Sulfate-Reducing Bacteria in Floating Macrophyte Rhizospheres from an Amazonian Floodplain Lake in Bolivia and Their Association with Hg Methylation

Darío Achá,[1]* Volga Iñiguez,[1] Marc Roulet,[2] Jean Remy Davée Guimarães,[3] Ruddy Luna,[1]
Lucia Alanoca,[2] and Samanta Sanchez[1]

*Instituto de Biología Molecular y Biotecnología, Facultad de Ciencias Puras, Universidad Mayor de San Andrés, C. 27 Campus
Universitario Cota Cota, La Paz, Bolivia[1]; Institut de Recherche pour le Developpement, HYBAM,
UMR154-LMTG, CP 9214, La Paz, Bolivia[2]; and Laboratorio de Traçadores, IBCCF,
Bloco G/CCS/Ilha do Fundão, Universidade Federal do Rio de Janeiro,
Rio de Janeiro, Brazil[3]*

Five subgroups of sulfate-reducing bacteria (SRB) were detected by PCR in three macrophyte rhizospheres (*Polygonum densiflorum*, *Hymenachne donacifolia*, and *Ludwigia helminthorriza*) and three subgroups in *Eichhornia crassipes* from La Granja, a floodplain lake from the upper Madeira basin. The SRB community varied according to the macrophyte species but with different degrees of association with their roots. The rhizosphere of the C_4 plant *Polygonum densiflorum* had higher frequencies of SRB subgroups as well as higher mercury methylation potentials (27.5 to 36.1%) and carbon (16.06 ± 5.40%), nitrogen (2.03 ± 0.64%), Hg (94.50 ± 6.86 ng Hg g^{-1}), and methylmercury (8.25 ± 1.45 ng Hg g^{-1}) contents than the rhizosphere of the C_3 plant *Eichhornia crassipes*. Mercury methylation in *Polygonum densiflorum* and *Eichhornia crassipes* was reduced when SRB metabolism was inhibited by sodium molybdate.

Mercury contamination in the Amazon basin constitutes a significant threat to human and ecosystem health. The most important human exposure to Hg is through fish consumption (17, 28). Mercury in fish is found mainly as methylmercury (MeHg), which is the most neurotoxic and bioaccumulable form of mercury. Mercury in the Amazon basin is believed to originate mainly from soil erosion and gold-mining activities (35, 36). In both cases, Hg is introduced to the ecosystem in its inorganic form and then transformed into methylmercury, which bioaccumulates through the food web until it arrives in fish and humans (26). Studies have demonstrated very high net mercury methylation potentials in the periphyton associated with floating macrophyte roots, up to 30 times higher than in the sediments (14, 29). However, it is not yet clear which microorganisms are responsible for this methylation and how the produced MeHg enters and bioaccumulates through the food web.

Several studies, mainly from the Northern Hemisphere, have demonstrated that most mercury methylation is mediated by sulfate-reducing bacteria (SRB) (3, 24, 27). These bacteria are a phylogenetically and physiologically diverse group with important roles in anaerobic environments (10). SRB are defined by their capacity to utilize sulfate as the final electron acceptor, reducing it in a disassimilatory manner (20). Despite the fact that SRB are considered anaerobic, they have been detected under aerotolerant and even aerobic conditions (19, 21, 30).

Although links between sulfate reduction and mercury methylation have been found in Amazonian sediments and in periphyton associated with floating macrophyte roots (16), no direct SRB determinations have yet been made in these matrixes. SRB have been found to be associated with marine macrophyte roots (19, 33). However, the floating macrophyte rhizospheres in these Amazonian lakes are far from sediments, are close to the surface, and are surrounded by highly aerobic water; they appear to be unusual environments for these mainly anaerobic bacteria. Therefore, we hypothesized that only a few, if any, SRB were present or viable in these micro-environments and, consequently, that they would not play a role in mercury methylation.

