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Research article

Key microbial populations involved in anaerobic degradation of phenol and p-cresol using different inocula

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ABSTRACT

Background: Anaerobic digestion is an alternative bioprocess used to treat effluents containing toxic compounds such as phenol and p-cresol. Selection of an adequate sludge as inoculum containing an adapted microbial consortium is a relevant factor to improve the removal of these pollutants. The objective of this study is to identify the key microorganisms involved in the anaerobic digestion of phenol and p-cresol and elucidate the relevance of the bamA gene abundance (a marker gene for aromatic degraders) in the process, in order to establish new strategies for inocula selection and improve the system's performance.
Results: Successive batch anaerobic digestion of phenol and p-cresol was performed using granular or suspended sludge. Granular sludge in comparison to suspended sludge showed higher degradation rates both for phenol (11.3 ± 0.7 vs 8.1 ± 1.1 mg l^{-1} d^{-1}) and p-cresol (7.8 ± 0.4 vs 3.7 ± 1.0 mg l^{-1} d^{-1}). After three and four re-feedings of phenol and p-cresol, respectively, the microbial structure from both sludges was clearly different from the original sludges. Anaerobic digestion of phenol and p-cresol generated an abundance increase in *Syntrophorhabdus* genus and bamA gene, together with hydrogenotrophic and aceticlastic archaea. Analysis of results indicates that differences in methanogenic pathways and levels of *Syntrophorhabdus* and bamA gene in the inocula, could be the causes of dissimilar degradation rates between each sludge.
Conclusions: Syntrophorhabdus and bamA gene play relevant roles in anaerobic degradation of phenolics. Estimation of these components could serve as a fast screening tool to find the most acclimatized sludge to efficiently degrade mono-aromatic compounds.
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1. Introduction

Phenols are the major organic constituents in effluents of coal conversion processes, coke ovens, petroleum refineries, phenolic resin manufacturing, herbicide manufacturing, fiberglass manufacturing and petrochemicals [1]. These pollutants are harmful for the environment, toxic to organisms and recognized as carcinogenic compounds [2]. Different biological technologies are available to treat effluents containing such compounds, one of these technologies is the anaerobic digestion.

The anaerobic digestion process is a complex procedure that involves different stages, each one catalyzed by different consortia of microorganisms. These steps involve the hydrolysis of complex molecules to monomers, which is followed by the steps of acidogenesis, acetogenesis, and methanogenesis [3].

The anaerobic technology has been implemented to treat phenols containing effluents due to the advantages it offers over other biological operations: withstanding high organic loading rates and low sludge generation, in addition to energy production [4]. Despite this, the stability and efficiency of anaerobic digestion depend on the microbial population, the biodegradability of the compounds and chemical characteristics [5]. The anaerobic treatment of wastewater containing toxic pollutants, can present low degradation rates of organic compounds, due to bacterial activity inhibition. Thus, the need of having bacteria with robust physiology is critical to the stability of the biological treatment process, because they can efficiently facilitate the removal of refractory organics, mitigate wastewater toxicity and thus, improve wastewater biodegradability [6].

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A way to have bacteria with robust physiology and activity, during anaerobic process, is by choosing the proper sludge that is going to be used as inoculum when starting the system, which should contain an adapted microbial consortium for a specific substrate. It has been reported that inoculum source is important for starting up anaerobic digesters, especially when treating organic compounds with low biodegradability [7]. For systems that work with retained biomass, such as sequential batch reactors (SBRs), it has been demonstrated that the inoculum composition determines the microbial functions in terms of chemical product generation [8], that is, different inocula have different performances under the same operating conditions. According to this, the inoculum selection process to carry out the anaerobic digestion of phenols is relevant to establish an efficient process.

