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Microbial diversity and the implications of sulfide levels in an anaerobic reactor used to remove an anionic surfactant from laundry wastewater

T.P. Delforno [a,*], A.G.L. Moura [b], D.Y. Okada [b], I.K. Sakamoto [b], M.B.A. Varesche [b]

[a] Microbial Resources Division, Research Center for Chemistry, Biology and Agriculture (CPQBA), Campinas University – UNICAMP, CP 6171, Campinas, SP CEP 13081-970, Brazil
[b] Laboratory of Biological Processes, Department of Hydraulics and Sanitation, Engineering School of São Carlos – University of São Paulo (EESC – USP) Campus II, São Carlos, SP CEP 13563-120, Brazil

HIGHLIGHTS

- Two specific LAS loading rates (1.0 and 2.7 mg LAS gVS^{-1} d^{-1}) were evaluated.
- S^{-2} greater than 20 mg S L^{-1} can inhibit microbiota involved with LAS removal.
- Microbial stratification and high diversity was observed in the reactor.
- By means Ion Torrent sequencing was observed an LAS-removing microbial core.

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ABSTRACT

The objective of this study was to evaluate the removal of linear alkylbenzene sulfonate (LAS) from commercial laundry wastewater using an expanded granular sludge bed (EGSB) reactor with two specific LAS loading rates (SLLRs), 1.0 and 2.7 mg LAS gVS^{-1} d^{-1}. The biomass was characterized using denaturing gradient gel electrophoresis (DGGE) and 16S Ion Tag sequencing. Higher LAS removal (92.9%) was observed in association with an SLLR of 1.0 mg LAS gVS^{-1} d^{-1} than with an SLLR of 2.7 mg LAS gVS^{-1} d^{-1} (58.6%). A relationship between the S^{-2} concentration in the effluent and the surfactant removal efficiency was observed. This result is indicative of the inhibition of LAS-removing microbiota at S^{-2} concentrations greater than 20 mg S L^{-1}. By using DGGE, microbial stratification was observed in the reactor in association with granule size, even though the reactor is considered to be a completely mixed regime. The RDP-classifier identified 175 genera, 33 of which were related to LAS degradation.

1. Introduction

Surfactants are one of the most versatile products. Their importance can be seen by the values in the global market (US$ 26.8 billion) and annual production (14,630 thousand tons) in 2012. The Brazilian market in 2012 reached US$ 1.5 billion with annual production of 522 thousand tons. Among all surfactants, the anionic surfactants receive special attention due to the high global (2942 thousand tons) and Brazilian (293 thousand tons) production, representing annual prices of US$ 7.5 billion and US$ 0.75 billion, respectively (Frost and Sullivan, 2013 *apud* Bain & Company and GasEnergy, 2014).

Linear alkylbenzene sulfonate is the most important anionic surfactant, with large worldwide production due to low-cost production and excellent properties. Because of its intense use, LAS can be found in domestic sewage (1–18 mg L^{-1}) and laundry wastewater (17–1024 mg L^{-1}; Braga and Varesche, 2014; Delforno et al., 2014). Therefore, previous studies have reported several problems associated with inappropriate disposal of wastewater contaminated with LAS, including dispersing pollution, foaming in rivers, oxygen diffusion problems, inhibition of microorganisms with regard to natural depuration and others (Garcia et al., 2005). To minimize these problems, certain studies have focused on LAS removal using physical–chemical treatment (Ge et al., 2004; Sostar-Turk et al., 2005) and anaerobic reactors (Braga and Varesche, 2014; de Oliveira et al., 2010; Delforno et al., 2014; Okada et al., 2014).

Accordingly, previous studies evaluated the LAS removal in laundry wastewater using fluidized bed reactors (FBR) (Braga and Varesche, 2011), expanded granular sludge bed reactors (EGSB)

* 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), isabel.sakamoto@gmail.com (I.K. Sakamoto), varesche@sc.usp.br (M.B.A. Varesche).

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(Delforno et al., 2014) and up-flow anaerobic sludge blanket reactors (UASB) (Okada et al., 2014).

In the FBR and EGSB reactors, the authors used influent supplementation (e.g., vitamins, co-substrates, or solutions of macro and micronutrients), whereas, in the UASB reactor, the authors operated without supplementation. In UASB reactor, Okada et al. (2014), noted that it is feasible the LAS removal in commercial laundry wastewater without supplementary feeding, only supplementation with buffering agent (sodium bicarbonate). The authors aforementioned observed that the concentration of volatile fatty acids and others compounds present in the laundry wastewater can support the microbial activity.

In addition, studies have been applied LAS influent concentration range from 9.5 ± 3.0 to 23.9 ± 5.0 mg L^{-1} in FBR (Braga and Varesche, 2011) and around 10.0 mg L^{-1} in EGSB and UASB reactor (Delforno et al., 2014; Okada et al., 2014). The higher removal rate was 82% and the lower was 68% for UASB and FBR, respectively.

Therefore, this study investigated the removal of LAS in commercial laundry wastewater with two specific LAS loading rates (SLLRs), 1.0 and 2.7 mg LAS gVS^{-1} d^{-1}, in an EGSB reactor (12.0 and 29 mg LAS L^{-1} in the influent, respectively) without supplementary feeding. The increase of SLLR was to evaluate the changes in microbial community under higher LAS concentration associate with laundry wastewater without supplementary feeding.

The biomass was characterized using two techniques: PCR–DGGE (polymerase chain reaction–denaturing gradient gel electrophoresis) and high-throughput sequencing. The objective for DGGE was to determine whether a possible microbial stratification along the reactor was present, and the goal of the high-throughput sequencing (Ion Torrent Platform) was to identify the microorganisms involved probably in LAS degradation.

