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Bioresource Technology 154 (2014) 114–121

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Effect of biomass adaptation to the degradation of anionic surfactants in laundry wastewater using EGSB reactors

T.P. Delforno *, A.G.L. Moura, D.Y. Okada, M.B.A. Varesche

Laboratório de Processos Biológicos (LPB), Departamento de Hidráulica e Saneamento, Escola de Engenharia de São Carlos (EESC), Universidade de São Paulo (USP), Engenharia Ambiental – Bloco 4-F, Av. João Dagnone, 1100 – Santa Angelina, 13563-120 São Carlos, SP, Brazil

HIGHLIGHTS

- Two EGSB reactors (adapted biomass and not adapted biomass) were operated.
- The biomass was adapted with standard LAS before being fed with real wastewater.
- The adaptation did not favor surfactant removal in real wastewater.
- With standard LAS the removal was 63% and with real wastewater was 76%.
- By means of pyrosequencing were identified genera that degrade aromatic compounds.

ARTICLE INFO

Article history:
Received 28 October 2013
Received in revised form 25 November 2013
Accepted 30 November 2013
Available online 16 December 2013

Keywords:
Linear alkylbenzene sulfonate (LAS)
Expanded granular sludge bed (EGSB)
Anaerobic reactor
Pyrosequencing

ABSTRACT

Two expanded granular sludge bed reactors were operated. R_{AB} (adapted biomass) was operated in two stages: Stage I, with standard LAS (13.2 mg L^{-1}); and Stage II, in which the standard LAS was replaced by diluted laundry wastewater according to the LAS concentration (11.2 mg L^{-1}). R_{NAB} (not adapted biomass) had a single stage, using direct wastewater (11.5 mg L^{-1}). Thus, the strategy of biomass adaptation did not lead to an increase of surfactant removal in wastewater (R_{AB}-Stage II: 77%; R_{NAB}-Stage I: 78%). By means of denaturing gradient gel electrophoresis, an 80% similarity was verified in the phases with laundry wastewater (sludge bed) despite the different reactor starting strategies. By pyrosequencing, many reads were related to genera of degraders of aromatic compounds and sulfate reducers (*Syntrophorhabdus* and *Desulfobulbus*). The insignificant difference in LAS removal between the two strategies was most likely due to the great microbial richness of the inoculum.

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1. Introduction

Linear alkylbenzene sulfonate (LAS) is an anionic surfactant with high global production and use, mainly in household cleaning products. Structurally, it consists of an alkyl chain (hydrophobic region), ranging from 10 to 14 carbon atoms, and another part (hydrophilic region) corresponding to a sulfonated aromatic ring.

The high production of LAS, combined with its recalcitrance under anaerobic conditions (Garcia et al., 2006), results in severe environmental problems, both physical (dispersion of pollutants by foam, oxygen diffusion) and biological (inhibition of the microorganisms responsible for the processes of natural purification). In domestic sewage, the LAS concentration may vary from 1 to 18 mg L^{-1} (Mungray and Kumar, 2009), and the concentration in laundry wastewater may vary from 17 to 1024 mg L^{-1} (Braga and Varesche, 2011).

Some studies have performed LAS removal in up-flow anaerobic sludge blanket (UASB) reactors to evaluate the effect of hydraulic retention time (HRT), co-substrates, bioavailability, stability (production of volatile acids) and temperature conditions (Lobner et al., 2005; Okada et al., 2013b); in expanded granular sludge bed (EGSB) reactors to evaluate the effect of HRT (Delforno et al., 2012); and in fluidized bed reactors to evaluate the effect of the support material and the applied LAS load (Oliveira et al., 2010).

Therefore, various studies suggest that LAS removal depends on several factors: HRT, presence of co-substrates, applied LAS load, process stability (low concentration of volatile acids) and the presence of bacterial consortia. Nevertheless, no study has evaluated the effect of biomass adaptation for LAS removal in real wastewater (e.g., laundry wastewater).

This study examined the effects of biomass adaptation on LAS removal in commercial laundry wastewater using EGSB reactors. In addition, the application of this technology has yielded satisfacting results compared to the use of UASB reactors for the treatment

* Corresponding author. Tel.: +55 16 33738356.
E-mail addresses: tiago.palladino@gmail.com (T.P. Delforno), alanamou@hotmail.com (A.G.L. Moura), dagokada@gmail.com (D.Y. Okada), varesche@sc.usp.br (M.B.A. Varesche).

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http://dx.doi.org/10.1016/j.biortech.2013.11.102

of recalcitrant compounds (Okada et al., 2013a). Associated with the operation of the reactors, the bacterial diversity in each reactor configuration was examined by polymerase chain reaction – denaturing gradient gel electrophoresis (PCR–DGGE) and pyrosequencing.

2. Methods

2.1. Experimental setup

Two EGSB reactors (R_{AB} – adapted biomass and R_{NAB} – not adapted biomass) were operated with a HRT of 38 h and in mesophilic conditions (30 °C). The reactors consisted of an acrylic apparatus with a volume of 1.40 L, height 1.0 m, diameter 0.04 m and six sample points. The up-flow velocity was constant (4 m h^{-1}) with effluent recirculation.

The reactors were fed with a modified mineral medium (adjusted $MgCl_2$ concentration to 25 mg L^{-1}; Angelidaki et al., 1990), vitamins (Touzel and Albagnac, 1983), sodium bicarbonate (400 mg L^{-1}) and a mixture of co-substrates. These co-substrates consisted of ethanol (250 mg COD L^{-1}), methanol (250 mg COD L^{-1}) and yeast extract (250 mg COD L^{-1}).

