**Influence of methanogenic populations in Holocene lacustrine sediments
revealed by clone libraries and fatty acid biogeochemistry**

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Running title: Influence of methanogens in Holocene sediments

Abstract

Methanogenic populations were investigated in subsaline Laguna Potrok Aike sediments, southern Argentina. Microbial density and activity were assessed via cell count and *in situ* ATP detection for the last ~11K years. Methanogen phylogenetics highlighted species stratification throughout depth, whereas CO₂ reduction was the major pathway leading to methane production. Organic substrates, characterized using pore water analysis, bulk organic fractions and saturated fatty acids, showed a clear link between sediment colonization and initial organic sources. Concentrations and $\delta^{13}\text{C}$

compositions of methane and fatty acids provided final evidence of a microbial imprint on Holocene organic proxies in the most colonized intervals.

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Keywords: methanogenesis, syntrophy, early diagenesis, substrate fractionation, fatty acids

Introduction

Methane and carbon dioxide (CO₂) in anoxic lake sediments are final products of organic matter (OM) degradation. Biogenic production of these end members depends mainly on OM lability and metabolic pathways used by microbial consortia. During early diagenesis, fermentation processes gradually degrade original organic mixtures into simple molecules, sometimes leading to a deviation from the initial signatures of the bulk fraction (Freudenthal et al. 2001; Lehmann et al. 2002). In anaerobic habitats, degradation of complex organic compounds is a stepwise process taking place via hydrolysis, acidogenesis, acetogenesis and methanogenesis (Haider and Schäffer 2009; Wüst et al. 2009), during which competitive and syntrophic relationships can occur between microbes with a clear dependence on local physico-chemical conditions (McInerney et al. 2008). For example, in environments with low nitrate and sulfate concentrations, methanogens can associate with chemoheterotrophic bacteria producing methane at various stages. In freshwater environments methyl fermentation is favored and proceeds unrestricted by sulfate reduction (Whiticar et al. 1986), whereas in cold environments acetogenesis appears to dominate (Kotsyurbenko 2005;

Nozhevnikova et al. 2007). This affects rates of methanogenesis (Lay et al. 1998) and the associated carbon isotope fractionation (Whiticar 1999; Conrad 2005). Thus, the produced amount of methane in sediments derives from variable organic substrata, bacterial activity and metabolic pathways (Conrad et al. 2010). Furthermore, microbes develop the ability to use refractory compounds (McInerney et al. 2009), while evolving toward energy conservation with depth (Schink 1997).

Currently, geomicrobiological investigations of methanogenesis in lakes remain limited to most surficial sediments. In autumn 2008, the ICDP-sponsored Potrok Aike Sediment Archive Drilling prOject (PASADO) dedicated a full core to geomicrobiological studies (Vuillemin et al. 2010) and allowed the investigation of microbial populations in deep lacustrine sediments for the first time. Climatic fluctuations, like those recorded during the Holocene (Recasens et al. 2012), may promote or restrain sediment colonization by microbes. In this context, bulk OM proxies were interpreted as reflecting initial OM sources and associated environmental changes, dismissing diagenetic effects within the uppermost 3 m of sediment (Mayr et al. 2009). More recently, geomicrobiological investigations along the uppermost meter have shown the impact of paleoenvironmental conditions on microbial communities (Vuillemin et al. 2013). On the contrary, paleoenvironmental studies rarely inspect the influence of microbes on proxies. The role of microbes as agents of early diagenesis can, however, manifest via degradation processes, leading to organic sources reworking and substrate fractionation. Moreover, the *in situ* biomass production associated with microbial activity is often neglected.

This study investigates methanogenic populations, combining fatty acids (FAs) and methane concentrations, with their corresponding $\delta^{13}\text{C}$ compositions, in the entire Holocene record of Laguna Potrok Aike. We focus on microbial species distribution throughout depth, linking methanogenic pathways to OM degradation and substrate evolution. We inspect local sediment conditions, such as pore water chemistry and organic sources, and their influence on microbial development inferred from adenosine-5'-triphosphate (ATP) detections and cell counts. We define the influence of microbial biomass in sediments using branched-chain and unsaturated FA concentrations and their $\delta^{13}\text{C}$ signatures. Finally, we discuss a potential imprint of microbial diagenesis on paleoenvironmental proxies.

Materials and Methods

Study area and field sampling

Laguna Potrok Aike is an endorheic maar lake in the Pali Aike volcanic field of southern Patagonia, Argentina (Zolitschka et al. 2006). The maar presents a very steep geometry with a water column of a maximal depth of 100 m (Gebhardt et al. 2011). Climatic parameters exert substantial control on the lake level (Ohlendorf et al. 2013) and can lead to important changes in organic sources and geochemical conditions recorded in the sediment (Recasens et al. 2012; Hahn et al. 2013). Presently, the lake is polymictic due to the persistent overturn caused by the Westerlies (average wind speed: 7.4 m/s) with subsaline conditions and water temperatures ranging from 4°C to 10°C throughout

the year. Oxidic conditions prevail in the water column (Zolitschka et al. 2006; Mayr et al. 2007). However, reducing conditions at the water/sediment interface have occurred over time (Haberzettl et al. 2007) and the oxygen penetration within surface sediment appears to be restricted (Vuillemin et al. 2013).

The investigations presented here were carried out in two hydraulic piston cores 5022-1A and 5022-1D of 65 mm diameter, and 87 and 97 m long, respectively (Ohlendorf et al. 2011), retrieved from the centre of the maar at 100 m water depth in autumn 2008. The cores were sampled at a resolution of one meter, avoiding felsic tephra layers and organic macroremains. A detailed description of the field sampling strategy and sample handling can be found in Vuillemin et al. (2010). Complete procedures for *in situ* ATP detection assessment, 4', 6-diamidino-2-phenylindole (DAPI) cell counts, and denaturing gradient gel electrophoresis (DGGE) analyses of bacterial and archaeal diversities are described in Vuillemin et al. (2013).

