Comparison of microbial and physicochemical behavior of expanded granular sludge bed system during methylparaben and triclosan removal

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ABSTRACT

Methylparaben and triclosan are antimicrobial agents widely used as preservatives in a variety of personal care and pharmaceutical products. Wastewater is considered the main source of these compounds in the environment. Expanded granular sludge bed (EGSB) reactors are a high rate technology for wastewater treatment based on biological processes and have been shown to be efficient in removing different types of compounds; however, little is known about the effect of contaminants such as methylparaben and triclosan on their behavior and effectiveness. In this study, we evaluate and compare the microbial and physicochemical behavior of EGSB systems during methylparaben and triclosan removal. The presence of different concentrations of pollutants had an influence on the cluster organization of microbial communities, especially bacteria. However, this did not affect the stability and performance of the EGSB systems. The banding patterns of the denaturing gradient gel electrophoresis of archaea demonstrated the constant presence and abundance of *Methanosaeta concilii* throughout all stages of operation, showing that this microorganism played a fundamental role in the stability of the reactors for the production of methane. The type of compound and its concentration influenced the expression of the *mcrA* and *ACAs* genes; however, these changes did not alter the stability and performance of the EGSB systems.

Key words | anaerobic digestion, emerging pollutants, microbial communities, water treatment

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INTRODUCTION

Methylparaben (methyl 4-hydroxybenzoate) (MPB) and triclosan (5-chloro-2-(2,4-dichlorophenoxy)-phenol) (TCS) are two antimicrobial agents widely used as preservatives in a variety of cosmetic, personal care, pharmaceutical, and food products (Vosátka *et al.* 2018; Hu *et al.* 2019). It has been calculated that approximately 1,500 t of TCS are produced annually worldwide (Chen *et al.* 2012), while MPB is used in more than 22,000 products (Andersen 2008). Due to their widespread use, these compounds have been routinely found in aquatic environments such as wastewater, surface water, and oceans (Steter *et al.* 2016; Orhon *et al.* 2017), at concentrations ranging between ng/L and μg/L (Luo *et al.* 2014). Although both compounds are considered to be of low toxicity, recent reports have revealed that MPB and TSC may possess endocrine disrupting effects (Ma *et al.* 2018), and be associated with breast tumors in women and sperm DNA damage (Wu *et al.* 2017), adversely affecting human health and aquatic ecosystems. For this reason, research into their occurrence, exposure, toxicity and removal from different water bodies has received global attention.

Wastewater treatment discharges are considered to be the main source of MPB, TCS and related compounds in the environment (Wu *et al.* 2017). Therefore, it is necessary to apply effective technologies to mitigate the risks of MPB and TCS in the environment. Expanded granular sludge bed (EGSB) reactors are a super high rate technology for wastewater treatment based on biological processes, whose popularity has increased in the last decade (Xu *et al.* 2018). However, because EGSB reactors use anaerobic digestion, their performance can be affected by the low cellular yields of methanogenic microorganisms. Several studies have reported the impacts of different compounds in wastewater on the performance of anaerobic reactors. It

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has been demonstrated that antibiotics such as tetracycline, cephalosporin and amoxicillin can cause changes in microbial communities, affecting the processes involved in organic pollutant removal and operation of the system, and may even lead to the collapse of the system (Meng *et al.* 2017; Li *et al.* 2019). MPB and TCS are recognized to have antimicrobial effects, so it is possible that they could have a variety of effects on anaerobic digestion processes, decreasing organic pollutant removal efficiency. There are some reports on the removal of MPB and TCS using EGSB systems, with removals close to 80% having been reached. However, little is known about the effect of these compounds on microbial community and metabolism, and few studies comparing the effect of different antimicrobial compounds on the behavior of EGSB systems have been carried out. In this study, we evaluate and compare the microbial and physicochemical behavior of EGSB systems during MPB and TCS removal. For this purpose, biogas production and removal efficiencies of MPB, TCS and chemical oxygen demand (COD) were monitored. Changes in microbial structure were evaluated using a polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) procedure, and the effect on microbial metabolism was evaluated based on the changes in expression of *mcrA* and *ACAs* genes through qRT-PCR (quantitative reverse transcription polymerase chain reaction).

MATERIALS AND METHODS

EGSB reactors system

Five EGSB reactors were built in acrylic (E1, E2, E3, E4, and E5), with an upper part that favored solid–liquid–gas separation, a body formed by a thin cylinder for the expansion of the mud, and a shaped support with a perforated plate for the input of the feed flow. The diameter of the cylinder was 4.4 cm, with a total height of 85 cm. An effective volume of 3.4 L and an expansion volume of the sludge mantle of 1 L were obtained. Five sampling points were installed in the reactors. Inflow to the reactor was via the lower part, and this was distributed uniformly by means of a perforated plate. The upper part had two output devices: one responsible for recirculating part of the effluent and the other for evacuating the excess effluent. The reactors were inoculated with granular sludge containing a volatile suspended solids concentration of 40 g/L obtained from an upflow anaerobic sludge blanket plant that treats effluent from a poultry slaughterhouse. The experimentation process was carried out under mesophilic conditions at room temperature (22 to 26 °C), using synthetic wastewater (SWW) prepared in the laboratory. The carbon source was sodium acetate and the aqueous matrix was enriched with the macronutrients and micronutrients necessary for anaerobic metabolism. With the aim of simulating the composition of domestic wastewater, the SWW was prepared according to the following composition in mg/L: peptone, 75; meat extract, 64; urea, 15; sodium acetate, 90. For each litre of wastewater, 1 mL of macronutrients and micronutrients was added, according to the ratio of chemical oxygen demand (COD)/N/P = 600/7/1. The SWW was prepared daily for the feeding of the reactors, with a COD of 1,000 mg/L.

Operational strategy

The operational strategy consisted of the use of five EGSB-type anaerobic reactors allowing the evaluation of the two compounds (MPB and TCS), two hydraulic retention times (HRTs) (8 and 24 h), and one control reactor fed only with SWW plus sodium acetate. The reactors were named EGSB 1 (control), EGSB 2 (MPB 8 h), EGSB 3 (MPB 24 h), EGSB 4 (TCS 8 h) and EGSB 5 (TCS 24 h). The EGSB systems were operated in four experimental stages over a total of 342 days. Stage I lasted 105 days, stage II 43 days, stage III 45 days and stage IV 99 days. Stage I consisted of acclimatizing the anaerobic granular sludge to a new source of substrate (SWW). For this, the reactors were fed with mixtures at different percentages of meat-poultry industry residual water and synthetic wastewater, at respective ratios of 90/10, 80/20, 60/40, 20/80, until reaching 100% synthetic wastewater. In the three stages that followed, the concentration of each of the compounds was increased. The average concentrations obtained in the influent for TCS were 30 μg/L at stage II, 100 μg/L at stage III and 300 μg/L at stage IV. MPB reached average concentrations of 30 μg/L at stage II, 300 μg/L at stage III and 1,000 μg/L at stage IV.