The R_{AB} reactor was operated in two stages: first, the biomass was allowed to adapt to standard LAS (Aldrich, CAS No. 25155-30-0, technical grade); second, the standard LAS was replaced by diluted laundry wastewater as a function of the concentration of LAS. The reactor R_{NAB} (not adapted biomass) had only one stage, with diluted laundry wastewater (Table 1).

The wastewater was collected from a commercial laundry located in São Carlos, SP, Brazil. The wastewater was collected after the first rinse in 10 or 20 L high-density polyurethane bottles. The bottles were stored at a temperature of 4 °C. After each collection, the commercial laundry wastewater was characterized.

The reactors were inoculated with a granular sludge (8.5 g TS L^{-1} and mean granular diameter of 4.05 mm) obtained from a full-scale UASB plant treating effluent from a poultry slaughterhouse (Avícola Dacar S/A, Tietê/SP, Brazil).

2.2. Physical chemical analysis

Analyses of pH (4500), total solids (2540D), total dissolved sulfide (4500-S2-D), chemical oxygen demand (COD; 5220D), Total Kjeldahl Nitrogen (TKN; 4500), and NH_4–N (4500-NH_3–C) were determined according to Standard Methods for Examination of Water and Wastewater (APHA-AWWA-WPCF, 2005).

The granular size distribution was determined by an image analysis technique according to Alphenaar et al. (1993). Granular sludge (ca. 5 mL, well mixed) was placed in a Petri plate. Pictures of the plates (more than 500 particles) were digitalized and analyzed by an image-analyzing software (Image-Pro Plus 4.5).

Nitrate, sulfate, fluoride, phosphate and chloride were quantified by ion chromatography (Dionex ICS-5000 with IonPAC AS23 (4 mm), eluent $Na_2CO_3/NaHCO_3$ (1 mL min^{-1}). Samples were previously purified in a C-18 column (Chromabond® C18ec) to remove surfactants.

Volatile fatty acids (VFAs), including caproic, valeric, isovaleric, butyric, isobutyric, propionic, acetic, formic, lactic, succinic, malic and citric acids, were quantified by HPLC using a Shimadzu system (Controller SCL10AVP, Pump LC-10ADVP, Oven CTO-20A and UV detector SCL10AVP) with an Aminex HPX-87H column (Biorad) (Penteado et al., 2013).

LAS was quantified by HPLC, in a Shimadzu system (SCL10AVP, LC-10ADVP, CTO-10A and RF-10AXL) with a reversed-phase C8 column (Supelco) and fluorescence detector (Duarte et al., 2006). For extracting adsorbed LAS, the solid samples (granular biomass) were collected at the end of the operation and washed three times with methanol, according to Duarte et al. (2006). The LAS mass balance included the surfactant added (influent), recovered in the effluent (liquid phase) and adsorbed on biomass in the reactor. At the end of each stage of operation, resazurin was added to the influent as a redox indicator.

Table 1
Feeding composition and stages of operation in the EGSB reactors.

	R_{AB}		R_{NAB}
	Stage I	Stage II	Stage I
Feeding			
Mineral medium	+	+	+
Ethanol (mg COD L^{-1})	250	250	250
Methanol (mg COD L^{-1})	250	250	250
Yeast extract (mg COD L^{-1})	250	250	250
Sodium bicarbonate (mg L^{-1})	400	400	400
Specific organic load (mg COD g VS^{-1} d^{-1})	71 ± 13	77 ± 16	77 ± 16
Standard LAS▲	+	–	–
Laundry wastewater□	–	+	+
LAS influent (mg L^{-1})	13.2 ± 2.3	11.2 ± 5.3	11.5 ± 5.4
Specific load (mg g VS^{-1} d^{-1})	1.2 ± 0.2	1.0 ± 0.7	0.9 ± 0.3
Hydraulic retention time (h)	38	38	38
Duration (days)	218	173	197

▲ Aldrich, CAS N°. 25155-30-0.
□ Diluted laundry wastewater.

2.3. Biological analysis

2.3.1. DNA extraction

Total DNA extraction for PCR–DGGE and sequencing was performed using a modified phenol–chloroform protocol described by Griffiths et al. (2000). DNA quality was assessed by a standard of 260/280 nm > 1.8, as measured by an ND-2000 spectrophotometer (Nanodrop Inc., Wilmington, DE) and agarose gel electrophoresis.

2.3.2. PCR–DGGE

At the end of the EGSB-R_{AB} and EGSB-R_{NAB} operation, samples were collected from the sludge blankets and phase separators for analysis by PCR–DGGE in the *Bacteria* and *Archaea* domains, as described in Duarte et al. (2008). For the *Archaea* domain, the primers 1100F (with a clamp GC) and 1400R (Kudo et al., 1997) were used. For the *Bacteria* domain, primers 968F (with a clamp GC) and 1392R (Nübel et al., 1996) were used.

DGGE banding patterns were analyzed using BioNumerics V.2.5. The similarity coefficients were determined according to the Jaccard coefficient and the dendrogram was determined by an unweighted pair group method with an arithmetic average (UPGMA) algorithm.

