Correlations between microbial population dynamics, bamA gene abundance and performance of anaerobic sequencing batch reactor (ASBR) treating increasing concentrations of phenol

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Correlations between microbial population dynamics, *bamA* gene abundance and performance of anaerobic sequencing batch reactor (ASBR) treating increasing concentrations of phenol

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ARTICLE INFO

Keywords:
Anaerobic digestion
bamA gene
Illumina sequencing
Microbial community
Phenol

ABSTRACT

The relevant microorganims driving efficiency changes in anaerobic digestion of phenol remains uncertain. In this study correlations were established between microbial population and the process performance in an anaerobic sequencing batch reactor (ASBR) treating increasing concentrations of phenol (from 120 to 1200 mg L^{-1}). Sludge samples were taken at different operational stages and microbial community dynamics was analyzed by 16S rRNA sequencing. In addition, *bamA* gene was quantified in order to evaluate the dynamics of anaerobic aromatic degraders. The microbial community was dominated by *Anaerolineae*, *Bacteroidia*, *Clostridia*, and *Methanobacteria* classes. Correlation analysis between *bamA* gene copy number and phenol concentration were highly significant, suggesting that the increase of aromatic degraders targeted by *bamA* assay was due to an increase in the amount of phenol degraded over time. The incremental phenol concentration affected hydrogenotrophic archaea triggering a linear decrease of *Methanobacterium* and the growth of *Methanobrevibacter*. The best performance in the reactor was at 800 mg L^{-1} of phenol. At this stage, the highest relative abundances of *Syntrophorhabdus*, *Chloroflexus*, *Smithella*, *Methanolinea* and *Methanosaeta* were observed and correlated positively with initial degradation rate, suggesting that these microorganisms are relevant players to maintain a good performance in the ASBR.

1. Introduction

Phenol is a widespread compound present in many industrial effluents such as coal conversion processes, petroleum refineries, herbicide manufacturing and petrochemicals. The concentration of phenolic compounds in this kind of effluents varies from 10 to 17,000 mg L^{-1} contributing to 40–80 % of its total chemical oxygen demand (COD) (Veeresh et al., 2005).

To treat effluents containing phenol, several physicochemical and biological treatments are available. Among biological processes, different anaerobic technologies that work with biomass retention such as upflow anaerobic sludge blanket reactor (UASB), expanded granular sludge bed reactor (EGSB) and anaerobic sequencing batch reactor (ASBR) have been implemented to cope with the presence of phenol. The advantages that these anaerobic systems offer compared to other biological technologies are: high organic loading rates capacity; methane production; low sludge generation and the capability of retaining microbes with special functions (e.g. phenolic compounds degradation) (Almendariz et al., 2005; Veeresh et al., 2005; Rosenkranz et al., 2013).

Due to the antimicrobial properties of phenol, this compound is toxic and inhibitory for most microorganisms (Chen et al., 2008a). In anaerobic sludge, half-maximal effective concentrations (EC_{50}) are reported to be between 120 and 225 mg gVS^{-1} (Hernandez and Edyvean, 2008; Wirth et al., 2015). Either in a phenol-acclimated and non-acclimated sludge, increasing concentrations of this compound affects the anaerobic digestion performance, deteriorating both the removal efficiency and methane production rate (Fang et al., 2004; Chapleur et al., 2016). According to this, the identification of the microbial populations resistant to high phenol concentrations is relevant to design and establish operational strategies capable to maintain such microbial members in order to improve the system´s stability. In line with this, one of the final goals of the microbial community studies in anaerobic digestion systems is to find the relationship between specific microorganims dynamics and system´s performance in order to determine if

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Table 1
Operational parameters and performance indicators of the phenol-fed ASBR reactor under the five operational stages.

Stage	I	II	III	IV	V
Inlet phenol concentration [mg L^{-1}]	120 ± 60	240 ± 4	500 ± 50	800 ± 7	1200 ± 43
Initial degradation rate (mg phenol · VSS^{-1} · d^{-1})	10	11	13	15	13
Elimination capacity (mg phenol · VSS^{-1} ·d^{-1})	26	28	19	31	11
Degradation time of last cycle (h)	13	20	45	50	215
Operational time (d) /number of cycles	39/22	18/7	21/6	33/7	85/6

this relationship could be useful to establish performance biomarkers that serves as early warning indicators of process failure or performance enhancement (Carballa et al., 2015).

Molecular approaches such as DNA fingerprinting and sequencing methods have been used to unravel relationships between microbial community dynamics and performance changes under variable phenol concentrations (Fang et al., 2004; Rosenkranz et al., 2013; Wirth et al., 2015; Chapleur et al., 2016; Madigou et al., 2016). In all these studies, the results show that *Clostridiales* is a highly abundant order during anaerobic digestion of this compound, together with -in some cases- *Anaerolinaceae* and *Syntrophaceae* (Rosenkranz et al., 2013; Wirth et al., 2015), suggesting that they play an important role in the process. However, the identification of high abundant microorganisms in the process not necessarily implies high activity from them (De Vrieze et al., 2016) and low abundance populations could even be more active than the most abundant ones, as demonstrated by metatranscriptomic and metaproteomic methods (Zakrzewski et al., 2012; Hanreich et al., 2013).

For this reason, a robust statistical analysis of the microbial community abundance dynamics must be done to establish which microorganisms are significantly correlated with a particular performance parameter of the process. Pearson correlation analysis tests have been used to identify key microbial players driving the performance/efficiency of anaerobic digesters (Regueiro et al., 2015; Shin et al., 2016). Regarding anaerobic digestion process of phenol, a correlation analysis between changes in the abundance of certain microorganisms and the performance of the reactor is still lacking.

Even though the sequencing analysis of microbial communities by 16S rRNA gene display more accurate results than fingerprinting methods (for example, DGGE band sequencing) (Pylro et al., 2016) this analysis also has some limitations due that the identity of microorganisms depends on reported sequences within databases. Because of that, it is recomendable to complement this information by studying functional genes (Oka et al., 2011).

