RESEARCH ARTICLE

Vertical distribution of bacterioplankton in Lake Averno in relation to water chemistry

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Abstract

The effects of environmental factors on bacterioplankton distribution along the water column of Lake Averno (Naples, Italy) have been investigated by means of denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR)-amplified 16S rRNA gene fragments, and multivariate analysis applied to molecular data and physico-chemical parameters. Bacterial richness, estimated from DGGE profiles, remains constant throughout the water column, whereas archaeal richness increases with depth. Moreover, archaeal richness was significantly correlated to most of the measured abiotic variables, whereas bacterial richness did not. Analysis of sequences from DGGE bands revealed that the dominant bacterial populations belong to *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, *Bacteroidetes* and *Firmicutes*, whereas sequences of the archaeal DGGE bands are affiliated to *Methanomicrobiales* and *Methanosarcinales* orders. Moreover, bacterial sequences affiliated to *Proteobacteria* and *Bacteroidetes* do not group closely to typical freshwater lineages/clades/tribes within these phyla. Bacterioplankton distribution along the water column was apparently correlated with the vertical gradient of physico-chemical parameters as a statistically significant relationship between most of them with grouping of specific taxonomic units was observed.

Introduction

Prokaryotes are key organisms in lakes as they play an important role in the biogeochemical cycling of elements such as carbon and nitrogen (Wetzel, 2001). Furthermore, these microorganisms play a relevant role in processes controlling the water quality of lacustrine ecosystems because they are involved in biodegradation of pollutants deriving from urban, industrial and agricultural activities (Briée *et al.*, 2007). Investigations on the relationships between the diversity of microbial community and environmental factors offer useful information that leads to a better understanding of the process of biogeochemical cycling of nutrient elements and offer the possibility to predict ecosystem responses to environmental changes (Fuhrman *et al.*, 2006). In fact, bacterioplankton community composition (BCC) is clearly affected by certain environmental factors, such as pH, water temperature, water chemistry, nutrient condition, geographical and seasonal variations (Lindström *et al.*, 2005; Hahn, 2006; Zeng *et al.*, 2009).

Several studies have investigated the depth-related changes in entire microbial communities in lake water columns (Øvreås *et al.*, 1997; Bosshard *et al.*, 2000; Hollibaugh *et al.*, 2001; Humayoun *et al.*, 2003; De Wever *et al.*, 2005; Dimitriu *et al.*, 2008; Jiang *et al.*, 2008). However, only a few studies have related these changes to the gradient of physicochemical properties taking into account a considerable number of environmental factors (Salcher *et al.*, 2008; Wei *et al.*, 2008; Zeng *et al.*, 2009) and using multivariate methods to correlate them with BCC composition (Lindström *et al.*, 2005; Wei *et al.*, 2008). Indeed, although most of these studies have investigated association between molecular community fingerprints and environmental parameters, little information about the effect of environmental variables on the presence of individual bacterial groups is available.

FEMS MICROBIOLOGY ECOLOGY

In the present study, the distribution and diversity of prokaryotes along the water column of Lake Averno in relation to environmental parameters were investigated. The microbial ecology of Lake Averno is of interest because it is an almost water closed basin, polluted by waste waters from nearby communities and agricultural activities, in which water overturn occurred occasionally in the past, causing an increase in toxic levels of H_2S with a consequent fish kill event (Caliro *et al.*, 2008). Data concerning depth-related changes in the physico-chemical parameters of Lake Averno water column indicate that it generally comprises two distinct water masses, an oxic epilimnion and an anoxic hypolimnion with evident zonation of the microbial processes along the entire water column (Caliro *et al.*, 2008). Breaking of the vertical/thermal stratification occurs in the winter periods when temperatures of epilimnetic waters remain below 7 °C over a long time (Caliro *et al.*, 2008). However, very little information regarding the microbial community of this lake is available. So far, only a study on the isolation of hydrogen-producing purple nonsulphur bacteria from samples taken at various depths in the Lake Averno was published (Bianchi *et al.*, 2010).

We examined the diversity of bacterial and archaeal communities along the stratified water column of Lake Averno by means of the denaturing gradient gel electrophoresis (DGGE) technique and subsequent sequencing of prominent DGGE bands. The association between specific taxonomic units to some physico-chemical parameters was investigated using nonlinear multidimensional scaling (NMDS) analysis to identify which factors have a significant impact on the bacterioplankton vertical distribution.

Materials and methods

Site and sampling

Lake Averno fills the mouth of one of several volcanic craters in the Phlegrean Field region, about 15 km West of Naples, in Southern Italy (40°50′ N, 14°04′ E, 1.10 m above sea level). It is almost elliptic (100 m long and 700 m wide) covering a surface of 0.54 km^2, and it is 34 m deep in the centre, with a total volume of *c.* 6×10^6 m^3. A canal, almost 1 km long, links the lake to the sea. The chemical and isotopic composition of its waters suggests that it originates from mixing of shallow waters with a Na–Cl hydrothermal component coupled with an active evaporation process (Caliro *et al.*, 2008). The lake is normally stratified, with an oxic epilimnion (from the surface to 6 m depth) and anoxic hypolimnion (from 6 to 33 m). The water overturn is an unusual phenomenon, occurring in the winter periods, when temperatures of epilimnetic waters remain below 7 °C for a long time (Caliro *et al.*, 2008). Over the last few years, the surrounding area has been subjected to considerable human impact, such as construction works, recreation facilities and agriculture. In addition, Lake Averno has been affected, for a long time, by intermittent organic enrichment through sewage discharges (Improta *et al.*, 2004).

Water samples (250 mL) were collected in May 2006 at different depths (1, 3, 5, 6, 9, 15, 21, 27, 32, 33 m below the surface) using a Niskin water sampler. Samples were kept in a cooler until they were filtered through 0.22-μm-pore-size membranes (diameter 50 mm; Millipore, MA; within 6 h from sampling) to recover the microbial biomass for subsequent DNA extraction. Filters were placed in cryovials and stored at −80 °C until processed.

Physico-chemical analyses of water column

The temperature (T), conductibility (Econd), redox potential (Eh) and pH were measured by means of a multiprobe logger (Hydrolab CTD). The nominal precisions are as follows: depth ± 0.05 m; T ± 0.01 °C; Eh ± 20 mV; pH ± 0.05 pH-units. The samples for nutrients and SO_4^{2-} analysis were immediately transferred to 500-mL flasks and taken to the laboratory in cool boxes, filtered through a 0.22-μm cellulose acetate membrane (GSW; Millipore) and stored at −18 °C until processed. Nutrient concentrations were measured with a spectrophotometric technique, using the automated colorimetric analysis Easychem Plus (Systea Scientific LCC, IL). In particular, orthophosphates were determined by ascorbic acid reduction, nitrites by the colorimetric method of sulphanylamide diazotization, nitrates by a cadmium-reducing column and ammonia by the phenate method (A.P.H.A., 2005). SO_4^{2-} concentration was assessed by ion chromatography. For sulphide analysis, oxygen-free 125-mL serum bottles, sealed with butyl rubber stoppers and containing 0.4 mL of a 2 M zinc acetate solution, were filled completely with water samples and stored at 4 °C. Sulphide was assessed by the iodometric method (A.P.H.A., 2005). Water sampling and analysis for the methane, CO_2 and HCO_3^- determination were performed using the method described by Caliro *et al.* (2008).

