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# Three-year survey of sulfate-reducing bacteria community structure in Carnoulès acid mine drainage (France), highly contaminated by arsenic

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## Keywords

sulfate-reducing bacteria diversity; *dsrAB*; acid mine drainage; T-RFLP; pH extreme environments; arsenic.

## Abstract

A 3-year survey on sulfate-reducing bacteria (SRB) was conducted in the waters of the arsenic-rich acid mine drainage (AMD) located at Carnoulès (France) to determine the influence of environmental parameters on their community structure. The source (S5 station) exhibited most extreme conditions with pH lowering to ~1.2; iron, sulfate, and arsenic concentrations reaching 6843, 29 593, and 638 mg L<sup>-1</sup>, respectively. The conditions were less extreme at the downstream stations SI (pH ~3.7; iron, sulfate, and arsenic concentrations of 1114, 4207, and 167 mg L<sup>-1</sup>, respectively) and COWG (pH ~3.4; iron, sulfate, and arsenic concentrations of 854, 3134, and 110 mg L<sup>-1</sup>, respectively). SRB community structures were characterized by terminal restriction fragment length polymorphism and library analyses based on *dsrAB* genes. The predominant *dsrAB* sequences detected were most similar to the family *Desulfobulbaceae*. Additionally, certain phylotypes could be related to spatio-temporal fluctuations of pH, iron, and arsenic species. For example, *Desulfohalobiaceae*-related sequences were detected at the most acidic sample (pH 1.4) with high iron and arsenic concentrations (6379 and 524 mg L<sup>-1</sup>, respectively). New *dsrAB* sequences, with no isolated representatives, were found exclusively in COWG. This study gives new insights on SRB community dynamics in AMD systems.

## Introduction

Mining of sulfide ores generally produces large volumes of pyrite-rich wastes (tailings). If oxygen-rich waters infiltrate the tailings, pyrite is quickly oxidized, producing acid water with high iron and sulfate contents, called acid mine drainage (AMD; Nordstrom & Alpers, 1999). This process involves a complex interplay between microorganisms, water, and minerals (Edwards *et al.*, 2000). AMD can occur naturally, but its occurrence is enhanced by mining activity that allows the contact of sulfidic rock with air or oxic water. The oxidation of sulfide minerals in mine waste material can produce high concentrations of dissolved sulfate and iron and generate acidic conditions that enhance mobility of dissolved metals/metalloids

(*e.g.* Cu, Pb, Zn, Hg, As) in the pore water. The high heavy metals concentration in the environment has toxic effects, such as the reduction on species richness and total biomass (Kelly, 1988). Many studies have investigated the use of sulfate-reducing bacteria (SRB) for AMD bioremediation purpose (Garcia *et al.*, 2001; Kappler & Dahl, 2001; Kaksonen *et al.*, 2003; Bijmans *et al.*, 2009, 2010) because SRB reduce sulfate to sulfide, which precipitates with metals and generates alkalinity. This reduction of sulfate is coupled with organic matter (CH<sub>2</sub>O) oxidation (1) followed by chemical reaction of metal (Me) precipitation (2). The balance Eqn. (3) conducts to the elimination of a strong acid (SO<sub>4</sub><sup>2-</sup>; pK<sub>a</sub> ~4) and the production of weak acid (H<sub>2</sub>CO<sub>3</sub>; pK<sub>a</sub> ~6.3):



Different metals, including Cd, Cu, Fe, Pb, Hg, Ni, and Zn, precipitate with sulfides, while metalloids such as As, Sb, and Mo form sulfide minerals. Previous studies showed efficient removal and precipitation of dissolved metals such as Cu and Zn in acidic liquors in pH-controlled bioreactors using sulfate-reducing bacteria (Garcia *et al.*, 2001; Kaksonen & Puhakka, 2007; Bijmans *et al.*, 2009). However, SRB optimum growth is between pH 5.0 and 9.0 (Grossman & Postgate, 1953; Widdel, 1988), but AMD pH is usually between 2.0 and 4.0 (Bechard *et al.*, 1994). The SRB able to precipitate metals in bioreactors could grow at pH 3.0 and above using glycerol or other non-ionic substrates as an electron donor (Lyew *et al.*, 1994; Kimura *et al.*, 2006; Johnson *et al.*, 2009; Behum *et al.*, 2011), demonstrating their metabolic versatility.

Sulfate-reducing bacteria have been isolated from oxic acidic as well as anoxic neutral tailings and are likely involved in sulfur cycling within those environments (Fortin & Beveridge, 1997; Blodau *et al.*, 1998; Friebe *et al.*, 1998; Wielinga *et al.*, 1999; Fortin *et al.*, 2000a; Kùsel *et al.*, 2009; Senko *et al.*, 2009; Alazard *et al.*, 2010). Although SRB are described as anoxic and neutrophilic bacteria, they can tolerate oxygen and low pH conditions (Dilling & Cypionka, 1990; Canfield & Desmarais, 1991). Moreover, the occurrence of SRB populations could be correlated with specific environmental conditions of pH, oxygen content, temperature, or redox potential (Edwards *et al.*, 1999; Fortin *et al.*, 2000a; Praharaj & Fortin, 2008). Recently, we demonstrated the presence of SRB in the Carnoulès AMD waters by 16S rRNA gene library analyses (Bruneel *et al.*, 2005). However, the SRB populations inhabiting the AMD environments have yet to be characterized. Therefore, to consider the potential role of SRB in natural bioremediation processes, it is crucial to describe the dominant SRB groups in AMD and heavy metal-polluted environments and to characterize their activity.

The aim of this article is to characterize the SRB community structure in the water of the Carnoulès AMD (France) and to correlate the community structure to changing environmental parameters. We performed a 3-year survey of SRB populations in the AMD waters originated from the abandoned Carnoulès mine, which, depending on the season, exhibit pH as low as 1.2 and extremely high arsenic concentrations (127–12 000 mg L<sup>-1</sup>). The SRB community diversity and dynamics were described by terminal restriction fragment

length polymorphism (T-RFLP) and library analyses targeting the *dsrAB* genes, specific to SRB. Physical-chemical parameters were monitored throughout the period, and the relationship between SRB populations and physical-chemical parameters were determined by multivariate analyses.

