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RESEARCH ARTICLE

# Bacterial periphytic communities related to mercury methylation within aquatic plant roots from a temperate freshwater lake (South-Western France)

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**Abstract** Macrophyte floating roots are considered as hotspots for methylmercury (MeHg) production in aquatic ecosystems through microbial activity. Nevertheless, very little is known about periphyton bacterial communities and mercury (Hg) methylators in such ecological niches. The ability to methylate inorganic Hg is broadly distributed among prokaryotes; however, sulfate-reducers have been reported to be the most important MeHg producers in macrophyte floating roots. In the present work, the periphyton bacterial communities colonizing *Ludwigia* sp. floating roots were investigated through molecular methods. Among the 244 clones investigated, anaerobic microorganisms associated with the sulfur biogeochemical cycle were identified. Notably, members of the sulfur-oxidizing prokaryotes and the anoxygenic, purple non-sulfur bacteria (*Rhodobacteraceae*, *Comamonadaceae*, *Rhodocyclaceae*, *Hyphomicrobiaceae*) and the sulfate reducers (*Desulfobacteraceae*, *Syntrophobacteraceae*, and *Desulfobulbaceae*) were detected. In addition, 15 sulfate-reducing strains related to the *Desulfovibrionaceae* family were isolated and their Hg-methylation capacity was tested using a biosensor. The overall results confirmed that Hg methylation is a strain-specific process since the four strains identified as new Hg-methylators were closely related to non-methylating isolates. This study highlights the potential involvement of periphytic bacteria in Hg methylation when favorable environmental conditions are present in such ecological micro-niches.

**Keywords** Periphyton · Bacterial diversity · Sulfate reducers · Mercury methylation · Biosensor

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## Introduction

Terrestrial and, to a lesser extent, wetland rhizospheres have been extensively studied with respect to microorganisms colonizing such niches. These ecosystems are characterized by intense interactions between plants and microorganisms that participate in plant health, crop production, and rhizoremediation processes (Rejmankova 2011; Berendsen et al. 2012). Microbial communities colonizing such niches are well known and sometimes considered as "the second plant genome" (Berendsen et al. 2012). In contrast, there are very few data concerning microbial diversity of aquatic rhizospheres, such as periphyton developing on aquatic roots (Stout and Nüsslein 2005; Matsuzawa et al. 2010; Wei et al. 2011). Aquatic roots from emergent, submersed, or free floating macrophytes and their associated bacterial communities are considered to be good metal accumulators and are studied for metal water removal strategies (Elifantz and Tel-Or 2002; Stout and Nüsslein, 2010). Periphyton is also considered as a pollutant entry point into the aquatic food web; this is the case for mercury (Chasar et al. 2009; Molina et al. 2010).

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Mercury (Hg) was recognized to be a major pollutant for human health and the environment at the "Minamata convention on Hg" (2013). The organic Hg compound, methylmercury (MeHg), constitutes the most toxic form because of its ability to bioaccumulate in living beings and biomagnify along the food webs. MeHg production in ecosystems is mostly caused by sulfate-reducing microorganisms (Compeau and Bartha 1985; Gilmour et al. 1992; King et al. 2001; Bridou et al. 2011) and, to a lesser extent, iron reducers (Kerin et al. 2006; Flemming et al. 2006, Yu et al. 2012) and methanogens (Pak and Bartha 1998; Hamelin et al. 2011). Some studies have demonstrated that biological Hg methylation was significantly higher in the periphyton of floating macrophyte roots in tropical areas (Mauro et al. 2002; Achá et al. 2005; Correia et al. 2011) and temperate lakes (Gentès et al. 2013a; Hamelin et al. 2011, 2015) as compared to sediment associated or not with roots (Yu et al. 2010; Gentès et al. 2013a). Recently, a specific gene cluster (hgcAB) has been linked to biotic Hg methylation activity (Parks et al. 2013) and was detected in a large variety of prokaryotes and environments (Gilmour et al. 2013; Podar et al. 2015). However, a direct correlation between the level of expression of these genes and the MeHg production by microorganisms could not be established (Goñi-Urriza et al., 2015; Bravo et al. 2016). Despite the great advance of Parks et al. (2013), the mechanism for Hg methylation is still to be elucidated. Thus, cultural approaches can still be useful to identify Hg methylators in ecosystems.

In lake Sanguinet, a temperate freshwater lake in South-Western France, some fish have shown Hg concentrations close to the International Marketing Limit (IML = 0.5 mg kg wet weight$^{-1}$) and above the World Health Organization guidelines (0.2 mg kg wet weight$^{-1}$; Gentès et al. 2013b, unpublished data). Another study in the same lake identified the periphyton of the invasive macrophyte *Ludwigia* sp. as the most important Hg methylation niche with the sulfate-reducing bacteria as main actors (Gentès et al. 2013a). The aim of the present work was to get a deeper insight into the bacterial communities associated with *Ludwigia* sp. floating roots, with a focus on sulfate reducers. The combination of molecular and cultivation based methods allowed for the compensation of the inherent biases for each approach and thus, resulting in more diversity. In addition, a new biosensor-based bioassay (Colin et al. submitted) allowed the evaluation of the Hg-methylation capacities of the sulfate-reducing isolates.

## Experimental procedures

### Study site and sampling procedure

Samples of *Ludwigia* sp. floating roots, an invasive macrophyte, were collected in the autumn (September 2012) (in triplicate for each analysis) from Lake Sanguinet, an oligotrophic lake in South-Western France (temperate ecosystem). Details of site characteristics are described in Gentès et al. (2013a). Root samples for molecular biology were collected in sterile cryotubes and immediately frozen on site in liquid nitrogen. They were stored at −80 °C in the laboratory until analysis. Samples for the isolation of sulfate-reducing bacteria were collected in sterile tubes, stored at 4 °C, and processed within 24 to 48 h.

