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Early diagenesis in the sediments of the Congo deep-sea fan dominated by massive terrigenous deposits: Part III – Sulfate- and methane- based microbial processes

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Abstract :

Geochemical profiles (SO42-, H2S, CH4, δ13CH4) and phylogenetic diversity of *Archaea* and *Bacteria* from two oceanographic cruises dedicated to the lobes sediments of the Congo deep-sea fan are presented in this paper. In this area, organic-rich turbidites reach 5000 m and allow the establishment of patchy cold-seep-like habitats including microbial mats, reduced sediments, and vesicomyid bivalves assemblages. These bivalves live in endosymbiosis with sulfur-oxidizing bacteria and use sulfides to perform chemosynthesis. In these habitats, unlike classical abyssal sediments, anoxic processes are dominant. Total oxygen uptake fluxes and methane fluxes measured with benthic chambers are in the same range as those of active cold-seep environments, and oxygen is mainly used for reoxidation of reduced compounds, especially in bacterial mats and reduced sediments. High concentrations of methane and sulfate co-exist in the upper 20 cm of sediments, and evidence indicates that sulfate-reducing microorganisms and methanogens co-occur in the shallow layers of these sediments. Simultaneously, anaerobic oxidation of methane (AOM) with sulfate as the electron acceptor is evidenced by the presence of ANMEs (ANaerobic MEthanotroph). Dissolved sulfide produced through the reduction of sulfate is reoxidized through several pathways depending on the habitat. These pathways include vesicomyid bivalves uptake (adults or juveniles in the bacterial mats habitats), reoxidation by oxygen or iron phases within the reduced sediment, or reoxidation by microbial mats.

Sulfide uptake rates by vesicomyids measured in sulfide-rich sea water (90 ± 18 mmol S m-2 d-1) were similar to sulfide production rates obtained by modelling the sulfate profile with different bioirrigation constants, highlighting the major control of vesicomyids on sulfur cycle in their habitats.

1 INTRODUCTION

Organic carbon mineralization rates in abyssal (4000-6000 m) sediments are often low (Middelburg 1997), mainly because the organic material reaching the seafloor is limited and of low reactivity (Wakeham *et al.* 1997). Aerobic respiration generally dominates organic matter remineralization in these sediments, implying deep oxygen diffusion in sediments (Glud 2008). Denitrification, metal oxide-reduction, dissimilatory sulfate-reduction (DSR), and methanogenesis are then often limited (Burdige 2006) due to the rapid disappearance of organic carbon at depth. Detecting high organic carbon concentrations (several %) at such depths, as in the terminal lobes of the Congo deep-sea fan (Rabouille *et al.* 2009, Baudin *et al.* 2010, Stetten *et al.* 2015) is thus highly unusual. This area is fed by turbidites carrying terrestrial material directly from the Congo River through an active channel-levee system (Vangriesheim et al. 2009, Babonneau et al. 2010). Massive loading of particles associated with reactive organic matter to the sediment are known to increase the relative importance of sulfate reduction in the sediment (Canfield 1989). During the first exploration of the terminal lobes of the Congo deep-sea fan in 2011 (WACS campaign in February 2011 (Olu 2011)), cold-seep like habitats such as bacterial mats and vesicomyid bivalves assemblages were discovered (Sen *et al.* 2017, Rabouille *et al.* this issue). These bivalves live in endosymbiosis with sulfur-oxidizing bacteria. These habitats are known to carry out chemosynthesis and are commonly fed by dissolved sulfides (Treude *et al.* 2009), such that the high input of terrestrial organic carbon through turbidites has been hypothesized to be responsible for high rates of sulfate-reduction and significant biogenic methane production in this area. Anaerobic oxidation of methane (AOM), using sulfate as electron acceptor, is also likely to occur and produce a large amount of sulfide available in these habitats. AOM is carried out by a consortium of anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria (SRB) that have been first reported in a cold seep at the Eel River Basin (Hinrichs *et al.* 1999) and other similar environments (Elvert *et al.* 1999, Pancost *et al.* 2000). Sulfate is not the only electron acceptor that can be used during AOM, however. Other electron acceptors such as nitrite and nitrate (Haroon *et al.* 2013), metal oxides Mn^{4+} and Fe^{3+} (Beal *et al.* 2009) could be used and be potentially energetically more favorable (Caldwell *et al.* 2008). AOM is thus a complex process that has yet to be evidenced in such a system.

The overall objective of this study was to gain insights in the carbon and sulfur cycles in such a dynamic system. Thus, in addition to the first exploration cruise conducted in 2011, a second cruise dedicated to the lobe sediments of the Congo deep-sea fan took place from December 2011 to January 2012 (Rabouille 2011). In this paper, we examine the chemical profiles of SO_4^{2-}, H_2S, CH_4, $\delta^{13}C\text{-}CH_4$ and identify the phylogenetic diversity of *Archaea* and *Bacteria* in sediment cores from three stations located along the main feeding channel. These data are presented and discussed in the context of the stations location and the type of habitats, including sediments with visible surface features such as black or/and white zones, the latter could be bacterial mats, and vesicomyid bivalves assemblages (Rabouille *et al.* this issue). This paper addresses two main objectives: 1) investigation of the potential processes dominating these habitats, including DSR, methanogenesis, and AOM; and 2) characterization of the phylogenetic diversity of bacterial and archaeal communities especially those involved in methane cycling in surficial (< 15 cm) organic-rich sediments where high concentrations of methane and sulfate co-exist.

2 MATERIALS AND METHODS

2.1 Study site

The Congo canyon, located on the west coast of Africa, is a unique example of a canyon directly connected to a major river (The Congo River). This giant canyon is presently connected to the river estuary through an incision in the continental shelf (Babonneau *et al.* 2010). This connection with the second largest river in the world by its water discharge (Milliman 1991) allows a massive transfer of particulate matter through the canyon as a result of strong turbidity currents (Savoye *et al.* 2000, Savoye *et al.* 2009). These turbidites are responsible for a large input of terrestrial organic matter

(Treignier *et al.* 2006, Baudin *et al.* 2010, Stetten *et al.* 2015, Baudin *et al.* this volume) at depths down to 5000 m, leading to the development of different types of habitats, including deeply reduced sediments, bacterial mats, and vesicomyid assemblages (Sen *et al.* 2017, Rabouille *et al.* this issue).

Six sampling sites were explored during two cruises as described in (Rabouille *et al.* this issue). Three sites, where most of the reduced habitats are located, were intensively sampled and are presented in this paper (Figure 1). Briefly, site A is located on the feeding channel, at the entrance of the lobe complex at 4755 m deep. Site B is located 10 km away from the active channel, at water depth of 4830 m, and is part of an older lobe. Those two sites are still fed by turbidites overflow out of the main channel. Finally, site C is located at the end of the main feeding channel at a depth of 4940 m. At this site the channel widens and the most recent and active lobe develops.

The vesicomyids habitats sampled within the three sites contains between 90 and 1200 individuals per meter square (ind m^{-2})) of vesicomyid bivalves (two species: *Christineconcha regab* and *Abyssogena southwardae) (Decker et al. this issue)*.

2.2 Sediment coring

Push cores (CT) with a diameter of 8 cm were retrieved precisely on selected habitats using the remotely operated vehicle (ROV) Victor 6000. The sediment core retrieved at site A (about 500 ind m^{-2}, predominantly *C. regab (92%)*) was taken within the assemblage. The core retrieved at site B (about 90 ind m^{-2}, mostly *A. southwardae (67%) (Teixeira et al. 2013)*) was not placed on visible bivalves. The core retrieved at site C (>1100 ind m^{-2}, mostly *C. regab (98%)* was taken within the assemblage, and an individual was retrieved from this core during sediment slicing (*C. regab*). On board sediment cores were immediately transferred into the cold room (~4°C). The pore waters were extracted every 1 cm using Rhizon soil moisture sampler (SMS) devices (5 cm length) and immediately split and preserved: One aliquot was transferred into a N_2-purged glass vial closed with a septum for CH_4 and $\delta^{13}C$-CH_4 analyses; another aliquot was fixed using $ZnCl_2$ (saturated) for H_2S analysis; and a last aliquot was acidified with 2µl of 69% nitric acid for SO_4^{2-} analyses. Another core was subsampled aseptically for molecular analyses in 2 or 5 cm thick layers using 2 mL cut-off syringes and immediately frozen at -80°C for nucleic acid extractions.