Analytical procedures and methods

Influent and effluent samples were collected twice a week and either analyzed immediately or temporarily stored at 4 °C. Analysis of COD, alkalinity, sludge volume index) and volatile fatty acids (VFA) was conducted in accordance with the standard method (APHA *et al.* 2012). The biogas volume was determined by the gas collecting devices at the determined sampling time. The biogas composition (CH_4, CO_2 and H_2S) was estimated using a 6890 plus FID-NPD gas chromatograph (Agilent Technologies) equipped

with a flame ionization detector and 19091P- Q04 HP-PLOT column. MPB and TCS were determined using an Acquity UPLC system (Waters Corporation), coupled to a Xevo TQD mass spectrometer (triple quadrupole) equipped with an electrospray ionization source.

DNA extraction and PCR amplification

For the microbiological analyses, four sludge samples of 15 mL were taken from each reactor at all operation stages. DNA was extracted from 1 g of centrifuged biomass, using an E.Z.N.A soil DNA kit (Omega Bio-tek, USA), in accordance with the manufacturer's protocol. The concentrations and quality of the extracted DNA were assessed by spectrophotometric analysis using a NanoDrop ND-2000 device (Thermo Fisher Scientific, USA) and electrophoresis on a 1.5% agarose gel.

PCR–DGGE was performed as described by Green *et al.* (2010): for the bacteria domain, primer 341F with a GC-clamp (5′-CCTACGGGAGGCAGCAG-3′) and primer 5′-ATTACCGCGGCTGCTGG-3′; and for the Archaea domain, primer PARCH340f (5′-CCCTACGGGG(C/T)GCA(G/C)CAG-3′) with a GC-clamp (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′) and PARCH519R (5′-TTACCGCGGC(G/T)GCTG-3′). Both amplifications were performed using Touchdown PCR in thermocycler TM100TM (Bio-Rad, USA).

PCR products were loaded on a 6 and 8% polyacrylamide gel for bacteria and archaea respectively, with a denaturing gradient from 30 to 70%. Electrophoresis was performed using DGGEK-2001 equipment (C.B.S Scientific, USA), at a constant voltage of 200 V for bacteria and 80 V for archaea, and a temperature of 60 °C, for 14 h. The gel image was captured on the ENDURO GDS Touch gel documentation system (EnduroTM Labnet, USA) under UV light. DGGE banding patterns were analyzed using GelCompar II (Applied Maths, Ghent, Belgium) software. The similarity matrix was calculated using the Pearson correlation coefficient. Cluster analysis was performed using the unweighted pair group method with an arithmetic average (UPGMA). Similarity analysis was used to examine the significant statistical differences between the DGGE profiles, and the results were finally presented as a dendrogram.

Sequencing and phylogenetic analysis

The DNA bands from DGGE gel with unique migration patterns, higher intensity and non-overlapping were excised, re-amplified by PCR and then purified. Re-amplified DNA bands were used as templates for sequencing. Sequencing was performed by Macrogen (Korea). The sequences were aligned and compared in the GenBank database using BLAST software from the National Center for Biotechnology Information (NCBI).

Evaluation of the expression of *mcrA* and *ACAs* genes by relative qRT-PCR

Total RNAs was extracted from 1 g sludge sample using the E.Z.N.A Soil RNA mini kit (Omega Bio-Tek, USA), following the manufacturer's protocol. The cDNAs synthesis was carried out using the SuperScrip® IV First-Strand Synthesis System (Invitrogen, UK), through reverse transcription polymerase chain reaction with hexamer primers. cDNA synthesis was operated for 10 minutes at 23, 50 and 80 °C in a thermo-cycler TM100TM (Bio-Rad, USA).

cDNAs were used for the quantification of the mRNA levels of the *mcrA*, *ACAs* and *gyrB* genes, by qRT-PCR with a LightCycler 1.5 (Roche, Switzerland). The *mcrA*, *ACAs* and *gyrB* gene primer pairs used have been reported previously. Thermal cycling conditions were: 95 °C for 10 min; followed by 45 cycles of 95 °C for 10 s; 60 °C for 20 s (*ACAs* gene); 55 °C for 20 s (*mcrA* gene); 50 °C for 20 s (*gyrB* gene); and a final extension step at 72 °C for 30 s. The expression of each gene was measured in triplicate, and the relative expression ratio was calculated by the Livak and Schmittgen method.

Statistical analysis

Statistical analysis was performed with the R software. Statistical variance between the different groups of physicochemical and sequencing data was determined using variance analysis (ANOVA), and the differences were significant when the P value was <0.05.

RESULTS AND DISCUSSION

Physicochemical behavior

Table 1 shows the results of pH and the intermediate alkalinity/partial alkalinity (IA/PA) ratio, which were evaluated throughout the different stages of the study. Although the VFA were evaluated, they were always below the detection limit of the method, probably due to the use of sodium acetate as a substrate. The general pH range for all reactors varied between 7.0 and 8.4. Reports in the

Table 1 | Behavior of the pH and the AI/AP ratio of the EGSB systems

	Stage I		Stage II		Stage III		Stage IV	
Reactor	**pH**	**IA/PA**	**pH**	**IA/PA**	**pH**	**IA/PA**	**pH**	**IA/PA**
E1	7.98	0.07	7.68	0.11	7.81	0.19	7.68	0.21
E2	8.05	0.21	7.69	0.24	7.85	0.23	7.59	0.26
E3	8.04	0.19	7.55	0.24	8.25	0.22	8.40	0.28
E4	8.10	0.23	7.88	0.24	7.85	0.23	7.81	0.24
E5	8.03	0.22	7.66	0.24	8.20	0.22	8.40	0.28

literature indicate that methane-producing microorganisms have an optimum growth in the pH range between 6.6 and 7.4, although once methane stability is reached they tolerate a wider pH range. This is consistent with the methane production results shown in Figure 1. The IA/PA ratio varied in the range of 0.07 and 0.28, this being the suggested range to guarantee the good operation of the reactors. The correlation analysis showed that the presence of the compound had an influence on the results of physicochemical analysis; however, no significant difference was established between reactors with MPB and TCS.

