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Characterizing phenol-removing consortia under methanogenic and sulfate-reducing conditions: potential metabolic pathways

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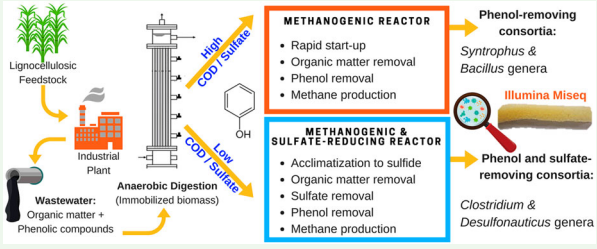
ABSTRACT

Phenol removal was investigated in anaerobic fixed-structured bed reactors, namely R1 and R2, treating synthetic wastewater simulating the soluble fraction of vinasse under strictly methanogenic (R1) and simultaneous methanogenic/sulfidogenic conditions (R2). Next-generation sequencing (Illumina MiSeq System) was used to further characterize the microbial communities in both systems. Phenol was completely and stably removed in R1 after a short operating period (≈55 days). Conversely, phenol removal in R2 required a longer period for biomass acclimation (≈125 days) to reach levels equivalent to R1. Volatile fatty acids (VFA) accumulation in R2, mainly due to the inhibition of the acetoclastic methanogenesis by sulfide, may have limited phenol removal in the initial operating phases, as intermediate steps from phenol degradation are thermodynamically dependent on the removal of acetate, hydrogen and bicarbonate. Overall, the potential for anaerobically removing phenol from complex wastewaters was confirmed, even at low phenol/COD ratios. 16S rRNA gene sequencing analysis showed a high correlation of taxonomic profile between R1 and the inoculum, whereas a lower correlation was observed between R2 and the inoculum samples. Functional inference further indicated that *Syntrophus* and *Bacillus* genera in R1 and *Clostridium* genus in both reactors possibly played a key-role in phenol degradation.



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Anaerobic digestion; fixed-structured bed reactor; phenol removal; microbial community; sulfide

1. Introduction

The removal of recalcitrant and toxic organic compounds in treatment systems is a key factor to enable safer discharge or reuse of wastewaters. Although aerobic processes have historically been considered the best (or only) approaches to efficiently degrade toxic compounds in wastewater treatment plants [1], studies have proved the capability of the anaerobic biomass to handle inputs of different toxics, such as surfactants [2], pentachlorophenol [3] and phenol [4]. Implementing appropriate operating strategies, aiming at acclimation of biomass to the toxics and preventing marked biomass losses, has been considered an imperative approach to enable the anaerobic biodegradation of toxic organic compounds [5].

Phenolic compounds comprise a group of toxic substances frequently released in residual streams from numerous industrial processes, including petrochemical, pharmaceutical, herbicide, pulp and paper, wood processing, wine and coffee production chains [6,7]. The toxicity of phenolic compounds results from their bactericide, carcinogenic, mutagenic and allergenic effects [6], which may directly impact the biogeochemical cycles in aquatic and terrestrial ecosystems, as well as affect populations supplied by contaminated resources [8].

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Potential negative effects from phenolic compounds may also be associated with the inadequate management of sugarcane vinasse, the primary wastewater from ethanol production. The accumulation of phenolic compounds in vinasse is due to the partial degradation of residual lignocellulosic structures during juice and/or molasses processes in distilleries [9,10], leading to phenol concentrations as high as 8000–10,000 mg L^{-1} in molasses-based production processes [7]. Therefore, the direct land application of vinasse via fertirrigation has the potential to trigger specific negative environmental effects resulting from the release of phenols into the environment [11]. Moreover, given the suitability of using anaerobic digestion (AD) as the core processing step for sugarcane vinasse [12,13], treatment performance losses may also result from this particular compositional aspect of vinasse, i.e. phenol accumulation.

The anaerobic biodegradation of phenol and phenolic derivatives has already been critically considered in reference studies [14–16]. However, specific characteristics of the anaerobic environments, primarily the source and acclimation level of the biomass and intrinsic compositional aspects of the wastewater may affect the biodegradation pathways and efficiency of selected compounds. Blum et al. [17] and Pearson et al. [18] associated significant inhibition of methane production (>50%) with phenol concentrations as high as 2250–3000 mg L^{-1}. Conversely, relatively low phenol concentrations (10 mg L^{-1}) may have inhibitory effects on microbial populations, especially when using non-acclimatized biomass [19]. In particular, the effects of phenolic compounds on the biomass in anaerobic systems applied to sugarcane vinasse have not been directly investigated, and therefore complementary studies are required to indicate possible potentials and limitations of the process.

