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KINETIC MODELING AND MICROBIAL ASSESSMENT BY FLUORESCENT *IN SITU* HYBRIDIZATION IN ANAEROBIC SEQUENCING BATCH BIOFILM REACTORS TREATING SULFATE-RICH WASTEWATER

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Abstract - This paper reports the results of applying anaerobic sequencing batch biofilm reactors (AnSBBR) for treating sulfate-rich wastewater. The reactor was filled with polyurethane foam matrices or with eucalyptus charcoal, used as the support for biomass attachment. Synthetic wastewater was prepared with two ratios between chemical oxygen demand (COD) and sulfate concentration (COD/SO_4^{2-}) of 0.4 and 3.2. For a COD/SO_4^{2-} ratio of 3.2, the AnSBBR performance was influenced by the support material used; the average levels of organic matter removal were 67% and 81% in the reactors filled with polyurethane foam and charcoal, respectively, and both support materials were associated with similar levels of sulfate reduction (above 90%). In both reactors, sulfate-reducing bacteria (SRB) represented more than 65% of the bacterial community. The kinetic model indicated equilibrium between complete- and incomplete-oxidizing SRB in the reactor filled with polyurethane foam and predominantly incomplete-oxidizing SRB in the reactor filled with charcoal. Methanogenic activity seems to have been the determining factor to explain the better performance of the reactor filled with charcoal to remove organic matter at a COD/SO_4^{2-} ratio of 3.2. For a COD/SO_4^{2-} ratio of 0.4, low values of sulfate reduction (around 32%) and low reaction rates were observed as a result of the small SRB population (about 20% of the bacterial community). Although the support material did not affect overall performance for this condition, different degradation pathways were observed; incomplete oxidation of organic matter by SRB was the main kinetic pathway and methanogenesis was negligible in both reactors.
Keywords: Anaerobic process; AnSBBR; Biofilm; FISH; Sulfate reduction; Support material.

INTRODUCTION

Sulfate is found in most wastewater, especially industrial effluent. Industries that generate sulfate-containing effluent include the production of organic peroxide, food oil, paper, photographic materials, mining, textiles and explosives (Lens et al., 1998; Silva et al., 2007; Liamleam & Annachhatre, 2007). The release of sulfate-rich wastewater into natural waters has serious environmental impacts and can disrupt the sulfur cycle.

Some anaerobic microorganisms, called sulfate-reducing bacteria (SRB), can use sulfate as the final electron acceptor in the oxidation of organic matter.

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This process, known as dissimilatory sulfate reduction, reduces sulfate to sulfide. These microorganisms are widely dispersed in anaerobic environments such as water and sediment (Madigan et al., 2009). SRB and other anaerobic microorganisms such as acidogenic bacteria and methane-producing *Archaea* (MPA) use organic compounds and hydrogen and, therefore, compete for common electron donors in sulfate-containing natural environments and in wastewater treatment plants (Liamleam & Annachhatre, 2007; Muyzer & Stams, 2008; Chou et al., 2008). An understanding of the interactions among these organisms will allow the effective control of SRB and MPA in reactors for the anaerobic treatment of wastewaters containing high sulfate concentrations (Domingues et al., 2002).

Several types of anaerobic reactors have been intensively studied and used for treating sulfate-rich wastewaters (Chuang et al., 2005; Silva et al., 2007; Damianovic & Foresti, 2007; Chou et al., 2008; Tang et al., 2009). Several configurations of continuous reactors have been proposed with suspended or immobilized biomass, the attached-growth reactors being the most used by researchers. This preference is based on the improvement of the relation between sulfidogenic and nonsulfidogenic microorganisms obtained in such heterogeneous configurations. Fixed-, moving- or fluidized-bed reactors have been proposed to treat sulfate-rich wastewaters, aiming at organic matter removal and sulfate reduction. Anaerobic sequencing batch reactors (Mohan et al., 2007; Sarti et al., 2009; Friedl et al., 2009; Sarti et al., 2010) have also been evaluated for treating sulfate-rich wastewater. This configuration can be operated with granular biomass or with microorganisms adhered to an inert material, being denominated, in this case, an anaerobic sequencing batch biofilm reactor (AnSBBR).

Because these reactors are designed to remove organic matter and sulfate, their rich microenvironments incorporate a broad spectrum of biological reactions that depend on the wastewater characteristics, electron donors and acceptors, and operational and environmental conditions. In these reactors, methanogenesis and sulfidogenesis occur through both cooperative and competitive relationships.

In general, sulfate is considered to be a problem for the application of anaerobic biotechnology for wastewater treatment. According to Lens et al. (1998), a complete suppression of the sulfate reduction and a complete conversion of the organic substrate into methane could be considered to be the most optimal process configuration. However, chemical removal of sulfate is expensive and generates large amounts of solid wastes and the suppression of sulfate reduction with inhibitors in biological reactors can be expensive or even impracticable. Moreover, the combination of anaerobic and micro-aerobic processes can be a sustainable alternative, since the sulfide generated in the anaerobic reactor can be partially oxidized to elemental sulfur in a micro-aerobic unit, thus permitting the recovery of sulfur in this combined process.