Different molecular approaches based on the analysis of 16S rRNA gene like fingerprinting, high throughput sequencing and quantitative PCR (qPCR) methods, have been used in order to identify the microbial populations present in anaerobic sludge on reactors treating phenolic compounds [4,9,10,11,12]. However, there are no studies regarding anaerobic digestion of phenols in which different sources of inocula are analyzed microbiologically and compared, in order to determine which are the microorganisms that effectively contribute to a better performance of the process.

Apart from 16S based methods, used to identify and estimate the microorganisms responsible for anaerobic degradation of phenols, complementary information can be gained by studying key functional genes [13].

In the anaerobic degradation of mono aromatic compounds (like phenol and p-cresol), via the 4-hydroxybenzoate to benzoyl-CoA pathway, the ring cleavage step of 6-oxocylcohex-1-ene-1-carbonyl-CoA is catalyzed by a hydrolase encoded by bamA gene [14,15]. This gene has been used as a biomarker of aromatic-degrading anaerobes under different redox conditions and has been correlated positively with the amount of degraded mono aromatic compounds, like toluene [16]. Therefore, the bamA gene amounts on different inocula could be related with different performances of these in terms of phenol degradation capabilities.

Based on the above discussion, the objective of this study was to evaluate the adaptation process of different inocula during the anaerobic digestion of phenol and p-cresol and determine, by high throughput sequencing of the 16S rRNA gene, what microorganisms are involved in the anaerobic digestion process of these compounds. In addition, the bamA gene was quantified in order to elucidate if initial amounts on the inocula are determinants of anaerobic digestion performance.

2. Material and methods

2.1. Experimental set-up

Batch anaerobic digestion tests were performed at 37 ± 1°C by adding mineral medium [4] and 183 ± 23 mg l^{-1} of phenol (three successive batches) or 106 ± 7 mg l^{-1} p-cresol (four successive batches) in 500 ml bottles. The concentrations of phenol and p-cresol chosen were similar to those used previously by Fang and Zhou [17] to emulate a wastewater containing medium-strength phenolic pollutants. As inoculum 4 g l^{-1} of volatile suspended solids (VSS) of sludge obtained from an up-flow anaerobic sludge blanket (UASB) reactor treating tobacco industry effluents or a continuous stirred-tank reactor (CSTR) treating aerobic sludge from a municipal treatment plant was used. The successive batches were carried out by re-feeding the inoculum with phenol or p-cresol once previous addition of the aromatic compound was depleted. Assays were performed in triplicate and included a negative control where only mineral medium was added.

2.2. Analytical methods

Degradation of phenolic compounds and methane production was monitored by sampling the liquid and gaseous phase, respectively every 1–3 d. Phenol and p-cresol concentration in liquid samples (1 ml) was determined by high performance liquid chromatography (HPLC) with an UV detector (Perkin Elmer, 200 series). The column used for chromatographic separation of compounds was a C_{18} Interstil® ODS 3 (250 x 4.6 mm, 5 um, GL Science Inc.). The mobile phase was composed of Milli Q water:acetonitrile, 50:50 *v*/v running at 25°C with a flow rate of 1.5 ml min^{-1}. The eluate was monitored at 280 nm and quantification was made by the external standard method using phenol and p-cresol as standards (Sigma-Aldrich, 99%).

2.3. Maximum rates of phenolic degradation and methane production

Maximum rates of phenolic degradation were estimated on the first and last batch of phenol and p-cresol by calculating the maximum slopes obtained from degradation curves over time for each compound.

2.4. Sludge sampling and total DNA extraction

Sludge samples were taken before inoculating the bottles (inoculum) and after the first and last batch for each compound. DNA was extracted from 0.5 g of pellet obtained after centrifuging the samples at 10,000 × g for 10 min at 4°C using the Power Soil DNA Isolation Kit (MoBio) following the manufacturer's guidelines. For sequencing analysis, extracted DNA from triplicate bottles were mixed in equal volumes in order to establish the mean microbial community present on samples.

