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ORIGINAL ARTICLE

Molecular analysis of the spatial distribution of sulfate-reducing bacteria in three eutrophicated wastewater stabilization ponds

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Abstract The spatial distribution of sulfate-reducing bacteria (SRB) within three eutrophicated wastewater stabilization ponds (anaerobic, facultative and maturation) was assessed by terminal restriction fragment (TRF) polymorphism targeting the dissimilatory (bi) sulfite reductase (*dsr*AB) gene. High sulfate reducing diversity was confirmed through the 93 and 78 TRFs found using *Sau3*A1 and *Taq* α1 restriction enzymes. Statistical analysis using Simpson (*D*) and Shannon (*H'*) diversity index and principal coordinate analysis revealed differential distribution of SRB at each treatment stage and between waste and sediment samples. Inversely to the distribution of purple phototrophic bacteria, the diversity of SRB decreased within sediment and increased within the water phase downstream of the plant.

Keywords Sulfate reducing bacteria · *dsrAB* gene · T-RFLP · Stabilization pond · Eutrophicated

Introduction

Sulfate-reducing bacteria (SRB) are major contributors to carbon and sulfur cycles (Widdel and Hansen 1992; Nealson 1997), and constitute a highly physiologically and phylogenetically diverse group (Rabus et al. 2001), characterized by their metabolic versatility (Barton and Tomei 1995), their resistance to fluctuating redox potential (Castro et al. 2002) and their ability to survive oxygen exposure. Sulfate reduction is considered as one of the main anaerobic processes in the bio-mineralization of organic matter. This biogeochemical process is widespread and takes place in many diverse environments, such as marine and freshwater sediments, and microbial mats (Fishbain et al. 2003; Purdy et al. 2003; Detmers et al. 2004). SRB are of increasing economic and industrial importance due to their bioremediation capacities, e.g., in heavy metal decontamination (Gibert et al. 2004; Johnson and Hallberg 2005). In wastewater systems, sulfate reduction can be the dominant terminal electron accepting process, accounting for up to 50% of organic matter mineralization (Kühl and Jørgensen 1992; Okabe et al. 2003). But undoubtedly, they are also responsible for many economic problems because of their involvement in bio-corrosion (Dinh et al. 2004), malodorous sulfide production, and their effects on chemical oxygen demand (COD) removal efficiency (Shayegan et al. 2005) and microbial activity inhibition (Reis et al. 1992; Cohen et al. 1977).

Several molecular methods targeting 16S rRNA or specific metabolic genes have been used to investigate the diversity of SRB, as well as the dynamics and behavior of this bacterial group within a variety of complex environments. Such methods include: cloning and sequencing (Dhillon et al. 2003), competitive PCR (Leloup et al. 2006), denaturing gradient gel electrophoresis (DGGE) (Geets et al. 2006; Miletto et al. 2007) and terminal restriction fragment length polymorphism (T-RFLP) (Castro et al. 2005), with the latter being one of the most frequently used high throughput fingerprinting methods. Because of its relative simplicity, T-RFLP analysis has been applied to the analysis of archaeal and bacterial 16S rRNA genes (Leybo et al. 2006; Lu et al. 2005; Wu et al. 2006; Hullar et al. 2006; Noll et al. 2005; Pérez-Piqueres et al. 2006; Schmidt et al. 2006; Thies et al. 2007) and to analyze

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specific functional genes (Mintie et al. 2003; Pérez-Jiménez and Kerkhof 2005) such as pmoA (Mohanty et al. 2006), dsrAB (Zhang et al. 2008) or pufM (Ranchou-Peyruse et al. 2006). While it shares the problems inherent to any PCR-based method (Acinas et al. 2005; Crosby and Criddle 2003; Kanagawa 2003; Kurata et al. 2004), T-RFLP has been shown to be one of the most rapid and powerful methods to assess changes in microbial community structure on temporal and spatial scales by monitoring the gain or loss of specific fragments from the profiles (Franklin and Mills 2003; Mummey and Stahl 2003; Wang et al. 2004).