### Molecular diversity of periphyton-associated bacterial communities

Total DNA was extracted from the triplicates (250 mg of periphyton) using the UltraClean Soil DNA Isolation kit (MoBio Laboratories, Inc.) and stored at −20 °C. Roots were subjected to successive pyrophosphate washings (1%) in order to recover the maximum of periphyton before DNA extraction as described in Gentès et al. (2013a). Afterwards, the triplicates were pooled before DNA amplification, cloning, and sequencing of the 16S rRNA gene fragments. PCR amplifications of the 16S rRNA gene were performed using the bacteria-universal primer set, 63F-1387R (Marchesi et al. 1998). Amplicons were cloned into the PCR®-2.1-TOPO® (TOPO TA Cloning® Kit, Invitrogen Corporation, USA) according to the manufacturer's instructions. The 16S rRNA gene fragment inserts were then re-amplified with vector-specific primers M13F (5′-CTGGCCGTCGTTTTAC-3′) and M13R (5′-GGTCATAGCTGTTTCCTG-3′), directly onto bacterial cells for each clone. The PCR mix was as follows: 1× PCR buffer, 1× BSA, 1.5 mM MgCl2, 0.4 mM of each dNTP, 0.2 μM of each primer, 2.5 U of Taq DNA polymerase (Eurobio), and DNA template (between 0.5 and 5 ng) in a final volume of 50 μL. PCR conditions for cloning were as follows: 15 min at 94 °C, 35 cycles consisting of 3 steps (45 s at 94 °C, 45 s at 54 °C, 1 min at 72 °C), and 10 min at 72 °C. Size and quantity of the 16S rDNA fragment were amplified and checked by electrophoretic migration on agarose gel 1%. All sequencing reactions were performed by GATC Biotech (http://www.gatc-biotech.com/en/index.html).

### Isolation of sulfate-reducing bacteria and genotyping

The isolations of sulfate reducers were conducted in a sterile, anoxic, synthetic, modified, multipurpose medium (Widdel and Bak 1992) that contained the following (in g L$^{-1}$, unless otherwise indicated): $KH_2PO_4$, 0.2; $NH_4Cl$, 0.25; $CaCl_2 \cdot 2H_2O$, 0.1; KCl, 0.5; $Na_2SO_4$, 4; NaCl, 1; $MgCl_2 \cdot 6H_2O$, 0.4; resazurin (7-hydroxy-3*H*-phenoxazin-3-one 10-oxide), 1 mL L$^{-1}$; hepes, 10 mM; lactate-Na, 10 mM; acétate-Na, 10 mM; trace element SL12, 1 mL L$^{-1}$; selenite/tungstate, 1 mL L$^{-1}$; and yeast extract, 0.1. The pH of the culture medium was adjusted to 7.0. Just before use, the following filtered-sterilized (0.2 μm) solutions were added: V7 vitamin solution

(Pfennig and Trüper 1992), 1 mL $L^{-1}$; $S_2O_4Na_2$, 100 μM; $FeSO_4 \cdot 7H_2O$, 1 mM. Isolations of sulfate-reducing bacteria were carried out by successive dilution in a 384-well microplate (starting concentration of periphyton: 13.2 g $L^{-1}$) under anoxic conditions (Bactron anaerobic chamber) according to Colin et al. (2012). Positive wells from the highest dilutions (iron sulfide precipitate) were re-cultivated in a sterile tube under anoxic conditions, and their purity was checked by phase-contrast microscopy.

For 16S rRNA gene sequencing, cells were taken from 5-mL well-grown liquid cultures. One milliliter of culture was centrifuged for 10 min at 10,000*g*, the pellet was suspended in 100 μL of sterile MilliQ water, and 1 μL of this solution was used for one PCR reaction. PCR amplifications were performed directly on culture with the universal bacterial primers 63F and 1387R according to Marchesi et al. (1998), as described in the previous section. After checking quality and quantity of amplicons by electrophorese migration, PCR products were sent to GATC Biotech (Germany) for sequencing.

### Phylogenetic analysis based on 16S rRNA gene sequences

The 16S rRNA gene partial sequences were corrected using Sequencher v4.1.4 software and compared to sequences deposited in the GenBank DNA database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) by the *Basic Local Alignment Search Tool* (BLAST) algorithm (Altschul et al. 1998). Closest sequences were downloaded in order to construct phylogenetic trees. Alignments of all sequences were achieved and assembled with Bioedit 7.1.3 software. Phylogenetic trees were constructed with MEGA v5.2 software (Tamura et al. 2007) using the neighbor-joining method (Saitou and Nei 1987). The confidence level of the phylogenetic tree topology was evaluated using 1000 bootstrap replications. 16S rRNA gene sequences obtained for the strains and environmental clones were deposited in the European Nucleotide Archive (EMBL-EBI) under accession numbers LT821089-LT821222.

### Biosensor assay of mercury methylation

The Hg-methylation capacities were assessed using a biosensor-based procedure (Colin et al. submitted). Briefly, sulfate-reducing isolates were grown in acid pre-cleaned tubes closed with a butyl stopper and flushed with $N_2$ to maintain anoxic conditions. The culture medium was unchanged as previously described, except that iron sulfate was removed in order to monitor bacterial growth by measuring optical density at 600 nm. The Hg-methylating strain *Desulfovibrio* BerOc1 was used as the positive control (Ranchou-Peyruse et al. 2009), while non-inoculated medium served as the control for abiotic methylation. Once in the late exponential growth phase, sulfate-reducing cultures were adjusted with 1 mM of $FeSO_4 \cdot 7H_2O$ in order to trap sulfides produced by sulfate respiration. Sulfate-reducing cultures were then spiked with 100 μg $L^{-1}$ of inorganic mercury Hg(II), and aliquots were sampled immediately (T0) and after 5 h of incubation at 30 °C (T5 hr) and stored at −20 °C until analysis.

In parallel, the biosensor *E. coli* MC1061 (Rantala et al. 2011; Ivask et al. 2001) was grown in an LB medium (37 °C, 200 rpm). Actively growing bacterial culture (optical density at 600 nm of 0.7–0.9) was centrifuged at 4000*g* for 10 min at 20–25 °C. LB media 2× was used to resuspend the cell pellet to an optical density of 1. Then, 100 μL of each aliquot (T0 and T5hr) and 100 μL of the *E. coli* culture were mixed in a microplate in triplicate and were incubated 1 h at 37 °C and 200 rpm. After the incubation period, bioluminescence was measured at 482 nm using a Multi-well Plate Reader (Series 4000 Perspective Biosystem). Results were given as light intensity (LI) and calculated as follows: LI T5h-LI T0)/OD; with T5hr: final time of incubation (5 h); T0: initial time of incubation; OD: optical density measured at T0 (just before the beginning of the Hg-incubation.