The functions of SRB and other anaerobic microorganisms can be assessed through a combination of kinetic modeling and microbial evaluation. These techniques facilitate a broad evaluation of the interactions between SBR and nonsulfidogenic micoorganisms (like MPA) in a reactor as well as the analysis of rational design procedures, process simulation and optimization. In this context, this paper reports the results achieved by combining kinetic analysis and fluorescent *in situ* hybridization **(**FISH) to evaluate the interactions between sulfidogenic and nonsulfidogenic microorganisms in two anaerobic sequencing batch biofilm reactors (AnSBBR) treating sulfate-rich wastewater.

MATERIAL AND METHODS

Characteristics and Operation of the Anaerobic Sequencing Batch Biofilm Reactors (AnSBBR)

Experiments were conducted with two anaerobic sequencing batch biofilm reactors filled with two specific supports for biomass attachment: polyurethane foam and eucalyptus charcoal. The bench-scale reactors were built in Plexiglas with total and working volumes of 10 L and 5.4 L, respectively. Mixing was achieved by three turbines and the support material filled a perforated basket inside the reactor (Figure 1). This configuration was based on that proposed by Ratusznei et al. (2000) and modified by Cubas et al. (2004).

The two reactors were operated for 70 consecutive 24 hours cycles with feeding and discharge times of 10 min. The temperature was set at 30±2ºC and the agitation intensity was 300 rpm. System performance was evaluated by monitoring COD removal, sulfate reduction efficiency and gas composition.

During the first 30 days of operation, sludge inoculum was added during feeding at a concentration of 300 mg L^{-1} as total suspended solids (STV) to promote the attachment of anaerobic microorganisms to the support materials.

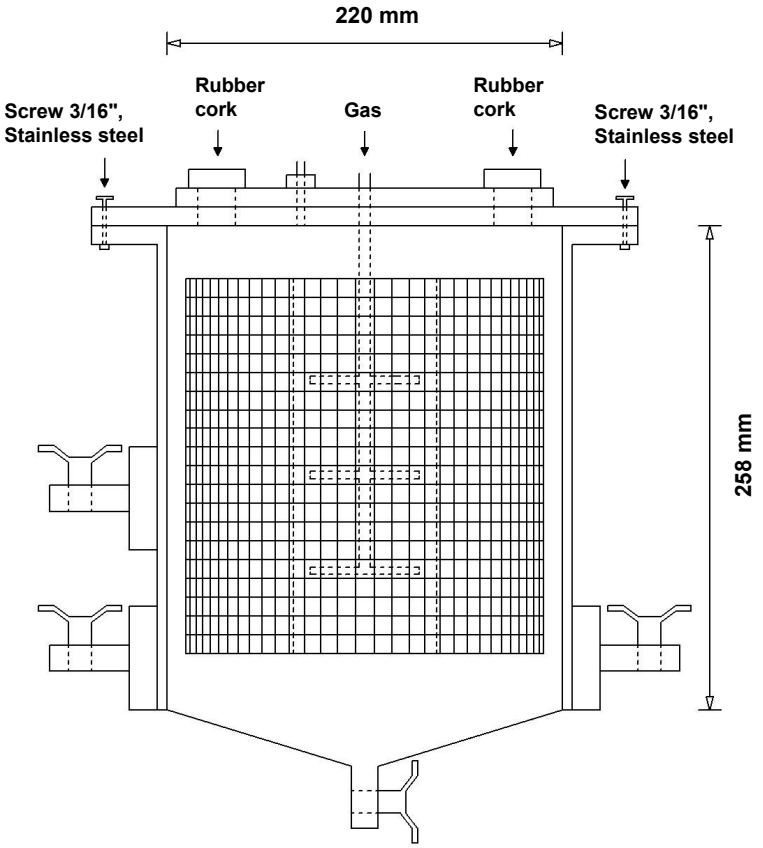

Figure 1: Bench-scale anaerobic sequencing batch biofilm reactor (AnSBBR).

Support Materials, Synthetic Wastewater and Inoculum

Polyurethane foam and eucalyptus charcoal were used as supports for biomass attachment. The main characteristics of each support material are presented in Table 1, which is adapted from Silva et al. (2006).

Table 1: Characteristics of the support materials

Support material characteristic	Polyurethane foam	Eucalyptus charcoal
Shape	**Cubic**	**Irregular pellet**
Apparent density (g mL^{-1})	0.023	0.51
Equivalent diameter* (cm)	0.6	0.5
Porosity	0.92	0.43
Mean pore diameter (μm)	543	1.9
Surface area (m^2 g^{-1})**	43.8	3.51

*Compared to an equal-volume sphere
** Measured by the multipoint BET method

The sulfate-rich synthetic wastewater employed in this study was composed of beef extract (0.42 g L^{-1}), starch (0.23 g L^{-1}), sucrose (0.07 g L^{-1}), soybean oil (0.11 mL L^{-1}), detergent (0.2 mL L^{-1}), sodium bicarbonate (0.4 g L^{-1}), sodium chloride (250 mg L^{-1}), magnesium chloride (7.0 mg L^{-1}) and calcium chloride (4.5 mg L^{-1}). This medium was prepared according to Sousa and Foresti (1996). The ratios between the chemical oxygen demand and sulfate concentration (COD/SO_4^{2-}) were adjusted to 0.4 and 3.2 by adding sodium sulfate. The synthetic wastewater was maintained at 4°C to avoid oxidation of the substrate; however, before entering the supply system of the reactor, the synthetic wastewater passed through a water bath at a working temperature of 30ºC.