Waste stabilization ponds (WSPs), which are used widely throughout the world, have proved to be an efficient and economical method of sewage treatment and are applied to the treatment of various wastewaters (Pearson et al. 1996; Saqqar and Pescod 1996). WSPs are particularly sensitive to environmental changes such as temperature, nutrient availability and organic loading instability (Abis and Mara 2006). Consequently, they frequently stratify and become eutrophic (Scheffer 1998). Under such operation conditions, WSPs constitute an appropriate environment for the development of sulfur bacteria, characterized by the blooming of photosynthetic purple sulfur bacteria (Veenstra et al. 1995; Villanueva et al. 1994; Belila et al. 2009). In order to understand the ecology of sulfur bacteria within these systems, the spatial distribution of the SRB community was assessed by targeting the dissimilatory sulfite reductase (*dsr*AB) genes using T-RFLP to assess the distribution and dynamics of SRB during red water phenomenon within wastewater treatment plants comprising three stabilization ponds.

Materials and methods

Wastewater stabilization plant

The stabilization pond system used in semi plants consists of three inter-connected ponds (Fig. 1). The shapes and depths of these ponds are summarized in Table 1. Primary treated wastewater fills up the first pond (Anaerobic pond: An) and then enters the secondary Facultative pond (F) through an outflow, before finally entering the Maturation pond (M). The study was carried out in a semi-industrial WSP pilot plant located in Mutuelleville urban City (36°49′ N, 10°10′ E). Effluents are essentially of domestic origin but can also contain detergents and heavy metals.

Physical and chemical parameters

In situ measurements of dissolved oxygen (DO), pH and temperature were performed in each pond. Temperature and pH were measured using WTW Handheld Meters 340i model (WTW, Weilheim, Germany). DO was measured using a Multiline F/set P4 universal meter (WTW). Sulfates, sulfides, 5-day biochemical oxygen demand (BOD_5), chemical oxygen demand (COD) and total suspended solids (TSS) determinations were carried out according to the analytical methods described in *Standard methods for the examination of Water and Wastewater* (APHA 1995). Chlorophyll *a* was estimated by the methanol extraction method described by Pearson (1986).

Sampling procedure

For molecular analysis, water and sediment samples were collected from the anaerobic, facultative and maturation ponds (Fig. 1). Sediment samples were extracted from the anaerobic and facultative ponds with a Plexiglas core tube; the maturation pond sediment layer was very thin and could not be sampled. Six samples were taken in May 2006 for molecular analysis (three sediments, and three water samples). Sediment samples were designated as follow: SA, SB as sediment samples from inlet and outlet point of the anaerobic pond, respectively, and SF as sediment sample from the facultative pond. Water samples WA, WF and WM taken from the anaerobic, facultative and maturation pond, respectively, were pre-filtered through polycarbonate filters (0.8 μm pore size) to minimize non-bacterial DNA, then filtered onto 0.45 μm Durapore membranes (Millipore, Billerica, MA). All samples were immediately frozen at −50°C until use.

DNA extraction

Total DNA was extracted from water and sediment samples with the UltraClean soil DNA isolation kit (Mo Bio Laboratories, Solana Beach, CA), as recommended by the manufacture. Extraction was optimized in order to maximize cell lysis and to avoid colored releases from sediment samples. In order to minimize the inherent T-RFLP random bias, replicate extractions were pooled (Kitts 2001).

PCR experiments

The *DsrAB* gene encoding dissimilatory (bi)sulfite reductase, which catalyzes the last step in the sulfate reduction pathway, was targeted using the primer set *Dsr*1F (ACSCACTG GAAGCACG) and *Dsr*4R (GTGTAGCAGTTACCGCA) (Peréz-Jiménez et al. 2001), with the forward primer being labelled with hexachlorofluorescein (Hex). PCR was opti-