Organic matter characterization

Stable isotope compositions of the homogenized bulk organic fraction were analyzed on decalcified ($\delta^{13}\text{C}_{\text{org}}$) and untreated ($\delta^{15}\text{N}$) samples, using an elemental analyzer (EuroVector[®], Euro EA[®]) linked in continuous flow to an isotope-ratio mass spectrometer (IRMS; Micromass, IsoPrime[®]). Isotope ratios are reported in δ -notation in per mil according to the following equation: $\delta = (R_{\text{sample}} / R_{\text{standard}} - 1) \times 1000$, where R is the measured ratio of $^{13}\text{C}/^{12}\text{C}$ of the sample and of the Vienna PeeDee Belemnite standard (V-PDB). Analytical precision of the isotope analysis is better than 0.1‰. Total

organic carbon (TOC) and total nitrogen (TN) were calculated from the yield of CO₂ and N₂ measured after sample combustion in the elemental analyzer. Analytical precision was $\pm 3\%$ (1σ) for C_{org} and $\pm 2\%$ (1σ) for N. TOC was recalculated to the content of the whole sample and results are presented in dry mass %. TOC and TN values were further used to calculate molar C_{org}/N ratios.

Methane headspace and methane carbon isotopic composition

The vials sampled in the field were first sonicated to homogenize sediments within the solution prior to methane headspace determinations. The gas fraction was sampled using a HP7694 chromatograph with a Headspace Sampler and separated by molecular weight on a GC column into methane, N₂O, and CO₂ and transported differentially with argon as the carrying gas to a coupled mass spectrometer (Agilent 6850 Series GC System). Methane peaks were detected in a flame ionization detector (FID) and appeared on the chromatogram at 2 min 11 sec. Methane spikes were then used to calibrate and transform detection intensities (pA) into volumes and millimolar by applying the ideal gas law $PV=nRT$ at standard conditions. Traces of nitrous and sulfidic gases were detected in the samples, but not measured any further.

The carbon isotope composition of methane ($\delta^{13}\text{C}_{\text{CH}_4}$) was determined on the same samples as for headspace chromatography, but only for three samples. Duplicate measurements were processed with an IRMS (Isoprime[®]) connected to a trace gas preconcentrator. The trace gas preconcentrator works at a helium flow of 17mL/min. H₂O is eliminated with a magnesium perchlorate trap and CO₂ in a Carbosorb[®] trap.

Methane of the cleaned gas flow is oxidized to CO₂ at 960°C with copper oxide. The evolved CO₂ is trapped and concentrated in a cryogenic trap. It is cleaned and separated in a porabond GC column before entering the IRMS. Results are given in the standard δ -notation relative to V-PDB and the precision for a duplicate $\delta^{13}\text{C}_{\text{CH}_4}$ analyses is 2-3 ‰ V-PDB.

Interstitial water chemistry

Pore water samples were obtained from core 5022-1A using soil moisture samplers (Rhizon SMS, Eijkelkamp) introduced into the sediment through small holes drilled in the core liners. Fluids were extracted using syringes screwed to the microrhizons and maintained under low pressure. After sampling, recovered waters were split for cation and anion analyses and flushed with helium gas immediately thereafter to avoid any shift in water chemistry. The transfer of pore water samples into sealed vials was performed under a N₂ protective atmosphere in small chamber. Samples for cation analyses were acidified with 100 μL HNO₃ (65%). Cations were measured by ICP-MS whereas anions were analyzed by ion-chromatography.

Fatty acid extraction

FA extractions, targeting compounds from 12 to 35 carbons, were carried out on fresh sediments preserved under anoxic conditions in hermetically sealed bags (Vuillemin et al. 2013). The extraction method was previously described by Naeher et al. (2012). Sediments (2 cm³) were ultrasonically extracted (40 min) with 2 mL methanol (MeOH)

and 6 mL dichloromethane (DCM), and heated (2 min at 330 W and 6 min at 500 W) in teflon tubes using a high performance microwave (Milestone[®]). Liquid and sediment fractions were separated by centrifugation. Sediments were retrieved, dried and weighed. Liquid extracts were combined in a separatory funnel with 20 mL milli-Q water DCM extracted NaCl solution (5%). The aqueous phase was extracted with 10 mL DCM twice, and the collected DCM fraction reduced by rotary evaporation (Rotavapor[®] R-210, Buchi). Samples were dissolved in DCM, dried with Na₂SO₄, blown down with N₂ at 30°C, redissolved in DCM, passed through a copper column to eliminate sulfur traces and blown down again. 3 mL KOH 6% was added and saponification carried out for 3 hours at 80°C. 1 mL of DCM extracted H₂O was added to stop the reaction. Neutral lipids were extracted with 1 mL n-hexane (4×), blown down, dissolved in DCM, dried with Na₂SO₄ and kept in the freezer. After acidification with HCl 6M (~0.85 mL), FAs were extracted with 1 mL n-hexane (4×), blown down, dissolved in 1 mL BF₃/CH₃OH 10% (Sigma Aldrich) to produce the methyl esters (FAMES). Derivatization was carried out for 30 min at 60°C, and the reaction stopped with 2 mL of DCM extracted H₂O. FAs were extracted with 1 mL n-hexane (4×), blown down, dissolved in DCM, dried with Na₂SO₄, blown down once more, and dissolved in 100 µL n-hexane. Aliquots (20 µL) were used for measurements. The precision for double injection is better than 0.5% and better than 8% for the whole method including extraction.

Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS)

The resulting fractions were measured using a GC System 6890 Series Plus (Agilent®) with a MultiPurposeSampler MPS2 (Gerstel®) and flame ionisation detection (GC-FID, Agilent 6890, with a 60 m VF-5 column x 0.25 mm inner diameter x 0.25 µm film thickness). Peaks quantification was achieved by using spikes of an external standard (C₃₆). The second run of analyses was performed on a GCMS-QP 2010 Ultra (Shimadzu®) with an autoinjector AOC-201 (Shimadzu®). The retention times of FAs detected in samples were compared with retention times of known compounds (FAME and BAME, Supelco®) on the GCMSSolution® version 2.70 software (Shimadzu®). Corroboration of FA identification was by comparison of mass spectra to those in the NIST 08 Mass Spectral Library. A third run of analyses was performed on a Network GC System 6890N (Agilent®) coupled to a Micromass IRMS (Isoprime®) to measure $\delta^{13}\text{C}$ composition of FAs. A nC_{25:0} alkane standard ($\delta^{13}\text{C} = -31.7\text{‰}$) was regularly measured to check for instrumental deviation. The precision of samples measured in triplicate is better than 0.5 ‰. The linearity of the IRMS is consistent from 0.5 to 2.0 nA. Samples were concentrated to reach at least 5 pA for a reliable measurement.

DNA extraction and PCR amplification

Sediment DNA extractions and purifications were performed using the commercial DNA extraction kit Mobio PowerSoil™ Isolation kit. The methodology was applied as recommended in the manufacturer's instructions.