2. Methods

2.1. EGSB – expanded granular sludge bed reactor

The reactor consisted of an acrylic apparatus with a volume of 1.40 L, a height of 1.0 m and a diameter of 0.04 m. At the upper end, there was a device to separate the solid, liquid and gas phases, and at the base, there was a distributor of flow. The up-flow velocity was constant (4 m h^{-1}) to generate effluent recirculation. Six sampling points were installed along the bed of the reactor, and one was installed on the phase separator. During the operation, the reactor was maintained at a mesophilic condition of 30 °C in a climatic chamber and with a hydraulic retention time (HRT) of 38 h. With the purpose of ensuring anaerobic systems, two procedures were adopted: the use of a water seal and the use of a siphon at the outlet of the system, which prevented the entry of oxygen via hose effluent. The reactor was inoculated with a granular sludge (7.0 gTVS L^{-1}) obtained from a full-scale UASB plant treating effluent from a poultry slaughterhouse.

2.2. Experimental setup

The EGSB reactor was operated in three stages. Stage I consisted of the reactivation of microorganisms because the granular sludge was kept under refrigeration (4 °C). Thus, in this stage, the reactor was fed with a synthetic medium (Touzel and Albagnac, 1983; Angelidaki et al., 1990). In Stages II and III, the synthetic medium was replaced with diluted laundry wastewater (only the sodium bicarbonate was kept to maintain a neutral pH) at two specific LAS loading rates (SLLRs): 1.0 ± 0.3 mg LAS $gTVS^{-1}$ d^{-1} (Stage II – 12.0 ± 3.0 mg LAS L^{-1} influent) and 2.7 ± 0.7 mg LAS $gTVS^{-1}$ d^{-1} (Stage III – 28.8 ± 6.4 mg LAS L^{-1} influent). Several studies have been evaluated anaerobic reactor with influent LAS concentration range from 10 to 15 mg L^{-1} (de Oliveira et al., 2010; Delforno et al., 2012, 2014; Okada et al., 2014). In most cases, when the influent concentration exceeds these concentrations the reactor becomes unstable. However, due to the satisfactory results obtained in Stage II, the concentration of LAS influent was doubled to evaluate the microbial response under higher LAS concentration. It is noteworthy that the LAS concentration in the Stage III is below of inhibition concentration (50–100 mg LAS L^{-1}) as determined by Angelidaki et al. (2004), Garcia et al. (2005).

2.3. Synthetic medium

The reactor was fed with a modified mineral medium (an adjusted $MgCl_2$ concentration of 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, which consisted of ethanol (250 mg COD L^{-1}), methanol (250 mg COD L^{-1}) and yeast extract (250 mg COD L^{-1}).

2.4. Laundry wastewater

The wastewater was collected from a commercial laundry located in São Carlos, SP, Brazil. During the washing process two compounds were added, (i) detergents and (ii) neutralizer-acidulant. The detergent composition consisted of LAS, nonionic surfactants, builders, bleaching agents, enzymes, fragrance, neutralizers and alkalizing agents (sodium hydroxide). The neutralizer-acidulant consisted of sodium sulfate, sodium metabisulfite and an alkaline vehicle. Thus, the source of LAS was one compound and the source of sulfate was another compound. It is noteworthy that all compounds were added manually by laundry employees, with low precision. The wastewater was collected after the first rinse in 10 or 20 L high-density polyurethane bottles, and the bottles were stored at a temperature of 4 °C. After each collection, the commercial laundry wastewater was characterized. In addition, due to the high concentration of LAS in the wastewater and the inhibitory concentration for methanogenic process, the laundry wastewater was diluted in public water supply as a function of LAS concentration determined in each stage. Thus, the dilution during the Stage II was around 1:9 (laundry wastewater: public water supply) while at Stage III was around 1:5 (laundry wastewater: public water supply).

2.5. Physical chemical analysis

Analyses of pH (4500), total solids (2540D), total dissolved sulfide (4500-S2-.D) and chemical oxygen demand (COD; 5220D) were determined according to the Standard Methods for Examination of Water and Wastewater (APHA-AWWA-WPCF, 2005).

Nitrate and sulfate were quantified by ion chromatography using a Dionex ICS-5000 with IonPAC AS23 (4 mm) and an eluent of $Na_2CO_3/NaHCO_3$ (1 mL min^{-1}). The 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 (Bio-Rad; Penteado et al., 2013).

LAS was quantified by HPLC using 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) .

2.6. Biological analysis

The analysis of the microbial community was performed by PCR–DGGE (domain Bacteria) and sequencing of the 16S rRNA gene. The analysis by PCR–DGGE was performed in two ways: (i) the first aimed to compare the microbial community of the inoculum and the end of Stages II and III in the reactor. For this purpose, homogeneous samples of the sludge blanket (SB) (Stages II and III) and the phase separator (PS) (Stages II and III) were sampled. (ii) The second method aimed to evaluate the community at different sampling points in the sludge blanket and the phase separator, thus verifying the existence of a possible microbial stratification along the reactor (only Stage III). For this purpose, samples were taken from the flow distributor (P1), three sludge blanket sites (P2, P3, and P4) and the phase separator (P5). For the phylogenetic characterization of the Bacteria domain, homogeneous samples were taken from the phase separator and the sludge blanket at the end of Stages II and III. Thus, four samples were sequenced using an Ion 318™ Chip Kit v2 400 bp.

2.6.1. DNA extraction

Total DNA extraction for PCR–DGGE and 16S rRNA sequencing was performed using a modified phenol–chloroform protocol described by Griffiths et al. (2000). The 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.

2.6.2. PCR–DGGE

PCR–DGGE was performed as described in Duarte et al. (2010). 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.