In the anaerobic degradation pathway of phenol this compound is first oxidized to benzoate, which is then activated and converted to benzoyl-coA by benzoyl-CoA ligase. This intermediate compound is dearomatized by the benzoyl-CoA reductase to form cyclohex-1,5-diene-1-carbonyl-CoA. The oxidation of this product follows a kind of beta-oxidation, which includes a hydrolytic opening of the alicyclic ring which is mediated by the 6-oxocyclohex-1-ene-1-carbonyl-CoA hydrolase. The product of this cleavage follows β-oxidation reactions to produce acetate (Levén et al., 2012; Fuchs, 2008). The *bamA* gene for example codifies the hydrolase which catalyse the ring cleavage step of 6-oxocylcohex-1-ene-1-carbonyl-CoA during the anaerobic degradation of phenol via the 4-hydroxybenzoate to benzoyl-CoA pathway (Kuntze et al., 2008, 2011; Laempe et al., 1999). This gene has been used as a biomarker of aromatic-degrading anaerobes and has been correlated positively with the amount of degraded mono aromatic compounds (Sun et al., 2014). Therefore, the study of the *bamA* gene dynamics in the anaerobic digestion process of phenol is an interesting approach to estimate the abundance of anaerobes effectively involved in the degradation of this compound.

In this context, the present work aims at studying the microbial community dynamics of an anaerobic sequencing batch reactor (ASBR) treating increasing phenol concentrations, and to assess the potential correlations between reactor performance parameters and abundances of specific phylotypes within the sludge community in order to find potential performance biomarkers within the process. The microbial community was analyzed both at the total diversity level by a taxonomic approach (through next-generation sequencing of 16S rRNA gene by Illumina MiSeq) and at the functional population level by targeting anaerobic phenol-degraders (through *bamA* gene quantification).

2. Material and methods

2.1. Experimental set-up

A 5 L laboratory-scale ASBR was operated using a granular sludge inoculum from a tobacco waste water treatment plant at 12 g of volatile suspended solids per liter (VSS L^{-1}), as previously described in Rosenkranz et al. (2013). Biomass concentration was kept approximately constant by doing sludge purges, every two weeks. The reactor was operated at 37 °C and fed with synthetic wastewater containing phenol (Sigma-Aldrich, 99%) as the sole carbon source and 21.4 mg L^{-1} of KH_2PO_4, 4.3 mg L^{-1} of NH_4Cl and $NaHCO_3$ to maintain alkalinity in a range of 2.5–3.0 g $CaCO_3$ L^{-1}. Each cycle of ASBR operation is constituted of 4 steps: feeding (20 min), reaction (the time needed to degrade at least 90% of phenol), biomass settling (1 h) and effluent discharge (20 min). The volume exchange ratio was 60%. After an initial acclimation to phenol at 120 − 240 mg L^{-1} that lasted 80 days (22 cycles), the inlet phenol concentration was progressively increased from 120 to 1200 mg L^{-1} during five operational stages (I to V), as described in Table 1.

2.2. Analytical methods

Volatile suspended solids were measured according to Standard Methods (APHA, 1995). Phenol was detected and quantified by a gas chromatograph GC-8A (Shimadzu, Kyoto, Japan), equipped with a 30″x4 mm ID packed column GP 60/80 Carbopack C/0.3% Carbowax 20 M/ 0.1% H_3PO_4 (Sigma Aldrich, St Louis, MO, US). The analysis was carried out at 120 °C, using nitrogen as carrier gas (50 mL/min) and a flame ionization detector at 200 °C.

2.3. Performance evaluation

All the following performance indicators excepting the elimination capacity (EC) were calculated during the last cycle of operation at each stage, once the degradation time got stabilized thus indicating the adaptation of the process to each concentration step. The EC was calculated as the total degraded phenol amount divided by the total degradation time over the entire stage, relative to the biomass content (mg phenol g^{-1} VSS^{-1} L^{-1} D^{-1}). The initial phenol degradation rate (IDR) was calculated as the slope of the first linear region of the degradation profiles and degradation time (DT) was defined as the time required to degrade at least 90 % of inlet phenol during the last cycle of the stage.

2.4. DNA extraction

The DNA extracts for this research were obtained from a previous study from our lab (Rosenkranz et al., 2013). At that moment the extracts were aliquoted in sterile DNAse free microtubes and stored at −80 °C for the 16S rRNA gene sequencing analysis and quantitative PCR assay (for this study). Briefly, in each stage of operation, sludge samples were collected at the end of the last cycle of operation, once phenol degradation efficiency reached at least 90%. Sludge samples were centrifuged at 10,000 *g* for 10 min and 0.5 *g* of pellet obtained were used for DNA extraction using the Powersoil DNA Isolation Kit (MO BIO Laboratories) following the manufacturer´s instructions. The integrity of DNA extracts was checked by 2% agarose gel electrophoresis. DNA concentration and purity were tested by UV spectrophotometric analysis at 260, 280 and 230 nm using a Nanodrop (Shimadzu Bio Spec-nano).

2.5. Quantitative PCR (qPCR)

The quantification assays were conducted in an AriaMX real-time PCR cycler (Agilent). Total bacteria and archaea abundances were assayed using the protocol described by Yu et al. (2006). The *bamA* gene quantification was performed using the primer set Bam-sp9 and Bam-asp1 targeting a 300 bp fragment (Kuntze et al., 2008). Each 20 u L PCR reaction contained 10 u L of Takyon Rox SYBR MasterMix dTTP Blue (Eurogentec, Köln, Germany), 0.9 u M of each primer and 2 u L of ten fold diluted DNA template. The thermal program consisted of an initial denaturation (95 °C, 3 min) and 44 amplification cycles (95 °C for 3 s; 60 °C for 40 s). Melting curves were constructed from 65 °C to 95 °C, read every 0.5 °C for 5 s. Calibration curves of *bamA* gene (10^{1}-10^{6} gene copies/uL) were prepared using genomic DNA from the anaerobic phenol-degrader *Thauera aromatica* (DSM 6984) assuming a genome size of 4.6 Mb (Kazy et al., 2010) and one copy number of this gene per genome. Quantification was carried out on triplicate samples. The qPCR efficiencies for all reactions were between 90–100 %. The results were expressed in gene copy number per gram of wet weight of sludge.