## Materials and methods

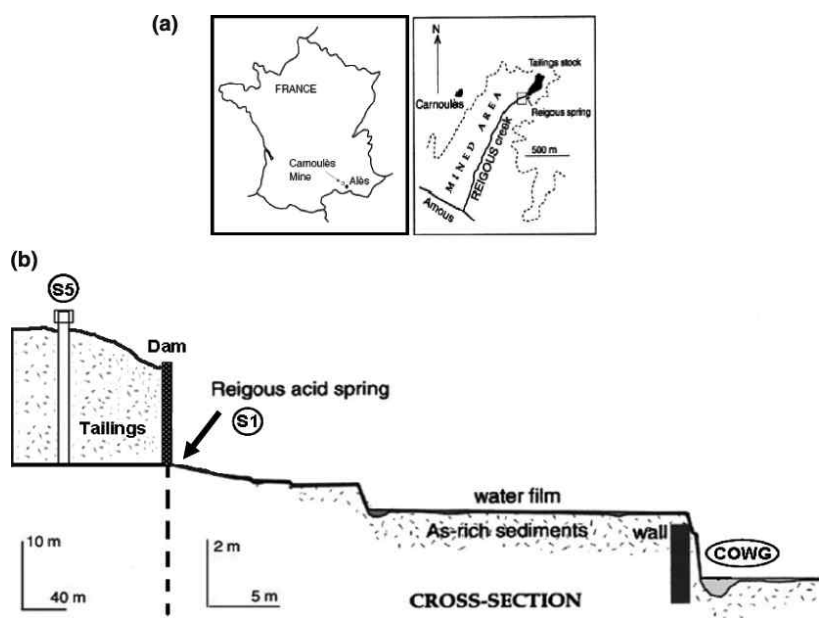
### Site description

The Carnoulès Pb–Zn mine [south France; (44°06' 41" N; 04°01' 27" E); Fig. 1a] is inactive since 1962. The exploitation of this mine generated approximately 1.5 Mt of tailings that are retained behind a dam. The tailings contain around 0.7% Pb, 10% FeS<sub>2</sub>, and 0.2% As (Leblanc *et al.*, 1996). Draining tailings waters exhibit strong variations of their chemical composition, which are controlled by dissolved oxygen concentrations, over the year (Casiot *et al.*, 2003a). At high oxygen levels, extremely low pH (pH < 1.8), high As (up to 12 000 mg L<sup>-1</sup>), and high Fe (up to 20 000 mg L<sup>-1</sup>) concentrations are reached, oxidized species being predominant. Under oxygen-deficient conditions, the pH increases, and the As and Fe concentrations decrease 10-fold due to As(V) and Fe(III) precipitation. An acidic (pH 2.2–4.0) anoxic spring results from the percolation of waters through the tailings (Casiot *et al.*, 2003b). This spring containing up to 350 mg As.L<sup>-1</sup>, 750–2700 mg Fe.L<sup>-1</sup>, and 2000–7500 mg SO<sub>4</sub><sup>2-</sup>.L<sup>-1</sup> (Leblanc *et al.*, 1996) emerges from the dam and generates the Reigous creek. Fe is present only in the reduced form Fe(II) in the Reigous spring water, while the mean proportion of As(III) reaches 60% (Egal *et al.*, 2010). During the first 30 m of downflow, Fe(II) is oxidized, which results in the removal of 20–60% As by coprecipitation with Fe(III) and formation of As(III)–Fe(III) and As(V)–Fe(III) phases (Casiot *et al.*, 2003b; Morin *et al.*, 2003).

### Sampling procedure

Waters from the Carnoulès AMD were collected in duplicates at three different stations (Fig. 1b): (1) at the center of the tailing tank to a borehole (S5 station), (2) at the source of the Reigous creek (S1 station), 600 m distance from S5, and (3) in the creek (COWG station), 30 m downstream from S1.

The main physical-chemical parameters (pH, temperature, and dissolved oxygen concentration) were measured monthly in the field. Temperature and pH were measured with an Ultrameter™ Model 6P (Myron L Company, Camlab, Cambridge). The concentration of dissolved oxygen was determined after reaction of O<sub>2</sub> with indigo



**Fig. 1.** (a) Location of the Carnoulès acid mine drainage study site and (b) cross-sectional view of the site showing sampling stations S5, S1, and COWG.

carmines (for the 0–12 mg O<sub>2</sub> L<sup>-1</sup> range) or with rhodazine D (for the 0–1 mg O<sub>2</sub> L<sup>-1</sup> range) using CHEMets® tests (CHEMetrics, Calverton, MD). The detection limit was 0.1 mg L<sup>-1</sup>. Some of the samples from the S5 station contained a high load of suspended particulate matter, which interfered in the measurement of oxygen concentration.

For chemical analysis, water samples were immediately filtered through 0.22-µm Millipore membranes using polycarbonate filter holders (Sartorius). Chemical analyses were performed in duplicates. For the determination of total Fe and As, filtered water was acidified to pH 1 with a 14.5 M HNO<sub>3</sub> solution and then stored at 4 °C until further analysis. To determine the As and Fe speciations, samples were preserved with EDTA and 1,10-phenanthroline chloride, respectively, as previously described (Bruneel *et al.*, 2008).

For dissolved organic carbon (DOC) analysis, waters were sampled in amber glass bottles and then filtered through glass-fiber filters GF6 (Whatman) when arriving to the laboratory. Bottles and filters used for sampling had been heated to 550 °C to remove organic residues. Filtered samples were stored in dark, preserved with H<sub>3</sub>PO<sub>4</sub> 1 : 1000 (v : v), and analyzed within 2 weeks.

The height of the water column in the borehole at station S5 was measured and registered automatically using a submerged pressure sensor (Druck-type PDCR) with data logger (Octopus, Hydreka).

For molecular analysis of sulfate-reducing bacterial community, single water samples were collected in

November 2004; January, April, August, September, and November 2005; April and July 2006; April, May, and July 2007. Three hundred millilitre of water was filtered through a sterile 0.22-µm nucleopore filter to retain all the bacteria whether adsorbed to particular matter or free in the water. The filters were flash-frozen in liquid nitrogen on site and stored at -20 °C until further analysis.

### Chemical analysis

Fe(II) concentrations were determined by the phenanthroline method (Tamura *et al.*, 1974). Briefly, an aliquot (20 µL) of filtered sample was buffered to pH 4.5 with 0.5 mL of an ammonium acetate/acetic acid buffer. Fe(II) complexes were formed by adding 1 mL of 1,10-phenanthroline chloride solution (0.5% w/v) and completed to 10 mL with deionized water. Absorbance was measured at 510 nm. The detection limit was 0.1 mg L<sup>-1</sup> and the analytical error <10% within the range 0.2–5 mg L<sup>-1</sup>. The concentration of total Fe was determined by Flame Atomic Absorption Spectrometry (Thermo Electron Corporation, France), with an analytical error <5%.

Sulfate concentrations were determined by nephelometry, after precipitation with BaCl<sub>2</sub>. The absorbance was measured at 650 nm (Rodier *et al.*, 1996). Dissolved organic carbon (DOC) was determined by the HTCO (high-temperature catalytic oxidation) method with a TOC-V<sub>CSH</sub> total organic carbon analyzer (Shimadzu).