2.3 Benthic fluxes measurements

Total oxygen uptake (TOU) fluxes as well as CH_4 fluxes were measured using a CALMAR benthic chamber as described previously (Khripounoff *et al.* 2015). Briefly, a Calmar benthic chamber was deployed on selected habitats by the ROV Victor 6000. Incubations lasted about 3 h with water sampling every 30 min. Oxygen was continuously recorded using an Aanderaa® optode placed within the chamber, and calibrated by chemical analysis of 10 ml of sampled water using the modified Winkler titration method (Carrit and Carpenter 1966). Fluxes were calculated by multiplying the slope of the linear regression by the chamber volume divided by the surface area.

2.4 Vesicomyids gills incubations

Specimens were collected at the terminal lobes of the Congo deep-sea fan at site C (Table 1, Figure 1), inside a vesicomyid assemblage called “vesicomyid heart” (Rabouille *et al.* this issue). This site was densely colonized by *Christineconcha regab* (Krylova and Von Cosel 2011, Decker *et al.* this issue). All specimens were sampled using a ROV-manipulated net. For this study, three specimens of *C. regab* were used (same size). They were morphologically identified (E. Krylova, pers. com), measured and dissected onboard. A part of the adductor muscle was frozen for DNA analysis to confirm morphological identification (S. Teixeira and S. Arnaud-Haond, unpubl. results). One gill per specimen was dissected for sulfide consumption measurements. Estimates of sulfide consumption by gill tissue were obtained using a protocol adapted from a previous study (Goffredi and Barry 2002). Sulfide consumption by intact gills was measured (on the day of collection) by immersing the gill tissue in sulfide-rich filtered seawater (FSW) solutions. We assumed that changes in oxygen storage capacity of single gill tissue was identical within the three specimens. Residual oxygen stored within the

hemolymph of gill tissue represents the main electron acceptor for presumed sulfur-oxidizing endosymbiotic bacteria. Sulfide solutions were prepared by dissolving Na_2S in N_2-bubbled FSW and transferred into anaerobic Penicillin vials of 50 mL (pH = 8). Gills were briefly rinsed in cold FSW before addition to the vials which were amended with 0.5 mM H_2S and capped with septa and crimped. Vials without tissue were included as controls. The Vials were rotated at 4°C for up to 15h. Incubation medium was sub-sampled using a syringe every 3h, and $ZnCl_2$ was added immediately to the samples for sulfide analyses. In the laboratory, sulfide concentrations were analyzed as described below. Rates of sulfide uptake were calculated from the slope of the linear regression obtained from sulfide concentrations versus time, basically after 6h for the gills and 15h for the control. Sulfide consumption was expressed in mmol per min and per gram gill or tissue dry weight after subtracting the H_2S loss observed in the controls. Tissues and gills wet/dry mass were determined to the nearest 0.1 g for individuals stored in 4% buffered formalin of the same size than those used for this study. Dry mass was obtained after tissues were dried for 24 h at 60°C.

2.5 Chemical analyses

On land, methane concentrations were measured by headspace gas chromatography using a PR2100 gas chromatograph equipped with a flame ionization detector (GC/FID Perichrom, France) connected to a headspace injector (dani HSS 86.50). The stable carbon isotopic ratio of methane ($\delta^{13}C\text{-}CH_4$) was determined with a G2201-i CRDS analyzer from Picarro. The average analytical precision was of 2 % and 0.9 ‰, for methane concentration and $\delta^{13}C\text{-}CH_4$, respectively. Sulfate were measured with an ion-exchange chromatograph Dionex ICS-5000 from Thermo Scientific®, equipped with Ionpac AS22-SC column, an Ionpac AG 22-SC precolumn and an electrical conductivity detector. Eluent was a mix of Na_2CO_3 – 5 mM and $NaHCO_3$ – 1.75 mM. IAPSO standard seawater was used as certified reference material for calibration. Sulfides were determined on-board by colorimetry using the methylene blue method (Cline 1969).

2.6 Microbial analyses

DNA extractions from sediments, PCR, cloning and sequencing. Total nucleic acids (DNA) were directly extracted in duplicate from one gram of sediment by using the PowerMax® soil DNA isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturers' instructions. DNA was purified and concentrated by adding 0.2 mL of NaCl 5M (SIGMA, St Louis, USA) and 10.4 mL of cold absolute ethanol (CARLOS EBRA, Val de Reuil, France). Samples were incubated for 10 min at room temperature following by 5 min in ice, and then centrifuged for 30 min at 2500 g at room temperature. The DNA pellets were washed with 5 mL of 70% ethanol and left to air-dry. The pellets were suspended in 200 µL of sterilized MilliQ water overnight at 4°C. Purified DNA extracts were stored at -20°C.

Partial 16S rRNA genes were amplified by PCR (Polymerase Chain Reaction) using the primer sets A24F/A915R (5'-CGC-TTG-ATC-CTG-CCG-3'/ 5'-GTC-CTC-CCC-CGC-CAA-TTC-CT-3'; Teske et al., 2002) and E8F/907R (5'-AGA-GTT-TGA-TCA-TGG-CTC-AG-3'/5'-CCG-TCA-ATT-CMT-TTR-AGT-TT-3' ; (Lane *et al.* 1985)) for archaea and bacteria domains respectively. PCR amplifications were performed in independent triplicates in a 25 µL reaction volume contained : 1X Green Go*Taq*® Buffer (Promega, Madison, USA), 2 mM $MgCl_2$ (Promega, Madison, USA), 0.4 mM dNTP mix, 0.2 µM of each primer (Eurogentec, Liège, Belgique), 0.6 U of Go*Taq*®G2 Hot start polymerase (Promega, San Luis Obispo, USA), and 1 µL of purified DNA template. The final volume was adjusted to 25 µL with sterile water. The amplifications were conducted in an initial 94°C denaturing step for 5 min, followed by 30 cycles of 94°C for 1 min, 57°C for 1 min 30 and 72°C for 2 min, and then a final 72°C elongation step for 6 min. Absence of contaminations was checked by negative controls. PCR reactions were carried out using a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA) thermal cycler, and PCR products were visualized using gel electrophoresis.

Prior to cloning, positively amplified PCR products from pooled triplicate reactions were purified on TAE agarose gel (0.8%) using NucleoSpin® gel and PCR Clean-up kit (Macherey Nagel, Düren,

Germany) according the manufacturer's instructions. The purified amplicons were ligated into pCR®TOPO TA vector (Invitrogen, Carlsbad, CA, USA) and transformed into *Escherichia coli* X-Gold® chemically competent cells (Stratagene) according to the manufacturer's recommendations. Cloned 16S rRNA gene fragments were sequenced using M13 universal primer with BigDye terminator chemistry and determined on an ABI3730xl Genetic Analyser (Applied Biosystems) (GATC Biotech, Konstanz, Germany).

Phylogenetic analysis. The quality of retrieved 16S rRNA sequences from electropherograms and base calls were manually verified using Geneious©R6 version 6.1.7 5 (Kearse *et al.* 2012), and the vector and priming sites were excluded. Sequences were imported into the ARB software package into a seed alignment (Ludwig *et al.* 2004). Sequences from other studies were retrieved from GenBank. The 16S rRNA sequences were then aligned against the entire database by using CLUSTALW (Thompson *et al.* 1994) implemented in ARB. The alignments were then refined manually. Phylogenetic trees were generated from distance matrices using the neighbor-joining method and the partial adjustment model (Wilbur, 1985) using ARB. The outgroup-rooted phylogenetic tree topology and node support (1000 bootstraps) were tested.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper have been submitted to GenBank/EMBL/DDBJ databases under the accession numbers (in review in genbank).

Modelling. The sulfate profile from sediments underlying vesicomyids at site C (Figure 2b) was fitted with the PROFILE software (Berg 1998) to determine the effect of biorrigation on sulfate reduction rates and compare these rates to sulfide uptake rates by vesicomyid gills. PROFILE assume that the concentration-depth profile represents a steady state, allowing the calculation of rate of net production or consumption as a function of depth together with the flux across the sediment-water interface. Bioirrigation, *i.e.* pumping of pore water by benthic fauna through their environment, can be included in the analysis as a vertical transport, and is expressed as an alpha coefficient (in yr^{-1}) that is considered constant over the assumed depth of influence of this irrigation. Three different bioirrigation scenarios were tested using data from the literature (Depth integrated rates of bioirrigation from 0 to 330 cm yr^{-1} (Haese *et al.* 2006, Fischer *et al.* 2012)) to obtain a range of depth-integrated sulfate consumption rates. A bioirrigation depth of 7 cm, corresponding approximately to the average length of vesicomyids in this site (60 ± 12 mm - (Decker *et al.* this issue)), was used for these calculations.