2.6. 16S rRNA amplicon gene sequencing

DNA samples were sequenced targeting the V4-V5 hyper variable region of the 16S rRNA gene with primers 515 F and 909R targeting both bacterial and archaeal communities (Wang and Qian, 2009). Sequencing was performed at MR DNA laboratory (Shallowater, TX, USA) on a MiSeq Illumina sequencer following the manufacturer's guidelines. Sequence data were processed using a proprietary 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 (Online Resource 1) were taxonomically classified using BLASTn against a curated GreenGenes database (DeSantis et al., 2006).

2.7. Statistical analysis of microbial community

Microbial community structure and correlation analysis between microbial populations and performance parameters at different stages of the reactor were computed with XLSTAT version 2014 (Addin soft). Beta diversity analysis of microbial community at different operational stages of reactor was carry out using the agglomerative hierarchical clustering (AHC) method, based on the relative abundances profiles of microorganisms at genus level (Eichorst et al., 2013). A dendrogram resulting from the AHC analysis was constructed using the mean link agglomeration method and the Bray-Curtis dissimilarity distance which quantifies how dissimilar a pair of samples is, based on specimen counts (Wong et al., 2016). Heat-map of genus dynamics over the different operational stages was performed within the Heat-map tool. Principal component analysis (PCA) was performed using the data analysis tool using the option Pearson correlation type and was constructed in order to visualize community structure distribution under different phenol concentrations and its effects on 3 performance parameters: initial degradation rate (IDR), elimination capacity (EC) and degradation time (DT). *BamA* gene abundance was also included as a biological variable in the PCA plot. Significant correlations between genus abundances, environmental and performance parameters together with *bamA* gene abundance were determined by Pearson correlation test analysis. The results from a Pearson correlation test having a p-value ≤ 0.05 were considered as significant.

3. Results and discussion

3.1. Effect of increasing phenol concentrations on process performance

After an acclimation period of 80 days, phenol concentration feeding in the ASBR was increased stepwise from 120 ± 60 up to 1200 ± 43 mg L^{-1} in a total process that was divided in five operational stages (Table 1). The detailed analysis of process performance in response to phenol increase was described previously (Rosenkranz et al., 2013) and the most relevant indicators of the process were used in this study to enlighten relationships with the microbial community structure and composition revealed by the 16S rRNA gene sequencing analysis. Briefly, the total degradation time (DT) necessary to degrade 90 % of the compound increased continuously as a result of higher substrate load. DT increase was linear during the first three stages (Table 1), got rather stable during Stage IV, and drastically increased during stage V (more than four times compared to the previous stage). As a result, the Elimination Capacity (EC) remained relatively constant (around 26 mg $_{phenol}$. VSS^{-1}. d^{-1}) from Stage I to IV, and dropped down to 11 mg $_{phenol}$ $\cdot VSS^{-1} \cdot d^{-1}$ in Stage V. Within each degradation profile, the Initial Degradation Rate (IDR) is indicative of the capacity of the community to withstand a new charge load. It increased progressively during the first four stages, and then decreased in the last stage. All together, these indicators show a maximal degradation efficiency at 800 mg L^{-1} of phenol (Stage IV) and suggest the existence of a threshold concentration between 800 and 1200 mg L^{-1} after which the reaction rate slowdowned, evidencing a deterioration of the reactor performance and possible partial inhibition.

Other studies performed in batch assays under methanogenic conditions reported a complete inhibition of phenol degradation at 2000 mg L^{-1} (Poirier et al., 2016; Chapleur et al., 2016). In our case, the maximal concentration applied (1200 mg L^{-1}) was below the complete inhibition limit: the degradation rate of the reactor is impaired but the adapted anaerobic biomass due to reactor configuration (ASBR) could tolerate and metabolize this load.

3.2. Total bacterial and archaeal abundances

As can be seen in Fig. 1A, the Bacteria and Archaea in this system represent on average 70% and 30%, respectively of the total community during the whole period, which is consistent with the operating conditions favoring favouring methanogenic activity. Despite reactor performance was negatively affected at the highest phenol concentration there was only a small decrease in total bacteria and archaea abundances only at Stage II (which was rapidly recovered at the subsequent stage), total microbial abundance stayed rather constant along the operation, independently of the phenol concentration, suggesting that the toxic phenol threshold implying mortality was not reached and that performance impairement was due to a change in microbial structure configuration rather than biomass losses.