Removal of COD and methane production

The average results of the reactors did not present significant differences in terms of COD reduction (Figure 1(b)) or methane production (Figure 1(a)) that would show an effect on the part of the contact with MPB and TCS, the increase in their concentrations or the operation at different HRTs (8 and 24 h). With regard to biogas, this has been reported to have typical composition of 55–70% methane and 30–40% CO_2, and to contain water vapor and traces of hydrogen sulfide, H_2, N_2 and other gases

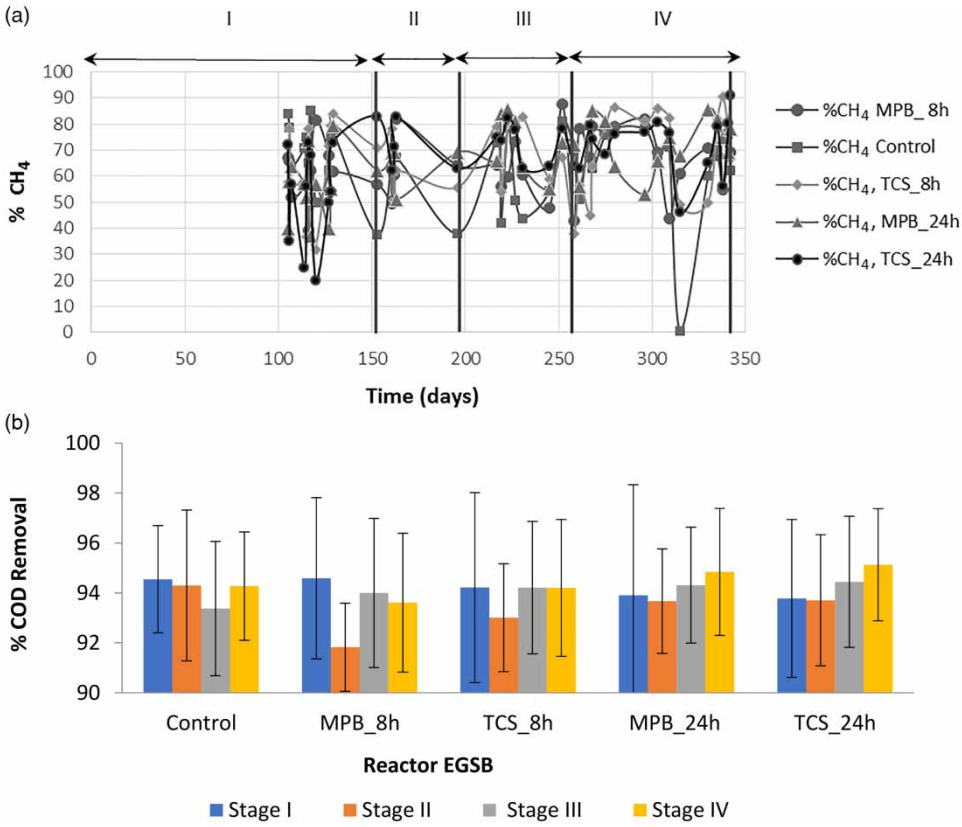

Figure 1 | Methane production and COD removal during different stages of experimentation. (a) Methane production. (b) COD removal.

(Arantes *et al.* 2017). Methane values in the systems operated in the present study oscillated very roughly within the ranges reported for this type of process.

The falls in the methane percentage values in the biogas composition observed in Figure 1(a) may be related to a specific failure in the operation of the system or to the condition of instability presented in stage I as a result of the initial starting process of the anaerobic systems. An interesting condition was presented in a general way in stage IV of the operation, where high stability in the percentage of methane in the composition of the biogas was observed. This condition was identified despite the presence of MPB and TCS in the highest concentration applied, which may indicate greater adaptation of methanogenic microorganisms to the presence of the compounds studied.

Meanwhile, analysis of the COD reduction performance of each of the reactors revealed high operation efficiency, independently of the stage or the type of compounds applied, a result expected due to the high correlation of these parameters. It is highlighted that the highest production of methane comes from the removal of the COD load, showing the stability of the EGSB system and the continuity of its performance, independently of the biological effect exerted by the presence of the pollutants on the microbiological populations responsible for the degradation of organic matter.

Different behaviors were observed for the EGSB systems through the operation under the application of increasing concentrations of TCS and MPB. TCS presented a sorption behavior and MPB showed a biodegradation behavior. In addition, HRT had a significant impact on TCS removal, but not on MPB removal. For the HRT of 24 h, a percentage of biodegradation was observed through the stages of operation in a range of 71–78% and sorption between 22 and 28% while for the HRT of 8 h the biodegradation was between 58 and 72% and sorption between 28 and 41%.

For MPB, the only significant removal mechanism was biodegradation, which was evidenced in the different stages and with the two HRTs evaluated. These removal percentages were above 98%. Similar removal percentages for this compound have been reported in different studies, in which they indicate that biodegradation is the main elimination mechanism with efficiencies between 94 and 99.9%

VARIATIONS OF BACTERIA AND ARCHAEA COMMUNITIES

Figure 2 shows the results obtained after analysis of the bacteria and archaea DGGE gels from the E1 reactor, which was used as a control. The results obtained from this reactor were used as a basis to analyze changes in the structure of archaea and bacteria communities in reactors exposed to MPB and TCS. The dendrogram obtained from the similarity matrix shows that the experimental stage had an influence

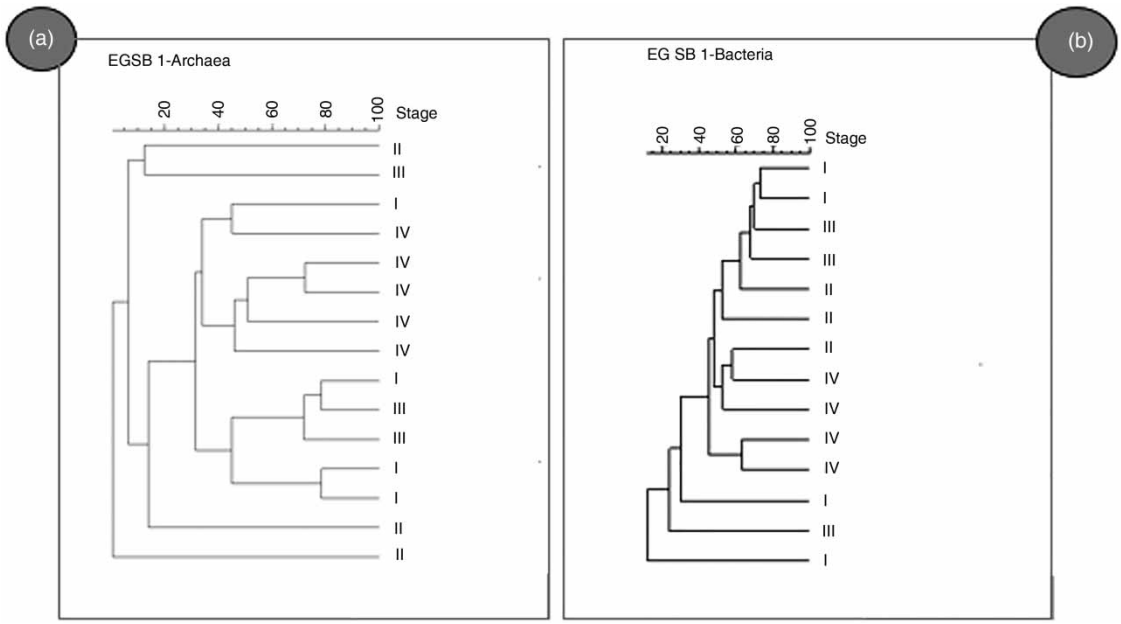

Figure 2 | Dendrogram of the similarity matrix using the Pearson correlation coefficient and the UPGMA grouping method for the DGGE profiles of E1 reactor: (a) archaea and (b) bacteria.

