

Effect of organic carbon and metal accumulation on the bacterial communities in sulphidogenic sediments

Matthieu Bueche¹ · Pilar Junier¹

Abstract A unique geochemical setting in Lake Cadagno, Switzerland, has led to the accumulation of insoluble metal sulphides in the sedimentary record as the result of past airborne pollution. This offers an exceptional opportunity to study the effect of these metals on the bacterial communities in sediments, and in particular to investigate further the link between metal contamination and an increase in the populations of endospore-forming bacteria observed previously in other metal-contaminated sediments. A decrease in organic carbon and total bacterial counts was correlated with an increase in the numbers of endospores in the oldest sediment samples, showing the first environmental evidence of a decrease in nutrient availability as a trigger of sporulation. Proteobacteria and Firmicutes were the two dominant bacterial phyla throughout the sediment, the former in an area with high sulphidogenic activity, and the latter in the oldest samples. Even though the dominant Firmicutes taxa were stable along the sediment core and did not vary with changes in metal contamination, the prevalence of some molecular species like *Clostridium* sp. was positively correlated with metal sulphide concentration. However, this cannot be generalized to all endospore-forming species. Overall, the community

composition supports the hypothesis of sporulation as the main mechanism explaining the dominance of endospore formers in the deepest part of the sediment core, while metal contamination in the form of insoluble metal sulphide deposits appears not to be linked with sporulation as a mechanism of metal tolerance in this sulphidogenic ecosystem.

Keywords Heavy metals · Nutrient deprivation · Starvation · Endosporulation · Firmicutes · Lake Cadagno

Introduction

Metals play an important role in many biochemical processes and are thus essential as micronutrients for all living organisms. However, at higher concentrations, they alter physiological functions, leading to detrimental effects on growth and reproduction (Olaniran et al. 2013). This is also true for microorganisms (Rial et al. 2011) and in many cases, contamination of an ecosystem by toxic levels of metals results in a dramatic decrease in microbial diversity, and therefore in the metabolic functions provided by the microbial community (Hemme et al. 2010).

Lake sediments are often a natural endpoint for metal contaminants. The sources of these are multiple. Natural sources include mineral alteration and transport as wind-borne particles. Heavy industry, burning of industrial and domestic waste or emissions by transportation vehicles can be mentioned as anthropogenic sources (Jarup 2003).

Understanding the impact of metal contamination on microbial communities in sediments and the reciprocal effect of microorganisms on metal mobility in aquatic ecosystems are key interlinked questions. In a previous study in Lake Geneva (Switzerland), we have established that endospore-forming Firmicutes (EFF) are predominant members of the bacterial

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communities in metal-contaminated sediments (Sauvain et al. 2013). The high metal concentration in these sediments has been associated to the release of treated effluents from a wastewater treatment plant on the shore. Natural resistance of EFF to metals was the initial explanation for this observation. In fact, endospore formation is a sophisticated survival mechanism allowing EFF to withstand unfavourable environmental conditions (Errington 2003). Endospores can last in this dormant state for a very long time until the conditions improve, leading to germination and a new vegetative cycle. In laboratory cultures, endospore formation has often been associated to nutrient deprivation (Nicholson 2002). Though this has not yet been demonstrated experimentally, toxic metals might also act as a physiological trigger of sporulation in sediments. However, there is also evidence against a direct link between high metal loads and a positive selective pressure on the native communities of EFF in sediments from Lake Geneva. The finding that Clostridia in human faeces are dominant in the most contaminated samples (Sauvain et al. 2013), suggested a common origin with the contaminating metals (i.e. simultaneous release in the treated effluent). Therefore, a lake model in which the source of contamination is not related to wastewater treatment was needed to better understand the relationship between sediment metal contamination and the selection of EFF.

The selected lake model is Lake Cadagno (Switzerland). This lake is one of the few examples of a crenogenic meromictic aquatic ecosystem. In the case of Lake Cadagno, sulphate-rich waters entering the lake through subaquatic springs contribute to maintaining a higher density in the deepest layers of the water column (monimolimnion), compared to the electrolyte-poor surface water (mixolimnion). This density difference prevents any seasonal mixing. Permanent anoxia prevails in the deepest layers (normally below 12 m deep) and dissolved sulphate feeds an intensive sulphur cycle (Bossard et al. 2001; Tonolla et al. 2004). The chemical conditions in the water column of Lake Cadagno make it an uncommon environment providing a unique opportunity to study geomicrobiological processes, especially those linked with the sulphur cycle and the transition between aerobic and anaerobic conditions in well defined, unmixed compartments within an ecosystem. This type of environments may also provide valuable information on global carbon cycling and other biogeochemical processes, including metal stability in sulphidogenic environments. In fact, a previous study in Lake Cadagno has shown the precipitation of discrete layers of highly insoluble metal sulphides in the sediment. This precipitation pattern has allowed the study of historical atmospheric metal contamination (Birch et al. 1996). The accumulation of these layers is explained by the formation of colloidal particles between metals originated from atmospheric deposition and organic matter or the formation of iron and manganese oxyhydroxides in the oxic layers of the

lake. These particles sink into the deepest anoxic layers of the water column and react with H_2S to produce a stable pattern of deposition of insoluble metal sulphides when reaching the sediment.

Previous microbiological studies in Lake Cadagno have focused on the changes of the bacterial communities along the water column (Bossard et al. 2000). In contrast, studies dealing with its sediments have focused primarily on geochemical processes (Anke Putschew et al. 1995; Birch et al. 1996; Lehmann and Bachofen 1999; Wirth et al. 2013). Recent publications have linked the chemical record with biomarkers of past bacterial activity (membrane lipids, fossil DNA, photosynthetic pigments) to reconstruct the paleoecological history of the lake (Niemann et al. 2012; Ravasi et al. 2012; Wirth et al. 2013). Concerning microbial populations in the sediment, only two publications had targeted anaerobic methane oxidizers (Schubert et al. 2011) and a particular taxonomical group (“morphotype R”) of Desulfobacteriaceae (Tonolla et al. 2005a). As far as we know, the present study constitutes the first attempt to characterize the bacterial communities from sediments of Lake Cadagno and to relate changes in community composition to the geochemistry of the sediment. In addition, considering the origin and mode of deposition of metal contaminants, this lake is an excellent model to evaluate the effect of metal sulphides on the same bacterial communities, and in particular the effect of metals on endospore formation as a response to sediment pollution.

Material and methods

Water column characterization

Lake Cadagno is a 21 m deep meromictic Alpine lake located in the southern Swiss Alps (46° 33' N, 8° 43' E) at 1921 masl. For the characterization of the water column, measurements of pH, oxidation-reduction potential (ORP), temperature, conductivity, salinity, dissolved oxygen and turbidity were made using a YSI 6000 profiler (Yellow Springs Inc., Yellow Spring OH, USA). To measure sulphide, water samples were collected in separate screw-cap tubes in which zinc acetate was previously added to attain a final concentration of 0.25 %. Tubes were stored on ice until sulphide determination by a colorimetric assay (Gilboa-Garber 1971; Tonolla et al. 2005b). Luminosity was measured using two LI-193SA spherical quantum sensors and a LI-1000 data logger (LI-COR, Lincoln NE, USA).

