Archaeal and bacterial community composition of sediment and plankton from a suboxic freshwater pond

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Abstract

We studied the composition of archaeal and bacterial communities present in the sediment and plankton of a shallow suboxic-to-anoxic freshwater pond with high organic matter input, as an example of a kind of inland freshwater system widely distributed in forests of temperate regions. Molecular surveys based on small subunit rRNA genes showed a remarkably high diversity of lineages within the Bacteria, with a total of 18 phyla or candidate divisions being detected, in addition to a few highly divergent phylotypes of unknown affiliation. We identified members of the five subdivisions of the Proteobacteria, as well as Acidobacteria, Verrucomicrobia, Planctomycetes, Bacteroidetes, Chlorobi, Actinobacteria, Firmicutes, Chloroflexi, Gemmatimonadetes, Spirochaetes, Fibrobacteres and the candidate divisions OD1, OP11, TM6, WS1, WS6 and Termite Group 1 (“Endobacteria”). Candidate division OD1 and beta-Proteobacteria were dominant in the environmental libraries of plankton and sediment, respectively. Archaea were also very diverse, but only members of the Euryarchaeota, including Methanosarcinales, Methanomicrobiales and some divergent lineages, were identified. The application of various species richness estimators confirmed the highly diverse nature of both plankton and sediment samples. The pond is a microbial-based complex ecosystem mainly fueled by the degradation of allochthonous organic matter that maintains tightly coupled carbon and sulfur cycles.

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1. Introduction

Inland freshwater ecosystems, including rivers and streams, lakes and ponds, wetlands and groundwater, are eminently microbial-based, relying on the activity of archaea, bacteria and protists for functioning. Many of these microorganisms are thought to be key components in the biogeochemical cycling of elements such as carbon and nitrogen and, therefore, to play a significant role in the biosphere. Furthermore, they intervene in the decontamination of wastewaters released into the environment from urban, industrial, and agricultural activities, thus contributing to maintaining water quality. In recent years, the use of cultivation-independent surveys based on PCR amplification of small subunit rRNA genes (SSU rDNAs) has considerably increased the information available for prokaryotic lake community compositions, leading, for instance, to the identification of several Actinobacteria and beta-Proteobacteria phylotypes that appear to be characteristic and abundant in freshwaters [18,49,51]. However, knowledge of the composition of microbial communities in these systems is still very fragmentary, given the heterogeneity of freshwater habitats in space and time. Various ecological factors, both physicochemical (size, water chemistry and retention time, temperature, irradiation) and biological (organic matter supply, primary producers, predation, viral dynamics) influence to various extents the composition of microbial communities [17,32].

Community composition in anoxic freshwater systems has been explored mostly in two types of reservoirs, the anoxic layers of meromictic lakes and geothermal hot or cold springs. Some diversity surveys have been carried out along the water column or in the chemocline of meromictic lakes (e.g. [5,23]).
Others have focused on particular metabolic types that appear particularly important in these anoxic systems, such as sulfate-reducing bacteria (SRB) and methanogenic archaea, (e.g. [26,27,30]). In oxygen-depleted environments bred by groundwater enriched in reduced gases, such as hot or cold sulfur springs, members of the Cyanobacteria, Chloroflexi and purple sulfur bacteria usually dominate the phototrophic component of microbial communities (e.g. [12,22]). However, other oxygen-depleted systems, such as small ponds that are suboxic or anoxic as a consequence of biological activity, remain largely understudied. These systems, permanent or not, may be very abundant in forests of temperate regions, thus accounting for a non-negligible fraction of freshwater reservoirs in these areas. The accumulation of large amounts of organic matter from leaves and other vegetal material in small confined basins where water circulation is very limited leads to rapid consumption of oxygen and nearly permanent anoxic conditions, particularly in the deepest layers. These ponds often display a vegetal cover of duckweed (Lemna minor), which further limits the diffusion of oxygen as well as the penetration of light. The result is oxygen deficit very close to the surface and anoxic conditions in the deepest regions, which favors the development of anaerobic communities that supplement the system with reduced gases such as methane and hydrogen sulfide.

In the present work, we studied the composition of bacterial and archaeal communities of the small planktonic fraction (0.22–5 μm) and the sediment from a suboxic pond of this type located in a wooded region in France. Our results show the presence of a wide spectrum of high-taxonomic-level groups within the Bacteria and the Euryarchaeota. Within the Bacteria alone, we detected members belonging to a total of 18 phyla or candidate divisions, and the existence of some very divergent phylotypes. The types of metabolic activities that can be inferred from sequence data suggest the occurrence of complex carbon and sulfur cycles in this ecosystem.

2. Materials and methods

2.1. Sampling

Samples were collected in January 2005 from a freshwater suboxic pond (CV, for Charca Verde) located within the forested university campus of Orsay (48°42' N, 2°10' E), which is classed as a botanical garden in France. Various physicochemical parameters, including temperature, pH, conductivity and dissolved oxygen were measured with a Multi-350i probe (WTW, Weilheim) (Table 1). Water was collected from the center and from approximately 50–70 cm beneath the surface with the help of a sampling holder to which a sterile 500-ml bottle was attached. Black anoxic sediments were collected close to the border of the pond by carefully inserting a sterile 50-ml Falcon tube into the substrate. Both plankton and sediment samples Smelt strongly of H2S and were immediately taken to the laboratory and processed. The water sample contained abundant particles of organic material, which was filtered away through several layers of sterile gauze. The filtrate was then passed through a 5-μm TMTP Millipore filter, and then through a 0.22-μm GTTP Millipore filter.