Inorganic arsenic species (As(III), As(V)) were analyzed by anion-exchange chromatography (25 cm × 4.1 mm

i.d. Hamilton PRP- $\times 100$  column with Varian ProStar gradient solvent delivery system) coupled to hydride generation (HG; VGS 200, FISONS, France) equipped with an Atomic Fluorescence Spectrometry (AFS) detector (Excalibur, PSAnalytical, GB) as described previously (Casiot *et al.*, 2005) or to ICP-MS (Thermo X7 Series). Total arsenic concentration was measured with ICP-MS (VG-Elemental PQ2 + Fisons and Thermo X7 Series), and the result generally matched the sum of As(III) and As(V) species within  $\sim 15\%$  of the measured amount.

### DNA extraction and PCR conditions

DNA extractions of bacteria retained in the filters were carried out with the UltraClean Soil DNA Isolation kit (MoBio Laboratories, Inc.) following the manufacturer's recommendations. Prior to extraction procedures, the filters were cut out with a sterile scalpel. Extracted DNAs were stored at  $-20^\circ\text{C}$ .

PCRs mixtures in  $1\times$  PCR buffer were as follows: 0.2 mM of dNTPs, 1.5 mM of  $\text{MgCl}_2$ , 0.2  $\mu\text{M}$  of both primers, 2.5 U of Taq DNA polymerase (Eurobio), and DNA matrix between 0.5 and 5 ng with a final volume of 50  $\mu\text{L}$ . A nested-PCR was performed using DSR1F/4R primers (Karkhoff-Schweizer *et al.*, 1995) for the first run and DSR619AF (5'-GYCCGGCVTTCCSTACAA-3') and 1905BR (5'-ATGTGCGGCGSGTDCAAY-3') for the second run as previously described (Giloteaux *et al.*, 2010). DNA amplification was performed with a minicycler PTC 200 (MJ Research) with an initial denaturation step at  $94^\circ\text{C}$  for 5 min, followed by 35 cycles of denaturation (45 s at  $94^\circ\text{C}$ ), annealing (45 s at  $55^\circ\text{C}$  for DSR1F/4R and  $54^\circ\text{C}$  for DSR619AF/1905BR), and extension (90 s at  $72^\circ\text{C}$ ). A final step of extension at  $72^\circ\text{C}$  for 10 min was applied. The PCR products were run in 1.0% (w/v) agarose gel electrophoresis in  $1\times$  TBE buffer and stained with ethidium bromide ( $0.2\ \mu\text{g mL}^{-1}$ ). DNA bands were detected under UV light.

### T-RFLP analyses

Amplicons for T-RFLP analyses were obtained as above described using 5'-labelled (FAM fluorochrome: 6-carboxyfluorescein) DSR916 primer. The T-RFLP conditions were performed as previously described (Bordenave *et al.*, 2007) using 3 U of Sau3AI and Taq $\alpha$ I (New England Biolabs) as restriction enzymes. Canonical Correspondence Analysis (CCA), performed using *MVSP* software (Multi-Variate Statistical Package 3.12d, Kovach Computing Services, 1985–2001, UK), allowed to determine the influence of physical–chemical parameters on the structure of sulfate-reducing bacterial communities.

### Cloning and analysis of *drsAB* sequences

Cloning procedures were performed as described in Giloteaux *et al.* (2010). Briefly, the unlabelled *drsAB* amplicons were cloned using the pCRT0PO2.1 TA cloning vector and competent *Escherichia coli* TOP 10F' cells (Invitrogen, the Netherlands). The inserts of the recombinant clones were directly amplified by PCR using M13 primers (M13F: 5'-GTAAAACGACGGCCAG-3'; M13R: 5'-CAGGAAACAGCTATGAC-3') and then analyzed by restriction fragment length polymorphism (RFLP), using the same restriction enzymes as those used in T-RFLP analyses. One clone of each RFLP pattern was sequenced using the Big Dye Terminator v 3.1 cycle sequencing kit (Applied Biosystem) with M13 primers following manufacturer's procedures. When necessary, the internal degenerate sequencing primers 1FI (5'-CAGGAYGARCTKCACCG-3') and 1RI (5'-CCCTGGGTRTGRAYRAT-3') (Dhillon *et al.*, 2003) were used to obtain full-length *drs* gene sequences. The phylogenetic analysis of *drsAB* sequences was performed as described in Giloteaux *et al.* (2010). Briefly, after databank query using the BLAST tools (Altschul *et al.*, 1998), ClustalX v1.83 (Thompson *et al.*, 1997) and PROSEQ v2.9 (Filatov, 2002) software were used for alignments. Phylogenetic tree was constructed with Mega v4 (Tamura *et al.*, 2007), using the neighbor-joining method (Saitou & Nei, 1987). 1000 bootstrap replications were performed to determine the confidence level of the phylogenetic tree topology.

The homologous coverage of *drsAB* gene libraries was calculated using the equation  $C_x = 1 - (N_x/n)$ , where  $N_x$  is the number of unique sequences (singletons), and  $n$  is the total number of sequences (Good, 1953). Rarefaction analysis was performed using PAST (Paleontological Statistics version 1.60) software from <http://folk.uio.no/ohammer/past/> website.

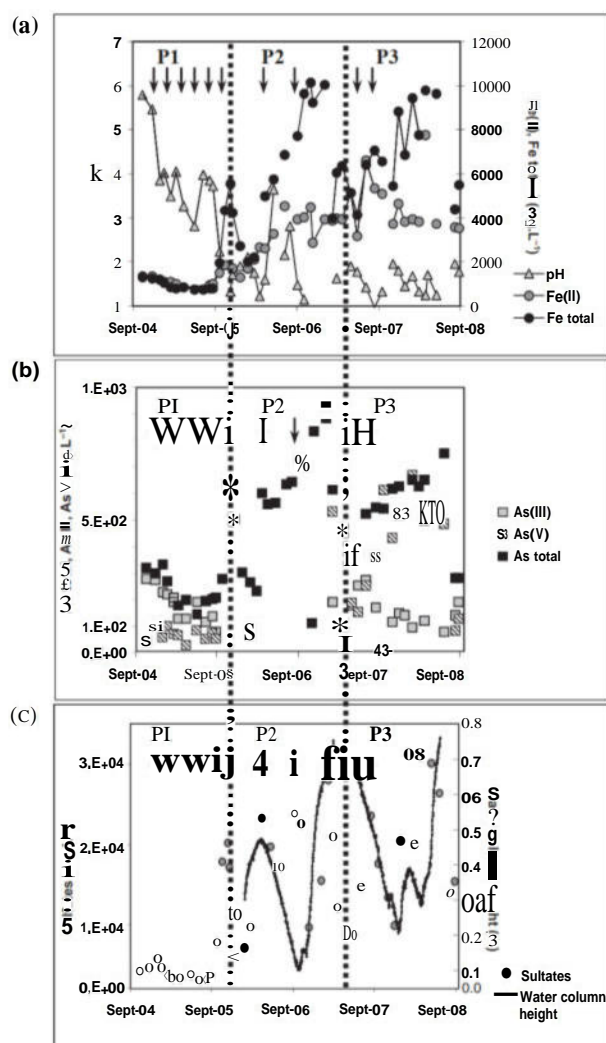
The *drsAB* sequences obtained in this study were deposited in the EMBL database under the accession numbers FN794274–FN794386.

