**Aquatic Sciences**

RESEARCH ARTICLE - BASED ON MIR INVESTIGATIONS IN LAKE GENEVA

# Endospore-forming bacteria as new proxies to assess impact of eutrophication in Lake Geneva (Switzerland–France)

Tina Wunderlin · Juan Pablo Corella · Thomas Junier ·
Matthieu Bueche · Jean-Luc Loizeau · Stéphanie Girardclos ·
Pilar Junier

**Abstract** Measurements of chemical composition and biological parameters of sediment cores are used as proxies for changes in past environmental conditions and more recently the human impact on ecosystem health. In this study, endospore-forming bacteria are proposed as a new biological proxy for such paleoecological reconstructions. A sediment core providing a record for the past 90 years ($^{137}$Cs and magnetic susceptibility dating) was retrieved from the Rhone Delta of Lake Geneva. X-ray fluorescence was analyzed at a 0.2-cm resolution, while DNA extracts, elemental geochemistry and grain size were obtained at 4-cm intervals. The total number of bacteria and endospore-forming bacteria were quantified by qPCR using the 16S rRNA gene and the endosporulation-specific *spo0A* gene. Furthermore, a *spo0A* fragment was subjected to amplicon sequencing to define OTUs (operational taxonomic units) and the phylogenetic affiliation of the endospore formers. The results showed that despite the fact that the quantity of extracted DNA decreased with the age of the sediment, the abundance of endospore-forming bacteria remained constant. However, the diversity of this group of bacteria changed significantly, reflecting the eutrophication of the lake from 1960 to 1990. The shift in community composition was linked to the dominance of anaerobic clostridia-like endospore formers. This trend has reversed in the last 10 years of the record, suggesting a recovery after perturbation. This study shows that the abundance and diversity of endospore-forming bacteria can be used as proxies to reconstruct lake history. We hereby

T. Wunderlin · T. Junier · M. Bueche · P. Junier (✉)
Laboratory of Microbiology, University of Neuchâtel,
Rue Emile Argand 11, 2000 Neuchâtel, Switzerland
e-mail: pilar.junier@unine.ch

T. Wunderlin
e-mail: tina.wunderlin@unine.ch

T. Junier
e-mail: thomas.junier@unine.ch

M. Bueche
e-mail: matthieu.bueche@unine.ch

J. P. Corella · S. Girardclos
Department of Geology and Paleontology, Institute for
Environmental Sciences (ISE), University of Geneva,
1205 Geneve, Switzerland
e-mail: pablo.corella@mncn.csic.es

S. Girardclos
e-mail: Stephanie.Girardclos@unige.ch

J. P. Corella
Museo Nacional de Ciencias Naturales (MNCN/CSIC), Serrano
115bis, 28006 Madrid, Spain

J.-L. Loizeau
Institute F. A. Forel, Earth and Environmental Sciences Section,
Faculty of Sciences, University of Geneva, Geneva, Switzerland
e-mail: Jean-Luc.Loizeau@unige.ch

successfully introduce a new strategy for paleoecology that could also be applied to ocean sediments and long sediment cores.

## Introduction

The chemical composition of lake sediments and its biological remains constitute an archive of historical data. Changes in chemical and biological parameters can be used to understand the relationship between ecological disturbances in lake systems and their impact on ecosystem health (Willard and Cronin 2007). Disturbances of lake systems can be physical or chemical. Examples of those are climate changes (rainfall, temperature), fires, storms, modification of land use (anthropogenic deviation of river inlets, deforestation) or pollution, acid deposition, and variations in nutrient input (eutrophication) or soil weathering. Many of the former are direct consequences of human activities.

In paleolimnology, changes in community composition of organisms producing identifiable fossilized remnants can be analyzed at different timescales from annual fluctuations to decades or centuries, depending on the resolution and length of a sediment core. The paleoecological record allows posing specific questions, such as: What is the natural variability and frequency of ecosystem differences? Are communities stable over time or do they change according to long-term trends? What are the properties of ecosystems prior to a disturbance and after recovery (Gorham et al. 2001)?

Lake sediments contain a variety of microorganisms that can be used as biological indicators for paleoecological studies (Gorham et al. 2001). To be a good paleoecological proxy, an organism, or at least a recognizable structure linked to it (e.g., pollen grains or siliceous microfossils), must remain unaltered in sediments for long periods of time. This might be the case for bacterial endospores, highly resistant cellular forms produced by certain genera belonging to the Firmicutes (Gram-positive low G+C content bacteria) (Onyenwoke et al. 2004), which are able to survive in a dormant state, with little to no in situ activity, for a long period of time. The potential of dormant bacteria to be used as paleoecological indicators was evaluated by Renberg and Nilsson (1992). They concluded that it was possible to isolate dormant bacteria (*Thermoactinomyces* spp., Bacilli and Clostridia endospores) from sediment and, by combining the analysis of biochemical and genetic data, to infer past environmental conditions. In addition, an interesting ecological feature within the group of endospore-forming bacteria is that there are aerobic, facultative anaerobic and strictly anaerobic ecotypes. With some exceptions, aerobic types cluster among the class Bacilli and anaerobes cluster mostly in the class Clostridia (Schleifer 2009).

There are few examples of research in paleoecology using endospores. Early reports suggested the presence of viable *Bacillus subtilis* spores in 320-year-old soil samples (Sneath 1962) and of *Thermoactinomyces* spp. in archeological excavations containing plant debris deposited between 85 and 125 AD (Seaward et al. 1976). Spores are commonly found in sediments and isolation and enumeration of viable cells and spores from sedimentary archives is an old technique (Renberg and Nilsson 1992). Viable endospores have been isolated from 5,800-year-old (Bartholomew and Paik 1966) and 9,000-year-old lake sediments (Renberg and Nilsson 1992). A detailed study of the survival and activity of bacteria in a sediment core of about 7 m deposited over the past 13,000 years in Lake Constance (Rothfuss et al. 1997) shows that below 25 cm all the viable heterotrophic bacteria were present as heat-resistant spores. Counts of viable spores decreased exponentially with depth and could not be detected below 6 m (about 8,900-year-old sediment). Furthermore, several publications have shown the isolation of thermophilic endospore-forming bacteria from cold marine sediments (Bartholomew and Paik 1966; Hubert et al. 2010; de Rezende et al. 2013). The results suggest that endospores are in most cases allochthonous and have been deposited at the time of sedimentation. All the above-mentioned studies used germination and culturing as the approach to establish the presence of viable endospores in the environment. However, culturing is biased toward a small fraction of the community (Staley and Konopka 1985; Amann et al. 1995). In a culture-independent study, the dipicolinic acid content of sediment was quantified to account for endospores in the sediment of the North Sea, where endospores have been found to make up to 3 % of the total prokaryotic community (Fichtel et al. 2007). In much older (deeper) sediment cores, the abundance of endospores has been estimated to be as high as the total abundance of vegetative cells (Lomstein et al. 2012). To our knowledge, a culture-independent assessment of endospore-forming community composition in sediments does not exist.