on the distribution of the archaea communities (Figure 2(a)), since the samples taken during the last stage are grouped in a single cluster. Figure 2(b) shows the dendrogram obtained for bacteria, in which similarity of bacterial communities can be observed throughout all stages of operation. However, the samples from stage IV are grouped in a single cluster, as are the archaea, which suggests that the organization of microbial communities was a function of time for both groups of microorganisms. The number of bands observed for bacteria varied from 42 in the initial stage to 26 in stage 4 (equivalent to the Shannon index of 2.5 ± 0.08 to 1.6 ± 0.08). For archaea, the number of bands (10 ± 2.8) remained constant during the first three stages of experimentation, equivalent to a Shannon index of 0.9 ± 0.1, while in stage 4 there was a band average of 7.5 ± 1.0, equivalent to a Shannon index of 0.8 ± 0.01.

Figure 3 shows the dendrograms obtained from analysis of E2 and E3 reactors. Unlike what is observed in the E1 reactor, there is no clear organization of bacterial communities according to the stage of experimentation (Figure 3(a) and 3(b)). This suggests that the compound had an effect on the organization of bacterial communities, favoring populations with the ability to tolerate its presence. The number of bands observed for bacteria varied on

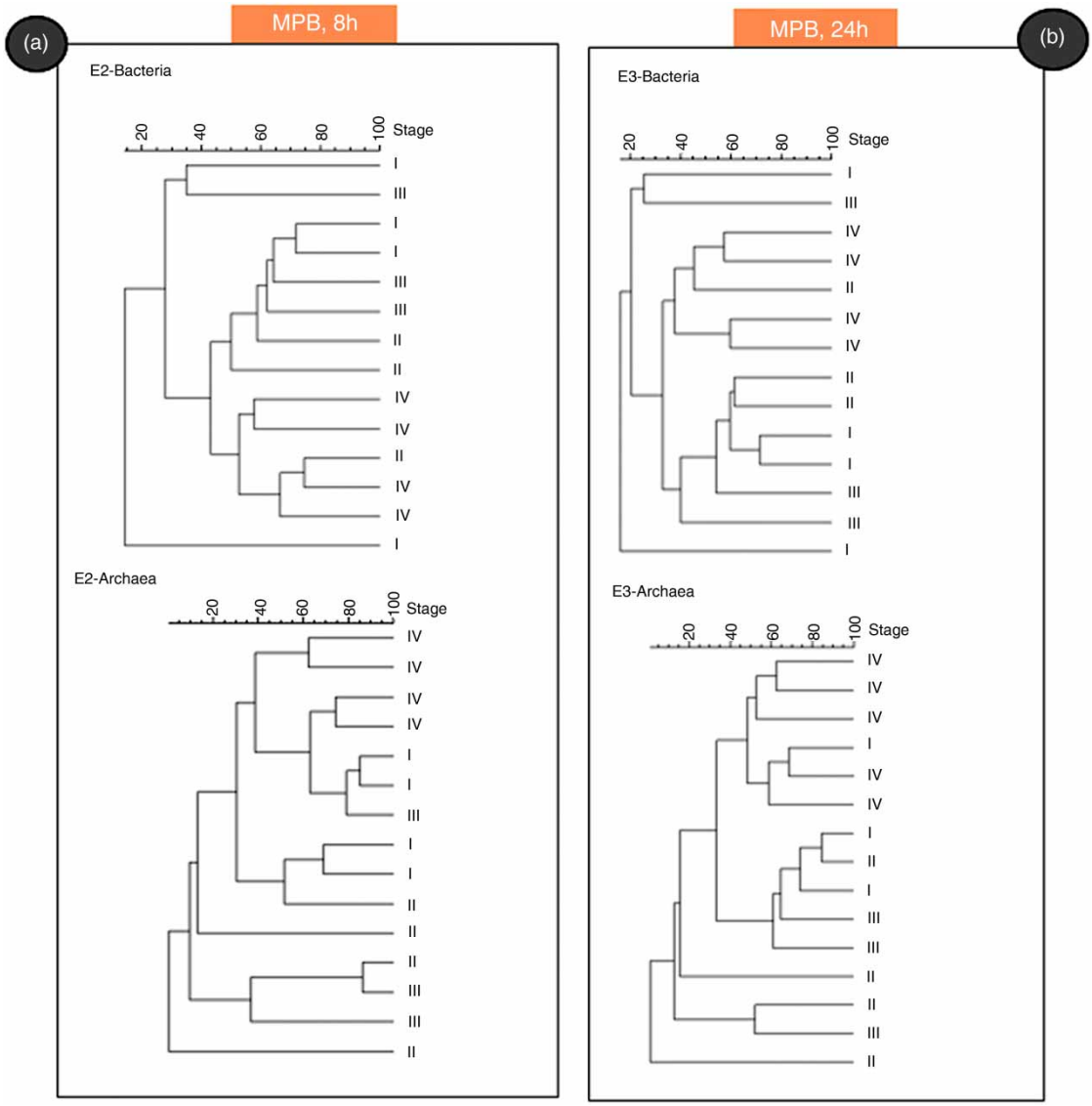

Figure 3 | Dendrogram of the similarity matrix using the Pearson correlation coefficient and the UPGMA grouping method for the DGGE profiles of bacteria and archaea: (a) E2, MPB 8 h and (b) E3, MPB 24 h.

average for both HRTs from 33 ± 0.6 in the initial stage to 21 ± 3.3 in stage 4. These results were expected, since MPB is a compound used as a preservative in various products due to its bactericidal and bacteriostatic action, which is linked to cell membrane damage (Lv *et al.* 2011). The Shannon index ranged from 1.5 ± 0.1 to 1.1 ± 0.2 on average for both HRTs, which may be related to the ability of this compound to act as an external pressure to select resistant species, thereby maintaining the ability to remove organic matter from the system. In this analysis, there is no evidence of an effect of HRT on bacterial communities, although it was expected that a longer time of exposure to the compound would increase its effects on bacteria.