## Results and discussion

### Total bacterial diversity associated with the periphyton of Ludwigia sp. floating roots

Periphyton bacterial communities developing on *Ludwigia* sp. floating roots were assessed through a cloning/sequencing approach based on the 16 rRNA gene. A total of 244 clones were sequenced and affiliated to their closest relatives at the phylum level (Fig. 1). The 16 rRNA gene sequences were aligned and grouped into 170 operational taxonomic units (OTUs) at a threshold of 98% similarity. The 16S rRNA gene clone library coverage was estimated to be 42% (Dotur software), suggesting that only a minor part of the bacterial community was investigated in the present study. The *Proteobacteria* dominated the bacterial community, representing 63% of the total number of clones (Fig. 1). Among them, 78% were affiliated to described bacteria and belonging to 15 different orders within *Alpha*, *Beta*, *Gamma*, and *Deltaproteobacteria* (Fig. 1). The remaining 22% could not be identified unambiguously, and this highlights the limited data available on periphytic bacterial diversity. In contrast to a previous study (Stout and Nüsslein, 2005), sequences related to the *Alphaproteobacteria* were dominant instead of the *Betaproteobacteria* (38 and 31% respectively, considering the identified clones). The aerobic heterotrophic bacterial community mainly related to the *Alphaproteobacteria* (Fig. 2), the *Betaproteobacteria* (Fig. 3), and the *Gammaproteobacteria* (Fig. 6 sup. data). They comprised microorganisms involved in the nitrogen cycle and were typical of plant rhizospheres such as *Azospirillum* (LSR126), *Bradyrhizobium* (LSR90, 120, 191, 145, 229, 37,