3 RESULTS

Sampled habitats were separated into two groups based on the presence or absence of bivalves. The first group gathers vesicomyid habitats from sites A, B and C (Figure 2). The second group gathers the other habitats at site C (bare sediment, reduced sediment, and bacterial mats) (Figure 3).

Pore water profiles of dissolved sulfate concentrations, ranging from a slight decrease from seawater concentrations around 15 cm in bare sediments close to vesicomyids in site C (Figure 3a) to a complete disappearance within 15 cm underneath a bacterial mat at site C (Figure 3d), suggest that sulfate-reduction occurs in all selected habitats. Various concentrations of sulfides are reported from a few µM (Figure 3a) to 6 and 15 mM underneath black (reduced) sediment and bacterial mat respectively, both located at site C (Figure 3c and 3d). Methane concentrations ranged from a few µM (<3 µM in bare sediments – Figure 3a) to several hundreds of µM (up to 400 µM underneath a bacterial mat at site C – Figure 3d), with $\delta^{13}C\text{-}CH_4$ between -92 and -67 ‰. Methane concentrations should be considered as lower values due to decompression that probably occurred during retrieval of the samples.

TOU fluxes ranged between 43 and 346 mmol m^{-2} d^{-1} in vesicomyid habitats, was as low as 9.6 mmol m^{-2} d^{-1} in the bare sediment at station C, and as high as 103 mmol m^{-2} d^{-1} in the reduced sediment

from station C ((Khripounoff *et al.* 2015, Khripounoff *et al.* this issue), Table 2). Methane flux, as directly measured within the Calmar benthic chamber, was moderate in the bare sediment (2.4 mmol m^{-2} d^{-1}), and much higher in vesicomyid habitats, with values as high as 139 mmol m^{-2} d^{-1}. The methane flux was also variable from one cruise to the other, and from one vesicomyid assemblage to the other. A flux of 1.8 mmol m^{-2} d^{-1} was recorded during the WACS cruise at station A on the Northern levee compared to 109 mmol m^{-2} d^{-1} recorded during the CONGOLOBE cruise on the Southern flank of this station within another vesicomyid assemblage.

The three Vesicomyids gills incubated displayed mean sulphide consumption rates of 0.14 ± 0.02 mmol g^{-1} DW tissue d^{-1} (Table 3). Controls showed a loss of only 0.2-0.3 µmol.h^{-1} (around 5%) and were subtracting to the gills consumption rates. Assuming a biomass at Station C of 629 g DW m^{-2} (Khripounoff *et al.* 2015), the mean rate of sulfide consumption is estimated at 90 ± 18 mmol S m^{-2} d^{-1}.

Modelling results. The sulfate profile from sediments underlying vesicomyids at site C was fitted using the PROFILE software using three values of alpha coefficient that reflects three bioirrigation scenarios (Figure 6). The first simulation includes no bioirrigation and led to an integrated sulfate consumption of 6 mmol S m^{-2} d^{-1}, the main consumption being maximum between 5.5 and 11 cm depth. The second simulation includes some bioirrigation with a depth integrated rate of bioirrigation of 33 cm yr^{-1}. This led to an integrated sulfate consumption of 14 mmol S m^{-2} d^{-1}, the main consumption being maximum between 4 and 8 cm depth. Finally, the third simulation includes an intense bioirrigation with a depth integrated rate of bioirrigation of 330 cm yr^{-1} and led to an integrated sulfate consumption of 85 mmol S m^{-2} d^{-1}, the main consumption being maximum between 4 and 8 cm depth.

Microbial diversity in Congo Lobes sediments of site C. In total, 334 bacterial clones (86 from microbial mats; 86 from the reduced sediment; 81 from vesicomyid habitats; and 81 from bare sediments) were analyzed. The composition of the bacterial 16S rRNA gene libraries of sediments associated with the microbial mat, reduced sediment, and vesicomyids were almost identical and showed the highest bacterial diversity (Figure 4A). The least bacterial diversity was observed in the bare sediments. Most of the 16S rRNA genes were closely related to sequences from uncultivated microorganisms observed in methane-rich sediments. By using phylogenetic affiliation, a total 12 bacterial phylotypes were found. *Proteobacteria* was the most dominant phyla over the entire samples with an average relative abundance of more than 50%. Of the *Proteobacteria*, Delta subdivision comprised 30% of the clone libraries except in bare sediments with 17%. A few sequences grouped within diverse phylogenetic lineages, namely the *Proteobacteria* subdivisions Gamma and Beta, *Planctomycetes*, *Actinobacteria*, *Spirochaetea*, *Firmicutes,* and *Atribacteria* (candidate division JS1).

Analysis of 16S rRNA genes revealed a bacterial assemblage typical of methane seep habitats and few sequences of bacteria likely involved in sulfur and methane cycling (Lazar *et al.* 2011, Vigneron *et al.* 2014). *Deltaproteobacteria*, potentially including SRB, were detected in all habitats (Figure 4A). Their diversity was relatively high in sediments underlying the microbial mats and the Vesicomyids and was the highest in the reduced sediment (7 phylotypes; 19 sequences). Four known genera of the *Desulfobacterales* order (*Desulfocapsa*, *Desulfobulbus*, *Desulfosarcina and Desulfobacula*) were represented. Two of the four uncultured, seep-specific putatively sulfate-reducing clades were detected: SEEP-SRB1 (*Desulfosarcina*/*Desulfococcus*-related); and SEEP-SRB3 (*Desulfobulbus*-related) from sediments below all habitats (Knittel *et al.* 2003). The sequences of potential SRB occurring in sediments colonized by microbial mats were affiliated only to the SEEP-SRB3.

In total, 329 archaeal clones (76 from microbial mats; 85 from the reduced sediment; 88 from the vesicomyids; and 80 from the bare sediment) were analyzed. Sequences were affiliated to 18 different archaeal phylotypes. Some members of these archaeal clades are typically found in anaerobic marine sediments (Teske and Sorensen 2008, Vigneron *et al.* 2015). *Bathyarchaeota (*MCG;

Miscellaneous Crenarchaeotal Group), representing 20% of total archaeal sequences, was the dominant phylum in microbial mats and the bare sediment (Figure 4B). These lineages are highly diverse and globally distributed in various marine and continental habitats (Kubo *et al.* 2012). Representative sequences of the class *Methanomicrobia*, mainly represented by methanogens and methanotrophs, were present in all habitats with similar relative abundance in clone libraries except for the vesicomyid habitats. Members of *Methanomicrobia* dominated the archaeal community in vesicomyid sediments (relative abundance of 95% of the clone library). In addition, two of the three major groups of anaerobic methanotroph (ANME), ANME-2 (a, b and c) and ANME-3, were detected in Congo Lobe sediments of site C. The reduced sediment and the sediment associated with the vesicomyids harbored more representatives' sequences of the ANME-2 and ANME-3. Only ANME-3 representative sequences were detected in the sediment underlying the microbial mats.

Some clones were associated with known methanogenic families: *Methanomicrobiaceae* and *Methanosarcinaceae*. Representative cultivated methanogens of the *Methanomicrobiaceae* use CO_2/H_2 or formate for methane production (Garcia *et al.* 2000). Cultivated methanogens belonging to the *Methanosarcinaceae* display the highest versatility with respect to the substrates they use for energy gain (Balch *et al.* 1979): methylated amine and methanol are generally preferred, but CO_2 reduction with hydrogen and also acetate may be used. Twenty four clones affiliated to methylotrophic methanogenic *Methanimicrococcus* genus (28% of the clone library) were detected in Vesicomyid sediments only. In addition, only two clones that were isolated from the sediments covered by the microbial mats were most closely related to the methanogenic family of the *Methanocellaceae*. Representative cultivated methanogens belonging to this family catabolize only acetate for energy (Garcia *et al.* 2000, Vigneron *et al.* 2015).