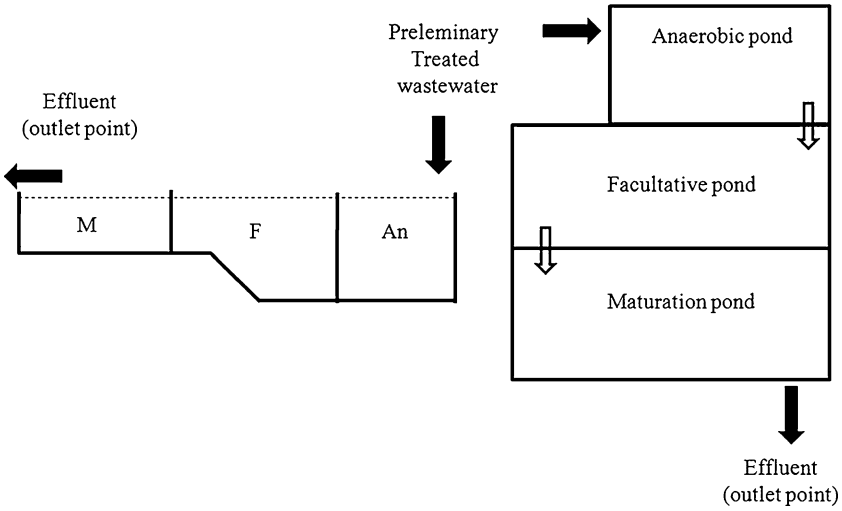

Fig. 1 Scheme of wastewater stabilization ponds: *An* anaerobic pond, *F* facultative pond, *M* maturation pond

mized by adjusting the amount of genomic DNA extract used for each sample. The PCR reaction mixture contained 200 μM each desoxynucleoside triphosphate (Qiagen, Hilden Germany), 1.5 mM $MgCl_2$, 1.25 U of *Taq* polymerase (Qiagen), 1X PCR Buffer (Qiagen), and 0.2 μM each primer in 50 μl final volume.

PCR was performed in a Peltier Thermal cycler 200 (MJ Research, Waltham, MA) using an initial denaturation step of 95°C for 15 min, followed by 35 cycles of a program consisting of denaturation at 95°C for 30 s, primer annealing at 55°C, and extension at 72°C for 30 s followed by final extension at 72°C for 7 min. PCR products were verified on 1% (w/v) agarose gel in 1X TBE buffer (89 mM Tris, 89 mM Boric acid, 2.5 mM EDTA pH 8.3) with ethidium bromide staining. PCR products were purified using GFX PCR DNA and a gel band purification kit (Amersham Biosciences, London, UK).

Restriction enzyme digestion

To better reflect the natural diversity of microbial populations within our samples, two restriction enzymes (*Sau3*AI and *Taq*αI) were used (Engebretson and Moyer 2003). After quantification of the purified PCR product by the "dots method" (Bordenave et al. 2004), purified PCR products were digested separately with restriction enzymes *Sau3*AI and *Taq*αI (New England Biolabs, Hitchin, UK). A 300-ng sample of amplified DNA was added to a final volume of 30 μl of restriction enzyme mix containing 10 U restriction enzyme, and incubated for 3 h at 37°C and 65°C for *Sau3*AI and *Taq*αI, respectively.

T-RFLP experiment

Two microliters of each digested DNA was mixed with 20 μl formamide and 0.5 μl size standard TAMRA™ 500 bp size

Table 1 Geometric characteristics of the three wastewater stabilization ponds (WSP) and wastewater quality parameters during the sampling campaign. *TSS* Total suspended solids, BOD_5 5-day biochemical oxygen demand, *COD* chemical oxygen demand

Parameter	Anaerobic pond	Facultative pond	Maturation pond
Surface (m^2)	30	100	122
Depth (m)	3.5	2.34–1.44	1.34
Volume (m^3)	96	180	164
Water depth (m)	3.3	2	1.15
TSS (mg/L)	251	184	88
Temperature (°C)	21	21	23
Chlorophyll *a* (μg/L)	50	2,600	3,980
Sulfates (mg/L)	320	245	45
Sulfides (mg/L)	40	25	9
COD (mg/L)	619	347	160
BOD_5 (mg/L)	327	209	169
COD/BOD_5 ratio	1.89	1.66	0.94
Dissolved oxygen (mg/L)	0	0.84	1.6
pH	7.2	7.8	8.2

standard (GeneScan, Applied Biosystems, Foster City, CA). Samples were denatured at 95°C for 5 min and placed directly on ice. DNA fragments were separated according to size by capillary electrophoresis at 15,000 V for 30 min on an ABI PRISM 310 Genetic Analyzer (GeneScan, Applied Biosystems). The 5′ terminal fragments were visualized by excitation of the hex molecule attached to the forward primer. Gel images were captured and analyzed using ABI Genescan version analysis software 3.1. Negative controls (no genomic DNA) were conducted with every PCR and run on Genescan gels. Outputs from the ABI Genescan software were transferred to Microsoft Excel for subsequent analysis. Duplicate samples were analyzed for replicated peaks. Nonreplicated peaks (i.e., peaks that were present in one duplicate but absent in the second run of the same sample) and peaks < 5 times the baseline fluorescence intensity were discarded. With these criteria, the practical detection limit for 1 TRF is ≈ 0.09% of the total amplified DNA (Hewson and Fuhrman 2004). The area under each peak was then averaged between replicates and expressed as a percentage of the total integrated area under the electropherogram after first removing the areas of nonreplicated peaks.