This study examined the presence and distribution of six SRB subgroups (*Desulfotomaculum*, *Desulfobacter*, *Desulfobacterium*, *Desulfococcus-Desulfonema-Desulfosarcina*, *Desulfobulbus*, and *Desulfovibrio-Desulfomicrobium*) (10) associated with four floating macrophyte rhizospheres (*Polygonum densiflorum*, *Hymenachne donacifolia*, *Ludwigia helminthorriza*, and *Eichhornia crassipes*) in the oxbow lake La Granja. This lake is located in the floodplain of the Beni River subbasin, which is situated in Bolivia and is part of the Amazon basin. The presence of SRB was examined along with mercury methylation potential and total mercury, methylmercury, nitrogen, and organic carbon contents in the root-associated periphyton.

Sampling and sample treatment. Most samples were collected in February 2003, at the end of the rainy season. Additional samples were collected in November 2004 and May 2005 for mercury methylation experiments. Floating roots from four macrophyte species were manually collected at a distance of 0.5 m or more from the edge of the macrophyte meadow and sealed in zip-lock bags filled with lake water. Care was taken during sampling to minimize losses of periphyton, fine detritus, and sediments attached to the roots. Physicochemical charac-

* Corresponding author. Mailing address: Watershead Ecosystems Graduate Programe, Trent University, Symons Campus, 1600 West Bank Drive, Peterborough, ON K9J 7B8, Canada. Phone: (705) 740-9030. Fax: (705) 748-1625. E-mail: darioacha@yahoo.ca.

TABLE 1. Comparison of levels of Hg and MeHg, percentages of MeHg, C and N concentrations, and ratios between
macrophyte periphyton[a]

Macrophyte species	n^b	Hg level (ng g^{-1})	MeHg level (ng Hg g^{-1})	MeHg (%)	C (%)	N (%)
Polygonum densiflorum	4	94.5 ± 6.9	8.3 ± 1.7	8.7 ± 1.5	16.1 ± 5.4	2.0 ± 0.6
Hymenachne donacifolia	3	70.7 ± 6.9	4.7 ± 1.7	6.7 ± 3.0	8.6 ± 5.0	1.2 ± 0.6
Ludwigia helminthorrhiza	2	72.0 ± 7.8	4.5 ± 1.0	6.3 ± 2.1	6.6 ± 4.8	1.0 ± 0.6
Eichhornia crassipes	3	71.7 ± 7.7	4.7 ± 0.7	6.6 ± 1.5	8.0 ± 3.7	1.2 ± 0.5

[a] Values are means ± standard deviations.
[b] *n*, number of samples analyzed.

teristics (pH, redox potential, conductivity, and dissolved oxygen) of the water surrounding the roots were measured with a series of specific electrodes. After collection, part of the root-associated periphyton was concentrated by a series of root washings and centrifugation at 1,000 rpm (for approximately 5 min) and used for methylation potential measurements. The remaining sample was kept at $-18°C$ until it was used for further manipulations (DNA extraction and geochemical analysis). Samples for DNA extraction were divided into three fractions. The first fraction consisted of whole roots with their associated material, the second of isolated periphyton, and the third of roots without periphyton.

At all locations, the water pH was about 7, and conductivity was between 199 and 250 $\mu s \cdot cm^{-1}$. Dissolved oxygen (1.52 to 4.37 mg $\cdot$ liter^{-1}) and redox potential ($+113$ to $+350$ mV) revealed that the environment surrounding macrophyte roots was highly aerobic and oxidative during the day. SRB, which were frequently related to highly reductive anaerobic environments (2), could have developed biochemical systems to protect themselves from oxygen exposure (4, 42) or could be protected inside anaerobic compartments (41). SRB can create an anoxic layer by producing H_2S that consumes oxygen (21) and have been detected inside roots (25), where they can escape from O_2. SRB may also develop in periphyton (19, 21), which has a biofilm-like structure, where conditions can be significantly different from the environment surrounding the roots (21, 37).