2.5. 16S rRNA amplicon gene sequencing

The 16S rRNA gene V4 variable region PCR primers 515/806 [18] with barcode on the forward primer were used in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. After amplification, PCR products are checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Samples are pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples are purified using calibrated Ampure XP beads. Then the pooled and purified PCR product is used to prepare DNA library by following Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines. Sequence data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). In summary, sequences were joined, depleted of barcodes then sequences <150 bp removed, sequences with ambiguous base calls removed. Sequences were denoised, OTUs generated and chimeras removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes [19].

2.6. qPCR of bamA gene and Syntrophorhabdus

The quantification assay was conducted in an AriaMX real-time PCR cycler (Agilent) using the following primer sets: Bam-sp9/Bam-asp1 for *bamA* gene [11] and primers 5′-GAAAGCCTGACCCAGCG-3′ (forward) and 5′-CCCACCTTCCTCCGCATTAA-3′ (reverse) for *Syntrophorhabdus* genus quantification [20]. Each 20 μl PCR reaction contained 10 μl of Takyon Rox SYBR MasterMix dTTP Blue (Eurogentec), 0.9 μM of each primer and 2 μl of DNA template. Thermal program consisted of an initial denaturation (95°C, 3 min) and 44 cycles of amplification (95°C,

3 s; 60°C, 40 s). Melting curves were constructed from 65°C to 95°C, read every 0.5°C for 5 s. Calibration curves (10^0–10^6 gene copies μl^{-1}) were prepared using different standards. Genomic DNA from *Thauera aromatica* (DSM 6984) was used to quantify *bamA* gene assuming a genome size of 4.6 Mb [21] and one copy number of this gene per genome. *Syntrophorhabdus* genus was quantified using as standard an 800 bp purified PCR product fragment of 16S rRNA gene from *Syntrophorhabdus aromaticivorans* (DSM 17771). Abundance results of bamA and *Syntrophorhabdus* were normalized against DNA (ng) and sludge mass (g), respectively.

2.7. Analysis of microbial communities

The structure of microbial communities in each sample was analyzed based on their genera relative abundances. For the analysis, those genera whose relative abundance in all samples were less than 0.5% were discarded. The Bray–Curtis similarity index of microbial communities was conducted using the Agglomerative Hierarchical Clustering (AHC) methodology using the XLSTAT statistical software (Addin Soft).

Determination of microorganisms whose presence would be strongly related to the degradation of phenol or p-cresol and subsequent conversion to methane, was inferred by observing the genera that increased their relative abundance in at least 10% on the last degradation batch compared to samples corresponding to their inoculum and negative control (without carbon source).

2.8. Statistical analysis

Maximum degradation rates and qPCR results were subjected to Analysis Of Variance (ANOVA) test to determine significant differences over tested samples. If ANOVA test gave significant differences (95% confidence interval) then Fisher's Protected Least Significant Difference (LSD) test was performed, in order to determine the significant differences (95% confidence interval) of each combination of samples. The statistical analysis was conducted using the XLSTAT software (Addin Soft).