Sediment sampling and conditioning

Sampling took place at the beginning of July 2014 using the facilities of the Alpine Biology Centre (<http://www.piora.org>). A 24 cm long sediment core was collected from the deepest

point (21 m) of the lake using a gravity corer and a 6 cm diameter polycarbonate tube. Once recovered, the core was subsampled every 1 cm. Hereafter, sediment depths will be referred to as the intermediate depth between the upper a lower depths (e.g. 0.5 cm corresponded to the layer between 0 and 1 cm). From each sediment slice, two 5 mL eppendorf tubes were completely filled and conserved at 4 °C in heat-sealed plastic bags with Anaerocult[®] A mini (Merck Millipore, Darmstadt, Germany) to preserve anaerobic conditions in order to measure H₂S, extract pore water and count microbial cells by flow cytometry. For DNA extraction, approximately 2 g of sediment was inserted in a BMT-20-G tube (IKA, Staufen, Germany) and completed with ten 6 mm glass beads. After addition of 10 mL of 1 % sodium hexametaphosphate solution, the mixture was homogenized twice for 1 min at 3000 rpm (inversion every 15 s) using an ultra-turrax[®] tube drive control (IKA) as described elsewhere (Sauvain et al. 2013). Supernatant was recovered after one hour of sedimentation and the homogenization process was repeated a second time with another 10 mL of sodium hexametaphosphate solution. At the end of the process, the two supernatants were pooled together and filtrated on two 0.22 µm cellulose-acetate sterile filters. Filters were immediately frozen at -20 °C and then at -80 °C (when back at the lab) until DNA extraction. To measure dipicolinic acid (DPA) concentration, approximately 5 g of sediment were inserted in 50 mL falcon tubes and immediately frozen at -20 °C, then at -80 °C (when back to the lab) and freeze-dried with a LyoLab AB device (LSL SecFroid, Aclens, Switzerland, liquidated) for about 24 h. To measure particulate metal concentration, total carbon and nitrogen (CHN) and water content, the remaining sediment was dried at 40 °C for 72 h and then weighed. Dried sediments were then conserved for further chemical analysis.

Sediment chemical analyses

Chemical analyses were performed on air-dried agate-ground samples as well as on interstitial water. Interstitial water was extracted from oxygen-protected samples by centrifugation 10 min at 4000×g. Recovered supernatant was then immediately collected and filtered through a 0.45 µm cellulose-acetate filters in a glove box (“La Petite” glove box, Plas-Labs, Lansing MI, USA) purged with N₂. Samples were then diluted with deionized water to a final volume of approximately 10 mL and acidified with low metal content HNO₃ to a final concentration of 1 %. These fixed samples were used for metal quantification by ICP-MS.

Solid sediment samples were weighted in tin capsules for CHN determination using an EA1108-elemental analyser (CE Instruments Ltd, Wigan, UK). Another set of samples was weighted in silver capsules and decarbonated by 6 h of HCl fumigation (Harris et al. 2001) for organic carbon determination prior to CHN analyses, and finally digested with aqua

regia (Förstner and Wittmann 1981) for total particulate metal determination by ICP-MS. ICP-MS measurements were performed with a HP4500 (Agilent technologies, Santa-Clara CA, USA) apparatus at the department of analytical chemistry of the University of Geneva (Switzerland). Finally, H₂S measurements were performed in the second set of oxygen-protected sediment samples by using a H₂S-100 microelectrode connected to the picoammeter PA2000 from Unisense (Unisense, Aarhus, Denmark).

Bacterial counting by flow cytometry

The protocol to count bacterial cells with the flow cytometer is an adaptation of two different procedures published elsewhere (Kallmeyer et al. 2008; Morono et al. 2013). For every layer, around 10 mg of sediment sample was weighted in a 2 mL eppendorf tube. After addition of 1 mL of carbonate dissolution solution (for 100 mL: 2 mL glacial acetic acid, 3.5 g sodium acetate, 0.9 g NaCl), samples were shaken during 2.5 h at 10 Hz. Supernatants were recovered after centrifugation (5 min at 3000×g) and the pellet was washed twice with 1 mL of physiological solution (supernatants were pooled with previous one). Pellets were re-suspended in a mixture of 300 µL of physiological solution, 150 µL of methanol and 150 µL of detergent solution (for 50 mL: 1.86 g disodium EDTA dehydrate, 1.33 g anhydrous sodium pyrophosphate, 0.5 mL Tween[®] 80, 0.45 mL NaCl). Samples were shaken during 12 h at 10 Hz. A four-layer density gradient containing 200 µL each of histodenz 30, 50, 80 %, and sodium polytungstate 70 % was carefully deposited under the samples. After centrifugation (30 min at 4500×g), supernatant and the three upper layers were recovered and pooled with previous supernatants. This procedure ensured the recovery of all the cells sucked into the density gradient by turbulent flow behind the denser mineral particles (Morono et al. 2013). The remaining pellet was washed with 1 mL of physiological solution. Supernatant was recovered after centrifugation (15 min at 5000×g) and pooled with previous one. Pellets were re-suspended in a mixture of 300 µL of physiological solution, 50 µL of methanol and 50 µL of detergent solution. Samples were then sonicated four times 10 s (power button set at 4) with a microtip sonicator (Sonifer 450, Branson Ultrasonics Corp., Danbury CT, USA), and the same four-layer density gradient was performed as described above. At the end of the centrifugation, supernatant as well as the four gradient layers were recovered and pooled with previous supernatants. The pellets were finally washed with 500 µL of physiological solution and supernatants were recovered by centrifugation (20 min at 5000×g) and pooled with previous one. The total volume recovered was approximately 6.9 mL.

For flow cytometry measurements, 20 µL of cell solution was diluted in 100 µL of physiological solution. This sample volume was determined experimentally in order to have a

good signal to noise ratio during the flow cytometry measurements. Finally, 30 μL of 100 \times concentrated SYBR[®] green I was added. After incubation in the dark at room temperature for 15 min, samples were diluted with 850 μL of physiological solution and filtrated through 30 μm cell tricks (Sysmex-Partec, Kobe, Japan). Flow cytometry measurements were performed with a CyFlow space instrument (Sysmex-Partec) equipped with a blue laser (488 nm/20 mW), a green filter (536/40 nm) as fluorescent parameter and a true volumetric facility (for inferring true cell concentration).

Endospore quantification

The quantification of bacterial endospores in sediments was performed using a DPA assay described elsewhere (Brandes Ammann et al. 2011). Briefly, 150 mg of freeze-dried sediments were placed into a screw-cap pyrex tube to which 2.7 mL of sodium acetate buffer (0.2 M, pH 5) and 300 μL of aluminium chloride (AlCl_3 , 0.5 M) solution were added. After mixing, caps were closed tightly and tubes were autoclaved (121 $^\circ\text{C}$, 103 kPa) for 20 min. For the fluorescence measurement, 1 mL of cool clear supernatant was recovered and mixed with 1 mL of terbium chloride solution (TbCl_3 , 30 μM) directly in the measurement quartz cuvette of the Perkin-Elmer LS50B fluorometer (Perkin-Elmer, Waltham, USA). Excitation wavelength was set at 272 nm with a slit width of 2.5 nm. Emission was measured at 545 nm (slit width 2.5 nm). The device was set in the phosphorescence mode (equivalent to time-resolved fluorescence) with a delay between emission and measurement set at 50 μs , a measurement frequency of 50 Hz (every 20 ms), and a signal integration time of 1.2 ms. Finally, values recovered for each measurement corresponded to the mean of the relative fluorescence unit (RFU) values given by the instrument within the 30 s following sample introduction in the device. To transform RFU units into DPA concentrations, an 8-point standard curve was established using increasing concentrations of DPA from 0.1 μM up to 10 μM . An amount of 1.5×10^{-16} mol of DPA per spore was used to convert DPA concentration to endospore numbers. This value was determined by a standard curve established with different concentration of endospores from *Lysinibacillus sphaericus*. The number of endospores in this series was also determined by direct counting in a Neubauer-improved counting cell (Paul Marienfeld, Lausa-Konigshofen, Germany).