## Results

### Physical–chemical conditions in Carnoulès AMD

At station S5, three periods were defined according to the physical–chemical parameters. The first period, P1, from November 2004 to December 2005, presented higher pH values with a decreasing trend of iron mainly in the reduced Fe(II) form. Arsenic concentrations were lower than  $340\ \text{mg L}^{-1}$ , with slight predominance of As(III) form (Fig. 2). From December 2005 to April 2007 (Period P2 in Fig. 2), pH was variable, and oxygen concentra-

tion increased over  $5 \text{ mg L}^{-1}$  (Table 1); a significant increase of Fe and As concentrations was evidenced until January 2007, followed by a decrease (Fig. 2). Iron concentration increased up to  $10\,114 \text{ mg L}^{-1}$ , with a significant contribution (up to 68% of total Fe) of Fe(III), while sulfate concentration increased up to  $29\,593 \text{ mg L}^{-1}$ . The last period, P3, from April 2007 to September 2008 (Fig. 2) was characterized by iron and arsenic concentrations similar to the P2 period but with lower pH (below pH 2.0) and a large predominance of As(V). Oxygen concentration analysis, when possible, revealed relatively high values (up to  $11 \text{ mg L}^{-1}$ ) during P2 and P3 periods and lower values (below  $5 \text{ mg L}^{-1}$ ) during P1 period.



**Fig. 2.** Variation of the pH, Fe(II) and total Fe (a), total As, As(V), and As(III) (b), sulfate and water column height (c) in the borehole (S5 station) over the course of the study. P1, P2, and P3: periods. Arrows indicate samples for T-RFLP analysis.

Temporal trends of the physical–chemical parameters at stations SI and COWG were similar and varied within a narrower range compared to S5 station (Table 1). A period between September 2005 and April 2006 was characterized by a decrease in iron and arsenic contents (Table 1).

Total arsenic and iron concentrations were significantly correlated for the entire data set as revealed by the high Pearson’s correlation coefficients ( $r = 0.83$ ;  $0.95$ ; and  $0.91$ ,  $P < 0.05$  for S5, SI, and COWG, respectively). At S5 station, sulfate and arsenite concentrations were strongly correlated ( $r = 0.87$ ,  $P < 0.05$ ), whereas sulfate and iron were inversely correlated with pH ( $r = -0.88$  and  $-0.78$ ,  $P < 0.05$  for iron and sulfate, respectively). No such correlations could be established at SI and COWG stations.

Four measurements of dissolved organic carbon concentration at each sampling stations over the studied period gave the following average values of  $14 \pm 4$ ,  $3.6 \pm 0.4$ , and  $3.0 \pm 0.8 \text{ mg L}^{-1}$  at S5, SI, and COWG, respectively, with no clear seasonal variations. The nature of organic molecules and their origin remains unknown. It might consist of humic substances, originating from litter degradation at the surface of the tailings impoundment.

### SRB distribution in Carnoulès AMD

The spatio-temporal distribution of sulfate-reducing bacterial community was determined on water samples from S5, SI, and COWG stations between November 2004 and July 2007 by T-RFLP-targeting *dsrAB* genes. Although these genes could be present in nonsulfate reducers, which could result in a misinterpretation of SRB community structure, the primers used here (DSR619AF/1905BR) exclusively target already published SRB *dsrAB* sequences (Giloteaux *et al.*, 2010) except for syntrophic *Bilophila dsrAB* sequences (data not shown), allowing a confident characterization of SRB communities. T-RFLP analysis is not suitable for bacterial identification but provides a fingerprint of the bacterial community, which allows to follow the dynamic of bacterial community structure (Paisse *et al.*, 2010). Throughout the entire experiment, the number of T-RFs ranged from 4 to 23 (average 12) and 4–24 (average 13) for Sau3AI and TaqI digestions, respectively.

The Canonical Correspondence Analysis (CCA, Fig. 3), correlating diversity data and physical–chemical parameters, showed that the SRB community structures from SI and S5 stations were affected by variations of environmental parameters. The samples were distributed along the axis 1, which is driven by all measured parameters, and explained 9.5% of the variation. In contrast, most samples from COWG clustered together (distributed

**Table 1.** Main physical-chemical parameters (pH, water temperature in °C, dissolved oxygen (DO) in mg L<sup>-1</sup>) and concentrations (mg L<sup>-1</sup>) of sulfate, iron, and arsenic (total concentrations and Fe(II), As(III), and As(V) species) in the dissolved phase (fraction below 0.22 µm) in samples used for SRB community survey. Tailings impoundment (S5) and Reigous creek: the source (S1); 600 m far from S5) and 30 m downstream (COWG)