A common approach proposed to eliminate negative effects from phenols in the treatment of sugarcane vinasse comprises the application of oxidative processes, such as ozonation [20,21], prior to AD. However, these processes are usually energy-intensive, potentially leading to energetically and economically unfeasible scenarios in full-scale applications [20]. In turn, specific strategies adopted in AD plants may be used to offset limitations from toxic compounds, and thus eliminate additional energy-intensive steps. Biomass immobilization, either via granulation or biofilm formation, has been successfully used in the AD of aromatic compounds [22] by using sludge-blanket [23], fixed-bed [3,4] and fluidized-bed reactors [24]. In particular, the anaerobic fixed-structured bed reactor (AFSBR) [25] has recently been effectively used to process sugarcane vinasse [12,26]. Nevertheless, removing specific organic toxic compounds, such as phenols, has not been assessed in such reactor configurations.

The high sulfate concentrations commonly observed in sugarcane vinasse [27] could also act favorably in phenol degradation, given the metabolic versatility of sulfate-reducing bacteria (SRB). SRB have the ability to use a wide range of organic compounds as electron donors [28], including hydrocarbons and aromatic compounds, such as phenol [14,29] and benzoate [30,31], the primary intermediate from the anaerobic biodegradation of phenol. Therefore, phenol degradation should be facilitated in anaerobic systems characterized by the concomitant establishment of methanogenic and sulfate-reducing conditions, preventing negative impacts on the biomass [32,33].

The aim of this study was to elucidate the biodegradation, as well as the effect of a given phenolic compound on the anaerobic treatment of carbohydrate-rich synthetic wastewater (CRSW) simulating the soluble fraction of sugarcane vinasse. Hydroxybenzene was used as the reference phenolic compound. Continuous experiments were carried out in two AFSBRs, i.e. one operated exclusively under methanogenic conditions and another associating methanogenic and sulfate-reducing conditions to assess the contribution of SRB on the performance of the process. Finally, for in-depth microbial characterization, molecular tools were further used to assess the taxonomic profile from both systems using next-generation sequencing (NGS, Illumina MiSeq System).

2. Materials and methods

2.1. Reactor design and operating conditions

Two identical bench-scale anaerobic fixed-structured bed reactors (FSBR), namely, R1 and R2, made from poly-methyl methacrylate (PMMA), were operated in parallel in continuous up-flow mode for 213 days under mesophilic temperature conditions (25°C). R1 was operated under strict methanogenic conditions, whilst methanogenic and sulfidogenic conditions were established in R2 due to the application of increasing sulfate concentrations throughout the operation. Polyurethane (PU) foam strips vertically arranged were used as the support material in both systems, performing a working volume of 1.65 L. Each reactor had five sampling ports along the height, characterizing different length/diameter (L/D) ratios or relative hydraulic retention times (HRTs). Characteristics of the basic layout of the experimental apparatus are presented in Figure S1 (supplementary data section). Additional constructive characteristics of the reactors are presented in Godoi et al. [34].

Table 1. Experimental conditions applied to the reactors.

Parameter	Reactor	Operating phase				
		I	II	III	IV	V
COD (mg L^{-1})	R1/R2	1050 ± 140	2170 ± 300	3890 ± 240[a]	4180 ± 150[a]	4200 ± 240[a]
Phenol (mg L^{-1})		120 ± 4	115 ± 7	125 ± 15	120 ± 10	120 ± 7
Phenol/COD		0.11	0.05	0.03	0.03	0.03
Sulfate (mg L^{-1})	R2	340 ± 130	740 ± 100	1050 ± 70[a]	875 ± 90[a]	1350 ± 60[a]
COD/sulfate		3.1	2.9	3.7[a]	4.8[a]	3.1[a]

[a]Data presented by Godoi et al. [34].

The reactors were inoculated using mesophilic granular anaerobic sludge collected from a full-scale sludge-blanket reactor treating poultry slaughterhouse wastewater. The granular sludge was blended and applied to the PU foam strips for 2 h at room temperature, as described by Mockaitis et al. [35]. The reactors were subjected to increasing organic loading rates (OLR), i.e. from 1.0 to 4.0 g COD L^{-1} day^{-1}, during phases I to III, whereas in phases IV onwards, similar OLR were applied (Table 1). The HRT was maintained in 24 h in all the studied conditions. The influent pH was set as approximately 8.0 by adding sodium bicarbonate ($NaHCO_3$) to the fresh synthetic wastewater at a $NaHCO_3$/COD ratio of 1.4.