DNA extraction

For every sample, DNA extraction was performed on one half of the frozen filters obtained at the end of the ultra-turrax homogenisation procedure. Small fragments of this half-filter were inserted into the lysing bead-beating tube of the FastDNA SPIN kit for soil (MP Biomedicals, Santa Ana,

USA). A modified protocol including three sequential bead-beating steps was used to recover DNA from resistant cells (e.g. bacterial endospores) as described elsewhere (Wunderlin et al. 2013). In this modified protocol the supernatant was recovered after each bead-beating step (three in total) in order to increase the bead-beating time without damaging DNA quality. Each supernatant was treated separately following the manufacturer's instructions (FastDNA SPIN kit for soil). At the end of the procedure, the three DNA extracts were pooled together and total DNA was quantified using Qubit[®] 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). To improve final DNA quality, but also to increase final concentration, an extra purification procedure was performed using a standard ethanol precipitation. Qubit quantification was also performed on the final product.

Quantitative PCR analyses

Total bacterial abundance was measured by classical qPCR assay on the 16S ribosomal RNA (rRNA) gene. A mixture of 0.3 μM of primers 338f (5'-ACT-CCTACGGGAGGCAGCAG-3') and 520r (5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al. 1993; Bakke et al. 2011), 1 \times Rotor-Gene SYBR Green PCR Master Mix (Qiagen) and PCR-grade water (up to final volume of 8 μL) was distributed in the reaction tubes prior to addition of 2 μL of DNA sample (around 1 to 2 ng). Corbett Rotor-Gene 3000 (Qiagen, Hilden, Germany) was used to perform the amplification and fluorescence measurement. The cycling was performed as follow: initial denaturation (15 min at 95 $^\circ\text{C}$), 40 cycles composed by a denaturation of 10 s at 95 $^\circ\text{C}$, an annealing of 15 s at 55 $^\circ\text{C}$ and an elongation of 20 s at 72 $^\circ\text{C}$. A standard curve was made of 10-fold dilutions of a known concentration of a plasmid obtained by cloning the 16S rRNA gene from an environmental bacterium.

To assess the abundance of endospore-forming Firmicutes (EFF), qPCR quantification of the *spo0A* gene (Bueche et al. 2013) was also performed. qPCRs were carried out in a final reaction volume of 10 μL with 5 μL Rotor-Gene SYBR green PCR master mix (Qiagen GmbH, Hilden, Germany), and 1 μM , respectively 0.45 μM of primers *spo0A655f* (5'-GGHGTDCGNCNCATATHAA) and *spo0A834r* (5'-CCAHGCGNACTTCWATNGCRT). Two-microliter aliquots of diluted samples (around 1 to 2 ng) were added to the reaction tubes. The Corbett Rotor-Gene RG-3000A thermocycler was used for the amplifications. An initial denaturation/polymerase activation step for 10 min at 95 $^\circ\text{C}$ was first performed, followed by 45 cycles consisted of denaturation 15 s at 95 $^\circ\text{C}$, annealing 15 s at 54 $^\circ\text{C}$, and elongation 10 s at 68 $^\circ\text{C}$. The standard curve was constituted by 10-fold dilutions of a known concentration of a plasmid obtained by cloning the *spo0A* gene from *Bacillus subtilis*.

Pyrosequencing and bioinformatics analysis

Twenty-four purified DNA samples were sent to Eurofins MWG Operon (Ebersberg, Germany) for amplicon-based 454-pyrosequencing (GS FLX++ technology, 454 Life Sciences, Branford, CT, USA). This sequencing technology allows for the sequencing of relatively large DNA fragments (about 1000 bp) and thus unidirectional sequencing was performed from adaptor primer A-side, targeting the almost entire 16S rRNA gene. This gene was amplified using the universal forward primer 27 F (5'-AGA GTT TGA TCM TGG CTC AG-3') and the universal reverse primer 1492R (5'-ACC TTG TTA CGA CTT-3') (Frank et al. 2008).

The dataset was analysed with Qiime (Caporaso et al. 2010) following the main pipeline for 454-pyrosequencing data. Briefly, sequences were first demultiplexed based on tag identifiers, then filtered to remove too long (>1000 bp), too short (<200 bp), bad quality (quality control threshold=25), as well as chimeric sequences using Usearch (Edgar 2010). Operational taxonomic units (OTU) were defined with Usearch using a 97 % identity threshold. A representative set of sequences from defined OTUs were then aligned using an external tool (Infernal aligner (Nawrocki et al. 2009) from the RDP pipeline (Cole et al. 2014)) and taxonomically assigned using a Greengenes (DeSantis et al. 2006) 16S rRNA gene database ending up with a BIOM (McDonald et al. 2012) format community matrix. Rarefaction curves were computed with CHAO1 metrics and bootstrapped beta-diversity analyses were computed to compare sample communities.

Starting from the BIOM matrix, constrained correspondence analyses (CCA, vegan library (Oksanen et al. 2013)) were performed with ad hoc R (Team RDC 2009) scripts (ESM 1: supplementary material) to visualize the link between microbial communities composition and chemical parameters measured in sediments. Specific scripts were also used to draw different plots for visualizing community composition.

To better identify the predominant organisms in the communities (OTUs representing more than 0.5 % of relative abundance), the corresponding sequences were gathered from the main Fasta file (resulting from pyrosequencing) and grouped together in a single Fasta file per dominant OTU. This treatment was performed using the information from Qiime (Caporaso et al. 2010) output files, EMBOSS (Rice et al. 2000) tools and low-level UNIX commands. OTUs corresponding fasta files were then individually blasted (Altschul et al. 1990) (blastn command) against a bacterial 16S rRNA reference database of 326,231 sequences (most complete database available, containing only good quality sequences of type and non-type strains) downloaded from the ribosomal database project web site (<http://rdp.cme.msu.edu/>). Finally, the more representative blast identifications were compared with the RDP identifications performed by the Qiime pipeline to keep the more relevant one.

Finally, to analyse the relationship between a particular portion of the Firmicutes community and the environmental parameters, different correlation analyses were performed using the pairs function (graphics library) from R.