2.6.3. 16S rRNA Ion Tag sequencing

The sequencing was performed at the GenOne Biotechnologies enterprise (Rio de Janeiro, Brazil) using 400-base chemistry and the Ion Torrent PGM technology (Life Technologies), specifically the 318 Chip. The rRNA genes were amplified using a primer set that flanked the V4 hypervariable region: primers 577F (5′-AYTG GGYDTAAAGNG-3′) and 924R (5′-CCGTCAATTCMTTTRAGT-3′), generating an amplified fragment with an average length of 347 bp. According to Neefs et al. (1990), the 16S rDNA gene is divided into nine variable regions (V1–V9), the V2, V4 and V6 regions are the most used to phylogenetic affiliation. In addition, Liu et al. (2007) observed low error rate for V2 and V4 region. Using the Ribosomal Database Project (RDP) RDP's Pipeline (http://pyro.cme.msu.edu/index.jsp; Cole et al., 2009), the sequences were processed as described in Delforno et al. (2014). The 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 the accession numbers ERS629278 (Stage II – PS), ERS629279 (Stage II – SB), ERS629280 (Stage III – SB) and ERS629281 (Stage III – PS). The project accession number is PRJEB8003.

3. Results and discussion

3.1. Reactor performance

In the total, five samples were taken to analyze the composition of the laundry wastewater (two samples during the Stage II and three samples during the Stage III), the average values found were 119 ± 53 mg LAS L^{-1}, 1582 ± 455 mg COD L^{-1} and 205 ± 104 mg S L^{-1} (sulfate). The pH ranged from 9.6 to 10.7, and the VFA average was 442 ± 556 mg COD L^{-1}. The primary acids detected were lactic acid (168.6 ± 174.8 mg L^{-1}) and malic acid (153.6 ± 326.7 mg L^{-1}). The laundry wastewater analyzed by Braga and Varesche (2014) possessed similar levels of LAS (162 ± 244 mg LAS L^{-1}) and COD (1710 ± 968 mg COD L^{-1}) but a lower sulfate concentration (7.0 ± 6.4 mg S L^{-1}) and a lower pH (5.6 ± 0.9). Interestingly, Braga and Varesche (2014) obtained a peak LAS concentration of 1024 mg L^{-1}, whereas the present results only reached 202 mg LAS L^{-1}. Delforno et al., 2014, who analyzed seven samples of laundry wastewater from a commercial laundry located in São Carlos, SP, Brazil, obtained similar values of 181 ± 82 mg LAS L^{-1}, 1603 ± 692 mg COD L^{-1} and 124 ± 74 mg S L^{-1} of sulfate. Moreover, the pH was basic and ranged from 9.0 to 10.0. Because of the high concentration of LAS, the laundry wastewater was diluted with a public water supply to obtain an influent LAS concentration range of 12.0–28.8 mg L^{-1} (as a function of each stage), which is below the inhibitory level for anaerobic processes (50 mg LAS L^{-1}; Angelidaki et al., 2004).

Stage I (26 days) was characterized by microbial reactivation because the granular sludge was refrigerated. The specific organic loading rate (SOLR) was 69 ± 9 mg COD gVS^{-1} d^{-1} (influent COD concentration of 755 ± 277 mg L^{-1}), and the removal efficiency was 89 ± 19% (Table 1).

In Stage II (only laundry wastewater by 65 days), the SOLR decreased to 21 ± 9 mg COD gVS^{-1} d^{-1}. The influent COD was

Table 1
Means and standard deviations of parameters analyzed in EGSB reactor.

Parameters	Stage I	Stage II	Stage III
COD			
Influent (mg L^{-1})	755 ± 277	221 ± 81	237 ± 114
Effluent (mg L^{-1})	90 ± 40	81 ± 30	123 ± 76
Removal (%)	89 ± 19	61 ± 15	48 ± 19
Specific organic load (mg COD gVS^{-1} d^{-1})	69 ± 9	21 ± 9	21 ± 11
LAS			
Influent (mg L^{-1})	–	12.0 ± 3.2	28.8 ± 6.4
Effluent (mg L^{-1})	–	0.9 ± 1.2	12.2 ± 7.7
Removal (%)	–	92.9 ± 10.3	58.6 ± 25.8
Specific load (mg gVS^{-1} d^{-1})	–	1.0 ± 0.3	2.7 ± 0.7
Specific removal (mg gVS^{-1} d^{-1})	–	0.9 ± 0.3	1.6 ± 0.8
Sulfate			
Influent (mgS L^{-1})	–	29.2 ± 10.1	53.8 ± 17.2
Effluent (mgS L^{-1})	–	12.5 ± 10.4	19.5 ± 14.4
Removal (%)	–	59.6 ± 29.1	62.6 ± 26.6
Sulfide effluent (mgS L^{-1})	–	1.8 ± 2.3	20.2 ± 25.1
Volatile fatty acids – VFAs			
Influent (mg Hac L^{-1})	–	42 ± 31	65 ± 36
Effluent (mg Hac L^{-1})	–	10 ± 10	25 ± 27
Final biomass (sludge blanket)			
Total solids (TS) g L^{-1}		6.2	
Total volatile solids (TVS) g L^{-1}		2.0	
Final biomass (phase separator)			
Total solids (TS) g L^{-1}		1.22	
Total volatile solids (TVS) g L^{-1}		0.56	
Duration (days)	26	65	114
HRT (hours)	39 ± 5	39 ± 4	39 ± 5

221 ± 81 mg L^{-1} with a removal efficiency of 61 ± 15%. The COD was related to volatile organic acids, surfactants, fibrous material and recalcitrant compounds present in the wastewater; thus, the lower COD removal was due to a portion of the material that was not readily degradable.

The specific LAS loading rate (SLLR) in Stage II was 1.0 ± 0.3 mg LAS gVS^{-1} d^{-1} (12.3 ± 3.2 mg LAS L^{-1} influent), and the average removal was 92.9 ± 10.3%. It is noteworthy that the feeding of EGSB reactor without synthetic medium (only laundry wastewater) did not result in system instability.