DNA extraction and PCR amplification

Total DNA was extracted from each filter using the phenol/chloroform/isoamyl alcohol extraction and sodium acetate–ethanol precipitation methods described by Vetriani *et al.* (2003). The pellet of nucleic acid was resuspended in sterile deionized water, to a final volume of 200 μL.

Crude extracts were further purified using the Wizard DNA clean-up system (Promega, WI) according to the supplier's instructions. Quantity and purity of DNA were checked fluorospectrometrically by NanoDrop (NanoDrop Technologies, DE).

A nested PCR approach was used to amplify bacterial and archaeal 16S rRNA gene fragments from total DNA samples. For members of the *Bacteria* domain, a first PCR amplification was performed with 20 ng of DNA using the forward primer 27F and the reverse primer 1495R (Table 1) as described by Di Cello *et al.* (1997). Dilutions 1 : 100 (2 μL) of PCR products of 1450 bp were then used as template for the second PCR amplification using the forward primer 63F-GC-clamp and the reverse primer 518R (Table 1) to produce 495 bp fragments suitable for DGGE analysis (El Fantroussi *et al.*, 1999). Both PCRs were performed in Qiagen *Taq* buffer (10X) containing 1.5 mM $MgCl_2$, with 150 ng of each primer, 250 μM (each) deoxynucleoside triphosphates and 0.5 U of *Taq* DNA polymerase (Qiagen, Hilden, Germany) in 25 μL reaction volume. Cycling parameters for PCR with primers 27F-1495R and with primers 63F-GC and 518R were as those previously described by Di Cello *et al.* (1997) and El Fantroussi *et al.* (1999), respectively.

The amplification of the almost-complete archaeal 16S rRNA gene was carried out with 20 ng of total DNA using the primers S-D-Arch-0025-a-S-17) and S-*-Univ-1517-a-A-21 (Table 1; Vetriani *et al.*, 1999). In contrast to the original procedure, a touchdown thermal program was used, with an initial denaturation step (95 °C, 2 min), followed by 30 cycles of touchdown amplification [denaturation (94 °C, 30 s), annealing (56 °C, 30 s with 0.5 °C decrease each cycle to 45 °C) and extension (72 °C, 30 s)], 5 cycles of amplification [denaturation (94 °C, 30 s), annealing (45 °C, 30 s) and extension (72 °C, 30 s)], and a final extension (72 °C, 7 min). A dilution 1 : 100 of this PCR product (1120-bp) was used as template (2 μL) for the amplification of a 456-bp fragment using the primers A934F and 1390R-GC (Table 1; Wu *et al.*, 2001). A touchdown thermal program to amplify archaeal 16S rRNA genes with primers A934F and 1390R-GC was used (Wu *et al.*, 2001). Both archaeal PCRs were performed in Qiagen *Taq* buffer (10X) containing 1.5 mM $MgCl_2$, with 0.1 μM (each) of primers, 200 μM (each) of deoxynucleoside triphosphates and 2.5 U of *Taq* DNA polymerase (Qiagen) in 25 μL reaction volume.

Table 1. Primers used in this study

Primer[†]	Sequence (5′ to 3′)	Specifity
27F	GAGAGTTTGATCCTGGCTCAG	Most *Bacteria*
1492R	CTACGGCTACCTTGTTACGA	Most *Bacteria*
63F-GC [‡]	AGGCCTAACACATGCAAGTC	Most *Bacteria*
518R	ATTACCGCGGCTGCTGG	Most *Bacteria*
S-D-Arch-0025-a-S-17	CTGGTTGATCCTGCCAG	Most *Archaea*
S-*-Univ-1517-a-A-21	ACGGCTACCTTGTTACGACTT	Most *Archaea*
A934F	AGGAATTGGCGGGGGAGCA	Most *Archaea*
1390R-GC[§]	ACGGGCGGTGTGTGCAA	Most *Archaea*

[†]Target gene of all primers used in this study is 16S rRNA gene.

[‡]This primer has the following GC clamp at its 5′end: 5′CGC CCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG3′ (Øvreås *et al.*,1997).

[§]This primer has the following GC clamp at its 5′end: 5′CGCCC GGGGCGCGCCCCGGGCGGGGCGGGGGC3′ (Wu *et al.*, 2001).

DGGE

Bacterial fragments were resolved by double-gradient-denaturing gradient gel electrophoresis (DG-DGGE), as described by Cremonesi *et al.* (1997), in a DCode universal mutation detection system (Bio-Rad, CA). A 6%–12% polyacrylamide (acrylamide : N,N-methylenebisacrylamide, 37.5 : 1) gel with denaturing gradient ranging from 30% to 60% was used. For the domain *Archaea*, PCR products were screened by DGGE as reported by Muyzer *et al.* (1993). PCR samples were loaded onto 6% polyacrylamide gel prepared with a denaturant gradient ranging from 40% to 60%. A 100% denaturing solution contained 7 M urea and 40% deionized formamide. Approximately 700 ng of the purified PCR products were loaded in each well. The gels were run for 16 h at 75 mV in 1X TAE buffer at 60 °C, stained with 50 μg mL^{-1} ethidium bromide for 30 min, destained in water and photographed with the UVIpro Platinum Gel Documentation System (GAS7500/7510; Eppendorf, Cambridge, UK).

Analysis of microbial community fingerprints and statistical analysis

The statistical comparison of the different DGGE patterns, run on the same gel, was made with Quantity One software package (Bio-Rad). The similarity between the band patterns was calculated using the Dice coefficient, and the clustering analysis was performed with the unweighted pair-group method using arithmetic averages (UPGMA) for dendrogram construction.

The number of DGGE bands present in each sample was used to measure the richness index (R).

Univariate Poisson regression models were used to assess the relationship between richness and physicochemical factors. Resulting *P*-values were adjusted for multiplicity using Benjamini and Hochberg correction (Benjamini & Hochberg, 1995).