Results and discussion

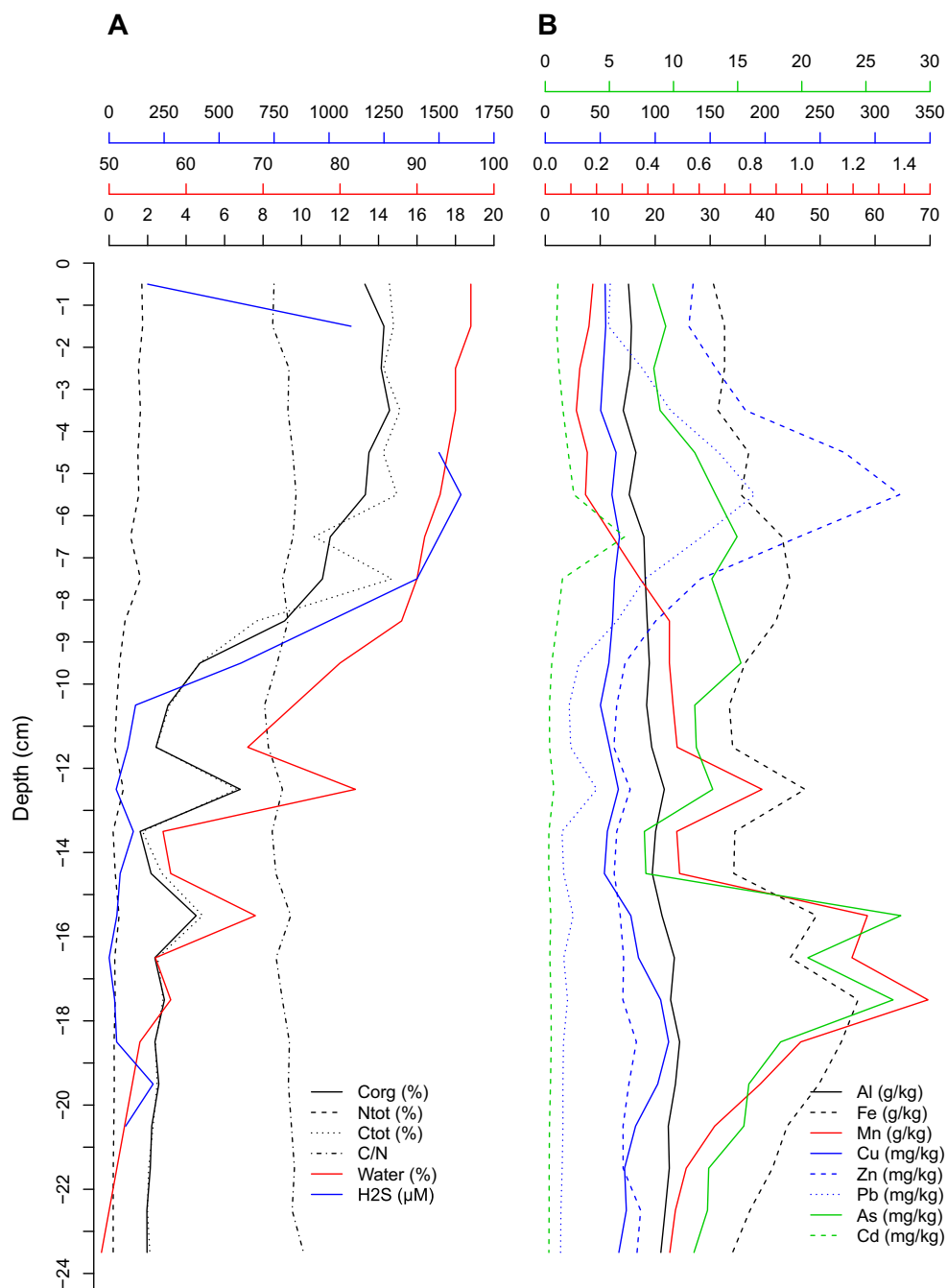
Water column

The water column of Lake Cadagno is thermally and chemically stratified (Birch et al. 1996; Tonolla et al. 2005b; Milucka et al. 2015). At the moment of sampling (ESM 1: Supplementary Figure. 1), surface water temperature was 12 °C, the thermocline stood at around 7 m deep and temperature in the lower part of the water column was close to 4 °C. Surface water (down to 7 m) was fully oxygenated (around 12 mg/L), while bottom water (below 11 m) was fully anoxic. The physicochemical parameters measured at 12 m, which corresponded to the chemocline, reflected the metabolic activity of anaerobic sulphur oxidisers (green and purple sulphur bacteria). These anoxygenic phototrophs survive in this narrow water layer where H₂S is available as electron donor and enough light penetrates to allow photosynthesis to occur. The increase in turbidity at this layer is due to their density and relatively large cellular size (e.g. in the case of *Chromatium okeani* (Tonolla et al. 2005b)). The intense metabolic activity of the microorganisms at this layer led also to an increase in ATP measured. Below 13 m light available is no longer sufficient to sustain anoxygenic photosynthesis. As H₂S is not consumed, concentrations increased below this depth.

Chemical characterization of the sediment samples

Different chemical parameters were measured every centimetre in the sediment core (Fig. 1a). Changes in total and organic carbon content mirrored each other. In the top layers (down to a depth of 3.5 cm) values remained almost constant at close to 15 %. Total and organic content dropped gently down to 8.5 cm with values reaching 11 %. This was followed by a sharp drop to around 3 % at a depth of 11.5 cm. Except for two peaks recorded at 12.5 and 15.5 cm, with 7 and 5 % of total and organic carbon, respectively, the content decreased further to a final value of 2 % at a depth of 23.5 cm. Total nitrogen followed a similar trend. Values ranged from 1.7 % in the top to 0.2 % for the lowest sediment layers. A rapid decrease was also recorded between 8.5 and 11.5 cm deep and the two peaks at 12.5 and 15.5 cm were also observed. These elevated values in the carbon and nitrogen content could probably be explained by the input of allochthonous material originated from landslides. Finally, a slight increase, from 8.5 to 10, was recorded for C/N ratios over the

Fig. 1 Physicochemical characterization in function of depth (in cm) of the sediment core taken at the deepest point of Lake of Cadagno. **a** Characterization of organic carbon (black solid line; %), total nitrogen (black dashed line; %), total carbon (black dotted line; %), C/N ratio (black dashed-dotted line; no units), water content (red solid line; %), and hydrogen sulphide (blue solid line; μM). For H_2S the points for 1.5, 2.5 and 3.5 cm are missing. **b**. Metal concentration in sediments (particulate) for aluminium (black solid line; g/kg), iron (black dashed line; g/kg), manganese (red solid line; g/kg), copper (blue solid line; mg/kg), zinc (blue dashed line; mg/kg), lead (blue dotted line; mg/kg), arsenic (green solid line; mg/kg), and cadmium (green dashed line; mg/kg)



entire core length, indicating the decrease of easily available organic carbon for heterotrophic metabolic activity.

The combination of organic carbon as electron source and sulphate as the most energetic electron acceptor in this anoxic environment, constitute the sulphide-producing conditions that characterize the lacustrine basin of Lake Cadagno (Luthy et al. 2000). The high concentrations of sulphate (reported in the literature to be as high as 2.1 mM; (Schubert et al. 2011)) and the sulphate-reducing activity observed in the first 10 cm of the core, led to measuring large amounts of H_2S (maximum value 1600 μM at a depth of 6 cm) in the upper

part of the sediment core. The concentrations measured are similar to those previously reported (Birch et al. 1996), but the extent of the sulphidogenic zone was compressed compared to previous studies suggesting H_2S production down to 40 cm. Finally, H_2S values measured in the lower sediment layers were near to 200 μM .

A decrease in water content has been used in the past as an indicator of the unperturbed nature of the sediment and of the settling of organic material from the water column as the main source of sediment material in Lake Cadagno (Birch et al. 1996). In the case of the sediment core recovered for this study,

such a slight decrease of the water content, from 97 to 90 %, is also reported for its upper part, down to 8.5 cm (Fig. 1a). Below this depth, the water content decreased sharply reaching 68 % at 11.5 cm, 57 % at 13.5 cm, and 50 % at 23.5 cm. In a previous study (Birch et al. 1996), similar local drops were also observed, and were attributed to changes in the compaction of the sediment due to turbidite formation events.