4 DISCUSSION

4.1 Aerobic remineralization/relative use of oxygen

Aerobic remineralization in the sediments of the Congo deep sea fan, outside the specific habitats studied here, is higher compared to usual abyssal plain and deep-sea sediments by a factor of 2-3 (Pozzato *et al.* This issue). Benthic oxygen consumption reaches very high values in specific habitats such as vesicomyids assemblages, with diffusive oxygen uptake (DOU) values up to 20.5 mmol m^{-2} d^{-1} at site A (Pozzato *et al.* This issue) and TOU fluxes up to 346 mmol m^{-2} d^{-1} at site C (Khripounoff *et al.* this issue). Reduced sediment also exhibit a large TOU (103 mmol m^{-2} d^{-1} – Table 2) despite the fact that no visible megafauna was identified, but where high density of macrofauna was recorded (around 65000 ind m^{-2}, Olu *et al.* This issue). The fluxes measured in dense habitats all fall in the range of fluxes measured in similar habitats of typical active seeps (see the review of Boetius and Wenzhoefer (2013) and references therein), where aerobic mineralization of carbon is generally not a major process. Pozzato *et al.* (2017) used *in-situ* and *ex-situ* oxygen microprofiles to calculate an averaged DOU of 2.6 mmol m^{-2} d^{-1} outside the dense habitats. They compared it to the averaged value of 7 mmol m^{-2} d^{-1} deducted from *in-situ* incubation with benthic chambers presented in (Olu *et al.* This issue), and suggested that the internal sedimentary remineralization carried out by meiofauna and microbes constitutes around 30% of the total recycling outside the habitats (as reflected by a TOU/DOU ratio of 2.7). Then, in bare sediment close to vesicomyid habitats in site C, this ratio is around 3.7. In specific dense habitats, the TOU/DOU ratio increases. In reduced sediment of site C, this ratio increases to 5, to 8 in sparse vesicomyid habitats of site B, up to 14 in vesicomyid dense patches from sites A and C. As no TOU was successfully recorded in bacterial mats from site C, this ratio is thus not available on this site but a value of 4.8 can be calculated in bacterial mats from another site (site F, between site B and C, unpublished data). This increase in TOU/DOU ratios likely reflects a larger contribution of macro- and megafauna in aerobic respiration processes, but also higher fluxes of reduced elements to the oxic zone. At cold-seeps, a large proportion of DOU is explained by the microbial oxidation of sulfide (de Beer *et al.* 2006, Gruenke *et al.* 2011) and the reoxidation of methane (Boetius and Wenzhoefer 2013). In our system, in habitats without large

megafauna, oxygen is likely used mainly for the reoxidation of these two reduced elements, as oxygen profiles often overlap with sulfide (Taillefert *et al.* this issue) and methane profiles (Figure 3). Other reduced elements (Mn^{2+}, Fe^{2+}, NH_4^+) can also be responsible for oxygen consumption (Taillefert *et al.* this issue). If we establish a quick budget for the patch of black sediment at site C, (Taillefert *et al.* this issue) found NH_4^+, Mn^{2+}, Fe^{2+} and $\sum H_2S$ diffusive fluxes of respectively 1.5, 0.95, 0 and 6.61 mmol m^{-2} d^{-1}. If we consider that respectively 2, ½, ¼ and 2 mol of oxygen are needed to oxidize 1 mol of NH_4^+, Mn^{2+}, Fe^{2+} and $\sum H_2S$, 16.7 mmolO_2 m^{-2} d^{-1} could be used to reoxidize these four molecules, which represents 81% of the DOU at this site and 16% of the TOU. If we take into account the large flux of methane outpassing the oxygenated zone in this patch (Table 2), then we can conclude that oxygen is likely used in majority for reoxidating reduced species, including methane. A large diversity of methanotrophs was detected in surficial sediments from those sites (S. Bessette, unpublished data) and supports this last assumption. In the particular case of vesicomyid habitats, bioirrigation as well as vesicomyids sulfide uptake have to be taken into account when budgeting the use of oxygen.

4.2 Anaerobic remineralization

Sulfate reduction

Dissimilatory sulfate-reduction (DSR) generally accounts for 25-50% of the mineralization of organic carbon in coastal sediments, and this relative contribution decreases three fold over the continental shelf from the shore to a depth of 200 m (Jorgensen, 1982). Congo lobe sediments are characterized by a total organic carbon of up to 5%, mainly composed of terrestrial but potentially degradable material (Baudin *et al.* 2010, Stetten *et al.* 2015, Baudin *et al.* this volume), and an oxygen depletion depth ranging from around one centimeter in the levees to millimeters in the habitats (Pozzato *et al.* This issue). The SO_4^{2-} and H_2S sedimentary profiles in the Congo lobe sediments suggest that DSR might be a major process for organic matter remineralization in the habitats. Sulfate is efficiently reduced within the first decimeters in sediments in all studied sites and dissolved sulfides are produced, though in much lower concentrations (Figures 2, 3 and 4A), suggesting that a significant fraction of sulfide is oxidized or precipitated as iron sulfide minerals. Within the active lobe (site C), four known potential SRB groups (SEEP-SRB1, SEEP-SRB3, and *Desulfocapsa*) are identified and are often found in anoxic methane-rich sediments (Dekas *et al.* 2016). Sequences isolated from the reduced, microbial mat, and vesicomyid-associated sediments are affiliated with the *Desulfocapsa* genus involved in sulfate reduction. The bacterial diversity data coupled to sulfate profiles suggest that DSR may occur, even if the methane concentrations measured at those sites suggests a significant use of sulfate by AOM (Sultan et al, 2016; Egger et al, 2016).

Methanogenesis

It is likely that methanogenesis occurs at depth within the lobes area, as demonstrated by the detection of methane in most of the sampled piston cores (L. Ruffine, pers. com.). The light $\delta^{13}C$ signatures of methane detected in the surficial sediment (Figure 2 and 3) indicate a biogenic origin (Whiticar 1999). The high CH_4 fluxes measured with *in-situ* benthic chambers (Table 2) together with the large number of *Methanomicrobia* and *Bathyarchaeota* (Miscellaneous Crenarchaeotic Group) sequences found in the surface sediments below the microbial mat, reduced sediment, and Vesicomyids may be indicative of methanogenesis. This process would then compete sulfate reduction, as relatively high sulfate concentrations are still detected at those depths. Methanogens can compete or coexist *via* syntrophic interaction with SRB in surface sediments (Treude *et al.* 2005, Maltby *et al.* 2016) if alternative catabolic substrates such as C_1 compounds (methanol, methylated nitrogen or sulfur compounds) are available (L'Haridon *et al.* 2014). Sequences within the genus *Methanomicrococcus* related to *Methanosarcinaceae* (Figure 4b) have been previously detected in methane seep sediment, mud volcano, or natural gas fields (Mochimaru *et al.* 2007, Beal *et al.* 2009). Cultured members of this genus are obligate methylotroph that could metabolize a variety of C_1 compounds, including methanol and methylated amines (Sprenger *et al.* 2000). Indeed,

hydrogenotrophic methanogenesis (i.e. reduction of CO_2 by H_2) in sediments with high sulfate concentrations is possible, for instance within the *Methanosarcinaceae* genus, if methanogens gain energy by growing syntrophically with propionate oxidizing bacteria (Kendall and Boone 2006). Methane concentrations, its stable isotope ratios, and microbial diversity analyses reveal that: 1) both DSR and methanogenesis are likely to co-exist within the habitats of the active lobe of the Congo deep-sea fan; and 2) anaerobic and organic-rich sediments found in the Congo Lobe ecosystems provide a suitable ecological niche for methanogens.