In this study, the community composition of endospore-forming bacteria is assessed as a paleoecological proxy to reconstruct the recent ecosystem history from a sedimentary record of Lake Geneva. Due to a high increase of phosphate release, Lake Geneva has seen a shift from oligotrophic waters toward eutrophication in the late 1960s. Measures to reduce the phosphate input from the early 1970s on have been effective and have decreased the nutrient level to nearly pre-1960 values (Lazzarotto and

Klein [2012]). Two sediment cores were retrieved from two inactive canyons in the Rhone Delta. Endospore-forming bacteria were quantified and used as biological markers of changes recorded in the sedimentary record. The contribution of endospore-forming bacteria to microbial communities at different depths was assessed using the gene coding for the sporulation transcription regulator *spo0A*. Environmental amplicons of *spo0A* were sequenced and annotated to determine the community composition of endospore-forming bacteria in the sediment. Here, the changes in the composition of the endospore-forming community were correlated with the chemical and physical characterization of the sediment. A shift in the composition and a sharp decrease of diversity reflected ecosystem changes due to eutrophication.

## Materials and methods

### Site description

Two Uwitec gravity sediment cores (CAN01, coordinates 559901–139859, 79 m depth, 105 cm; and CAN02, coordinates 559405–140504, 96 m depth, 107 cm) were retrieved in August 2011 using *La Licorne* research vessel (Institute A. Forel, University of Geneva, Switzerland) in two canyons (C1 and C2, Supplementary Figure 1) on the eastern side of the Rhone delta in Lake Geneva (Switzerland), which are inactive Rhone canyons since the river was channeled around 1870 (Sastre et al. [2010]). Both canyons constitute paleoreliefs with smooth lateral slopes and without any connection to a modern river, although C1 was likely connected to the local Eau Froide River in the past (Sastre et al. [2010]). The cores were stored in a cold room at 4 °C.

### Sedimentological description

Measurements of the physical properties every 5 mm were carried out with a Geotek multi-sensor core logger (MSCL) at the ETH Zurich Limnogeology Laboratory. The cores were then split into two lengthwise halves to proceed with the sedimentological description. Pictures were obtained using a digital camera and controlled light conditions. X-ray fluorescence was analyzed in CAN02 core using an AVAATECH XRF core scanner (2000 A, 10 and 30 kV) every 2 mm at the University of Barcelona. CAN01 sediment core was sampled every 4 cm for total carbon (TC) and total nitrogen (Vonlanthen et al. [2012]) using an elemental analyzer (Hekatech Euro EA, Germany) and for total inorganic carbon (TIC) using titration coulometry (Coulomat 5015 CO2-Coulometer, Coulometric Inc., USA) at Eawag (Switzerland). Total organic carbon (Gioia et al.)

was calculated as the difference between TC and TIC. In addition, grain size distribution was measured with a 4-cm resolution using a Mastersizer 2000 particle-size analyzer (Marlvern instruments Ltd, USA) at the Pyrenean Institute of Ecology (Zaragoza, Spain).

### Geochronology

CAN01 was dated by the $^{137}$Cs activity method on dry sediment by gamma spectrometry using HPGe well detectors (Ortec, GWL series, USA) at the Institute Forel (University of Geneva, Switzerland). Core correlation with CAN02 sediment core was carried out by visual description, sediment color and texture and by comparing magnetic susceptibility (MS) and density core profiles. Dating was based on the first fallout of $^{137}$Cs in 1954–1955, the peak of atmospheric nuclear tests in 1963–1964 and the peak of the Chernobyl accident in 1986. Additional dates were obtained by the correlation of the MS signal with dated sediment cores published previously (Loizeau et al. [1997]). Dating was based on a length depth scale and not cumulated sediment mass scales, because porosity did not change drastically with depth; therefore no significant bias was introduced by porosity variations.

### DNA extraction

For DNA extraction, sediment core CAN01 was sub-sampled aseptically every 2 cm and samples were stored at −20 °C until processing. The DNA extraction protocol was optimized on preliminary trials with endospores from *B. subtilis*. Endospore preparations of 99 % spores were subjected to successive DNA extractions following protocols for three commercially available kits (Supplementary Figure 2). Between each extraction, the freed DNA was separated from the remaining cell pellet by centrifugation for 5 min at $14,000 \times g$. Sediment DNA extractions were finally performed using the MP FastDNA® SPIN kit for soil (MP Biomedicals, Santa Ana, CA, USA) with the following modifications: the sediment was subjected to three repetitive extractions. Briefly, 0.5 g sediment was subjected to in situ cell lysis using bead beating at 50 strokes per second with the TissueLyser LT (QIAGEN, Hilden, Germany) for 10 min. The sample was then centrifuged for 5 min at $14,000 \times g$ and 900 μl of supernatant fluid containing the initial fraction of free DNA was collected in a separate tube. To the pelleted sediment, lysis buffer was added two additional times for a second and third round of bead beating for 5 min. Each time, between the bead-beating steps the supernatant fluid was collected in a separate tube. The three DNA-containing supernatants were then processed individually for the remaining steps of the extraction protocol following the manufacturer's

guidelines. The three purified DNA samples were in the end pooled together and DNA precipitated with 0.3 M Na-acetate and ethanol (99 %) and washed with ethanol (70 %) before re-suspending in sterile water. DNA yield for the pooled extracts was measured with a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) using the Quant-iT dsDNA BR assay kit, following the manufacturer's instructions. DNA quality was also verified by agarose gel electrophoresis.

Quantification of total bacteria

Quantification of bacterial DNA in sediment extracts was carried out by real-time quantitative PCR of the V3 region of the 16S rRNA gene with primers 338f and 520r (Ovreås et al. 1997). The qPCR mix contained 0.5 ng of DNA template, 0.3 μM of each primer and 10 μL of QuantiTect SYBR® Green PCR Kit (QIAGEN). Total reaction volume of 20 μL was reached with PCR-grade water. The qPCR was run with a Rotor-GeneTM 6000 instrument (QIAGEN) with the following program: enzyme activation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 5 s, annealing at 55 °C for 15 s and extension at 72 °C for 20 s. Thresholds (Th), Ct values and derivatives of melting curves were determined using Rotor-Gene 6 software. All the qPCR reactions were run in three technical replicates. For quantification, three independent plasmid standards series with 300 to $3 \times 10^6$ gene copies/μL of the 16S rRNA gene of an environmental clone were included.

Quantification of endospore-forming bacteria

Quantification of *spo0A* gene was done as mentioned above for the 16S rRNA gene, but with the primers *spo0A*655f and *spo0A*923r (Bueche et al. 2013). The qPCR mix contained 5 ng DNA template, 0.76 μM of each primer and 1 × QuantiTect SYBR® Green PCR Kit. Total reaction volume of 20 μL was reached with PCR-grade water. The program differed by an annealing temperature of 52 °C for 30 s and extension 72 °C for 30 s. All qPCR reactions were run in three technical replicates. For quantification, three independent plasmid standards series with 30 to $3 \times 10^5$ gene copies/μL of *spo0A* gene of *B. subtilis* were included.

