were dried over anhydrous Na_2SO_4, dissolved in 100 µl *n*-hexane, and stored at −20°C until further analysis.

The double-bond positions in methylated FAs were determined by analysis as dimethyl disulfide (DMDS) adducts, according to previous publications (Nichols et al. 1986, Elvert et al. 2003). In brief, the aliquot of methylated samples was treated with 100 µl DMDS and 20 µl iodine solution (6% w/v in diethyl ether) at 50°C for 48 h. After cooling, 500 µl *n*-hexane was added, and excess iodine was reduced with 500 µl sodium thiosulfate solution (5% w/v in water). Combined organic phases were diluted with 50 µl *n*-hexane prior to further analysis. Methylated FAs were further purified over Ag+-impregnated silica columns to separate saturated, monounsaturated, and polyunsaturated fractions using hexane:DCM (1:1 v/v), hexane:ethyl acetate (4:1 v/v), and acetone, respectively.

2.6. Fatty acid analysis

Methylated FAs were first analyzed by GC using the Sherlock microbial identification system (MIDI) consisting of an Agilent 7890 Series and a flame ionization detector (FID) equipped with an HP Ultra 2 phenyl methyl silicone fused capillary column (25 m length, 0.2 mm inner diameter [i.d.], 0.33 µm film thickness), an automated sampler, and computer with associated software (Sherlock Pattern Recognition Software, MIDI). The methylated FAs were identified based on their retention times and relatively quantified.

Concentrations of methylated FAs were also determined by GC analysis using a DB-5 silica capillary column (60 m length, 0.25 mm i.d., 0.25 µm film thickness) in an Agilent 7890 Series with a split/splitless injector operated in splitless mode and an FID. The initial oven temperature was set to 60°C, subsequently increased to 130°C at a rate of 20°C min^{-1}, raised again to 320°C at a rate of 4°C min^{-1}, and held for 40 min. The carrier gas was He at a constant flow rate of 1.0 ml min^{-1}. The injector temperature was set at 290°C and the detector at 300°C. A concentration for each FA compound was calculated relative to the internal standard present in the FA fraction.

Methylated FAs were identified by GC-MS using an Agilent GC interfaced to a 5977E MS. The GC-MS was operated in electron impact mode at 70 eV with a full scan mass range of m/z 50 to 800 (cycle time of 0.9 s, resolution of 1000). The GC was equipped with a fused silica capillary column (HP-5MS, 25 m length, 0.25 mm i.d., 0.10 µm film thickness) with carrier gas (He, 1.2 ml min^{-1}). The samples were injected in the splitless mode and subjected to the same temperature program given for GC analysis. Molecular structures were determined by comparing their mass spectral fragmentation patterns and retention times with previously published data (Lösekann et al. 2008).

2.7. Compound-specific stable carbon isotope analysis

The $\delta^{13}C$ values of methylated FAs were determined by GC combustion isotope ratio MS (GC-C-IRMS). An isotope ratio mass spectrograph (visION, Isoprime) was connected with a GC (Hewlett Packard 7890 N series, Agilent Technologies) via a combustion interface (a glass tube packed with copper oxide and operated at 850°C). The samples were subjected to the same temperature conditions and capillary column described for the GC-MS analyses. Calibration was performed by injecting several pulses of reference gas CO_2 of known $\delta^{13}C$ value at the beginning and end of each sample run. Isotopic values are expressed as $\delta^{13}C$ values in per mille (‰) relative to VPDB. The correlation coefficients (r^2) of the known $\delta^{13}C$ values of certified isotope standards with the average values of the measured samples were higher than 0.99. In the case of methylated FAs, $\delta^{13}C$ measurements needed to be corrected for the additional carbon atoms for the derivatization with BF_3–MeOH. In this study, the $\delta^{13}C$ value of the introduced methyl group was −45.6 ± 0.4‰. To monitor the accuracy

and precision of the GC-C-IRMS performance, standards with known $\delta^{13}C$ values were repeatedly analyzed every 5 or 6 sample runs. Standard deviations of carbon isotope measurements were generally better than ±0.4‰, as determined by repeated injections of the standard. Finally, the analytical precision of FAs was better than ±0.5‰ as determined by repeated (more than 3) measurements of each sample.