4.3 Anaerobic oxidation of methane

AOM occurs in the commonly called sulfate-methane transition zone (SMTZ), where the methane profile crosses the sulfate profile (Sultan et al, 2016). Except for vesicomyids associated-sediments, where methane was absent in the top few centimeters, sulfate and methane were present in high concentrations and generally all the way to the first few centimeters of the sediment cores. A strict SMTZ is difficult to identify in these habitats, probably because the sedimentation dynamics in this area disrupt the steady state. It has indeed been proposed that the rates of carbon burial, methane production, and downward diffusion of sulfate control the depth and the thickness of this SMTZ (Regnier *et al.* 2011, Meister *et al.* 2013). Thus, the combination of high organic matter content deposited by turbidites, methane production at shallow depths, and diffusion in the sediments of the Congo lobe complex may generate a SMTZ within a few centimeters from the sediment/water interface. This hypothesis is supported by the microbial community composition (Figure 4 and 5). Generally, most of the methane is consumed in the SMTZ by AOM mediated by ANME and SRB partners (Boetius and Wenzhoefer 2013). The distribution of ANMEs in a diffusive environment is then restricted to the SMTZ where both methane and sulfate are available (Knittel and Boetius 2009). In the present study, ANME are found within the first 12 cm of sediment in all habitats. Members of SEEP-SRB1 are found together with ANME-2 and ANME-3 archaea beneath the vesicomyids and reduced sediment. SEEP-SRB3 within the *Desulfobulbaceaea* are typically associated with relatives ANME (Niemann *et al.* 2006, Losekann *et al.* 2007) and are found in the present study in sediments underlying bacterial mats and, more importantly, vesicomyids (Figure 4 and 5). SEEP-SRB3 relatives are found beneath vesicomyid assemblages along with relatives ANME-2 and ANME-3: their potential association could be linked to the AOM process as shown in other environments (Boetius *et al.* 2000, Niemann *et al.* 2006, Losekann *et al.* 2007, Vigneron *et al.* 2013) or to the fact that these sediments accumulate quickly (>10cm yr^{-1} in station C, (Rabouille *et al.* this issue)) and during catastrophic turbidity events, which may reset geochemical conditions to more oxidized conditions and mix microbial populations. Furthermore, sulfate may not be the only electron acceptor in the AOM process, as high concentrations of iron and manganese oxides were reported in the Congo deep-sea fan sediments (Beckler *et al.* 2016, Taillefert *et al.* this issue) that are more energetically favorable terminal electron acceptors than sulfate for AOM (Beal *et al.* 2009). Indeed, iron-dependent AOM in mesocosm studies with sediment slurries has been observed (Beal *et al.* 2009, Sivan *et al.* 2011, Egger *et al.* 2015). In the present study, a large diversity of iron-reducing bacteria represented by different phylogenetic groups is described. For instance at site C, sequences affiliated to *Bacteroidetes* and *Proteobacteria* were identified. Representative sequences of these phyla were already detected in heavy-metal contaminated fields and hydrothermal ecosystems (Ellis *et al.* 2003, He *et al.* 2015). Further investigations on activity rates measurements of AOM coupled to iron, manganese, and sulfate-reduction in these deep marine ecosystem are needed to better discuss this last hypothesis.

4.4 DSR versus AOM

To better constrain the relative contribution of DSR and AOM in the sulfate reduction process, two approaches are commonly used. The first approach is direct rate measurements by isotopic labeling (Jørgensen 1978). In the Congolobe project we did not measure those rates. The second approach is modelling. Using the same modelling approach we used here, Fossing *et al.* (2000) calculated the rates of SR on two piston cores from the continental slope off Namibia and compared them to direct

measurements. They discussed the limitation of the curve fitting approach that only provides information on net sulfate consumption rates. Then, within the SMTZ, the two approaches gave similar results whereas above this zone, SR was underestimated. This bias was linked to bioirrigation that was at first not included in the model. Injection of sea water within the sediment provides sulfate at depths and thus increase the gross rates of SR. Then, non-linear sulfate profile, as observed in our study, could be representative of sulfate reduction above the SMTZ (Fossing *et al.* 2000). In figure 6, typical kinked sulfate profile within a vesicomyids habitat was modelled with increasing bioirrigation depth-integrated rates, resulting in an increase in global SR rates, especially visible in the top 5 cm of sediments. We here suppose that in most habitats studied here, sulfate is probably consumed by SRB in surficial sediments (0-10 cm), and by AOM deeper in the sediment.

4.5 How can we explain the differences of sulfides concentrations observed within the habitats?

Bare sediments, characterized by low methane concentrations, presented the lowest bacterial diversity compared to the black reduced sediments, white microbial mats, and vesicomyid-associated sediments which harbored similar bacterial and archaeal community composition, mainly involved in sulfur and methane cycles (DSR and AOM). Simultaneously, known methanogenic clades dominated the microbial mats and vesicomyid-associated sediments (Figure 4). These microbial processes led to a high production of sulfides that could be reoxidized through different pathways in these habitats. Beneath bacterial mats, sulfide reoxidation is likely coupled to the bacterial mats activity as reported elsewhere (Losekann *et al.* 2007), or/and to the sulfide consumption by juveniles of vesicomyids colonizing these mats (Olu *et al.* This issue). Within black reduced sediments, no bacterial mat nor vesicomyid juveniles are found and thus sulfide reoxidation is probably mainly driven by oxygen (large TOU – Table 2) or iron (Taillefert *et al.* this issue). Sediments from the Congo lobes are rich in reactive manganese and iron oxides such as ferrihydrite (Beckler *et al.* 2016, Taillefert *et al.* this issue). These authors concluded that outside the habitats, and in surficial sediments, organic mineralization is mainly driven by microbial iron reduction, whereas within the habitats, sulfate reduction prevails and produces high sulfide concentrations that rapidly reduce Mn(IV) and Fe(III) oxides abiotically. Finally, superficial sediments (0-10cm) underlying vesicomyid assemblages contained less sulfides, especially within the first centimeters of sediment where biorrigation by vesicomyids and sulfide uptake by chemosynthetic symbionts are significant (Fischer *et al.* 2012). Indeed, incubations of vesicomyid gills in sulfide-rich media, though not representative of the *in-situ* consumption by vesicomyids, established that much more sulfide can be consumed by these chemosynthetic organisms than sulfide produced by sulfate reduction. This modeling exercise indicates that a depth integrated rate of bioirrigation of around 330 cm yr^{-1}, probably realistic in habitats with large vesicomyids (Fischer *et al.* 2012), may be able to satisfy sulfide uptake rates by the chemosynthetic symbiont of the vesicomyids. These findings indicate that dissolved sulfide should be below detection in most surficial sediments of the habitats populated by vesicomyids and suggest that sulfate reduction provides enough sulfides due to enhanced supply to deeper sediments by bioirrigation.

5 CONCLUSION

The Congo deep-sea fan is a unique environment where organic-rich turbidites reach 5000 m and allow the establishment of discrete, cold-seep like habitats along the main channel. This is the first time that a deep-sea fan system is reported to display similar characteristics than active cold-seeps. Several conclusions can be outlined from the present findings:

1) The high total oxygen uptakes measured with benthic chambers within dense habitats and the overlap of oxygen profile with reduced species (mainly sulfides, methane and dissolved iron) suggest that a large share of oxygen is used for the reoxidation of reduced species. Then, carbon mineralization processes could be mostly dominated by anaerobic processes such as bacterial sulfate reduction and methanogenesis. This differs from bare

sediments taken outside these dense habitats, where oxygen mineralization seems more efficient (Pozzato *et al.* This issue) and where iron reduction dominates the suboxic/anoxic processes (Taillefert *et al.* this issue).

2) Bacterial and archaeal diversity together with geochemical profiles provided evidence for the occurrence of AOM with sulfate as the electron acceptor in the top 12 cm of reduced sediments, sediments underlying bacterial mats, and sediments colonized by vesicomyid assemblages at site C. The SMTZ associated with this process was not clearly defined, probably because of the sedimentation dynamics that disrupt the steady state of this system.
3) Measured sulfide concentrations were highly variable within the habitats. In bacterial mats sediments, sulfide was likely reoxidized by the bacterial mat itself and also by vesicomyid juveniles that require sulfide to grow. Sulfide consumption rates by adult vesicomyid gills are in the same range than sulfide production rates calculated from sulfate profiles in the sediments, provided large bioirrigation rates are able to enhance this production of sulfide. These findings suggest that sulfide consumption by Vesicomyids may compete with precipitation of iron sulfide in controlling sulfide concentration in these sediments.

This study, in conjunction with the other two companion papers (Pozzato *et al.* This issue, Taillefert *et al.* this issue) provide insights in the biogeochemical functioning of the cold-seep like habitats of the Congo deep-sea fan. More studies on the temporal evolution of such habitats would help understand how these patchy habitats are formed and colonized by meio-, macro-, and megafauna. Simultaneously, the biogeochemical reasons for the existence of methanogenesis in these shallow sediments and the possible involvement of manganese and/or iron oxides in the AOM process should be investigated further.

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Figure 1: General map of the distal part of the Congo turbidite system with location of the 3 sites

Figure 2: Depth profiles of sulfate, sulfide, methane, and 13C-methane in sediment pore waters underlying vesicomyid assemblages

Figure 3: Depth profiles of sulfate, sulfide, methane, and 13C-methane in the pore waters of the sedimentary features observed at Station C

Figure 4. Relative abundance in percentage of (A) bacterial and (B) archaeal taxa based on the 16S RNA gene analysis derived from all sediment habitats of the Congo Lobe Site C.

Figure 5. Phylogenetic tree showing the affiliations of selected 16S rRNA sequences within the *Deltaproteobacteria* from the Congo Lobes sediments of the site C. The tree was generated from 694 nucleic acid sequences by neighbour-joining analysis with 1000 bootstraps. Clone sequences from sediments habitats of the Congo Lobe site C are in boldface type. The bar indicates 10% estimated phylogenetic divergence.