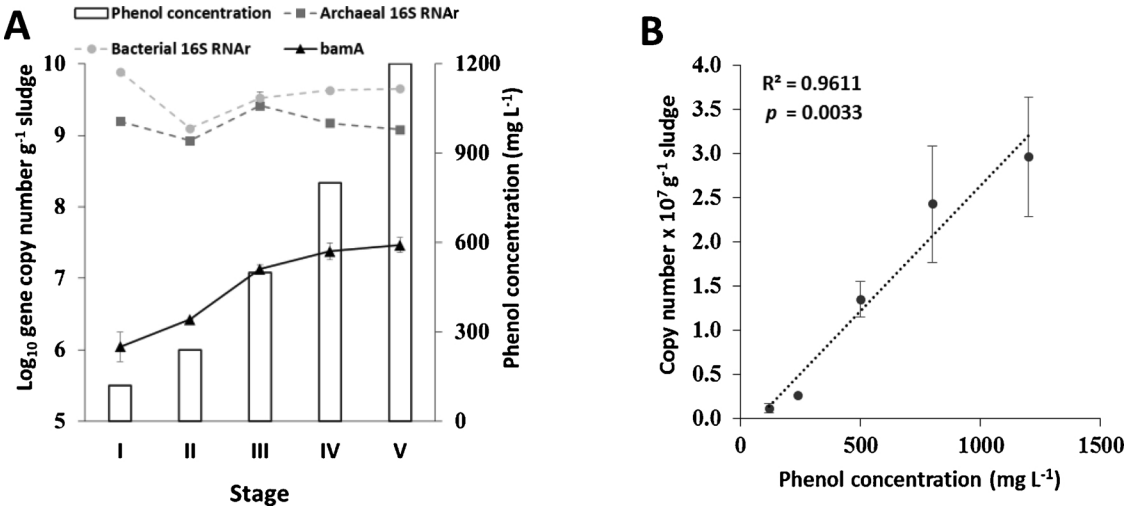

Fig. 1. BamA gene dynamics in ASBR. In A, the abundance of bamA gene, total bacteria and total archaea at different operational stages in ASBR. In B, correlation between phenol concentration applied to inlet feeding in ASBR and bamA gene abundance. The dotted line correspond to the linear model describing the significant correlation between variables. Error bars represents one standard deviation arround the mean.

3.3. Evolution of bamA gene abundance at different ASBR stages

During the ASBR process the *bamA* gene levels had an incremental trend as phenol concentration increase (Fig. 1A), suggesting that microorganisms with the capacity to degrade phenol accumulated throughout the process. In our work, phenol is removed through the process and *bamA* levels increased in a significant positive linear correlation ($p < 0.01$) with phenol concentration (Fig. 1B). Inlet phenol concentration is therefore a strong and direct selective pressure/driving force stimulating the growth of phenol-degraders.

The *bamA* gene copy number was 2–4 orders of magnitude lower than the total bacterial and archaeal 16SrRNA gene copy number, confirming that the functional community effectively involved in phenol degradation via the benzoyl-CoA pathway represents only a minor fraction (0.3% maximum) of the total community. Variations in 16S rRNA gene copy number per genome between microorganisms also could originate an underestimation of the relative abundance of *bamA* gene harbouring populations. This result demonstrates the importance to consider the rare biosphere in anaerobic digestion ecosystems, where subdominant species can act as keystones and impact the overall function despite their low abundance, as highlighted in fermentative ecosystems (Rafrafi et al., 2013).

There is only one study that employed *bamA* gene quantification to enumerate anaerobic aromatic degraders under methanogenic conditionns and that suggest a correlation between the increase of *bamA* gene levels and the removal percentage of an aromatic compound (toluene) (Sun et al., 2014). Based on this, our result confirms that quantification of *bamA* gene could be useful as a monitoring variable capable of describe the progression of degradation process of mono aromatic compounds.

3.4. Microbial community analysis

3.4.1. Effect of phenol concentration at overall microbial community structure in ASBR

Within Bacteria, Miseq amplicon sequencing analysis shows that *Anaerolineae*, *Bacteroidia*, *Clostridia* and *Deltaproteobacteria* were the dominant bacterial classes in almost all operational stages, except for Stage I (Fig. 2), where *Alphaproteobacteria* and *Bacilli* were dominant at the expense of *Deltaproteobacteria* abundance. The *Bacteroidia* and *Clostridia* classes have been reported as dominant in anaerobic batch reactors treating phenol as the sole carbon source at different concentrations (Wirth et al., 2015). The *Deltaproteobacteria* class has been found dominant in mesophilic methanogenic phenol degrading enrichments at 500 mg L^{-1} (Chen et al., 2008b). *Anaerolineae*, on the other hand, is regarded as an important class from the *Chloroflexi* phylum, which is part of the "core group" of organisms always found in anaerobic digesters independently of substrate nature (Cabezas et al., 2015).

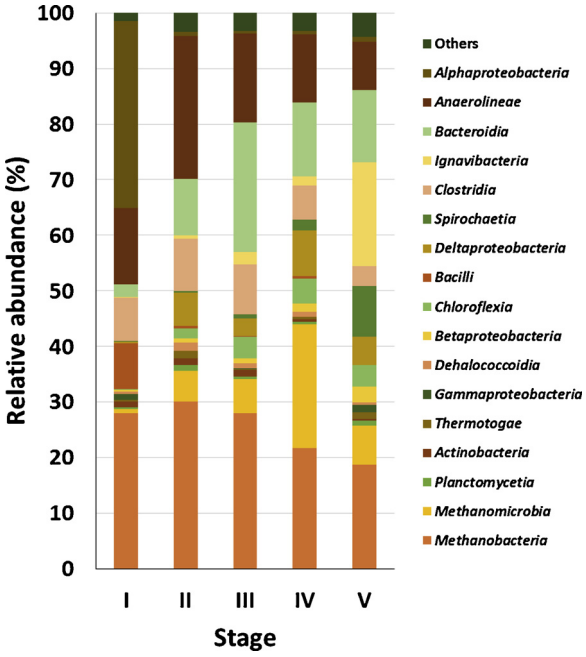

Fig. 2. Relative abundance at class level of microbial communities in the ASBR at the last cycle of each operational stage. Only classes having abundance ≥ 1% in at least one sample are represented otherwise were considered as "others".

Within the archaeal domain, *Methanobacteria* was the dominant class in all operational stages followed by *Methanomicrobia*. According to these results, it is likely that anaerobic digestion of phenol in this system was carried out mainly via the hydrogenotrophic methanogenesis pathway.