Statistical analysis

Terminal-restriction fragment (TRF) peaks identified by GeneScan 3.1.2 software from replicate sample profiles were compiled, aligned and normalised by calculating the fluorescence intensity of each TRF as a percentage of the total fluorescence intensity of all TRFs in the profile. TRF richness (S), Shannon diversity index (H′) (Atlas and Bartha 1987; Legendre and Legendre 1998) and Simpson index (D) based on the size and the number of TRFs, were calculated as an estimate of TRF diversity and evenness within the microbial community (Dunbar et al. 2000). All these diversity indexes were also used to analyze changes in composition of the purple phototrophic bacteria at each treatment stage (Clarke and Warwick 2001). Principal coordinates analysis (PCoA) was applied to ordinate the T-RFLP profiles (Blackwood et al. 2007; Deirdre and Clipson 2008). A similarity matrix was calculated to determine similarity among T-RFLP fingerprints. All statistical analyses were performed with MVSP 3.12d software (Kovach Computing Service, Anglesey, Wales) (Kovach 1999). Statistical analysis was performed by one-way ANOVA using Biostat 2008 (AnalystSoft, Vancouver, CA).

Results

Pond performances

Three wastewater stabilization ponds were investigated between December 2005 and January 2007. Temperature measurements showed that anaerobic and facultative wastewater stabilization ponds stratify and destratify intermittently. In the absence of artificial aeration or mixing devices, and under warm climatic conditions, both ponds were affected by thermal stratification between April and October. The chlorophyll *a* (Chla) concentration reached relatively high levels within the facultative and maturation ponds, at 2,600 and 3,980 μg/L, respectively. All these biological and chemical proprieties gave evidence of the eutrophic state within the wastewater stabilization plant, as defined by Urban Wastewater Treatment Directive 91/271/ECC (Doering et al. 2006). The concentrations of both DO and hydrogen sulfide (Table 1) indicate the expansion of anaerobic conditions within the first and the second ponds. Remarkably, the DO concentration increased markedly within the next facultative and maturation WSPs, reaching 0.84 and 1.6 mg/L, respectively, in spite of the algal bloom (Table 1).

As advanced by Metcalf and Eddy (1991), influent wastewater is characterized as ‘strong’—the COD/BOD_5 ratio was 1.89 as for typical degradable municipal wastewater (Markantonatos 1990). The data showed that, after the first anaerobic treatment step, the BOD_5 and COD removal percentages were 36% and 43.9%, respectively, as the anaerobic pond has tendency to remove COD better than BOD (Papadopoulos et al. 2001). Within the facultative pond, both BOD and COD were reduced by 19% and 46%, respectively. The fact that COD removal is still higher than BOD_5 is probably due to the degradation of treatment performances and the proliferation of anaerobic conditions induced by eutrophication, which also explain the decrease of the COD/BOD_5 ratio from 1.89 to 1.66 within facultative pond (Table 1). On the other hand, the increase of COD removal rate within the maturation pond may be explained by the fact the WSPs treats essentially municipal sewage where raw sewage is rarely contaminated by industrial wastewater. The COD/BOD_5 ratio for biological wastewater treatment plant effluents ranges between 4 and 12 depending on the unbiodegradable soluble COD fraction and effluent suspended solids concentration (Wentzel et al. 2003). The COD/BOD_5 ratio value for municipal raw wastewater is in the range of 1.25 to 2.5, whereas for industrial wastewater it can be up to 10 or more (Markantonatos 1990). The soluble unbiodegradable fraction, relative to the COD_{TOT}, is reported to range between 10–54% in raw wastewater and 7–34% in primary sewage (Xu and Hultman 1996; Satoh et al. 2000; Ekama et al. 1986; Roeleveld and van Loosdrecht 2002). The sulfate reduction rate increased downstream of the WSPs; 23.4% of sulfates were reduced within the anaerobic pond while 81% were reduced following the wastewater facultative treatment step. Despite the increased sulfate reduction rate, only 25 and 9 mg/L hydrogen sulfide (H_2S) were detected