Due to the small sample size, we could not perform further inferential analyses; thus, a multivariate descriptive analysis was carried out to correlate the presence of DGGE bands to environmental parameters. A cluster

analysis on presence/absence of each DGGE bands, based on Jaccard distance and complete linkage, was performed. The resulting tree was cut based on the optimal number of clusters according to the gap statistics (Tibshirani *et al.*, 2001). To visualize cluster analysis and to describe relationships between environmental factors and presence/absence of DGGE bands, a non-metric multidimensional scaling (NMDS) analysis was carried out using the Bray–Curtis dissimilarity index (Minchin, 1987). To evaluate the ordination, correlation between fitted vectors and ordination values (R^2) was calculated, and several iterations of the NMDS procedure were performed to obtain the lowest stress value possible (the best goodness of fit) based on different random initial positions of the objects in the ordination space. The fitted environmental vectors and centroids, that is, mean and standard deviation for each variable and each cluster, were overlain on the NMDS ordination by fitting smooth surfaces using generalized additive models with thin plate splines (Wood, 2000). To evaluate significance of the environmental factors on the variability in the NMDS, biplot permutation tests were used (Minchin, 1987).

When deriving biplots from the NMDS, if there was an overlap of band codes, priority was given to labels of most abundant bands, that is, those whose presence was observed more often overall.

To aid interpretation of the cluster analysis, the mean and the standard deviations of the environmental factors based on locations in which at least one band in the cluster was present were computed. The analyses were performed using the software R 2.13.0 (R Development Core Team, 2011).

Extraction, sequencing and phylogenetic analysis of DGGE bands

Prominent DGGE bands were excised from the gels with a sterile scalpel, transferred into 50 μL sterile water and incubated overnight at 4 °C to allow diffusion of the DNA. Eluted DNA from each band was reamplified as described previously and submitted again to a DGGE run to confirm electrophoretic mobility.

Sequencing reactions were prepared from PCR products amplified with unclamped primers using an Applied Biosystem BIG DYE® Terminator sequencing kit version 3.1, according to the manufacturer's instructions and analysed using a 3730 DNA Analyzer Applied Biosystem apparatus. Each sequence was submitted to the CHECK_CHIMERA program of the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu) to detect the presence of possible chimeric artifacts. The sequence similarity search tools BLAST (Altschul *et al.*, 1990) were used to obtain a first indication of the closest relative for each retrieved sequence. Hierarchical taxa assignment was performed by means of RDP naïve Bayesian rRNA Classifier (Wang *et al.*, 2007), and phylogenetic reconstruction was carried out using the ARB software package (Ludwig *et al.*, 2004). Partial sequences obtained in this study were added to a database *c.* 50 000 homologous prokaryotic 16S rRNA gene primary structures using the alignment tool of the ARB software package and subsequently corrected manually. Sequences of typical freshwater lake bacteria, assigned to specific lineages, clades and tribes as described by Newton *et al.* (2011), are included in the ARB database. For the stability of phylogenetic trees, backbone trees that comprised sequences of only $\geq$ 1000 nucleotides were first calculated. Validity of branching patterns of the trees was checked by applying three phylogenetic reconstruction methods – neighbour-joining, maximum parsimony and maximum likelihood – to the appropriate sets of sequences. Partial DGGE sequences were added to the trees afterwards according to maximum parsimony criteria. This tool does not correct for evolutionary distances and does not allow changes in overall tree topology.

Nucleotide accession number

The sequences generated in this study have been deposited in the Gen Bank database under accession numbers GU224058 to GU224089.

Results

Physico-chemical parameters of the lake

Results of the physico-chemical analysis of the Lake Averno water column are reported in Table 2. The vertical profiles of T, pH and dissolved oxygen (DO) indicate that this lake, in May 2006, was made up of two distinct water masses, the epilimnion (1–9 m) and the hypolimnion (9–33 m). The DO concentration decreased rapidly from 3 to 9 m depth. However, complete hypolimnetic anoxia was not reached at the bottom of the lake. The interface between the oxic and anoxic layer was characterized by strong variations in Eh, T and pH. The nitrate concentration was *c.* 1 μM in the entire water column with the exception of a maximum value at 5 m, where the vertical concentration of DO decreased, and at 9 m, where the vertical concentration of Eh slightly increased. Nitrite concentration was detectable only at a depth of 9 and 33 m. Ammonia concentration increased towards the bottom with the highest value being 643 μM at 33 m, and it displayed a concave up distribution between 15 and 5 m. Inorganic phosphorus and ammonia showed the highest value at 33 m and a step gradient between 9 and 15 m. Methane concentration showed a progressive increase from the upper part of the water column

Table 2. Physico-chemical properties along Lake Averno water column

	Samples									
	1 m	3 m	5 m	6 m	9 m	15 m	21 m	27 m	32 m	33 m
T (°C)	26.0	22.5	14.6	11.9	9.8	9.0	8.8	8.7	8.8	9.1
pH	9.19	9.15	8.01	7.70	7.73	7.59	7.56	7.55	7.54	7.00
Eh (mV)	117.2	128.9	178.9	−184.9	−165.8	−298.0	−300.0	−300.00	−301.8	−305.2
DO (mM)	352.8	531.2	98.3	14.24	10.05	7.89	7.11	6.65	5.94	4.69
E.cond.(mS cm^{-1})	2.84	2.85	2.96	3.00	3.02	3.08	3.08	3.08	3.08	3.48
NO_2^- (μM)	0.0	0.0	0.0	0.0	3.0 ± 0.03	0.0	0.0	0.0	0.5 ± 0.02	1.5 ± 0.05
NO_3^- (μM)	1.0 ± 0.02	0.8 ± 0.01	1.5 ± 0.01	0.8 ± 0.01	3.8 ± 0.03	1.1 ± 0.02	0.8 ± 0.02	0.9 ± 0.03	0.8 ± 0.02	1.2 ± 0.03
$NO_2^- + NO_3^-$	1.0	0.8	1.5	0.8	6.8	1.1	0.8	0.9	1.3	2.7
NH_4^+ (μM)	3.2 ± 0.01	3.1 ± 0.11	6.3 ± 0.22	16.5 ± 0.27	77.0 ± 1.01	171.0 ± 16.5	160.6 ± 8.18	179.0 ± 2.39	328.4 ± 12.8	643.1 ± 15.1
Nox/Nrid	0.31	0.26	0.23	0.05	0.09	0.01	0.00	0.01	0.00	0.00
PO_4^{3-} (μM)	0.3 ± 0.01	0.3 ± 0.01	0.4 ± 0.02	0.4 ± 0.01	0.4 ± 0.03	3.3 ± 0.01	3.4 ± 0.01	3.4 ± 0.01	9.2 ± 0.01	25.7 ± 0.23
H_2S(μM)	11.3	92.8	59.9	5.75	5.81	76.9	65.5	79.8	347.8	1507.7
SO_4^{2-} (mM)	1.8	1.7	1.8	1.8	1.8	1.8	1.8	1.9	1.4	0.3
HCO_3^- (mM)	4.3	4.3	5.4	6.1	6.3	6.4	6.5	6.5	7.2	11.2
CH_4 (μM)	0.37	0.54	0.9	1.46	2.08	17.4	22.6	31.3	304.2	2958.8
pCO_2 (mb)	0.22	0.21	1.72	4.61	8.18	14.6	6.29	6.60	10.5	45.7

to the bottom. Sulphide concentration increased with depth as well reaching a maximum value of 1507.7 μM at 33 m, whereas sulphate concentrations showed constant values with the exception of a slight decrease at 3 m and a sharp decrease at 32 and 33 m. HCO_3^- also showed a slight increasing concentration, from 1 to 6 m, in the epilimnion, a constant concentration from 6 to 22 m, and a sharp increase at 33 m. pCO_2 values varied between 0.22 and 4.61 mbar in the epilimnion and between 8.18 and 10.5 mbar, from 9 to 32 m, with the exception of a value of 14.6 mbar measured at the depth of 15 m. The maximum pCO_2 value (45.69 mbar) was observed at 33 m.