3.4.2. Structural changes at genus level during ASBR operation

Fig. 3B shows the AHC analysis of microbial community profiles at the genus level (only those with abundances equal or over 0.5% in at

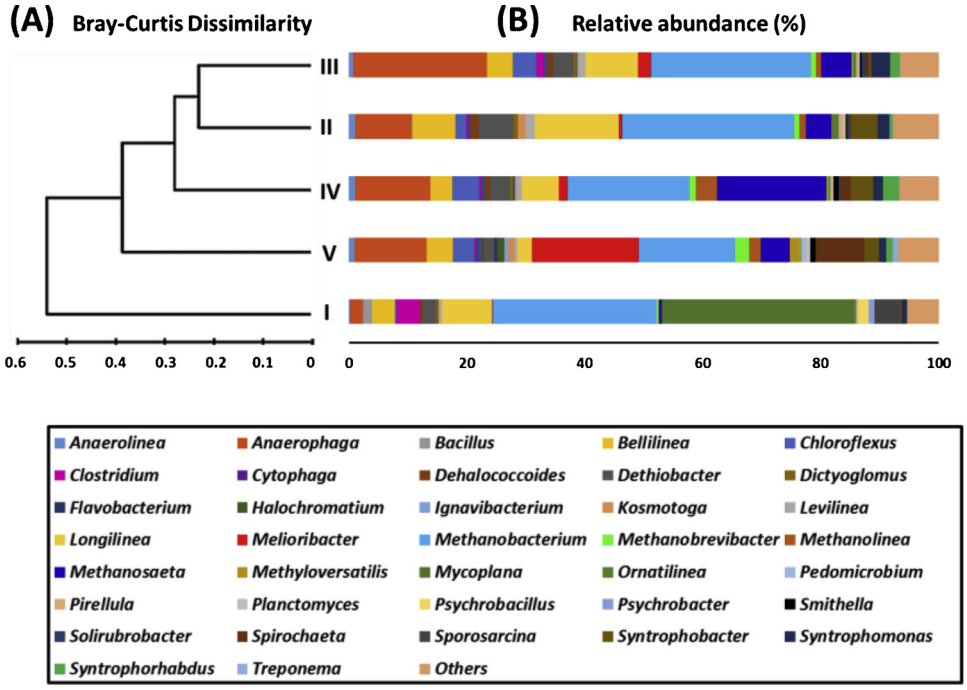

Fig. 3. Microbial community structure at the genus level in ASBR operating at different feeding phenol concentrations (I = 120, II = 240, III = 500, IV = 800 and V = 1200 mg L-1). In A, community clustering analysis based on Bray-Curtis dissimilarity between samples. In B, relative abundance of genera at each stage. Only genera having abundance ≥0.5% in at least one sample are represented otherwise were considered as "others".

least one sample) and the relative abundances of each genus at different operational stages in ASBR. The dendrogram (Fig. 3A) shows the strongest differentiation between community from stage I (corresponding to the lowest phenol concentration at 120 mg L^{-1}) and the other stages, highlighting the sludge acclimatation process to new environmental conditions imposed by phenol feeding. This differentiation was caused mainly by the highest relative abundance of *Mycoplana* which dominated the bacterial community (33% of total bacteria), as well as the presence of *Clostridium* (4%) and *Sporosarcina* (4%), while they were all almost negligible in the following stages (Fig. 4B). As far as we know, this is the first report of *Mycoplana* presence in an anaerobic digester treating phenol. The capacity of *Mycoplana* to degrade aromatic compounds has been reported previously in pure culture experiments (Lakshmi et al., 2013) and was found at low abundance levels in a microcosm treating nonylphenol at 100 ppm under nitrate reducing conditions (Wang et al., 2015). Thus, *Mycoplana* was presumably the main phenol degrader at this stage, followed by *Clostridium* and *Sporosarcina*, which have been reported at high abundance in methanogenic reactors treating phenolic compounds (Tartakovsky et al., 2001; Hernandez et al., 2013). The drastical decrease of *Mycoplana* at the following stages suggest that this genus is not adapted to growth efficiently at higher phenol concentrations under methanogenic conditions.

Regarding dominant archaeal genera, *Methanobacterium* (an hydrogenotrophic methanogen) was the most abundant followed by *Methanosaeta* (acetotrophic) and *Methanolinea* (hydrogenotrophic). *Methanobacterium* dominance, in anaerobic digestion of phenol, has been reported previously (Madigou et al., 2016), but its high abundance is not a necessary requirement to carry out the process efficiently, since other studies involving phenol degradation reported that *Methanosaeta, Methanosarcina* and *Methanoculleus* can be dominant too (Poirier et al., 2016; Zhang et al., 2005).

After Stage I, the community structure followed a clear progressive evolution along the phenol concentration gradient applied (between 240 and 1200 mg L^{-1}), as evidenced in the dendrogram representation, where the successive stages cluster together. Within this group, the medium-concentration samples (between 240 and 800 mg L^{-1}) clustered together (at ~70% of similarity), clearly apart from stage V sample, in which the highest concentration of phenol was applied. This last stage is characterized by the highest abundance of *Melioribacter* (18%) (which dominated the community), and the presence of *Spirochaeta, Methyloversatilis* and *Methanobrevibacter*, in contrast with the previous stages where they were almost negligible, suggesting that these genera were able to tolerate phenol concentration as high as 1200 mg L^{-1}. Within these genera, *Methyloversatilis* is the only microorganism capable of metabolizing phenol (Smalley et al., 2015). *Melioribacter* and *Spirochaeta* on the other hand, were reported to be present in methanogenic systems treating aromatic compounds but their effective role in phenol degradation is not clear (Li et al., 2014; Xiong et al., 2015). Within all archaeal members, *Methanobrevibacter* was the only genus that had its maximum abundance at this stage indicating that this hydrogenotrophic methanogen can growth better than other methanogens under this environmental condition.