within the facultative and maturation ponds, respectively, probably because of release of H_2S into the atmosphere. The increase in the sulfate reduction rate downstream of the system might have been stimulated by the algal bloom within the facultative and maturation ponds, since algae constitute a carbon source for biological sulfate reduction (Nedergaard et al. 2002; Boshoff and Rose 2004.

T-RFLP fingerprint similarity

The number of TRFs obtained from each restriction enzyme digestion varied between 11 and 35 using *Sau3*AI and between 11 and 23 using *Taq* α I (Table 2). T-RFLP profiles were compared to detect similarities within and between each pond. With *Sau3A1*, the Shannon and Simpson diversity indices ranged between 2.535 to 3.863 and between 0.77 and 0.903, respectively. The highest SRB diversity indices were detected within the facultative pond water phase (WF) (Table 3).

The highest similarity value (55.73%) was detected between SB and SF (Table 4). The sediment sample similarity indices ranged between 16.57% and 55.73%, and between 8.95% and 43.1% for water samples (Table 4). In the anaerobic pond, the SRB community composition in SA was more similar to the water phase WA (20.4%) than SB (2.1%), reflecting a shift in SRB community composition. The mean species richness (S) was 22; 26 and 32 TRFs within the anaerobic, facultative and maturation ponds, respectively indicating an unexpected increase in SRB diversity downstream of the wastewater stabilization plant. The Shannon diversity index (*H*′) indicated significant differences between sediment samples (SA, SB and SF) ($P<0.05$) and water samples (WA, WF, WM) ($P<0.05$); indeed, SRB diversity decreased from SA (H′= 3.863) to SB (H′=2.535). Conversely, it increased within the water phase of the facultative and maturation pond (H'_{WF}=3,551; H'_{WM}=3,543). The Shannon diversity index varied significantly ($P<0.05$) between water and sediment samples within the anaerobic and facultative ponds (Table 3), assuming a shift of the SRB community between both solid and liquid phases. To discriminate T-RFLP profiles, PCoA using combined data recovered from *Sau3A1* and *Taq* α*1* enzymes digestion was used as an ordination method to visualize multivariate patterns. Three axes explained 68.9% of the total variability (Fig. 2). Axis 1 explained a total variability of 54.05%, while axis 2 explained 8.75% with a cumulative percentage of 62.8%. Differential distribution between water and sediment samples plots was observed throughout the PCoA results using both restriction enzymes (Fig. 2). In particular, two distinct communities characteristic of sediment and water phases can be distinguished through axis 1, although no significant differences due to the choice of restriction enzyme were observed (Fig. 2).

Table 2 Terminal restriction fragment (TRF) length richness obtained with *Sau3*A1 and *Taq* α 1 enzyme digestion. Samples: sediment samples from anaerobic (*SA* inlet, *SB* outlet) and facultative (*SF*) ponds; water samples from anaerobic (*WA*), facultative (*WF*), and maturation (*WM*) ponds

	SA	SB	SF	WA	WF	WM
Sau3A1	25	11	31	35	18	32
Taq α *1*	18	11	18	22	16	23

Table 3 *Sau3A1* diversity indices for the sulfate-reducing bacteria (SRB) community within each pond. *H*′ Shannon index, *D* Simpson's diversity index D = Σ (Pi)2, *S* OTU richness

Sample[a]	*H'*	*D*	Evenness[b]	*S*
SA	3.863	0.903	0.940	25
SB	2.535	0.770	0.847	11
WA	2.815	0.794	0.841	31
WF	3.551	0.867	0.892	35
SF	2.786	0.770	0.816	18
WM	3.543	0.858	0.897	32

[a] Sample definitions as in Table 2

[b] Shannon evenness: (*J*′) = H′/ln(S), where H = Σ Pi (ln Pi), where (Pi) is the fraction of the total integrated area in each peak