Metal concentrations in pore water (ESM 1: Supplementary Figure 2) were several orders of magnitude lower than those of the particulate metals (Fig. 1b), indicating that the latter are bound to the sediment particles. Concentrations of particulate zinc, lead and cadmium showed a large peak, extending from 3.5 to 8.5 cm, reaching maximal concentrations of 322, 190 and 6.2 mg/kg of dry sediment, respectively (Fig. 1b). These metals accumulated in sediment layers corresponding to the H₂S peak because of the formation of highly insoluble metal sulphides. These metal sulphide deposits have been used as chronological markers of human and industrial activity in the region surrounding Lake Cadagno. The distribution pattern has been attributed to the development of an important metal industrial factory in 1947. After the installation of new filters in 1985, atmospheric pollution decreased, leading to lower atmospheric deposition in the catchment of Lake Cadagno (Birch et al. 1996). This decrease is also observed in the sediment record.

Concentration peaks in iron, manganese and aluminium, which originate from rock erosion, were observed in the past at 20.5–22.5 cm and have been used as an indication of an avalanche occurring in 1951 in the shores of the lake (Birch et al. 1996). With the exception of aluminium, which did not vary in our study, the other accumulation peaks were observed between 15.5 and 21.5 cm (57 g/kg of Fe and 1.5 g/kg of Mn at 17.5 cm) (Fig. 1b). Changes in the concentration of copper and arsenic were also measured. Copper content ranged from 50 to 110 mg/kg of dry sediment, forming a wide peak located between 15.5 and 21.5 cm deep. Concentrations of arsenic were 28 and 27 mg/kg of dry sediment, at 15.5 and 17.5 cm, respectively (Fig. 1b).

Although establishing the age of the sediment was not the aim of this study, estimating the time frame of the sedimentary record studied here is relevant to consider the overall levels of metal contamination compared to pre-industrial values. Using as reference the peaks of zinc, lead and cadmium (5.5 cm) for the year 1981 and of iron and manganese (17.5 cm) for 1951, the sedimentation rate between these two events corresponded to 4 mm/year, which is slightly lower than the values reported previously (Birch et al. 1996) but would be high for an Alpine lake (Arnaud et al. 2002; Gilli et al. 2003). Using the peaks of zinc, lead and cadmium to estimate the sedimentation rate of the upper part of the core gives a sedimentation rate of 2 mm/year, which is compatible with a recent study published for Lake Cadagno (Wirth et al. 2013). However, this will indicate a considerable change in the sedimentation rate in the past 20 years. Nonetheless, if the more conservative value of

4 mm/year is used to estimate the age of the oldest sediment layer, the core studied here comprised a record of nearly all the latest 80 years. Based on the values at 24 cm and using as reference sediments dated to 1850 (Birch et al. 1996), the pre-industrial levels of iron, manganese, zinc, copper, lead and cadmium, corresponded to background levels of these elements in sediments from other remote lakes (ESM 1: Supplementary Table 1). This also shows the remarkable stability of the overall metal content and the contamination related to atmospheric industrial pollution of the sediments studied here. It is worth mentioning that an inexplicable increase in copper concentration (110 mg/kg of dry sediment) was registered below the peaks of iron and manganese, but the sudden origin of this element other than mineral weathering could not be found.

Abundance of bacteria, endospore formers and endospores in the sediment

Two different methods, flow cytometry and qPCR of the 16S rRNA gene, were used to estimate total bacterial abundance. Although factors such as the multiple copies of the 16S rRNA gene in bacterial cells (4.3 in average, Lee et al. 2009) and biases in DNA extraction (Feinstein et al. 2009) or in cell extraction for flow cytometry cannot be disregarded, the two measurements were remarkably similar. An exponential decrease of cell concentration from the top (5e10 cells/g of dry sediment) to the bottom of the sediment core (1.5e9 cells/g) was observed (Fig. 2). This exponential decay was significantly correlated (correlation coefficient of 0.82) to the decrease in organic C (ESM 1: Supplementary Figure 3), and can be due to a decrease in available nutrients to sustain microbial activity. As indicated previously, the increase in C/N ratio also supports the decrease of readily available organic matter as a nutrient source (Yamakura and Sahunalu 1990).

Abundance of EFF evaluated by quantitative PCR of the *spo0A* gene followed a similar trend of exponential decay, with a flattening at around the same depth (10 cm). Copy numbers varied from 1.4e8 EFF/g of dry sediment in the top layers, down to 8.7e6 EFF/g in the bottom. As in the case of total bacteria, the decay in EFF abundance was significantly correlated to the decrease in organic carbon (correlation coefficient of 0.911; ESM 1: Supplementary Figure 3). On the other hand, the abundance of endospores, evaluated by the concentration of DPA, increased linearly with depth. In the top layers, around 1.8e7 endospores/g of dry sediment was measured whereas 1e8 endospores/g were found in the bottom. The increase in the proportion of endospores correlates negatively with organic C (correlation coefficient of -0.959), *spo0A* (correlation coefficient of -0.884) and 16S rRNA gene (correlation coefficient of -0.819) copy numbers. This is the first environmental evidence, to the best of our knowledge, of the formation of spores as a response to starvation. Indeed,

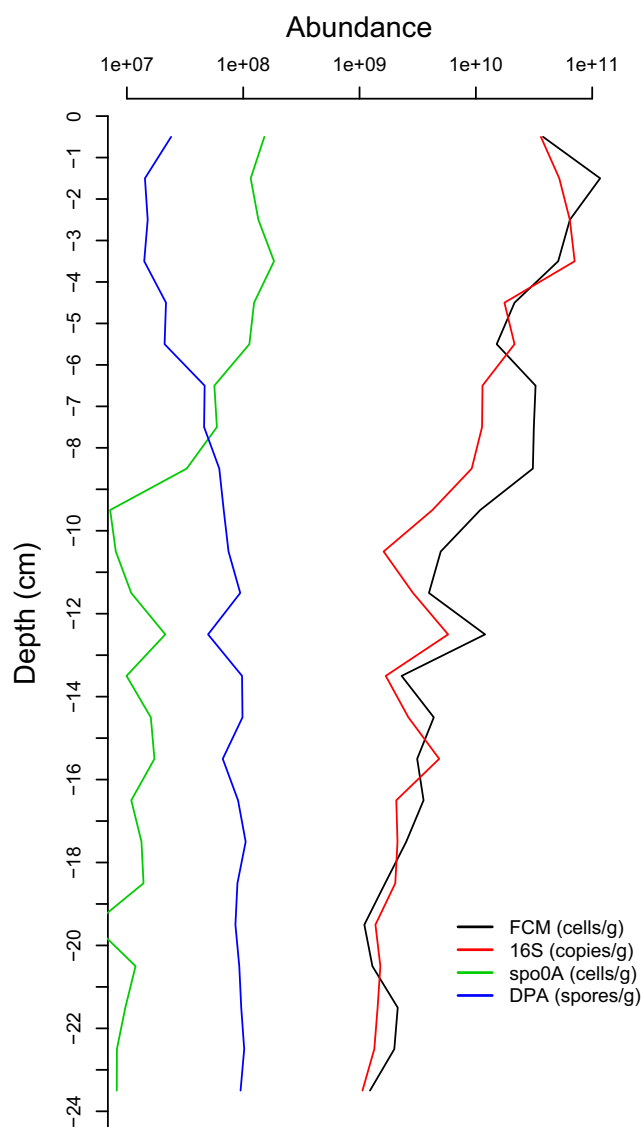


Fig. 2 Total bacterial abundance quantified by flow cytometry (*black solid line*) or by qPCR on the 16S rRNA gene (*red solid line*). Endospore-forming Firmicutes quantified by qPCR of the endospore-specific gene *spo0A* (*green solid line*). Endospore numbers quantified by dipicolinic acid (DPA) fluorimetric assay (*blue solid line*)

endosporation has often been associated to the deprivation of nutrients, but this has only been shown under laboratory conditions (Nicholson et al. 2000; Nicholson 2002).