2.8. Statistical analysis

Based on the fractional abundances of FAs obtained from the MIDI, principal component analysis (PCA) was performed using R software version 3.4.2 (package information, FactoMineR, Lê et al. 2008) to provide a general view of the variability of the FA profile distributions. For all statistical analyses, the fractional abundances of selected FAs were transformed using *z*-score normalization, which was calculated with data having a mean of zero and an SD of 1. We also performed a non-parametric Mann-Whitney *U*-test, which does not meet the normality assumption of the 1-way analysis variance to evaluate the differences in mean values between 2 different groups.

3. RESULTS AND DISCUSSION

3.1. Bulk organic carbon profiles

The bulk $\delta^{13}C$ values of each sample (e.g. tube sections and worms) ranged from −57.1 ± 1.2 to −49.9 ± 2.6‰ (Fig. 2), mostly overlapping for the given samples, except for the top of the tube sections. Among them, the bulk $\delta^{13}C$ values of worms were the most ^{13}C depleted compared to organic sources (−10 to −35‰) derived from photosynthetic origin or dissolved chemical species in sea-

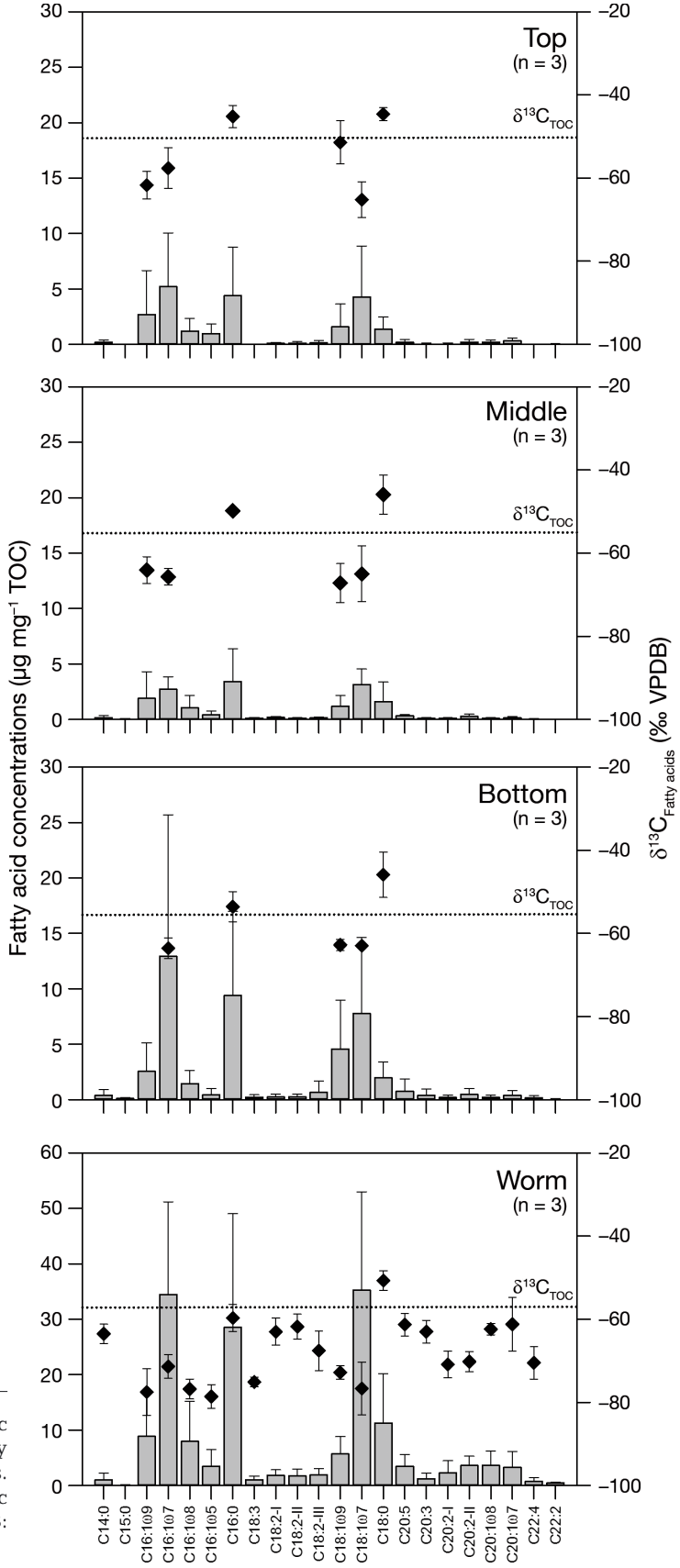

Fig. 2. Concentrations (gray bar) and carbon isotopic composition ($\delta^{13}C$) values (closed diamond) of fatty acids (FAs) extracted from the tube sections and worms. Black dotted lines indicate $\delta^{13}C$ values of total organic carbon (TOC) in each sample. Error bars: SD. VPDB: Vienna Pee Dee Belemnite