2.3.3. Pyrosequencing

The 16S rRNA pyrosequencing was performed for two samples from the sludge blanket: (i) R_{AB}-Stage II and (ii) R_{NAB}-Stage I. DNA was purified with Illustra GFX PCR DNA and Gel Band Purification (GE Healthcare). rRNA genes were amplified for pyrosequencing using a primer set that flanked the V4 hypervariable region of the 16S rRNA gene at corresponding *Escherichia coli* positions 563 and 802: primers 563F (5′-AYTGGGYDTAAAGNG-3′) and 802R (5′-CAGGAAACAGCTATGACC-3′). The pyrosequencing was performed at the Instituto de Agrobiotecnologia Rosario (INDEAR) (Rosario, Argentina) using a 454 Genome Sequencer FLX (Roche). Barcodes that allow sample multiplexing during pyrosequencing

were incorporated between the 454 adapter and the forward primers.

Sequences were processed with the Ribosomal Database Project (RDP) Pyrosequencing Pipeline (http://pyro.cme.msu.edu/index.jsp) (Cole et al., 2009).

Sequences were first trimmed to remove the adaptor, barcodes, primers and sequences containing ambiguous 'N' or shorter than 200 bp (Pipeline Initial Process). Chimera sequences were removed using the DECIPHER program (http://decipher.cee.wisc.edu/index.html; Wright et al., 2012).

For alignment of the sequences, the tool "secondary structure aware Infernal aligner" was used. To determine the operational taxonomic units (OTU), "hierarchical clustering" was used, with 97% similarity. OTUs with singleton sequences that may represent sequencing errors (Dickie, 2010) were removed.

Then, the sequences representing each OTU were selected (Dereplicate Sequence).

RDP-Classifier was used for the taxonomic classification of sequences representative of each OTU. The confidence threshold adopted was 80% for genus and 50% for other taxonomic levels (Phylum-Family). Alfa (Chao1, Shannon, Simpson and Dominance) diversity was quantified using Past software.

The sequences were submitted to the European Nucleotide Archive (http://www.ebi.ac.uk) under accession numbers ERS361032 (R_{AB}-Stage II) and ERS361032 (R_{NAB}-Stage I); the project accession number is PRJEB4790.

3. Results and discussion

3.1. Laundry wastewater characterization

The characteristics of the domestic laundry wastewater obtained in this experiment are shown in Table 2. The average LAS value was 181 ± 82 mg L^{-1}. Braga and Varesche (2011) analyzed laundry wastewater and observed LAS concentrations of 162 ± 244 mg L^{-1}. Owing to the high concentration of LAS, the laundry wastewater was diluted at a ratio of 1:12, to obtain an influent LAS concentration of approximately 12 mg L^{-1}, below the inhibitory value in anaerobic processes (50 mg LAS L^{-1} – Angelidaki et al., 2004). COD concentration ranged from 800 to 2665 mg COD L^{-1}, with an average of 1603 ± 692 mg L^{-1}. At the same time, just 346 ± 427 mg COD L^{-1} of VFAs was measured, with a predominance of lactate acid (96 ± 93 mg L^{-1}), malic acid (85 ± 143 mg L^{-1}) and isobutyric acid (48 ± 87 mg L^{-1}). The high concentration of lactate acid has been related to detergent formulations (Narayanan et al., 2004). The difference between VFAs and total COD was most likely due to the amount of anionic surfactant, builder and synthetic fibers present in the wastewater.

The presence of neutralizers and alkalizers resulted in an average pH value of 10 ± 1, with a high concentration of sulfate at 372 ± 223 mg S L^{-1} due to the addition of laundry products such as sodium sulfate and sodium metabisulfite. Meanwhile, the concentration of sulfate and pH varied depending on the products added in the washing process. Braga and Varesche (2011) obtained a sulfate concentration of 7 ± 6 mg L^{-1} and a pH of 6 ± 1.

The total solid concentration was 4.53 ± 2.83 g L^{-1}, composed of 2.90 ± 2.23 g L^{-1} fixed solids and 1.63 ± 0.86 g L^{-1} volatile solids. The high concentration of fixed solids was most likely due to inorganic ions such as phosphate (27 ± 42 mg L^{-1}), nitrate (360 ± 674 mg L^{-1}) and sulfate (372 ± 223 mg L^{-1}).

Table 2
Physical–chemical parameters analyzed in laundry wastewater.

Parameters	Value
Dilution	1:12 ± 5
LAS (mg L^{-1})	181 ± 82
COD (mg L^{-1})	1603 ± 692
Volatile fatty acids – VFA	
Acetic acid equivalent (mg HAc L^{-1})	323 ± 399
COD equivalent (mg COD L^{-1})	346 ± 427
Citric acid (mg L^{-1})	19 ± 32
Malic acid (mg L^{-1})	85 ± 143
Succinic acid (mg L^{-1})	4 ± 5
Lactic acid (mg L^{-1})	96 ± 93
Formic acid (mg L^{-1})	13 ± 20
Acetic acid (mg L^{-1})	9 ± 6
Propionic acid (mg L^{-1})	48 ± 87
Isobutyric acid (mg L^{-1})	54 ± 130
Isovaleric acid (mg L^{-1})	2 ± 4
Alkalinity (mg $CaCO_3$ L^{-1})	
Partial	399 ± 256
Total	489 ± 279
pH	10 ± 1
Chloride (mg L^{-1})	89 ± 42
Fluoride (mg L^{-1})	27 ± 42
Phosphate (mg L^{-1})	196 ± 136
Total Kjeldahl nitrogen (mgN L^{-1})	32 ± 8
Nitrate (mgN L^{-1})	360 ± 674
Ammonia (mg L^{-1})	5 ± 4
Sulfate (mg S L^{-1})	372 ± 223
Solids (g L^{-1})	
Total suspended solids (TSS)	0.12 ± 0.05
Fixed suspended solids (FSS)	0.02 ± 0.01
Volatile suspended solids (VSS)	0.09 ± 0.05
Total solids (TS)	4.53 ± 2.83
Total fixed solids (TFS)	2.90 ± 2.23
Total volatile solids (TVS)	1.63 ± 0.86