At stage IV, in which performance of ASBR was better, *Methanosaeta* and *Methanolinea* were the methanogens that showed their highest abundance (19 and 4%, respectively) in comparison to other stages as can been seen in the heat-map (Fig. 5). *Methanosaeta* is a well-known microorganism that produces methane via the acetotrophic pathway (Smith and Ingram-Smith, 2007). This genus probably uses the acetate generated from phenol conversion by direct phenol or benzoate degraders. Regarding bacterial members, the genera that had their highest abundance value at this stage (compared to the other stages) were *Smithella*, *Syntrophorhabbdus* and *Chloroflexus* (0.9, 2.7 and 4.6%, respectively) as can be seen in the heat-map (Fig. 5). From those, *Syntrophorhabdus* is the only known phenol degrader that acts in syntrophy with a hydrogen scavenger microorganism to grow (Qiu et al., 2008). Based on this, the highest abundance of the syntrophic phenol degrader *Syntrophorhabdus* coupled to a greater abundance of the hydrogenotrophic methanogen *Methanolinea* could be the reason of the increase in phenol degradation performance at this stage. An increase in phenol degradation must lead to an increment on acetate production rate, which would explain the greater abundance of *Methanosaeta.*

3.4.3. *Identification and dynamics of core community*

The analysis of the microbial populations also allowed us to establish a group of genera that maintained relative abundances of at least 2% in all operational stages, which implies that they can be of great importance to carry out the process successfully. This group was formed

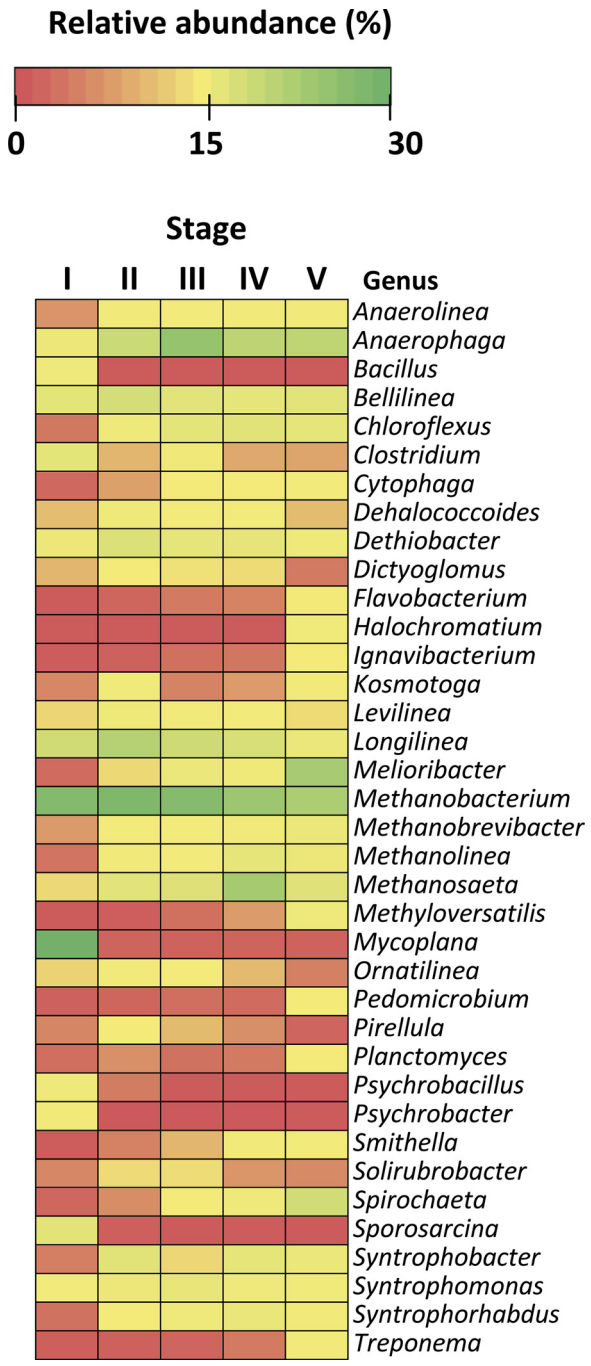

Fig. 4. Heat map of abundance dynamics for each genera at different ASBR operational.
stages. Only genera having a relative abundance ≥ 0.5% in at least one operational stage are represented.

by the genera *Bellilinea* (3.7–7.4%), *Longilinea* (2.5–14.3%), *Anaerophaga* (2.1–22.7%) and *Methanobacterium* (16–29%). *Bellilinea* and *Longilinea* are filamentous fermenters that produce volatile fatty acids. They lack the ability to use phenol as carbon source (Yamada et al., 2007) and it is suggested that they may be relevant to maintain the structure of granules in the anaerobic sludge (Yamada et al., 2005), an essential feature needed to work properly in SBR type reactors. On the other hand *Anaerophaga* is a strict anaerobe fermenter and its presence has been reported previously in microcosms enriched with benzoate (Herrmann et al., 2008) an intermediate compound produced in the anaerobic phenol degradation (Ju and Zhang, 2014), thus *Anaerophaga* could be using (at least) part of the benzoate generated as carbon source during the phenol degradation process.

As can be seen in Fig. 4, the increase of phenol concentration negatively affected the abundances of *Dethiobacter*, *Longilinea* and *Methanobacterium*. All these three genera reducted their abundances to the minimum at the highest phenol concentration. A drop in members belonging to the core group could be one of the causes for performance impairment at this operational stage. As can be noted in the heat-map, the abatement in *Methanobacterium* abundance was compensated by a raise of *Methanolinea* and *Methanobrevibacter*, both hydrogenotrophic methanogens that share the same ecological function as *Methanobacterium* (i.e. hydrogenotrophic methanogenesis) and have been found in the same ecological niches (Yu et al., 2015; Martin Vincent et al., 2018; Prajapat et al., 2019). Abundance changes of co-ocurring archaeal members sharing the same function is caused by an environmental condition that favors the growth of one archaeal member over another. In this case, phenol concentration increase fauvored the growth of *Methanolinea* and *Methanobrevibacter* over *Methanobacterium*. Martin Vincent et al. (2018) reported that during an increase in organic loading in a methanogenic digester *Methanobrevibacter* increased its relative abundance over *Methanobacterium*. The latter suggests that the increase in organic load caused by a phenol concentration increment in the ASBR promoted the growth of *Methanobrevibacter* as well as *Methanolinea*.