The dendrogram obtained for archaea (Figure 3(a) and 3(b)) shows that the organization of the communities was a function of time, which is similar to what was found in the E1 reactor. The number of bands in the gel remained constant during the four stages of experimentation, as did the Shannon index. Due to the conformation of the anaerobic granule, it is unlikely that the archaea were in direct contact with the compound, which would explain why there is no decrease in the number of archaea bands (Blasco *et al.* 2014). It is also necessary to consider that the mechanism of action commonly described for this compound is cell membrane damage due to lipid damage. This could provide some resistance to archaea due to differences in the composition of their membranes to those of bacteria. These results could explain why methane production was not impaired despite the presence of MPB.

The analysis of microbial communities exposed to different concentrations of TCS is shown in Figure 4. Unlike what was observed in E1, no clear grouping was observed as a function of time for the bacteria group (Figure 4(a) and 4(b)). The average number of bands for both HRTs was 34 ± 1.1 during the first three stages, decreasing to 21 ± 3.2 in stage 4, indicating an effect of concentration on the number of bacterial species. The change in the Shannon index was similar to that observed with the MPB. These results allow it to be stated that this compound had a clear effect on the structure and composition of the bacterial community of the reactor. Figure 4 also shows the dendrogram obtained for archaea. The results are similar to those obtained with the E2 and E3 reactors, where for both HRTs the samples are observed to be distributed in different clusters, with different levels of similarity, while the samples of stage 4 are organized in a single cluster.

Of the two compounds evaluated, TCS has been studied more in terms of its effect on bacteria. It is known that its mechanism of action is based on the inhibition of RNA synthesis, especially of proteins responsible for the construction of the cellular membrane (Vosátka *et al.* 2018). Another property of TCS is that its effectiveness is minimally inhibited by the presence of organic matter (Healy *et al.* 2017). These characteristics could explain why this compound had a more evident effect on the organization of the bacterial communities according to what is shown in the dendrograms. However, as was the case with MPB, these changes were not reflected in the functioning of the reactor, indicating the utility of this system for the removal of this type of pollutant.

As with the MPB, it was expected that a longer retention time would be more toxic to the bacteria and archaea, and that this would be reflected both in the analysis by DGGE and in the monitoring of the control parameters of the reactors. However, the results show that the values for removal and production of methane were similar to those of the control, suggesting that the selected bacterial populations adapted quickly and did not affect the operation of the reactor.

When we compare the results of the organization of the bacterial populations of the reactors with MPB and TCS with those of the control, a clear effect is observed in the decrease in the bands and the index of diversity related to the presence of the compounds. While in the E1 reactor the populations are organized as a function of time, which may be related to a normal process of microbial succession, in the reactors with MPB and TCS there is no clear organization by clusters, which may be related to the decrease in the bands and diversity index. When comparing the results between the reactors with pollutant, it was not possible to establish a significant difference between them. This suggests that, although the compounds have different mechanisms of action, the effect of these was similar for sensitive bacterial populations. However, it is necessary to carry out studies with sludge from different sources and with different microbial compositions in order to verify this.

Identification of the taxonomic groups of the microbial community from the cut of DGGE bands

The determination of the most characteristic taxonomic groups in the reactors was carried out by cutting the most intense and frequent bands, with subsequent sequencing and comparison of the sequences obtained using the GenBank database. To corroborate the appropriate taxonomic allocation, the largest number of possible bands observed in the same position of the gel was cut.

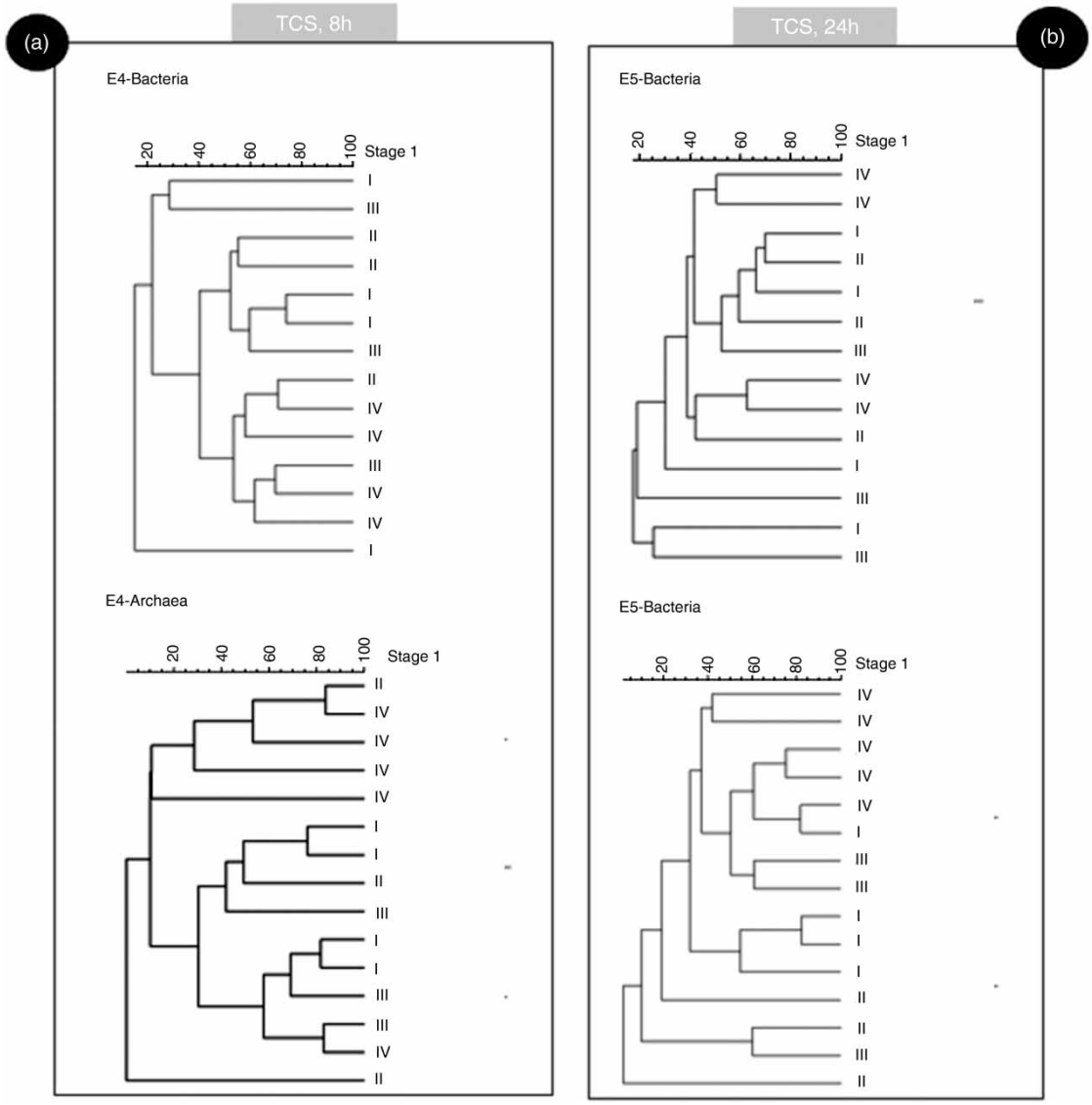

Figure 4 | Dendrogram of the similarity matrix using the Pearson correlation coefficient and the UPGMA grouping method for the DGGE profiles of bacteria and archaea: (a) E4, TCS 8 h and (b) E5, TSC 24 h.