water (Paull et al. 1985, Thurber et al. 2010, Feng et al. 2015). This may be partially attributable to methane oxidizers (below ca. –66‰) using a carbon source highly depleted in ^{13}C such as biogenic methane (Paull et al. 1985, 2015, MacAvoy et al. 2002, Thurber et al. 2010). However, sulfur oxidizers can also have similar isotopic values ($\delta^{13}C \approx -72$‰) by incorporating ^{13}C-depleted DIC derived from anaerobic oxidation of methane (AOM) and by further fractionation in autotrophic assimilation pathways (Nelson & Fisher 1995, Boetius et al. 2000, Lösekann et al. 2008, Thurber et al. 2010). With respect to the previous study for symbiotic associations in *Oligobrachia haakonmosbiensis* and the presence of thiotrophic symbionts (Lösekann et al. 2008), it may be inferred that *O. haakonmosbiensis* synthesizes organic carbon produced by the metabolism of potentially thiotrophic symbionts. However, the overlapping values of bulk $\delta^{13}C$ among samples seem to reflect mixed contributions between methane- and sulfur-oxidizing bacterial sources. Accordingly, to distinguish the intrinsic bacterial sources in relation to the bulk $\delta^{13}C$ signatures in our samples, we further investigated FA compositions and their $\delta^{13}C$ signatures.

3.2. Fatty acid profiles

In total, 26 FAs were identified based on the MIDI with the dominance of C16:1ω7 (31.6 ± 2.4%) followed by C18:1ω7 (24.3 ± 7.1%) (Table S1 in the Supplement). The results of the MIDI were similar to those obtained from the GC and GC-MS analyses (Fig. 2, Fig. S2 in the Supplement). The FA concentrations of the worms were, in general, much higher than those of the tube samples, indicating predominance of C16:1ω7 (34.4 ± 16.7 µg mg^{-1} total organic carbon [TOC]) and C18:1ω7 (35.3 ± 17.7 µg mg^{-1} TOC) (Fig. 2, Table S2 in the Supplement). The MIDI FA compositions of all samples were further examined by PCA (Fig. 3). The first PC component (PC1) accounted for 48.5% of the total variance, and the second PC component (PC2) explained 18.4% of the total variance. The unsaturated FAs (especially C16:1ω7 and C18:1ω7) were responsible for the separation along the PC1 axis. All worms with the highest abundance of C16:1ω7 (34.6 ± 1.1%) and C18:1ω7 (33.9 ± 2.9%) were found on the left side of the biplot (Fig. 3), while a few tube sections with some branched FAs (on average 0.8%) were positioned on the right side (Fig. 3). Of the saturated forms, even-carbon-numbered FAs (e.g. C16:0 and C18:0) were responsible for the distribution along the PC2 axis. One of the bottom sections with relatively high abundances of these acids (23.2–25.5%) was found in the upper right part of the biplot (Fig. 3).

Interestingly, the PCA results showed that the worm groups were close to each other and apart from the tube samples (Fig. 3). Our results are therefore consistent with previous findings that distinctive bacterial communities thrive in the worm in accordance with the use of specific carbon sources during their endosymbiotic metabolisms (Hilário et al. 2011). However, tube sections did not show a consistent FA pattern within each section (Fig. 3). Such patterns may provide evidence for contributions of mixed types of bacterial communities (e.g. nitrifying and sulfur-oxidizing bacteria) inhabiting the tube (Blumer et al. 1969, Lipski et al. 2001, Inagaki et al. 2004, Zhang et al. 2005). Furthermore, chemoautotrophic organisms could be metabolized via different carbon fixation