Sampling station	Sampling Period	pH	T (°C)	DO	SO <sub>4</sub> <sup>2-</sup> (±SD)	Fe (total) (± SD)	Fe(II) (±SD)	As (total) (±SD)	As(III) (±SD)	As(V) (±SD)
Tailing Stock	11/2004	5.78	15.50	2.00	4204 (±420)	1318 (±66)	1345 (±135)	318 (±16)	277 (±28)	42 (±4)
	1/2005	3.86	14.20	0.60	5811 (±581)	1202 (±60)	1229 (±123)	332 (±17)	228 (±23)	59 (±6)
	4/2005	4.05	17.30	0.15	3267 (±327)	793 (±40)	1021 (±102)	179 (±9)	128 (±13)	66 (±7)
	8/2005	3.84	20.60	4.50	3284 (±328)	809 (±40)	973 (±97)	204 (±10)	139 (±14)	76 (±8)
	9/2005	3.73	20.60	ND	3634 (±363)	789 (±39)	997 (±100)	207 (±10)	78 (±8)	129 (±19)
	11/2005	ND	ND	ND	17 831 (±1783)	4355 (±218)	1864 (±186)	638 (±32)	102 (±10)	536 (±26)
	4/2006	1.22	15.70	11.00	29 593 (±2959)	ND	2692 (±269)	600 (±30)	49 (±4)	552 (±28)
	7/2006	2.15	18.00	ND	17 226 (±1722)	6843 (±342)	4526 (±453)	633 (±32)	92 (±9)	541 (±27)
	4/2007	ND	ND	11.00	12 095 (±1209)	6335 (±317)	3933 (±393)	470 (±23)	16 (±2)	69 (±7)
	5/2007	1.92	17.10	ND	8839 (±884)	5135 (±257)	5127 (±513)	353 (±18)	177 (±18)	186 (±19)
Reigous Creek	7/2007	1.44	18.60	ND	14 654 (±1465)	6379 (±319)	6601 (±660)	524 (±26)	273 (±27)	251 (±25)
	11/2004	3.40	16.00	2.00	3106 (±311)	998 (±50)	1119 (±112)	119 (±6)	93 (±9)	19 (±2)
	1/2005	3.94	13.50	0.60	5019 (±502)	1233 (±62)	1320 (±132)	209 (±10)	122 (±12)	44 (±4)
	4/2005	4.27	17.50	0.35	4505 (±451)	1317 (±66)	1643 (±164)	219 (±11)	195 (±20)	64 (±6)
	8/2005	4.32	19.10	0.05	4336 (±434)	1424 (±72)	1767 (±177)	264 (±13)	201 (±20)	68 (±7)
	9/2005	2.53	16.60	4.50	4177 (±418)	924 (±46)	914 (±91)	118 (±6)	42 (±4)	48 (±5)
	11/2005	2.50	16.60	4.50	4390 (±439)	892 (±45)	1177 (±118)	108 (±5)	30 (±3)	39 (±4)
	4/2006	4.22	12.80	0.35	3444 (±344)	840 (±42)	721 (±72)	125 (±6)	46 (±5)	23 (±2)
	7/2006	3.65	15.80	0.25	6677 (±668)	1397 (±70)	1370 (±137)	215 (±11)	147 (±15)	55 (±6)
	4/2007	4.13	13.70	5.50	3558 (±356)	1235 (±62)	1284 (±128)	194 (±10)	163 (±16)	13 (±1)
COWG	5/2007	4.20	15.60	0.70	4605 (±460)	873 (±44)	1164 (±116)	119 (±6)	80 (±8)	19 (±2)
	7/2007	3.92	17.20	0.30	2465 (±246)	1123 (±56)	1516 (±152)	155 (±8)	158 (±16)	8 (±1)
	11/2004	3.49	16.10	ND	2480 (±248)	739 (±37)	770 (±77)	86 (±4)	81 (±8)	25 (±3)
	1/2005	3.58	11.50	3.00	4328 (±433)	1012 (±51)	1039 (±104)	150 (±7)	70 (±7)	24 (±2)
	4/2005	3.20	14.80	3.50	3972 (±397)	1044 (±52)	1135 (±114)	147 (±7)	102 (±10)	45 (±5)
	8/2005	3.65	21.40	1.50	3713 (±371)	1096 (±55)	1295 (±130)	172 (±9)	102 (±10)	63 (±6)
	9/2005	2.76	16.90	7.00	3310 (±331)	730 (±37)	467 (±47)	98 (±5)	16 (±2)	37 (±4)
	11/2005	2.74	ND	ND	1793 (±179)	508 (±25)	553 (±55)	58 (±3)	22 (±2)	22 (±2)
	4/2006	3.28	12.90	3.50	2688 (±269)	618 (±31)	559 (±56)	78 (±4)	70 (±7)	36 (±4)
	7/2006	3.48	19.00	3.50	1758 (±176)	1115 (±56)	1120 (±112)	141 (±7)	77 (±8)	59 (±4)
4/2007	3.95	13.69	3.50	4651 (±465)	960 (±48)	1013 (±101)	137 (±7)	82 (±8)	23 (±2)	
	3.83	15.10	8.30	3209 (±321)	625 (±31)	918 (±92)	81 (±4)	71 (±7)	16 (±2)	
	3.48	17.60	5.05	2581 (±258)	956 (±48)	1032 (±103)	87 (±4)	78 (±8)	11 (±1)	

Standard deviation (SD) determined from the analysis of samples analyzed in duplicate, both within the same run and on separate days. ND, not determined.



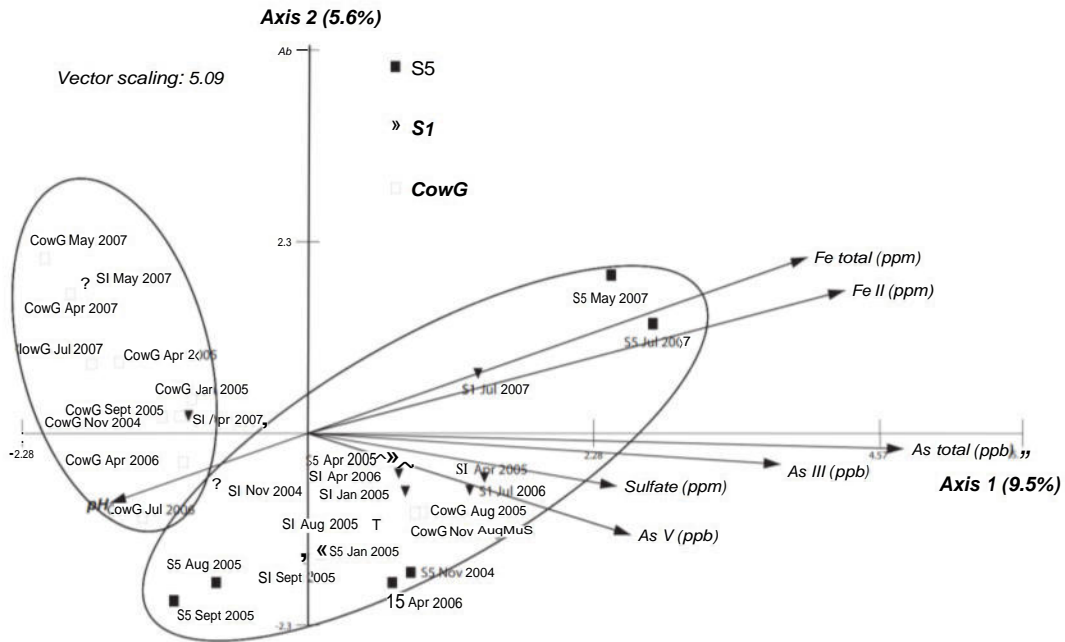


Fig. 3. Canonical Correspondence Analysis (CCA) correlating the SRB community structures from all of stations with As total, As(V), As(III), Fe total, Fe (II), sulfate, and pH in waters. The bacterial community structures correspond to T-RFLP profiles.