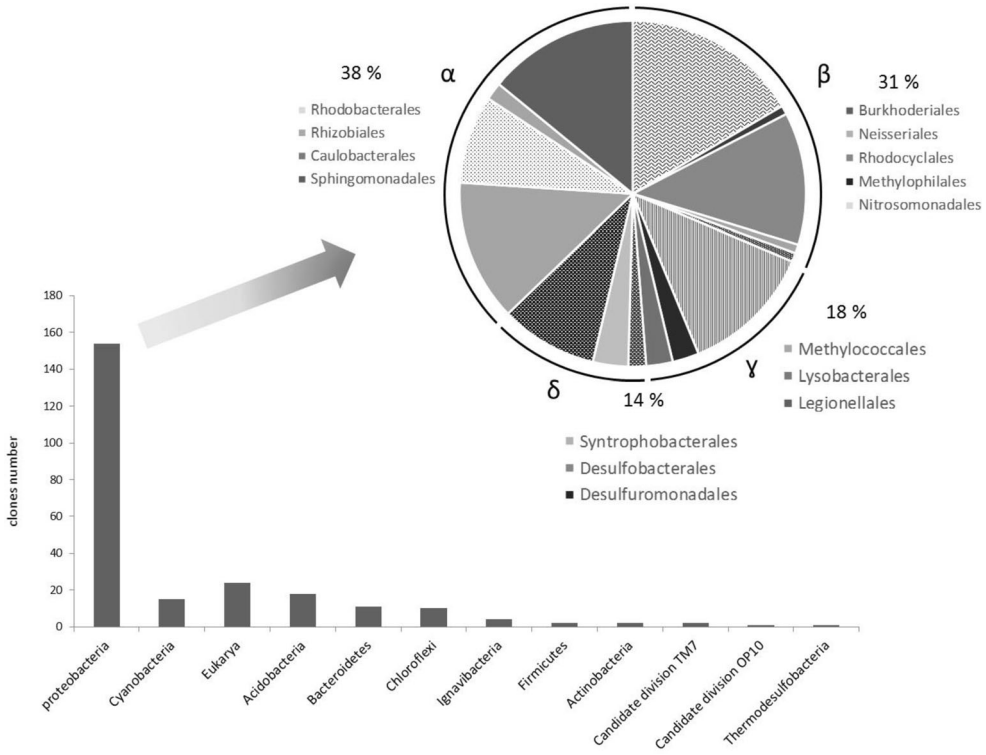


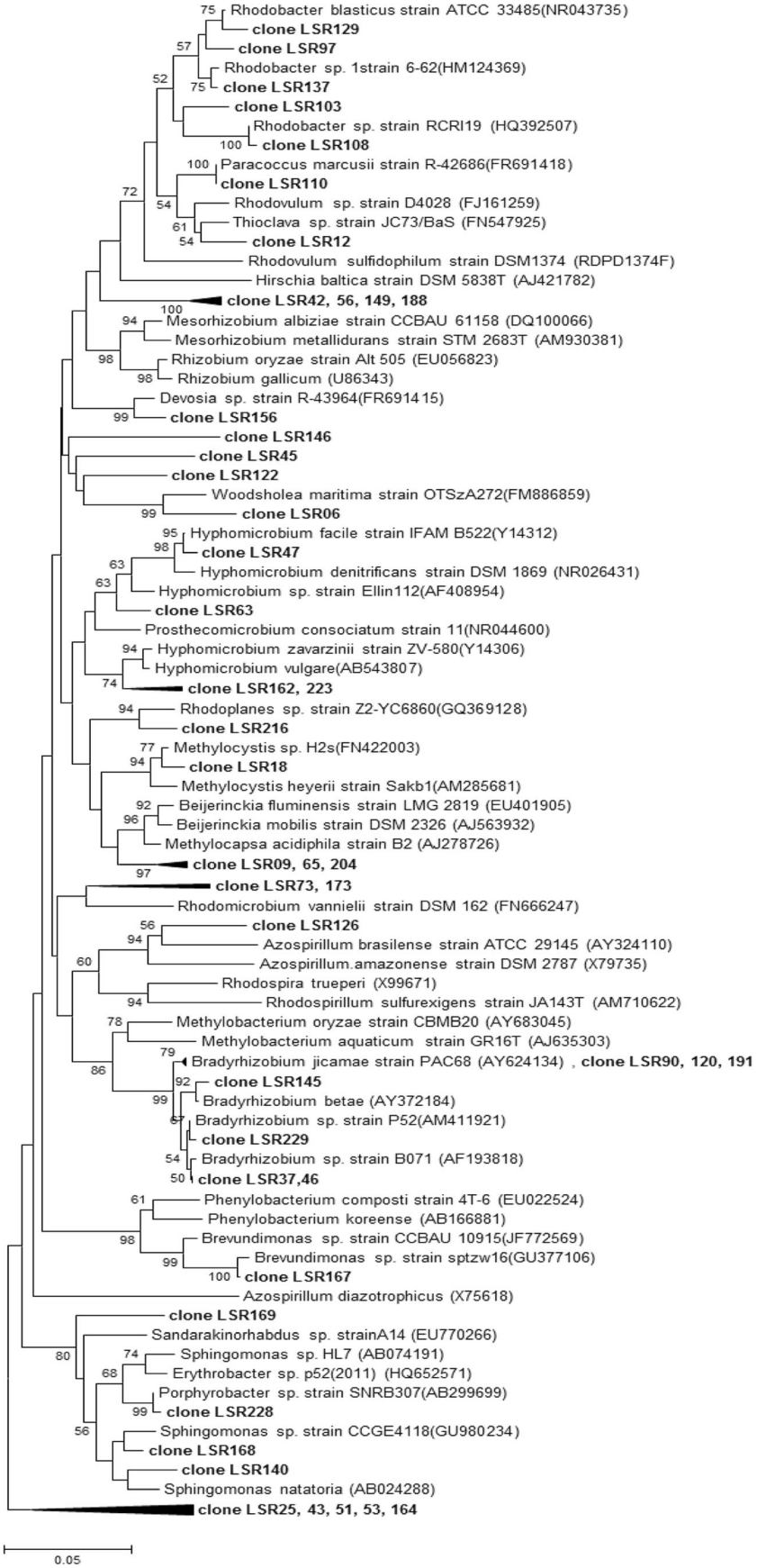

**Fig. 1** Abundance of clones (total = 244) from bacterial divisions present in the 16S rRNA gene clone libraries from the periphyton of *Ludwigia* sp. roots (Sanguinet lake) with focus on the *Proteobacteria* phylum

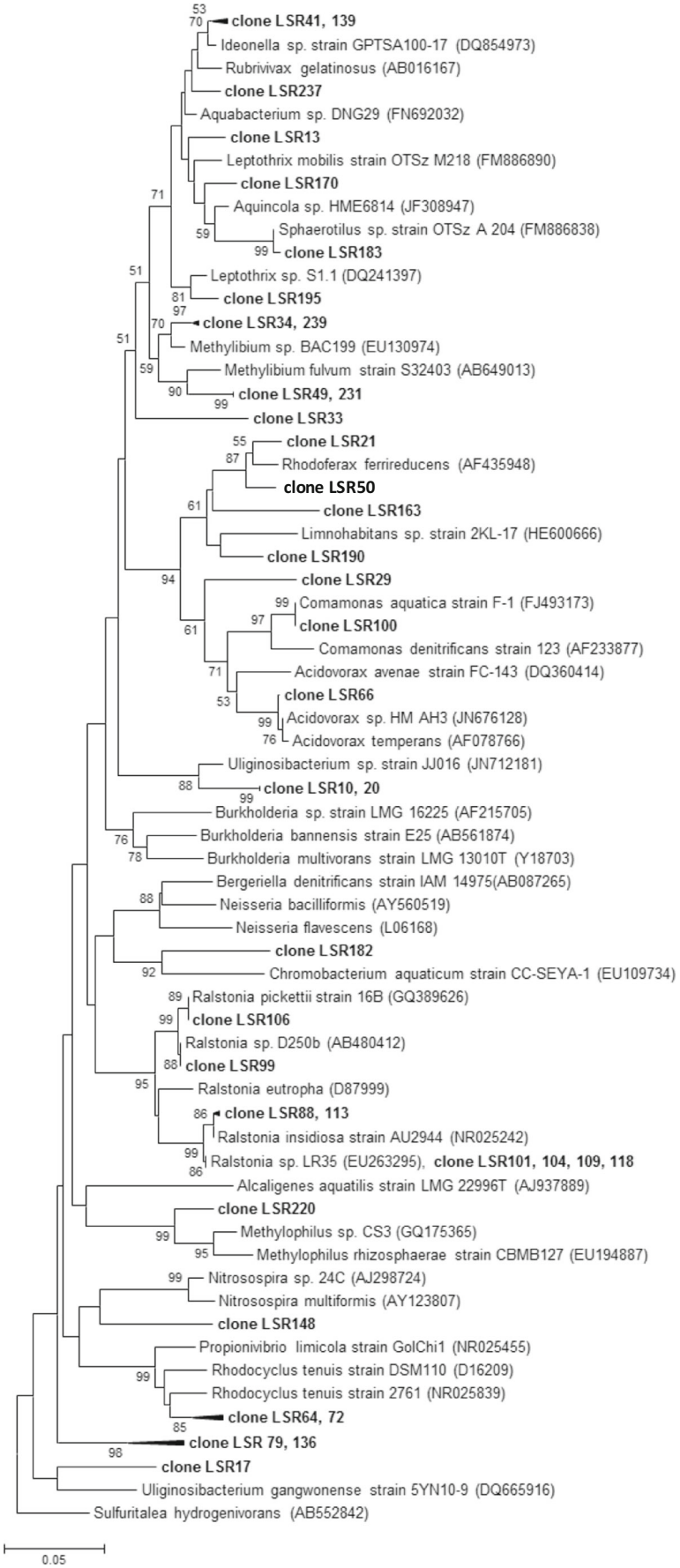
46), and to a lesser extent *Nitrospira* (LSR148) related populations known as indigenous inhabitants of aquatic rhizospheres (Beier et al. 2010; Wei et al. 2011). About 13% of the clones were related to known aerobic methylotrophic bacteria, some of them known as methanotrophic microorganisms within the *Gammaproteobacteria* (15 clones affiliated to *Methylococcaceae*; Fig. 6, sup. data) and the *Alphaproteobacteria* (LSR18 affiliated to *Methylocystis* sp.; Fig. 2). Wetlands and the rhizosphere of emergent aquatic plants are known as methane production systems, and methane oxidation activity is reported in such niches (Gerard and Chanton 1993; Calhoun and King 1997; Conrad 2009). Even if not investigated in this study, methanogens are probably present in the periphyton of *Ludwigia* sp. and were, for example, detected through 16S rRNA-based amplification in periphyton in other freshwater environments and are responsible for MeHg production (Hamelin et al. 2011).