Phenol concentration was maintained at approximately 120 mg L^{-1} by the dosage of phenol (C_6H_5OH – JT Baker, reagent grade) to the fresh wastewater in all conditions. Different phenol/COD ratios (0.11 to 0.03) were assessed in each operating condition due to the variations in the influent COD (Table 1). In particular, R2 was also subjected to different COD/sulfate ratios by varying the influent sulfate concentrations (340–1350 mg L^{-1}; Table 1). Sodium sulfate (Na_2SO_4) was used as the sulfate source. Conversely, sulfate was dosed only as a nutrient in R1 (25 mg Na_2SO_4 L^{-1}).

2.2. Synthetic wastewater

A carbohydrate-rich synthetic wastewater (CRSW) simulating the soluble fraction of sugarcane vinasse was used to feed the reactors. Sucrose and ethanol were the primary organic constituents of the CRSW, with concentrations in the ranges of 560–2700 mg L^{-1} and 60–380 mg L^{-1}, respectively. Compositional details of the CRSW throughout the operating period are presented in the supplementary material section (Table S1).

2.3. Analytical methods and performance assessment

The following parameters were measured when monitoring the reactors: pH, COD, sulfate, total dissolved sulfide (TDS), volatile fatty acids (VFAs) and phenol concentrations. COD, pH, sulfate and TDS concentrations were determined according to the Standard Methods for the Examination of Water and Wastewater [36]. Zinc sulfate ($ZnSO_4$) was used to remove interferences from dissolved sulfide in COD analyses for R2. In this case, $ZnSO_4$ was added in excess to the samples prior to centrifugation to precipitate as zinc sulfide (ZnS). VFA concentrations were assessed by gas chromatography with flame ionization detection according to Adorno et al. [37]. Phenol measurements were carried out according to the 4-aminoantipyrine methodology modified by Buchanan and Nicell [38]. Prior to phenol analyses, sulfide interference was removed from the samples, as proposed by Gordon [39]: sulfide precipitation by adding silver nitrate ($AgNO_3$) 1 M to the samples, precipitation of the excess Ag^+ ions by adding 1 g of sodium chloride (NaCl) and separation of the precipitate through sample centrifugation (5000 rpm, 10 min). Since other interferences were absent in the sample matrix, the pre-treatment step by distillation was omitted, thus minimizing phenol losses by volatilization throughout the process [39]. Overall, the primary response-variables used to assess the performance of the reactors included: COD removal efficiency (ER_{COD}, in %), phenol removal efficiency (ER_{PHENOL}, in %) and sulfate removal efficiency (ER_{SO4}, in %).

2.4. Statistical analyses

Statistical analyses were performed using the BioEstat 5.0 software. Phenol removal efficiency values obtained for both systems were initially subjected to the Lilliefors test to assess the normality of the distribution. Performance data were further compared using the one-way analysis of variance (ANOVA) followed by Tukey's test at 5% significance.

2.5. Molecular analysis: sampling, sequencing and bioinformatics

16S rRNA gene amplicon sequencing was carried out to characterize the microbial community established in both reactors. Biomass sampling methodology included the removal of one PU foam strip from each reactor by

the end of the experimental run (phase V). The attached biomass was removed from the foam by successive washing with phosphate-buffered saline solution (PBS 1X: 130 mM NaCl; 7 mM Na_2HPO_4; 3 mM NaH_2PO_4; pH = 7.2). The samples were further centrifuged (8000 rpm at 4°C for 10 min) and stored at −20°C prior to analysis. DNA extraction was carried out according to the modified phenol–chloroform protocol described by [40], and the platform Illumina MiSeq 2 × 250 bp was used for next-generation sequencing. The 16S rRNA genes were amplified using the primer sets S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′), flanking the V3 and V4 hypervariable regions [41].

The MG-RAST pipeline (Version 3.3.7.3) performed the analysis of the 16S rRNA gene amplicon sequencing data, which included the quality filter, trimming, clustering and taxonomic assignment. Quality filter and trimming were performed according to [42], whereas the low-quality sequences were removed using the modified Dynamic Trim [43] by adopting default parameters. The remaining sequences were clustered in operational taxonomic units based on the similarity threshold of 97%. The representative sequence of each cluster was used to assign the taxonomy using RDP database. The parameters adopted were a maximum E-value of $1e^{-5}$, minimum percent identify cutoff of 60% and minimum alignment length cutoff of 15. The best hit classification was used to visualize the results.