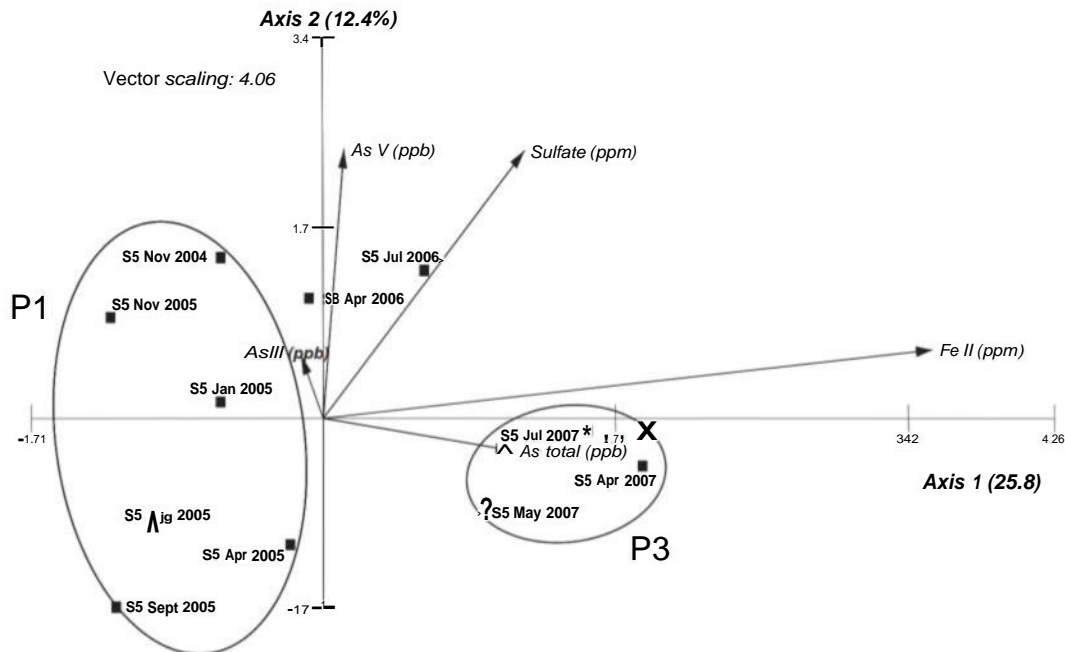


Fig. 4. Canonical Correspondence Analysis (CCA) correlating the SRB community structures from station S5 (tailings) with As total, As(V), As(III), Fe II, and sulfate. P1 and P3 are the three periods highlighted in Fig. 2. The bacterial community structures correspond to T-RFLP profiles.

along the axis 2, explaining 5.6% of the variation) probably because the measured environmental parameters are most stable at this station.

When focusing on S5 station, the CCA (Fig. 4) showed a clear separation between samples from 2004 to 2005 (P1 period) and samples from 2007 (P3 period) as deter-



mined by the physical–chemical parameters. Samples from 2006 corresponded to a transition between these two periods. Samples from 2004 to 2005 were distributed along axis 2 (12.4% of the variation) and were mainly influenced by the different forms of arsenic, whereas the 2007 samples were distributed along axis 1 (25.8% of the variation) and were influenced by iron and total As content. Thus, the temporal variations observed within the SRB community structure at S5 station, followed the modifications of physical–chemical parameters from P1 to P3, that is, increasing concentrations of Fe, As, and sulfate, and decreasing pH. Similar analysis on S1 and COWG stations showed no particular trend (data not shown).

T-RFLP profiles showed that no T-RF was common to all samples; nevertheless, 10 T-RFs (51a, 55a, 56a, 58a, 61a, 114a, and 116a for TaqI digestion and 58, 68, and 106 for Sau3AI digestion) were frequently found and observed at the three stations (data not shown). The relative abundances of these T-RFs were variable. Five of them (61a, 116a, 58, 68, and 106) represented more than 50% of the relative abundance (e.g. T-RF 58 Sau3AI represented 90% of relative abundance in COWG 08/05 sample).

In contrast, specific T-RFs were observed indicating that environmental and chemical parameters influenced the bacterial community structure. For example, two T-RFs (153 and 200a) were exclusively found in samples with pH ranging from 2.7 to 4.2, irrespective of the station and period. The As content also determined the presence of some T-RFs, but differentially depending on As speciation. T-RF 105 was exclusively found in samples with As(III) concentration below 102 mg L<sup>-1</sup>, whereas T-RF 75a was only found in samples with As(III) content over 92 mg L<sup>-1</sup>. In contrast, T-RF 75a was present in samples containing more than 42 mg L<sup>-1</sup> of As(V), while T-RFs 185 and 255 were detected only in samples containing <36 mg L<sup>-1</sup>. Finally, T-RFs 117 and 154 were present in samples with total Fe content below 1044 mg L<sup>-1</sup>, and T-RF 75a was detected in samples with Fe contents over 1100 mg L<sup>-1</sup> (data not shown).

SRB diversity in Carnoulès acid mine drainage was analyzed by constructing 10 *dsrAB* gene libraries. Samples for this analysis were selected according to T-RFLP profiles, in order to assure a maximum of divergence in the SRB community structure, but also to be able to compare the community at the three stations. A total of 96 clones per library were analyzed by RFLP. For all libraries, the high coverage (0.94–0.98) and rarefaction analysis (which revealed that the saturation plateau was reached for each library; data not shown) indicated that a sufficient number of clones were analyzed. Species richness of *dsrAB* gene libraries was greater in S5 than in S1 or COWG.

Library analysis showed six clusters (Fig. 5). The dominant phylotype was related to *Desulfofustis glycolicus*, member of the *Desulfobulbaceae* family (cluster I), which was found in all samples, regardless of the station or time period (Fig. 5), except for S5 07/07 library. The relative abundance of sequences affiliated with this cluster varied from 28 to 100% depending on the libraries (Fig. 6). Sequences affiliated with clusters II, III, and IV were present in specific samples according to either the season or the station. They were related to *Desulfomicrobium* spp. (cluster II), to sequences from the environment affiliated to the *Desulfobalobiaceae* family (cluster III) and the *dsrAB* Xenologue group (cluster IV). All sequences from S5 07/07 library were specifically related to cluster III. Moreover, sequences related to environmental clones with no isolated representatives (cluster V) were found in COWG station in all sampling periods and dominated the composition of the microbial community in April 2005 (Fig. 6). Although cluster V has been proposed as a new lineage of SRB (Dhillon *et al.*, 2003), further evidence is needed to determine whether these sequences are found within SRB species.

Predictive digestions revealed that TaqI was not discriminative enough to correlate T-RFs to sequences, while Sau3AI was able to detect sequences from clusters I, II, III, and IV. Sequences from cluster I were related to T-RF of 106 by *in silico* digestion. This T-RF was found at relatively high abundance in most samples. Sequences from cluster II were related to T-RF 268, which was a dominant T-RF (5.3–37%) during period 1 at S5 and S1 stations. Sequences from cluster III were related to T-RF 222, which was found to be dominant in sample S5 during the third period, exclusively (23–58%).