Determination of OTUs of endospore-forming bacteria

Degenerate primers amplifying a 602 bp fragment of the *spo0A* gene (Wunderlin et al. 2013) were used for determination of the phylogenetic affiliation (OTUs) of endospore-forming bacteria. PCR reactions were performed with 0.5 ng DNA template, 1× reaction buffer (TaKaRa Bio, Shiga, Japan), 3 mM MgCl₂, 10 μg bovine serum albumin (BSA; New England Biolabs, Ipswich, MA, USA), 1 U of the proofreading Ex Taq Polymerase (TaKaRa), 200 μM of each dNTP and 1 μM of each primer in a total reaction volume of 50 μl, completed with PCR-grade water. Negative controls (1 μl PCR-grade water) and positive controls (1 ng *Paenibacillus alvei* DNA template) were included in all reactions. Reactions were done with the Arktik Thermo Cycler (Thermo Fisher Scientific, Vantaa, Finland) with the following temperature program: initial denaturation at 94 °C for 5 min; then 10 cycles of denaturation at 94 °C for 30 min, touchdown annealing starting at 55 °C with decrease of 0.3 °C per cycle for 30 s and elongation at 72 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 30 min, annealing at 52 °C for 30 s and elongation at 72 °C for 1 min; and a final extension at 72 °C for 5 min. Amplified fragments were sent for barcode amplicon sequencing with Roche GS FLX + (Eurofins MWG Operon, Ebersberg, Germany). Sequences were binned according to their barcode and the corresponding sample.

A size distribution with the entire set of sequences was computed. The length distribution of the amplicons showed a peak at around 600 bp (95 % of the sequences), corresponding to the expected fragment size. Sequences shorter or larger than that were removed for further analysis. The remaining sequences were then curated to establish operational taxonomic units (OTUs). Briefly, the amplicons were subjected to the following steps: removing of duplicates, de-noising (removing sequences containing sequencing errors) and removing chimeras. The remaining sequences were clustered with the UCLUST algorithm (Edgar 2010). Putative OTUs were defined based on over 97 % nucleotide sequence identity (uclust default parameters) in the same way as commonly done for the 16S rRNA gene.

A classifier to define the genus affiliation of the OTUs for the *spo0A* amplicons was developed in analogy to the naïve Bayesian classifier used by the Ribosomal Database Project (RDP) (Wang et al. 2007). Classification was based on a training set of 238 Spo0A sequences representing all 17 genera of Firmicutes available from the European Bioinformatics Institute (EMBL-EBI) database (Kanz et al. 2005). The genera included *Alicyclobacillus, Alkaliphilus, Bacillus, Brevibacillus, Caldicellulosiruptor, Clostridium, Desulfitobacterium, Desulfotomaculum, Eubacterium, Exiguobacterium, Geobacillus, Halanaerobium, Paenibacillus, Ruminococcus, Sulfobacillus, Thermoanaerobacter* and *Thermoanaerobacterium.* Unfortunately, classification down to the species level was not possible because the reference data required for a reliable assignment is currently unavailable and biased toward medically relevant species.

Statistical analysis and display

The distribution of the most abundant OTUs (over 40 sequences per OTU) per depth was analyzed using the

heatmap function in R (R 2012). Correlations with depth were calculated using linear or exponential regression with the program SigmaPlot 12.0 (Systat Software, San Jose, CA, USA). Significance of differences of parameters before and after 1960 was calculated using Mann–Whitney rank sum test or Student's $t$ test, when data were normally distributed. Correlations between community structure (only OTUs shared between at least two samples) and environmental parameters were determined by canonical correspondence analysis (CCA) with the program R using the package vegan (Oksanen et al. 2007) and BiodiversityR (Kindt and Coe 2005). For the CCA, total phosphorus values (mean weighted concentrations in μg/L measured at the center of the lake at station SHL2) were retrieved from Lazzarotto and Klein (2012); www.cipel.org, Annex 1, p. 46.). Phosphorus values before 1957 were assumed to be constant (average values from 1957 to 1959).

## Results

### Sedimentary facies

The description of CAN01 and CAN02 sediment revealed unique sedimentary facies corresponding to hemipelagic sediments as previously shown in Loizeau (1991) and Corella et al. (2011). This background sedimentation consisted of alternating triplets of (1) mm-thick organic debris layers (2) calcite-rich white laminae and (3) mm- to cm-thick allochthonous detrital layers mostly transported within the river plume and dispersed as interflow. Grain size profile (Fig. 1) revealed that sediments were fine silts with a mean grain size of 17 μm, although an interval between 15 and 42 cm with coarser material (mean grain size 26 μm) can be seen. The lack of turbidities along the sediment core revealed the absence of underflow processes during the last decades in these inactive canyons and makes this emplacement adequate to carry out the paleoecological reconstruction proposed in this study.

### Chronology of the sedimentary sequence

$^{137}$Cs activity in core CAN01 ranged from 7 to 297 Bq kg$^{-1}$ (Fig. 1). The lack of $^{137}$Cs in the sediment from 44-cm depth downwards suggests that deposition below this depth predates AD 1954. Two well-defined peaks were found at 37 and 19.5-cm depth (149 and 247 Bq kg$^{-1}$, respectively), most probably corresponding to the 1963–1964 atmospheric nuclear tests maximum fallout and the 1986 Chernobyl accident. According to these two peaks, sedimentation rate since 1963 has been quite stable at 0.77 ± 0.05 cm year$^{-1}$. As there is no $^{137}$Cs signal below 44 cm, MS measurements were used to date the lower part of the core. MS shows a peak

at a depth of 65.5 cm, which can be correlated with peak # 8, dated to 1943 ± 1.4, from a previous study in the Rhone delta area (Loizeau et al. 1997). This suggests a higher sedimentation rate before 1950 corresponding to ~1.83 cm year$^{-1}$, similar to the increase observed in the more distal area of the delta (Loizeau et al. 1997). Assuming constant sedimentation rate, the dating of the bottom of the core can be extrapolated to ~1920 AD. Correlation between both sediment cores enables estimating similar sedimentation rates for CAN02 (Fig. 1).

### Sediment geochemistry

The XRF data shown in Fig. 1 reflect biogeochemical relations in the lake and its catchment area (Corella et al. 2011). The downcore XRF profile in the studied sediment core (CAN02) revealed a large limnological change in the lake in the years from 1953 to 1991 (15–47-cm sediment depth) (shaded area in Fig. 1), with a decrease in K and Ti and a significant increase in Ca above 47 cm (1953) ($p = <0.001$, Whitney rank sum test). The Fe/Mn ratio fluctuated strongly in this time period and displayed significantly higher values since 1953 ($p = <0.001$, Whitney rank sum test). Organic matter content in the sediment was relatively low with TOC values ranging from 0.4 to 2.2 % and C/N ratio from 10.9 to 42.4. The data of TIC, TOC as well as C/N ratio reflect the shift in lake conditions around 1960, after which TIC is significantly higher (average of 2.1 %, $p = 0.007$, Whitney rank sum test); TOC is also significantly higher (average 1.17 %, $p = 0.002$) and C/N ratio is significantly lower (average 14.4, $p = <0.001$).

### DNA yields and quantification of total bacteria

The DNA extraction method was optimized to target endospores as well as vegetative cells. Three different commercial DNA extraction kits were tested on an endospore preparation of B. subtilis to decide which was the most efficient in extracting DNA from endospores. The results show that a mechanical disruption including three rounds of extended bead beating is needed to obtain DNA from hard-to-break structures such as spores (Supplementary Figure 2).

Using the modified extraction method, the obtained DNA ranged from 0.8 to 16.4 μg g$^{-1}$ sediment (Fig. 2a). The highest DNA yield was not obtained at the top, but at 5-cm sediment depth and two other peaks could be identified at depths of 17 and 43 cm. Throughout the entire depth of the core, there is a significant exponential decay in DNA (correlation $r^2 = 0.89$, $p = <0.001$).