3.5. Correlations between microorganisms, bamA gene and performance parameters

A principal component analysis was performed in order to visualize how changes in feeding phenol concentration affects the microbial community structure variability during the different operational stages in ASBR (Fig. 5). In the PCA plot each sample point has an specific position based on its particular genera abundance profile. This position was represented as circles whose size was proportional to the inlet phenol concentration applied to each operational stage. As can be noted, PC1 (X axis) and PC2 (Y axis) explains a great part of the samples variability (47 % and 33 %, respectively). In addition, performance and environmental variables were represented as arrows, whose direction and length indicate to which sample the increase of this variable is explaining the microbial abundance patterns and the magnitude of that variable in explaining the microbial profiles variation, respectively. Based on the aforementioned, it is possible to observe that phenol concentration (PC) is related with the X axis principal component which means that microbial community structure was shaped at a great extent by the change of phenol feeding concentration. Genera correlated significantly with a specific variable is presented in Table 2 and were also plotted in the PCA. Significant positive correlations between the abundance of *bamA* gene with abundances of *Cytophaga* and *Smithella* were observed. These could mean that (i) they are directly involved in phenol degradation (either through *bamA* pathway, or another gene not targeted in our assay) or (ii) they are indirectly related for example through consumption of by-products of phenol degradation. Althought there is no information reported regarding the metabolic capacity of *Smithella* to degrade aromatic compounds, this genus is capable of syntrophically oxidize butyrate and propionate to acetate (Zhang et al., 2017) supporting the hypothesis (ii) of indirect relationship with phenol degraders. On the other hand, *Cytophaga* have been found in anaerobic reactors treating trichlorophenol (Collins et al., 2005) and clones belonging to this genus have been reported to be involved in polyaromatic hydrocarbon degradation under methanogenic conditions (Braun et al., 2015). Based on this and knowing that *Cytophaga* members harbour the *bamA* gene (by consulting Kyoto Encyclopedia of Genes and Genome) (Kanehisa and Goto (2000)), we infer that *Cytophaga* might be a direct phenol degrader.

Analysis between reactor parameters and microorganisms reveals that there are six genera that correlated positively with phenol concentration and ten with degradation time (Table 2). Of these,

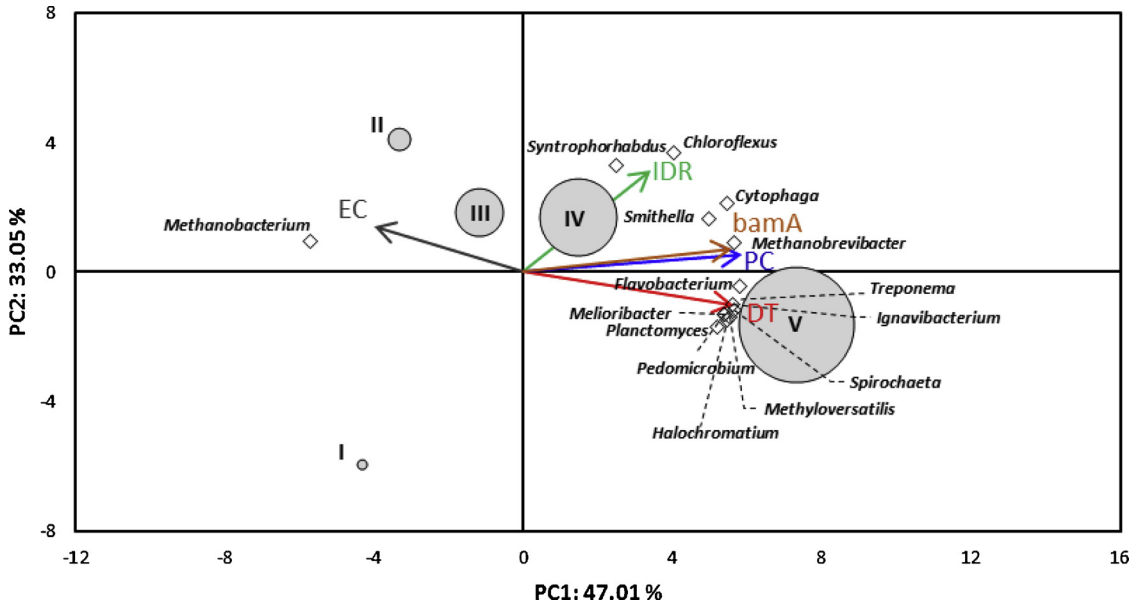

Fig. 5. Principal component analysis of the microbial structure distribution at genus level and correlations with bamA gene abundance, phenol concentration (PC) and the performance parameters; Elimination capacity (EC), Initial degradation rate (IDR) and degradation time (DT). For each operational stage, the circle size is proportional to feeding phenol concentration. Genera that correlated significantly with one or more variables were presented in the plot together with their specific positions denoted by diamonds.

Table 2
Pearson correlation coefficients (r) between significant correlated genera and Initial degradation rate (IDR), degradation time (DT) (performance variables), phenol concentration (PC) (environmental variable) and *bamA* gene (biological variable). The p-value is indicated: * < 0.05, ** < 0.01.