Relevant differences in the taxonomic profiles between the two samples were identified using the Statistical Analysis of Metagenomic Profiles (STAMP) software v 2.1.3. The differences were assessed by the two-sided Fisher's Exact test, and Storey's FDR method was used for multiple test corrections as recommended by STAMP developers.

Sequences obtained in this study were submitted to the European Nucleotide Archive (http://www.ebi.ac.uk) under the project accession number of PRJEB21843.

3. Results and discussion

3.1. Anaerobic removal of phenol

The anaerobic removal of phenol from a carbohydrate-rich wastewater at strictly methanogenic (R1) and simultaneous methanogenic/sulfate-reducing (R2) conditions was assessed in continuous fixed-structured bed reactors at different phenol/COD ratios (Table 1). Characteristics of the COD removal patterns and metabolite production in both systems are presented in Figure 1a-c, whilst overall performance data are summarized in Table 2.

Regarding the overall performance of the two reactors, COD removal in R1 was higher than in R2 throughout the entire operating period (Figure 1a). This discrepant pattern most likely resulted from inhibitory effects associated with sulfide production in R2, with TDS concentrations ranging from 85 mg L^{-1} (minimum) to 345 mg L^{-1} (maximum) throughout the entire experimental run. In fact, sulfide may exert relevant toxic effects on the biomass [44] to such a degree that a wide range of TDS concentrations (100–800 mg L^{-1}) was reported to be potentially inhibitory in anaerobic reactors [45]. On the other hand, although TDS concentrations in phase V were higher (320 mg L^{-1}; Table 2), COD removal efficiencies in R2 reached similar levels as in R1 (94% and 97%, respectively; Table 2), which indicates the successfully acclimatization of the involved microorganisms to sulfidogenic conditions [44]. Further information on COD removal, sulfate reduction and biogas production rates during phases III to V may be found in [34].

Both reactors were continuously fed with CRSW containing approximately 120 mg L^{-1} of phenol (Table S1) (or 286 mg L^{-1} as COD, based on the stoichiometric ratio for the complete oxidation of hydroxybenzene, i.e. 2.38 g COD g^{-1}phenol) throughout the entire operating period, irrespective of the applied OLR (1.0–4.0 g COD L^{-1} day^{-1}). Figure 2 presents the temporal profiles for influent and effluent phenol concentrations in both reactors throughout the experimental run. Phenol removal under methanogenic conditions (R1) rapidly increased during phase I (Figure 2), reaching an average value of 67% (Table 2). During phase II, phenol removal levels markedly increased to 96% (Table 2) in R1, leading to further residual phenol concentrations below 1 mg L^{-1} from phase III onwards (Figure 2), i.e. ER_{PHENOL} over 99%.

Previous studies reported on the effective phenol biodegradation under anaerobic conditions via the carboxylation route of phenol to benzoate, which is further converted to acetate, bicarbonate and hydrogen (Reaction 1), and then to methane [23,46]. However, it is imperative to maintain adequate hydrogenotrophic and acetoclastic activities (Reactions 2 and 3, respectively) in such systems, in order to maintain the viability of the conversion of benzoate to acetic acid (Reaction 1), since this is a thermodynamically unfavorable reaction [23]. The high COD removal efficiencies (Figure 1a), as well as the minimal residual VFA concentrations (Figure 1b) suggested the establishment of an anaerobic consortium able to maintain low hydrogen partial pressures in R1. This characteristic most likely enabled the achievement of high phenol removal efficiencies (>99%) in a few days of operation, even during temporary increases in the applied phenol load during phases III and IV