DGGE fingerprints and statistical analysis

DGGE was carried out on nested bacterial and archaeal PCR fragments as the nested PCR-based approach gave a higher resolution (more bands) than the PCR-based approach (data not shown). The bacterial and archaeal communities recovered at various depths showed different DGGE profiles based on the number and distance of migration of the PCR products (Fig. 1a and b). In the DGGE profiles, the distinct bands correspond to 16S rRNA gene fragments that differ in nucleotide sequence

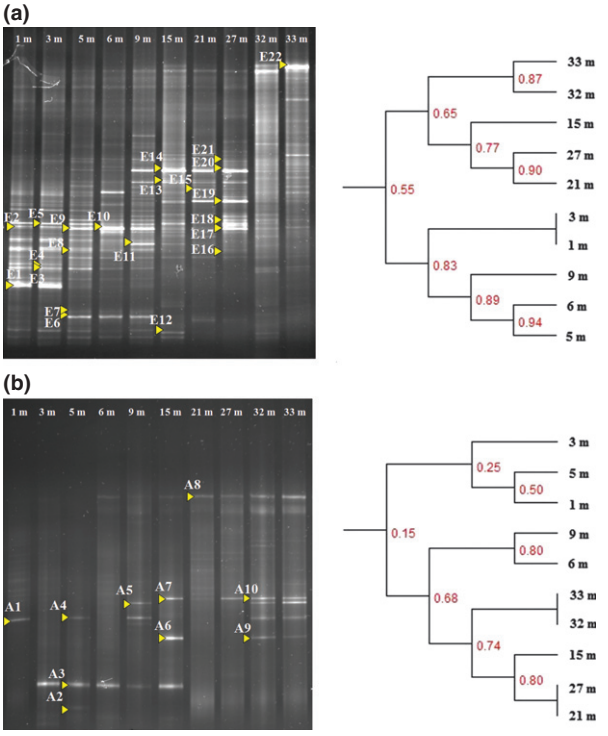

Fig. 1. DGGE fingerprints of 16S rRNA gene fragments and cluster analysis by UPGMA of bacterial (a) and archaeal (b) communities from different depths of Lake Averno water column. Arrows indicate sequenced bands.

and reflect distinct numerically dominant operational taxonomic units (OTU) in the community.

For both domains, the microbial communities were divided into two main groups as a function of depth: bacterial patterns of the upper samples (1–9 m) clustered together and were separated from those of the zone from 15 to 33 m, archaeal profiles of the anoxic zone (9–33 m) were grouped into a cluster distinct from that comprising samples from 1, 3 and 5 m depth (Fig. 1a and b). The averages of similarity coefficients among bacterial and archaeal communities were 0.66 ± 0.16 and 0.45 ± 0.33, respectively.

The richness index (R) from all the DGGE patterns was calculated (Table 3). R values ranged from 29 to 39 in the bacterial communities, while lower values, from 1 to 7, were observed in the archaeal communities. Archaeal richness was significantly associated with most environmental parameters in univariate analysis (Table 4). For example, depth-related decrease in oxygen was accompanied by a significant increase in the number of archaeal bands. No significant association between bacterial richness and environmental parameters was found (Table 4).

Cluster analysis was also performed to group bacterial and archaeal DGGE bands according to measured environmental variables. All bands were grouped into six clusters independently from the domain they belonged (Supporting Information, Table S1). The results of the cluster analysis were visualized in the NMDS ordination on the two-dimensional plot in which representative bands belonging to each group (Fig. 2a) were reported. NMDS ordination fits data very well ($R^2 = 0.99$), so that biplots are a reliable visual representation of the data. The relationship of clusters with environmental variables was investigated by superimposing the environmental variables on the two-dimensional plot generated by NMDS ordination and reporting centroids. As shown in Fig. 2b, clusters 1, 3 and 4 are markedly separated from the other ones. Cluster 1 comprises bands mainly located in the superficial layers of the lake that are characterized, on average, by high values of DO, T, Nox/Nrid and low values of PO_4^{3-}, NH_4^+, HCO^-_3 pCO_2. Cluster 3 is separated from the other clusters due to the lowest variability of mean values of most environmental parameters. Its bands are found only in deep layers of the lake (21 and 27 m) characterized by highest SO_4^{2-} concentration and lowest values of Eh, T, DO, NO_3^-, NO_2^-, as well as H_2S and CH_4 (Table 5). Bands of cluster 4, also mainly present in the deep layers of the lake (32 and 33 m), are more dispersed in the plot than those of cluster 3 and are associated on average to highest values of almost all the analysed parameters, with the exception of temperature, Nox/Nrid and Eh, and to lowest values of DO and SO_4^{2-}. Clusters 2, 5 and 6, including bands present in the different sampling sites, are intermediate to clusters 1, 3 and 4; moreover, bands of cluster 5 are associated with higher DO values than those observed for bands of clusters 2 and 6. The latter displayed a slightly lower variability of some parameters, such as pH, E cond, NH_4^+, SO_4^{2-} and HCO_3^-. Despite the large standard deviations of some parameters (Table 5), we still assert a separation among clusters due to the substantial fold changes among means.

Table 3. Richness indexes calculated from bacterial and archaeal DGGE fingerprints obtained at different depths

	Richness indexes (R)	
Depth	*Bacteria*	*Archaea*
1 m	29	1
3 m	29	1
5 m	36	3
6 m	34	4
9 m	39	6
15 m	31	6
21 m	29	3
27 m	29	4
32 m	35	7
33 m	34	7

Table 4. Univariate poisson regressions on bacterial and archaeal richness indices

	Bacterial richness		Archaeal richness	
Parameter	Parameter	*P*-value	Log-odds	*P*-value
Depth	0.0005	0.9438	0.0614	0.0010
Temperature	−0.0142	0.2973	−0.1616	0.0001
pH	−0.1357	0.2618	−1.4532	0.0001
DO	−0.0006	0.2087	−0.0054	0.0002
Eh	0.0000	0.9136	−0.0042	0.0004
E cond	0.3121	0.5228	4.9956	0.0005
N-NO_3^-	0.1927	0.0495	0.2831	0.2311
N-NO_2^-	0.1758	0.0483	0.5308	0.0208
$NO_2^- + NO_3^-$	0.0983	0.0424	0.2204	0.0678
Nox/Nrid	−0.2876	0.6828	−7.3152	0.0002
NH_4^+	0.0002	0.7095	0.0040	0.0011
PO_4^{3-}	0.0042	0.6994	0.0826	0.0070
H_2S	0.0001	0.6535	0.0011	0.0261
SO_4^{2-}	−0.1061	0.5585	−1.0650	0.0272
CH_4	0.0000	0.6414	0.0005	0.0379
HCO_3^-	0.0374	0.4063	0.4817	0.0004
pCO_2	0.0039	0.5515	0.0580	0.0023

P-values are adjusted for multiplicity according to Benjamini & Hochberg correction.