The sludge used as inoculum was taken from an upflow anaerobic sludge blanket (UASB) reactor used to treat poultry slaughterhouse wastewater. The microbial characterization of this sludge can be found in Hirasawa et al. (2008).

Kinetic Model

The two major routes of organic matter consumption (methanogenesis and sulfidogenesis)

were evaluated by kinetic analysis, following the model depicted in Figure 2.

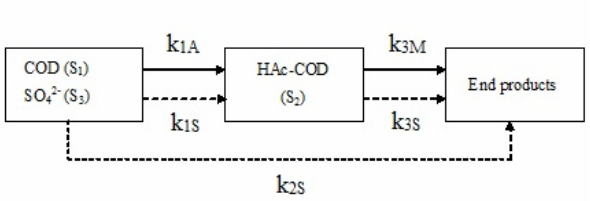

Figure 2: Diagram of the proposed kinetic model. S_1: primary organic matter as COD, S_2: acetic acid (COD equivalent) and S_3: sulfate concentration.

Temporal profiles of COD, organic acids and sulfate concentrations were obtained for the reactors under stable operational conditions, when the effluent values of the COD and sulfate did not vary between cycles.

A simplified kinetic model is depicted in Figure 2. This model considers the degradation of primary organic matter (S_1) as the COD through three pathways. Two of the pathways are sulfidogenic; one is acidogenic and does not use sulfate as an electron acceptor. The only intermediate considered was acetic acid (S_2), which can be degraded by either MPA or SRB. The acidogenic and methanogenic conversions were assumed to follow a first-order model, and the sulfidogenic bioreactions were assumed to follow a second-order kinetic model with sulfate as one of the substrates. In this way, the model is completely described by five kinetic constants that were estimated by adjusting the equations derived from the mass balance for organic matter (S_1), acetate (S_2) and sulfate (S_3) in the batch reactor to match the experimental temporal profiles of the COD, acetic acid and sulfate.

The mass balances for S_1, S_2 and S_3 resulted in the following equations:

$$\frac{\partial C_{S_1}}{\partial t} = -k_{1A}C_{S_1} - k_{1S}C_{S_1}C_{S_3} - k_{2S}C_{S_1}C_{S_3} \quad (1)$$

$$\frac{\partial C_{S_2}}{\partial t} = k_{1A}C_{S_1} + k_{1S}C_{S_1}C_{S_3} - k_{3S}C_{S_2}C_{S_3} - k_{3M}C_{S_2} \quad (2)$$

$$\frac{\partial C_{S_3}}{\partial t} = -k_{1S}C_{S_1}C_{S_3} - k_{2S}C_{S_1}C_{S_3} - k_{3S}C_{S_2}C_{S_3} \quad (3)$$

In equations 1 through 3, k_{1A} is the first-order kinetic constant for primary organic matter consumption by acidogenic bacteria (AB), k_{1S} is the second-order kinetic constant, which represents the incomplete conversion of primary organic matter to acetate by SRB, k_{2S} is the second-order kinetic constant for complete oxidation of primary organic matter through a non-acetogenic pathway by SRB, k_{3M} is the first-order kinetic constant for acetate consumption by MPA, and k_{3S} is the second-order kinetic constant for acetate consumption by SRB.

Fourth-order Runge-Kutta method was used to solve numerically the system of ordinary differential equations (1 to 3) and the kinetic constants k_{1A}, k_{1S}, k_{2S}, k_{3S} and k_{3M} were estimated using the solver command of the Microsoft Excel® software.

Chemical and Chromatographical Analysis

The chemical oxygen demand (COD) and sulfide and sulfate levels were analyzed according to the Standard Methods for the Examination of Water and Wastewater (1998). Excess zinc sulfate was added to the samples to eliminate sulfide interference with the COD analysis. Total volatile fatty acids (as acetic acid, HAc) and bicarbonate alkalinity were analyzed according to the methodology proposed by Dilallo and Albertson (1961). Methane was determined by gas chromatography using a Porapak-Q column (2m x ¼ in; 80/100 mesh) and a Gow-Mac chromatograph (Gow-Mac Instrument Co., Shannon, Ireland) equipped with a thermal conductivity detector. Hydrogen at 50°C was used as the carrier gas; the injector, oven and detector were kept at 80°C.

Acetic acid concentrations were determined using a Hewlett Packard 6890 gas chromatograph equipped with a HP INNOWAX column (30 m x 0.25 mm) and a flame ionization detector. Hydrogen (2.0 mL min^{-1}) was used as the carrier gas. The injector temperature was 250ºC with a split ratio of 1:20, and the detector temperature was 300ºC. The oven temperature was held at 100ºC for 3 min, heated at 5ºC min^{-1} to 180ºC, and then held at that temperature for 5 min.