The number of 16S rRNA gene copies (Fig. 2b) ranged from $8.2 \times 10^9$ copies g$^{-1}$ sediment in the upper part of the core (at a depth of 5 cm) to $3.8 \times 10^8$ copies g$^{-1}$
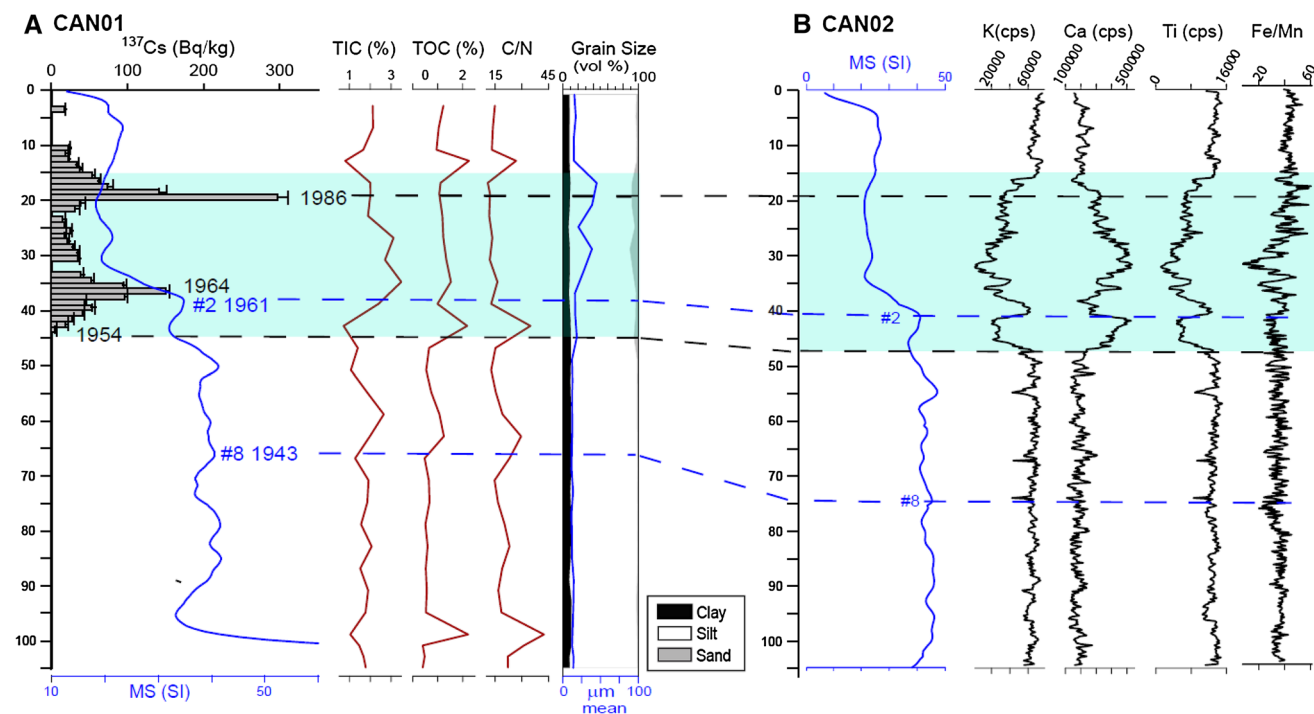
**Fig. 1** Sediment cores analyzed in this study. **a** From *left to right*: [14]Cs activity and magnetic susceptibility (MS) profiles and geochemical profiles of sediment core CAN01 (*TIC* total inorganic carbon; *TOC* total organic carbon; *C/N* organic carbon/nitrogen ratio), grain size (*blue line* indicates the mean grain size). **b** From *left to right*: magnetic susceptibility (MS) and XRF profiles in cps (counts per second) (K, Ca, Ti, Fe/Mn ratio) in core CAN02. *Blue lines* correspond to correlation horizons based on sedimentological and magnetic susceptibility profiles. *Colored area* corresponds to a period with a large limnological change (color figure online)
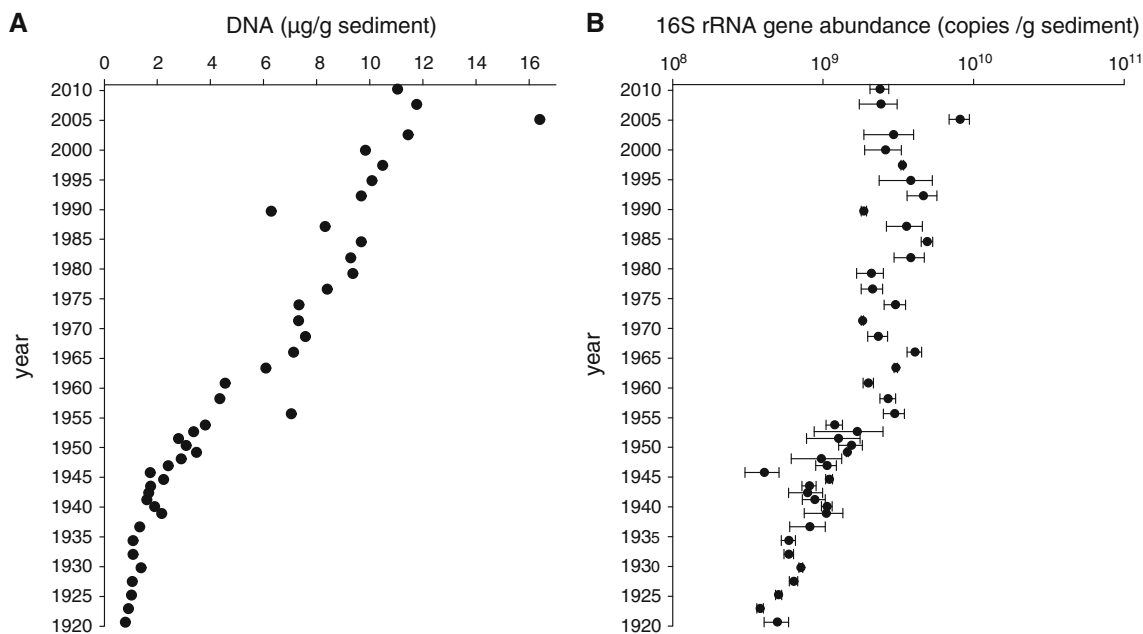


**Fig. 2 a** DNA content (μg g$^{-1}$ sediment) with depth corresponding to calendar year timeline. **b** Distribution of 16S rRNA gene abundance (copies g$^{-1}$ sediment) with depth. *Error bars* correspond to independent technical qPCR replicates

sediment at a depth of 99 cm. The decrease with depth can be fitted to an exponential decay ($r^2 = 0.56$, $p = <0.001$). The results for the 16S rRNA gene abundance from the

core could be divided into three sections, with each showing a different trend. In the top section of the sediment core, there is no clear trend of decreasing or increasing

gene abundance with depth, although the variation between samples is considerably higher than below. At 5 cm, coinciding with the high value of DNA yield, the highest 16S rRNA gene numbers of $8.2 \times 10^9$ copies $g^{-1}$ sediment were measured, which was substantially higher than all other values. In the middle section (between 15 and 47 cm), there was a trend toward an increasing abundance when moving upward, although there was some variation between samples, especially in the upper half of this section. From 47 cm downward, gene abundance was relatively constant, but one order of magnitude lower than those above.