Phylogenetic analysis

Twenty-two (from E1 to E22, Fig. 1a) and 10 (from A1 to A10, Fig. 1b) of 44 bacterial and 22 archaeal excised bands, respectively, produced useful and reliable

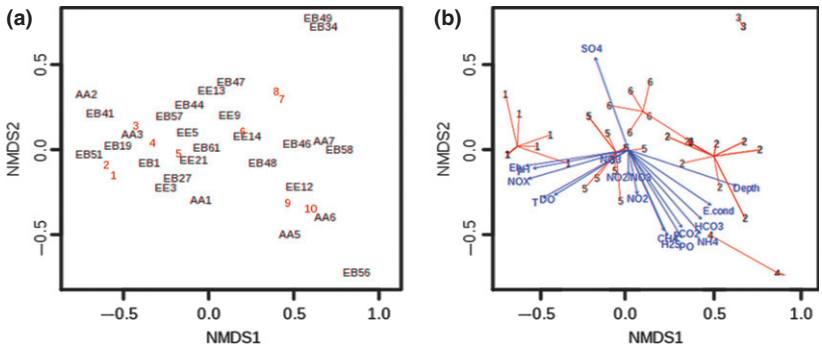

Fig. 2. NMDS ordination (a) and cluster analysis (b) of DGGE bands and environmental parameters. Proximity is nonmetric. In (a), numbers from 1 to 10 correspond to increasing depth levels. EE and AA stand for bacterial and archaeal sequenced bands, respectively. In (b), numbers represent bands belonging to a defined cluster. When two DGGE bands are overlapping, the one most frequently occurring at different lake depths is shown. In (b), the arrows represent discriminant power and direction of covariates: the longer the arrow, the stronger the discriminant power; two bands at opposite sides of an arrow are discriminated by the corresponding covariate.

Table 5. Centroid and standard deviation for each variable and each cluster obtained by NMDS

	Clusters					
Parameters	1	2	3	4	5	6
Depth	10.14 ± 10.65	18.50 ± 11.41	24.00 ± 4.24	24.67 ± 13.58	15.20 ± 12.25	16.43 ± 10.58
Temperature	14.66 ± 6.92	10.09 ± 2.11	8.75 ± 0.07	9.23 ± 0.51	12.92 ± 6.31	10.23 ± 2.24
pH	8.13 ± 0.73	7.58 ± 0.28	7.55 ± 0.01	7.42 ± 0.38	7.90 ± 0.71	7.67 ± 0.17
DO	145.77 ± 211.33	19.36 ± 32.03	6.88 ± 0.33	6.89 ± 2.8	103.89 ± 185.26	21.45 ± 34.00
Eh	−75.07 ± 209.95	−209.6 ± 167.02	−300.00 ± 0.00	−257.00 ± 79.52	−143.07 ± 203.41	−195.94 ± 175.51
E cond	2.98 ± 0.10	3.10 ± 0.16	3.08 ± 0.00	3.19 ± 0.25	3.05 ± 0.18	3.04 ± 0.05
$N\text{-}NO_3^-$	1.40 ± 1.09	1.36 ± 1.02	0.85 ± 0.07	1.93 ± 1.63	1.27 ± 0.92	1.39 ± 1.09
$N\text{-}NO_2^-$	0.50 ± 1.12	0.62 ± 1.09	0.00 ± 0.00	1.67 ± 1.26	0.50 ± 1.00	0.50 ± 1.12
$NO_2^- + NO_3^-$	1.9 ± 2.18	1.99 ± 2.04	0.85 ± 0.07	3.60 ± 2.86	1.77 ± 1.86	1.89 ± 2.18
Nox/Nrid	0.14 ± 0.13	0.05 ± 0.08	0.00 ± 0.01	0.03 ± 0.05	0.10 ± 0.12	0.06 ± 0.08
NH_4^+	86.5 ± 123.13	197.74 ± 207.69	169.8 ± 13.01	349.5 ± 283.64	158.82 ± 200.70	134.11 ± 11.99
PO_4^{3-}	2.04 ± 3.34	5.77 ± 8.56	3.40 ± 0.00	11.77 ± 12.84	4.68 ± 7.89	2.93 ± 3.14
H_2S	85.75 ± 120.97	268.64 ± 512.28	72.65 ± 10.11	620.44 ± 787.19	225.33 ± 461.32	91.64 ± 117.20
SO_4^{2-}	1.73 ± 0.15	1.57 ± 0.54	1.85 ± 0.07	1.17 ± 0.78	1.61 ± 0.48	1.76 ± 0.16
CH_4	46.71 ± 113.71	417.28 ± 1032.05	26.68 ± 6.53	1088.36 ± 1626.88	333.91 ± 926.99	54.20 ± 110.87
HCO_3^-	5.71 ± 1.10	6.95 ± 1.79	6.50 ± 0.00	8.23 ± 2.61	6.42 ± 1.93	6.34 ± 0.54
pCO_2	5.72 ± 5.56	12.28 ± 14.04	6.45 ± 0.22	21.46 ± 21.02	9.86 ± 13.39	7.50 ± 4.16

sequences without ambiguous positions, showing close similarity (sequence identity ≥ 95%) to 16S rDNA gene sequences present in the NCBI databases, in most cases belonging to uncultured relatives (Table 6). These sequences, corresponding for each lane to an average of 40% and 85% of the total intensity of bacterial and archaeal bands, respectively, have been included in the phylogenetic trees (Fig. 3a and b).

Bacterial 16S rRNA gene sequences were affiliated to *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, *Bacteroidetes* and *Firmicutes* phylogenetic groups. Most sequences could be successfully allocated only to family level because of low genus bootstrap confidence levels (< 80%). A great number of sequences were allocated near reference sequences from diverse origin obtained by BLAST search rather than near sequences of typical freshwater lineages/tribes/clades within *Alphaproteobacteria*, *Betaproteobacteria* and *Bacteroidetes* phyla (Table 6 and Fig. 3a).