3. Results and discussion

3.1. Anaerobic degradation of phenol and p-cresol in different sludge types

Successive batches of anaerobic degradation of phenol and p-cresol were performed using different sludge sources as inoculum. The granular sludge came from an UASB reactor which treats wastewater from tobacco industry and the suspended sludge was obtained from a CSTR used to stabilize aerobic sludge generated from activated sludge process. During batch assays, both sludge were able to degrade completely the tested compounds (Fig. S1, Fig. S2). The granular sludge had significantly higher degradation rates for phenol and p-cresol, in comparison to the suspended sludge (Fig. 1). The maximum degradation rates of phenol during the first and last batch using granular sludge were 9.4 ± 1.4 and 11.3 ± 0.7 mg l^{-1} d^{-1}, respectively, higher than maximum degradation rates of suspended sludge (2.7 ± 0.8 and 8.1 ± 1.1 mg l^{-1} d^{-1}). In p-cresol degradation, granular sludge had maximum degradation rates of 4.0 ± 0.1 and 7.8 ± 0.4 mg l^{-1} d^{-1} for first and last batch, respectively, in comparison to suspended sludge where maximum degradation rates were 1.7 ± 0.3 and 3.7 ± 1.0 mg l^{-1} d^{-1}. These suggest that granular sludge has a microbial community better adapted to degrade aromatic compounds in comparison to the suspended sludge. This could be explained by the source from which granular sludge was obtained. Tobacco wastewaters are known to contain high concentrations of compounds difficult to degrade, like nicotine and polycyclic aromatic hydrocarbons [22] thus, the microbial consortia present in granular sludge used to treat tobacco wastewater was already acclimated to the presence of aromatic compounds.

On the other hand, it is observed that the maximum degradation rates tend to be higher in latest batches compared to the first ones for each compound and sludge. The increase in degradation rate on last batches were significant for all cases excepting in the granular sludge degrading phenol. The increased degradation rates on last batches suggests a selection process of microbial configuration, where degradative bacteria having a proper metabolic activity to catabolize these compounds increased their abundance.

3.2. Microbial structure changes during anaerobic digestion of phenol and p-cresol

By sequencing the 16S rRNA gene, it was possible to identify and estimate the relative abundances of different bacteria and archaea present in the samples taken during anaerobic digestion of phenol and p-cresol (Table S1). In order to group the samples based on its microbial community composition, statistical analysis of microorganism's relative abundances by Agglomerative Hierarchical Clustering (AHC) was performed. Results of this analysis are represented by a dendrogram (Fig. 2) which reveals how much the community composition differs in comparison to the inocula or after the first and last degradation batches of phenol and p-cresol. Unsurprisingly, two main branches of the dendrogram were generated which divided samples with the

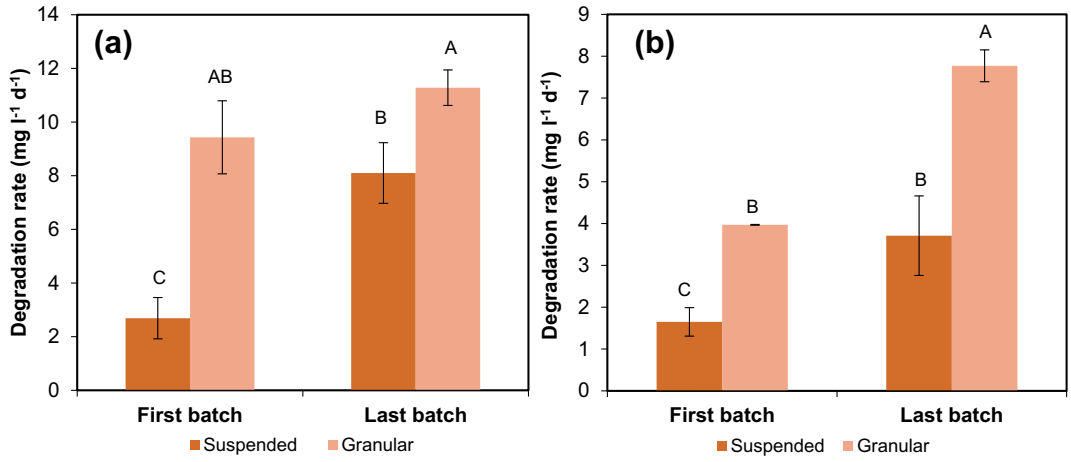

Fig. 1. Maximum degradation rates of phenolic compounds using suspended and granular sludge as inocula. In (a) rates for phenol degradation and in (b) for p-cresol degradation. Error bars represents the standard deviation from the mean of triplicate samples. Different letters above bars indicates significant differences ($p \leq 0.5$) between samples.