Delforno et al. (2014), operating an EGSB reactor with a similar SLLR (1.0 ± 0.7 mg LAS gVS^{-1} d^{-1}) of laundry wastewater and SOLR (71 ± 13 mg COD gVS^{-1} d^{-1}) of synthetic medium, obtained an average of 76.4 ± 18.1%. Thus, the absence of influent supplementation (present study) showed an increase around 15% comparing to Delforno et al. (2014) which supplemented with synthetic medium.

Probably, the SOLR was the key parameter for obtaining high surfactant removal rates with values of 21 ± 11 and 71 ± 13 mg COD gVS^{-1} d^{-1} for the present study (Stage II) and Delforno et al. (2012), respectively.

In UASB reactor Okada et al. (2013) for an SOLR of 30 ± 1 mg COD gVS^{-1} d^{-1}, the LAS removal efficiency was 76%, whereas for 70–180 mg COD gVS^{-1} d^{-1}, the removal values ranged from 37% to 53%. The organic components that comprised the SOLR were methanol, ethanol and yeast extract in the synthetic medium. Thus, Okada et al. (2013) verified that decreasing SOLR increased LAS removal.

At Stage III, the SOLR was similar to Stage II (21 ± 11 mg COD gVS^{-1} d^{-1}) but the COD removal was 48 ± 19%. The difference between Stage II and Stage III was due to the COD related to sulfide (Stage II: 1.76 ± 2.33 mg S L^{-1}; Stage III: 20.2 ± 25.1 mg S L^{-1}). The SLLR was 2.7 ± 0.7 mg LAS $gSTV^{-1}$ d^{-1} (LAS influent: 28.8 ± 6.4 mg L^{-1}), and the LAS removal rate decreased to 58.6 ± 25.8%. A possible relationship was observed between increases in the concentration of S^{-2} in the effluent (due to sulfate in the laundry wastewater) and decreases in surfactant removal. During Stage II, the sulfide effluent concentration was not higher than 10 mg S L^{-1}, and the LAS removal was greater than 70%. However, with the beginning of Stage III (the 91st day of operation), an increase in sulfide concentrations in the effluent was observed (<20 mg S L^{-1}), and the LAS removal efficiency dropped below 40% (Fig. 1). The increase in sulfide concentrations was due to the increase in laundry wastewater input into the reactor. Accordingly, the sulfate concentration increased (Table 1), resulting in a higher concentration of sulfide generated by the sulfate reduction.

Although increasing concentrations of LAS can be connected to the reduction removal efficiency (Delforno et al., 2012), a high removal of LAS was observed during a period of Stage III (days 151–185) in which the sulfide levels were lower. During days 151–185, the LAS removal was 85% in association with a sulfide concentration of 1 ± 2 mg S L^{-1}. During days 91–150 and 186–217,

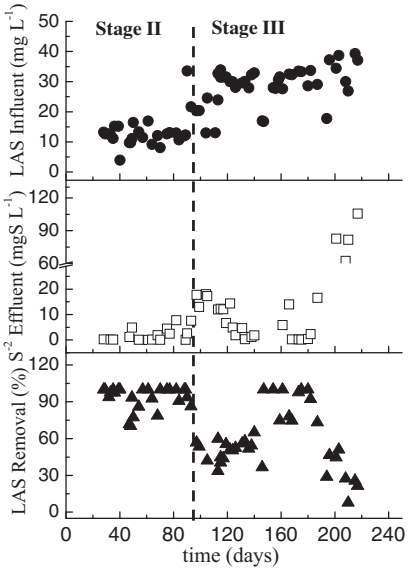

Fig. 1. Temporal variation in LAS removal (▲), sulfide concentration (□) and influent LAS concentration (●).

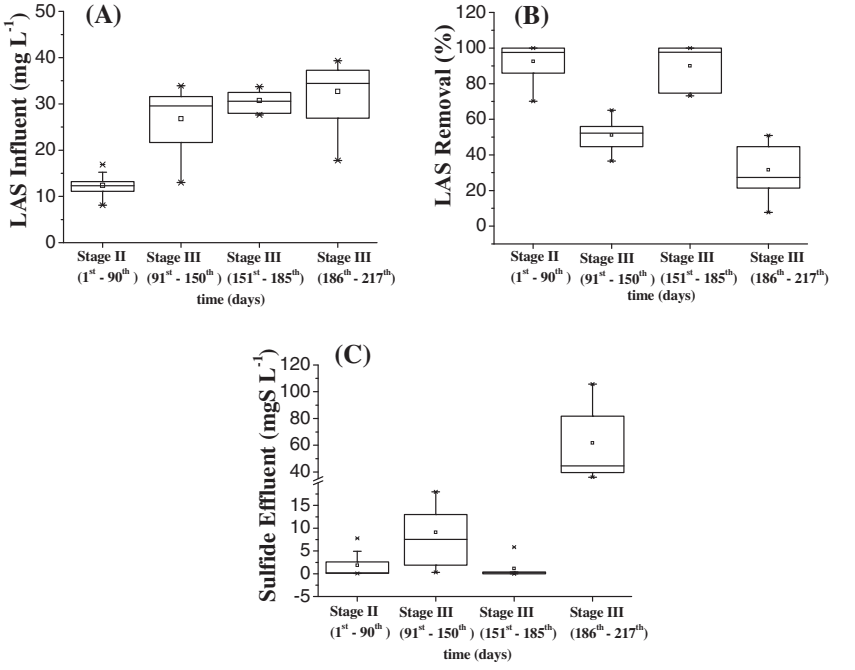

Fig. 2. Box plot of LAS influent (A), LAS removal (B) and sulfide effluent (C).

the sulfide concentrations were 9 ± 6 and 62 ± 25 mg S L^{-1}, and the LAS removal rates were 50% and 30%, respectively (for LAS influent concentrations of 32 ± 7 and 30 ± 2 mg LAS L^{-1}, respectively). Therefore, the sulfide levels in the reactor were related to the LAS removal rate (Fig. 2).