Mercury methylation potential, total mercury, and methylmercury. All periphyton samples were incubated for 24 h in the dark at in situ water temperature (22 to 28°C), in Teflon-lined, screw-cap, 50-ml borosilicate tubes with 30 ml of filtered lake water. Duplicate or triplicate samples and a control killed with 1 ml of 4 N HCl received 10 nCi (370 Bq) $^{203}HgCl_2$, obtained from Isotope Products Laboratory, Valencia, CA (February 2003), and Georgia State University, Atlanta, GA (November 2004 and May 2005). The fresh, incubated samples were equivalent to 0.5 g (dry weight), and the total added Hg concentrations ranged from 40 to 800 ng Hg g^{-1} (dry weight). Hg methylation was stopped by the addition of 1 ml of 4 N HCl, and the samples were frozen until MeHg extraction. Mercury was extracted using a simplified technique described in more detail by Guimarães et al. (15). Mercury methylation was ≥ 36 times greater in the C_4 plant *Polygonum densiflorum* (31.83%) than in the C_3 plant *Eichhornia crassipes* ($\leq 0.02\%$), and the latter also showed low or undetectable mercury methylation in the November 2004 and May 2005 experiments. Sediment surface methylation potential (6.39%) (1) at the center of the lake, an area not covered by macrophytes, was also significantly lower than methylation in *Polygonum densiflorum* periphyton

($P < 0.05$), which is consistent with other studies in the Amazon (14, 29). Additionally, inhibiting SRB by adding sodium molybdate (20 mM final concentration) during the incubation of *Eichhornia crassipes* and *Polygonum densiflorum* rhizospheres and sediment samples reduced mercury methylation potentials to 12, 0.7, and 15% of the untreated samples, respectively. This suggests an important role of SRB in mercury methylation in these matrixes.

The Hg and MeHg analyses were performed by cold-vapor, atomic fluorescence spectrometry following a modification of the method developed by Bloom and Fitzgerald (6). The technique is described in greater detail by Pichet et al. (31). The levels of total Hg and MeHg and the percentage of MeHg were significantly higher in *Polygonum densiflorum* periphyton than in the periphyton of the other macrophytes (Table 1). The periphytons from the other three macrophytes had approximately the same concentrations of Hg and MeHg and the same percentages of MeHg. Similarly, carbon and nitrogen were more abundant in *Polygonum densiflorum* periphyton than in any other macrophyte, and the organic matter quality (atomic C/N ratio) was significantly different in this macrophyte (Table 1).

The consistency in the difference in methylmercury concentration, mercury methylation, and carbon and nitrogen content between *Polygonum densiflorum* and *Eichhornia crassipes* is not surprising because MeHg concentrations have previously been linked to organic matter quality and quantity (24, 34). Organic matter in the rhizosphere comes mainly from dead leaves, root exudates (25, 38), and photosynthetic periphyton. These carbon sources plus anatomical and physiological variations relevant to periphyton growth may explain the observed differences in MeHg production between macrophytes (29). Furthermore, some studies have found SRB to be closely associated with macrophytes (19, 25), and therefore, a direct impact of the macrophyte carbon contribution could be expected, at least for some of the SRB subgroups. Nevertheless, it should be pointed out that significantly higher methylation potentials have been reported for *Eichhornia crassipes* (14, 29) than were found in this study, suggesting that factors other than the macrophyte carbon contribution play a role in controlling mercury methylation potentials and MeHg concentrations. Differences between mercury methylation potentials reported could also be attributed to the growth cycle of the plant (12, 19, 23) or to the surrounding conditions.

Strain culture and sample incubation. *Polygonum densiflorum* roots of 1 cm^3 and their associated material were mixed with 10 ml of sterile water. The mixture was diluted by a factor of 10^{-2} and 10^{-3} in Widdel and Pfenning medium (45) supplemented with lactate, acetate, ethanol, benzoate, and propi-

TABLE 2. Comparison of the frequencies of SRB subgroups detected in different macrophyte species and fractions of the sample

Plant or sample type	Macrophyte	n^a	Frequency of SRB subgroup					
			Desulfoto-maculum	Desulfo-bulbus	Desulfo-bacterium	Desulfo-bacter	Desulfococcus-Desulfonema-Desulfosarcina	Desulfovibrio-Desulfomicrobium
C$_4$ plant	*Polygonum densiflorum*	12	0.55	0.55	0.00	0.27	1.00	1.00
	Hymenachne donacifolia	6	1.00	1.00	0.00	0.33	1.00	1.00
C$_3$ plant	*Ludwigia helminthorrhiza*	6	0.83	0.17	0.00	0.17	0.83	1.00
	Eichhornia crassipes	6	0.50	0.00	0.00	0.17	0.83	1.00
Roots only		10	0.60	0.40	0.00	0.30	0.90	1.00
Periphyton		10	0.80	0.60	0.00	0.10	1.00	1.00
Whole^b		10	0.60	0.30	0.00	0.30	0.90	1.00