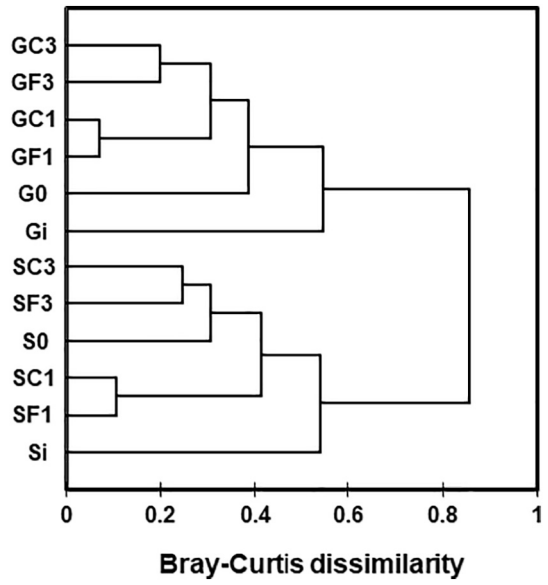

Fig. 2. Clustering of samples based on the Bray–Curtis dissimilarity. The letters and numbers on the left of the dendrogram indicates the identity of each sample located in a specific branch. The samples with "S" and "G" corresponds to suspended and granular sludge, respectively. C and F indicates if sample was exposed to p-cresol or phenol, i = inoculum, 0 = control, 1 = first batch and 3 = last batch.

same type of sludge. This indicates that each sludge has a distinctive microbial structure (86% of Bray–Curtis dissimilarity between them) and is not greatly altered after being exposed to phenol or p-cresol. However, it is possible to observe that microbial structure of samples from the first and last batches had a Bray–Curtis dissimilarity of 54%, when comparing with their respective inocula. Therefore, phenol and p-cresol degradation made changes in community composition but maintaining the distinctive structure of each sludge. On the other hand, in control samples, in which no substrate was added, a microbial differentiation is also observed with respect to the inocula, which suggests that the incubation time, by itself, is also an important factor in the differentiation of sludge microbial structure. The latter agrees with a previous experiment in which the microbial composition of a methanogenic inoculum was affected by storage time at different temperatures (including room temperature) [23]. Thus, the main three factors that shape the microbial configuration are the sludge origin, incubation time and exposure to phenolic compounds. Finally, it can be seen that there are few differences between microbial structures corresponding to the samples obtained from the latest additions of phenol and p-cresol, for each sludge, indicating that a similar microbial configuration is involved in the degradation of both compounds.

3.3. Relevant microorganisms in the anaerobic digestion of phenol and p-cresol

After calculating relative abundances of each microorganism in the samples, the populations that increased by 10% or more (in comparison to the inoculum and control) were determined in order to identify the microorganisms that effectively grow by the addition of phenolics and therefore, possessing the catabolic machinery to utilize these compounds or their degradation products as carbon and energy sources. As shown in Fig. 3, *Syntrophorhabdus* genus considerably increases its relative abundance in both sludges and for both compounds, indicating that their presence is crucial for the degradation of tested phenols. Previous degradation experiments of phenol using a co-culture of *Syntrophorhabdus* with *Methanospirillum hungatei* (hydrogenotrophic methanogen) showed that phenol is degraded by 31% during 100 d of incubation generating 3 mol of acetate per mol of aromatic compound degraded. Also it was