Archaea sequence analysis

Eleven bands were observed in DGGE gels, but only nine could be identified by sequencing. Table 2 shows the assignments of the sequenced bands corresponding to the Archaea domain in the samples analyzed by DGGE. The correlation analyses did not establish a direct relationship between the presence of a specific microorganism and the MPB or TCS, which does not indicate that these compounds did not influence the microbial populations since, as mentioned above, a decrease in the number of bands was observed in the presence of both compounds. However, with this analysis it was possible to detect the microorganisms that were constant throughout the different stages and that were probably responsible for the maintenance of stable reactor conditions. This suggests that investigation of these microorganisms and their ecological and metabolic function during the removal of compounds like MPB and TCS should be deepened.

The band with the most intensity in all the reactors was classified taxonomically as *Methanosaeta concilii* with 100% identity. This microorganism was present in all the gels analyzed during the different stages for all compounds, and presented the highest intensity in all the samplings. These results suggest that this microorganism was fundamental for the stability of the system since, despite the

Table 2 | Identification of the sequences of archaea obtained from DGGE with primers PARCH340f and 519r

No.	Sequence number	Access number
1	*Thermogymnomonas acidicola* strain IC-189 16S	NR_041513.1
2	*Methanobacterium petrolearium* strain Mic5c12 16S ribosomal RNA gene, partial sequence	NR_113044.1
3	*Methanobacterium kanagiense* strain 169 16S ribosomal RNA gene, partial sequence	NR_112749.1
4	*Methanomassiliicoccus luminyensis* strain B10 16S ribosomal RNA gene, partial sequence	NR_118098.1
5	*Methanosaeta concilii* strain GP6 16S ribosomal RNA gene, complete sequence	NR_102903.1
6	*Methanomethylovorans uponensis* strain EK1 16S ribosomal RNA, partial sequence	NR_133781.1
7	*Caldisphaera draconis* strain 18U65 16S ribosomal RNA gene, partial sequence	NR_115941.1
8	*Methanosaeta* sp. 16S ribosomal RNA gene, complete sequence	NR_102903.1

change in the microbial populations, the functioning of the system remained unaffected, especially in terms of methane production. *Methanosaeta concilii* is an acetotrophic methanogen, so its predominance can be also explained by the composition of the water used in the experimentation.

Bacterial sequence analysis

Despite the number of bacterial bands observed in the different gels, due to the quality of the sequences, it was only possible to identify four. However, these bands were the most intense and frequent in the different stages and reactors, which suggests that they are important within the system. The distribution of the bands was similar for all the reactors and it was not possible to identify specific reactor bands. Band No. 1 was characterized by being present with a greater intensity than the rest of the bands in most of the samples. The bacterium *Flavobacterium* sp., a strict aerobic bacterium that belongs to the phylum *Bacteroidetes* and was isolated from a wastewater treatment plant, was identified as associated with this band. Other species in this same genus have been reported in wastewater, and play an important role in the degradation of organic matter because they have a wide variety of enzymes (Fujii *et al.* 2014).

Syntrophorhabdus sp., belonging to the *Deltaproteobacteria* class, was associated with band No. 2. This obligate anaerobic and anaerobic microorganism can use aromatic compounds such as phenol, p-cresol, isophthalate, benzoate and 4-hydroxybenzoate, which it converts into acetate in syntrophic association with the hydrogenotropic methanogens (Qiu *et al.* 2008). In recent years this microorganism has been studied to elucidate the degradation of aromatic compounds in anaerobic environments. Although one of the main mechanisms of removal of micropollutants is their adsorption in sludge, the presence of microorganisms that can degrade aromatic compounds indicates that this could be related to some biotransformation mechanism of the compounds evaluated and explain to some extent the removal percentages obtained. *Hymenobacter* sp., belonging to the phylum *Bacteroidetes*, was identified in band No. 3. This genus is characterized by the production of a large amount of extracellular polymeric substances (Kang *et al.* 2016), which play a fundamental role in the sludge granulation processes.

The last band, No. 4, was identified as *Desulfovibrio vulgaris*, a sulfate-reducing bacterium that is widely studied in anaerobic environments. This particular species has also been reported as a predominant member in anaerobic digesters (Jabari *et al.* 2016). The relations of competition and cooperation between sulfate-reducing bacteria and methanogens have been of interest, since both are responsible for the final reactions in the catabolism of organic compounds in anaerobic environments and share a limited group of substrates such as acetate and hydrogen.

Changes in relative expression of the *mcrA* and *ACAs* genes

Figure 5 shows the results of the expression of the *ACAs* and *mcrA* genes in the five reactors. The results of the expression indicate that E2 (Figure 5(b)) presented behavior similar to E1 (Figure 5(a)) at all stages with respect to the expression of the *ACAs* and *mcrA* genes. When evaluating the effect of HRT on the mrcA gene, significant differences were found ($p \leq 0.05$) between the two reactors, possibly due to the evident increase in the expression of both genes in stage 4 of E3 (Figure 5(c)). When the data related to expression of the *mcrA* and *ACAs* genes and MPB concentration was analyzed, a very strong positive correlation was found between these two variables (R^2 0.9). Despite the concentrations evaluated in this study, which reached 1,000 μg/L for MPB in stage IV, no complete inhibition of methanogenesis was observed. One possible explanation for this could be that the gradual exposure to contaminants in increasing concentrations favored the adaptation of the