[a] Number of samples analyzed.
[b] Roots with periphyton.

onate and incubated for 2 weeks. Then the consortia were cultured in medium supplemented with only one of the electron donors mentioned. Growth was observed in medium with lactate, ethanol, and benzoate and less clearly in medium with acetate. Each consortium was tested for its capacity to reduce sulfate in a Postgate B medium (32). The consortia grown with lactate and ethanol were positive by this sulfate reduction assay. The consortium grown in medium supplemented with all electron donors was evaluated by PCR, detecting three (*Desulfotomaculum*, *Desulfococcus-Desulfosarcina-Desulfonema*, and *Desulfovibrio-Desulfomicrobium*) of the six SRB subgroups tested.

Although most sulfate-reducing bacteria are not cultivable (18, 22) and these data could not be fully comparable to data from culture-independent assays, the growth in Widdel and Pfenning medium and the sulfate reduction in Postgate medium are direct probes of SRB presence. Furthermore, SRB viability shows that they are potentially active and may play a role in mercury speciation, as suggested by the above-mentioned reduction in mercury methylation potentials in the presence of SRB inhibitors such as sodium molybdate. Still, the relative role of different SRB strains from Amazonian macrophytes in mercury methylation is yet to be established.

Nucleic acid extraction. DNA was extracted directly from each sample fraction with a modification of the soil DNA extraction method described by Zhou et al. (46). In this study, the sample mass was reduced to 300 mg, but the final concentration of the reagents was maintained.

PCR conditions, SRB detection, and dot blot hybridization. The 16S rRNA gene was amplified by PCR with fD1 and Rp2 primers (44). The reaction tubes (20 μl) contained 1× of reaction buffer B (Promega), 1.5 mM of MgCl$_2$, 0.2 mM of deoxynucleoside triphosphates (Promega), 0.5 μM of each primer, 0.04 U · μl^{-1} of *Taq* polymerase (Promega), 0.25 μg · μl^{-1} of bovine serum albumin (BSA), and 5 to 15 ng · μl^{-1} of DNA template. Amplification products were diluted 40-fold into a fresh reaction mixture containing one pair of the six pairs of SRB group-specific primers (for *Desulfomaculum*, *Desulfobacter*, *Desulfobacterium*, *Desulfococcus-Desulfonema-Desulfosarcina*, *Desulfobulbus*, and *Desulfovibrio-Desulfomicrobium*) (see supplementary table A at http://ca.geocities.com/darioacha

/tablea.pdf) (10). Each reaction comprised preheating at 95°C for 2 min, 30 cycles of denaturalization at 95°C for 1 min followed by annealing for 1 min and 72°C for 1 min, and finally 72°C for 5 min. All PCR products were electrophoresed in 1% agarose gel with ethidium bromide in 0.5× Tris-borate-EDTA buffer and then visualized by UV illumination. Reference strains for the six SRB subgroups, provided by Richard Devereux, were used as positive controls (*Desulfovibrio desulfuricans* ATCC 27774, *Desulfococcus multivorans*, *Desulfobulbus propionicus*, a *Desulfobacterium* sp., a *Desulfobacter* sp., and a *Desulfotomaculum* sp.)

Previously described hybridization oligonucleotides (see supplementary table B at http://ca.geocities.com/darioacha /tableb.pdf) (10) for each of the six SRB groups tested were used to verify the PCR products. Oligonucleotides were 3′ end labeled with nonradioactive dUTP-fluorescein with the Gene Images 3′-oligonucleotide module (Amersham) according to the manufacturer's instructions. PCR products were diluted and transferred to a positively charged nylon membrane (Pharmacia). After hybridization at optimized temperatures, dUTP-fluorescein-labeled PCR products were detected using the Gene Images CDP-Star detection module (Amersham). Hybridization was visualized by exposing the membranes to X-ray film. Temperatures were optimized with the same reference strains for PCR.