3.2. Performance of EGSB reactors (R_{AB} and R_{NAB})

The COD removal efficiency was high, with average values of approximately 90–92% (all reactors); the specific organic loading rate (SOLR) supplied ranged from 71 ± 13 mg COD g VS^{-1} d^{-1} (R_{AB}-Stage I) to 77 ± 16 mg COD g VS^{-1} d^{-1} (R_{NAB}-Stage I). Moreover, the addition of diluted laundry wastewater did not affect COD removal (R_{AB}-Stage II; 92%) compared to R_{AB}-Stage I (Standard LAS-91%; Table 3). Along with high COD removal, a low concentration of VFAs was observed in the effluent. The highest VFA concentration was observed in the R_{AB}-Stage I (11.4 ± 13.5 mg HAc L^{-1}) fed with standard LAS (Table 1). On the other hand, in the R_{AB}-Stage II and R_{NAB}-Stage I reactors (both with dilute laundry wastewater), the VFA concentration was lower, with 3.2 ± 4.9 mg HAc L^{-1} and 7.2 ± 13.6 mg HAc L^{-1}, respectively. A relation between VFA concentration and LAS removal has been previously reported by Lobner et al. (2005), in which concentrations lower than 50 mg L^{-1} favor LAS removal. Propionic acid (4 mg L^{-1}) and isobutyric acid (4 mg L^{-1}) were prevalent in R_{AB}-Stage I. However, in R_{AB}-Stage II- and R_{NAB}-Stage I, propionic acid (5 mg L^{-1}) dominated. The recalcitrance of propionic and isobutyric acid in the presence of LAS has been previously reported (Angelidaki et al., 2004). Angelidaki et al. (2004) observed 100% inhibition of microorganisms that are consumers of propionic acid in the presence of 50 mg LAS L^{-1}.

With standard LAS (R_{AB}-Stage I), the removal was 63 ± 10% with a specific LAS loading rate (SLLR) of 1.2 ± 0.2 mg g VS^{-1} d^{-1}. Delforno et al. (2012) observed similar removal (69 ± 8%) with an EGSB reactor supplied with an SLLR of 1.5 mg LAS g VS^{-1} d^{-1}. In R_{AB}-Stage II (diluted laundry wastewater; SLLR 1.0 ± 0.7 mg LAS g VS^{-1} d^{-1}), the removal reached 76 ± 18%. Thus, the greatest LAS removal rate was obtained by replacing standard LAS with laundry wastewater. The presence of sequestrants may change the adsorption mechanism of LAS most likely contributed to this difference (between Stage I and II of R_{AB}).

Table 3
Physical–chemical parameters analyzed in EGSB reactors.

Parameters	R_{AB} – adapted biomass		R_{NAB} – not adapted biomass
	Stage I	Stage II	Stage I
COD (mg L^{-1})			
Influent	755 ± 102	813 ± 75	815 ± 73
Effluent	61 ± 24	72 ± 26	84 ± 34
Removal (%)	92 ± 3	91 ± 3	90 ± 4
Specific organic load (mg COD g VS^{-1} d^{-1})	71 ± 13	77 ± 16	77 ± 16
LAS (mg L^{-1})			
Influent	13.2 ± 2.3▲	11.2 ± 5.3□	11.5 ± 5.4□
Effluent	4.8 ± 1.6	2.4 ± 1.7	2.1 ± 1.8
Removal (%)	63.5 ± 10.3	76.4 ± 18.1	78.6 ± 16.7
Specific load (mg g VS^{-1} d^{-1})	1.2 ± 0.2	1.0 ± 0.7	0.9 ± 0.3
Specific removal (mg g VS^{-1} d^{-1})	0.8 ± 0.2	0.8 ± 0.7	0.7 ± 0.3
Partial alkalinity (mg $CaCO_3$ L^{-1})			
Influent	238 ± 24	293 ± 39	297 ± 49
Effluent	268 ± 34	347 ± 62	347 ± 74
Total alkalinity (mg $CaCO_3$ L^{-1})			
Influent	315 ± 26	407 ± 49	413 ± 59
Effluent	367 ± 48	473 ± 88	478 ± 101
pH			
Influent	7.4 ± 0.2	7.5 ± 0.2	7.5 ± 0.2
Effluent	7.0 ± 0.1	7.1 ± 0.1	7.1 ± 0.1
Sulfide (mg S L^{-1})			
Effluent	–	3.47 ± 3.65	3.03 ± 3.63
Sulfate (mg L^{-1})			
Influent	–	17.4 ± 11.9	17.4 ± 11.9
Effluent	–	2.1 ± 3.8	2.8 ± 7.6
Removal (%)	–	84.9 ± 17.0	87.5 ± 25.0
Volatile fatty acids			
Effluent (mg HAc L^{-1})	11.4 ± 13.5	3.2 ± 4.9	7.2 ± 13.6
Final biomass (sludge blanket – g L^{-1})			
Total solids	8.28		5.72
Total volatile solids	6.73		4.37
Final biomass (phase separator – g L^{-1})			
Total solids	1.49		0.46
Total volatile solids	1.24		0.30
Duration (days)	218	173	197

▲ Standard LAS (Aldrich, CAS N°. 25155-30-0, technical grade).
□ Diluted Laundry Wastewater.