Distribution of TRFs

DsrAB-based T-RFLP results revealed high SRB diversity within the three investigated ponds, with 93 and 78 TRFs detected. Statistical analysis confirmed the differential distribution between each pond, and between water and sediment samples within the anaerobic and facultative pond. The highest SRB diversity was detected within the water phase of the three ponds, with 31, 35 and 32 TRFs within WA, WF and WM, respectively. Species richness (*S*)

Table 4 Similarity matrix calculated from *Sau3A1* terminal restriction fragment length polymorphism (T-RFLP) profiles of *DsrAB* amplified from water and sediments from anaerobic, facultative and maturation ponds

Samples[a]	SA	SB	WA	WF	SF	WM
SA	100					
SB	16.566	100				
WA	20.390	2.085	100			
WF	4.508	0.000	43.097	100		
SF	21.128	55.729	2.520	0.217	100	
WM	13.553	30.367	8.950	22.258	0	100

[a] Samples defined as in Table 2

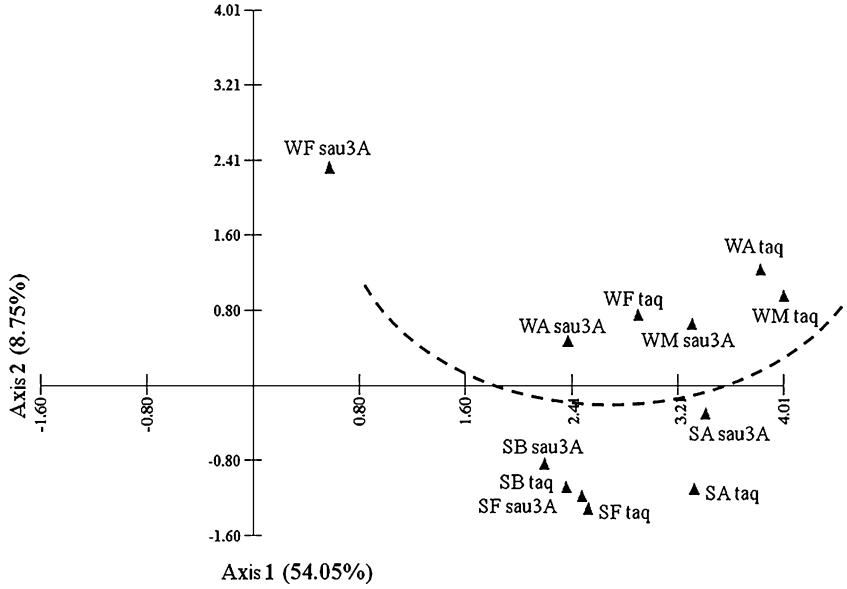

Fig. 2 Principal coordinate analysis (PCoA) results extracted from terminal restriction fragment length polymorphism (T-RFLP) profiles of *dsr*AB gene digestion with *Sau*3A1 and *Taq*α1 restriction enzymes

(Table 3) revealed an increase of SRB community richness downstream of the plant, with 25 TRFs detected within the anaerobic sediment phase (SA), and 35 and 32 TRFs within facultative and maturation water phases (WF and WM), respectively. Remarkably, the diversity of the SRB community within the sediment sample decreased downstream of the anaerobic pond, from 25 TRF detected in SA to 11 in SB and 18 in SF (Table 2). Comparison of SRB distribution within anaerobic and facultative pond revealed that 53.7% of TRFs were localized in the sediment samples, while 66% were localized within the water sample in the facultative pond. Some TRFs exhibited a restricted distribution, e.g., TRFs of 200, 211and 335 bp were detected only in the maturation pond, TRFs of 109, 142 and 266 bp were detected in the anaerobic pond, and TRFs of 468, 471 and 456 bp were restricted to the facultative pond. Of all the TRFs released, only one phylotype (TRF 221 bp) exhibited a ubiquitous distribution, being detected in all three wastewater stabilization ponds, and dominant within water phases WA and WF (Table 5).