2.2. DNA extraction, PCR amplification, cloning and sequencing

Nucleic acids were purified from the GTTP filters (picoplankton and small nanoplanktonic fractions) and from sediment samples (~100 μl volume). Filters were washed extensively with phosphate saline buffer (130 mM NaCl, 10 mM phosphate buffer, pHe 7.4, PBS) to resuspend the retained biomass. Subsequently, lysis proceeded by the addition of 80 μg/ml proteinase K, 1% SDS, 1.4 M NaCl, 0.2% β-mercaptoethanol and 2% CTAB (final concentrations) and incubation of the samples overnight at 55 °C. Lysates were extracted once with hot phenol (65 °C), once with phenol–chloroform–isoamylalcohol, and once with chloroform–isoamyl–alcohol.

Nucleic acids were concentrated by salt and ethanol precipitation. Sediment nucleic acids were extracted using both the above classical protocol and the Epicenter SoilMaster™kit, using the recommendations of the manufacturer. Nucleic acids were resuspended in 10 mM Tris–HCl, pH 8. In order to minimize PCR-derived bias and to potentially increase the diversity recovered, 16S rRNA genes were subsequently amplified by PCR using different combinations of bacterial (B-), archaeal (A-) and prokaryotic-specific primers: B-27F (AGAGTTTGATCCTCAG), A-23FLP (CCGGAATCC CGGCCGCTGCAGAYCTGGTYGATYCTGCC), A-21F (TTCCGTTTGCCTGCCGGA), A-20F (TCCCGTTGATCCT GCC), A-ANMEF (GGCTCAGAACACGTGGA), B-1387R (GGGCCGCTGCAGAYCTGGTYGATYCTGCC), A-21F (TT CCGTTTGCCTGCCGGA), A-20F (TCCCGTTGATCCT GCC), A-ANMEF (GGCTCAGAACACGTGGA), B-1387R (GGGCCGGWTGTCAAGGC) and 1492R (GTTACACCT GTTACGACTT). Polymerase chain reactions (PCR) were performed under the following conditions: 30 cycles (denaturation at 94 °C for 15 s, annealing at 50 °C to 55 °C for 30 s, extension at 72 °C for 2 min) preceded by 2 min denaturation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CV rim (center)</th>
<th>CV surface (center)</th>
<th>Center of CV pond ~50 cm depth*</th>
<th>500 m distant pond</th>
<th>L’Yvette (permanent stream nearby)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>9.8–9.9</td>
<td>9.5</td>
<td>8.8</td>
<td>7.8</td>
<td>8.5</td>
</tr>
<tr>
<td>pH</td>
<td>7.08</td>
<td>7.08</td>
<td>7.07</td>
<td>7.8</td>
<td>8.2</td>
</tr>
<tr>
<td>Oxygen concentration (mg/ml)</td>
<td>0.55</td>
<td>2.28</td>
<td>0.23</td>
<td>9.59</td>
<td>9.28</td>
</tr>
<tr>
<td>Conductivity (μS/cm)</td>
<td>1025</td>
<td>1023</td>
<td>1026</td>
<td>809</td>
<td>660</td>
</tr>
</tbody>
</table>

* Plankton samples were taken exactly at this point.
at 94 °C, and followed by 7 min extension at 72 °C. Dimethyl sulfoxide was added to a final concentration of 3–5% to the PCR reaction mix and, in the case of archaea, semi-nested PCR reactions were also performed. 16S rDNA clone libraries were constructed using the Topo TA cloning system (Invitrogen) following the instructions provided by the manufacturers. A total of 9 16S rDNA libraries were constructed for the archaea (7 plankton, 2 sediment) and 7 for bacteria (5 plankton, 2 sediment). After plating, positive transformants were screened by PCR amplification of inserts using M13R and T7 flanking vector primers. Only high-quality partial sequences were screened by PCR amplification of inserts using M13R and T7 flanking vector primers. Only high-quality partial sequences were sequenced, together with their closest homologues completely sequenced, and the selection of clones for complete sequencing. This allowed the identification of identical or nearly identical sequences and the selection of clones for complete sequencing. The 239 representative clones that were found were full-length-sequenced in order to carry out detailed phylogenetic analyses: 117 and 83 bacterial clones, and 29 and 10 archaeal ones, from plankton and sediment, respectively.

2.3. Biodiversity estimates

Rarefaction curves and different biodiversity indices were estimated from our sequence data using the program DOTUR [40]. Prokaryotic high-quality partial sequences were aligned using ClustalX [46] for plankton and sediment samples independently, and the respective distance matrices were generated in Phylip format after excluding gaps. The resulting matrices were used as input for DOTUR in order to generate rarefaction curves at different sequence identity levels (100%, 97%, 95% and 80%), lineage-through-time plots, and different species richness indicators (Table 2).