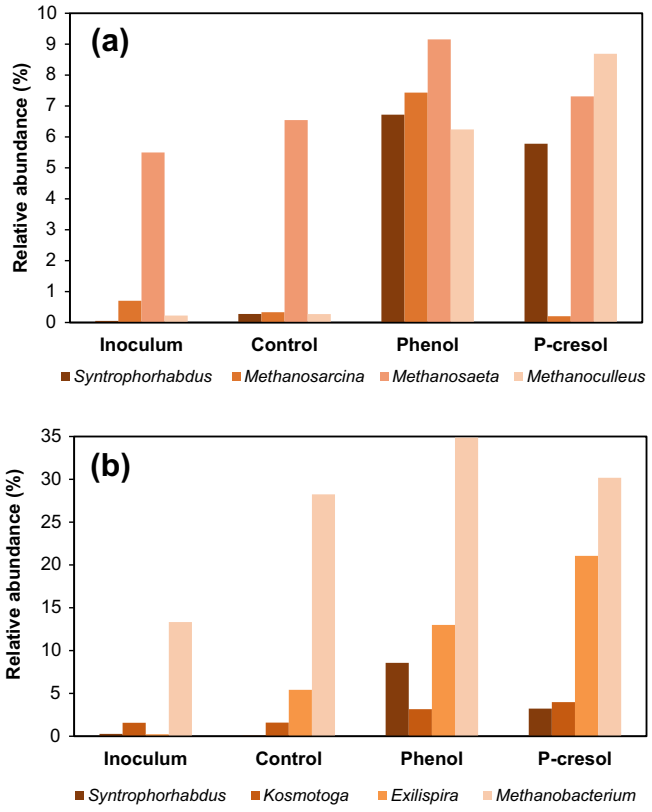

Fig. 3. Relative abundances of genera which increased their abundances after phenol and p-cresol degradation. In (a) genera from suspended sludge and (b) from granular sludge.

confirmed that *Syntrophorhabdus* is a bacterium capable of converting phenol and p-cresol into other products (acetate and hydrogen) in a syntrophic manner, in the presence of an hydrogen consumer [24]. Based on this, the increase of *Syntrophorhabdus* is consistent with the increase of hydrogenotrophic archaea in both sludge, *Methanoculleus* in the suspended sludge and *Methanobacterium* in the granular sludge, which enables the metabolic activity of this aromatic degrader bacterium. These results are in concordance with those obtained by Na et al. [25] where *Syntrophorhabdus* together with *Methanobacterium* became dominant in an UASB reactor treating phenol. On the other hand, there were microorganisms that increased their abundances in one sludge or another. In granular sludge, *Exilispira* and *Kosmotoga* genus increased considerably. *Exilispira* had a negligible abundance in inoculum but increased to 13.0% and 21.1% on last batches of phenol and p-cresol, respectively. *Kosmotoga*, in other hand, increased its abundance from 1.6% (inoculum) to 3.2% and 4.0% in phenol and p-cresol last batches, respectively. Regarding the possible roles that these two bacteria may have in anaerobic digestion of these compounds, it is worth to mention that *Exilispira* belongs to the phylum *Spirochaetes* and there is evidence that strongly suggests that members belonging to this phylum could be involved in syntrophic acetate oxidation generating hydrogen and carbon dioxide as metabolic products [26]. *Kosmotoga*, on the other hand, is a genus that is phylogenetically related to *Mesotoga* of which has been also reported its capacity to oxidize acetate [27]. Thus, both the increase of *Exilispira* and *Kosmotoga* genus could be explained by a putative acetate oxidation process occurring during degradation. This, gives rise to the hypothesis that in the granular sludge, anaerobic digestion of phenol and p-cresol is given (at least to some extent) by the conversion of these compounds into acetate, then into hydrogen and carbon dioxide and finally into methane.

In suspended sludge, apart from *Methanoculleus*, two additional archaea increased their abundances, *Methanosarcina* and *Methanosaeta*.