TRF abundance variability

Assessment of the SRB distribution within three eutrophicated WSPs showed that, apart from their differential distribution, the abundance of each TRF varied at each treatment stage. The sediment samples from anaerobic pond were characterized by dominant TRFs of 73 bp (31.76%), 267 bp (31.92%) and 109 bp (18.92%), while TRFs of 114 bp (36.78%), 221 bp (17.65%) and 268 bp (14.11%) dominated the anaerobic pond water sample (W_A) (Table 5). Phylotypes 66, 73 and 77 bp dominate within facultative pond where relative abundance accounts for 12.03%, 39.44% and 23.1% respectively. Finally, TRFs of 267 and 149 bp dominate within maturation pond with relative abundance of 30.43% and 10.11%. TRF (77 bp) was detected in all sediment samples SA, SB and SF, and dominates the sediment of the facultative pond (SF) where relative abundance reaches 23.1%. TRF 114 bp was depicted in both sediment and water phases of the anaerobic pond, but dominates within the water sample with 36.78% of relative abundance.

Discussion

SRBs play significant ecophysiological roles in anaerobic bio-mineralization pathways, especially in wastewater treatment systems (Oude Elferink et al. 1994; Dar et al. 2007). Our sampling regime monitored the physical and chemical parameters of the WSPs and confirmed the decrease in wastewater treatment performance with total removal percentages of 27, 36 and 43% for total suspended solid (TSS), BOD5 and COD, respectively. The high chlorophyll *a* concentration recorded within facultative and maturation ponds, as well as the blooming of purple photosynthetic bacteria (red water), gave evidence of the eutrophic state within the three WSPs. Within such an ecosystem, the terminal stages of the anaerobic mineralization of organic matter is catalyzed by SRB and methanogens and their competitive and cooperative interactions (Oude Elferink et al. 1994), but during this eutrophic state, the relatively high concentrations of sulfates and sulfides measured, and the SRB richness detected by T-RFLP (93 phylotypes) favor the hypothesis that sulfate reduction was the dominant terminal electron accepting process. Statistical analysis of the T-RFLP data revealed differential distribution of the SRB community within each treatment stage

Table 5 *Sau3A1* TRF distribution and their relative frequencies in the three WSPs

TRF relative density (%)						
TRF (bp)	SA[a]	SB	WA	WF	SF	WM
37	-[b]	-	0.39	0.22	0.37	-
47	-	-	-	-	0.40	-
57	-	-	-	-	0.60	-
60	-	1.05	-	-	-	-
61	1.39	-	-	-	5.38	-
63	-	0.67	-	-	-	-
64	0.81	-	-	-	2.78	-
66	1.96	10.28	-	-	12.03	-
68	-	-	-	-	3.08	-
69	-	-	-	-	3.09	-
70	-	1.74	-	-	-	-
73	9.55	31.76	0.94	-	39.44	-
77	3.69	11.32	0.70	-	23.10	-
80	-	-	-	-	0.99	-
83	-	-	-	-	1.03	-
84	0.56	-	-	-	-	-
87	-	-	-	-	-	1.33
88	-	4.10	-	-	-	1.40
89	-	2.03	-	-	1.13	-
94	-	-	-	-	-	1.30
95	-	-	-	0.74	-	1.21
98	0.75	3.07	0.33	-	1.15	-
101	-	-	-	-	-	1.32
103	-	-	2.39	2.54	-	-
105	-	-	-	19.20	-	-
109	18.92	-	0.87	-	-	-
111	8.33	-	-	-	-	8.77
114	15.02	-	36.78	-	-	-
115	-	-	-	23.11	-	14.22
131	-	-	-	-	1.85	-
142	-	2.07	-	-	-	-
143	-	-	-	0.20	-	-
148	-	-	0.30	-	-	-
149	2.63	-	-	0.55	-	10.11
151	1.25	-	-	-	-	-
156	-	-	-	-	-	1.13
164	-	-	-	-	-	1.24
167	-	-	-	-	-	1.40
168	-	-	0.29	-	-	-
177	-	-	-	0.19	-	-
178	-	-	0.33	-	-	-
182	-	-	-	-	-	1.67
189	-	-	-	-	-	1.39
190	-	-	-	0.21	-	-
200	-	-	-	-	-	2.14
201	-	-	-	-	-	1.52
211		-	-	-	-	1.39
219	2.12	-	-	-	-	-
221	2.29	-	17.65	11.41	-	6.46
266	11.84	-	-	-	-	
267	-	31.92	-	-	-	30.43
268	-	-	14.11	13.61	-	-
271	0.97	-	-	-	-	-
272	-	-	2.03	-	-	2.53