2.4. Phylogenetic analyses

Alignment and preliminary distance phylogenetic analysis of the 316 partial 16S rDNA sequences was done using ClustalX. This allowed the identification of identical or nearly identical sequences and the selection of clones for complete sequencing. The 239 representative clones that were completely sequenced, together with their closest homologues in GenBank (http://ncbi.nlm.nih.gov/) detected by BLAST [1], were aligned automatically with an alignment containing ~16,000 16S rDNA sequences using BABA (Philippe, pers. commun.). The multiple alignment was then manually edited using the program ED from the MUST package [38]. Neighbor-joining (NJ) trees were constructed for the different prokaryotic taxa in order to choose representative subsets of sequences for further phylogenetic analyses. Gaps and ambiguously aligned positions were excluded from our analyses using strict criteria, which resulted in alignments of variable length (positions). Nine different subsets of 16S rDNA sequences were selected to have good taxonomic coverage of different regions in phylogenetic trees. These subsets were: Archaea (96 sequences, 1009 positions), overall bacterial phylogeny including candidate divisions (134 sequences, 1018 positions), Gram-positives + Acidobacteria (64 sequences, 1094 positions), Planctomycetes + Verrucomicrobia (46 sequences, 1074 positions), Chlorobi + Bacteroidetes (50 sequences, 876 positions), beta-Proteobacteria (70 sequences, 1272 positions), alpha- + gamma-Proteobacteria (65 sequences, 1154 positions), delta-Proteobacteria (78 sequences, 1000 positions) and epsilon-Proteobacteria (33 sequences, 1252 positions). These datasets were analyzed by maximum likelihood (ML) using TREEFINDER [24] applying a general time reversible model of sequence evolution (GTR), and taking among-site rate variation into account by using an eight-category discrete approximation of a Γ distribution (invariable sites are included in one of the categories). ML bootstrap proportions were inferred using 1000 replicates. Phylogenetic trees were viewed using the program TREEVIEW [37]. The sequences reported in this study were submitted to GenBank with accession numbers DQ676351 to DQ676481 (see also Figs. 3–6).

3. Results and discussion

The CV pond is located in a wooded area, and receives substantial amounts of leaves and other plant debris from the surroundings (Supplementary figure S1). The dissolved oxygen concentration measured with an oxygen probe at the plankton sampling depth (~50 cm below surface) was around ten times lower than that at the surface of the same CV pond, and forty times lower than that of normally oxygenated waters located at close proximity (Table 1). The CV pond is suboxic, and very likely it is truly anoxic in the deepest layers. According to the

Table 2

<table>
<thead>
<tr>
<th></th>
<th>MVP (plankton)</th>
<th>MVS (sediment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of sequences</td>
<td>203</td>
<td>113</td>
</tr>
<tr>
<td>No. of unique sequences</td>
<td>120</td>
<td>100</td>
</tr>
<tr>
<td>Diversity indices</td>
<td></td>
<td></td>
</tr>
<tr>
<td>97% identity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>± CI (5%)</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>95% identity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>± CI (5%)</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>80% identity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>± CI (5%)</td>
<td>0.09</td>
<td>0.24</td>
</tr>
<tr>
<td>Simpson</td>
<td>3.97 ± 0.2</td>
<td>3.9 ± 0.19</td>
</tr>
<tr>
<td>Shannon</td>
<td>3.77 ± 0.15</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>Jackknife</td>
<td>209 ± 45</td>
<td>265 ± 102</td>
</tr>
<tr>
<td>ACE</td>
<td>226 ± 169</td>
<td>160 ± 72</td>
</tr>
<tr>
<td>Chao1</td>
<td>198 ± 81</td>
<td>170 ± 90</td>
</tr>
<tr>
<td></td>
<td>130 ± 46</td>
<td>100 ± 42</td>
</tr>
</tbody>
</table>

When applicable, 5% confidence intervals (CI) are given.
dissolved oxygen concentration, freshwater environments are usually classified as oxic (6–12 mgO₂/L), suboxic (0.1–6 mgO₂/L) and anoxic (<0.1 mgO₂/L), at 20 °C and atmospheric pressure. Optical microscopy of the CV water showed a wide range of prokaryotic and eukaryotic microbial morphologies. In particular, many purple sulfur bacterial colonies composed of large rods with intracellular sulfur granules, most closely resembling members of the genus *Chromatium*, were observed. These colonies appeared to be surrounded by a relatively thick mucous coating (Supplementary figure S1). These purple sulfur bacteria utilize H₂S as electron donor for CO₂ reduction producing elemental sulfur [34] that was clearly visible within cells. Large cyanobacterial filaments of *Oscillatioria*-like spp. were also abundantly observed (Supplementary figure S1). Members of this genus can carry out anoxygenic photosynthesis using photosystem I alone and H₂S instead of H₂O as electron donor, producing elemental sulfur that is secreted out of the cells [34]. Although phylotypes belonging to other genera of the purple sulfur bacteria of the family Chromatiaceae (gamma-proteobacteria) were detected in subsequent work, *Chromatium* spp. SSU rDNAs were not. Similarly, cyanobacterial phylotypes were not detected (see below). This was most likely due to the fact that, owing to their large sizes (>5 μm), members of these abundant prokaryotes in the CV pond plankton were filtered away during the prefiltration steps.