As already reported in similar studies based on about 100 clones (Stout and Nusslein 2005; Matsuzawa et al. 2010), the other main bacterial phyla represented were the *Acidobacteria* (7% of total clones; mainly the genera *Geothhrix* sp.), the *Bacteroidetes* (5% of total clones; mainly *Cytophagaceae* and *Sphingobacteriaceae*), and the *Chloroflexi* (4% of total clones; *Anaerolineaceae)*. Sequencing revealed that 10% of total clones were affiliated to Eukarya (algae chloroplasts from diatoms). The root treatment prior to DNA extraction (washing with pyrophosphate) probably prevented the detection of the plant DNA. Nevertheless, the primer set used here (Marchesi et al. 1998) allowed the detection of eukaryotic primary producers, known to colonize periphyton. The presence of multicellular filamentous *Cyanobacteria* (6% of total clones) belonging to the genera *Phormidium*, *Calothrix*, and *Oscillatoria* were also detected.

Since the floating roots are found at the water surface, sufficient light is provided for primary production through microbial photosynthesis and influence the global activity of the community. The role of phototrophs, including algae and bacteria, in the Hg cycle is largely neglected, whereas they constitute the dominant part of the periphytic biofilms, influence Hg bioavailability (redox state, sequestration) (Grégoire and Poulain, 2014), and have the ability to accumulate Hg (Mason et al. 1996; Miles et al. 2001). Lefebvre et al. (2007) highlighted the biotransformation of $Hg^{II}$ into meta-cinnabar (β-HgS)—a stable complex of Hg, by cyanobacteria, including one belonging to *Phormidium* (*Phormidium limnetica*). Direct methylation also seems possible by algae, even if the mechanisms are unknown (Deng et al. 2013; Pongratz and Heumann, 1998). Leclerc et al. (2015) have recently demonstrated that exudates produced by algae in a periphytic biofilm could increase Hg bioavailability, showing the indirect effect

**Fig. 2** *Alphaproteobacteria* phylogenetic relationships based on the alignment of 16S rRNA gene sequences (680 pb) of clones (accession numbers: LT821141-LT821185) isolated from the periphyton of *Ludwigia* sp. roots (Sanguinet lake). Tree was constructed using the neighbor-joining method. Bootstrap percentages above 50% (1000 bootstrap replicates) are indicated at the branching points. The *scale bar* represents nucleotide substitutions. GenBank accession numbers are shown in *parentheses*. Tree was rooted with *ECORRDB E. coli* (M25588)


Rhodobacter blasticus strain ATCC 33485(NR043735)
clone LSR129
clone LSR97
Rhodobacter sp. 1strain 6-62(HM124369)
clone LSR137
clone LSR103
Rhodobacter sp. strain RCRI19 (HQ392507)
clone LSR108
Paracoccus marcusii strain R-42686(FR691418)
clone LSR110
Rhodovulum sp. strain D4028 (FJ161259)
Thioclava sp. strain JC73/BaS (FN547925)
clone LSR12
Rhodovulum sulfidophilum strain DSM1374 (RDPD1374F)
Hirschia baltica strain DSM 5838T (AJ421782)
clone LSR42, 56, 149, 188
Mesorhizobium albiziae strain CCBAU 61158 (DQ100066)
Mesorhizobium metallidurans strain STM 2683T (AM930381)
Rhizobium oryzae strain Alt 505 (EU056823)
Rhizobium gallicum (U86343)
Devosia sp. strain R-43964(FR691415)
clone LSR156
clone LSR146
clone LSR45
clone LSR122
Woodsholea maritima strain OTSzA272(FM886859)
clone LSR06
Hyphomicrobium facile strain IFAM B522(Y14312)
clone LSR47
Hyphomicrobium denitrificans strain DSM 1869 (NR026431)
Hyphomicrobium sp. strain Ellin112(AF408954)
clone LSR63
Prosthecomicrobium consociatum strain 11(NR044600)
Hyphomicrobium zavarzinii strain ZV-580(Y14306)
Hyphomicrobium vulgare(AB543807)
clone LSR162, 223
Rhodoplanes sp. strain Z2-YC6860(GQ369128)
clone LSR216
Methylocystis sp. H2s(FN422003)
clone LSR18
Methylocystis heyerii strain Sakb1(AM285681)
Beijerinckia fluminensis strain LMG 2819 (EU401905)
Beijerinckia mobilis strain DSM 2326 (AJ563932)
Methylocapsa acidiphila strain B2 (AJ278726)
clone LSR09, 65, 204
clone LSR73, 173
Rhodomicrobium vannielii strain DSM 162 (FN666247)
clone LSR126
Azospirillum brasilense strain ATCC 29145 (AY324110)
Azospirillum.amazonense strain DSM 2787 (X79735)
Rhodospira trueperi (X99671)
Rhodospirillum sulfurexigens strain JA143T (AM710622)
Methylobacterium oryzae strain CBMB20 (AY683045)
Methylobacterium aquaticum strain GR16T (AJ635303)
Bradyrhizobium jicamae strain PAC68 (AY624134) , clone LSR90, 120, 191
clone LSR145
Bradyrhizobium betae (AY372184)
Bradyrhizobium sp. strain P52(AM411921)
clone LSR229
Bradyrhizobium sp. strain B071 (AF193818)
clone LSR37,46
Phenylobacterium composti strain 4T-6 (EU022524)
Phenylobacterium koreense (AB166881)
Brevundimonas sp. strain CCBAU 10915(JF772569)
Brevundimonas sp. strain sptzw16(GU377106)
clone LSR167
Azospirillum diazotrophicus (X75618)
clone LSR169
Sandarakinorhabdus sp. strainA14 (EU770266)
Sphingomonas sp. HL7 (AB074191)
Erythrobacter sp. p52(2011) (HQ652571)
Porphyrobacter sp. strain SNRB307(AB299699)
clone LSR228
Sphingomonas sp. strain CCGE4118(GU980234)
clone LSR168
clone LSR140
Sphingomonas natatoria (AB024288)
clone LSR25, 43, 51, 53, 164
0.05