First PCR amplifications were performed for Bacteria and Archaea with 3 µL DNA template, 1× PCR-buffer (Takara), 0.4 µmol/L of each of the primers, 200 µmol/L of

each of the desoxynucleotide triphosphates, 1.25 units Ex-Taq polymerase (Takara) in a 50- μ L PCR reaction mixture with molecular grade water. Negative controls were added to all PCR sets with 1 μ L of molecular grade water as template to provide a contamination check. Bacterial 16S rRNA gene amplifications were performed using the bacterial universal primer pair 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). Reaction mixtures were held at 95°C for 2 min followed by 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 90 s plus 1 s per cycle, with a final extension step of 5 min at 72°C (Webster et al. 2003). For archaeal gene amplifications, a nested PCR approach with overlapping primers was selected to avoid an enrichment step by cultures (Vissers et al. 2009). 4F (5'-TCY GGT TGA TCC TGC CRG-3') with Univ1492R (5'-CGG TTA CCT TGT TAC GAC TT-3') were used as the initial primer pair (Dong et al. 2006). Archaeal PCR amplifications were performed as follows: 94°C for 5 min, 30 cycles at 94°C for 1 min, 53°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 5 min (modified after Ye et al. 2009). 2 μ L of PCR product were used in the second PCR round with the overlapping forward primer 3F (5'-TTC CGG TTG ATC CTG CCG GA-3') associated with 9R (5'-CCC GCC AAT TCC TTT AAG TTT C-3') as the reverse primer.

Cloning procedure and phylogenetic analyses

PCR products were purified using the High Pure[®] PCR Product Purification Kit (Roche Diagnostics SA), measured with a Nanodrop[®] ND-1000 Spectrophotometer (Witec AG), and diluted to a concentration of 10 ng/ μ L. 2 μ L PCR template were ligated to the

pCR[®]4-TOPO[®] vector (Invitrogen[™] by life technologies[™]) and cloned into competent *Escherichia coli* cells. Cloning procedure was performed using the TOPO[®] TA Cloning[®] Kit (Invitrogen[™] by life technologies[™]) following the manufacturer's recommendations. Transformed cells were incubated at 37°C for 20 hours on a LB medium containing 1g/L NaCl, 1 g/L Bactotryptone, 0.5 g/L Bactoyeast, 1.5 g/L Bactoagar and 2 mL/L ampicillin. Archaeal clones were selected from sediment samples at 0.3, 0.6, 1.9, 2.5, 5.0, 7.8, 9.4 m depth to constitute the libraries. Sequencing cycles were performed using the BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied BioSystems) with vector primers D4 and R5. Sequencing was performed on an ABIPRISM[®] 3130xl Genetic Analyzer (Applied BioSystems, Hitachi).

Sequences were assembled with CodonCode Aligner[®] v.3.7.1 (CodonCode Corporation), aligned on Seaview[®] v.4.3.0 (Gouy et al. 2010) using the clustalW2 algorithm, and primers selectively cut off with VecScreen (www.ncbi.nlm.nih.gov/VecScreen). Chimeras were detected using the online program Bellerophon[®] (Huber et al. 2004) (<http://comp-bio.anu.edu.au/bellerophon/>). 16S ribosomal DNA gene sequences were identified using the megx[®] Geographic-BLAST (<http://www.megx.net>) and SILVA[®] comprehensive ribosomal RNA databases (Pruesse et al. 2007). Rarefaction curves (not shown) were established for 5 and 10% divergence, using the Mothur[®] v. 1.25.1 software (Schloss et al. 2009). Sequences related to methanogens were selected for phylogenetic analyses. References were obtained from the SILVA[®] browser (<http://www.arb-silva.de>) and a phylogenetic tree produced on the Ribosomal Database Project (<http://rdp.cme.msu.edu>) using the

Weighbor Joining method (Bruno et al. 2000), including results from a bootstrap test using 100 replicates.

The sequences generated in this study have been deposited in the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>) under accession numbers JX272064 to JX272122.

Results

Bulk organic matter proxies and organic remains (diatoms)

TOC contents (Fig. 1A) average 0.6%, with values over 1% at 0.3, 0.7, 6.6 m depth and a maximum (2%) at 11.3 m depth. Atomic C_{org}/N ratios (Fig. 1A) display variations with depth, with maxima at 0.7, 4.0 and 10.4 m depth and minima at 0.6, 2.5 and 5.0 m depth. Organic carbon and nitrogen isotope compositions of the bulk fraction show opposite trends. $\delta^{13}\text{C}_{\text{org}}$ decreases gradually from -23.0‰ at the surface to -27.8‰ at 6.2 m depth (Fig. 1A) and increases to -24.1‰ at 11.3 m depth. Conversely, the $\delta^{15}\text{N}$ values are 4.0‰ in the uppermost sediments, increase to 6.6‰ at 3.0 m depth (Fig. 1A), decrease to 5.5‰ at 5.0 m depth, increasing to 6.6‰ at 7.8 m depth and decreasing to 3.2‰ at 11.3 m depth. A sharp peak in diatom concentration, an indicator of algal paleoproductivity, is observed between 9.4 and 11.3 m depth (Fig. 1B; Recasens et al. 2012).

Pore water chemistry

Salinity concentrations (i.e. $\text{Na}^+ + \text{Cl}^-$) range between 22.62 (5.6 m depth) and 18.53 mM (11.6 m depth), reflecting subsaline conditions (Fig. 1B). Sulfate concentrations (Fig. 1B) are maximal (267 μM) in surficial sediments, decrease to 63 μM at 2.0 m depth, increase to 184 μM at 4.2 m depth, with sulfate depletion (ca. 20 μM) occurring below 5.0 m depth. Nitrate concentrations are all below 5 μM , while phosphate oscillates between 60 and 100 μM in the uppermost 8 m of sediment and is depleted (ca. 10 μM) at 9.2 m depth (Fig. 1B).

Total methane content and its carbon isotopic composition ($\delta^{13}\text{C}_{\text{CH}_4}$)

Methane content (Fig. 1C) displays a constant increase from the sediment surface to 1.9 m depth, reaching 100 mM. It decreases subsequently to 50 mM at 4.0 m and fluctuates between 60 and 85 mM thereafter. The carbon isotope compositions of methane ($\delta^{13}\text{C}_{\text{CH}_4}$) were measured on selected samples at 1.3, 7.8 and 10.3 m depth. $\delta^{13}\text{C}_{\text{CH}_4}$ values correspond to -24.0‰ in shallow sediments, decreasing to -68.3‰ and -65.9‰ in bottom sediments.