Quantification of endospore-forming bacteria

Endospore-forming bacteria in the sediments were quantified by counting the copies of the *spo0A* gene. The pattern of *spo0A* gene abundance with depth (Fig. 3a) followed a different trend than the pattern from the 16S rRNA gene. Gene abundance ranged from $5.4 \times 10^3$ copies $g^{-1}$ sediment at the bottom of the sediment core to $1.9 \times 10^5$ copies $g^{-1}$ at the top. The highest value did not coincide with the sample that had the highest 16S rRNA gene abundance. There is a weak negative correlation between *spo0A* gene abundance and depth (regression $r^2 = 0.14$, $p = 0.01$). When splitting the core in the three sections mentioned above, the pattern looked different for each segment. In the deepest segment of the sediment core (50–106 cm), no overall trend could be seen. There were two samples with higher values than the others in this section (at 87-cm depth

and in the area between 65 and 75 cm). In the middle section of the core (15–50 cm), there was high variation between the samples and, contrary to the 16S rRNA gene numbers, there was a slight decreasing trend when moving upward in the core. Finally, in the top section of the core (0–15 cm), the variation between samples was high, but overall a trend could be seen shifting toward increasing values at the top of the core. The highest value of the *spo0A* gene abundance was found at a depth of 3 cm.

The ratio between the 16S rRNA gene and the *spo0A* gene abundance was in the range of $0.7 \times 10^{-3}$ % to $8 \times 10^{-3}$ % and averaged $3.2 \times 10^{-3}$ % (Fig. 3b). The ratios were significantly higher in the bottom section of the core (below 50 cm) than in the upper half (Student's *t* test; $p = <0.001$).

Determination of phylotypes of endospore-forming bacteria

A partial sequence of the *spo0A* gene from a selection of samples was amplified and subsequently sequenced. The samples were selected (1) to represent depths spanning through the entire core and (2) from interesting points where trends were shifting or values were particularly high or low. The sequences of *spo0A* were then used to determine the phylotypes and abundance of endospore-forming bacteria. After curation of the sequences, 5,144 sequences could be clustered into 552 OTUs. The results showed that despite that the quantity of extracted DNA decreased with the age of the sediment, the richness of endospore-forming
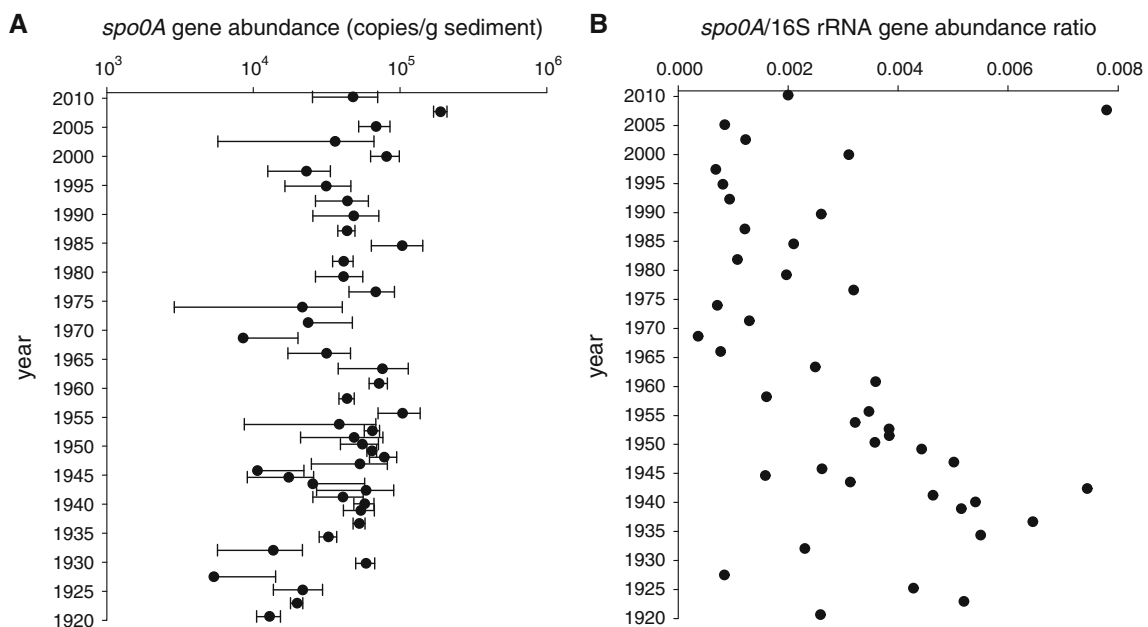


**Fig. 3 a** Depth distribution of *spo0A* gene abundance (copies $g^{-1}$ sediment). *Error bars* correspond to independent technical qPCR replicates. **b** Ratio of 16S rRNA/*spo0A* gene abundance

**Fig. 4** Heatmap of the most dominant phylotypes obtained from *spo0A* sequences (>40 sequences per OTU) with clustering of samples from different depths according to similarities in community structure (OTU presence and abundance in the *vertical axis*). The frequency of OTUs is indicated by the color with *red shades* standing for no sequence and *yellow* indicating a high number of sequences. The depth, corresponding dating and number of OTUs (Richness) are shown on the *right* (color figure online)
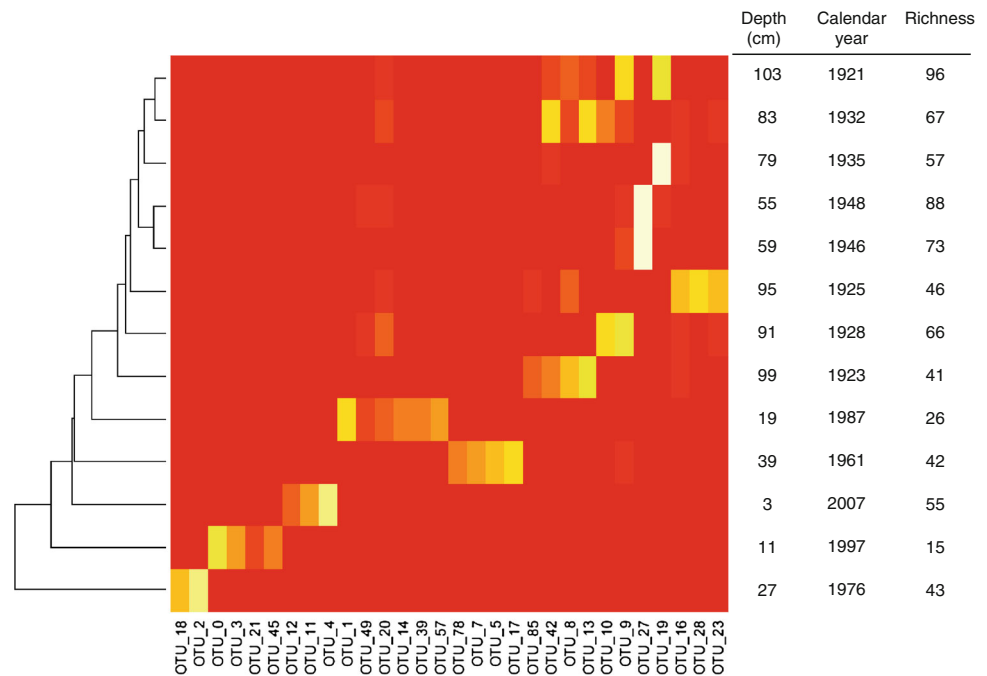