clone LSR41, 139
Ideonella sp. strain GPTSA100-17 (DQ854973)
Rubrivivax gelatinosus (AB016167)
clone LSR237
Aquabacterium sp. DNG29 (FN692032)
clone LSR13
Leptothrix mobilis strain OTSz M218 (FM886890)
clone LSR170
Aquincola sp. HME6814 (JF308947)
Sphaerotilus sp. strain OTSz A 204 (FM886838)
clone LSR183
Leptothrix sp. S1.1 (DQ241397)
clone LSR195
clone LSR34, 239
Methylibium sp. BAC199 (EU130974)
Methylibium fulvum strain S32403 (AB649013)
clone LSR49, 231
clone LSR33
clone LSR21
Rhodoferax ferrireducens (AF435948)
clone LSR50
clone LSR163
Limnohabitans sp. strain 2KL-17 (HE600666)
clone LSR190
clone LSR29
Comamonas aquatica strain F-1 (FJ493173)
clone LSR100
Comamonas denitrificans strain 123 (AF233877)
Acidovorax avenae strain FC-143 (DQ360414)
clone LSR66
Acidovorax sp. HM AH3 (JN676128)
Acidovorax temperans (AF078766)
Uliginosibacterium sp. strain JJ016 (JN712181)
clone LSR10, 20
Burkholderia sp. strain LMG 16225 (AF215705)
Burkholderia bannensis strain E25 (AB561874)
Burkholderia multivorans strain LMG 13010T (Y18703)
Bergeriella denitrificans strain IAM 14975(AB087265)
Neisseria bacilliformis (AY560519)
Neisseria flavescens (L06168)
clone LSR182
Chromobacterium aquaticum strain CC-SEYA-1 (EU109734)
Ralstonia pickettii strain 16B (GQ389626)
clone LSR106
Ralstonia sp. D250b (AB480412)
clone LSR99
Ralstonia eutropha (D87999)
clone LSR88, 113
Ralstonia insidiosa strain AU2944 (NR025242)
Ralstonia sp. LR35 (EU263295), clone LSR101, 104, 109, 118
Alcaligenes aquatilis strain LMG 22996T (AJ937889)
clone LSR220
Methylophilus sp. CS3 (GQ175365)
Methylophilus rhizosphaerae strain CBMB127 (EU194887)
Nitrosospira sp. 24C (AJ298724)
Nitrosospira multiformis (AY123807)
clone LSR148
Propionivibrio limicola strain GolChi1 (NR025455)
Rhodocyclus tenuis strain DSM110 (D16209)
Rhodocyclus tenuis strain 2761 (NR025839)
clone LSR64, 72
clone LSR 79, 136
clone LSR17
Uliginosibacterium gangwonense strain 5YN10-9 (DQ665916)
Sulfuritalea hydrogenivorans (AB552842)
0.05

◀ **Fig. 3** *Betaproteobacteria* phylogenetic relationships based on the alignment of 16S rRNA gene sequences (468 pb) of clones (accession numbers: LT821104-LT821140) isolated from the periphyton of *Ludwigia* sp. roots (Sanguinet lake). Tree was constructed using the neighbor-joining method. Bootstrap percentages above 50% (1000 bootstrap replicates) are indicated at the branching points. The *scale bar* represents nucleotide substitutions. GenBank accession numbers are shown in *parentheses*. Tree was rooted with *ECORRDB E. coli* (M25588)

of such periphytic phototrophic microorganisms on potential MeHg production by other microorganisms.

Periphyton is characterized by oxygen fluxes coming from the roots themselves (Christensen et al. 1994), diffusion from the water column and in situ production. Organic material also originated from the roots (exudates) and indigenous primary production. The periphyton is thus an organic rich matrix and the amount of organic matter can be estimated to be about 40% dry weight (Gentès et al. 2013a). This large amount of organic material and the concomitant presence of oxygen enhance aerobic respiration and carbon degradation in the periphyton. Ecoplates revealed high activity potentials within these periphytic communities (data not shown) as compared to sediment and water. Periphyton can be considered as a hotspot for microbial activity within the water column. Many heterotrophic aerobes or facultative anaerobes were detected in the clone library, most of them within *Proteobacteria* (see Figs. 2, 3, and 4). Aerobic activity may lead to micro-oxic conditions in the periphyton, and as it occurs in microbial mats for example, the anoxia may be transient during the day-night cycle (Van Gemerden 1993).

In addition to methanogens, whose presence is highly suspected, some other anaerobes were detected within the bacterial community. They comprise many purple non-sulfur bacteria (PNSB) belonging to the genera *Rhodobacter* (LSR129, 97, 137, 103, 108), *Rhodoplanes* (LSR216),