Composition of the bacterial communities

Bacterial community composition was analysed by sequencing of the 16S rRNA gene. After quality filtering a total of 312,485 sequences were retained, which corresponded to 23,711 OTU based on 97 % of sequence identity. The average length of the sequences with a quality score above 25 was around 450 bp (ESM 1: Supplementary Figure 4). For the set of sequences identified as Firmicutes, the total number of

sequences was 77,498, with 7112 OTU defined. For total bacteria, except for samples at 1.5, 16.5, 19.5 and 23.5 cm, which have a number of sequences below 6533 (average absolute deviation for the entire dataset; ESM 1: Supplementary Table 2), all the remaining samples have sufficient coverage, although sample from 0.5 cm is at the lower limit. Sufficient coverage can be seen on the Good's coverage index as well as on the ratio richness and chao1 metrics (Sobs/Schao1). The same index confirmed the lack of coverage for the four samples indicated above (values below 0.75 and 0.5, respectively; ESM 1: Supplementary Table 2). This was also verified by rarefaction curves on chao1 metrics presented in ESM 1: Supplementary Figure 5. For the fraction of the community identified as Firmicutes and based on the same alpha-diversity criteria mentioned (ESM 1: Supplementary Table 2), communities from the samples at 0.5, 1.5, 9.5, 11.5, 12.5, 15.5, 16.5, and 23.5 cm should be considered under sequenced. This however does not seem to be so marked when looking at the rarefaction curves in which only communities from the samples at 0.5 and 1.5 cm are clearly under sequenced (ESM 1: Supplementary Figure 5).

Community composition investigated at the phylum level (Fig. 3) showed that two main phyla dominated through the samples. Proteobacteria dominate in the upper part of the sedimentary record (0.5 to 3.5 cm), as well as between samples at 12.5 and 15.5 cm, while decreasing steadily at the deepest samples. Firmicutes gain in importance from 4.5 to 5.5 cm, while clearly dominating the communities from the deepest part of the core (below 16.5 cm). Among the five most frequent phyla, the other groups noteworthy are OD1 and Chloroflexi, which displayed a relatively constant frequency in all depths, and Caldiseica that was more relevant in the sample at 9.5 cm. For the latter it is important to indicate that so far the only culture of a representative of the phylum Caldiseica corresponds to a thermophilic thiosulfate-reducing bacterium (Mori et al. 2009), suggesting a potential role of these organisms with the sulphur cycle in Lake Cadagno.

Although one must be careful when drawing functional conclusions from an analysis based on a phylogenetic marker such as the 16S rRNA gene, some interesting tendencies could be observed based on the community composition. For example, the most abundant Proteobacteria detected in the samples, corresponded to Syntrophobacterales, which dominated between 1.5 to 15.5 cm, and Desulfobacterales (Fig. 3). The two most important genera found within the Syntrophobacterales corresponded to reported sulphate-reducing bacteria such as *Desulfomonile* sp. (Tonolla et al. 2005a; DeWeerd et al. 1990) and *Desulfobacca* (Oude Elferink et al. 1999), both of which dominated the community even below the peak of H₂S detected in pore water (down to sample at 15.5 cm). In the case of Desulfobacterales, the dominant genus was *Desulfococcus*, a genus of sulphate reducers that has been previously identified as a key player in the

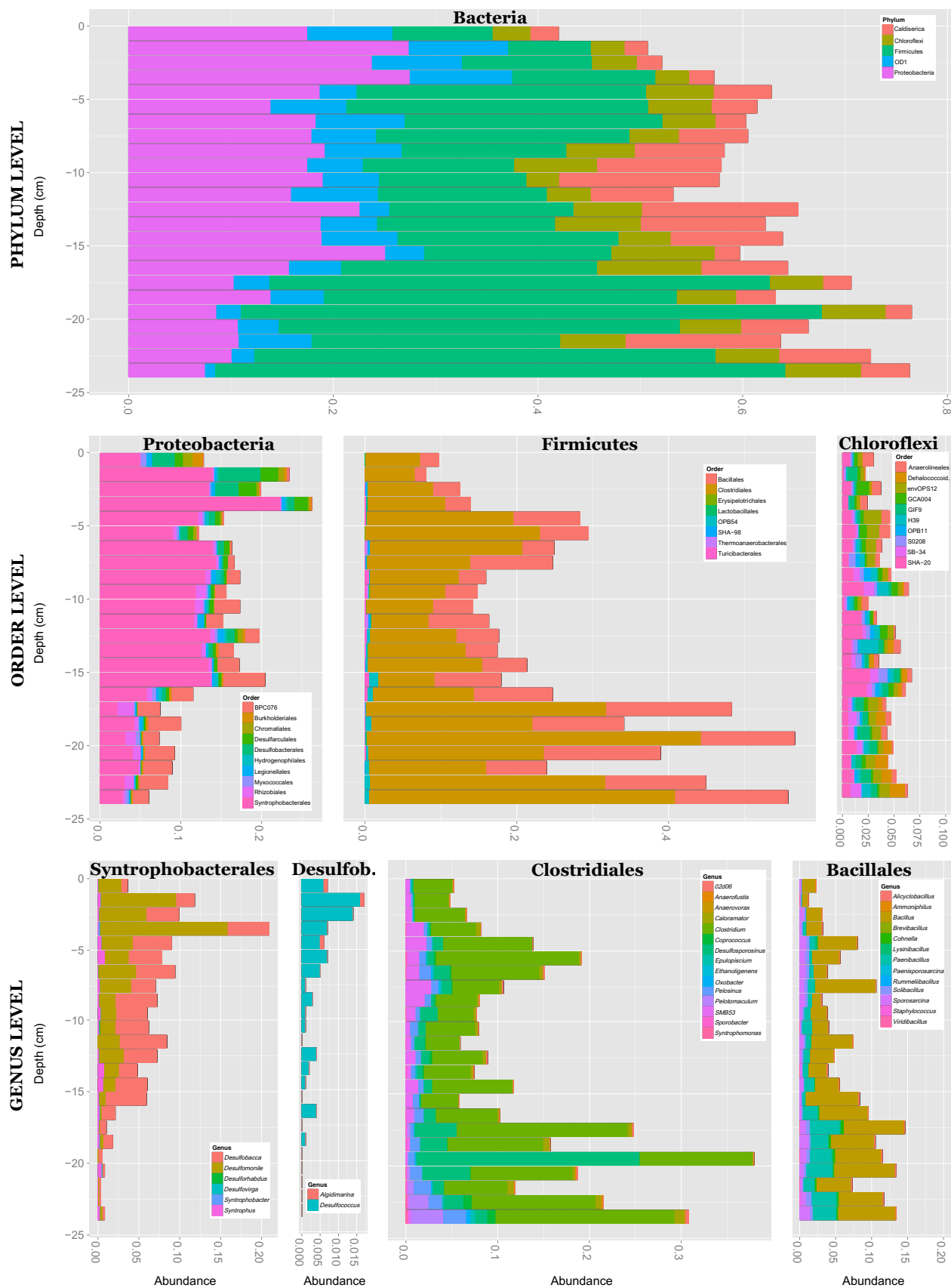


Fig. 3 Bacterial community composition at phylum, order and genus level for the most abundant members of the community