3.1. Overall phylogenetic diversity and species richness of small plankton and sediment

We extracted DNA from the suboxic 0.22–5 μm planktonic fraction and from anoxic sediment. Subsequently, we amplified SSU rDNAs from the two samples using different primer combinations of archaea-, bacteria- and prokaryote-specific primers, and constructed several SSU rDNA libraries to minimize potential PCR bias. Thus, 7 and 2 archaeal libraries, and 5 and 2 bacterial libraries were constructed for plankton and sediment, respectively. A total of 316 high-quality partial (∼800 bp) sequences from plankton (clones MVP) and sediment (clones MVS) were compared to sequences in databases by BLAST to have a first look at the overall diversity recovered. This was remarkably wide, both at the high rank taxonomic level (phyla) and also within taxa. In the case of bacteria, members of 15 and 13 different phyla/candidate divisions were identified for plankton and sediment, respectively. A total of 316 high-quality partial sequences from plankton (clones MVP) and sediment (clones MVS) were compared to sequences in databases by BLAST to have a first look at the overall diversity recovered. This was remarkably wide, both at the high rank taxonomic level (phyla) and also within taxa. The figure 1 shows the distribution in major phylogenetic groups of 16S rDNA bacterial clones of plankton and sediment from the suboxic freshwater pond under study (CV pond, Orsay, France).
Broad diversity was observed for the Archaea as well, although in this case only members of the subkingdom Eur-yarchaeota were identified (see below). Two independent sequence alignments for plankton and sediment were used to estimate the level of phylotype redundancy (rarefaction curves) and to calculate various species richness indices at different levels of sequence similarity. We considered three levels of operational taxonomic units (OTUs) that were defined at 97%, 95% and 80% sequence identity, as rough approximations to the species, genus and phylum levels [40]. Rarefaction curves are shown in Fig. 2A and B. Saturation was only reached at the 80% sequence identity level, but not at 97% or even 95% thresholds, showing that both sediment and plankton samples are very diverse beyond the genus level. A similar trend could be observed in the lineage-through-time plots, where the occurrence of OTUs having more than 10 or even 20% divergence at the sequence level was relatively abundant (Fig. 2C and D).

We used various methods to estimate species richness (Table 2). Species richness indices are based on the proportional abundance of species (OTUs). The Shannon index, which is widely used to compare OTU richness in different samples, was very similar at the three OTU levels considered both for sediment and plankton samples, and remarkably high (close to 4) at the 97% threshold (Table 2). Other species richness estimators, such as non-parametric Chao1 and ACE, are widely used to evaluate the number of OTUs in a given sample. Non-parametric methods have the general advantage of using small sample sizes (OTUs in SSU rDNA libraries in our case) without assuming any underlying model of OTU distribution. However, they may reflect experimental PCR bias and they only provide a lower boundary for diversity (since they do not assume any model of distribution), which may thus lead to underestimation of the actual diversity. Furthermore, in our case, because we excluded gaps from calculation of distance matrices to avoid differences due to the various
Fig. 3. Maximum likelihood phylogenetic tree showing the positioning of archaean 16S rDNA phylotypes retrieved from sediment and plankton of the suboxic CV pond. Names in italics correspond to cultivated species or strains, while the rest correspond to 16S rDNA clones unless otherwise specified (fosmid). Names in bold correspond to clones obtained in this study. Accession numbers are given in brackets only for environmental clones or non-described species. Only bootstrap values higher than 50% are given at nodes. Clones labeled CV1-A2 were retrieved from the sediment of the same pool in a previous study [41].
Fig. 4. Maximum likelihood phylogenetic tree showing the position of bacterial phylotypes retrieved from sediment and plankton of the suboxic CV pond belonging to the Chloroflexi, Fibrobacteres, Gemmatimonadetes, Spirochaetes and various candidate divisions. Names in italics correspond to cultivated species or strains, while the rest correspond to 16S rDNA environmental clones. Names in bold correspond to clones obtained in this study. Accession numbers are given in brackets only for environmental clones or non-described species. Only bootstrap values higher than 50% are given at nodes. Cand. Div., candidate division; TG-1, termite group 1.
sequence lengths, we may have eliminated some true differences between sequences, and thus our species richness estimates are conservative. Nevertheless, the differences introduced in this way are not expected to affect comparisons at the level of 97% sequence identity. The Chao 1 estimator is often used for environmental gene or genome libraries, since it is based on the presence of singletons and doubletons, which frequently account for most of the phyotypes observed in this kind
Fig. 6. Maximum likelihood phylogenetic trees showing the position of bacterial phylotypes retrieved from sediment and plankton of the suboxic CV pond belonging to different subdivisions of the Proteobacteria. Names in italics correspond to cultivated species or strains, while the rest correspond to 16S rDNA environmental clones. Names in bold correspond to clones obtained in this study. Accession numbers are given in brackets only for environmental clones or non-described species. Only bootstrap values higher than 50% are given at nodes.
of library [4]. Chao1, Jackknife and ACE estimates for sediment and plankton CV pond samples overlapped considering the respective confidence intervals, but the latter were quite large at the 97% and 95% identity thresholds (Table 2). Interestingly, in contrast to rarefaction curves, the Chao1 richness estimate collector’s curves reached a plateau for the 97% and 95% sequence identity thresholds after having sampled ~150 plankton OTUs (Fig. 2E) and ~100 sediment OTUs (Fig. 2F). This phenomenon is in agreement with previous observations that richness estimators stabilize at smaller sampling effort than rarefaction curves [28]. The use of these curves could thus be used to determine relatively objectively when a given library or sample has been sufficiently explored to yield stable estimates of OTU richness, although, since potential bias can affect these measurements, a cautious interpretation is needed [28].

3.2. Archaeal community composition

In order to reconstruct detailed phylogenetic trees, we completely sequenced 39 archaeal SSU rDNA clones that represented the diversity observed in the 64 partial archaeal sequences previously analyzed. All of them were euryarchaeotal sequences (supplementary table S1 and Fig. 3). A large proportion of phylotypes, both from plankton and sediment, belonged to the Methanomicrobiales, and they were more closely related to sequences retrieved from freshwater sediment or groundwater aquifers (Fig. 3). A few phylotypes belonged to the Methanosarcinales. Both euryarchaeotal orders contain methanogenic species, but within the Methanosarcinales, uncultured lineages forming various subgroups generally known as the ANME-2 are methanotrophic. ANME-2 Archaea form syntrophic consortia with sulfate-reducing bacteria (SRB) [3], and are likely able to oxidize methane by a reverse methanogenesis pathway [19]. However, although sequences clearly belonging to the ANME-2 were not identified, our phylotype MVP-9A-24 branched as a sister group to the ANME-2 and Methanosarcinales (Fig. 3), and the possibility that it might perform anaerobic methanogenesis could be considered. Interestingly, although known ANME-2 sequences thus far have originated from marine or estuarine sites and none is available from freshwater lakes, the presence of ANME-like anaerobic methanotrophs has been recently demonstrated, and was shown to co-exist with aerobic methanotrophs in one freshwater lake [10].