Fluorescent *In Situ* Hybridization (FISH)

Microbial characterization was performed on samples collected from different portions of the reactor: top (height, h=18 cm), center (h=9 cm) and bottom (h=0). Samples were collected after reaching operational equilibrium for each condition studied, when the COD, the sulfate and organic acid concentrations and the pH did not vary between cycles.

The oligonucleotide probes used for fluorescent *in situ* hybridization (FISH) were EUB338 (*Bacteria* domain) (Amann et al., 1990), NON338 (negative control) (Manz et al., 1992), ARC915 (*Archaea*

domain) (Stahl & Amann, 1991) and SRB385 (sulfate-reducing bacteria of the δ-*Proteobacteria* subclass) (Amann et al., 1990). The probes were 5' end-labeled with the fluorescent dyes rhodamine or CY3. The fixation protocol and hybridization conditions used have been described by Araújo et al. (2000).

The samples were examined under an Olympus BX60-FLA fluorescence microscope using filter sets specific for DAPI and rhodamine or CY3. Cell counts were conducted according to a previously described methodology (Snaidr et al., 1997). For each probe and sample, 800 to 1000 DAPI-stained cells and the respective hybridized cells were counted in 20 to 25 randomly selected microscopic fields. The percentage of cells hybridized with specific probes was calculated in relation to the total number of cells stained with DAPI. The SRB cells were calculated in relation to total bacterial cells detected by the EUB338 probe.

RESULTS AND DISCUSSION

Performance of the AnSBBR

Data for COD removal efficiency (E_{COD}), sulfate reduction efficiency ($E_{sulfate}$), pH, effluent bicarbonate alkalinity (BA) and effluent concentration of volatile fatty acids (VFA) are presented in Table 2.

The AnSBBR fed with a COD/SO_4^{2-} ratio of 0.4 showed operational stability after the 38th cycle for both the support materials, while the reactor fed with a COD/SO_4^{2-} ratio of 3.2 achieved stability after the 61st and 63rd cycles when filled with polyurethane foam and eucalyptus charcoal, respectively.

No methane was detected in the biogas generated in either of the reactors fed with a COD/SO_4^{2-} ratio of 0.4, indicating that methanogenesis was not effective. However, a small amount of methane production was indicated in the reactors fed with a COD/SO_4^{2-} ratio of 3.2; average methane concentrations in the reactors filled with polyurethane foam and charcoal were 11 and 110 $mg.L^{-1}$, respectively.

The application of Student-*t*-test with a 5% significance level (α=0.05) indicated that there was no significant difference between the support materials in the two reactors with COD/SO_4^{2-} ratios of 0.4 with respect to organic matter removal or sulfate reduction. However, for COD/SO_4^{2-} ratios of 3.2, this statistical test found that the reactor filled with charcoal demonstrated better organic matter removal performance than the reactor filled with polyurethane foam.

Kinetic Modeling

The kinetic model (Figure 2) was fitted to temporal profiles of organic matter (as COD) and sulfate and acetate (COD equivalent) concentrations (Figure 3) and a good fit was observed (R^2 = 0.959±0.03). The kinetic constants were estimated and their values are presented in Table 3.

For the reactor operated with a COD/SO_4^{2-} ratio of 0.4 and filled with polyurethane foam, the kinetic model indicated that the primary organic matter was converted by complete- or incomplete-oxidizing SRB, in addition to acidogenic microorganisms, without the use of sulfate as the electron donor. However, the incomplete-oxidizing SRB processed organic matter at higher rates. In this operation, acetate was converted via bioreactions that were mediated only by SRB because k_{3M} was nil.

Table 2: Performance of the AnSBBR

	COD/SO_4^{2-} of 0.4		COD/SO_4^{2-} of 3.2	
	Polyurethane Foam	**Eucalyptus Charcoal**	**Polyurethane Foam**	**Eucalyptus Charcoal**
E_{COD} (%)	62±14%	69±5%	67±9%	81±8
$E_{sulfate}$ (%)	31±6%	34±7%	94±4%	98±1
pH	7.8±0.4	8.1±0.3	7.3±0.2	7.2±0.2
BA (mg $CaCO_3.L^{-1}$)	788±129	1132±230	564±104	746±94
VFA (mg $HAc.L^{-1}$)	69±61	43±22	146±28	47±19

E_{COD} - COD removal efficiency,
$E_{sulfate}$ - sulfate reduction efficiency,
BA - bicarbonate alkalinity,
VFA - volatile fatty acids concentration