Although the anaerobic microbiota can support high S^{-2} concentrations (100–150 mg L^{-1}, at a pH of 6.8; Speece, 1983), the results demonstrate that the microbiota involved with LAS removal do not thrive in concentrations greater than 20 mg L^{-1}. In other words, these LAS-removing microbiota exhibit a low tolerance to S^{-2}. In actuality, this susceptibility could be the result of many factors, such as competition for the same substrates between sulfate-reducing bacteria (SRB) and the LAS-removing microbiota, non-competitive inhibition caused by SRB-generated sulfide and/or toxicity of S^{-2}. These results represent a new challenge in the application of anaerobic reactors for the removal of LAS present in laundry wastewater with high sulfate concentrations.

According to the mass balance of the total amount of LAS added to the reactor (4460 mg LAS), 7% and 2% were adsorbed to the biomass in the sludge blanket and phase separator, respectively. Biological degradation accounted for 52%, whereas adsorption and biological removal removed 61%. The mass balance was realized at the end of the reactor operation (Stage II and Stage III), and Stage III showed low LAS removal rates, resulting in a decrease in the percentages of the mass balance.

3.2. PCR/DGGE analyses

Based on the biomass DGGE profile taken along the reactor (Fig. 3C and D), the bacterial populations differed as a function of the sampling site. Because the reactor is considered completely mixed (i.e., a high ratio of recirculation flow to feed flow), the nutrient distribution does not differ along the reactor (Seghezzo et al., 1998). However, stratification was observed in relation to the size of the granules. Larger and denser granules were observed at the base of the reactor, and lower density granules were observed in the upper region of the sludge blanket. In the phase separator (P5), no granules were found, only flocculent biomass attached to the acrylic. The P4 biomass was 78% similar to the phase separator sample (P5), both of which lack granular structure. A 73% similarity existed between the populations of P2 and P3, both of which contained well-defined granules. However, a similarity coefficient of only 30% existed between these two groups (i.e., biomass structure in the form of granules (P2 and P3) and flocculent material (P5 and P4).

The biomass of the phase separator region P5 and P4 exhibited lower similarity coefficients (<34%) when compared to the biomass from the inoculum. These regions likely experienced the largest changes to the original biomass. According McHugh et al. (2003), two of the main advantages of the microbial granule arrangement are the collective defense with regard to toxic compounds and optimization of the survival of microbial populations. For this reason, the absence of the granular structures results in greater susceptibility of the microbiota to environmental changes. In the biomass DGGE profiles taken at the end of Stages II, III and the inoculum, large-amplitude variations in similarity values and clustering with the operation stages were observed. The highest similarity coefficient (60%) was between the inoculum and Stage II biomass sludge blanket.

The biomass of the flow distribution (P1) was 52% similar to the points P2 and P3 (granular biomass) and <40% similar to the points P4 and P5 (flocculent biomass). This pattern was likely due to the presence of a dead zone with fragmented granules.

3.3. Microbial composition

Using the Ion Torrent PGM technology for massive sequencing, it was obtained 396,818 sequences of 16S rRNA genes (4 samples;

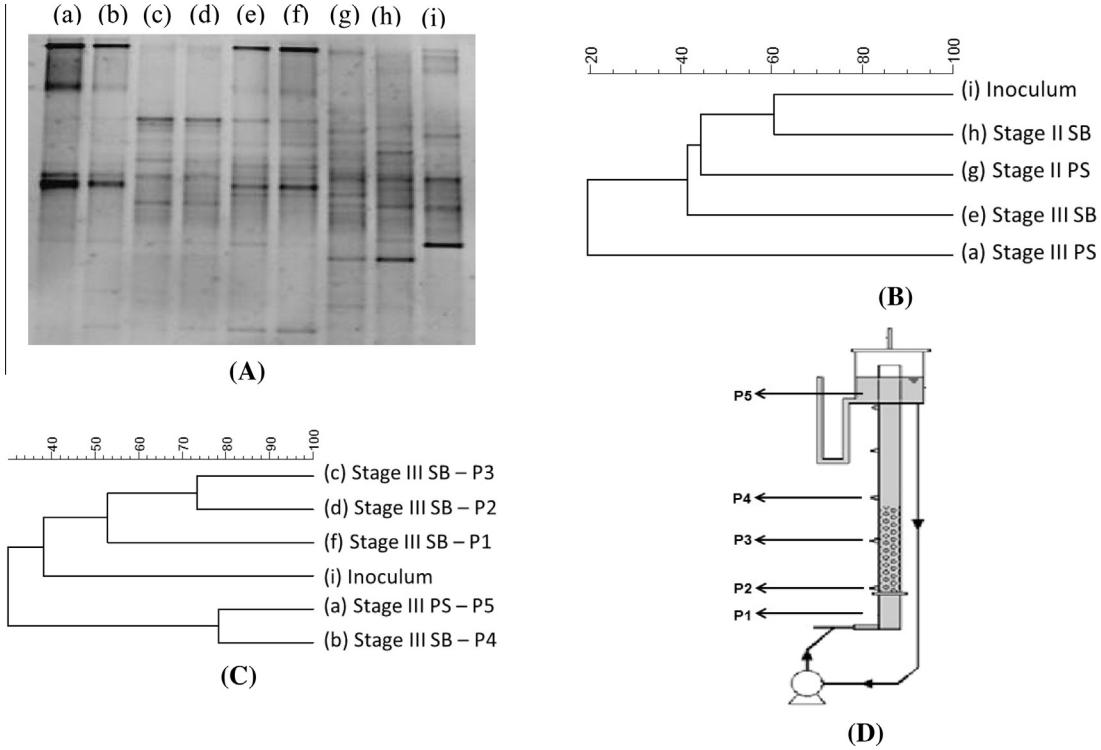

Fig. 3. Cluster analysis based on the DGGE profiles for the domain Bacteria. SB represents the sludge blanket and PS the phase separator. (A) Photo of DGGE with all samples. (B) Homogeneous samples collected at the end of Stage II (12 mg LAS L^{-1}) and Stage III (29 mg LAS L^{-1}). (C) Samples were taken from different areas of the reactor in Stage III. (D) Sampling sites along the reactor in Stage III: flow distributor (P1), three sludge blanket sites (P2, P3 and P4) and the phase separator (P5).