## Discussion

This study aimed to characterize the SRB population in Carnoulès mine in order to bring new insights into the diversity and the shift of SRB communities in response to naturally occurring physical–chemical parameters, prevalent at this site. This information could be useful to exploit SRB capacities for AMD bioremediation. SRB activity is influenced by environmental factors, such as temperature, pH, redox potential, substrate availability as well as sulfate and sulfide levels (Widdel, 1988). SRB are generally active in anoxic waters and produce hydrogen sulfide reacting with dissolved heavy metals to form metal sulfide precipitates (Senko *et al.*, 2009).

Here, we describe the diversity of SRB populations that inhabit diverse ecological niches, encompassing a wide range of physical–chemical conditions, including pH as acidic as 1.2, and sulfate, iron, and arsenic concentrations as high as 29 593, 6845, and 638 mg L<sup>-1</sup>, respectively.

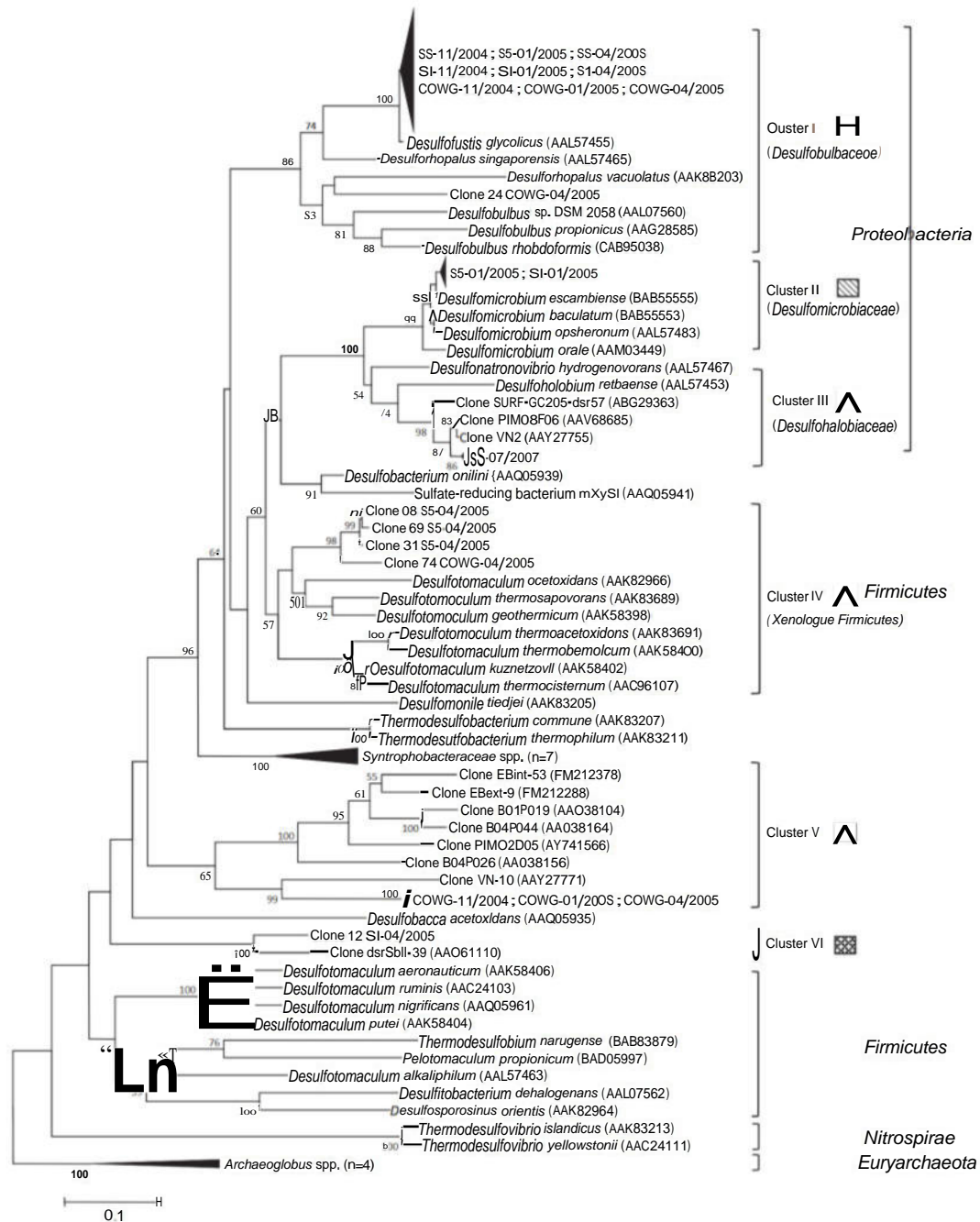


Fig. S. Phylogenetic tree based on the translated (a subunit) amino acid sequences of PCR-amplified *dsrAB* genes from S5, S1, and COWG environmental libraries with sequences from other different environmental sources and formally described species. Scale bar corresponds to 0.1 substitutions per site. Percentages of 1000 bootstrap resamplings that supported the branching orders in each analysis are shown above or near the relevant nodes. Bootstrap values are shown for branches with more than 50% bootstrap support.

The presence of SRB in such extreme conditions has already been described at the Kam Kotia, Broulan, and Kidd creek mines (pH 2.0) (Fortin *et al.*, 2000b), in natural waters (Herlihy & Mills, 1985; Koschorreck *et al.*, 2003), in sediments (Sanchez-Andrea *et al.*, 2011), and in

anaerobic reactors (Lyew *et al.*, 1994; Elliott *et al.*, 1998; Kimura *et al.*, 2006; Johnson *et al.*, 2009; Bijmans *et al.*, 2010) with pH lower than 3.0, showing that SRB are able to survive under acidic pH conditions (for a review, see Koschorreck, 2008). A sulfate-reducing activity was also

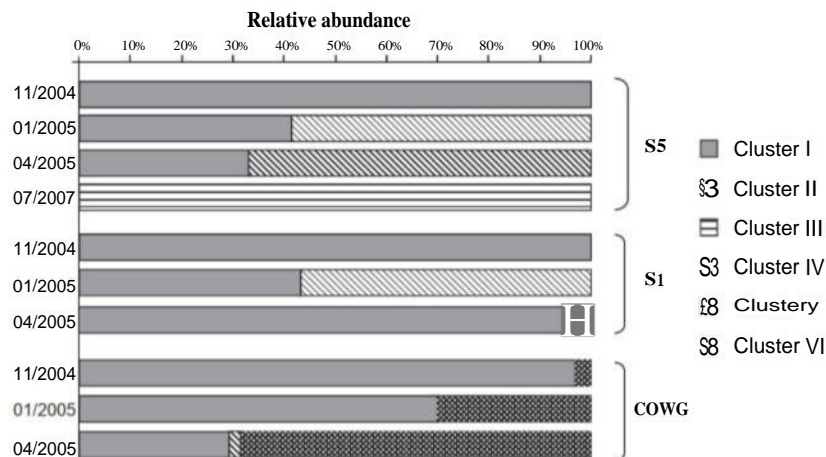


Fig. 6. Relative abundance of cluster clones recovered from *dsrAB* environmental libraries from S5, SI, and COWG station.

described in acidic mine tailing and was found dependent on the type of tailings, carbon availability, and temperature (Fortin *et al.*, 2000b; Praharaj & Fortin, 2004, 2008).