An uneven distribution of sequences related to *Alphaproteobacteria* (bands E1, E3, E4, E8, E9, E10, E16, E17, E18 and E19) and *Betaproteobacteria* (bands E2, E5, E11, E13, E14, E15, E20 and E21) throughout the water column was observed. Sequences related to *Deltaproteobacteria* (bands E6 and E7) were present in the upper part of the Lake (from 1 to 9 m), while sequences affiliated to the *Firmicutes* and *Bacteroidetes* groups (bands E12 and E22) were found only in the deepest part of the water

Table 6. Similarity of the 16S rRNA gene sequences from bacterial and archaeal DGGE bands to sequences retrieved from database and their phylogenetic affiliation

Bands	Closest relative*	Accession no.	Identity (%)	Band phylogenetic affiliations (%)†		
				Order	Family	Genus
E1	*Roseomonas fridaquae* strain CW67	EU290160	98	*Rhodospirillales* (100)	*Acetobacteraceae* (99)	*Roseomonas* (58)
E2	Uncultured bacterium clone S23_830	EF572731	97	*Burkholderiales* (100)	*Incertae sedis* (100)	*Ideonella* (70)
E3	Unidentified bacterium clone K2-S-3	AY344379	100	*Rhodobacterales* (100)	*Rhodobacteraceae* (100)	*Rhodobacter* (49)
E4	Unidentified bacterium clone K2-S-3	AY344379	100	*Rhodobacterales* 100)	*Rhodobacteraceae* (100)	*Rhodobacter* (74)
E5	Uncultured bacterium clone S23_830	EF572731	97	*Burkholderiales* (100)	*Incertae sedis* (100)	*Ideonella* (67)
E6	Uncultured bacterium clone T106D	AM158388	99	*Myxococcales* (78)	*Nannocystaceae* 27)	*Plesiocystis* (21)
E7	Uncultured bacterium clone T106D	AM158388	99	*Myxococcales* (87)	*Polyangiaceae* (44)	*Sorangium* (23)
E8	Uncultured bacterium clone FRC-FBR-69d-10.31-96	DQ646440	99	*Rhodobacterales* (100)	*Rhodobacteraceae* (99)	*Paracoccus* (78)
E9	Uncultured bacterium clone 3C003151	EU801779	100	*Rhodobacterales* (100)	*Rhodobacteraceae* (100)	*Rhodobacter* (90)
E10	Uncultured bacterium clone 3C003151	EU801779	100	*Rhodobacterales* (100)	*Rhodobacteraceae* (100)	*Rhodobacter* (95)
E11	Uncultured bacterium clone X-132	AM905635	99	*Burkholderiales* (100)	*Comamonadaceae* (100)	*Malikia* (69)
E12	Uncultured bacterium clone E07	EF590062	98	*Clostridiales* (96)	*Ruminococcaceae* (96)	*Ruminococcaceae Incertae Sedis* (41)
E13	Uncultured bacterium clone RB7C2	AF407381	99	*Burkholderiales* (100)	*Comamonadaceae* (100)	*Rhodoferax* (100)
E14	Uncultured bacterium clone RB7C2	AF407381	99	*Burkholderiales* (100)	*Comamonadaceae* (100)	*Rhodoferax* (100)
E15	Uncultured bacterium clone d123	AF422672	100	*Burkholderiales* (100)	*Comamonadaceae* (100)	*Simplicispira* (96)
E16	Uncultured *Rhodobacteraceae* TDNP_Bbc97_72_2_135	FJ516819	99	*Rhodobacterales* (100)	*Rhodobacteraceae* 100)	*Rhodobacter* (69)
E17	Uncultured bacterium clone 3C003151	EU801779	99	*Rhodobacterales* (100)	*Rhodobacteraceae* (100)	*Rhodobacter* (89)
E18	Uncultured bacterium clone 3C003151	EU801779	98	*Rhodobacterales* (100)	*Rhodobacteraceae* (100)	*Rhodobacter* (96)
E19	Uncultured bacterium clone TH_a72	EU272948	99	*Sphingomonadales* (100)	*Sphingomonadaceae* (100)	*Novosphingobium* (92)
E20	*Rhodoferax antarcticus* strain Fryx1	AY609198	98	*Burkholderiales* (100)	*Comamonadaceae* (100)	*Rhodoferax* (100)
E21	Uncultured bacterium clone LE201B11	FJ694328	99	*Burkholderiales* (100)	*Comamonadaceae* (100)	*Rhodoferax* (100)
E22	Uncultured bacterium D242_27F_BAC2_013	AB447719	97	*Bacteroidales* (100)	*Porphyromonadaceae* (100)	*Paludibacter* (100)
Archaea						
A1	Uncultured archaeon clone F5	EU910621	99	*Methanomicrobiales* (100)	*Methanomicrobiaceae* (89)	*Methanoculleus* (48)
A2	Uncultured archaeon clone L7A6	EF644786	100	*Methanosarcinales* (94)	*Methanosaetaceae* (54)	Methanothrix (54)
A3	Uncultured archaeon clone L7A4	EF644784	100	*Methanosarcinales* (99)	*Methanosarcinaceae* (66)	*Methanolobus* (26)
A4	Uncultured euryarchaeote clone F5	EU910621	99	*Methanomicrobiales* (100)	*Methanomicrobiaceae* (92)	*Methanoculleus* (47)

Table 6. Continued

Bands	Closest relative*	Accession no.	Identity (%)	Band phylogenetic affiliations (%)†		
				Order	Family	Genus
A5	Uncultured euryarchaeote clone F18	EU910620	99	*Methanomicrobiales* (100)	*Methanomicrobiaceae* (99)	*Methanoculleus* (80)
A6	*Methanomethylovorans* sp. Z1	EF174501	100	*Methanosarcinales* (100)	*Methanosarcinaceae* (100)	*Methanomethylovorans* (100)
A7	Uncultured *Methanospirillaceae* ME-17	AB288683	96	*Methanomicrobiales* (100)	*Methanomicrobiaceae* (81)	*Methanoculleus* (34)
A8	Uncultured bacterium clone mbII-a9	AB426163	100	*Methanosarcinales* (100)	*Methanosaetaceae (*100)	*Methanothrix* (100)
A9	*Methanomethylovorans* sp. Z1	EF174501	99	*Methanosarcinales* (100)	*Methanosarcinaceae* (100)	*Methanomethylovorans* (100)
A10	Uncultured euryarchaeote clone F5	EU910621	96	*Methanomicrobiales* (100)	*Methanomicrobiaceae* (77)	*Methanofollis* (31)

*Sequence similarities between rRNA sequences of DGGE bands and those of the closest relatives retrieved from NCBI database.
†Identification performed using the RDP Classification Algorithm. Bootstrap confidence values are given in brackets (classification is well supported for confidence > 80%).

column at a depth of 32 and 33 m. All the archaeal DGGE bands analysed were affiliated to sequences belonging to the phylum *Euryarchaeota*. Phylogenetic analysis divided the sequences into two groups of methanogens, which were affiliated to the *Methanomicrobiales* (bands A1, A4, A5, A7, A10) and *Methanosarcinales* (bands A2, A3, A6, A8, A9) orders (Fig. 3b). Most of the sequences belonging to the two orders could not be allocated to genus level because of the low bootstrap confidence level < 80% (Table 6). Vertical changes in archaeal communities were observed as bands A6, A7 and A8 (affiliated with *Methanomethylovorans* genus, *Methanomicrobiaceae* family and *Methanotrix* genus, respectively) were found only in the anaerobic layers, while bands A1, belonging to the *Methanomicrobiaceae* family and bands A2 and A3 (showing close similarity to the uncultured relatives not identified at genus level) disappeared in the deepest part of the water column.