Microbial indexes

ATP is only produced by living organisms and its measurements are presently used as an indicator of ongoing microbial activity. Background readings measured on milli-Q water varied between 25 and 30 relative luminescence units (RLU). 30 RLUs were, thus, systematically removed from every reading. Average values increase from 50 at 0.3 m depth to 217 RLUs at 4.0 m depth (Fig. 1C), with sharp peaks occurring at 0.6

and 4.0 m depth. ATP values then decrease to a minimum at 7.8 m depth, with a minor final increase at 9.4 m depth. DAPI cell counts (Fig. 1C) varies throughout depth, displaying three maxima at 0.6, 5.0 and 7.8 m depth, local minima at 2.5 and 7.0 m depth and a minimum in the lowermost sediment. The number of DGGE bands indicates the dominance of Bacteria over Archaea (Fig. 1C), but both trends are similar, with maxima and minima at identical depths. Low band numbers are found at 0.71, 3.41, and 7.0 m depth and maximal band numbers at 0.3, 2.5, 5.0, and 9.4 m depth.

Fatty acid concentrations and their $\delta^{13}\text{C}$ isotopic signatures

For each sample, we identified 27 compounds from $\text{C}_{14:0}$ to $\text{C}_{30:0}$. Concentrations are expressed in % of dry sediment normalized to TOC. $\delta^{13}\text{C}$ compositions of unsaturated FAs, medium-chain saturated fatty acids (MC SFAs) and long-chain saturated fatty acids (LCFAs) represent mean values normalized by each compound concentration (Fig. 2A). Total FAs reach 1% of the TOC content at 5.0, 7.8 and 10.3 m depth (Fig. 2A), and display an overall increase with depth. Unsaturated and iso/antesio FA concentrations show minor maxima at 2.5, 5.0 and 7.8 m depth. Their corresponding $\delta^{13}\text{C}$ signatures (Fig. 2A) display values between -34 and -32‰ from 4.0 to 7.0 m depth, a maximum (-27.0‰) at 7.8 m depth, followed by a minimum (-34.5‰) at 9.4 m depth. MC SFAs represent a substantial proportion of FAs from 4.0 to 7.0 m depth, but clearly dominate in lowermost sediments (7.0 to 11.3 m depth). Their $\delta^{13}\text{C}$ values gather around -32‰ with few variations (Fig. 2A), with a maximum (-29.1‰) followed by a minimum (-34.5‰) in the lowermost record. LC SFAs are substantial at 1.3 and 5.0 m

depth and display an opposite $\delta^{13}\text{C}$ trend compared to unsaturated FAs and MC SFAs (Fig. 2A), with maximal values (-25.2 and 28.0‰) at **0.7** and 6.4 m depth and minimal values (-34.8 to 35.9‰) from 7.0 to 9.4 m depth.

Concentrations for $\text{C}_{14:0}$, $\text{C}_{16:0}$ and $\text{C}_{18:0}$ (Fig. 2B) are high in lowermost sediments, indicating the substantial presence of labile OM. Their $\delta^{13}\text{C}$ signatures are identical (Fig. 2C), showing an increase with depth from a minimum of -33.6‰ to a maximum of -27.7‰ at 7.8 m depth. In contrast, $\delta^{13}\text{C}$ profiles for $\text{C}_{20:0}$ and $\text{C}_{22:0}$ values (Fig. 2C) slightly decrease from -27.7‰ to -36.7‰ with depth, reaching a minimum (-41.3‰) at 7.8 m depth. Refractory OM is represented by $\text{C}_{24:0}$ to $\text{C}_{30:0}$. $\text{C}_{24:0}$ and $\text{C}_{26:0}$ concentrations (Fig. 2B) are substantial at 1 m and from 4.0 to 7.0 m depth, with respective $\delta^{13}\text{C}$ values ranging from -23.0 to -33.8‰ (Fig. 2C), whereas $\text{C}_{28:0}$ and $\text{C}_{30:0}$ constitute a minor fraction, displaying low $\delta^{13}\text{C}$ values (-30.5 to -37.8‰).

Bacterial FAs constitute 2 to 23% of total FAs, and 8 to 37% including all unsaturated FAs. Generally, microbial FA concentrations (Fig. 2B) are maximal at 1.9, 5.0, 7.8 and 10.3 m depth. Peaks associated with specific bacterial compounds (Fig. 2D) occur at: 5.0, 7.8 and 10.3 m depth for $\text{C}_{15:0}$ iso and $\text{C}_{15:0}$ anteiso; 1.9 and 4.0 m depth for $\text{C}_{16:0}$ iso and $\text{C}_{16:1}$; 1.9 m depth for $\text{C}_{17:0}$ iso and $\text{C}_{17:0}$ anteiso; 1.9, 5.0 and 7.8 m depth for $\text{C}_{18:2}$ and $\text{C}_{18:1}$. Because detection was not achieved for compounds of concentrations below 100 $\mu\text{g/gTOC}$ (Fig. 2C), these $\delta^{13}\text{C}$ composition results are partial (Fig. 2D). A first trend referring to bacterial chains from $\text{C}_{15:0}$ iso to $\text{C}_{18:1}$ was detected, with values included in a 5‰ interval (Fig. 2C, dotted lines) with decreasing tendencies at 1.9, 4.0 and 5.0 m depth and a maximum value (-24.9‰) at 7.0 m depth. At 1.9 m

depth, C_{17:0} anteiso displays a very low $\delta^{13}\text{C}$ value (-37.5‰). Bacterial chains over twenty carbons show a linear decrease with depth from -31.6‰ to -45.0‰ (Fig. 2C, dashed line), indicating important fractionation processes linked to methane production.

Archaeal phylogenetics

Archaeal clone libraries were established from samples at 0.25, 0.55, 1.91, 2.51, 5.0, 7.84 and 9.4 m depth. Sequences in uppermost sediments are mainly related to the Marine Group 1 and Marine Benthic Groups B and D (Fig. 3) with few methanogens, whereas methanogens and the Miscellaneous Crenarchaeotic Group (MCG) dominate in samples from 1.9 to 9.4 m depth. South African Gold Mine Group (SAGMEG) sequences appear below 7.0 m depth.