bacteria did increase; richness was significantly less in the samples from depth 11, 19, 27 and 39 cm (*t* test, $p = 0.007$) with a mean of 31.5 OTUs per sample than in the other samples with a mean of 65.4 OTUs per sample. Richness ranged from 15 (at 11-cm depth) to 96 OTUs at 103-cm depth (Fig. 4). The majority (471) of the total identified phylotypes (554) only appeared as a single copy sequence. The heatmap (Fig. 4) shows the relationship of the most abundant phylotypes, with representatives that have >40 sequences per OTU. The lighter the color in the heatmap, the more sequences per OTU were found. The samples from the top of the core (1961–2007) clustered separately from the older half of the core (1921–1948) and have distinct OTUs that are only found at one depth. On the contrary, in the lower half of the core a number of dominant phylotypes were detected at several depths (OTUs Nr. 8, 9, 13, 20, 42) (Fig. 4).

To determine the community structure, OTUs were classified into genera on the basis of Spo0A protein sequence belonging to a known training dataset of 17 endospore-forming genera. This resulted in the classification of 552 OTUs into seven genera (*Alkaliphilus* 4 OTUs, *Bacillus* 76, *Brevibacillus* 5, *Clostridium* 104, *Desulfitobacterium* 12, *Desulfotomaculum* 7 and *Paenibacillus* 112). The remaining 232 OTUs could only be classified as far as the classes Bacilli or Clostridia (data not shown). The number of individual sequences per genus and samples are displayed in Fig. 5. Contrary to the trend of richness, the abundance values in the upper section of the core (years 1997, 1987, 1976 and 1961) with an average of 464 sequences per sample is significantly higher than in the rest of the samples with an average abundance of 363.3

sequences per sample (*t* test; $p = 0.01$). On the contrary, the number of genera present in those samples is significantly reduced (*t* test; $p = 0.008$). The mean number of genera for years 1997, 1987, 1976 and 1961 was 2.7, while other samples had on average 4.6 genera per sample. Between years 1961 and 1987, the reduction in richness coincided with an increase in the representation of anaerobic ecotypes represented by the genera *Clostridium* (years 1987 and 1976) and *Desulfitobacterium* (year 1961), and a reduction in the representation of *Paenibacillus*, in particular in 1976. In the year 1997, the reduction in richness was due to the dominance of the genus *Bacillus*.

## Correlation between physico-chemical and microbiological parameters

The main changes in biological parameters could be seen in the core section of 15–47 cm, where also the chemical and physical parameters showed greatest variation.

Correlation of both types of parameters is shown as a biplot of a canonical correspondence analysis (CCA), based on the abundance dataset (containing all OTUs with more than five sequences) of all samples and the parameters Ca, K, Ti, Fe, Mn, Fe/Mn, depth and total P (Fig. 6). The community composition of endospore-forming bacteria from the samples of 1976, 1987, 1997 and 2007 shows divergence from the cluster of samples from the older part of the core (1921–1948). The direction of the divergence (particularly for sample 1976) is on the same axis as the significant contribution of Fe/Mn ($p = 0.01$), P ($p = 0.01$) and Ca ($p = 0.03$). The ordination of Ca is directly opposite of K, Ti, Fe and Mn. The direction of highest

**Fig. 5** Endospore-forming community structure represented by *spo0A* sequences and their genus affiliation at different depths. The length of the *bar* corresponds to total sequence abundance. Different genera are depicted in different *colors*. *Gray bars* are sequences that could not be classified to genus level (unclassified) but corresponded to Bacilli or Clostridia (color figure online)
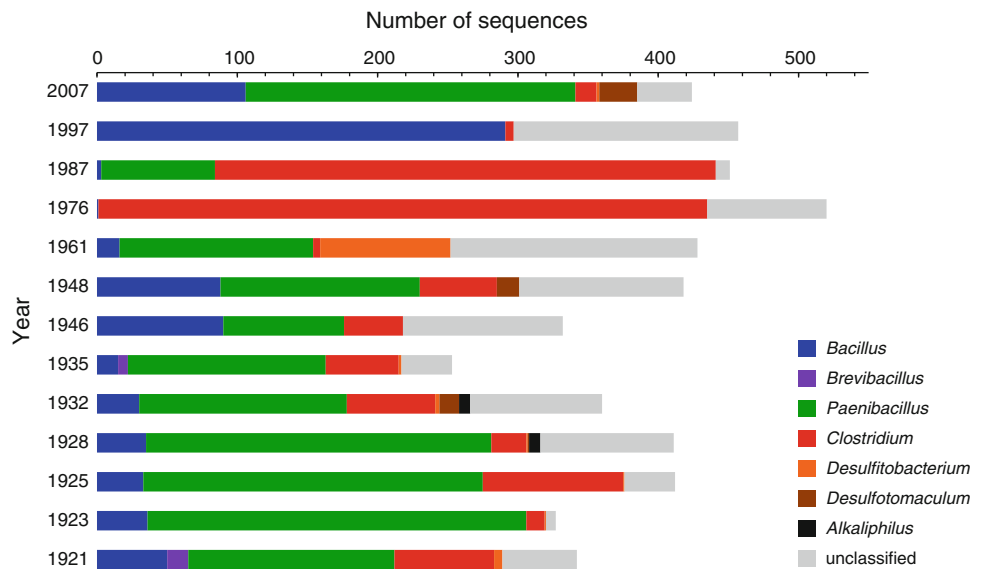


**Fig. 6** Canonical correspondence analysis (CCA) of the community matrix with all OTUs composed of a minimum of five sequences and the environmental matrix with values of depth, total P, Ca, K, Ti, Fe, Mn and Fe/Mn as constraining parameters. The total P ($\mu g\ L^{-1}$) values are average yearly values from the Grand Lac (CIPEL, 2012). *X*-axis explained 15.3 % and *Y*-axis explained 15.0 % of importance. The positions of samples are depicted with *black circles* and the positions of individual OTUs with a *red plus* (color figure online)
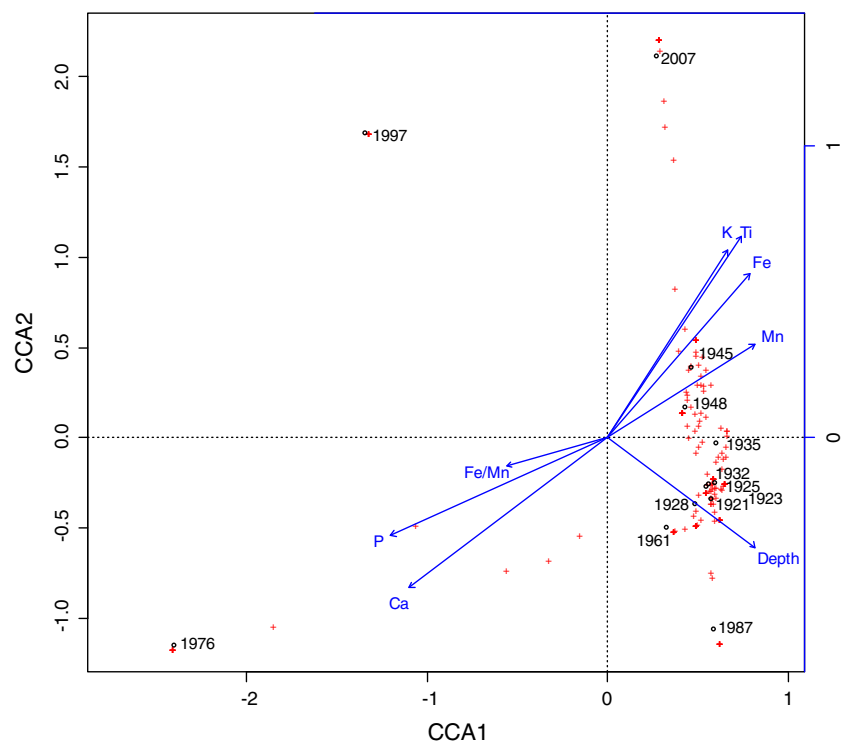