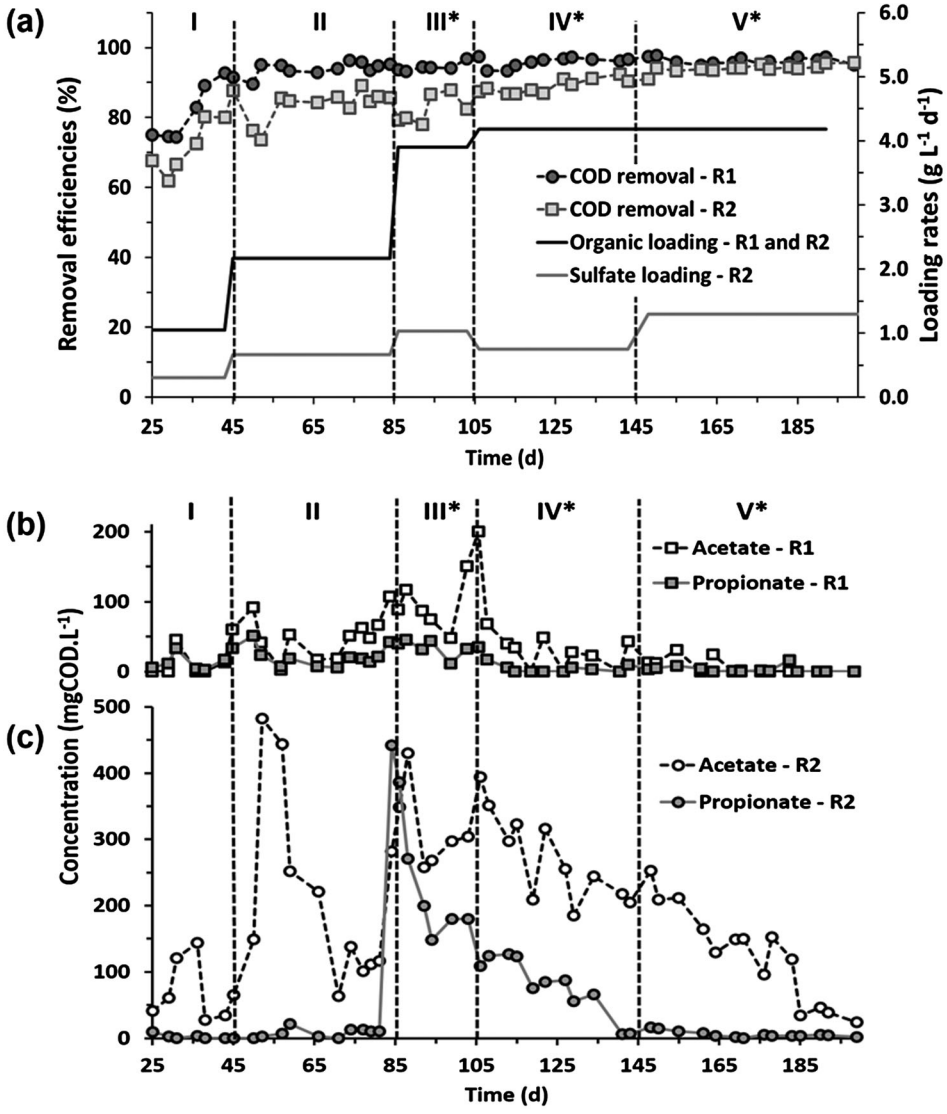

Figure 1. Overall performance of the reactors: COD removal efficiency and organic and sulfate loading rates (a); and monitoring of VFA concentrations in the effluent samples from R1 (b) and R2 (c). Error bars represent the standard deviation ($n > 10$ samples). (*) Data previously presented Godoi et al. [34].

(Figure 2). Unfortunately, the analytical methods used herein were not able to determine the phenol degradation intermediates in the anaerobically treated CRSW.

$$C_7H_5O_2^- + 6H_2O \rightarrow 3CH_3COO^- + 3H_2 + CO_2 + 2H^+ \quad (1)$$

$$\Delta G = +53.0\,\text{kJ mol}^{-1}$$

$$12H_2 + 3HCO_3^- + 3H^+ \rightarrow 3CH_4 + 9H_2O \quad (2)$$

$$\Delta G = -406.8\,\text{kJ mol}^{-1}$$

Table 2. Overall performance of the reactors according to COD, phenol and sulfate removal levels and total dissolved sulfide (TDS) production.

Response-variable	Reactors	Operating phase				
		I	II	III	IV	V
ER_{COD} (%)	R1	79 ± 9	94 ± 2	95 ± 1[a]	96 ± 2[a]	96 ± 2[a]
ER_{PHENOL} (%)		67 ± 16	96 ± 4	100 ± 1	100 ± 1	99 ± 1
ER_{COD} (%)	R2	73 ± 5	84 ± 5	83 ± 4[a]	89 ± 2[a]	94 ± 1[a]
ER_{PHENOL} (%)		39 ± 16	36 ± 15	76 ± 15	95 ± 2	97 ± 1
ER_{SO4} (%)		88 ± 1	79 ± 13	85 ± 5[a]	93 ± 5[a]	97 ± 3[a]
TDS (mg L^{-1})		96 ± 11	242 ± 22	195 ± 15[a]	190 ± 7[a]	320 ± 25[a]

[a]Data presented by Godoi et al. [34]. Abbreviations: ER_{COD} – COD removal efficiency, ER_{PHENOL} – phenol removal efficiency and ER_{SO4} – sulfate removal efficiency.