A phylogenetic tree was built with 58 methanogen sequences (Fig. 4) from Laguna Potrok Aike sediments, evidencing a majority of *Methanomicrobiales* and *Methanosarcinales* with an overall low diversity of species. The defined clusters highlight a stratification of methanogenic populations in Holocene sediments. Most sequences at 0.3, 0.6 and 2.5 m depth are identified as *Methanolinea* sp., whereas sequences at 1.9 m depth relate to *Methanosarcinales* Gulf of Mexico Archaea 1 (GOM Arc 1). Sequences at 5.0 and 7.8 m depth comprise *Methanoregula* sp. exclusively. The few sequences at 9.4 m depth are identified as *uncultured Methanomicrobiaceae* branching in between *Methanosphaerula palustris* and *Methanolinea tarda*.

These results include two bacterial sequences retrieved at 5.0 m depth, because they relate to *Syntrophus aciditrophicus* (Table 1), which is known to establish syntrophic relationships with methanogens (Jackson et al. 1999).

Discussion

Bulk organic matter characteristics and microbial development

OM sources in bulk sediments are often traced using C_{org}/N ratio, $\delta^{13}C_{org}$ and $\delta^{15}N$. Although these parameters generally allow the distinction between terrestrial and aquatic contributions, they can hardly discriminate individual sources (Das et al. 2008). As algal OM is preferentially degraded during sinking and sedimentation, source interpretations are often biased towards macrophytes and land plants (Meyers 1994). Diagenesis generates the preferential preservation of refractory OM with a preferential removal of nitrogen over carbon (Giani et al. 2010). Moreover, ammonium adsorption in clay lattices often leads to a decrease in C_{org}/N ratios with depth and over time (Freudenthal et al. 2001). Still, high C_{org}/N ratios can indicate sporadic terrestrial inputs into Laguna Potrok Aike sediments (Fig. 1A), and high diatom concentrations attest of high algal productivity (Fig. 1B).

However, microbial processes can extend deep into the sediments, as shown by the ATP values at 4.0 m depth (Fig. 1C). DAPI cell counts and DGGE (Fig. 1C) also indicate that microbial populations have preferentially colonized and proliferated in those horizons with low atomic C_{org}/N ratios (Fig. 1A). Additionally, microbial

development appears to be strongly correlated with phosphate concentrations in pore water (Fig.1B), which are directly dependent on OM degradation. Microbial colonies, with regard to their alteration potential (Lehmann et al. 2002) and biomass production (Freudenthal et al. 2001), influence and become part of the labile organic sources. Beside the fact that bacterial growth is higher when associated with such labile OM (Amon and Benner 1996), the conversion of refractory components into methane can be carried out via trophic links between fermenting microbes and methanogens (Wüst et al. 2009). However, methanogenesis is considered a late stage of degradation as it normally occurs after depletion of available oxidants by layered microbial communities (Nealson 1997; Konhauser 2007). In Laguna Potrok Aike sediments, methanogenic activity should start around 0.7 m depth to correspond with the transition to CO₂ as the main oxidant (Vuillemin et al. 2013). Coexistence can nevertheless be achieved via mutualistic interactions and syntrophy (Wang et al. 2010), which are essential to the complete conversion of natural polymers (McInerney et al. 2009). In lacustrine anoxic sediments, syntrophic populations are widespread and present a strong potential for carbon fractionation (Borrel et al. 2011).

Depth distribution of methanogenic species

Archaeal distribution with depth (Fig. 3) shows that methanogens become dominant from 1.9 m depth, coinciding with the highest methane concentration (Fig. 1C). The phylogenetic tree (Fig. 4) emphasizes four depth-dependent clusters, revealing the stratification of methanogenic populations in the sediment. The first cluster (ca. 0.3-2.5

m depth downward) is related to *Methanolinea tarda* (Fig. 4), a species that tolerates a 1.5% salinity and grows using C1 compounds exclusively (e.g. formate, H₂/CO₂), thus referring to the methyl fermentation and CO₂ reduction pathways (Imachi et al. 2008). A syntrophic relationship with sulfate-reducing bacteria (SRB) is often observed under sufficient sulfate concentrations (Fig. 1B; Imachi et al. 2006 and 2008). The second cluster (ca. 1.9 m depth) is composed of *GOM Arc 1 Methanosarcinales*. These methanogens have been documented in relation to the anaerobic oxidation of methane (AOM) and are referred to as AOM- associated Archaea (AAA; Borrel et al. 2011; Schubert et al. 2011). Growth and methane production are based on fermentative products, such as methanol, methylamines, H₂/CO₂ and possibly acetate (Simankova et al. 2001). Methanogenesis derived from methylamines leads to the production of ammonium (Hedderich and Whitman 2006), which can be critical for microbial development under nitrogen-limiting pore water conditions (Fig. 1B). Substrate competition does not occur with *Methanolinea* or SRB (Borrel et al. 2011). The third cluster (ca. 5.0 to 7.8 m depth) is composed of *Methanoregula sp.* (Fig. 4), a halotolerant but sulfide sensitive species producing methane from H₂/CO₂ exclusively (Bräuer et al. 2011), thus indicating the CO₂ reduction pathway. The presence at the same depth of *Syntrophus*-related sequences (Table 1) provide evidence for a syntrophic relationship with *Methanoregula* (Jackson et al. 1999; Garcia et al. 2000), thus potentially enabling an advanced metabolization of refractory polymers by microbes (McInerney et al. 2009; Wüst et al. 2009). The fourth cluster (ca. 10.3 m depth) corresponds to *uncultured Methanomicrobiaceae*, which have been previously

reported in syntrophic acetate oxidation processes (Schnürer et al. 1994; Nüsslein et al. 2001). Nearest related species use mainly formate and H_2/CO_2 during methanogenesis, but require acetate for growth (Asakawa and Nagaoka 2003; Cadillo-Quiroz et al. 2009).

Methanogenic populations at Laguna Potrok Aike are dominated by hydrogenotrophs, with possible syntrophic partnerships involving *Methanoregula* and *Methanolinea*, which is in agreement with many documented freshwater environments of low trophic levels (Borrel et al. 2011). Conditions in Laguna Potrok Aike water column and sediment (Zolitschka et al. 2006) also suggest that these species are halo and psychrotolerant (Simankova et al. 2001; Simankova et al. 2003). The stratification of methanogenic species with depth may derive from the gradual degradation of OM which provides successive substrates to each species (Fig. 5). Methanogenesis starts with the methyl fermentation pathway and shifts to CO_2 reduction at 2.5 m depth (Fig. 5). The transition depth is colonized by *Methanosarcinales*, perhaps due to their ability to use variable fermentative products as substrate. In lowermost sediments, we propose that the *uncultured Methanomicrobiaceae* perform syntrophic oxidation of acetate as a late stage of methanogenesis (Fig. 5).