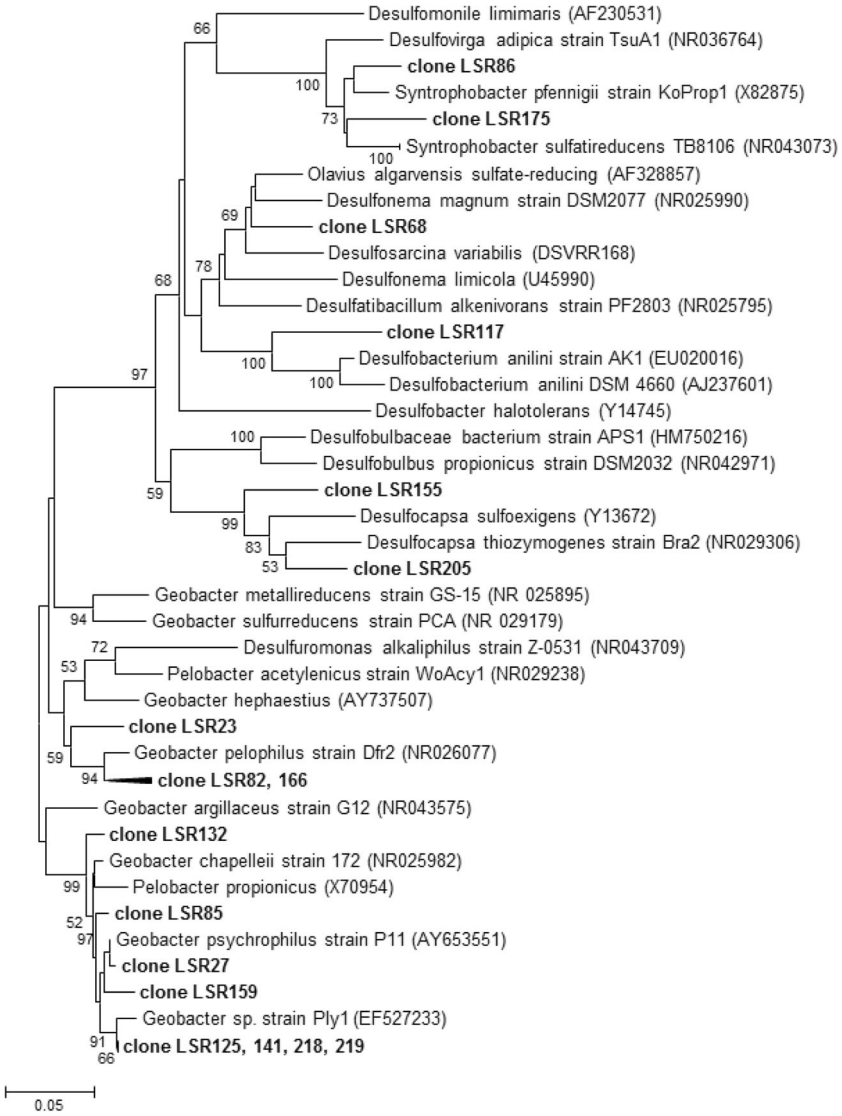



**Fig. 4** *Deltaproteobacteria* phylogenetic relationships based on the alignment of 16S rRNA gene sequences (676 pb) of clones (accession numbers: LT821206-LT821222) isolated from the periphyton of *Ludwigia sp.* roots (Sanguinet lake). Tree was constructed using the neighbor-joining method. Bootstrap percentages above 50% (1000 bootstrap replicates) are indicated at the branching points. The *scale bar* represents nucleotide substitutions. GenBank accession numbers are shown in *parentheses*. Tree was rooted with *ECORRDB E. coli* (M25588)

*Rhodomicrobium* (LSR73, 173) (Fig. 2), and *Rhocyclus* (LSR64,72) and *Rhodoferax* (LSR21,50,163) (Fig. 3). PNSB can use either reduced sulfur compounds or small organic molecules (fatty acids, alcohol, and sugars) as electron donors and/or carbon sources during anoxygenic photosynthesis. Some PNSB are also able to use $Hg^{II}$ as an electron sink when no other electron donor is present, showing their capacity to modify Hg availability and demonstrating that Hg can fulfill a physiological function in bacteria (Grégoire and Poulain 2016). Other anaerobes also comprise representatives of the sulfate and iron-reducing bacteria within *Deltaproteobacteria* (Fig. 4). Iron-reducing members related to the *Desulfuromonadales* were represented by *Geobacter*-related populations (11 clones representing about 9% of identified *Proteobacteria*; Figs. 1b and 4) and may be involved in Hg methylation (Kerin et al. 2006; Flemming et al. 2006; Yu et al. 2010). In order to determine the Hg methylation potential among these iron-reducing populations, specific isolation procedures should be conducted and the isolated strains tested for MeHg production. In this study, this was done for the sulfate-reducing bacteria whose key role in Hg methylation had been demonstrated in such environmental niches (Gentès et al. 2013a).

## Sulfate-reducing bacteria in the periphyton of *Ludwigia* sp. floating roots

Among the 244 clones sequenced in this study, just four clones were unambiguously related to the *Desulfobacter*ales, including two *Desulfobacteraceae* (LSR68 and LSR117) and two *Desulfobulbaceae* (LSR155 and LSR205) (Figs. 1b and 4). A dominance of representatives of these two families through the molecular approach is in good agreement with previous molecular studies, based on clone libraries, applied on intertidal sediment (Purdy et al. 1997, 2002; Joulian et al. 2001; Bahr et al. 2005; Mussmann et al. 2005). Such results also agreed with a RT-PCR study applied to macrophyte floating roots that detected *Desulfobacteraceae* among the main sulfate-reducing microorganisms (Acha et al. 2011). The same study also revealed that *Desulfovibrionales* represented up to 6.8% of the total bacterial community. In our study, members of *Desulfovibrionales* were exclusively detected through the cultivation method (15 strains), suggesting that the microplate isolation technique complemented the molecular approach for the description of sulfate-reducing populations. The differences between the techniques are probably due to technical limits of (i) the in vitro cultivation (Colin et al. 2012) favoring