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \quad (3)$$

$$\Delta G = -135.6\,kJ\,mol^{-1}$$

The establishment of high phenol removal levels under simultaneous methanogenic/sulfidogenic conditions (R2) was much slower compared to R1 (Figure 2). In fact, phenol removal efficiency values obtained for both reactors were statistically different during phases I to III (p value < 0.05). These marked discrepant patterns contradicted the initial hypothesis of enhanced removal of aromatic compounds under sulfate-reducing conditions, given the better performance of R1. Reference studies report on the direct participation of SRB in the conversion of phenol [14] and its derivatives, such as benzoate [29]. Phenol may be directly degraded by some SRB (Reaction 4), whilst the conversion of benzoate in sulfidogenic environments may occur either through complete (Reaction 5) or incomplete oxidizing (Reaction 6) pathways [28].

$$2C_6H_5OH + 7SO_4^{2-} + 6H_2O + 2H^+ \rightarrow 12HCO_3^- + 7H_2S \quad (4)$$

$$C_6H_5COO^- + 3.75SO_4^{2-} + 4H_2O \rightarrow 7HCO_3^- + 3.75HS^- + 2.25H^+ \quad (5)$$

$$4C_6H_5COO^- + 3SO_4^{2-} + 16H_2O \rightarrow 12CH_3COO^- + 4HCO_3^- + 3HS^- + 9H^+ \quad (6)$$

Phenol removal in R2 reached 39% during phase I, further decreasing to 36% in phase II (Table 2 and Figure 2). This pattern is opposite to the COD removal, which increased from 73% (phase I) to 84% (phase II) (Table 2) after a temporary drop (Figure 1a). A marked acetate accumulation in R2 at the beginning of phase II (Figure 1c) showed the partial inhibition of the acetoclastic archaea metabolism most likely by the higher sulfide concentrations [47], which increased from 96 to 242 mg L^{-1} (as TDS) following the higher applied sulfate concentration (Table 1). In particular, this behavior may have hindered the metabolic pathways associated to the degradation of phenol via the benzoate pathway (Reaction 1), given the unfavorable thermodynamic character of this reaction. Previous studies have also indicated performance losses in terms of phenol biodegradation in methanogenic systems subjected to the accumulation of acetate [16]. In addition, toxic effects from sulfide may have also exerted inhibitory effects over phenol-degrading [48] and/or benzoate-degrading [31] microorganisms in R2, given the marked increase in TDS levels in phase II (Table 2).

The increase in ER_{PHENOL} (76%; Table 2) in R2 during phase III seems to be associated with the decrease in TDS levels (242 to 195 mg L^{-1}; Table 2), despite the relevant accumulation of VFAs (Figure 1c), which resulted primarily from the increase in the OLR. Moreover, despite the imbalanced conditions in R2 during phase III, VFA concentrations presented a decreasing pattern over time, indicating the gradual acclimatization of acetoclastic methanogenic microorganisms [44]. The increase in the COD/sulfate ratio (from 2.9 to 3.7; Table 1) also enabled a greater participation of methanogens in the overall organic matter conversion