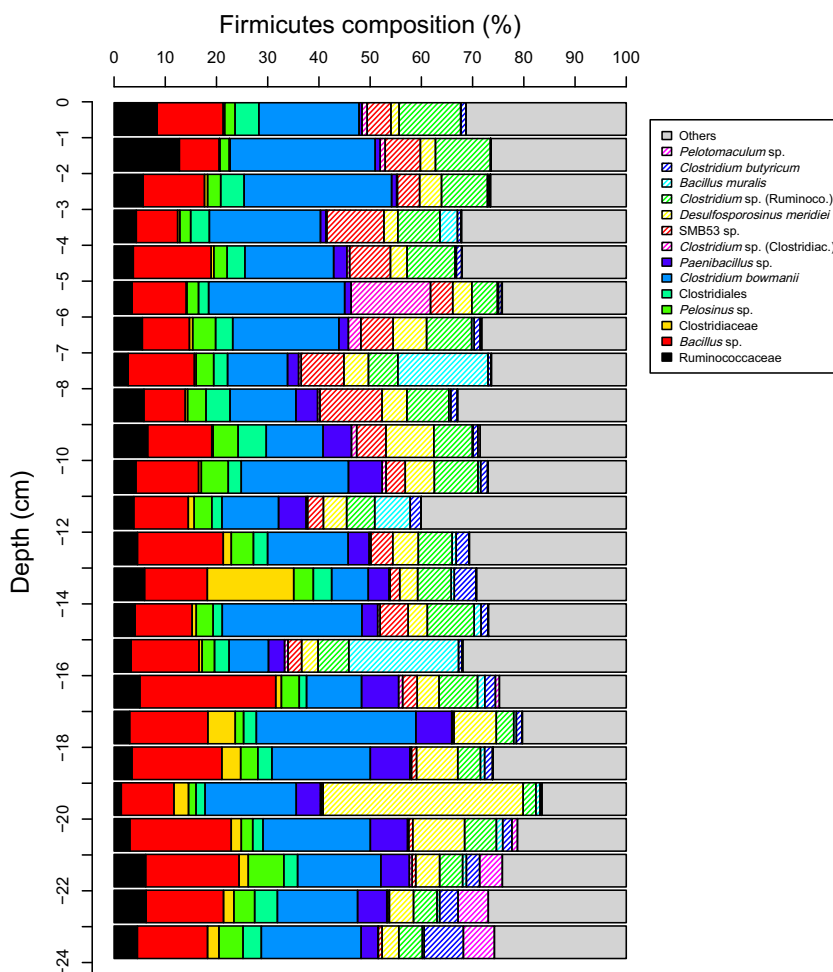
anaerobic degradation of non-methane hydrocarbons in marine seeps (Kleindienst et al. 2014). Regarding the most abundant genera of Chloroflexi detected in the samples, a very diverse community composition was observed. Unfortunately for many of those, relatively little metabolic information could be obtained.

Based on the relative abundance of sequences assigned to the phylum level, a general trend towards an increase in the relative abundance of Firmicutes was observed as a function of depth, starting from around 10 % in the top sediment layers to around 50 % in the bottom (Fig. 3). A small peak, reaching 29 % of Firmicutes relative abundance, was however registered for samples from 3.5 to 7.5 cm. Two additional peaks were also observed at 17.5 and 19.5 cm, with respective relative abundances of 51 and 58 %. Changes in the relative abundance of 14 dominant taxonomical assignments (relative abundance greater than 2 % in at least one sample) within the sub-community of Firmicutes are shown in Fig. 4. In general, more than 60 % of the sub-community of the Firmicutes was composed by a few taxa consistently present throughout the samples like Ruminococcaceae, *Pelosinus* sp., Clostridiales, and *Clostridium bowmanii*, which are found in

every sample with no clear trend in function of depth. Relative abundance of other taxa, such as *Bacillus* sp., *Paenibacillus* sp. and *Clostridium butyricum* slightly increased with depth, whereas *Pelotomaculum* sp. suddenly appeared in the last four samples. Relative abundance of *Clostridium* sp. (belonging to the Ruminococcaceae family) and of members from the SMB53 genus slightly decreased with depth. Finally, peaks of abundance were observed for some taxa, like *Clostridium* sp. (belonging to the Clostridiaceae family, at 5.5 cm deep), Clostridiaceae (at 13.5 cm), *Bacillus muralis* (at 7.5 and 15.5 cm), and *Desulfosporosinus meridiei* (at 19.5 cm). Despite the fact that sulphate-reduction can be found among certain endospore-forming Firmicutes such as *Desulfotomaculum* (Junier et al. 2010), sequences related to those could not clearly assigned to those even for the sulphidogenic part of the sediment core.

Given our global aim of investigating a correlation between EFF and metal accumulation, we analysed the correlation between changes in relative abundance of the dominant Firmicute taxa with the environmental parameters studied (ESM 1: Supplementary Figure 6). *Clostridium* sp. (belonging to the Clostridiaceae) was the only molecular species

Fig. 4 Relative abundance of dominant Firmicutes taxa (relative abundance greater than 2 %). The relative abundances of the OTUs with the same taxonomical assignment were pooled together. The “Others” category represents the remaining pool of the OTU assigned to taxa that represented a relative abundance lower than 2 %



correlated with metal sulphide concentration (zinc as representative; correlation coefficient = 0.708). This increase in Clostridia for sample at 4.5 to 7.5 cm, could be attributed to higher concentration of metals, as it was demonstrated before (Sauvain et al. 2013), but cannot be generalized to all groups since, for example, the relative abundances of *Clostridium* sp. (belonging to the Ruminococcaceae family) and of SMB53 sp. taxon were negatively correlated to copper concentration (correlation coefficients = -0.695 and -0.602, respectively). In addition, other molecular species were either positively or negatively correlated with other chemical parameters measured. For example, *Paenibacillus* sp. is negatively correlated with the concentration of organic carbon and H₂S (correlation coefficient = -0.819 and -0.624, respectively). In contrast, the SMB53 sp. taxon was positively correlated with organic carbon and H₂S (correlation coefficient = 0.66 and 0.688, respectively).