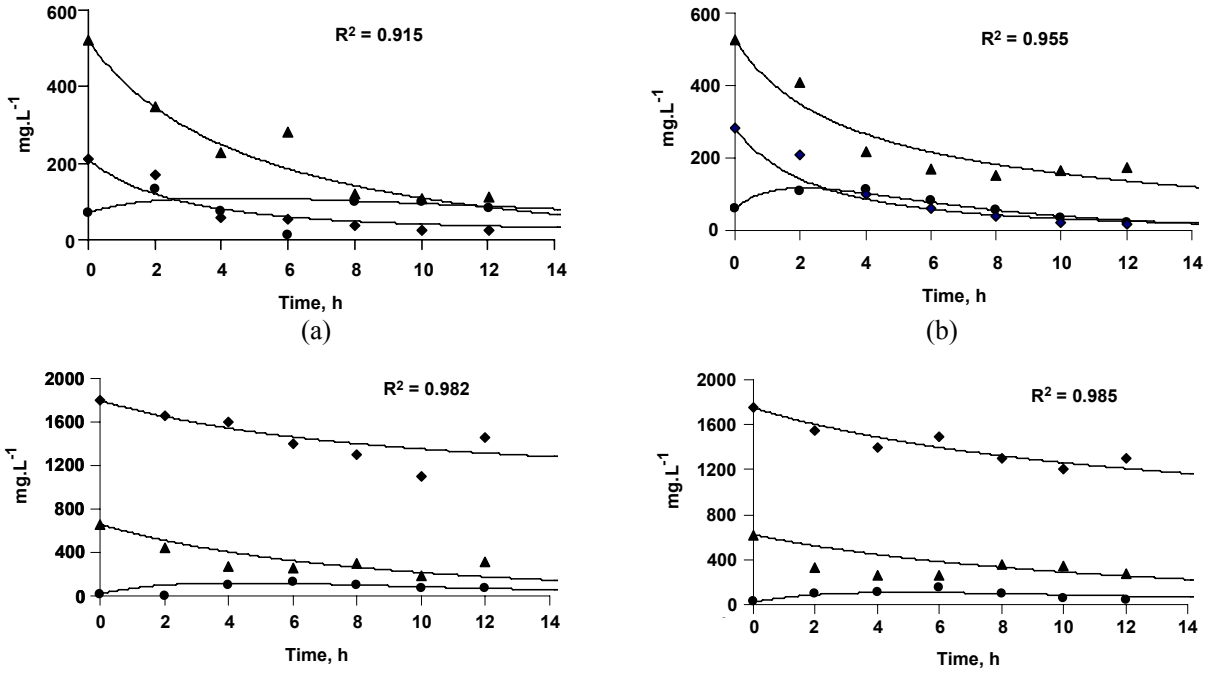

Figure 3: Kinetic model adjusted to the temporal profiles of organic matter-COD (▲), acetic acid-COD (●) and sulfate concentration (♦) for (a) COD:SO_4^{2-} = 3.2, polyurethane foam reactor; (b) COD:SO_4^{2-} = 3.2, eucalyptus charcoal reactor; (c) COD:SO_4^{2-} = 0.4, polyurethane foam reactor; and (d) COD:SO_4^{2-} = 0.4, eucalyptus charcoal reactor.

Table 3: Kinetic constants for different support materials and COD/SO_4^{2-} ratios

Kinetic constant	Values (x 10^{-4})			
	COD/SO_4^{2-} of 0.4		COD/SO_4^{2-} of 3.2	
	Polyurethane Foam	Eucalyptus Charcoal	Polyurethane Foam	Eucalyptus Charcoal
k_{1A} (h^{-1})	0.010	0	0	0
k_{1S} ($L.mg^{-1}.h^{-1}$)	0.617	0.535	3.07	6.13
k_{2S} ($L.mg^{-1}.h^{-1}$)	0.119	0	3.66	1.99
k_{3S} ($L.mg^{-1}.h^{-1}$)	0.004	0.244	0	0
k_{3M} (h^{-1})	0	0	518	2,540

When the reactor was filled with charcoal at a COD/SO_4^{2-} ratio of 0.4, only incomplete-oxidizing SRB metabolized primary organic matter with acetate, which is converted only by sulfidogenic bacteria. This was the only experimental condition in which SRB activity was the exclusive and dominant process.

Kinetic differences between the reactors filled with polyurethane foam and with charcoal at a COD/SO_4^{2-} ratio of 0.4 did not influence the overall conversion because no significant statistical difference was observed between the two reactors with respect to organic matter removal or sulfate reduction. In both reactors, the main kinetic pathway (according to the k_{1S} values) was the conversion of organic matter by incomplete-oxidizing SRB; methanogenic activity was negligible (nil values of k_{3M}). Complete-oxidizing SRB activity was important only for the reactor filled with charcoal (k_{3S} = 0.244 x 10^{-4} L mg^{-1} h^{-1}).

At a COD/SO_4^{2-} ratio of 3.2, organic matter consumption occurred only by complete- or incomplete-oxidizing SRB, and k_{1A} was nil. For the reactor filled with polyurethane foam, the conversion rates for complete- and incomplete-oxidizing SRB were balanced (k_{1S}/k_{2S} = 0.84), but the conversion rate for incomplete-oxidizing SRB was higher in the reactor filled with charcoal (k_{1S}/k_{2S} = 3.08). In both reactors, acetate was consumed exclusively by methanogenic archaea with nil values of k_{3S} because the constant k_{3M} obtained for the reactor containing

charcoal was five-fold higher than that observed for the reactor filled with polyurethane foam. As a consequence, the methane concentration in the biogas of the reactor filled with charcoal was higher, as previously presented.