variance (tip of the arrow) of the ratio of Fe/Mn is close as for total P values, suggesting a strong correlation between these two parameters.

## Discussion

From a paleoecological point of view, the sedimentary record studied here is relatively recent, with the bottom of the core being assigned to AD 1920. Nonetheless, the results show that nucleic acids in the sediment decrease exponentially with time, so does the number of bacteria assessed as 16S rRNA gene abundance, which is significantly reduced with depth. Decrease of DNA with sediment depth is more prominent than the decrease in 16S rRNA gene abundance, which can be explained by the contribution of eukaryotic DNA to the total yield. Quantification of bacteria cannot directly be correlated with DNA yields in this sediment core. However, at the bottom of the core, there are still $3.8 \times 10^8$ copies per gram sediment of the 16S rRNA gene detectable. Considering that bacteria can house up to 15 copies of the 16S rRNA gene per cell

(Klappenbach et al. 2000), this accounts for $25 \times 10^6$ cells per gram sediment of an age of about 90 years. However, sediments with such high numbers of bacterial cells most likely house a large diversity of species and the communities are too complex to analyze for trends over time. Neither DNA nor molecular quantification and community assessment of total bacteria can therefore serve as ecological proxies in this sediment core.

Endospores have been considered as ideal candidates to survive in the sediment for long periods of time and to potentially serve as paleolimnological proxies (Renberg and Nilsson 1992). Endospores are very resistant structures, built with the purpose of protecting its enclosed DNA from degradation (Nicholson et al. 2000). In this study, we confirm this hypothesis with the constant if not increasing quantification of the spo0A gene abundance with sediment depth. Indeed, the decreasing trend observed in DNA and 16S rRNA gene abundance with depth of the sediment core is independent of the spo0A gene abundance determined throughout the sediment core, as seen by the ratio of spo0A and 16S rRNA gene increasing with depth (Fig. 3b). The ratio between spo0A and 16S rRNA genes is low with an average of 0.003 %. By assuming an average of 15 16S rRNA gene copies per cell (Klappenbach et al. 2000), roughly 1 in 2,000 bacterial cells is an endospore-forming bacteria. Targeting such a small fraction of the bacterial community results in reduced complexity and higher resolution. Because of low complexity and the stability of DNA retrievable from this group of bacteria over time, the endospore-forming bacterial community is a promising proxy for paleoecology.

The composition of the endospore-forming community at different depths of the sediment reflect prominent shifts in the community in the years between 1961 and 1997. These shifts correspond to reduced species richness, while total abundance of endospore-forming bacteria increased during the same time period. In samples from 1976 to 1987, the members from the genus Clostridium are most abundant, and in the sample from 1997 members from Bacillus are most abundant. The classification of the OTUs into these groups is important as it can be linked to some of the ecological features known for cultured species, especially concerning aerobic or anaerobic growth conditions. The genera Clostridium (Schleifer 2009), Desulfotomaculum (Stackebrandt et al. 1997) and Desulfitobacterium (Utkin et al. 1994) are strict anaerobic heterotrophic bacteria. Bacteria from genera Bacillus (Schleifer 2009) and Brevibacillus (Shida et al. 1996), on the contrary, grow aerobically, while Paenibacillus (Shida et al. 1997) is composed of members that grow as facultative anaerobes. Therefore, shifts in community composition for endospore-forming bacteria from 1961 to 1987 appear to be linked to decrease availability of oxygen in the sediment or at its surface at the moment of sedimentation.

Three issues should be considered for the interpretation of these results. The first one is the specificity of the spo0A as molecular marker to target endospore-forming bacteria. The second is the origin of endospore-forming bacteria (i.e., autochthonous versus allochthonous) and the extent to which endospore-forming communities reflect the environmental conditions at the moment of sedimentation. The third issue is the highly dynamic nature of sediments and the potential changes in the paleoecological signature that will result from in situ microbial activity.

Specificity of the spo0A gene in an important issue. This gene is conserved among all endospore-forming species known so far (Galperin et al. 2012; Abecasis et al. 2013; Traag et al. 2013), and is notably absent from exospore-forming groups such as Streptomyces spp. and Mycobacteria (Abecasis et al. 2013), as well as in the developmental cascade at the origin of the fruiting bodies in Myxococcus (Kroos 2007). A previous study has shown similar sequences to spo0A being present in nonspore-forming species, even though at low similarity scores (Onyenwoke et al. 2004). However, a more recent profile analysis of spo0A in 626 genomes has found only one putative ortholog in a non-endospore-forming genome (Traag et al. 2013). This suggests that spo0A can be considered as a specific molecular marker for endospore-forming bacteria. In addition, all of our attempts to amplify nonspore-forming strains with our spo0A primer have been negative (data not shown). In addition, although not all the OTUs could be assigned to the genus level because of a lack of references, a significant score and e value for spo0A were obtained for all the curated sequences, and a classification into the classes Bacilli or Clostridia was achieved.

Regarding the origin of the endospore-forming bacteria, previous studies based on culturing of specific endospore-forming species from lake or marine sediments (Bartholomew and Paik 1966; Robles et al. 2000; Hubert et al. 2010) have suggested that endospores are in most cases allochthonous and have been deposited at the time of sedimentation followed by little in situ activity. In the present study, this might not be the case. The high C/N ratio shown in Fig. 1, with values close to 19, underlines a mixed origin of terrestrial and aquatic organic matter (Meyers 2003). However, the fact that DNA yields are high at the same depth that have peaks in C/N ratio (13 and 43 cm) imply terrestrial input of organisms at these same time points, potentially due to floods. The quantity of endospore formers at these depths does not correlate with the C/N ratio. This means that likely the majority of the endospore-forming community in the sediments is from an autochthonous source and is less influenced by transport from rivers. An example for this could be members from Paenibacillus, facultative anaerobes known to hydrolyze complex carbohydrates such as chitin (Shida et al. 1997). Both properties of Paenibacillus indicate

potential advantages to actively thrive at the sediment surface, which is confirmed by their dominance in the majority of samples. The difference between previous reports and the results obtained in the present study could be due to the biases introduced by culturing, which are omitted in the molecular approach taken here since DNA can also be recovered from non-viable spores of both, autochthonous and allochthonous origin.