Methane $\delta^{13}C$ signature and organic matter degradation

Methane production is mainly based on methyl fermentation in freshwater shallow sediments, in which CO_2 reduction plays a minor role (Whiticar 1999). In Laguna Potrok Aike, methane content and microbial activity do not correlate (Fig. 1C), mainly because methanogenesis depends on non-competitive substrates (Whiticar 1999; Conrad et al.

2010), whereas microbial assemblages are dominated by Bacteria (Fig. 1C). The linear increase of methane content in shallow sediments may reflect gas diffusion toward the surface from a zone of maximal production around 2 m depth. The $\delta^{13}\text{C}_{\text{CH}_4}$ composition at 1.3 m depth is relatively high (-24.0‰), corresponding with the bulk $\delta^{13}\text{C}_{\text{org}}$. AOM processes are known to occur in freshwater sediments in link to nitrate, iron, manganese and sulfate reduction processes (Deutzmann and Schink 2011; Schubert et al. 2011). During this process, the uptake of the light carbon isotope by methane oxidizing microbes results in more positive $\delta^{13}\text{C}_{\text{CH}_4}$ values of the residual methane fraction (Holler et al. 2009). Nitrate in pore water is presently depleted (Fig. 1B) while iron and manganese were below detection limits (not shown). Conversely, sulfate concentration in pore water is sufficient to allow AOM processes (Boetius et al. 2000). In the absence of anaerobic methane-oxidizing Archaea (ANME), this $\delta^{13}\text{C}_{\text{CH}_4}$ value can be interpreted as an early stage of methyl fermentation performed by *Methanolinea* or as due to some microaerobic oxidation of methane. Still, the potential uptake of ^{12}C by SRB in association with AAA methanogens during AOM processes has to be considered (Borrel et al. 2011). These *Methanosarcinales* can use additional substrates (Fig. 4), leading to maximal methane concentration at 1.9 m depth (Fig. 1C). Thereafter, methane concentrations decrease sharply, which may reflect the shift of methanogenic pathway to CO_2 reduction (Whiticar 1999). Indeed, methanogenic populations are essentially composed by *Methanoregula* sp. (Fig. 4) which grows on H_2/CO_2 exclusively (Bräuer et al. 2011; Fig. 5). Syntrophy with *Methanoregula* can explain the current sustainability of microbial populations (Fig. 1C) because it allows an efficient conversion

of polymers into methanogenic substrates (McInerney et al. 2009; Fig. 5). The very negative $\delta^{13}\text{C}_{\text{CH}_4}$ value at 7.8 m depth (-68.3‰) can correspond to an advanced stage of CO_2 reduction (Whiticar et al. 1986) derived from the use of refractory OM by syntrophic consortia (Penning et al. 2005). Deeper in the sediment (ca. 9.4-11.3 m depth), methanogenesis relies possibly on H_2/CO_2 produced during anaerobic oxidation of acetate (Fig. 5), which tends to accumulate during acetogenesis and degradation of FAs (Schink 1997). This is only partially inferred from the $\delta^{13}\text{C}_{\text{CH}_4}$ value (-65.9‰) and presence of *uncultured Methanomicrobiacea*. These methanogens have been reported from deep lacustrine sediments in which they outcompete acetoclastic methanogenesis via syntrophic acetate oxidation (Nüsslein et al. 2001; Hattori 2008).

Fatty acid sources

According to Mayr et al. (2009), the $\delta^{13}\text{C}_{\text{org}}$ composition of the bulk organic fraction measured in sediment traps of Laguna Potrok Aike deep waters varies between -24.2 and -25.5‰. Several OM sources were identified in the OM fraction, whose relative proportions explained the $\delta^{13}\text{C}_{\text{org}}$ bulk values: Diatom oozes (mean: -25.6‰), cyanobacteria (mean: -21.8‰), terrestrial vegetation (mean: -26.0‰), aquatic mosses (mean: -32.2‰), macrophytes (mean: -13.0‰) and soils (mean: -25.0‰).

Terrestrial and planktonic sources are normally traced using LC SFAs and MC SFAs, respectively (Naraoka and Ishiwatari 2000; Niggemann and Schubert 2006). High concentrations of $\text{C}_{16:0}$ to $\text{C}_{20:0}$ FAs along with diatom valves, observed at 10.3 m depth (Figs. 1B and 2B), provide evidence for a substantial contribution of algal sources. Such

preservation of labile OM can be attributed to an increased sedimentation rate associated with redeposition events during the Early mid-Holocene (Anselmetti et al. 2009). On the contrary, concentrations of LC SFAs do not directly correlate with high C_{org}/N ratios (Figs. 1A and 2B). The comparison of ^{13}C compositions between LC SFAs and initial organic sources shows that $C_{24:0}$ and $C_{26:0}$ (Fig. 2C) reflect terrestrial sources values coming from the Patagonian steppe, whereas $C_{26:0}$ and $C_{30:0}$ correspond to aquatic mosses values presently growing on the lake shores. This shows that the ^{13}C signature is best preserved in refractory compounds (Naraoka and Ishiwatari 2000).

Their $\delta^{13}C$ shift towards more positive values (up to 6‰) in between 4.0 and 7.0 m depth coincides with the maximal density, activity and diversity of microbial populations (Fig. 1C) and likely indicates a ^{12}C uptake by microbes and partial reworking of the bulk OM sources. At 1.3 m depth, similar features are observed for the $C_{20:0}$ to $C_{26:0}$ profiles (Fig. 2C), emphasizing the necessity of syntrophy partnership in the degradation of long carbon chains (McInerney et al. 2009). Additionally, $\delta^{13}C$ values for $C_{14:0}$ to $C_{18:0}$ are too ^{13}C -depleted (-27.7 to -34.2‰) as regards possible algal sources (Sun et al. 2004), with even more depleted $\delta^{13}C$ values for $C_{20:0}$ and $C_{22:0}$ in lowermost sediments (from -36.9 to -41.2‰). The contrasting $\delta^{13}C$ trends displayed by unsaturated FAs and LC SFAs (Fig. 2A) further imply that some FAs get ^{13}C -depleted and some ^{13}C -enriched during diagenesis (Gong and Hollander 1997; Freudenthal et al. 2001), with a significantly higher turnover of labile compounds (Amon and Benner 1996). As microbes discriminate against the heavy isotope, FAs produced by microbes tend to be depleted

in ^{13}C in comparison to the initial substrate, while residual FAs are normally enriched in ^{13}C (Niggemann and Schubert 2006).