Kinetic differences observed in the two reactors operated at a COD/SO_4^{2-} ratio of 3.2 influenced the overall conversion process; organic matter was more efficiently removed in the reactor filled with charcoal (COD removal efficiency of 81%) than in the reactor filled with polyurethane foam (COD removal efficiency of 67%). Because sulfate reduction was similar in both reactors, methanogenic activity seems to have been the determining factor in these results. In the reactor filled with charcoal, the preferential conversion of primary organic matter by incomplete-oxidizing SRB (k_{1S}/k_{2S} = 3.08) stimulated acetoclastic methanogenic activity and thus improved organic matter removal.

A comparison between the operations with different COD/SO_4^{2-} ratios indicates that the reaction rates were higher at a COD/SO_4^{2-} ratio of 3.2; the kinetic constants for this condition were five- to thirty-fold higher. The poor performance of the reactors with COD/SO_4^{2-} ratios of 0.4, which had COD and sulfate removal efficiencies of approximately 65% and 32%, respectively, can be partially explained by lower bioreaction rates because the 24-h cycle time was insufficient to provide better efficiencies. However, this performance was also affected by other factors such as the relatively high sulfate concentration used with respect to organic matter, which changed the equilibrium of the microbial population and resulted in higher sulfide concentrations.

Microbial Assessment by Fluorescent *In Situ* Hybridization (FISH)

FISH analyses showed that the bacterial community growth in the reactor filled with polyurethane foam was favored at a COD/SO_4^{2-} ratio of 0.4 over the ratio of 3.2 (Figure 4a). The SBR community was dominant under this condition. The percentages of cells hybridized by the EUB338 probe were 90.6% (Standard Error, SE=0.9), 83.5% (SE=1.5) and 81.4% (SE=1.1) in samples from the bottom, center and top of the reactor, respectively, for a COD/SO_4^{2-} ratio of 0.4. For a COD/SO_4^{2-} ratio of 3.2, these values were 72.6% (SE=2.0), 70.7% (SE=-1.6) and 80.4% (SE=1.3) at the bottom, center and top of the reactor, respectively. Observed morphologies among the organisms belonging to the general bacteria group were similar to curved and oval rods.

In the three evaluated portions of the reactor with a COD/SO_4^{2-} ratio of 0.4, the percentages of cells hybridized with the SRB385 probe were 22.4% (SE=1.6), 18.9% (SE=1.5) and 9.7% (SE=1.2) at the bottom, center and top of the reactor, respectively. For a COD/SO_4^{2-} ratio of 3.2, these values were 63.8% (SE=2.2), 68.8% (SE=1.9), and 63.3% (SE=2.3), respectively (Figure 4a). Morphologies similar to curved and oval rods predominated for the general SRB group in these reactors.

O'Reilly & Colleran (2006) also observed high microbial diversity in granular sludge from the inoculum of a UASB reactor used to treat wastewater from citric acid processing. At a COD/SO_4^{2-} ratio of 4, they observed predominantly curved rods (similar to *Desulfovibrio* sp.), as well as bulb-shaped coccobacilli that resembled a *Desulfobulbus*-type species. Based on FISH, they noted that this diversity persisted at a COD/SO_4^{2-} ratio of 2, but became dominated by *Desulfovibrio* sp.

The archaeal communities detected by the ARC915 probe accounted for 22.4% (SE=1.3), 29.3% (SE=2.0) and 19.6% (SE=1.2) of the populations at the bottom, center and top of the reactor, respectively, for a COD/SO_4^{2-} ratio of 3.2 (Figure 4a). The dominant archaeal cells under this condition were similar to *Methanosaeta*. This community could not be characterized or counted for a COD/SO_4^{2-} ratio of 0.4, which was probably due to the low concentration ($<10^4$ cells mL^{-1}) of these microorganisms in the samples (Amann et al., 1995).

Figure 4b presents the FISH analysis results for the samples taken from the reactors filled with charcoal. *Bacteria* domain cells again dominated, returning values of 81.0% (SE=1.5), 79.6% (SE=1.3) and 78.9% (SE=0.7) at the bottom, center and top of the reactor, respectively, for a COD/SO_4^{2-} ratio of 0.4; for a COD/SO_4^{2-} ratio of 3.2, the equivalent results were 85.2% (SE=1.2), 85.3% (SE=2.0) and 85.3% (SE=2.9).

The cells hybridized with the EUB338 probe were cocci and curved, oval, straight, and thin. At the same points, the percentages of cells hybridized with the SRB385 probe were 25.4% (SE=1.7), 17.3% (SE=1.4), and 14.7% (SE=1.0) for a 0.4 COD/SO_4^{2-} ratio and 64.8% (SE=1.5), 72.3% (SE=1.4), and 70.1% (SE=1.3) for a 3.2 COD/SO_4^{2-} ratio (Figure 4b). Based on these results, the SRB community represented 69.1% of the total bacterial cells detected by the EUB338 probe for the reactor fed with a COD/SO_4^{2-} ratio of 3.2. This percentage was slightly lower for the polyurethane-foam-filled reactor, where it was about 65.3%.