	Variables			
	Performance		Environmental	Biological
Genus	**IDR**	**DT**	**PC**	**bamA**
Chloroflexus	**0.957***	0.458	0.771	0.844
Cytophaga	0.782	0.802	**0.955***	**0.953***
Flavobacterium	0.413	**0.992****	**0.941***	0.862
Halochromatium	0.113	**0.982****	0.802	0.678
Ignavibacterium	0.297	**1.000****	**0.897***	0.799
Melioribacter	0.198	**0.995****	0.845	0.732
Methanobacterium	−0.596	−0.875	**−0.962****	**−0.946***
Methanobrevibacter	0.411	**0.960****	**0.919***	0.826
Methyloversatilis	0.241	**0.995****	0.873	0.769
Pedomicrobium	0.187	**0.994****	0.841	0.725
Planctomyces	0.107	**0.960****	0.786	0.650
Smithella	**0.885***	0.625	**0.902***	**0.956***
Spirochaeta	0.295	**0.994****	**0.899***	0.804
Syntrophorhabdus	**0.983****	0.118	0.541	0.683
Treponema	0.212	**0.992****	0.858	0.750
DT	0.296	–	**0.896***	0.798
PC	0.683	**0.896***	–	**0.980****
bamA	0.799	0.798	**0.980****	–

Flavobacterium, *Ignavibacterium*, *Spirochaeta* and *Methanobrevibacter* had positive correlations with both parameters. *Ignavibacterium* was found to be a dominant aniline-degrader in methanogenic microcosm (Sun et al., 2015). Accordingly, this genus has the metabolic machinery to degrade monoaromatic compounds and, based on these results, the capability to tolerate high phenol concentrations. No aromatic degrading capacity was reported for the other genera. However, spirochaetal organisms were found in granular sludge of an EGSB-AF reactor treating 50 mg L^{-1} of trichlorophenol and were capable to tolerate the presence of this highly toxic compound (Collins et al., 2005) thus, it is expected that members of *Spirochaeta* genus are also able to cope with high phenol concentrations. On the other hand, higher degradation time in ASBR observed in stage V indicate that microbial members positively correlated with these variables were able to maintain the anaerobic digestion process stable (due to their capacity to tolerate high phenol concentration) but less efficiently either because a partial inhibition phenomenon or lower total degradation activity of the microorganisms which leads to display a higher degradation time. Correlation tests also showed that the genera *Syntrophorhabdus*, *Chloroflexus* and *Smithella* (maximum abundances at stage IV) have a significant positive correlation with the initial degradation rate. *Syntrophorhabdus* was widely reported in methanogenic systems treating phenolic compounds like phenol and p-cresol (Chen et al., 2009; Levén and Schnürer, 2010; Ju and Zhang, 2014; Na et al., 2016; Franchi et al., 2018a). Ju and Zhang (2014) reported the presence of *Syntrophorhabdus* in two methanogenic batch reactors treating phenol concentrations between 400 and 1000 mg L^{-1} displaying different degradation rates and suggested that a higher proportion of this microorganism could explain the higher rate of one reactor. At archaeal level, *Methanobacterium* showed a significant negative correlation with phenol concentration while *Methanobrevibacter* had a positive correlation with that parameter. Therefore, the concentration of this compound has a negative linear effect on the growth of *Methanobacterium* and, conversely, promotes in a linear manner the abundance of *Methanobrevibacter*. One possible explanation of this result, could be the presence of a competition relationship between these archaeal members, a phenomenon which offen occurs in closely related OTUs (both genus belongs to *Methanobacteriaceae* family) requiring similar resources for growth (i.e. carbon dioxide and hydrogen) (Wu et al., 2016). According to this, the phenol concentration increment, could be a relevant environmental variable in promoting the competition strength of *Methanobrevibacter* to the detriment of *Methanobacterium*. On the other hand, although *Methanolinea* and *Methanosaeta* had positives correlations with initial degradation rate of ASBR the p-values of these correlations were just above the significance threshold (0.052 and 0.054, respectively). Despite this, our results show that the abundance increase of these archaeal members tends to improve the phenol degradation performance in a linear manner. The theorical reason of this trend could be explained first by an increase of the hydrogen scavenging capacity of the system due to a higher abundance of *Methanolinea* thus favouring *Syntrophorhabdus* degrading activity and secondly by an increase in the acetate consumer *Methanosaeta* which would be contributing to prevent

the ASBR acidification. Notably, two paralel metabolic pathways may be co-occuring during the stage with highest efficiency, which suggest a positive synergic contribution of these archaeal members to the process. These results agree with our previous findings regarding the positive interactions (cooperation) between *Syntrophorhabdus*, *Methanosaeta* and hydrogenotrophic archaeas in a context of anaerobic digestion of phenolic compounds (Franchi et al., 2018b).

4. Conclusions

The increase of phenol concentration at more than 800 mg L^{-1} in ASBR system triggered a performance impairment on degradation process that was associated with an abundance reduction of a core group of microorganisms. At 800 mg L^{-1} of phenol, the degradation performance of ASBR reached its maximum in correspondence with highest abundances of *Syntrophorhabdus*, *Chloroflexus*, *Smithella*, *Methanolinea* and *Methanosaeta* which in turn were positively correlated with the initial degradation rate, indicating that the synergistic growth of these microorganisms promoted a better performance of the anaerobic degradation of phenol in the system. On the other hand, *bamA* gene dynamics showed that aromatic degraders targeted by the qPCR assay were accumulating through the process and correlated possitively with inlet feeding phenol concentration, suggesting that *bamA* gene quantification could be a useful monitoring variable capable to describe the growth progression of phenol degrading bacteria.

5. Author contributions

Author 1: Oscar Franchi
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Performed the analysis
Wrote the paper
Author 2: Rolando Chamy
Conceived and designed the analysis
Author 3: Léa Cabrol
Performed the analysis
Author 4: Francisca Rosenkranz
Conceived and designed the analysis
Collected the data
Contributed data or analysis tools

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was funded by CONICYT Project 781302010 and FONDECYT Project 1151161 from CONICYT – CHILE.

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