Table 2
Results of 16S rRNA Ion Tag sequencing.

	Stage II – SB	Stage II – PS	Stage III – SB	Stage III – PS
Result analysis				
Good's estimated coverage (%)	95	96	96	96
Total sequences (raw data)	140,686	96,442	81,282	78,408
Total sequences (trimmed data)	63,957	44,330	40,273	31,260
Sequence length (bp)	329 ± 44	313 ± 47	327 ± 45	316 ± 42
Total OTUs	7277	4955	4254	3489
Singletons	3320	1798	1571	1384
Total OTUs (taxonomical classification)	3957	3157	2683	2105
Richness estimation				
Chao1	6214 ± 523	4732 ± 1125	4256 ± 1331	3072 ± 1833
Rarefaction	4623 ± 602	3613 ± 832	3164 ± 998	2268 ± 1269
Diversity index				
Shannon (*H*)	6.24 ± 0.56	6.90 ± 0.16	6.45 ± 0.42	6.10 ± 0.61

Table 2). During the processing of sequences, 216,998 sequences were removed due to the low quality of the bases (Phred > 20), lengths less than 200 bp, chimeras or ambiguous bases. Thus, 56.6% of the total sequences were discarded. Many of the sequences were removed due to formation of a dimer between the forward and reverse primers, resulting in a fragment of approximately 35 bp (data not shown). After processing, the average size of the sequenced fragments was 313–329 bp.

By analyzing of coverage (using the Good formula), it was determined that 95–96% of all the microbial communities were accessed. Furthermore, the analyses of the rarefaction curves were used to infer that the phylum taxonomic level (80% similarity) and the number of sequences obtained were sufficient to represent all the diversity in the biomass (Supplementary Fig. 1). Stabilization occurred after 10,000 sequences. However, the rarefaction curves demonstrated that even with a large number of sequences, it was not possible to access the entire diversity at the genus (95%) and species (97%) level. Stabilization of the rarefaction curves did not occur at the genus and species levels.

The values of richness estimators (Chao1) and rarefaction ranged from 3072 to 6214 and 2268–4623, respectively. The highest values of Chao1 and rarefaction were obtained in Stage II with an SLLR of 1.0 ± 0.3 mg LAS $gSTV^{-1}$ d^{-1}, whereas the lowest values were obtained in Stage III with an SLLR of 2.7 ± 0.7 mg LAS $gSTV^{-1}$ d^{-1}. The inhibition of microbiota likely occurred due to the SLLR. Similar results were obtained in the DGGE analysis, which showed large changes in the populations in the biomass of Stage III.

The Shannon diversity index yielded values greater than 6.0 for all samples, which indicate high diversity. Furthermore, the observed Simpson values (1-D) near 1 indicate high diversity and equitable distribution.

For the comparison of the four samples, the Bray–Curtis index was used (Fig. 4A). The biomass grouped according to the operation stages of the reactor. This pattern was observed for the biomass in Stage II (PS, SB) and Stage III (PS, SB), which exhibited similarity values of 25% and 45%, respectively. With increased operation time in the reactors, the similarity between the biomass of different regions increased. Furthermore, a similarity value of only 10% was observed between the biomass of Stage II and Stage III, a result of the increased concentration of LAS and reactor operation time. By means of the presence/absence of genera in the Venn diagram, it was determined that 22% of the identified genera were present in all four samples. In contrast, 5–11% of the genera were unique to each sample (Fig. 4B).

By using the RDP-Classifier, over 95% of the sequences were classified at the phylum level. At the genera level, the percentage ranged from 16% to 75% of the classified sequences. Among the major phyla, the *Proteobacteria* phylum represented greater than 20% (relative abundance) of the samples from Stage III and 65–70% of the sludge blanket and phase separator samples. Except for the sample from Stage II SB, in which the phylum *Bacteroidetes* was higher, the phylum *Proteobacteria* was the most abundant (Fig. 5A).

The 20 most abundant genera, excluding unclassified ones, represented 99% (Stage II – SB), 84% (Stage II – PS), 93% (Stage III – SB) and 95% (Stage III – PS) of the relative abundances (date not shown). Including the unclassified sequences, the values were 15% (Stage II – SB), 24% (Stage II – PS), 50% (Stage III – SB) and 71% (Stage III – PS). Genera such as *Geobacte*r, *Syntrophobacter*, *Smithella*, *Phenylobacterium* and *Legionella* were found in four samples (Fig. 5B). From these five genera, only *Geobacter* was directly related to the degradation of LAS and/or its intermediaries because this type of Bacteria uses aromatic compounds, such as benzene and toluene, as a carbon source. *Geobacter* genus is a strict anaerobe and has the metabolic capacity to perform β-oxidation, a step in the LAS degradation (Brenner et al., 2005).

3.3.1. Microbial composition regarding to LAS degradation

In the total, 175 genera were obtained. Of these, 33 genera, representing 2.0–19.6% of the total sequences obtained from the samples, were related to the degradation of LAS and/or aromatic compounds. Higher percentages were obtained in Stage III (15.9–19.6% for 2.7 ± 0.7 mg LAS gVS^{-1} d^{-1}), which the SLLR was higher than in Stage II (2.0–6.1% for 1.0 ± 0.3 mg LAS gVS^{-1} d^{-1}). However, during the stage III the fluctuations of sulfide levels resulted in a decrease of LAS removal. But, the specific LAS removal rate (SLRR) increased from 0.9 ± 0.3 mg gVS^{-1} d^{-1} (Stage II) to 1.6 ± 0.8 mg gVS^{-1} d^{-1} (Stage III) supporting the higher percentage of microorganism related to the degradation of LAS and/or aromatic compounds. It is noteworthy that the SLRR between Stage II and Stage III showed statistical difference (ANOVA and the post hoc Tukey, $p < 0.05$).