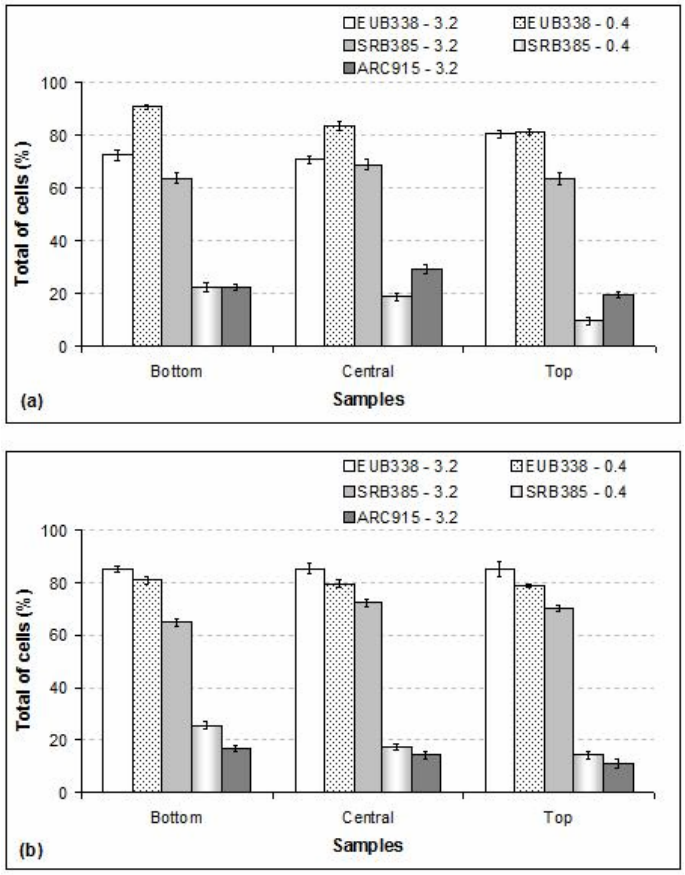

Figure 4: Microbial community composition determined by FISH at the bottom, center and top of the reactors with COD:SO_4^{2-} = 3.2 or 0.4 for (a) polyurethane foam and (b) eucalyptus charcoal. Relative abundances of the *Bacteria* domain (EUB338), *Archaea* domain (ARC915) and SRB group of the δ-*Proteobacteria* subclass (SRB385). Bars indicate the standard error (SE).

The archaeal communities detected by the ARC915 probe accounted for 16.8% (SE=1.4), 14.3% (SE=1.3) and 11.0% (SE=1.7) of the populations at the bottom, center and top of the reactor, respectively, for the reactor fed with a COD/SO_4^{2-} ratio of 3.2 (Figure 4b). The dominant archaeal cells under this condition were similar to *Methanosaeta*. For the aforementioned reasons, *Archaea* domain microorganisms were not detected in the reactor with a COD/SO_4^{2-} ratio of 0.4.

FISH analyses also showed that there was no significant difference among samples collected from different portions (bottom, center or top) of the reactors for all conditions studied (Figure 4a, b), which confirms that the reactor presented a complete-mix system under the applied conditions. However, lower percentages of archaeal cells were observed at the top of the reactor; this probably resulted from microaeration of this portion of the reactor, which is not completely isolated and can therefore have contact with the atmosphere.

For the reactors filled with polyurethane foam and charcoal at a COD/SO_4^{2-} ratio of 0.4 (Figure 4), the FISH data indicate that SRB represented only about 20% of the bacterial community. The low reaction rates are therefore attributed to sulfate reduction and poor sulfate removal efficiencies, which were 31±6% for the reactor filled with polyurethane foam and 34±7% for that filled with charcoal. The majority of the SRB detected by FISH in the two reactors may have been represented by incomplete-oxidizing SRB, in accordance with the

kinetic model (higher values of k_{1S}), and, to a minor extent, by complete-oxidizing SRB (only for the reactor filled with polyurethane foam) and acetate-oxidizing SRB (mainly in the reactor filled with charcoal).

Because only 20% of the bacterial cells in the reactors with a COD/SO_4^{2-} ratio of 0.4 were SRB, non-sulfate-reducing bacterial groups were predominant in these reactors. For the reactor filled with polyurethane foam, the activity of these organisms was detected because the conversion of organic matter by acidogenic bacteria (without the use of sulfate as an electron donor) was predicted by the mathematical model ($k_{1A} = 0.01 \times 10^{-4}$ h^{-1}). Acidogenic bacteria were found to be more competitive for organic substrates than SRB. On the other hand, according to mathematical adjustments, SRB dominated the process in the reactor filled with charcoal. It is possible that the acidogenic, non-sulfate-reducing bacteria were inactive in this reactor or that the mathematical model failed to predict only sulfidogenic activity under this experimental condition.