Global analysis of the chemical parameters and the bacterial community composition

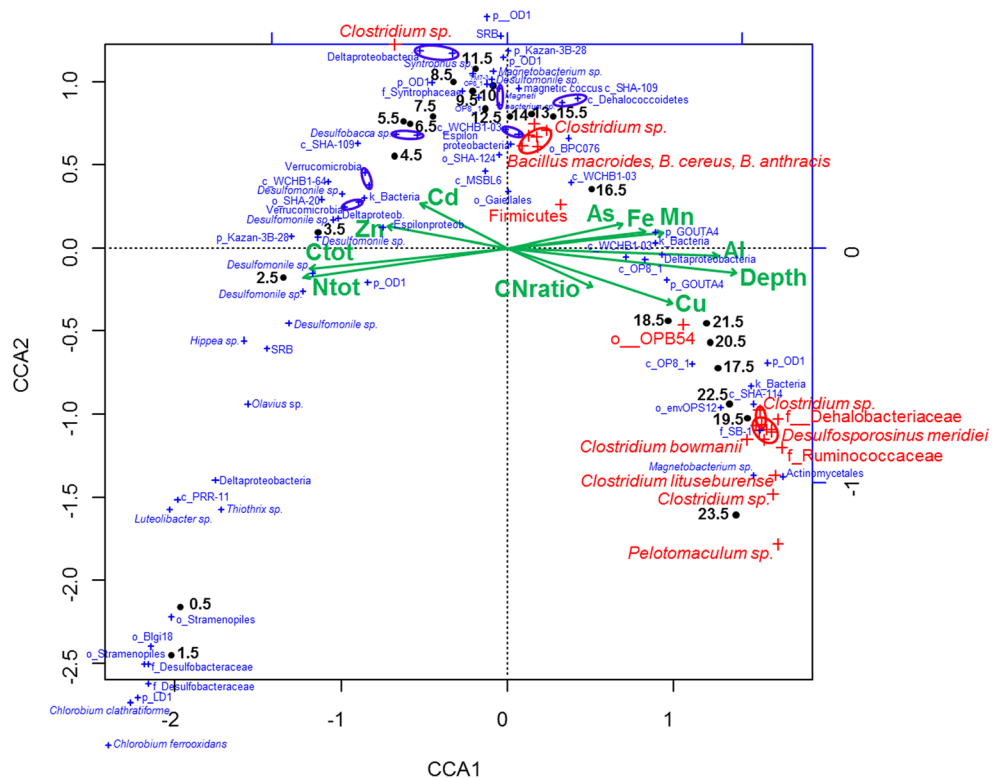
A multivariate analysis allowed a deeper interpretation of the correlation between the chemical parameters measured and the structure of the bacterial communities. In the ordination plot (Fig. 5) four distinct groups of samples could be identified. The position of these groups along the first CCA axis was clearly related to sediment depth.

Group one, composed by samples at 0.5 and 1.5 cm correspond to the top layers of the sediment core. In these samples, sequences related to photosynthetic taxa such as *Chlorobium* sp. as well as sequences related to the chloroplast of the stramenopiles were found. In this case the presence of such taxa could be expected as the results of deposition of dead cells from the upper layers of the water column where photosynthetic activity occurs and where the activity of these organisms has been reported (Tonolla et al. 2005b). This finding also supports the hypothesis of organic matter (cell debris) as the main source of material for sediment formation (Birch et al. 1996).

Group two, composed by samples at 2.5 and 3.5 cm is mainly characterized by the increase in the relative abundance of *Desulfomonile* sp., *Hippea* sp., members of the phyla Verrucomicrobia, Kazan-3B-28 and OD1, members of the sub-phyla delta- and Epsilonproteobacteria, members of the class WCHB1-64 and of the order SHA-20.

Group three, composed by samples from 4.5 to 15.5 cm were characterized by the detection of sulphate-reducing taxa like *Desulfobacca* (Oude Elferink et al. 1999), *Desulfomonile* sp. (Tonolla et al. 2005a; DeWeerd et al. 1990) and members of the phylum OD1 (Wrighton et al. 2012), explaining the very high sulphidogenic activity in these layers. In addition, some Firmicutes species, like *Bacillus macroides*, *Bacillus cereus*, *Bacillus anthracis* and *Clostridium* spp. were found in these layers. In the deepest layers, starting from 10.5 cm deep, nutrients become limiting and taxa capable to thrive

Fig. 5 Constrained ordination plot of the bacterial communities based on 97 % identity for OTU clustering. The plot is constrained by the environmental variables particulate zinc, lead, cadmium, arsenic, copper, and depth (green arrows). Identified OTUs appear either in blue for non-Firmicutes taxa or in red for Firmicutes. Only taxa with a relative abundance higher than 0.5 % in one or more samples are displayed. Black dots correspond to the samples (i.e. layers of the sediment core)



with low energy metabolisms or to form resistance structures were characteristic. This includes bacteria displaying syntrophic metabolism such as members of the order envOPS12, which belong to the Anaerolineae (some taxa were successfully co-cultivated with a hydrogenotrophic methanogen; Yamada et al. 2007), Syntrophaceae which can only survive in close association with hydrogen-consuming microorganisms such as methanogens (Madigan et al. 2012), Dehalococcoidetes that use electrons from H₂ to reduce chlorinated compounds (Siddaramappa et al. 2012; Löffler et al. 2013). Finally, resistance structure formation is represented within Firmicutes and non-Firmicutes taxa. For non-Firmicutes one can consider members of the class WCHB1-03 which belong to the Caldiserica phylum characterized by the formation of an outer protective envelope (Mori et al. 2009). In the case of endospore-forming Firmicutes, *Clostridium* sp., *Desulfosporosinus* sp. (Vos et al. 2011) and members of the Dehalobacteriaceae family were found. In this same group of samples several sequences related to magnetotactic bacteria were identified. These bacteria are known by their capacity to navigate within the gradients of the different available substances, to meet their needs (Yan et al. 2012). The sample at 16.5 cm corresponded to a transitional area characterized by sequences related to the WCHB1-03 class and high levels of particulate arsenic, iron, and manganese.

Group four, composed by samples from 17.5 to 23.5 cm, was characterized by an increase of the C/N ratio, the highest depth, an increase in particulate copper content and a decrease of nutrients (Corg and Ntot). The bacterial community was clearly characterized by the predominance of anaerobic Firmicutes taxa, such as *D. meridiei*, *C. bowmanii*, *Clostridium lituseburense*, *Clostridium* sp., *Pelotomaculum* sp., members of the order OPB54, and of the family Dehalobacteriaceae and Ruminococcaceae. Other non-firmicute taxa found in this group are *Magnetobacterium* sp., members of the phyla OD1 and GOUTA4, and of the order Actinomycetales. All the other taxa correspond to uncultured bacteria inhabiting extreme environments, such as members of the *Candidatus* phylum OP (Organisms from the obsidian pool in Yellowstone) (Rohini Kumar and Saravanan 2010), of the order BPC076 found in a hydrocarbon seep (Reed et al. 2002), of the family SB-1 (Sphingobacteriales) also found in Armenian hot springs (Hedlund et al. 2013).

Overall, the community composition supports the hypothesis of sporulation as the main mechanism explaining the dominance of endospore formers in the deepest part of the sediment core (below 10.5 cm). In contrast, metal contamination in the form of insoluble metal sulphide deposits appears not to be linked with the role of sporulation as a mechanism of metal tolerance, suggested in our study in Lake Geneva (Sauvain et al. 2013). However, it is worth indicating that in

Lake Geneva no correlation was found between total particulate sulphur and toxic metals, indicating another mechanism for metal accumulation on those sediments and likely another effect on the composition of microbial communities and in endospore formers.

Finally, an important effort is still needed to enlarge reference datasets for the identification of the bacterial communities. In the future the description of cultivation-refractory species using tools like single-cell genomic approaches (Binga et al. 2008) may help to better understand the link between specific endospore-forming taxa (i.e. SMB53 or *Clostridium* spp.) and particulate metals.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing of interests.

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