The relative contribution of bacterial biomass to sedimentary OM can be estimated by branched chain and unsaturated FA concentrations (Fig. 2D; Bechtel and Schubert 2009; Volkman et al. 2008), although the use of these sole compounds sometimes results in an underestimation (Teece et al. 1999). Firstly, bacterial FAs appear more abundant in horizons of low $\text{C}_{\text{org}}/\text{N}$ ratios (Figs. 1A and 2B), implying that labile OM derived from algal sources promotes a better development of heterotrophic bacteria. Secondly, depth distribution of bacterial compounds can account for differences in microbial populations (Boschker and Middelburg 2002). Peaks in $\text{C}_{16:1}$ and $\text{C}_{17:0}$ anteiso concentrations (ca. 1.9 and 4.0 m depth) coincide with higher sulfate concentrations (Fig. 1B), possibly indicating the production of these compounds by SRB (Volkman et al. 2008; Bechtel and Schubert 2009). From 9.4 to 11.3 m depth, high concentrations of $\text{C}_{15:0}$ iso/anteiso, MC SFAs and diatoms (Figs. 1B, 2B and 2D) show that algal OM is the main substrate in the microbial production of these compounds (Niggemann and Schubert 2006). Moreover, the $\text{C}_{18:2}$ peak at 7.8 m depth, which is associated with the decrease in diatom concentration, shows little difference with the $\delta^{13}\text{C}$ signature of diatomaceous oozes (Figs. 1B and 2D). In addition, main bacterial FAs display $\delta^{13}\text{C}$ values ranging from -24.9 to -34.3‰ (Fig. 2C, dotted lines), which possibly correspond with a gradual fractionation of algal matter. Although most ^{13}C signatures of FAs produced and/or reworked by microbes average $\sim -31\text{‰}$, fractionation varies for each compound and can be influenced by different metabolic types and

substrates. SRB growing heterotrophically produce certain compounds that are sometimes depleted in ^{13}C by 12‰ compared to their substrates (Londry et al. 2004). Moreover, methane can presently act as an additional source of carbon, leading to highly ^{13}C -depleted FAs when oxidized (Yang et al. 2011). Thus, $\delta^{13}\text{C}$ values of the $\text{C}_{17:0}$ anteiso (-37.5‰) and methane (-24.0‰) at 1.9 m depth may indicate the production of this compound by SRB along with some microaerobic oxidation of methane (Deutmann and Schink 2011). However, AOM processes related to AAA methanogens or other oxidants cannot be excluded (Schubert et al. 2011). On the contrary, unsaturated compounds of more than 20 carbons were previously linked to methanic environments and possibly to methanotrophs (Stefanova and Disnar 2000; Pancost et al. 2000). Such microbial compounds that are methane-derived show even more ^{13}C -depleted (Pancost et al. 2000). Thus, the linear $\delta^{13}\text{C}$ decrease of $\text{C}_{20:1}$ and $\text{C}_{24:1}$ with depth may be connected with methanogenic activity (Yang et al. 2011) as this trend is parallel to the evolution of the methane ^{13}C composition (Figs. 1C and 2C).

Bacterial FAs represent a small fraction of the sedimentary TOC, but can constitute more than 30% of FA sources within sediments. Moreover, these results do not include long chain FAs constituting methanogen membranes (Boschker and Middleburg 2002) as their extraction requires another protocol. Such ^{13}C -depleted archaeal compounds have been reported to accumulate in horizons showing prolonged methanogenic activity (Noble and Henk 1998; Pancost et al. 2000). Microbial synthesis under anaerobic conditions also produces saturated FAs, sometimes falsely attributed

to terrestrial sources as they can be depleted by up to 12‰ relatively to the substrate (Teece et al. 1999; Niggemann and Schubert 2006).

Imprints on bulk organic proxies

From 3.4 to 7.0 m depth, methanogenic populations are highly active, as emphasized by the ATP results (Fig. 1C) and involve syntrophic partnerships (Table 1). Cell counts and microbial FA concentrations (Figs. 1C and 2D) denote a significant microbial biomass relatively enriched in ^{12}C (Fig. 2C), which is often not taken into account when using organic proxies for paleoenvironmental reconstructions. Excursions in $\delta^{13}\text{C}_{\text{org}}$ and $\delta^{15}\text{N}$ seen in the same interval (Fig. 1A) are difficult to explain using organic sources modeling (Mayr et al. 2009). Conversely, methanogenic populations, as the ones identified in Laguna Potrok Aike, can potentially fractionate nitrogen from the substrate (Lojen et al. 1997). Because *Methanosarcinales* drive OM ammonification (Hedderich and Whitman 2006), microbial biomass could get enriched in ^{15}N via the assimilation of ammonium (Macko and Estep 1984) leading to more positive $\delta^{15}\text{N}$ values (Lojen et al. 1997). The capacity to grow solely on CO_2 and ammonium has been documented for several methylotrophs and hydrogenotrophs (Kenealy et al. 1982; Raemakers-Franken et al. 1991). As syntrophic OM degradation goes on, the recycling of byproducts into microbial biomass can turn into an additional factor of isotope fractionation (Freudenthal et al. 2001).

At 7.8 m depth, microbial activity, diversity and density start decreasing, while $\delta^{13}\text{C}_{\text{org}}$ and $\delta^{15}\text{N}$ compositions converge showing a positive correlation again (Figs. 1A

and 1C). Although possible early diagenetic imprints on organic fractions were inspected down to 3 m depth, alteration and production of successive substrates by microbes (McInerney et al. 2009) are factual means of their prolonged activity (Fig. 1C). Hence, we propose that microbial reworking of bulk OM initial signatures is occurring within specific sedimentary layers that are sheltering dense and active methanogenic populations.