Among the analyzed sequences, 0.16–10.79% of the sequences were related to the following genera that perform desulfonation reactions: *Acinetobacter*, *Aeromonas*, *Comamonas*, *Desulfovibrio*, *Hydrogenophaga* and *Pseudomonas*. Of the obtained sequences, 0.36–12.30% were related to the following genera with the metabolic capacity to perform β-oxidation: *Azoarcus*, *Geobacter*, *Parvibaculum*, *Pseudomonas*, *Rhodopseudomonas*, *Synergistes* and *Syntrophomonas*. Of the obtained sequences, 0.01–9.80% were related to two genera (*Pseudomonas* and *Parvibaculum*) with the metabolic capacity to perform ω-oxidation reactions. Only *Pseudomonas* has the ability to perform both β- and ω-oxidation, in addition to desulfonation. Thus, *Pseudomonas* plays a necessary role in the microbial consortium that mineralizes LAS (Brenner et al., 2005).

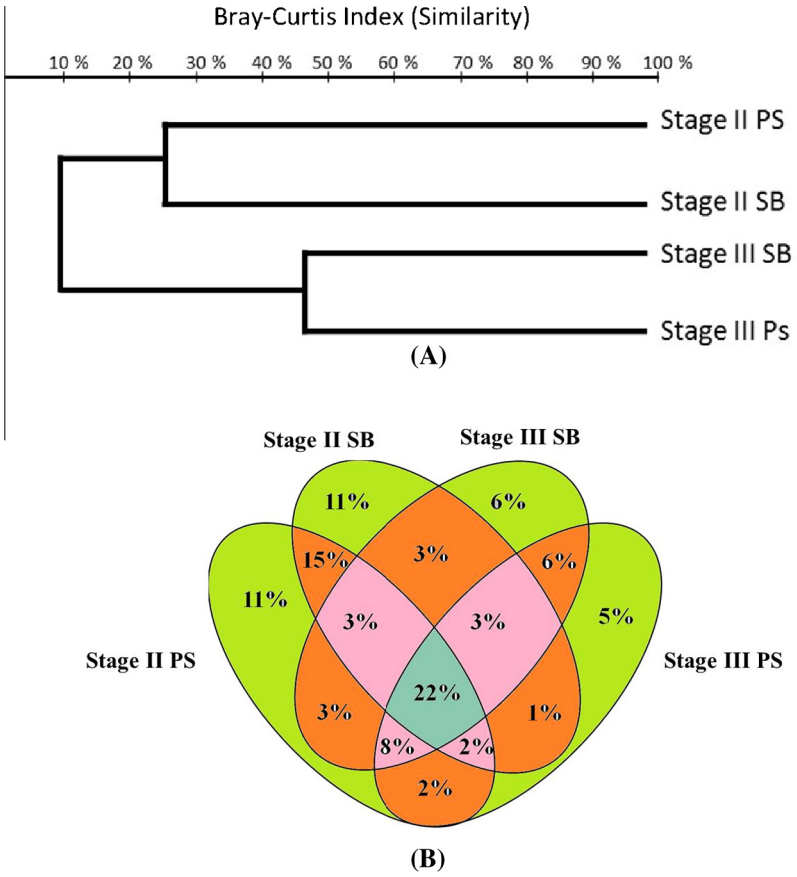

Fig. 4. Bray–Curtis index (A). Venn diagram of the presence and absence of genera (B).

In the four samples, 14 identified genera were related to anaerobic degradation of aromatic compounds: *Acinetobacter*, *Comamonas*, *Desulfobulbus*, *Desulfomonile*, *Geobacter*, *Geothrix*, *Holophaga*, *Mycobacterium*, *Pseudomonas*, *Rhodopseudomonas*, *Sporomusa*, *Stenotrophomonas*, *Sulfuritalea* and *Syntrophorhabdus*. With the exception of Sulfuritalea, the other genera have been identified in another studies related to LAS degradation (de Oliveira et al., 2009; Duarte et al., 2010; Delforno et al., 2012; Duarte et al., 2010). This result shows the existence of a microbiological core in common among the four samples and, consequently, the role of these microorganisms in LAS degradation. Probably, the presence of Sulfuritalea genus was favored to sulfur and aromatic compounds. This genus grows chemolithoautotrophically under anoxic conditions by the oxidation of reduced sulfur compounds and hydrogen. Moreover, this genus cans growth in heterotrophic conditions using organic acids and aromatic compounds such as benzoate (Kojima and Fukui, 2011).

The microbial community inside the reactor can be affected due to several reasons. Some of these reasons are the composition of the support material, for example in FBR (de Oliveira et al., 2009), presence of toxic compounds (sulfophenyl carboxylic acids – SPC) during the process of LAS biodegradation (Lara-Martin et al., 2010) and presence of toxic compounds in the laundry wastewater (Braga and Varesche, 2014). In addition, metabolized byproducts from compounds present in the laundry wastewater can represent one more factor toxic to microbial community. For example, sulfide generated from laundry wastewater rich in sulfate as previously discussed (Section 3.1 – reactor performance).

On the other hand, there are compounds in laundry wastewater which favor the degradation of the LAS such as sequestrants (e.g., EDTA) due to decrease the amount of LAS adsorbed in the biomass (Delforno et al., 2014; Okada et al., 2014).