The rest of the archaeal sequences were much more distant from culturable archaeal species and taxa than those mentioned above, with several phylotypes closely related to sequences retrieved from deep-sea vents or sediments, and cold seeps (Fig. 3). They interspersed within the environmental groups DHVE1, DHVE4 and DHVE5, initially defined based on the diversity of deep-sea hydrothermal vent clones [44]. This is most likely due to the similar redox conditions in both types of environments. Phylotypes belonging to the DHVE1, which is related to the Thermoplasmatales, were all retrieved from pond plankton, as was the case for the only phylotype clustering with DHVE4. Phylotypes belonging to the DHVE5 clustered not only with deep-sea sequences, but with a group of quite diverse phylotypes that had been retrieved in a previous environmental survey from the CV pond sediment (CV1-A2-10 and -20) and from another pond located a few kilometers away (clones CH1-S2) using eukaryotic primers [41]. This suggests that this group related to the DHVE5 may also be typical of freshwater sediments and/or anaerobic freshwater plankton.

3.3. Candidate divisions of bacteria and some bacterial phyla moderately represented in SSU rDNA libraries

In the case of bacterial clones, we completely sequenced 200 bacterial SSU rDNA clones representative of the diversity observed in the 252 partial bacterial sequences initially analyzed. We then constructed various maximum likelihood (ML) phylogenetic trees covering the diversity of the identified taxonomic groups (Fig. 1). Fig. 4 represents an ML tree covering the general diversity of bacteria and showing the position of CV pond phylotypes affiliating with various phyla with moderate representation in our SSU rDNA libraries, as well as sequences clustering with the candidate divisions identified in the CV pond.

A few clones were too divergent from members of recognized taxa or candidate divisions to be affiliated with any of them. Clone MVP-21 was isolated and far from any known sequences, although it appeared at the base of a large group of candidate divisions (Fig. 4). The other two very divergent phylotypes clustered with other divergent environmental sequences, and could thus represent novel high-rank taxonomic groups. Thus, MVP-15 was closely related to a phylotype retrieved from a low-temperature freshwater biodegraded oil reservoir in Canada [16], and MVS-104 formed a cluster with a clone from salt marsh sediment and a sequence from peculiar, likely chemosulfotrophic, microbial filamentous structures at the Nullarbor Cave, Australia [21] (Fig. 4).

In addition to uncertain lineages, a relatively high proportion of bacterial clones belonged to six candidate divisions, lacking cultivated members so far. With the exception of the candidate division WS1, identified initially in a hydrocarbon- and chlorinated-solvent-contaminated aquifer [8] and encompassing the CV pond sediment phylotype MVS-77, all candidate divisions were retrieved from plankton (Figs. 1 and 4). A few phylotypes belonged to candidate division TM6, whose members have been identified in soil, anaerobic digesters and anaerobic trichlorobenzene (TCB)-transforming communities [48]. Interestingly, a variety of phylotypes were affiliated with members of the candidate division Termite Group 1 (TG-1). Members of the TG-1 bacteria are endosymbionts in the cytoplasm of strict anaerobic flagellate protists of the genera *Pyronemphya* (oxymonad) and *Trychonympha* (parabasalid) living in guts of termite and wood-feeding cockroaches. This is the reason why the phylogenetic group of TG-1 has been proposed as the candidate phylum "Endomicrobia" [43]. The function of these symbionts is not clear. They may be involved in cellulose digestion, but nitrogen fixation, ammonium assimilation, supplying of essential amino acids and vitamins or a role in hydrogen metabolism (e.g.
reductive acetogenesis from CO₂ are also envisaged [43]. As parabasalids were previously identified in the CV pond during a eukaryotic SSU rDNA survey [41], it is possible that these strict anaerobic flagellates harbor TG-1 endosymbionts. Indeed, environmental conditions in the CV pond and termite guts are similar in terms of large cellulose input and practically anoxic conditions.

In our ML tree, a large supergroup encompassing the candidate divisions WS6, OP11 and OD1 plus one unclassified lineage emerged (Fig. 4). Only one phylotype, MVP-31, belonged to candidate division WS6, which was first identified in a contaminated aquifer [8]. Clones MVP-22, MVP-74, MVP-112 and MVP-115 identified in the plankton of the CV pond can be considered as bona fide members of candidate division OP11 (Fig. 4), first detected in a Yellowstone hot-spring [22]. However, although environmental clone ko116 from a nitrogen-removing biofilm appears classified as OP11 in GenBank (accession number AJ224539), this clone together with our clones MVP-16 and MVP-37 and a clone from deep-sea sediment (BD1-5) formed an independent cluster from OP11 with high statistical support (OP11-like in Fig. 4).

Therefore, this lineage likely constitutes an independent high-rank taxonomic group. The last candidate division present in CV pond plankton, OD1, was outstandingly diverse and abundant in our SSU rDNA libraries, by far the most frequently represented in the plankton sample if we consider the different proteobacterial subdivisions independently (Fig. 1).