Figure 6. Sulfate depth profile fitted with the PROFILE software in the pore waters underlying a vesicomyid assemblage at site C. The methane profile at this site is shown in the first graph. Calculated sulfate consumption rates (line) as well as depth integrated effect of irrigation (dots) are plotted for different bioirrigation scenarios.

Table 1: Sampling sites description. CoL stands for the Congolobe cruise. CT stands for pushcore.

	Long (°S)	**Lat (°E)**	**Depth (m)**	**Sampling dives**	**Sampling cores**	**Comments/description**
Site A	6°28'S	6°02'E	4765	CoL:PL484	CT 3, CT 8 & CT 12	Inside a vesicomyidae bed
Site B	6°25'S	5°49'E	4840	CoL:PL492	CT 8 & CT 11	At the border of a vesicomyidae bed
Site C	6°42'S	5°29'E	4950	WACS:PL436	CT 1 & CT 5	Inside a patch of "black and white" sediment
				CoL:PL490	CT 8	Inside a patch of "black and white" sediment
					CT 11 & CT 12	Inside a patch of black sediment
				CoL:PL491	CT 8 & CT 11	Inside a vesicomyidae bed
					CT 16 & CT 17	In bare sediment far from a vesicomydae bed

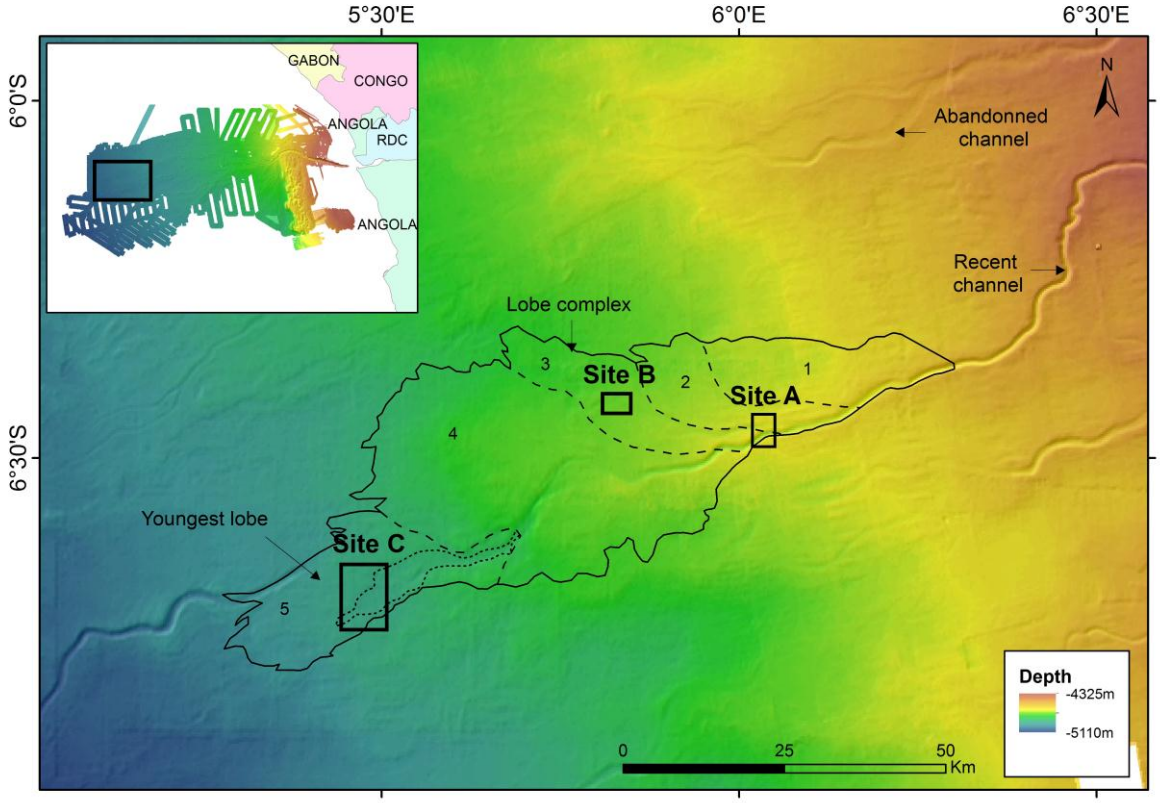
Table 2: Total oxygen uptake (TOU) and methane (CH_4) flux at the different study sites.

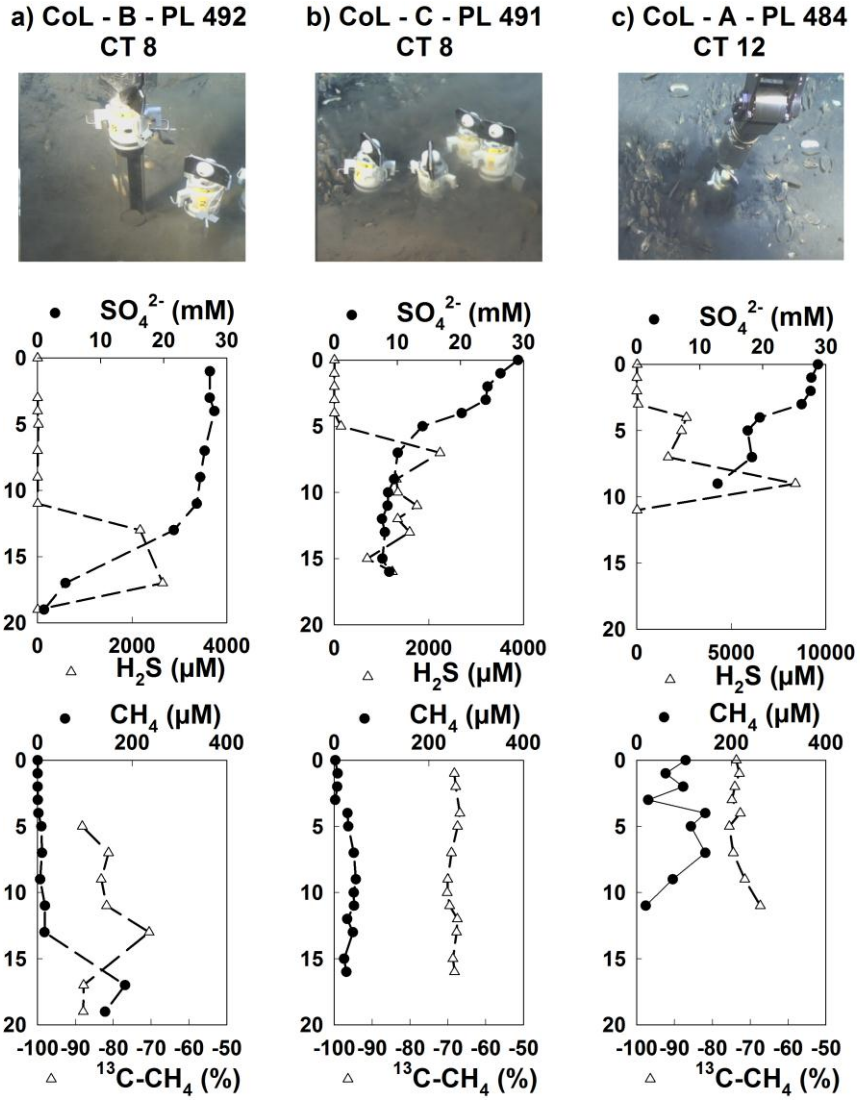
Station	Dominant species + mean density under the CALMAR	TOU (mmol m^{-2} d^{-1})	CH_4 flux (mmol m^{-2} d^{-1})
WACS PL 435 St A[1]	*Christineconcha regab (506)*	157	1.8
WACS PL 437 St C[1]	*C.regab (748)*	346	139
CoL PL 492 St B[2]	*C.regab (97)*	43	8.4
CoL PL 484 St A[2]	*C.regab (335)*	161	109
CoL PL490 St C [3]	reduced sediment	103	93
CoL PL491 St C [3]	bare sediment	9.6	0.1