Nested PCR and dot blot hybridization revealed that five (*Desulfomaculum*, *Desulfobulbus*, *Desulfobacter*, *Desulfococcus-Desulfonema-Desulfosarcina*, and *Desulfovibrio-Desulfomicrobium*) of the six SRB subgroups were present in macrophyte roots. The *Desulfovibrio-Desulfomicrobium* subgroup was detected in all samples and the *Desulfococcus-Desulfonema-Desulfosarcina* subgroup was detected in most of them, while *Desulfobacterium* (mainly reported in saline environments) was never detected. *Desulfomaculum*, *Desulfobulbus*, and *Desulfobacter* were more frequently detected in *Hymenachne donacifolia* roots (Table 2), and subgroups *Desulfobulbus*, *Desulfobacter*, and *Desulfococcus-Desulfonema-Desulfosarcina* were, in general, more frequent in roots from C$_4$ plants *(Polygonum densiflorum* and *Hymenachne donacifolia)* (Table 2). Subgroups *Desulfomaculum*, *Desulfobulbus*, and *Desulfococcus-Desulfonema-Desulfosarcina* were more frequent in the per-

iphyton fraction, while *Desulfobacter* was more frequent in samples with whole roots with their associated material and in roots without periphyton.

The *Desulfovibrio-Desulfomicrobium* subgroup, detected in all samples, constitutes the *Desulfovibrionaceae* family (7, 11), which is the most studied (43) and is frequently detected in a large variety of environments (3, 19, 21). There are several reports of its capacity to tolerate aerobic or nearly aerobic conditions (4, 9, 39), which could make it ideal for inhabiting these particular macrophyte rhizospheres. Additionally, *Desulfovibrio* is considered to be an important group responsible for mercury methylation activity (3, 8, 24), and its presence could explain the high Hg methylation rates in macrophyte periphyton but not the observed differences between macrophytes. In fact, in this study, *Desulfovibrio-Desulfomicrobium* organisms were detected even in the rhizosphere of *Eichhornia crassipes* (Table 2), where mercury methylation potential was close to or below detection limits. Similarly, organisms of the *Desulfococcus-Desulfonema-Desulfosarcina* subgroup were detected in *Polygonum densiflorum* as well as in *Eichhornia crassipes*, despite the difference in mercury methylation potential. This is consistent with the fact that none of the genera of the *Desulfococcus-Desulfonema-Desulfosarcina* subgroup are considered to be highly important for MeHg production (24).

The *Desulfotomaculum* subgroup was also frequently detected, which is consistent with the fact that members of this subgroup have often been detected in freshwater environments (13). This subgroup has been detected in association with rice roots (40), where redox potential and diluted oxygen levels are likely similar to those found around macrophyte roots.

The *Desulfobulbus* subgroup was reported to be highly abundant and active in both anaerobic and aerobic layers of biofilms (30). It has been proposed that *Desulfobulbus* is probably one of the first SRB subgroups to colonize biofilms, creating suitable conditions for other SRB (37). Moreover, *Desulfobulbus* was isolated from oxygenated layers (30, 41), and O_2 seems to stimulate *Desulfobulbus* (19). Although *Desulfobulbus* is not considered to be one of the main mercury methylators in marine environments, there is evidence of its ability to produce MeHg (5). *Desulfobulbus* is significantly more abundant in association with C_4 plants, where the percentage of MeHg is also higher (Table 1).

Desulfobacter is more frequently detected in marine environments because of its apparent preference for high concentrations of NaCl and $MgCl_2$ (45). It might not be able to tolerate aerobic conditions (37, 41), which could explain its low frequency of detection in the macrophytes. In fact, it was rarely detected in periphyton samples but more frequently in root samples (Table 1). This is in agreement with *Desulfobacter*'s sensibility to oxygen exposure and suggests that *Desulfobacter* organisms live in or more closely attached to macrophytes than those of the other SRB subgroups. There are several reports that identify this group as one of the most active Hg methylators (3, 24, 27).

SRB methylate mercury at variable rates (24), and differences in the SRB community could explain the differences in the mercury methylation potential and MeHg concentrations between *Polygonum densiflorum* and *Eichhornia crassipes*. However, to our knowledge, SRB have been identified as main mercury methylators only in anaerobic environments (3, 8, 24),

and the possibility that other groups are the main Hg methylators in aerobic environments remains open. No direct relation between SRB and mercury methylation is established, but the presence of a diverse SRB community and the implication of SRB involvement in mercury methylation in this unusual environment was demonstrated. Further quantitative studies of the SRB community may help to clarify the picture.

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