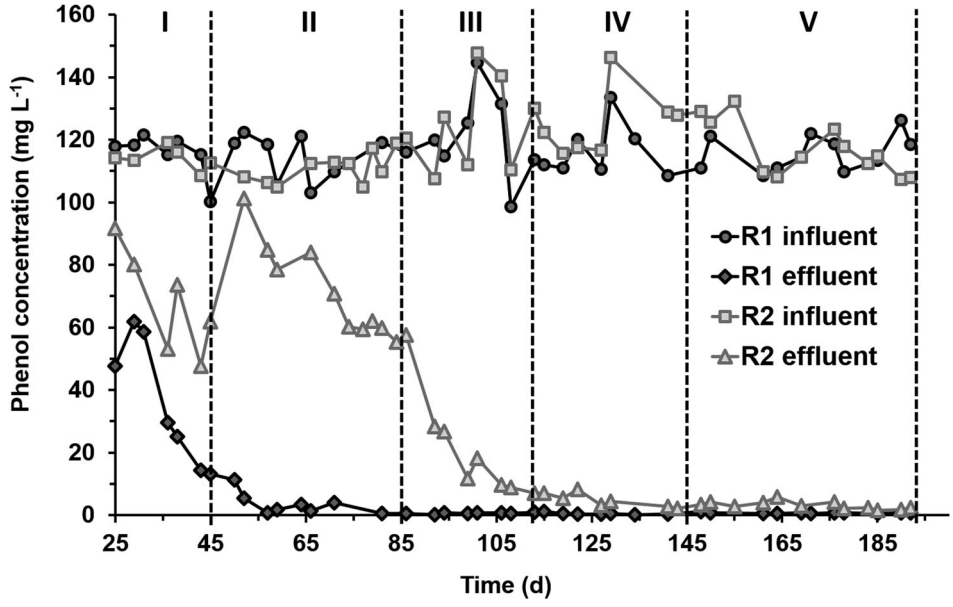

Figure 2. Temporal profiles of the influent and effluent phenol concentrations in both reactors throughout the entire operating period.

in R2 [34], which may have positively affected phenol biodegradation. Previous studies corroborate this hypothesis, as methanogenesis drives the degradation of benzoate in COD/sulfate ratios greater than 3 in sulfate-rich environments, whereas sulfate-reducing pathways are decisive only for COD/sulfate ratios less than 1.5 [31].

The further increase in the COD/sulfate ratio to approximately 5 (phase IV; Table 1) most likely enhanced phenol removal in R2 (Table 2 and Figure 2). In this case, ER_{PHENOL} reached 95% (Table 2), which was statistically similar to the removal efficiency obtained in R1 (p value > 0.05). Phenol removal in R2 during phase V (97%; Table 2) was also statistically equivalent to R1 (p value > 0.05), despite the increase in TDS levels (190 to 320 mg L^{-1}; Table 2) caused by the decrease in the COD/sulfate ratio (3.1; Table 1). These results suggest the phenol biodegradation potential of sulfidogenic systems operating under stable conditions, based on the attainment of equivalent performances compared to strictly methanogenic reactors.

Moreover, the low applied phenol/COD ratios, which simulate the usually diluted concentrations of phenols into the high organic content of sugarcane vinasse [27], could be stimulated the effective removal of this particular aromatic compound. In fact, the more readily degradable organic compounds (e.g. carbohydrates), which were present in the wastewater, may have been used as co-substrates [23,49], enhancing the phenol removal in both systems and avoiding negative effects over the biomass [50].

The results confirm the suitability of removing aromatic compounds from complex wastewaters, such as vinasse, directly in the anaerobic treatment stage, without impairing the overall efficiency of the process, as long as the reactor is operated under stable conditions. Moreover, energy-intensive oxidation pre-treatment processes, such as ozonation, may be efficiently replaced, without additional costs.

3.2. Biomass characterization

The microbial composition of the two reactors treating CRSW under strictly methanogenic (R1) and simultaneous methanogenic/sulfidogenic conditions (R2) was analyzed by Illumina MiSeq sequencing. The number of sequences generated were 237,345 (R1) and 244,283 (R2), grouped into 1188 and 1036 OTUs for R1 and R2 samples, respectively. The sequences obtained were compared with the microbial composition of the inoculum (235,800 sequences grouped into 841 OTUs), previously reported [51]. The characterization of the microbial structure of the reactors at the genus level is depicted in Figure 3a-c. The extended error bar plots (Figure 3) show the statistically significant differences in the taxonomic profiles of the biomass from the two reactors and the inoculum. Relative abundance values indicated the predominance of the *Desulfonauticus* genus (9.3%; Figure 3a) in R2, suggesting that this particular genus was the primary microbial group conducting the dissimilatory sulfate reduction in this system.