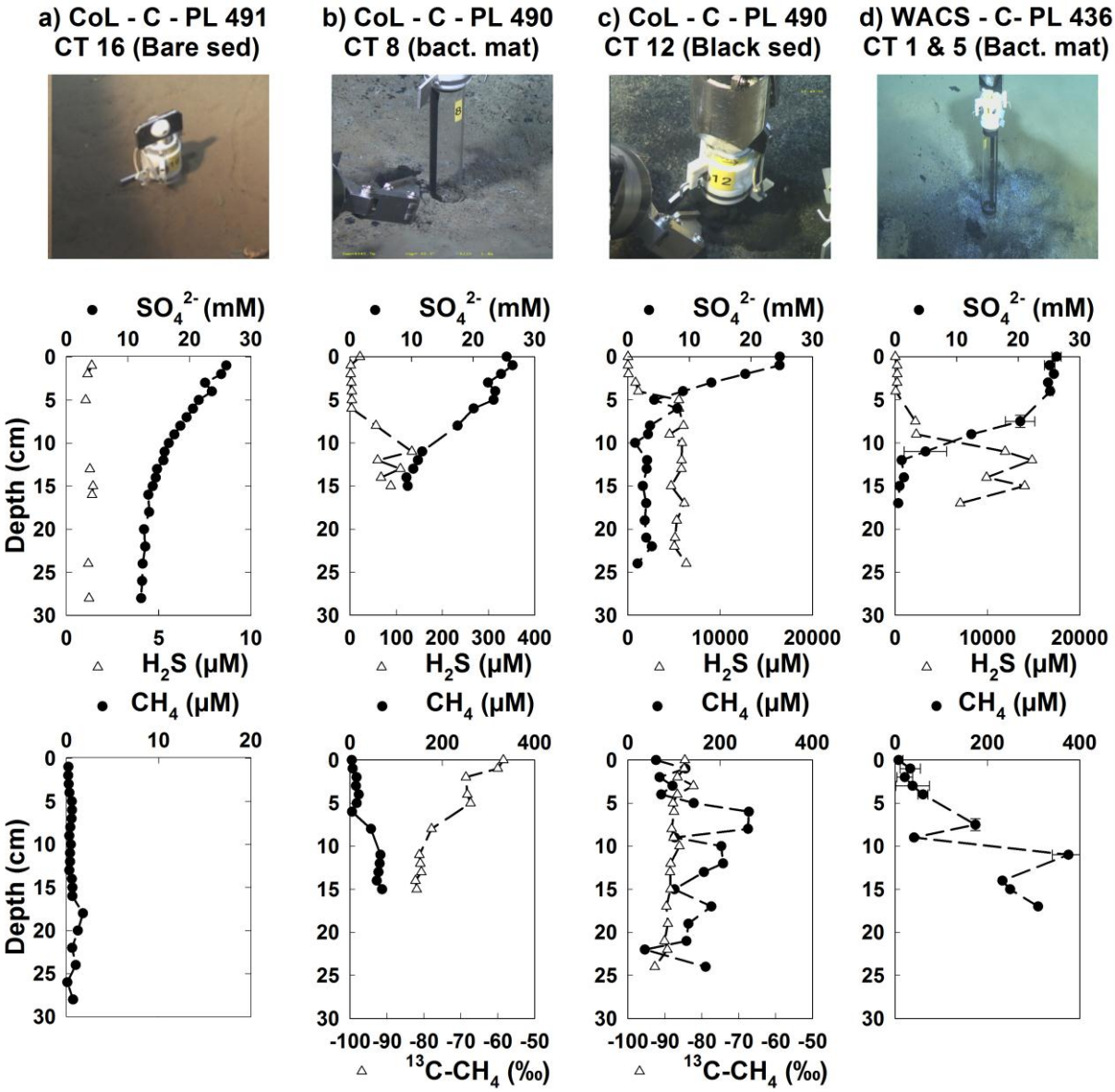
[1] (Khripounoff *et al.* 2015); [2](Khripounoff *et al.* This issue); [3] This study

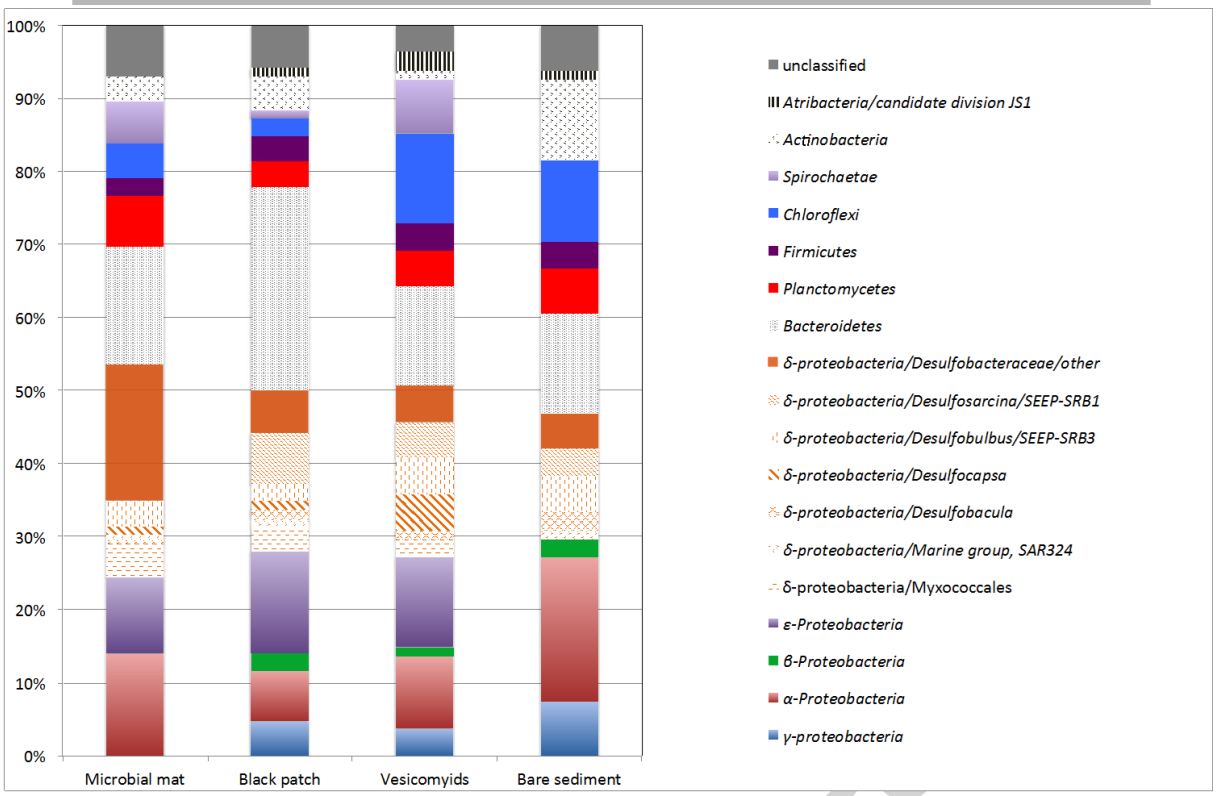
Table 3: Vesicomyids (V1, V2 and V3), their gill characteristics, and incubation results. Final rates in mmol m^{-2} d^{-1} were calculated using a dry density of 629gDW tissue m^{-2}.