In laundry wastewater, it is common to detect the presence of sequestrants (Jaworska et al., 2002) that can complex ions such as Ca^{2+} and Mg^{2+}, influencing the adsorption of LAS. According to Westall et al. (1999), the presence of these ions can promote the adsorption of LAS due to the reduction of electrostatic repulsion. Thus, the products present in the wastewater may have slowed the adsorption of LAS in the biomass.

Therefore, the concentration of adsorbed LAS on the sludge blanket was higher in R_{AB}-Stage I (14.6 ± 2.4 mg LAS g TS^{-1}) than in R_{AB}-Stage II (7.9 ± 0.1 mg LAS g TS^{-1}). In addition, the value obtained in R_{AB}-Stage I is near the inhibitory limit of 14 mg g VSS^{-1}, obtained by Gavala and Ahring (2002) when operating an anaerobic batch assay, that may result in the instability of the system. Furthermore, a smaller granule size was obtained in R_{AB}-Stage I (Standard LAS; 3.63 ± 0.75 mm), while in R_{AB}-Stage II (with wastewater), the mean value was 4.22 ± 0.59 mm (Fig. 1).

Another point is the presence of an electron acceptor (SO_4^{2-}) only in R_{AB}-Stage II. Sulfate may contribute to the removal of COD and according to Okada et al. (2013b), a low residual COD favors LAS removal. However, the sulfidogenic pathway contributes little to the total COD removal (<5% of total COD added) and does not support the difference in LAS removal between Stage I and II of R_{AB}. R_{AB}-Stage II showed concentrations of 17.4 ± 11.9 mg S L^{-1} and 2.1 ± 3.8 mg S L^{-1} of SO_4^{2-} in the influent and effluent, respectively, with an average reduction of 84.9 ± 17.0%. According to Lens et al. (1998), the theoretical ratio of COD/sulfate is 0.67. Thus, a reduction of ~14.7 mg S L^{-1} consumed ~33 mg L^{-1} of COD by the sulfidogenic pathway, which is a low value.

The LAS removal in R_{NAB}-Stage I was 78.6 ± 16.7% (SLLR of 0.9 ± 0.3 mg g VS^{-1} d^{-1}), similar to that of R_{AB}-Stage II, which reached 76 ± 18% (both with laundry wastewater).

Therefore, no improvement in surfactant removal was observed in the reactor using laundry wastewater (R_{AB}) following its long exposure to standard LAS (R_{AB}-Stage I; 218 days).

The main factor that may have contributed to this result is the high microbial richness of the inoculum. The inoculum (granular sludge) was obtained from a full-scale UASB treating effluent from a poultry slaughterhouse. According to Hirasawa et al. (2008), the bacterial community present in this granular sludge showed high richness. The high richness results in a metabolic response to different influent conditions.

Furthermore, Lee et al. (1995) reported that for complete LAS degradation, it is necessary to involve a microbial consortium, due to the limited metabolic capacity of single anaerobic species. Anionic surfactant degradation is faster with the use of mixed cultures than with isolated cultures (Goudar et al., 1999). In addition, the greater concentration of LAS adsorbed on the sludge blanket (near the inhibitory concentration; R_{AB}-Stage I) may have acted as a stress factor for the biomass.

According to the mass balance (Table 4), the total amount of LAS added in reactor R_{AB} was 3829 mg LAS, 9% and 7% were adsorbed in

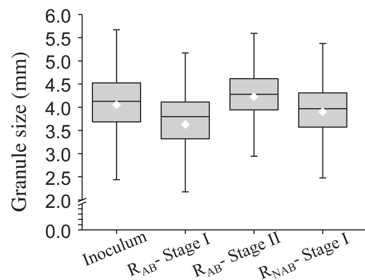

Fig. 1. Box plot of the distribution of granule size in the inoculum, R_{AB} and R_{NAB}. The bars represent upper and lower limits, and (□) represents the mean values.

Stage I and II, respectively, with high values adsorbed to the biomass in the sludge blanket. Similar results were obtained for R_{NAB}-Stage I, in which 3% of the LAS added was adsorbed. The percentages related to biological degradation (removal of adsorbed LAS) were similar in R_{AB}-Stage II (73%) and R_{NAB}-Stage I (78%), both fed with laundry wastewater. On the other hand, the value observed in R_{AB}-Stage I was only 56% (fed with standard LAS). A similar value (57% biological degradation) was obtained by Delforno et al. (2012) using an EGSB reactor for standard LAS removal with 14 mg LAS L^{-1} influent and 237 days of operation. The values of biological degradation correspond with the LAS removal percentages (Table 3), and consequently, the higher removal of LAS with laundry wastewater than with standard LAS.

3.3. PCR–DGGE analyzes

According to the Jaccard similarity coefficient of PCR–DGGE banding patterns, the *Archaea* domain showed a higher range of coefficients (40–90%) than the *Bacteria* domain (50–82%; Fig. 2) for all samples. For the *Archaea* domain, the samples collected from the laundry wastewater stages, R_{NAB}-Stage I SB, R_{AB}-Stage II PS and R_{AB}-Stage II SB**,** showed 74% similarity, except for the sample from R_{NAB}-Stage I PS, which showed a 64% similarity with R_{AB}-Stage I PS (standard LAS). Moreover, the coefficients of sludge blanket samples R_{AB}-Stage I SB and R_{AB}-Stage II SB were closer to the inoculum. Similar results were obtained for the *Bacteria* domain, with a 74% similarity coefficient between R_{AB}-Stage I SB and the inoculum.