Both genera are known to carry out methane production through acetate conversion [28], but *Methanosarcina*, in addition, can also produce methane by the hydrogenotrophic pathway [29]. *Mehanosarcina* reached a relative abundance of 7.4% only when phenol was degraded, which meant an increase of more than 10 times with respect to the inoculum (0.7%). *Methanosaeta* genus on the other hand, was already present at high abundance on the inoculum (5.5%) and increased to 9.2% and 7.3% after phenol and p-cresol degradation, respectively. As compared to *Methanosarcina*, *Methanosaeta* genera have lower growth rates [30] thus, less noticeable increase is expected from this genus in comparison to *Methanosarcina* during anaerobic digestion process. Based on methanogens that were increased in the suspended sludge, it is presumed that mineralization of phenolic compounds into methane, is carried out mainly by the aceticlastic pathway, in which first *Syntrophorhabdus* degrades phenols and produces acetate and hydrogen, as intermediate metabolites and then, acetate is converted into methane by *Methanosaeta* in the case of p-cresol digestion, or by both methanogens *Methanosaeta* and *Methanosarcina*, in the case of phenol digestion. Unlike granular sludge and due to the fact that it was not observed any potential acetate oxidizing bacteria, it seems that the hydrogenotrophic methanogens present in the suspended sludge would be acting mainly as hydrogen scavengers consuming the hydrogen derived from phenol degradation performed mainly by *Syntrophorhabdus*. According to these, we suggest that granular and suspended sludges could display preferential methanogenic pathways during anaerobic digestion of phenols, and the use of one pathway or another could be one of the factors contributing to the performance differences between both sludges during the anaerobic digestion of phenols. In this case, the better performance in anaerobic digestion of phenols by the granular sludge could be related with a preferential use of the hydrogenotrophic pathway. As reported previously, depending on environmental conditions, hydrogenotrohic pathway could display higher rates of methane production (thus higher organic matter degradation) in comparison to acetoclastic pathway [31,32].

3.4. Absolute abundance of Syntrophorhabdus

Relative abundance increase of *Syntrophorhabdus* genus on both sludges, treating phenol and p-cresol, revealed the key role of this genus in the degradation process. However, the relative abundance increase could be originated by two ways: by an important decrease occurred in the absolute abundance of some dominant bacteria, in conjunction with a maintenance of *Syntrophorhandus* abundance, or indeed, there is a an absolute growth of this genus over other microorganisms, confirming the capability of this genus to use phenol and p-cresol as carbon sources to grow. To clarify by which way relative abundance increased, absolute abundance of *Syntrophorhabdus* genus was determined by qPCR analysis on the different sludge samples. As can be seen in Fig. 4a, absolute abundance of *Syntrophorhabdus* increased significantly in more than one order of magnitude after last phenol degradation, on both sludge types. After p-cresol degradation, *Syntrophorhabdus* also increased its absolute abundance (at least two fold) in comparison to inoculum and control, although not significantly. Notably, inoculum of granular sludge showed higher abundance of *Syntrophorhabdus*, in comparison to suspended sludge, which could be associated with the higher adaptation level of this sludge to degrade aromatic compounds. Although these last results were not statistically significant, similar inferences were suggested by Ju and Zhang [33] in which a higher degradation rate of a methanogenic reactor treating phenol, in comparison to other similar, was explained by the higher proportion of *Syntrophorhabdus*. Therefore, the results obtained in our study establishes that *Syntrophorhabdus* relative abundance increase was related with the absolute growth of this genus, during the anaerobic digestion process, and confirms that its levels are one of the reasons why one sludge performed better than the other.