The candidate division OD1 appears globally distributed in marine and freshwater settings, having been identified in anaerobic digesters [7,20]. Recently, a fosmid containing an OD1 SSU rDNA was identified in a metagenomic library from a mesophilic sulfur spring and sequenced. Although clones have been retrieved from soils, although not exclusively [39]. Clones from the CV pond were most closely related to sequences from soil, deep-sea sediment and anaerobic consortia (supplementary table S1 and Fig. 5).

A few phylotypes ascribed to the recently described phyla Gemmatimonadetes and Fibrobacteres were exclusively retrieved from CV sediment (Figs. 1 and 4). There is only one cultivated species of this phylum, Gemmatimonas aurantiaca, which was isolated from an anaerobic-aerobic sequential batch reactor, but it appears very diverse (19% SSU rDNA intraphylogenetic sequence divergence) and widely distributed in nature [50]. Fibrobacter spp. are fibrolytic and have been isolated from ruminal systems [2]. Their presence in the CV pond appears justified, as in the case of the TG-1 lineage, by the similarities of this freshwater system to the rumen and other anaerobic bioreactors previously discussed. Finally, phylotypes belonging to the Chloroflexi and the Spirochaetes, typically associated with some anoxic settings, were found both in plankton and sediment (Fig. 4). The Chloroflexi are anoxygenic phototrophic filamentous bacteria that can be quite thermophilic and are frequently encountered in hot springs [34], but environmental surveys have revealed many diverse phylotypes of these green non-sulfur bacteria in anoxic zones of stratified freshwater systems [13].

3.4. Gram-positive bacteria, Acidobacteria, Planctomycetes, Verrucomicrobia, Chlorobi and Bacteroidetes

Various phylotypes retrieved both from sediment and plankton were ascribed to the two Gram-positive bacterial phyla, Actinobacteria and Firmicutes. Although the Actinobacteria appear to be common in freshwater lakes [18], they were poorly represented in our libraries (Fig. 1). Nevertheless, their closest representatives in GenBank were sequences from meromictic lakes and an anaerobic reactor (supplementary table S1 and Fig. 5). The Firmicutes were more abundant than the Actinobacteria, at least in CV plankton libraries, and were frequently related to phylotypes from anoxic environments, such as anoxic soil or the chemocline of the meromictic Lake Cadagno [5] (Fig. 5). Clone MVP-17 was related to an oral clone affiliated with Selenomonas, a genus characteristic of rumen starch and lactate decomposers that is also found in gingival crevices [34]. The Acidobacteria were also identified in sediment and plankton of the CV pond. However, acidobacterial phylotypes were quantitatively much more frequent in sediment samples (Fig. 5). The Acidobacteria form a coherent taxonomic group, although very diverse, thus far containing very few cultivated species that are chemoorganotrophic. Most environmental clones have been retrieved from soils, although not exclusively [39]. Clones from the CV pond were most closely related to sequences from soil, deep-sea sediment and anaerobic consortia (supplementary table S1 and Fig. 5).

The phyla Planctomycetes and Verrucomicrobia were also well represented in CV SSU rDNA libraries; Planctomycetes were slightly more abundant in sediment, whereas Verrucomicrobia were more prolific in plankton samples (Fig. 1). CV planctomycete phylotypes were related to environmental sequences retrieved from aquifers, marine sediments and anaerobic digestors (Fig. 5 and supplementary table S1). This is not surprising, as the Planctomycetales have been identified in different habitats including hot springs and lakes [9]. Many Planctomyces species are facultative aerobic chemooorganotrophs, which grow by fermentation or respiration, but others are strict anaerobic autotrophs that carry out the anaerobic oxidation of ammonia using nitrite (anammox) [47]. Since the CV pond phylotypes are distant from cultivated members of this phylum, it is very difficult to advance their possible physiology. However, given the environmental oxygen-depletion, they likely perform some kind of anaerobic metabolism. Verrucomicrobia CV clones were varied and were often related to phylotypes retrieved from meromictic freshwater lakes, groundwater systems or deep-sea sediment (Fig. 5 and supplementary table S1). This phylum was recognized only recently and counts only a few cultivated members. However, environmental verrucomicrobial sequences have been recovered from large biotope spectra, from freshwater and marine aquatic systems to forest and agricultural soil [34]. Known species are able to ferment a variety of sugars, and hence, it is possible that they participate in the degradation of organic material in the CV pond in this way.
Various green sulfur bacterial phylotypes were identified both in the plankton and sediment (probably benthic) of the CV pond. The Chlorobi, green sulfur bacteria, are a group of anoxygenic photosynthetic bacteria frequent in anoxic layers of microbial mats and meromictic lakes, usually in deeper layers than the purple sulfur bacteria, since they support higher $H_2S$ concentrations and require less light intensity. They photosynthesize using $H_2S$ as electron donor, although they can also grow photoheterotrophically, and some of them form symbiotic consortia with heterotrophic partners that are widely distributed in chemoclines of meromictic lakes throughout the world [14]. In fact, our phylotype MVS-13 was relatively close to chlorobial sequences from dechlorinating consortia (Fig. 5). The rest of the green sulfur bacterial clones were related to environmental sequences from other freshwater systems, including hot springs. The CV pond libraries also contained a relatively important number of clones affiliated with the Bacteroidetes (Cytophaga-Flexibacter-Bacteroides and related genera), particularly in sediment samples, where they were quantitatively comparable to the delta-Proteobacteria and the Acidobacteria (Fig. 1). Bacteroidete phylotypes were very diverse and their closest relatives were sequences from anaerobic digestors, freshwater lakes, deep-sea sediment, sulfidic caves and aquifers (Fig. 5 and supplementary table S1). Members of this phylum are typical degraders, hydrolyzing complex organic molecules (cellulolytic or chitinolytic) or fermenting under strict anaerobic conditions [34]. Remarkably, some anaerobic flagellates living in termite guts entertain a symbiotic relationship with Bacteroidetes bacteria, which tightly cover their surface. However, the mutual benefit for the two partners is not yet understood [42]. The presence of anaerobic flagellates thriving in the CV pond [41] opens up the possibility that certain Bacteroidetes could establish some kind of metabolic symbiosis with them.