Table 5 (continued)

TRF relative density (%)

TRF (bp)	SA[a]	SB	WA	WF	SF	WM
303	-	-	-	-	0.88	-
321	-	-	-	-	-	1.43
329	-	-	-	-	-	1.41
335	-	-	-	-	-	5.11
338	-	-	-	-	-	1.11
347	-	-	-	0.57	-	-
351	-	-	-	0.48	-	-
353	5.11	-	-	-	2.12	-
360	-	-	-	0.49	-	-
364	-	-	-	0.60	-	-
365	-	-	-	0.64	-	-
375	2.68	-	-	-	-	-
376	2.70	-	-	-	-	-
378	-	-	-	2.24	-	-
380	0.87	-	-	-	-	-
382	-	-	1.90	-	-	-
408	-	-	-	1.90	-	-
432	-	-	11.60	8.33	-	-
445	2.61	-	-	-	-	-
446	-	-	5.59	3.51	-	-
452	-	-	0.30	-	-	-
453	0.98	-	-	-	-	-
454	0.97	-	-	-	-	-
455	-	-	3.50	2.44	-	-
456	-	-	-	-	0.58	-
459	-	-	-	0.36	-	-
461	-	-	-	0.80	-	-
463	1.02	-	-	0.89	-	-
464	0.98	-	-	0.46	-	-
466	-	-	-	0.46	-	-
468	-	-	-	0.38	-	-
470	-	-	-	0.51	-	-
471	-	-	-	0.55	-	-
472	-	-	-	0.54	-	-
477	-	-	-	0.41	-	-
478	-	-	–	0.44	–	-
482	-	-	-	0.33	-	-
483	-	-	-	0.35	-	-
484	-	-	-	0.34	-	-

[a] Samples defined as in Table 2

[b] Not detected

(Table 5), and in particular, two distinct communities characteristic of sediment and water phases were distinguished using PCoA (Fig. 2). The structure of the SRB community changed within each pond, and the results showed that richness, and relative abundance of all detected phylotypes varied between water and sediment samples within anaerobic and facultative ponds (Table 2). Inside the latest pond, the diversity of the SRB community decreased in the sediment samples from 25 TRFs (SA) to 18 TRFs (SF) and, remarkably, the highest SRB richness was observed within water samples WA and WF, where the highest number of SRB ecotypes was detected with 35 TRFs (Table 2).

Unlike purple phototrophic anoxygenic bacteria, SRB distribution seems unaffected by wastewater strength during the treatment process since the richness of the SRB community increased downstream of the plant. Abiotic parameters play a major role in the ecology of the SRB group. Since the activity and distribution of SRB is

controlled mainly by physical and chemical parameters (Leloup et al. 2006), temperature, organic matter supply and the availability of utilizable organic carbon influence the variation of SRB abundance. Sulfate level and availability also play significant role in structuring the SRB community (Purdy et al. 2002). In our study, the distributive abundance of the SRB in the water samples (WA, WF and WM) was not affected by levels of oxygen. Indeed, the spatial distribution of the SRB group within the three WSPs increased downstream of the WSPs despite the increase in oxygen level, as 35 and 32 TRFs were detected in the facultative and maturation ponds, respectively. While strict anaerobes, SRB can survive under oxic stress by developing molecular defense strategies, such as migration and aggregate formation, that allow them to create favorable zones for their metabolism and possible growth (Dolla et al. 2006).

Conclusions

SRB are synergistically associated with photosynthetic bacteria by sharing a mutualistic relationship with sulfide-oxidizing phototrophic bacteria in an endosymbiotic sulfur cycle. This complementary study revealed that, under a eutrophic state, SRB exhibit different ecological patterns from the purple anoxygenic phototrophic bacteria; their diversity and richness decreased within sediments and increased within water phase downstream of the plant. Since they are the only bacteria known to reduce sulfate, SRB play a key role in the initiation of the sulfur cycle and, consequently, the blooming of the purple photosynthetic bacteria responsible for the red water phenomenon. Our study also confirms T-RFLP as an efficient and rapid method with which to detect spatial changes in bacterial community structure under specific environmental conditions.

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