T-RFLP and *dsrAB* gene library analyses of biomass filtered from water samples confirmed the presence of different types of SRB populations along the Carnoulès AMD, even when water samples contained up to 11 mg L<sup>-1</sup> of dissolved oxygen. This was not surprising as previous studies showed that SRB are able to survive in microenvironments, associated with particles (Fukui & Takii, 1990; Fortin & Beveridge, 1997) that provide protection from the surrounding acidity and oxygen (Fortin *et al.*, 1996), or are capable of removing oxygen via active respiration (Krekeler *et al.*, 1998).

Some *dsrAB* sequences, which included *dsrAB* sequences affiliated to *Desulfobulbaceae*, were detected in almost all samples irrespective of the physical-chemical conditions. These sequences are closely related to *D. glycolicus*, which is a complete glycolate oxidizer (Friedrich *et al.*, 1996). Glycolate can be excreted by *Euglena* sp., algae and many autotrophic and heterotrophic prokaryotes, such as *Thiobacillus neapolitanus* (Lord *et al.*, 1970; Cohen *et al.*, 1979; Yokota and Kitaoka, 1982; Bateson & Ward, 1988), which were detected in Carnoulès (Casiot *et al.*, 2004; Bruneel *et al.*, 2005) and other AMDs (Cohen *et al.*, 1979; Mann *et al.*, 1987; Brake *et al.*, 2001). Nevertheless, these sequences could not be retrieved from S5 waters in July 2007, when the conditions were the more extreme (pH 1.4, highest sulfate, iron, and arsenate concentrations).

Other *dsrAB* sequences were specifically found in sampling stations depending on environmental conditions. A general increase of oxygen concentration and a decrease in iron, sulfate, and arsenic concentrations were observed between SI and COWG stations, as a result of well-characterized iron oxidation and precipitation processes

taking place along the Reigous creek from station SI to station COWG (Egal *et al.*, 2010). The variation of chemical parameters was found to be related to weather conditions. In driest months, dissolved concentrations of Fe, As, and SO<sub>4</sub><sup>2-</sup> variations could be associated with typical summer rainstorms. At the Reigous creek source (SI), the highest dissolved oxygen concentration was observed after rainfall events (Egal *et al.*, 2010). At the mine-tailing site (S5), a strong correlation between sulfate and arsenite concentrations was observed.

According to physical-chemical parameters, three main periods were defined during the 3-year survey. Distinctive SRB community structure (Fig. 3) could be attributed specifically to a station or a period suggesting that the diversity of SRB community in Carnoulès AMD water was dependent on the fluctuating physical-chemical parameters. The seasonal fluctuations were more notable on SRB community structures at the mine-tailing site (S5), with a clear influence of As, Fe, and pH. When conditions were more extreme (S5, P3, July 2007), the more abundant *dsrAB* sequences were affiliated to *Desulfohalobaceae*, with closest relatives belonging to sequences or strains obtained from salt marsh, estuarine, or hypersaline sediments (Friedrich, 2002; Bahr *et al.*, 2005; Leloup *et al.*, 2006; Lloyd *et al.*, 2006). The capacity of SRB to develop under such acidic conditions is yet to be described.

In waters with the highest pH and relatively low As concentrations, beyond *D. glycolicus* dominance, the BSR community was dominated by *Desulfomicrobiaceae*-related sequences that were specific to period PI (S5 and SI). In a previous study, a *Desulfomicrobium* sp. strain, which can perform arsenate respiration, was isolated from an arsenic-contaminated mine (Macy *et al.*, 2000). Moreover, a sequence, closely related to a novel type of dissimilatory (bi) sulfite reductase, was detected only at SI station. This

sequence was previously detected in acidic fens (pH~4.0) (Loy *et al.*, 2004) and uranium mill-tailing soils (Chang *et al.*, 2001).

COWG station was characterized by the presence of sequences specifically found in this station. These sequences were related to the dominant cluster V, unrelated to known cultured SRB, and clustered together with environmental sequences detected in hydrothermal site (e.g. clone B04P026) (Dhillon *et al.*, 2003). These sequences presented a major deletion in the  $\alpha$ -subunit of the *dsrA* gene (from position 259–273, data not shown), similar to those found within *archaeal* sulfate reducers and some Gram-positive SRB (Klein *et al.*, 2001). An environmental sequence (clone TOYOdsr-1), which shares the same deletion, was not represented in the DSRA tree (Fig. 5) and has been retrieved from hydrothermal waters within a Cu–Pb–Zn mine in Toyoha, Japan (Nakagawa *et al.*, 2002). The abundance of these sequences increased at COWG station when pH decreased and Fe and As increased showing the ability of this SRB population to develop under adverse conditions.

In April 2005, S5 and COWG waters showed similar physical–chemical conditions with pH around 4.0 and intermediate concentrations of sulfate and arsenic. Sequences affiliated to the spore-forming Xenologue *Firmicutes Desulfotomaculum* spp. were found exclusively in these samples. *Desulfotomaculum* strains, spore-forming bacteria, have been found in a large range of pHs and under slightly oxic conditions (Bryukhanov *et al.*, 2011). Some species also share the ability to use Fe(III) or As(V) as sole terminal electron acceptor for growth such as *Desulfotomaculum reducens* and *Desulfotomaculum auripigmentum* (Tebo & Obraztsova, 1998) suggesting that they may play a crucial role in AMD functioning.

In this study, SRB populations were detected by molecular methods, but no conclusions can be made regarding their activity. Attempts to isolate SRB strains from the S5, S1, and COWG Carnoulès waters were unsuccessful. However, anoxic enrichment experiments were performed adding sulfate to Carnoulès waters. The observed FeS precipitates, as a consequence of sulfide production, indicated that SRB could be alive in Carnoulès water with the ability to perform an active sulfate reduction.

The 3-year survey proposed here describes the dynamics of SRB community, which was mainly influenced by seasonal fluctuations of pH, iron, and arsenic species. The extreme conditions prevailing at the Carnoulès AMD and their seasonal fluctuations exerted a strong selection pressure on SRB populations. As a result, certain SRB phylotypes were time- and site-specific able to cope with extreme conditions such as acidic and oxygenated waters. Further investigation is required to establish SRB activity in mine tailings, to understand the role of SRB in the

AMD of the Carnoulès mine. SRB representatives from the Carnoulès AMD should be isolated, and deciphering their genomic and ecophysiological characteristics will provide new insights into the structural and functional relationships of resident SRB that ensures their survival in acidic and heavily arsenic-contaminated habitats.

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