Discussion

A study to assess how bacterioplankton composition of Lake Averno varies along the gradient of environmental factors was carried out by means of DGGE analysis of *Bacteria* and *Archaea* communities and multivariate analysis applied to molecular and physico-chemical data to correlate the presence of DGGE bands with specific environmental parameters.

Despite the rapid development of high-throughput molecular technologies in recent years, DGGE continues to be a popular tool to track differences in microbial community composition over time and space as it allows to process multiple samples more rapidly and economically than sequencing.

Cluster analysis of DGGE fingerprints of *Bacteria* and *Archaea* showed that microbial composition shifts with depth. These results are in agreement with those of previous studies carried out in other stratified lakes showing vertical heterogeneity of the microbial assemblages (Konopka *et al.*, 1999; Casamayor *et al.*, 2000; Dominik & Höfle, 2002; Koizumi *et al.*, 2004). However, high similarity values of some bacterial DGGE profiles indicate that certain populations may not be restricted to either aerobic or anaerobic locations. On the contrary, the low similarity values between archaeal communities of the oxic and anoxic zone indicate that different archaeal populations occur at different depths.

The lack of a significant correlation between the number of bacterial OTUs and the physico-chemical parameters indicates that vertical changes of environmental variables do not influence the bacterial richness of Lake Averno. On the contrary, the significant correlation between environmental variables and the number of archaeal OTUs suggests that some defined parameters could influence the richness of the archaeal community that increases with depth. Variation in the number of DGGE bands of prokaryotes along the water column of lakes was observed in previous studies. Øvreås *et al.* (1997) observed a higher number of individual bands in bacterial and archaeal DGGE profiles of superficial oxic layer samples compared with those of anoxic deep layers of a stratified lake, whereas De Wever *et al.* (2005) observed that the number of bands generally increased from lake surface to deep water in Lake Tanganyika, a meromictic lake characterized by permanent vertical temperature stratification, suggesting that it could be

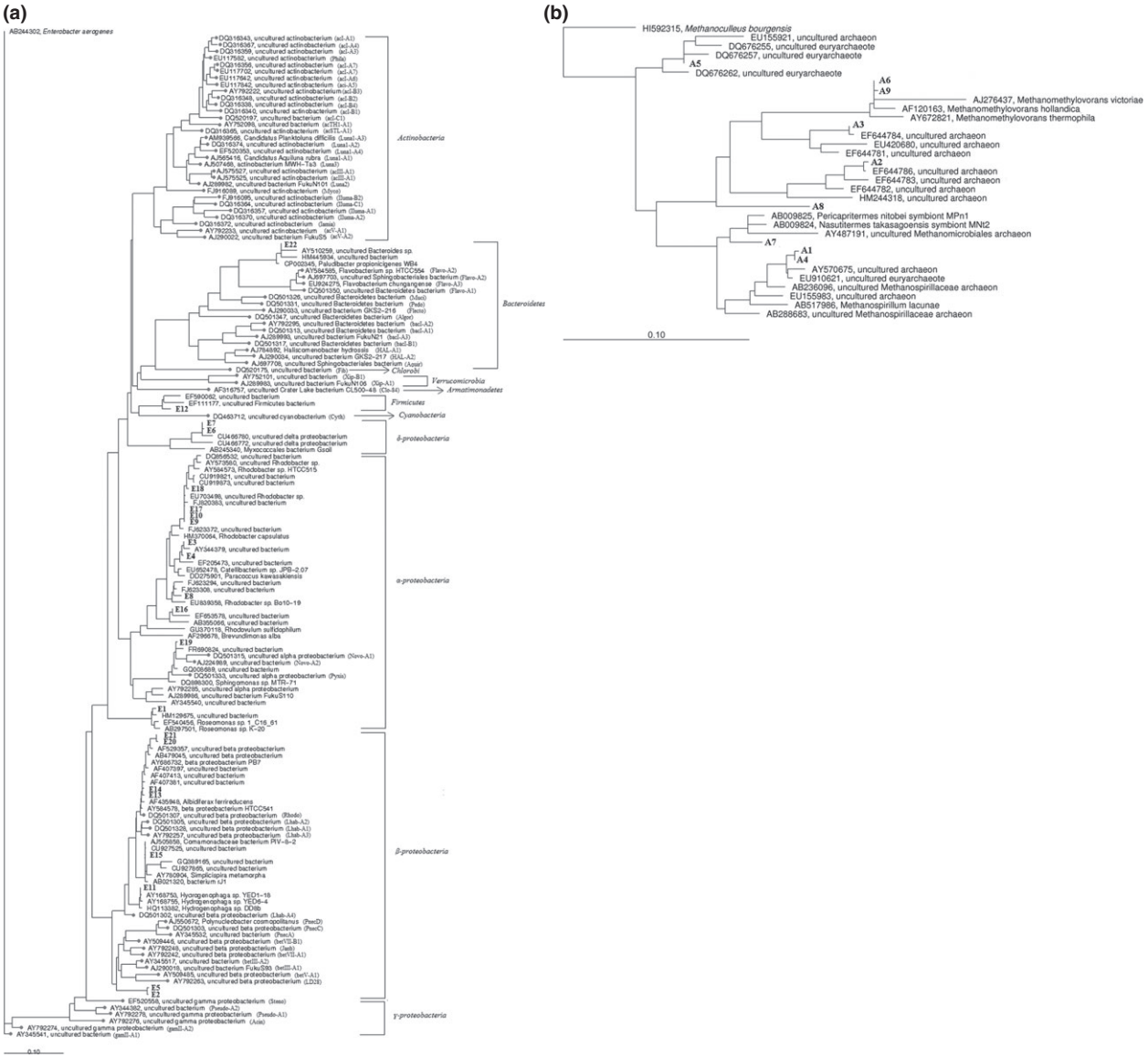

Fig. 3. Phylogenetic affiliation of bacterial (a) and archaeal (b) DGGE bands obtained from Lake Averno samples. The phylogenetic tree was generated using maximum likelihood method with the ARB program. The bar indicates a 10% estimated sequence divergences. The sequences from Lake Averno are indicated in boldface type, while reference sequences that belong to an established freshwater lineage/clade/tribe as defined by Newton *et al.* (2011) are evidenced by a grey circle. Accession numbers of Lake Averno sequences are given in Table 6. The sequences of *Enterobacter aerogenes* and *Methanoculleus bourgensis* were used as outgroup in (a) and (b), respectively.

related to differences of oxygen and/or nutrients along the water column.