Unlike the *Archaea* banding pattern, in the *Bacteria* domain, the samples were grouped according to sampling site (sludge blanket or phase separator). The greatest coefficient (82%) was between R_{AB}-Stage II SB and R_{NAB}-Stage I SB (both fed with laundry wastewater), whereas R_{AB}-Stage I SB (standard LAS) showed a 78% similarity with the inoculum. The samples collected from the phase separator of reactor R_{AB} showed a 78% similarity with standard LAS – Stage I and laundry wastewater – Stage II, whereas the sample from R_{NAB}-Stage I PS showed low similarity (<50%). Although the reactors have different starting strategies (the R_{AB}-Stage I uses standard LAS and the R_{AB}-Stage I laundry wastewater; R_{NAB} uses only laundry wastewater), the bacteria communities from the sludge beds were 80% similar (both with laundry wastewater). On the other hand, lower coefficients (<50%) were found between the communities of bacteria from the phase separator. These results reinforce that the granules were functioning as a protective structure preventing major modifications to the bacterial community. Nevertheless, in the phase separator with flocculent biomass, significant changes occurred over the operating time.

3.4. Pyrosequencing

By using 454 pyrosequencing, 3161 and 6442 raw sequences were generated with an average length of 225 bp (Table 5). After trimming, 82% of the sequences (both samples) were used to determine the OTUs with 97% similarity. A total of 39% (R_{AB}-Stage II) and 38% (R_{NAB}-Stage I) of the OTUs were represented by single sequences (singletons) and were not used in taxonomical classification. Estimated values of Good's coverage were 94.0–96.2% for R_{AB}-Stage II and R_{NAB}-Stage I, respectively.

The R_{NAB}-Stage I sample showed a higher estimated richness value (Chao1and Rarefaction) than the sample from R_{AB}-Stage II. However, the Chao1 and Rarefaction estimations are strongly influenced by the number of singletons (OTU with unique sequence), doubletons (OTU with two sequences) and sequences per sample. The number of sequences in the R_{NAB}-Stage I sample was twice that of the R_{AB}-Stage II sample.

The diversity index values (Shannon and Simpson) indicated a slight difference between the R_{AB}-Stage II sample (4.92 and 0.98, respectively) and R_{NAB}-Stage I (4.56 and 0.96, respectively). Moreover, the dominance index in the R_{NAB}-Stage I sample (0.04) was higher than in R_{AB}-Stage II (0.02). The dominant genus was related to *Desulfobulbus* (RDP-Classifier).

By using the *RDP-Classifier*, 57% (R_{AB}-Stage II) and 77% (R_{NAB}-Stage I) of sequences were classified by Phylum, whereas for Genus, only 11% (R_{AB}-Stage II) and 35% (R_{NAB}-Stage I) were classified. Sequences were found to be affiliated with 14 phyla (Fig. 3 and Supplementary Table 1). The most prevalent were the phyla *Proteobacteria* (15–35%), *Firmicutes* (12–17%), *Synergistetes* (4–7%), *Verrucomicrobia* (4–7%) and *Chloroflexi* (5–6%).

The highest prevalence of the phylum *Proteobacteria* was due mainly to the presence of four families, *Desulfobulbaceae* (3–27%), *Syntrophorhabdaceae* (3–6%), *Syntrophacea* (1.8–2.0%), and *Syntrophobacteraceae* (0.6–1.0%). Within the *Desulfobulbaceae* family, 3% (R_{AB}-Stage II) and 27% (R_{NAB}-Stage I) of the reads were related to the *Desulfobulbus* genus. This genus has the ability to use sulfate as an electron acceptor, reducing it to sulfide. A high bioavailability of sulfate was observed in the laundry wastewater characterization (1115 mg L^{-1}; Table 2), with an influent concentration of 52 mg L^{-1} (both reactors; Table 3). Moreover, the difference between R_{AB}-Stage II and R_{NAB}-Stage I is most likely related to the operational strategy of each reactor (Table 1). In R_{AB}-Stage I, the

Table 4
Final LAS mass balance.

	R_{AB} adapted biomass				R_{NAB} not adapted biomass	
	Stage I		Stage II		Stage I	
	mg LAS	%	mg LAS	%	mg LAS	%
Mass added	2313	–	1516	–	1313	–
Mass in effluent	820	35	287	19	239	18
Mass degraded	1289	56	1111	73	1025	78
Mass adsorbed – sludge blanket	174	8	66	4	44	3
Mass adsorbed – phase separator	29	1	52	3	5	0
Adsorbed on the sludge blanket (mg LAS g TS^{-1})	14.6 ± 2.4		7.9 ± 0.1		7.6 ± 0.9	
Period with LAS (d)	218		173		197	