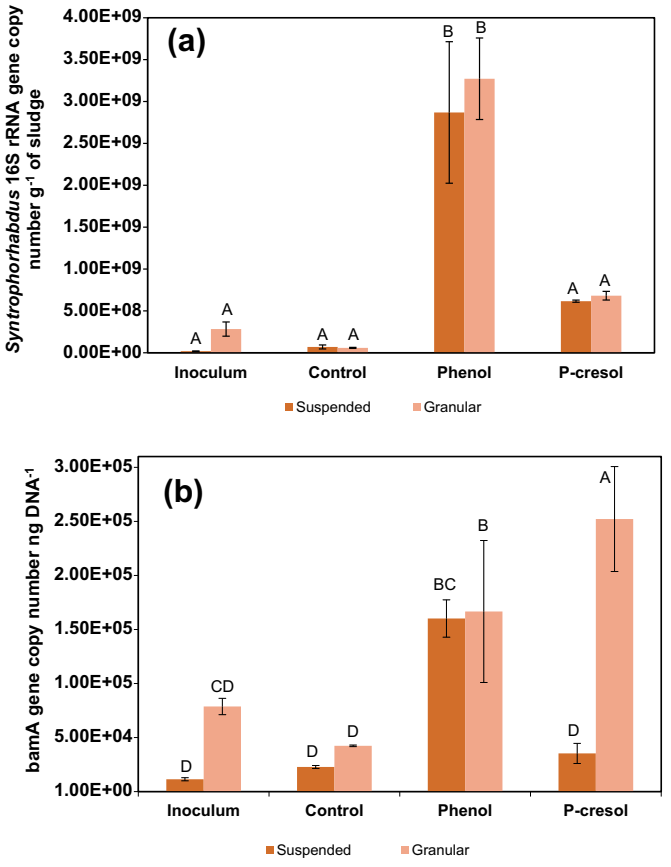

Fig. 4. Abundances of *Syntrophorhabdus* and bamA gene determined by qPCR. In (a) *Syntrophorhabdus* abundance and (b) bamA gene abundance. Error bars represents the standard deviation from the mean of triplicate samples. Different letters above bars indicates significant differences ($p \leq 0.5$) between samples.

3.5. BamA gene abundance increase during degradation of phenols

Estimation of bamA gene has been reported to be useful to enumerate anaerobic aromatic degraders on different redox conditions [30]. Based on this, it is expected that bamA gene increases its abundance after degradation of aromatic compounds. To confirm that, qPCR analysis was performed in order to quantify the bamA gene in the inocula, controls and after the degradation of phenol and p-cresol (Fig. 4b). In the case of granular sludge, bamA gene increased its abundance significantly from 7.9×10^4 copies ng^{-1} of DNA in the inoculum to 1.7×10^5 and 2.5×10^5 copies ng^{-1} of DNA, after phenol and p-cresol degradation, respectively. On the other hand, in suspended sludge, bamA gene tended to be higher after degradation of phenolic compounds, but this was only significant in the case of phenol degradation with values of 1.1×10^4 copies ng^{-1} of DNA in the inoculum and 1.6×10^5 copies ng^{-1} of DNA in the final sludge. In general, these results confirm that bamA gene assay is capable to describe the increase in total aromatic degraders on anaerobic sludge and in this case, the increase in bamA gene tended to explain the higher degradation rate increase by sludge. On the other hand, bamA gene abundance did not correlate with *Syntrophorhabdus* abundance, as it was expected. This suggests that anaerobic aromatic degraders targeted by bamA gene, are not only members of *Syntrophorhabdus* genus, in fact, even though *Syntrophorhabdus* was the only known aromatics degrading bacterium that increased its abundance after degradation of phenols, this may have been not the only genus present in the samples with the capacity to degrade aromatic compounds, which would explain the non-correlation between bamA and *Syntrophorhabdus*.

4. Conclusions

Sludge of different sources conserved a distinctive microbial structure despite suffering an adaptation process due to phenolic compounds exposure. Independently of sludge origin, the genus *Syntrophorhabdus* together with hydrogenotrophic archaea increased their relative abundance after degradation of phenol and p-cresol, indicating the important role of these microorganism during the anaerobic process. Higher initial abundances of *Syntrophorhabdus* genus and bamA gene, were probably the main causes of the higher degradation rates displayed. Therefore, the abundance estimation of *Syntrophorhabdus* and bamA gene in anaerobic sludge, could serve as a fast screening parameter to find the most acclimatized sludge to efficiently degrade mono-aromatic compounds and thereby improve the anaerobic process kinetics.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejbt.2018.08.002.

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