NMDS analysis revealed that the presence of some bacterial and archaeal bands was significantly correlated with some environmental variables such as DO, T, Nox/Nrid, SO_4^{2-}, Eh (clusters 1, 3 and 4) and pH and Econd (clusters 2, 5, 6; see Results section). Lindström *et al.* (2005) reported pH and T as environmental factors that significantly affect the appearance of individual bacterial groups in lakes. It is worth noting that bands belonging to cluster 3 present in the deep layers of the lake are correlated with high values of SO_4^{2-} and low values of H_2S, suggesting that this cluster is not characterized by sulphate-reducing bacteria. Cluster 4, where all but one bands are present in the deepest layers of the lake, showing high NO_3^- and NO_2^- values despite high H_2S and CH_4 concentrations, is not characterized by denitrifying bacteria. Unfortunately, we could not verify these hypotheses because it was not possible to sequence DGGE bands belonging to clusters 3 and 4 except one included in cluster 4.

Proteobacteria and *Bacteroidetes* have been reported as the most abundant bacterial groups in eutrophic freshwater ecosystems (Jasper *et al.*, 2001; Trusova & Gladyshev, 2002) where they are supposed to play an important role in global biogeochemical cycling. In our study, most of the sequenced bacterial DGGE bands retrieved from the various sampling depths were affiliated to the *Alphaproteobacteria* and *Betaproteobacteria* and grouped in several different phylogenetic clusters, thus, confirming the ecological success and the extreme metabolic diversity of these phyla in freshwater ecosystems (Newton *et al.*, 2011). A recent study performed in a deep meromictic lake located in High Arctic Canada shows that *Proteobacteria* represent about one-third of the sequences at most depths (Comeau *et al.*, 2012).

It is noteworthy that in our study, most of these sequences are not affiliated to typical freshwater taxa described by Newton *et al.* (2011) suggesting that the bacterioplankton in Lake Averno is quite unusual for a freshwater lake. Moreover, it could also be speculated that most sequences affiliated to *Alphaproteobacteria* and *Betaproteobacteria* are included in the groups of bands correlated with Econd according to NMDS results. Wu *et al.* (2006) reported a higher relative abundance of members of *Alphaproteobacteria* in high mountain lakes with increasing salinity concentration. By contrast, the relative abundance of *Betaproteobacteria* decreased with increasing salinity.

In contrast to Jasper *et al.* (2001) and Kondo *et al.* (2009), who reported that members of the phylum *Bacteroidetes* were the dominant bacterioplankton populations in eutrophic and meromictic lakes, we found only one sequence among those analysed, recovered from a deep location, belonging to this group. This sequence was related to the novel genus *Paludibacter*, a strictly anaerobic, chemo-organotrophic bacterium that uses various sugars and produces useful bioactive compounds, such as acetate and propionate (Ueki *et al.*, 2006). We cannot exclude, however, the presence of other *Bacteroidetes* species, in this lake, below the DGGE threshold limit (0.5–1% of the total targeted gene pool, Casamayor *et al.*, 2000), because several faint bands could not have been recovered from the gels and sequenced. We did not detect members of *Actinobacteria*, which are common in freshwater ecosystems (Kolmonen *et al.*, 2004; Hahn, 2006; Martínez-Alonso *et al.*, 2008; Newton *et al.*, 2011). This absence could be explained by the fact that Lake Averno is mainly anoxic and often the abundance of this bacterial group decreases with decreasing oxygen concentration (Newton *et al.*, 2011). We retrieved members of the *Firmicutes* and *Deltaproteobacteria* groups, which have infrequently been recovered in freshwater samples (De Wever *et al.*, 2005; Newton *et al.*, 2011). Sequences belonging to *Deltaproteobacteria* subphylum were recovered from the upper part of Lake Averno, and, according to NMDS results, they were included in the group of bands positively correlated with DO and Eh. The low bootstrap confidence values obtained for their classification at the family level, by the RDP Classification Algorithm, suggested that they could belong to previously uncharacterized taxonomic units. Although most of the *Deltaproteobacteria* present in lakes are expected to be sulphate reducers restricted to the anoxic layer (Karr *et al.*, 2005; Lehours *et al.*, 2005), we were unable to identify this functional microbial group among sequences belonging to this subphylum.

The archaeal sequenced bands retrieved from the various sampling depths were related to methanogen sequences belonging to the orders of *Methanosarcinales* and the *Methanomicrobiales*. This finding is consistent with the results of earlier studies on lake water samples (Øvreås *et al.*, 1997; Lehours *et al.*, 2005, 2007). In the present study, methanogen-related sequences were found not only in the anaerobic methane-rich waters but also in the aerobic layer as observed in previous studies (Øvreås *et al.*, 1997; Schulz *et al.*, 2001; Grossart *et al.*, 2011). Grossart *et al.* (2011) detected potentially methanogenic *Archaea* in the oxygenated, methane-rich epilimnion of a dimictic oligotrophic lake, suggesting that their attachment to photoautotrophs might allow for anaerobic growth and direct transfer of substrates for methane production. Moreover, although the finding of these microorganisms in the oxic layer could be explained also by the presence of transient anoxic microzones (De Long, 1992) or endosymbiotic niches (Vogels *et al.*, 1980), the low bootstrap values for the phylogenetic placement, at genus level of these sequences and the fact that they exhibit a strong similarity only with sequences of uncultured organisms of unknown physiology and metabolism, support the hypothesis that they could represent unknown mesophilic aerobic members of *Archaea*. In contrast with the considerable contribution of *Archaea* to the methanogenesis process occurring through decomposition of organic matter, neither sequencing of DGGE bands nor taxonomic units-environment correlations, revealed the presence of *Archaea* involved in the reduction of sulphate.

In summary, the results of the present study offer the first description of the bacterial and archaeal community in the Lake Averno. Sequencing of the predominant bands, in the DGGE gels, revealed the presence of bacterial groups commonly found in freshwater ecosystems, but also the presence of members not included in typical freshwater clusters. Moreover, our results suggest the presence of archaeal and bacterial populations not previously characterized. Furthermore, the use of NMDS demonstrated the relationships between BCC and environmental factors providing information on the influence of some environmental variables on the presence of indi-

vidual bacterial groups. In future, high-throughput next-generation sequencing technologies could shed further light on the whole bacterial and archaeal community of Lake Averno as well as on the presence of individual organisms at specific sampling depths.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. NMDS clustering, localization and affiliation of DGGE bands.