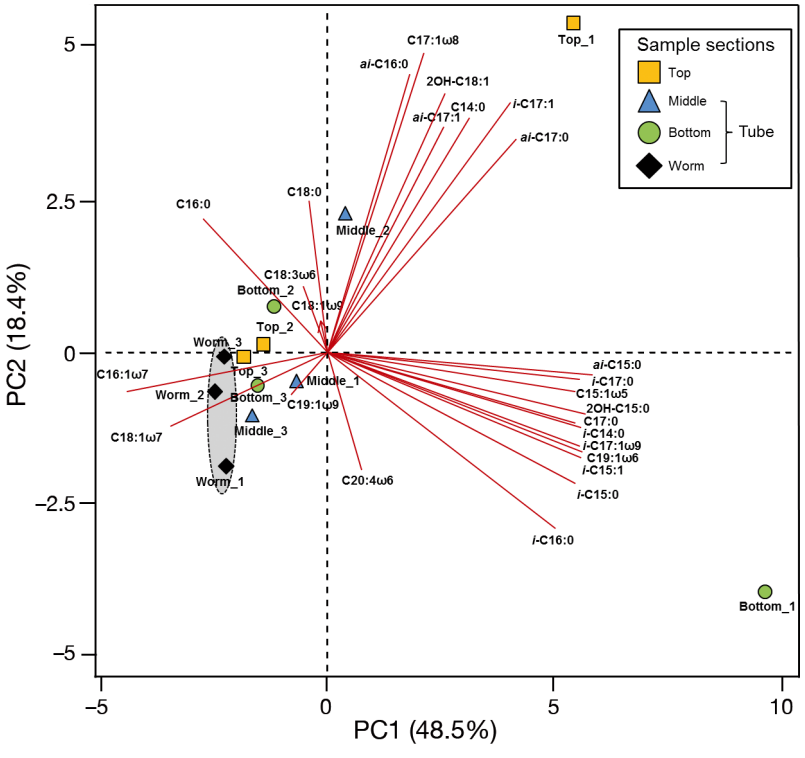

Fig. 3. Principal component analysis for fatty acid compositions obtained from the Sherlock microbial identification system. The grey shaded ellipse highlights 3 worm samples as a group

pathways, causing large differences in carbon isotopic fractionation (Hügler & Sievert 2011). In this regard, we further investigated $\delta^{13}C$ values of FAs to explore potential carbon sources utilized by different bacterial communities in more detail.

3.3. Specific carbon sources utilized by bacterial communities

Although it is difficult to assign all FAs to specific microbial groups or processes, some specific compounds are indicative of certain taxonomic groups (Kharlamenko et al. 1995, Van Gaever et al. 2009), especially when they occur at relatively high abundances. The FA patterns showed similar compositions in all samples (Fig. 3), but they differed significantly in the FA amounts (Fig. 2). The predominant FAs were C16:1ω7 and C18:1ω7, especially in the worms (Fig. 2, Table S2), probably resulting from discriminative metabolisms of the related bacterial communities. The high amounts of monounsaturated FAs, including ω7, may reflect specific habitat conditions because the FA desaturation pathway in bacteria preferentially leads to the production of ω7 isomers in anaerobic conditions (Alexandrino et al. 2001). The $\delta^{13}C$ values of C16:1ω7 and C18:1ω7 were on average −71.4 ± 2.9 and −76.7 ± 4.3‰, respectively (Fig. 4, Table S3 in the Supplement), consistent with other generally ^{13}C-depleted FA values in the worms (Table S3, Fig. S3 in the Supplement). These unsaturated FA isotopic values were ~42 and ~70‰ lower than $\delta^{13}C_{DIC}$ values in sediment porewater (anoxic layer) and bottom seawater, respectively, which were measured at the Beaufort mud volcano (Figs. 4 & 5). Previous studies reported that the *O. haakonmosbiensis* symbionts possessing the genes RubisCO form I and form II were associated with the potential metabolism for autotrophic CO_2 fixation from sulfur oxidizers in habitats connected to the active AOM process (Niemann et al. 2006, Lösekann et al. 2008). Compared to the isotopic fractionations (as negative as −95‰) of methanotrophs taking place during lipid syntheses (Summons et al. 1994), RubisCO form I and form II have a fractionation effect of 18 to 30‰ (Guy et al. 1993, Robinson et al. 2003, Scott et al. 2004). Thus, the $\delta^{13}C$ values of unsaturated FAs in the worms may provide a clue for chemoautotrophic fixation utilizing much lighter ambient porewater DIC coupled to the isotopic fractionation of RubisCO form I and form II. Possibly, *Oligobrachia* sp. harbors dominant sulfur oxidizers that utilize AOM-derived DIC rather than methane as a carbon source. However, the $\delta^{13}C$ values of unsaturated FAs (−79.2 to −70.4‰) were more depleted than those expected for RubisCO form I and form II (Fig. 4, Fig. S3). Thus, the