With respect to the influence of the in situ activity in the paleoecological signature of the microbial community, it is not clear if endospore-forming bacteria grow actively on the lake bottom or in the sediment. From the TOC values (top sediment at 1.23 %) that stay stable through the core, it can be implied that the available carbon is quickly turned over and that in the sediment the carbon available for heterotrophic growth is very small. Also, a recent study from Lake Geneva has shown ATP to strongly decrease in the top few centimeters of the sediment to almost zero at 10 cm, suggesting little microbial activity below this depth (Thevenon et al. 2011). When conditions get oligotrophic and in particular if the carbon is low, endosporulation sets in due to starvation (Hageman et al. 1984). Based on this, our current interpretation is that burial of endospores happens for the most part at this stage.

A way to distinguish between active and inactive members of the community could be the differentiation between vegetative cells and endospores. However, the current techniques to quantify endospores [e.g., dipicolinic acid content as in (Fichtel et al. 2007)] are destructive and thus identification cannot be coupled to quantification. Further studies including differentiation between cells and endospores and a characterization of the metabolic properties of these endospore formers could give additional insights into this issue.

Signals of ecosystem changes in the period between 1960 and 1990 are also reflected in some chemical and physical parameters of the sediment cores in this study. The sediment cores are composed of silt-sized hemipelagic sediment, suggesting no direct influence by the Rhone River hyperpycnal flows. But as most of the particle input to Lake Geneva, including clay- and silt-sized minerals, is due to the Rhone River and as the general Lake Geneva circulation deflects stratified inflows to the right, the overall sediment signature at the sampling site mostly reflects changes in the lake's catchment. However, authigenic calcite production is mainly influenced by the lake's productivity, which adds an in situ signature to the sediment record. The results of the elemental analysis in the sediment core (CAN02) of this study therefore mainly reflect changes in runoff, autogenic production in the water column or changes at the sediment–water interface.

K and Ti are known to be linked to allochtonous input by runoff. As K and Ti counts follow an inverse trend to grain size, they seem to be related to fine sediment, as seen in Corella et al. (2011). Ca counts profile shows an opposite trend to K and Ti, and is thus interpreted as mainly due to endogenic productivity and larger-sized particles. Even though Ca and the published values for total P (measured at the center of the lake over 20 different depths) are linked, when looking at our CCA it is difficult to disentangle endogenic Ca precipitation due to increased primary production from the inputs due to changes in runoff. However, recent studies show the link between photosynthetic microorganisms and the formation of low-Mg calcite in freshwater (Plee et al. 2008; Pacton et al. 2012), favoring the hypothesis of a link between Ca and eutrophication. Fe and Mn depth profiles are directly linked to K and Ti counts in the CCA biplot; they are therefore also interpreted as influenced by allochthonous inputs. Neither of the above single elements can therefore clearly be linked to changes solely due to lake eutrophication.

On the contrary, the Fe/Mn ratio, frequently used as an indicator of the redox condition in lake hypolimnia (Koinig et al. 2003; Corella et al. 2012), point to changes linked to eutrophication. An increase in Fe/Mn ratio may reflect hypoxia at the sediment water interface due to preferential resolubilization of Mn over Fe linked to differences in redox kinetics. Mn is more easily reduced and transported away from the sediment, therefore depleting the Mn in respect to the residual Fe content in the sediment (Schaller and Wehrli 1996). At site CAN02, the increase in the Fe/Mn ratio since 1960 (Fig. 1) can be associated with hypoxia at the sediment water interface. A decrease in the oxygen content at lake bottom conditions has also been connected to lower C/N ratio and higher TOC content due to higher primary production in the water column (Corella et al. 2011). These three parameters have significantly changed since 1960 in this sediment record, coinciding with the shift in the structure of anaerobic endospore-forming bacterial communities and the dominance of anaerobic genera such as *Clostridium*.

If the changes in the community structure of endospore-forming bacteria and the redox proxies in the sediment during the years 1961–1997 are indeed a record of the variation in oxygen availability, evidence for this environmental fluctuation should also be found in the history of this lake. In Lake Geneva, in the last 100 years, anthropogenic pressures such as the release of nutrients via wastewater or agricultural runoff have had an important impact on the ecosystem. The lake has been closely monitored since 1957 and data are made publicly available by the 'Commission Internationale de la Protection des Eaux du Léman' (CIPEL) in the form of yearly published reports (http://www.cipel.org), with the most recent one released in 2012 (Lazzarotto and Klein 2012). Long-term trends show a steady increase of total phosphorus since 1957 with a

peak in 1979. These values, together with phosphate data since 1970, indicate a shift in the trophic status of the lake from oligotrophic to eutrophic taking place in the late 1960s. The system has since recovered, even though total phosphorus levels are still double the values before 1960. Eutrophication of Lake Geneva is one of the environmental disturbances with the best ecological record (Anneville and Pelletier 2000; Gerdeaux and Perga 2006; Thevenon et al. 2012). High nutrient levels increased primary production, which together with warm winters and successive incomplete mixing of the lake, resulted in low oxygen levels in deeper waters. Long-term lake bottom hypoxia (delimited as $<4$ mg $O_2$ $L^{-1}$) have been registered in the deepest part of the lake (300 m) in the years from 1973 to 1978 and 1986 to 1998, by CIPEL (Lazzarotto and Klein 2012). Important changes have been seen in the composition of pelagic primary and secondary producers as a consequence of higher phosphorus concentrations as well as a warming climate (Molinero et al. 2006). Changes at higher trophic levels had also occurred such as the extinction of whitefish (Vonlanthen et al. 2012).

Interestingly, the most recent part of the record showed a trend toward the recovery of endospore-forming communities, although indicating a delay between the decrease of water nutrients and the response of the community. The community composition in the most recent sample (2007) is changing back to a similar diversity that was found in samples dating from 1928 to 1932, demonstrating an intrinsic resilience of the system for these bacteria. This is demonstrated by eight OTUs that are shared between the samples from 2007 and samples prior to 1948. In samples from the years 1976, 1987 and 1997, none, three and one OTU, respectively, are shared with samples from before 1948. This result is interestingly opposite to *Daphnia* population evolution in unproductive Swiss lakes, where eutrophication led to partly irreversible species changes (Rellstab et al. 2011).

In conclusion, the research presented here breaks new ground for the use of specific groups of bacteria as proxies for changes found in sedimentary records. In the past, other microbial groups have been used in freshwater paleoecology. A good example of this is the study of siliceous microfossils from diatoms, which are preserved in sediments as amorphous biogenic silica (Hobbs et al. 2010). However, the dissolution of diatoms within lake sediments may compromise the interpretation of the sediment record (Ryves et al. 2006). In this sense, bacterial endospores have the advantage of being biological structures with the specific role of resisting environmental stress for long time periods (Nicholson et al. 2000), and therefore can be expected to remain unaltered within the sediment record. In addition, nowadays, with new molecular techniques and the possibility to directly sequencing metagenomes from environmental samples, the discovery and use of even older endospores as paleoecological proxies is plausible, since viability will no longer be a major issue. Although issues related to the dynamic nature of sediments and the origin and in situ activity of endospore-forming bacteria need to be studied further, this study is a proof of concept that endospore-forming community reflects changes in oxygen conditions in this lake.

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