Additionally, the reactor was subjected to high influent sulfate concentrations (30–54 mg S L^{-1}). Therefore, although there is evidence in the literature that the microbial community may have been affected by the presence of toxic compounds during the LAS biodegradation or toxic compounds present in the laundry wastewater, probably, the main modifier of LAS biodegradation and microbial community were metabolized byproducts from sulfate (eg., sulfide). This conclusion is supported by physical–chemical results (Table 1 and Figs. 1 and 2) and the several sulfur cycle genera found which will be discussed below.

3.3.2. Microbial composition regarding to sulfur cycle

Due to the high concentration of sulfate in the wastewater (205 ± 104 mg S L^{-1}), genera associated with the sulfur cycle were highly abundant (4.8%–55.3%; Supplementary Fig. 2). The relative abundance of these genera increased between Stage II (SB-4.8% and PS-6.2%) and Stage III (SB-20.3 and PS-55.3%).

The genera related to sulfate reduction include *Desulfatirhabdium*, *Desulfocapsa*, *Desulfomicrobium*, *Desulfomonile*, *Desulforhabdus*, *Desulfovibrio* and *Syntrophobacter*. Of these, certain genera (*Desulfatirhabdium*, *Desulfomicrobium*, *Desulfomonile* and *Desulfovibrio*) are related to aromatic compound degradation and have already been identified in previous studies (Cook et al., 1998; Brenner et al., 2005; Duarte et al., 2010; Delforno et al., 2014). The relative abundance in Stage II ranged from 0.01% to 0.30% in association with an influent sulfate concentration of 29.2 ± 10.1 mg S L^{-1}; whereas the relative abundance in the Stage III ranged from 3.54% to 4.75% in association with an influent sulfate concentration of 53.8 ± 17.2 mg S L^{-1}.

In addition, it was observed specific microbiota related to sulfide and elementary sulfur (Brenner et al., 2005). These genera include *Bosea*; *Desulfuromonas*; *Petrimonas* and *Sulfurovum*. None

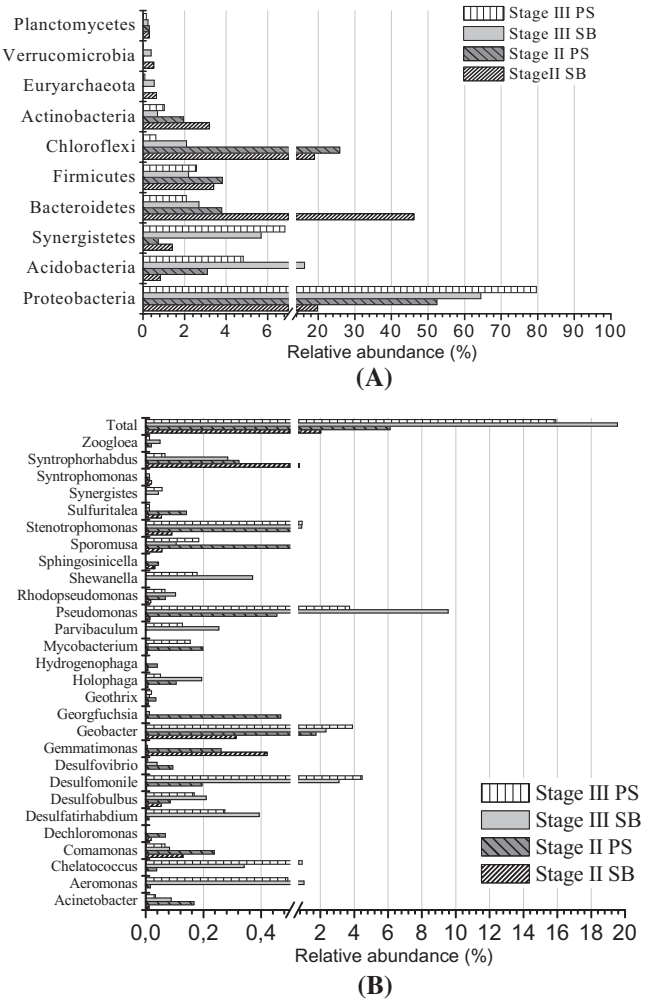

Fig. 5. Taxonomic affiliation and relative abundance of phyla (A). Taxonomic affiliation and relative abundance of genera with regard to aromatic compound degradation/LAS degradation (B). The confidence threshold adopted in the RDP Classifier was 50% for phylum/family and 80% for genus.

of these genera are related to the LAS degradation and/or its intermediaries; however, they do perform important functions associated with reactor stability.

The highest relative abundances are associated with higher sulfide concentrations (Stage II: 1.8 ± 2.3 mg S L^{-1}, 0.05%; Stage III: 20.2 ± 25.1 mg S L^{-1}, 46.8%). Moreover, the *Sulfurovum* genus is a chemolithoautotroph that uses elemental sulfur or thiosulfate as an electron donor and oxygen and nitrate as electron acceptors, with CO_2 as the carbon source (Kleinsteuber et al., 2008). A high relative abundance of this genus was observed in the region of the phase separator in Stage III (40.3%). This region is favorable due to its greater susceptibility to the presence of oxygen, availability of CO_2 and elemental sulfur.

4. Conclusions

The highest LAS removal was observed for an SLLR of 1.0 mg LAS gVS^{-1} d^{-1} (92.9 ± 10.3%). The higher SLLR, 2.7 mg LAS gVS^{-1} d^{-1}, resulted in a lower LAS removal efficiency (58.6 ± 25.8%). Based on the observations, the primary factor responsible for this lower removal efficiency was the increased sulfide levels in the reactor due to higher sulfate reduction activity. S^{-2} concentrations less than 20 mg S L^{-1} were associated with high LAS removal rates, even for an SLLR of 2.7 mg LAS gVS^{-1} d^{-1}. The molecular tools showed that microbial stratification in the reactor due to the granule size and a microbial core related to LAS removal.

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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.2015.05.050.

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