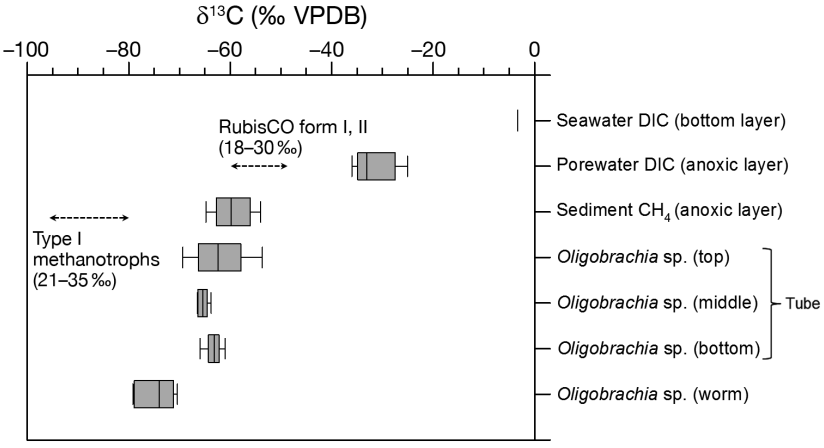

Fig. 4. Carbon isotopic composition ($\delta^{13}C$) signatures of specific fatty acids (FAs) (C16:1ω7 and C18:1ω7) for each sample and different carbon sources in the Beaufort mud volcano. The midpoint of a boxplot is the median; the 25 and 75% quartiles define the hinges (end of the boxes); the difference between the hinges is the spread; lines are drawn from each hinge to 1.5× the spread. The potential ranges for $\delta^{13}C$ signatures for autotrophic CO_2 fixation (based on RubisCO form I and form II fractionation) and methane oxidation (based on type I methanotroph fractionation) are shown as dotted lines. Note that the values indicate the fractionation factors for each process, reported by Guy et al. (1993) and Summons et al. (1994). VPDB: Vienna Pee Dee Belemnite; DIC: dissolved inorganic carbon

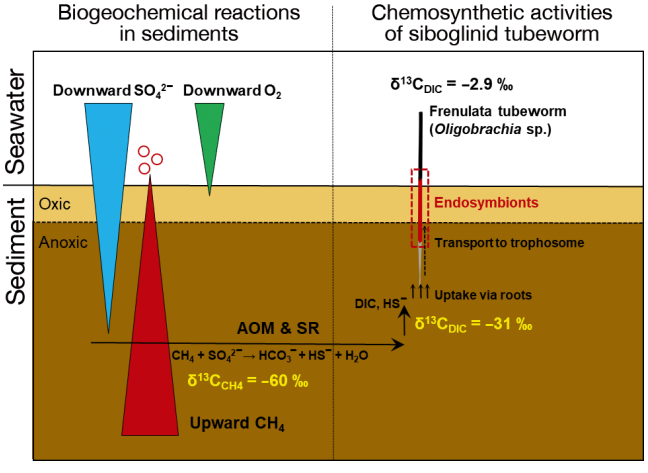

Fig. 5. Schematic mechanisms for the metabolism of chemosynthetic bacterial communities inhabiting siboglinid tubeworms. Fluid flows are represented by triangles (red for methane, blue for sulfate, and green for oxygen). Black arrows denote biogeochemical reactions and energy flows. DIC: dissolved inorganic carbon; AOM: anaerobic oxidation of methane; SR: sulfate reduction

depleted isotopic signatures observed in the worms cannot be solely explained by chemoautotrophic carbon fixation. Given that the frenulate could take up dissolved organic compounds (e.g. amino acids) through its epidermis (Southward & Southward 1981), isotopically light dissolved organic carbon produced from tube- or sediment-inhabiting bacteria could be used as an additional or alternative carbon source in the worms. Alternatively, this may be associated with an unexpected isotopic fractionation during an additional autotrophic carbon fixation, such as reductive tricarboxylic acid cycle (Hügler & Sievert 2011). However, with the current data set, it is difficult to elucidate a responsible process, and more research is needed.