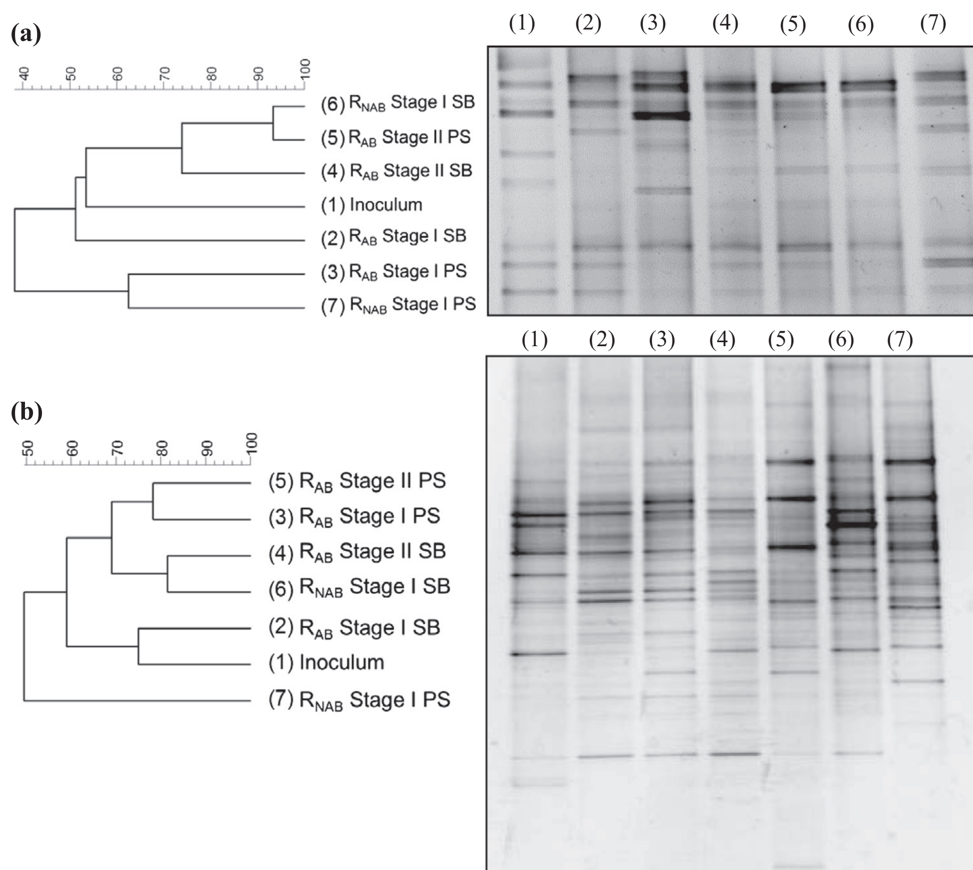

Fig. 2. Cluster analysis based on the DGGE profiles for the *Archaea* domain (a) and *Bacteria* domain (b). SB indicates sludge blanket, and PS indicates phase separator.

Table 5
Pyrosequencing result analysis, richness estimator and diversity index from R_{AB} Stage II and R_{NAB} Stage I samples.

	R_{AB} – Stage II	R_{NAB} – Stage I
Pyrosequencing result analysis		
Good's estimated coverage (%)	94.0	96.2
Total sequences (raw data)	3161	6442
Total sequences (trimmed data)	2598	5318
Sequence length (bp)	225 ± 1.0	225 ± 1.3
Total OTUs	382	505
Singletons	150	193
Total OTUs (taxonomical classification)	232	312
Richness estimation		
Chao1	544 ± 74	749 ± 92
Rarefaction	382 ± 20	505 ± 19
Diversity index		
Shannon (*H*)	4.92 ± 0.11	4.56 ± 0.16
Simpson (1 − *D*)	0.98 ± 0.01	0.96 ± 0.01
Dominance	0.02 ± 0.01	0.04 ± 0.01

longer operation time (218 days) without a high concentration of sulfur acted as a selective pressure on the microbial community, decreasing organisms related to the reduction of sulfate compounds. However, R_{NAB} was fed with a high sulfate concentration from the first day. Additionally, *Desulfobulbus* has enzymatic mechanism for the cleavage of aromatic compounds and actively participates in the removal of C compounds (by dissimilative sulfate reduction), favoring the removal of LAS.

Other genera related to dissimilative sulfate reduction and the degradation of aromatic compounds were found, such as *Desulfomicrobium* (0.08%, only R_{NAB}-Stage I; *Desulfomicrobiaceae* family) and *Desulfomonile* (0.08%, only R_{AB}-Stage II; *Syntrophaceae* family). *Syntrophorhabdus* (3.6% R_{AB}-Stage II and 2.0% R_{NAB}-Stage I) and *Parvibaculum* (0.17%, only R_{AB}-Stage II) were also related to aromatic compound degradation. *Syntrophorhabdus* shows the ability to oxidize benzoate (e.g., *Syntrophorhabdus aromaticivorans*), mainly in syntrophy with hydrogenotrophic methanogens (Qiu et al., 2008). *Parvibaculum* is characterized as Gram-Negative rods with the capacity to perform β and ω-oxidation, which can start the catabolism of LAS molecules (Schleheck et al., 2004). In addition, the family *Synergistaceae* (Phylum *Synergistes*), found in 7% of sequences from sample R_{AB}-Stage II and 4% of sequences from R_{NAB}-Stage I, has the enzymatic machinery to perform ω-oxidation under anaerobic conditions (Allison et al., 1992).

Within the phylum *Firmicutes*, two genera were observed: *Acetobacterium* (0.08%, only R_{NAB}-Stage I; *Eubacteriaceae* family) and *Sporomusa* (0.54% R_{AB}-Stage II, 0.26% R_{NAB}-Stage I; *Veillonellaceae* family). *Sporomusa* is strictly anaerobic and performs reactions with methoxylated aromatic compounds (Breznak, 2006), and it has been detected in reactors treating LAS (Delforno et al., 2012).

Two genera belonging to the phylum *Chloroflexi* and family *Anaerolineaceae* were related to the structure of granular sludge (*Leptolinea* and *Longilinea*; 0.21–0.30% – R_{AB} and 0.01–0.12% – R_{NAB}). According to Yamada et al. (2006), *Leptolinea*, is filamentous bacteria that grow in strictly anaerobic conditions, commonly found on the surface of granular sludge. The same is observed with *Longilinea*: they are multicellular and filamentous, growing under strictly anaerobic conditions (Yamada et al., 2006). Thus, both the genera related to *Anaerolineaceae* family are associated with the granular structure.