### 3.5. Proteobacteria

The ecologically successful Proteobacteria were the most abundant phylum in plankton and sediment libraries of the CV pond. However, important differences were observed in the relative distribution and abundance of the different proteobacterial subdivisions (Fig. 1). Thus, beta- and alpha-Proteobacteria were much more abundant in sediment than in plankton libraries, with the alpha-Proteobacteria nearly absent from water samples. In contrast, the epsilon-Proteobacteria were more abundant in plankton than in sediment, while the relative proportions of gamma- and delta-Proteobacteria in both sample types were comparable. Most proteobacterial phylotypes branched within the recognized subdivisions, but clones MVP-120 and MVS-98 were deeply branched between the gamma- and beta-Proteobacteria and, to a lesser extent, MVS-4, with the latter apparently closer to the beta-Proteobacteria (Fig. 6).

The beta-Proteobacteria appear to be numerically important in freshwater lakes [17,51]. In our case, beta-proteobacterial phylotypes were by far the most abundant in sediment libraries, in a proportion comparable only to that of the candidate division OD1 in plankton libraries (Fig. 1). They were also very diverse, including some divergent phylotypes that branched at the base of the group. In addition to the above-mentioned MVS-4, clone MVS-8 formed a robust early-branching cluster with a sequence retrieved from magnesite mine drainage (Fig. 6). The rest of the beta-proteobacterial clones took part in a large and varied monophyletic cluster that included very different species and environmental sequences of various origins. Based on proximity to cultivated species of known physiology, at least five different metabolic types could be hypothesized for the beta-Proteobacteria inhabiting the CV pond: hydrogen-oxidizers (e.g. *Hydrogenophilus* and *Aquaspirillum/Hydrogenophaga* relatives), $H_2S/SO_4$-oxidizers (*Thiobacillus*-like), nitrifying bacteria (*Nitrosospira* relatives), dechlorinating bacteria (*Dechloromonas*) and methyloths (*Methylphilus*). It may even be possible that some of the beta-Proteobacteria establish symbiotic consortia with anoxygenic green sulfur bacteria, since the latter are frequent epibions of a motile beta-proteobacterium in phototrophic consortia widely distributed in meromictic lakes [14].

Compared to beta-Proteobacteria, alpha and gamma subdivisions were less abundant. Most alpha-proteobacterial clones arose from the sediment, with some being related to soil or lake clones. Two phylotypes, MVS-73 and MVS-76, were related to purple phototrophic bacteria of the genus *Rhodo bacter*. As mentioned above, *Chromatium* spp. were detected by microscopic observation (Supplementary figure S1), but due to their larger size they were most likely excluded from the small plankton studied. Gamma-Proteobacteria from CV sediment and plankton were also very diverse phylogenetically, but most of the clones were closely related to cultivated gamma-Proteobacteria that oxidize reduced sulfur species (e.g. *Thiocystis, Thiothrix*). Therefore, most of them could in fact be oxidizing $H_2S$, $SO_4$ or thiosulfate in this environment. One phylotype, MVP-79, was nearly identical to the SSU rDNA of the parasitic strains *Haemophilus piscium* and *Vibrio parahaemolyticus*. However, in the absence of fish in this small pond, it is possible that this organism parasites other small animals (frogs or perhaps invertebrates).

Finally, delta- and epsilon-Proteobacteria were also abundant in CV pond libraries (Fig. 1). Delta-Proteobacteria were very diverse, and included, with strong statistical support, the very divergent phylotype MVP-7 (Fig. 6). Most delta-Proteobacteria are sulfate reducers; they are abundant and play a cardinal role in anoxic settings, including meromictic and anoxic lakes [27,30]. In addition to sulfate-reducing bacteria, various phylotypes were related to the myxobacteria, usual degraders and fermentors, and to syntrophic genera (*Syntrophus*) that typically establish interspecies $H_2$-transfer symbioses with methanogenic archaea. For instance, the CV pond clone MVS-101 was very closely related to FA-PB5, a sequence obtained from a biodegraded-oil reservoir syntrophic consortium. FA-PB5 (*Syntrophus*-like) establishes a syntrophic association with a *Methanoseta*-like methanogen, as it is able to degrade long-chain fatty acids [15]. The epsilon-Proteobacteria are naturally associated with sulfide-rich environments, as they are dominant in the surroundings of deep-sea vents [33,45] and
sulfur springs [12]. Although they are absent or rare in common freshwater lakes [9], they appear particularly abundant at oxic/anoxic interfaces (redox clines) in marine environments [31]. Their preference for these transitional redox zones may explain why epsilon-proteobacterial clones were abundant in the CV pond, particularly in planktonic libraries. Most of our clones were closely related to environmental clones from deep-sea vents, caves and groundwater or to cultivated sulfur-oxidizing species (Fig. 6). They are likely involved in sulfur cycling by oxidizing sulfide or sulfur using small amounts of oxygen or nitrate. Some epsilon-Proteobacteria can also reduce elemental sulfur to sulfide [45].