The overall FA composition patterns and their $\delta^{13}C$ values observed at all tube sections appear to be ambiguous when it comes to the identification of bacterial communities inhabiting these sections (Fig. 3). Nevertheless, the isotopic signatures of FAs slightly differed from each other (Fig. S3). Corresponding to the bulk $\delta^{13}C$ values observed in the top tube, the most enriched $\delta^{13}C$ values of saturated (–44.9 ± 2.0‰) and unsaturated (–59.6 ± 6.4‰) FAs showed differences in comparison with those of other tube sections (i.e. middle and bottom) and worms. Moreover, these FA isotope values were similar to the identified $\delta^{13}C$ values of thiotrophic endosymbionts in benthic organisms (e.g. *Bathymodiolus aduloides*) with little isotopic variation (Becker et al. 2010, Feng et al. 2015). A possible carbon source related to the relatively enriched $\delta^{13}C$ values of identified FAs is likely DIC in bottom seawater taken up by chemoautotrophic bacterial communities (e.g. nitrifying or thiotrophic bacteria) under aerobic conditions. Considering that these bacterial groups fractionate isotopic values up to ~30‰ during carbon fixation (Hügler & Sievert 2011), we may expect $\delta^{13}C$ values of –30.2‰ for FAs in the top tube, if bottom seawater is the main carbon source. However, the measured FA $\delta^{13}C$ values (–54.7 ± 6.4‰) were more depleted than expected, indicating the presence of an additional source, such as sediment porewater (Fig. 4, Fig. S3). These results suggest that chemoautotrophic bacterial communities use DIC supplied from sediment porewater for the chemoautotrophic CO_2 fixation rather than from bottom seawater.

Overall, branched FAs were less abundant in the worms than in the tube sections (Fig. 3, see also Table S1 in the Supplement). Branched FAs are generally reported as indicators in anaerobic bacteria and sulfate-reducing bacteria (Volkman & Johns 1977, Kaneda 1991, Elvert et al. 2003, Niemann et al. 2006). In this regard, iso- and anteiso-C15:0, iso- and anteiso-C17:0, and iso-C17:1ω9 are the FA signatures for sulfate-reducing bacteria (*Desulfobacter* spp.) (Smith et al. 1986, Rajendran et al. 1992). Therefore, the isotopic signatures of identified FAs in bottom sections appear to be derived from autotrophic and/or heterotrophic bacterial communities in the sediments (Fig. 4, Fig. S3). Considering that the tube itself acts as a direct conduit for seawater sulfate down to the subsurface environment (Olu-LeRoy et al. 2007), this mechanism may lead to enhanced sulfate reduction and, as a consequence, sustained production of dissolved sulfide under oxygen-depleted conditions in sediment (Duperron et al. 2009). Our results suggest that the bottom tube sections (including roots) may be able to create suitable conditions for the growth of sulfate-reducing bacteria. Ultimately, it implies that they might influence the *Siboglinidae* endosymbiotic system via their metabolic interactions (Fig. 5). The potential contribution of heterotrophic bacterial communities may also be considered from the presence of other branched FAs (Dalsgaard et al. 2003), which were detected in the bottom tube, but these possibilities require further investigation.

4. CONCLUSIONS

We investigated FA compositions and their $\delta^{13}C$ signatures in the tube and the worm of the frenulate siboglinid *Oligobrachia* sp. to obtain some hints on the distributions of bacterial communities. The depleted $\delta^{13}C$ values (ca. –74.1‰) in both FAs (C16:1ω7 and C18:1ω7) indicated that sulfur-oxidizing symbionts were dominant in the worm, utilizing AOM-derived DIC (–31.6 ± 4.2‰) rather than methane (–59.5 ± 3.9‰) as a carbon source. Thus, our results suggest that AOM-derived DIC may be involved in carbon uptake of endosymbionts inhabiting *Oligobrachia* sp. Additionally, the enriched-^{13}C values (–44.9 ± 2.0 and –59.6 ± 6.4‰, respectively) of saturated and unsaturated FAs in the top tube sections differ from the depleted values (–64.6 ± 8.7‰) of overall FAs detected in other tube sections, including the worm, indicating a carbon fixation from mixed sources of bottom seawater and sediment porewater. The presence and related metabolisms of other bacterial communities were inferred from putative isotope fractionations, reflecting chemoautotrophic processes using ambient carbon sources under oxic–anoxic conditions occurring from the top to bottom of the tube sections. Moreover, it is expected that the tube

may also host aerobic bacterial groups and sulfate-reducing bacteria, which could supply additional energy sources for tubeworm metabolism, although the contribution may be minor. Consequently, different tube sections appear to function differently to support the Siboglinidae symbiotic system by possessing diverse bacterial communities. Given that the tubeworm host incorporates the locally adapted microbial communities, further studies need to compare *in situ* microbial processes occurring in siboglinid tubeworm-dominated sediments to identify key factors controlling the tubeworm endosymbiotic systems.

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