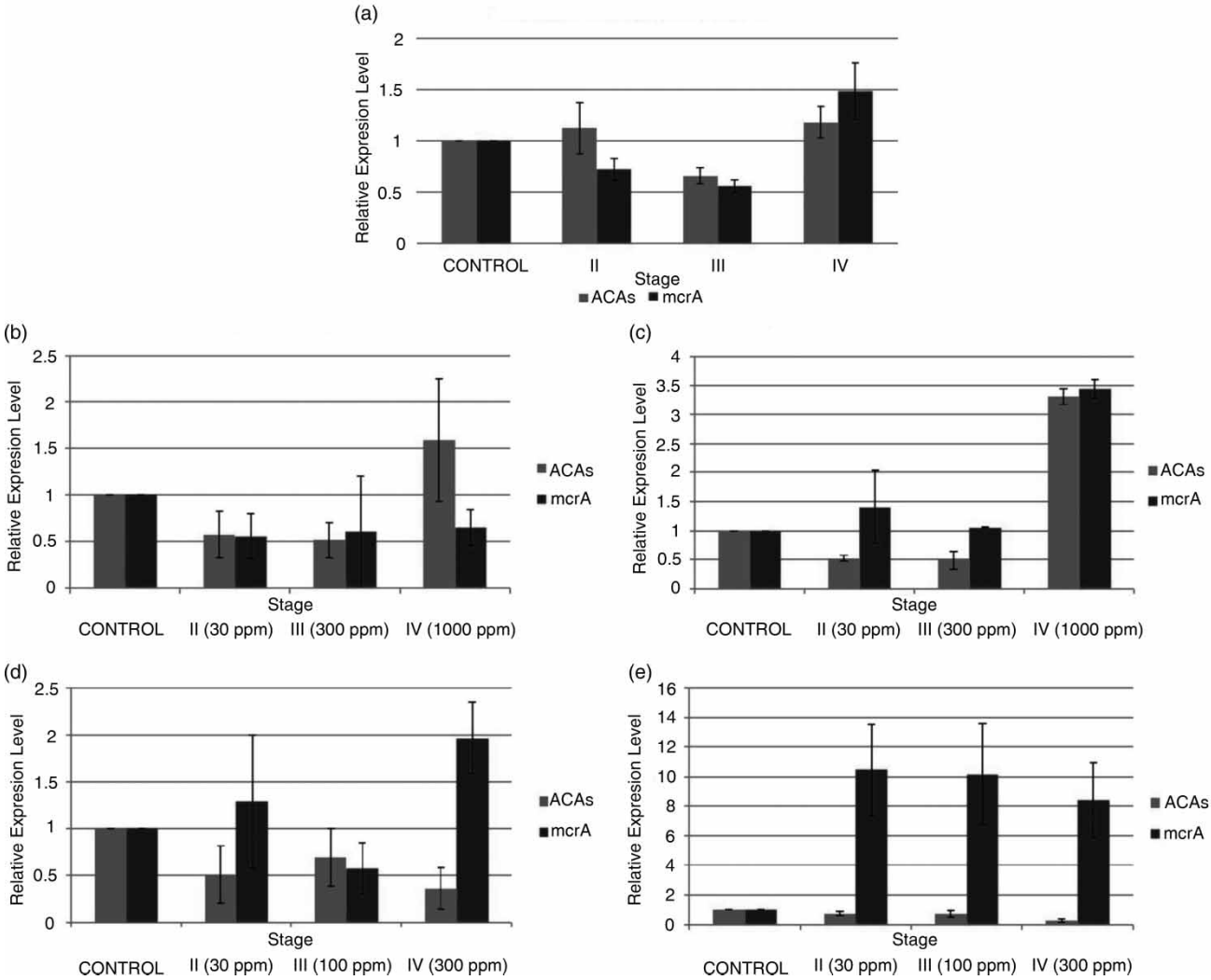

Figure 5 | Relative expression of the *ACAs* and *mcrA* genes in the different stages of experimentation. (a) E1 control, (b) E2 MPB 8 h, (c) E3 MPB 24 h, (d) E4 TCS 8 h, (e) E5 TCS 24 h.

microorganisms to the compound. The presence of a carbon source, such as sodium acetate, allowed the mitigation of the toxic effects of compounds (Fernandez-Fontaina *et al.* 2014).

For the reactor with TCS, a decrease in expression was observed for the *ACAs* gene, compared to E1, E2 and E3. In contrast, the *mcrA* gene had a higher expression in the presence of TCS. The highest values of relative expression were obtained for the *mcrA* gene with an HRT of 24 h (Figure 5(e)). These were higher than those observed in the other reactors, suggesting that this compound influenced the expression of this gene. For the *mcrA* gene, a positive correlation was found between gene expression and TCS concentration.

The concentration of methane for the reactor with TCS shows an increase in methane production, from 25% in stage I, to 62% in stage II, 68% in stage III and 66% in stage IV. According to the results for gene expression, methane production was also supported by hydrogenotrophic methanogens, which were found in the reactor samples. In general terms, a tendency was observed for the *mcrA* gene to decrease expression with an HRT of 8 h and increase expression with an HRT of 24 h, independently of the compound. Other studies have reported a similar tendency, with greater abundance of hydrogenotrophic methanogens obtained in samples collected at longer HRTs. Therefore, HRT seems to be an important factor influencing the metabolism of microbial communities (Krakat *et al.* 2010).

The different expression levels evidenced between *ACAs* and *mcrA* in stage IV could account for the fact that acetoclastic methanogenesis was not the only metabolic pathway for methane production in reactions with TCS. This is especially interesting for *Methanosaeta concilii*, considering that this

microorganism was identified as the predominant operational taxonomic unit in the DGGE analyses conducted, which suggests that its abundance is not directly related to greater metabolic activity, as found by De Vrieze *et al.* (2016).

CONCLUSION

The organization of microbial communities in the sludge under natural conditions was conditioned by time. This is shown by the analysis of the organization of the different groups of microorganisms in the control reactor without contaminants, which were in a clearly defined cluster throughout all stages, most notably in the last. The presence of different concentrations of TCS and MPB had an influence on the cluster organization of microbial communities, especially bacteria. However, this did not affect the stability and performance of the EGSB systems, which was demonstrated by the removal of COD and methane production. As the concentration of contaminants increased, a decrease in the bacteria band detected in the DGGE gel was observed in most cases, suggesting that the compounds could affect the most sensitive species. However, no differences were found between the two compounds. The banding patterns of the DGGE of archaea and the subsequent sequencing of the cleaved DNA demonstrated the constant presence and abundance of *Methanosaeta concilii* throughout all stages of operation, showing that this microorganism played a fundamental role in the stability of the reactors for the production of methane. The type of compound and its concentration influenced the expression of the *mcrA* and *ACAs* genes; however, these changes did not alter the stability and performance of the EGSB systems. Despite the difference between the mechanism of action and the structure of both compounds, no significant differences were observed in the microbial dynamics and removal capacity of the reactors, which indicates the adaptability of this type of system. However, taking into account the limitations of the techniques used, it is necessary to carry out more research to clearly identify which microorganisms contribute to maintaining the stability of the system, regardless of external disturbances.

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CONFLICT OF INTEREST

The authors report no potential conflict of interest.