3.6. Metabolic and ecological considerations

Microbial communities thriving in suboxic-to-anoxic waters and anoxic sediments of the CV pond were very diverse, encompassing lineages belonging to many different phyla within Archaea, Bacteria and also eukaryotes. This phylogenetic diversity reflects a variety of metabolic activities co-existing in a complex ecosystem that is fundamentally microbial, containing primary producers and degraders, but also an active population of protistan grazers [41], and sustaining complex intertwined carbon and sulfur cycles.

The community of photosynthesizing organisms is diverse, including duckweed on the pond surface, some microalgae [41] and also anoxicogenic photosynthesizers (green non-sulfur, green sulfur and purple sulfur bacteria). However, most of the metabolic activity of the pond seems to be driven by the degradation of external organic matter derived from plant debris (leaves, branches, etc) that feeds the pond recurrently, and hence, from external primary production. Paradoxically, the activity of anoxicogenic photosynthesizers is rather the consequence of that external primary production (oxygenic photosynthesis), since the H$_2$S they require originates from sulfate or sulfur reduction during terminal degradation of organic matter, only a small fraction of which can be explained by anoxicogenic photosynthesizing activity. In this sense, anoxicogenic photosynthesis in such a system, contrary to that occurring in sulfur springs where there is a geological supply of H$_2$S, can be considered as secondary primary production [35]. In fact, anoxicogenic phototrophs appear to contribute efficiently together with the anaerobic food chain to the conversion of allochthonous organic carbon into easily degradable bacterial biomass by fueling the sulfur cycle [35], which, in turn, accelerates the carbon cycle in the system.

The degradation of allochthonous organic matter in the predominantly anaerobic pond ecosystem requires the interplay of various microbial partners. Complex polymers such as cellulose, lignin or long fatty acids are probably initially degraded by specialized microorganisms. Subsequently, fermenting bacteria degrade simpler organics to small molecules, such as acetate that can be utilized by acetogenic methanogens. There are various CV pond lineages that could perform such decomposition functions; in fact, many phylotypes have as closest relatives sequences retrieved from anaerobic digestors or the rumen, where these activities are dominant. In addition, syntrophic associations likely play an important role in many of these hydrolytic activities, for example, as discussed above, symbiosis between syntrophic delta-Proteobacteria and methanogens [15] or between "endomicrobia" (termite group I bacteria) and anaerobic flagellates [43]. Other types of symbioses that could exist in the CV pond, such as phototrophic consortia involving green sulfur bacteria and beta-Proteobacteria [36], or Bacteroidetes and anaerobic flagellates [42], are less well understood from the point of view of metabolic exchange. Hydrogen, a major end product of fermentation, is likely a key metabolite exchanged among certain syntrophic partners, but is also likely released into the environment, as there are various phylotypes related to hydrogen-oxidizing bacteria (e.g. Hydrogenophilus). A major sink for hydrogen is probably constituted by methanogenic archaea. Indeed, three types of methanogens, whose relative dominance may vary seasonally as a function of temperature and population dynamics [29], may be operating in the system: acetogenic and hydrogenoclastic methanogens, which would respectively take up acetate and hydrogen derived from fermentation; and C$_1$-methanogens, which would methanol or methylamines derived from the hydrolysis of complex organic matter.

Curiously, no typical aerobic methanotrophic bacteria were identified in the CV pond samples, whether type I, II or X (belonging to the alpha- and gamma-Proteobacteria). This is surprising given the presence of methanogenic archaea supplying methane to the system. Saturation curves indicate that not all the diversity in the pond has been explored (Fig. 2), and therefore, aerobic methanotrophs could still be retrieved. Other possible explanations would be that aerobic methanotrophs are confined to the uppermost layers, which provide microaerophilic conditions (Table 1), or that, since oxygen is insufficient in the system, methanotrophy could be carried out anoxogenically by euryarchaeotal lineages distantly related to the ANME-2 and the Methanosarcinales (Fig. 3). In any case, organisms metabolizing C$_1$ compounds (methanol, methylamines) appear to exist in the pond, since lineages related to beta-proteobacterial methylotrophs were observed (Methylphilus-like). Microorganisms utilizing C$_1$-compounds may be more diverse than previously thought, probably including some Planctomycetes and some lineages of uncertain affiliation [25].

In addition to a complex carbon cycle, the CV pond system possesses an active sulfur cycle that appears to be maintained by sulfate-reducing bacteria, mostly delta-Proteobacteria, and sulfide, sulfur or thiosulfate oxidizers belonging to various bacterial phyla, including green and purple sulfur bacteria, gamma-Proteobacteria (Thiocystis, Thiobrix) and epsilon-Proteobacteria (Arco bacter, Sulfolomanas, Thiomicrospira). The diversity of S-oxidizing bacteria is comparable to that found in systems where there is a geographical supply of reduced sulfur species, such as continental hot or cold springs and deep-sea hydrothermal vents [6]. In contrast, the origin of reduced sulfur in the CV pond system is biological and intimately linked to the decomposition of organic matter and hence the carbon cycle. It thus appears that there is a positive feedback from the carbon cycle to the sulfur cycle and vice-versa.
Finally, although many CV pond lineages belong to groups for which some kind of metabolic information is available, there are also numerous phylotypes belonging to divergent phylogenetic groups for which metabolic information does not exist. Some of them appear to be important to the system, such as the OD1 candidate division, which accounts for a large proportion of phylotypes in the plankton of the suboxic pond (Fig. 1). Studying the seasonal dynamics of such organisms, and investigating conditions that reign in CV plankton, should increase our knowledge of their physiology and elucidate their ecological role.

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Appendix. Supplementary material


References