Conclusions

Our combined bulk OM, pore water, fatty acids, isotopic and phylogenetic study of Laguna Potrok Aike sediments show that methanogenic species stratify over depth depending on the substrate. Syntrophic interactions along with a stepwise degradation of OM allow microbial populations to sustain their activity in deep sediments, leading to the partial reworking of refractory compounds. The succession of methanogenic pathways with depth gradually increases carbon fractionation, resulting in very negative $\delta^{13}\text{C}$ values of methane and specific FAs. The capacity of methanogens to grow on fermentation byproducts solely makes them an additional source of biomass enriched in ^{12}C and ^{15}N within sediments. Microbial biomass sometimes accounted for a third of FA sources within sediments. As specific sedimentary horizons have been exposed to the prolonged influence of these methanogenic populations, the isotope compositions of the bulk OM fraction can be considered partially overprinted by microbial diagenesis. Thus, these results demonstrate that diagenesis of labile organic fractions mediated by

microbes should be considered a potential bias of paleoenvironmental reconstructions using bulk OM proxies.

Acknowledgements

This study was financially and logistically supported by ICDP, Swiss National Science Foundation (Grant 200020-119931/2 to D. Ariztegui), University of Geneva, University of Bremen, Deutsche Forschungsgemeinschaft, Eawag, Natural Sciences and Engineering Research Council of Canada, University of Buenos Aires, Secretaria de Ciencia y Tecnologia of Cordoba, Vetenskapsrådet of Sweden. The following persons are kindly acknowledged for their help and advice on microbiology methods: J. Pawlowski, J. Fahrni, J. Guiard, P. Junier and their research partners for PCR and DGGE procedures. We also thank M. Holzmann, F. Lejzerowicz, L. Perret-Gentil and their research partners for their help during cloning procedures. We are also indebted to C. Mayr and S. Becker for sampling and performing pore water analyses on the ICP-MS, A. Lücke and H. Wissel for carrying out the isotope measurements, W. Klöti for processing methane concentration analyses, and P. Achleitner for organizing the accommodation at the EAWAG.

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Table and figures captions

Fig. 1. Multiproxy dataset displaying bulk OM characteristics, pore water concentrations and microbial indexes. **Top row (A) left to right:** TOC [% dry weight]; atomic C_{org}/N ratio; $\delta^{13}\text{C}_{\text{org}}$ and $\delta^{15}\text{N}$ of the bulk fraction [‰]. **Medium row (B) left to right:** Diatom concentration [million valve/gram dry sediment]; pore water Na + Cl concentrations [mM]; pore water sulfate concentrations [μM]; pore water nitrate and phosphate concentrations [μM]. **Bottom row (C) left to right:** Methane content [mM] and some of its respective $\delta^{13}\text{C}$ values [‰]; *in situ* ATP detections [RLU]; DAPI cell counts [million cell/gram dry sediment]; number of DGGE bands for Bacteria (white squares) and Archaea (black dots with grey ones indicating cloned samples).

Fig. 2. Multiproxy dataset displaying FA concentrations and their respective carbon isotope signatures. **First row (A) left to right:** Cumulative total concentrations of FAs displayed as specific fractions (iso/anteiso, unsaturated, MC SFAs, LC SFAs) [mg/gTOC]; $\delta^{13}\text{C}$ of branched and unsaturated FAs [‰]; $\delta^{13}\text{C}$ of MC SFAs [‰]; $\delta^{13}\text{C}$ of LC SFAs [‰]. **Second row (B) left to right:** Concentrations of FAs $\text{C}_{14:0}$, $\text{C}_{16:0}$ and $\text{C}_{18:0}$ [mg/gram TOC]; concentrations of FAs $\text{C}_{20:0}$, $\text{C}_{22:0}$ and $\text{C}_{24:0}$ [mg/gram TOC]; concentrations of FAs $\text{C}_{26:0}$, $\text{C}_{28:0}$ and $\text{C}_{30:0}$ [mg/gram TOC]; cumulative FAs concentrations indicative of microbial biomass [mg/gram TOC]. **Third row (C) left to right:** $\delta^{13}\text{C}$ of FAs $\text{C}_{14:0}$, $\text{C}_{16:0}$ and $\text{C}_{18:0}$ [‰]; $\delta^{13}\text{C}$ of FAs $\text{C}_{20:0}$, $\text{C}_{22:0}$ and $\text{C}_{24:0}$ [‰]; $\delta^{13}\text{C}$ of FAs $\text{C}_{26:0}$, $\text{C}_{28:0}$ and $\text{C}_{30:0}$; $\delta^{13}\text{C}$ of various microbial fatty acids [‰]. **Fourth row (D) left to right:** $\text{C}_{15:0}$ iso/anteiso concentrations [$\mu\text{g}/\text{gram TOC}$]; $\text{C}_{16:0}$ iso and $\text{C}_{16:1}$ concentrations [$\mu\text{g}/\text{gram TOC}$]; $\text{C}_{17:0}$ iso/anteiso concentrations [$\mu\text{g}/\text{gram TOC}$]; $\text{C}_{18:2}$ and $\text{C}_{18:1}$ concentrations [mg/gram TOC].

Fig. 3. Histogram of all retrieved archaeal sequences and their respective identifications.

Fig. 4. Phylogenetic tree (Weighted Neighbor Joining) of partial 16S rRNA gene sequences (900 bp) related to methanogenic species. Sequences were retrieved at various depths from Laguna Potrok Aike anoxic sediments. *Methanosarcinales* present in Laguna Potrok Aike sediments are related to the AAA lineage (i.e. AOM-associated Archaea).

Fig. 5. Schematic summary showing the stepwise OM degradation. Successive steps are hydrolysis, acidogenesis (or fermentation), acetogenesis and methanogenesis. Methanogens from the present study are placed with respect to their substrates and depths. It shows that methanogenesis takes place during different degradation stages. Final products can reenter the cycle by being reduced (CO_2 reduction) or oxidized (CH_4 anaerobic oxidation).

Table 1. Closest matches of bacterial 16S rRNA gene sequences, retrieved at 4.97 m sediment depth, to cultured and environmental syntrophic organisms.

Clone No.	Length (bp)	Maximum score in Genbank database	Query coverage	Maximum identity	Accession N°
POTROK_AIKE_Bact D7/Clone 03	1425	<i>Syntrophus aciditrophicus</i> (GU993263)	99%	87%	JX272064
		<i>Syntrophaceae uncultured</i> (FJ484515)	SILVA	SILVA	
POTROK_AIKE_Bact D7/Clone 39	1437	<i>Syntrophus aciditrophicus</i> (GU993263)	99%	94%	JX272065
		<i>Syntrophaceae uncultured</i> (AM086113)	SILVA	SILVA	