Like the kinetic model, the FISH analysis indicated that methanogenic reactions did not occur under these conditions. In fact, methane was not detected in the biogas from either reactor. Most likely, the methane-producing *Archaea* (mainly acetotrophic) were inhibited by the sulfide concentrations in the liquid medium, which were around 120 mg L^{-1} for the reactor filled with polyurethane foam and 114 mg L^{-1} for the reactor filled with charcoal. However, Maillacheruvu & Parkin (1996) observed that acetate-utilizing SRB in a microbial consortium are more sensitive to the presence of sulfide than other microorganisms. Therefore, the thermodynamics and kinetics of microbial reactions can better explain the prevalence of sulfate reduction via acetic acid over methanogenesis. According to Isa et al. (1986), the $\Delta G^{o\prime}$ for sulfate reduction is -47 kJ per reaction in contrast to -31 kJ per reaction for methane production when acetate is the electron donor. Moreover, Bhattacharya et al. (1996) found that sulfate-reducing bacteria present a lower half-saturation constant (K_S) in the Monod kinetic model (102 mg L^{-1}) than methane-producing *Archaea* (116 mg L^{-1}).

The FISH analytical data for the samples taken from the reactors with a COD/SO_4^{2-} ratio of 3.2 (Figure 4) indicate that SRB represented 69.1% of the bacterial population in the reactor filled with charcoal and 65.3% of the population in the reactor filled with polyurethane foam. This finding demonstrates the predominance of sulfate-reducing bacteria and the formation of a sulfidogenic community in both reactors, which resulted in higher rates for the bioreactions involving sulfate reduction (higher values of k_{1S} and k_{2S}) and, consequently, better average removal efficiencies for sulfate and COD (94±0.4% and 67±9% for polyurethane foam and 98±1% and 81±8% for charcoal, respectively).

The predominance of SRB in the two reactors with COD/SO_4^{2-} ratios of 3.2 was predicted by the kinetic model, which indicates that the organic matter was converted only by complete- or incomplete-oxidizing SRB. However, incomplete-oxidizing SRB were dominant in the reactor filled with charcoal ($k_{1S}/k_{2S} = 3.08$), whereas an equilibrium between complete- or incomplete-oxidizing SRB was observed in the reactor filled with polyurethane foam ($k_{1S}/k_{2S} = 0.84$).

The kinetic model also indicates acetoclastic methanogenic activity at a COD/SO_4^{2-} ratio of 3.2. However, sulfidogenesis was predominant and minimal methane was produced. According to Mizuno & Noike (1998), SRB play an important role in the interspecies transfer of hydrogen gas (H_2). It is possible that the primary organic matter had been converted into intermediate products such as propionate, butyrate and acetate and that, in this case, SRB competed with methanogenic archaea by consuming hydrogen. SRB present a thermodynamic advantage in hydrogen competition over methanogenic archaea and homoacetogenic bacteria. The free energy variation values under standard conditions ($\Delta G^{o\prime}$) for hydrogen consumption reactions by these microorganisms are -151.9, -135.6 and -104 kJ per reaction, respectively.

CONCLUSIONS

Based on results obtained from operating anaerobic sequencing batch biofilm reactors (AnSBBR) filled with different support materials and at different COD/SO_4^{2-} ratios, the following conclusions can be drawn:

- Sulfate-rich wastewater with a COD/SO_4^{2-} ratio of 3.2 could be suitably processed in anaerobic sequencing batch biofilm reactors filled with charcoal, with sulfate reduction and COD removal efficiencies above 90% and 80%, respectively. However, even for this best operating condition, a post-treatment would be required to remove residual organic matter and to provide a partial oxidation of sulfide to elemental sulfur;
- The type of support material used for biomass

attachment (polyurethane foam or eucalyptus charcoal) did not affect the overall performance of the AnSBBR at a COD/SO_4^{2-} ratio of 0.4. Low values of sulfate reduction (around 32%) and organic matter removal (around 65%) under this condition resulted from the low reaction rates associated with the small SRB population (about 20% of the bacterial community). However, the different support materials led to diverse degradation routes and the conversion of organic matter by incomplete-oxidizing SRB was the main kinetic pathway;

- The support material affected the overall performance of the AnSBBRs at a COD/SO_4^{2-} ratio of 3.2, with organic matter removal efficiencies of 67% and 81% and similar sulfate reductions of 94% and 98% for the reactors filled with polyurethane foam and charcoal, respectively. Although both reactors had higher reaction rates and dominant SRB populations (more than 65% of the bacterial community), their kinetic pathways were quite distinct: an equilibrium between complete- and incomplete-oxidizing SRB was observed in the reactor filled with polyurethane foam and incomplete-oxidizing SRB were dominant in the reactor filled with charcoal. Moreover, methanogenic activity seems to have determined the observed differences in reactor performance;
- The combination of kinetic modeling and fluorescent *in situ* hybridization (FISH) was valuable for completely evaluating the operation of anaerobic reactors with complex interactions between different microorganisms.

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