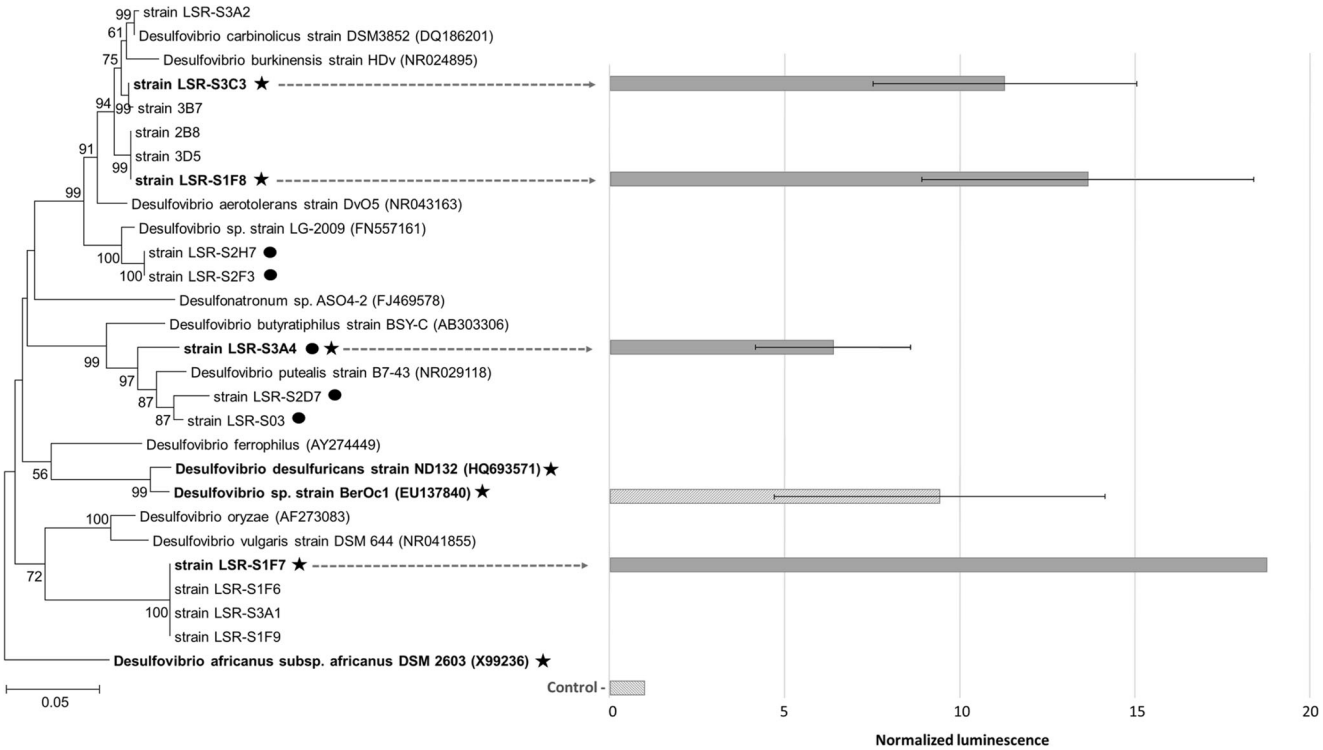



**Fig. 5** Phylogenetic tree based on the alignment of 16S rRNA gene sequences (541 pb) of strains (accession numbers: LT821089-LT821103) isolated from the periphyton of *Ludwigia* sp. roots (Sanguinet lake) belonging to Desulfovibrionales. Their Hg methylation potential was evaluated with the biosensor MC1061. *Stars* indicate methylator strains (through luminescence intensity or literature), *circles* indicate new taxa. *D. strain BerOc 1*: positive control for Hg methylation; culture media: negative control. Tree was constructed using the neighbor-joining method. Bootstrap percentages above 50% (1000 bootstrap replicates) are indicated at the branching points. The *scale bar* represents nucleotide substitutions. GenBank accession numbers are shown in *parentheses*. Tree was rooted with *ECORRDB E. coli* (M25588)

*Desulfovibrio* species in a low sulfate environment (Vladar et al. 2008; Laanbroek and Pfennig, 1981, Laanbroek et al., 1984) and (ii) the PCR primer specificity that favors *Desulfobacteraceae* representatives (Leloup et al. 2005; Purdy et al. 1997; Detmers et al. 2004). Calculations from the dilution-to-extinction microplate technique showed that *Desulfovibrio* accounted for around $1.5.10^4$ cells g periphyton and therefore is an important functional bacterial group in aquatic rhizospheres. The question of their participation in Hg methylation can be answered once they are isolated in pure culture.

### Mercury methylation capacities of the sulfate-reducing isolates

The discovery by Parks et al. (2013) of two genes required for Hg methylation is great and promising progress for understanding Hg transformation mechanisms. However, the lack of relationship between the gene expression levels and Hg methylation potential (Goñi-Urriza et al., 2015; Bravo et al. 2016) requires the use of other tools to identify Hg methylating bacterial strains. The biosensor-based procedure developed by Colin et al. (submitted) appears to be a reliable and relatively accurate tool for studying such capacities of microorganisms.

Some of the isolates obtained here probably represent new taxa among the family *Desulfovibrionaceae* (strains LSR-S2H7, LSR-S2F3, LSR-S2D7, LSR-S3, 3A4; Fig. 5, black circles) and should be further characterized and described. All the sulfate-reducing isolates have been tested for their methylation capacities using a biosensor that detects the MeHg produced. Among the 15 strains tested, four gave a positive result (strains LSR-S3C3, LSR-S1F8, LSR-S3A4, LSR-S1F7; Fig. 5, black stars). The remaining 11 strains did not show Hg methylation capacities or the MeHg produced was below the detection limit.

As already proposed (Ranchou-Peyruse et al. 2009; Gilmour et al. 2011), the methylation capacity is strain dependent since closely related strains exhibited different responses. For example, strain LSR-S1F7 showed high methylation capacity whereas other strains from this group (LSR-S1F6, LSR-S3A1, LSR-S1F9) did not. It is therefore impossible to predict methylation capacity (i.e., risk assessment) by studying sulfate-reducing bacteria diversity and we lack information on hgcA-hgcB diversity studies. The isolations of microorganisms harvesting the genes required for Hg methylation are necessary to achieve this goal. Effort must be placed on (i) the isolation procedures in order to gain a better view of Hg methylation distribution among the phylogenetic spectrum, (ii) epigenetics to better understand relations between hgcAB genes and environmental factors, and (iii) study the biodiversity of hgcA and the link between hgcAB expression level and Hg methylation.

The occurrence of organic matter from the plant exudates and from autochthonous production (oxygenic and anoxygenic photosynthesis, chemosynthesis) makes the periphyton extremely reactive in the ecosystem. This biogeochemical environment and organisms/community interactions are central for Hg methylation capacity of such strains. It is important to keep in mind that culture medium and growth conditions used in this study are far too mimic natural processes occurring in periphyton. One could be assumed that the metabolic activity of these microorganisms, including mercury methylation, may differ under natural conditions. The measurement of Hg methylation potential for the periphyton with the biosensor is not achievable for the moment. Indeed, the presence of inhibitors (sulfur compounds) tends to prevent the biosensor functioning (Colin et al. submitted). However, in the study of Gentès et al. (2013a), high Hg methylation potentials were measured in the periphyton of *Ludwigia* sp. aquatic roots from Sanguinet lake and were demonstrated to be principally due to sulfate reducers.

## Conclusion

Periphyton harvest complex bacterial and archaeal communities which participate in biogeochemical cycles, including metal cycles such as Hg (through transformations and complexation). The high abundance of organic material, the presence of anoxic micro niches, the optimal sulfate concentrations in this lake, and the capacity of biofilms to concentrate metals together provide the conditions for a hotspot of Hg methylation within the periphyton.

In this study, the 16S rRNA diversity revealed the presence of several families involved in the sulfur biogeochemical cycle, and four *Desulfovibrio* strains were identified as new Hg-methylators, with some of them belonging to new cultivated taxa. However, the role played by those *Desulfovibrio* strains in mercury methylation processes in situ remains difficult to determine. The amplification of the HgcAB genes from sulfate-reducing isolates will probably help us in the future to link HgcAB expression level and Hg methylation in periphyton and to better characterize microbes playing a key role in these transformations.

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