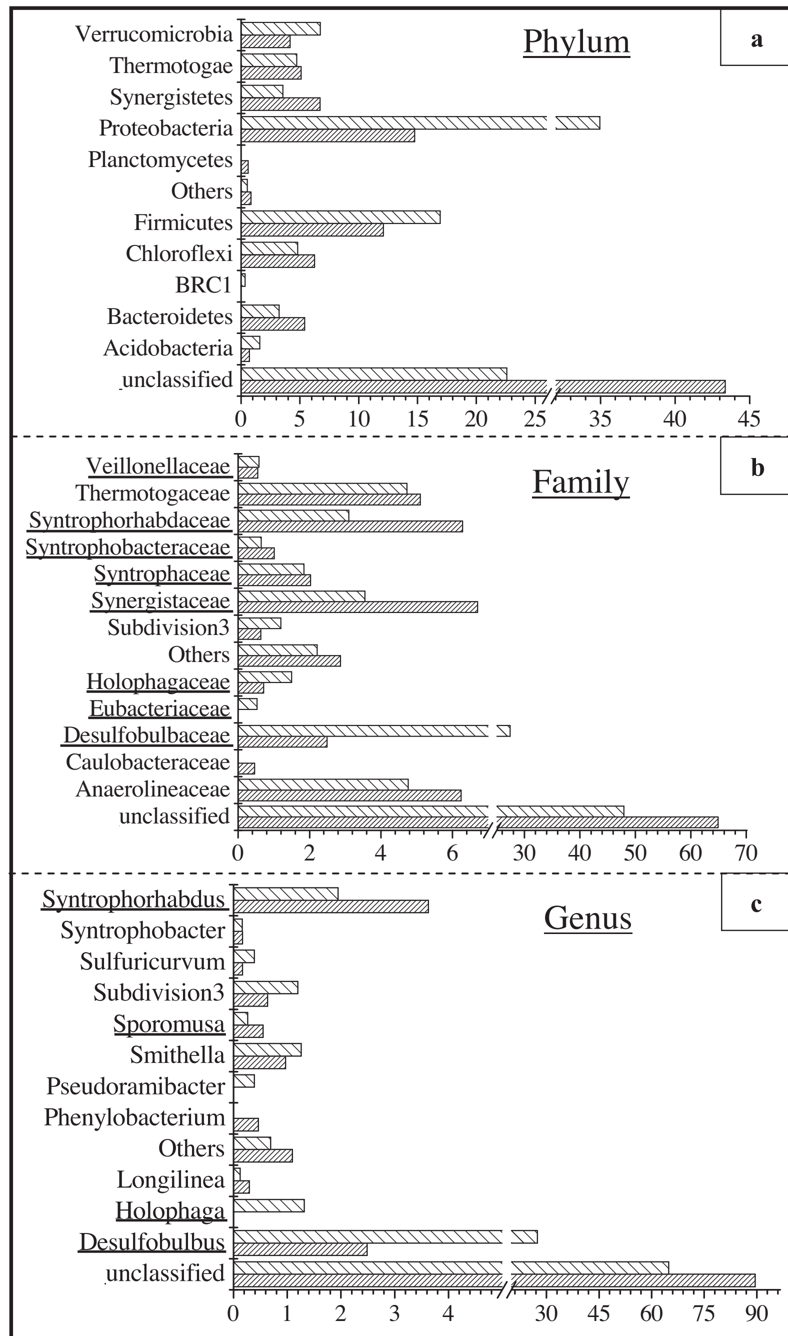

Fig. 3. Relative abundance of (a) Phyla, (b) Family and (c) Genera found in samples taken from R_{AB} – Stage II (hatched) and R_{NAB} – Stage I (hatched), at 97% similarity level. The samples were classified using RDP Classifier at a confidence threshold of 50% for Phyla/Family and at 80% for Genera. Underlined families and genera are related to the degradation of aromatic compounds.

Apart from the genera *Desulfobulbus*, *Sporomusa* and *Syntrophorhabdus*, the genus *Holophagae* (0.08% R_{AB}-Stage II and 1.36% R_{NAB}-Stage I), related to the phylum *Acidobacter* and family *Holophagacea*, has the capacity to degrade aromatic compounds (Krieg et al., 2010). In fact, approximately, 7% (R_{AB}-Stage II) and 31% (R_{NAB}-Stage I) of the sequences (genus taxonomical level) found are associated with the degradation of aromatic compounds and/or intermediate LAS molecules. Moreover, 85–95% of the sequences were related to strictly anaerobic microorganism, supporting the resazurin test (colorless upon addition).

4. Conclusion

The strategy of biomass adaptation, first with standard LAS (R_{AB}-Stage I; 64% surfactant removal) and second by replacing standard LAS by laundry wastewater (R_{AB}-Stage II; 76%), did not result in an increase of surfactant removal, when compared to the reactor fed directly with laundry wastewater (R_{NAB}-Stage I; 78%). The richness of the microbial community of the inoculum increases the robustness of the process; changes in feed resulted in changes in the bacterial community. Moreover, the pyrosequencing led to

the identification of genera related to the degradation of aromatic compounds and sulfate reduction in both reactors.

Acknowledgement

The present study was funded by the *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP), Process no. 2011/06783-1.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.11.102.

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