REFERENCES

Andersen, A. 2008 Final amended report on the safety assessment of methylparaben, ethylparaben, propylparaben, isopropylparaben, butylparaben, isobutylparaben, and benzylparaben as used in cosmetic products. *International Journal of Toxicology* **27**, 1–82.

APHA/AWWA/WEF 2012 *Standard Methods for the Examination of Water and Wastewater*, 22nd edn. American Public Health Association/American Water Works Association/Water Environment Federation, Washington, DC, USA.

Arantes, M. K., Helton, J. A., Rodrigo, S. & da Silva, E. A. 2017 Treatment of brewery wastewater and its use for biological production of methane and hydrogen. *International Journal of Hydrogen Energy* **42** (42), 26243–26256.

Blasco, L., Kahala, M., Tampio, E., Ervasti, S., Paavola, T., Rintala, J. & Joutsjoki, V. 2014 Dynamics of microbial communities in untreated and autoclaved food waste anaerobic digesters. *Anaerobe* **29**, 3–9.

Chen, X., Richard, J., Liu, Y., Dopp, E., Tuerk, J. & Bester, K. 2012 Ozonation products of triclosan in advanced wastewater treatment. *Water Research* **46** (7), 2247–2256.

De Vrieze, J., Regueiro, L., Props, R., Vilchez-Vargas, R., Jáuregui, R., Pieper, D. H. & Carballa, M. 2016 Presence does not imply activity: DNA and RNA patterns differ in response to salt perturbation in anaerobic digestion. *Biotechnology for Biofuels* **9**, 244.

Fernandez-Fontaina, E., Carballa, M., Omil, F. & Lema, J. M. 2014 Modelling cometabolic biotransformation of organic micropollutants in nitrifying reactors. *Water Research* **65**, 371–383.

Fujii, D., Nagai, F., Watanabe, Y. & Shirasawa, Y. 2014 *Flavobacterium longum* sp. nov. and *Flavobacterium urocaniciphilum* sp. nov., isolated from a wastewater treatment plant, and emended descriptions of *Flavobacterium caeni* and *Flavobacterium terrigena*. *International Journal of Systematic and Evolutionary Microbiology* **64** (5), 1488–1494.

Green, S. J., Leigh, M. B. & Neufeld, J. D. 2010 Denaturing gradient gel electrophoresis (DGGE) for microbial community analysis. In: *Handbook of Hydrocarbon and Lipid Microbiology*. Springer, Berlin, Germany, pp. 4137–4158.

Healy, M. G., Fenton, O., Cormican, M., Peyton, D. P., Ordsmith, N., Kimber, K. & Morrison, L. 2017 Antimicrobial compounds (triclosan and triclocarban) in sewage sludges, and their presence in runoff following land application. *Ecotoxicology and Environmental Safety* **142**, 448–453.

Hu, Y., Li, Z., Yang, J. & Zhu, H. 2019 Degradation of methylparaben using BiOI-hydrogel composites activated peroxymonosulfate under visible light irradiation. *Chemical Engineering Journal* **360**, 200–211.

Jabari, L., Gannoun, H., Khelifi, E., Cayol, J.-L., Godon, J.-J., Hamdi, M. & Fardeau, M.-L. 2016 Bacterial ecology of abattoir wastewater treated by an anaerobic digestor. *Brazilian Journal of Microbiology* **47** (1), 73–84.

Kang, J. W., Baik, K. S., Im, W.-T. & Seong, C. N. 2016 *Hymenobacter coalescens* sp. nov., isolated from wetland freshwater. *International Journal of Systematic and Evolutionary Microbiology* **66** (9), 3546–3551.

Krakat, N., Schmidt, S. & Scherer, P. 2010 Mesophilic fermentation of renewable biomass: does hydraulic retention time regulate methanogen diversity? *Applied and Environmental Microbiology* **76** (18), 6322–6326.

Li, Z., Hu, Y., Liu, C., Shen, J., Wu, J., Li, H. & Zuo, J. 2019 Performance and microbial community of an expanded granular sludge bed reactor in the treatment of cephalosporin wastewater. *Bioresource Technology* **275**, 94–100.

Luo, Y., Guo, W., Ngo, H. H., Nghiem, L. D., Hai, F. I., Zhang, J. & Wang, X. C. 2014 A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Science of The Total Environment* **473–474**, 619–641.

Lv, F., Liang, H., Yuan, Q. & Li, C. 2011 In vitro antimicrobial effects and mechanism of action of selected plant essential oil combinations against four food-related microorganisms. *Food Research International* **44** (9), 3057–3064.

Ma, X., Wan, Y., Wu, M., Xu, Y., Xu, Q., He, Z. & Xia, W. 2018 Occurrence of benzophenones, parabens and triclosan in the Yangtze River of China, and the implications for human exposure. *Chemosphere* **213**, 517–525.

Meng, L.-W., Li, X.-K., Wang, S.-T., Liu, L.-L., Ma, K.-L. & Zhang, J. 2017 The long-term impact of cefalexin on organic substrate degradation and microbial community structure in EGSB system. *Chemosphere* **184**, 215–223.

Orhon, K. B., Orhon, A. K., Dilek, F. B. & Yetis, U. 2017 Triclosan removal from surface water by ozonation – kinetics and by-products formation. *Journal of Environmental Management* **204**, 327–336.

Qiu, Y.-L., Hanada, S., Ohashi, A., Harada, H., Kamagata, Y. & Sekiguchi, Y. 2008 *Syntrophorhabdus aromaticivorans* gen. nov., sp. nov., the first cultured anaerobe capable of degrading phenol to acetate in obligate syntrophic associations with a hydrogenotrophic methanogen. *Applied and Environmental Microbiology* **74** (7), 2051–2058.

Steter, J. R., Brillas, E. & Sirés, I. 2016 On the selection of the anode material for the electrochemical removal of methylparaben from different aqueous media. *Electrochimica Acta* **222**, 1464–1474.

Vosátka, R., Krátký, M. & Vinšová, J. 2018 Triclosan and its derivatives as antimycobacterial active agents. *European Journal of Pharmaceutical Sciences* **114**, 318–331.

Wu, Y., Sun, Q., Wang, Y., Deng, C. & Yu, C.-P. 2017 Comparative studies of aerobic and anaerobic biodegradation of methylparaben and propylparaben in activated sludge. *Ecotoxicology and Environmental Safety* **138**, 25–31.

Xu, H., Liu, Y., Gao, Y., Li, F., Yang, B., Wang, M. & Sand, W. 2018 Granulation process in an expanded granular sludge blanket (EGSB) reactor for domestic sewage treatment: impact of extracellular polymeric substances compositions and evolution of microbial population. *Bioresource Technology* **269**, 153–161.

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