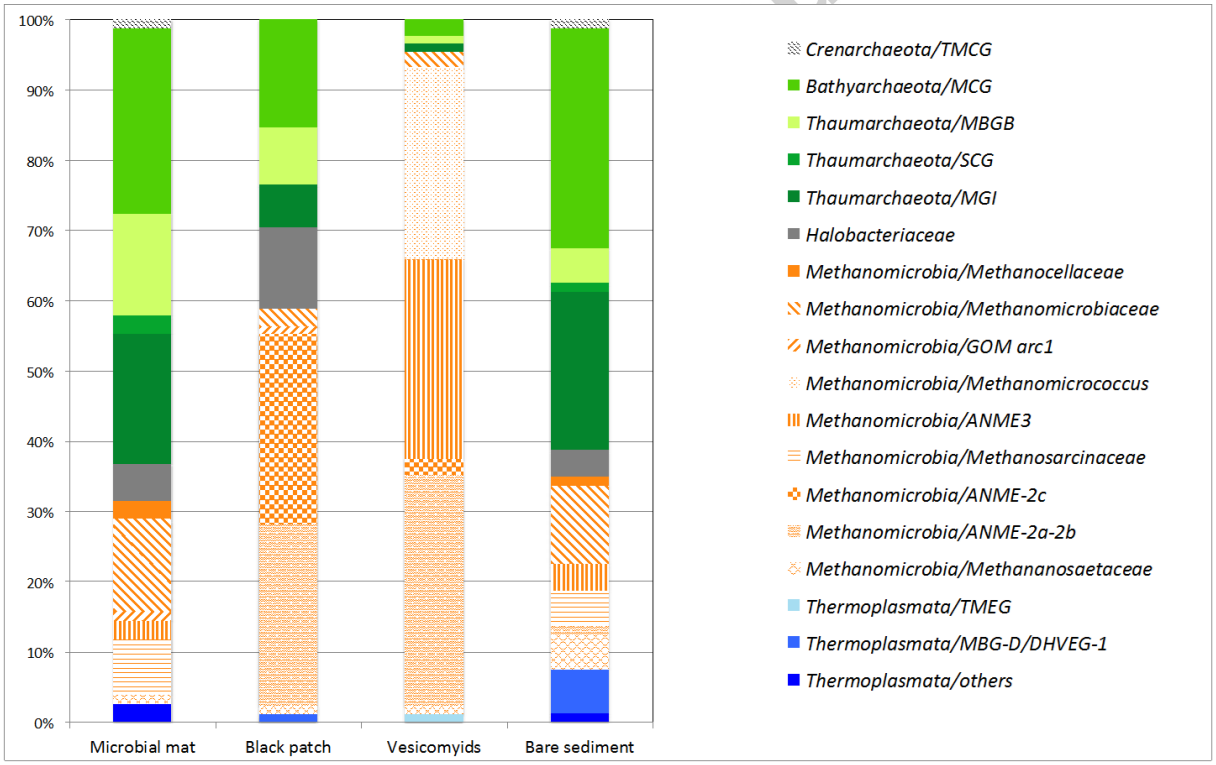
	V1	V2	V3	Mean	SD
Vesicomyid length (mm)	68.0	72.0	57.0	65.7	7.8
Wet weight tissue (g)	9,11	10,19	6,44	8,58	1,93
Dry weight tissue (g)	1,98	2,28	1,29	1,85	0,51
Wet weight gill (g)	1,59	1,78	1,12	1,50	0,34
Dry weight gill (g)	0,43	0,49	0,28	0,40	0,11
rate (mmol d^{-1})	0,16	0,18	0,07	0,14	0,06
rate (mmol g^{-1} DW tissue d^{-1})	0,16	0,15	0,11	0,14	0,03
rate (mmol m^{-2} d^{-1})	103	97	70	**90**	**18**

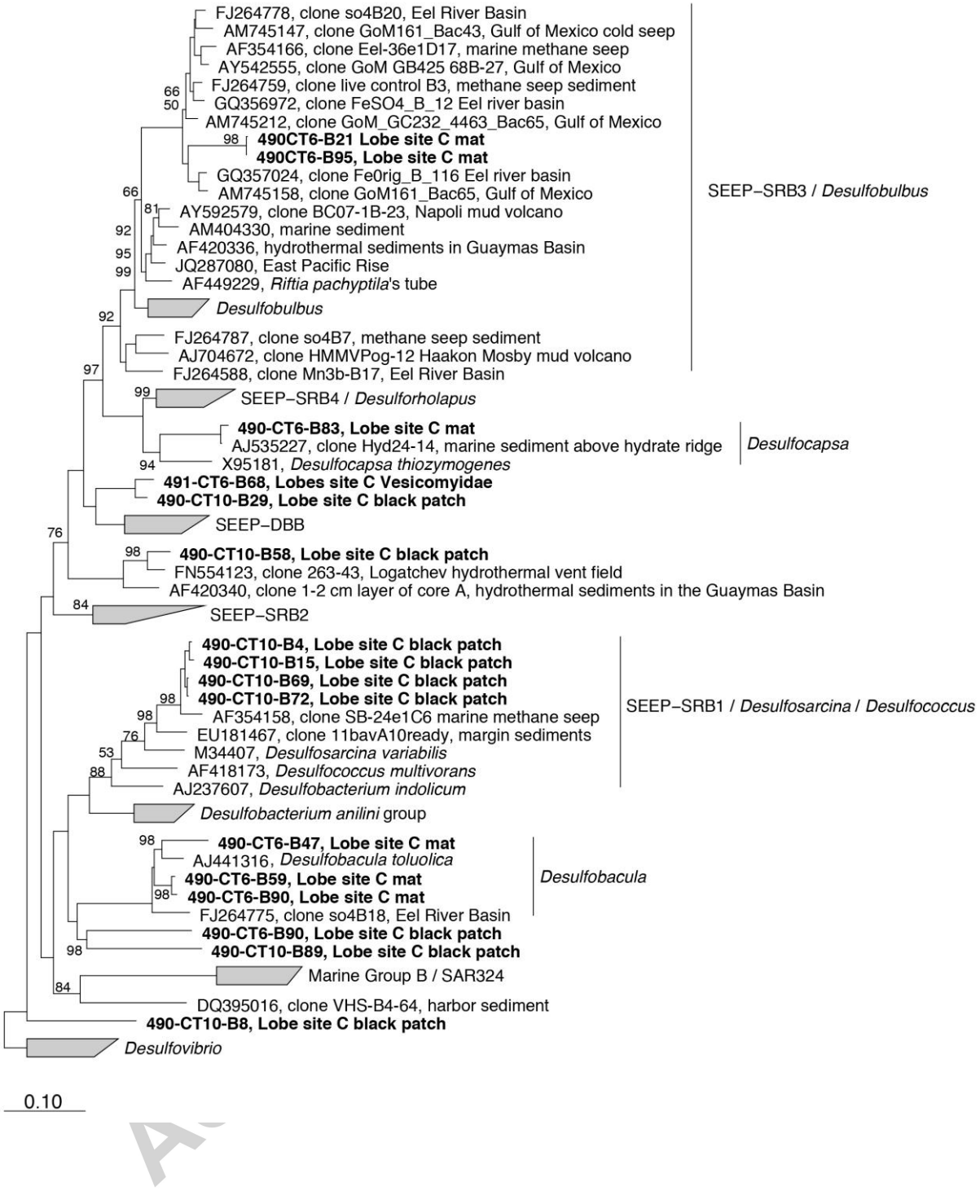








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AF354166, clone Eel-36e1D17, marine methane seep
AY542555, clone GoM GB425 68B-27, Gulf of Mexico
FJ264759, clone live control B3, methane seep sediment
GQ356972, clone FeSO4_B_12 Eel river basin
AM745212, clone GoM_GC232_4463_Bac65, Gulf of Mexico
490CT6-B21 Lobe site C mat
490CT6-B95, Lobe site C mat
GQ357024, clone Fe0rig_B_116 Eel river basin
AM745158, clone GoM161_Bac65, Gulf of Mexico
AY592579, clone BC07-1B-23, Napoli mud volcano
AM404330, marine sediment
AF420336, hydrothermal sediments in Guaymas Basin
JQ287080, East Pacific Rise
AF449229, Riftia pachyptila's tube
Desulfobulbus
FJ264787, clone so4B7, methane seep sediment
AJ704672, clone HMMVPog-12 Haakon Mosby mud volcano
FJ264588, clone Mn3b-B17, Eel River Basin
SEEP–SRB3 / Desulfobulbus
SEEP–SRB4 / Desulforholapus
490-CT6-B83, Lobe site C mat
AJ535227, clone Hyd24-14, marine sediment above hydrate ridge
X95181, Desulfocapsa thiozymogenes
Desulfocapsa
491-CT6-B68, Lobes site C Vesicomyidae
490-CT10-B29, Lobe site C black patch
SEEP–DBB
490-CT10-B58, Lobe site C black patch
FN554123, clone 263-43, Logatchev hydrothermal vent field
AF420340, clone 1-2 cm layer of core A, hydrothermal sediments in the Guaymas Basin
SEEP–SRB2
490-CT10-B4, Lobe site C black patch
490-CT10-B15, Lobe site C black patch
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490-CT10-B72, Lobe site C black patch
AF354158, clone SB-24e1C6 marine methane seep
EU181467, clone 11bavA10ready, margin sediments
M34407, Desulfosarcina variabilis
AF418173, Desulfococcus multivorans
AJ237607, Desulfobacterium indolicum
Desulfobacterium anilini group
SEEP–SRB1 / Desulfosarcina / Desulfococcus
490-CT6-B47, Lobe site C mat
AJ441316, Desulfobacula toluolica
490-CT6-B59, Lobe site C mat
490-CT6-B90, Lobe site C mat
FJ264775, clone so4B18, Eel River Basin
Desulfobacula
490-CT6-B90, Lobe site C black patch
490-CT10-B89, Lobe site C black patch
Marine Group B / SAR324
DQ395016, clone VHS-B4-64, harbor sediment
490-CT10-B8, Lobe site C black patch
Desulfovibrio
0.10