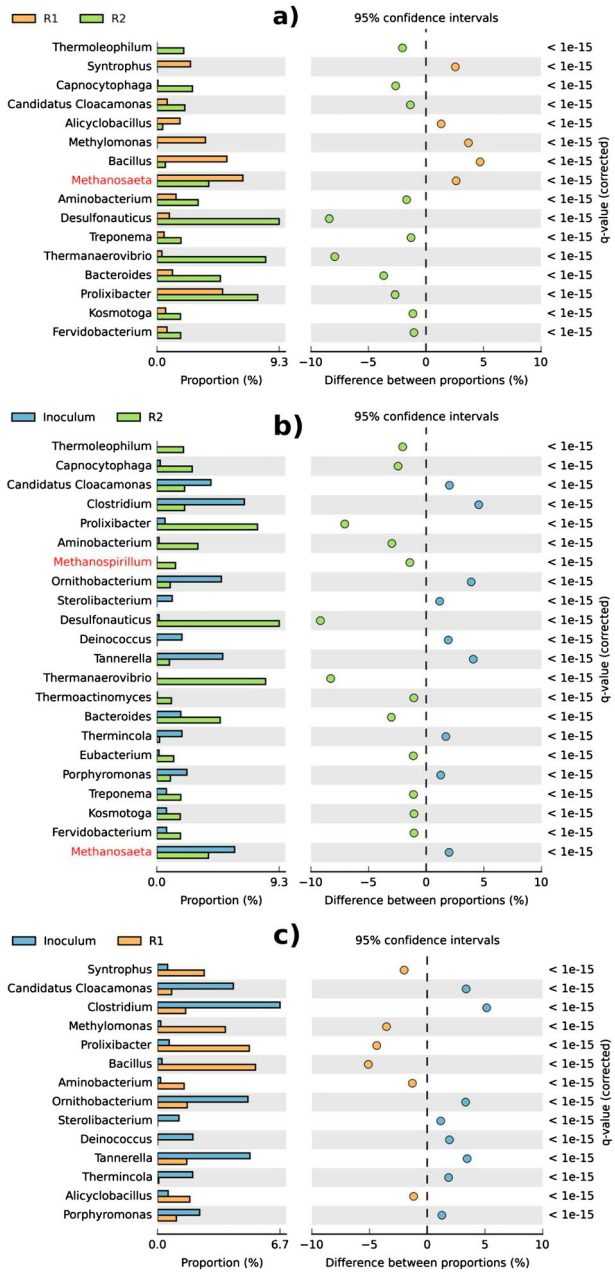

Figure 3. Extended error bar plot identifying significant differences between mean proportions of genera comparing Inoculum (blue), R1 (orange) and R2 (green) samples. The differences were assessed by the two-sided Fisher's Exact test and Storey's FDR method was used to multiple test corrections using the STAMP software. Corrected p values are shown on the right and genera belonging to the Archaea domain are highlighted in red.

Species belonging to *Desulfonauticus* genus, such as *D. submarinus*, use hydrogen as an electron donor in the reduction of sulfate, sulfite, thiosulfate or elemental sulfur [52]. Organisms belonging to the *Thermanaerovibrio* genus (8.3%; Figure 3a) were also identified in R2, and were characterized by their ability to convert several organic substrates into hydrogen. The growth of this particular group is enhanced in the co-cultivation with hydrogen scavengers [53], such as SRB.

The *Methanosaeta* genus was the most representative group of the Archaea domain found in both systems (Figure 3a). Individuals belonging to this genus (e.g. *M. concilli*) use acetate as their sole energy and carbon source [54]. Relative abundance values for the *Methanosaeta* genus were 6.6% and 3.9% in R1 and R2, respectively (Figure 3a), indicating that sulfidogenic conditions were more restrictive for the establishment of these organisms. Higher relative abundances were also observed for the *Prolixibacter* genus in both systems compared to inoculum (5.0% and 7.7% in R1 and R2, respectively; Figure 3a-c). The high influent sucrose concentrations (560–2700 mg L^{-1}) most likely favored the establishment of this group, which is characterized by facultative anaerobes able to ferment sugars [55].

Specific genera, namely, *Methylomonas, Syntrophus* and *Bacillus* (Figure 3a) were identified at higher relative abundances in R1 compared to R2. The *Methylomonas* genus is formed by aerobic methylotrophic organisms, which are able to survive under limited oxygen availability [56]. The more reduced conditions resulting from the presence of dissolved sulfide most likely hindered the establishment of this particular microbial group in R2. In turn, individuals from the *Syntrophus* genus, such as *S. acidotrophicus*, are characterized as strict anaerobes able to degrade benzoate and VFAs in syntrophy with hydrogenotrophic organisms [57]. This finding suggests the biodegradation of phenol via benzoate, as previously discussed (Section 3.1). The higher relative abundance observed for the *Syntrophus* genus in R1 than in R2 (2.6% *vs.* 0.001%; Figure 3a) most likely resulted from the prompt maintenance of low hydrogen partial pressures in the strictly methanogenic reactor, based on the rapid establishment of stable operating conditions as shown by the COD (Figure 1a) and phenol (Figure 2) removal profiles. Microorganisms associated with the *Bacillus* genus are also able to degrade phenol (but no benzoate), as well as molecular hydrogen and acetate [58]. This group may have also effectively participated in phenol degradation in R1, only marginally contributing to this process in the methanogenic/sulfidogenic reactor, as shown by its lower relative abundance in R2 (Figure 3a). Individuals

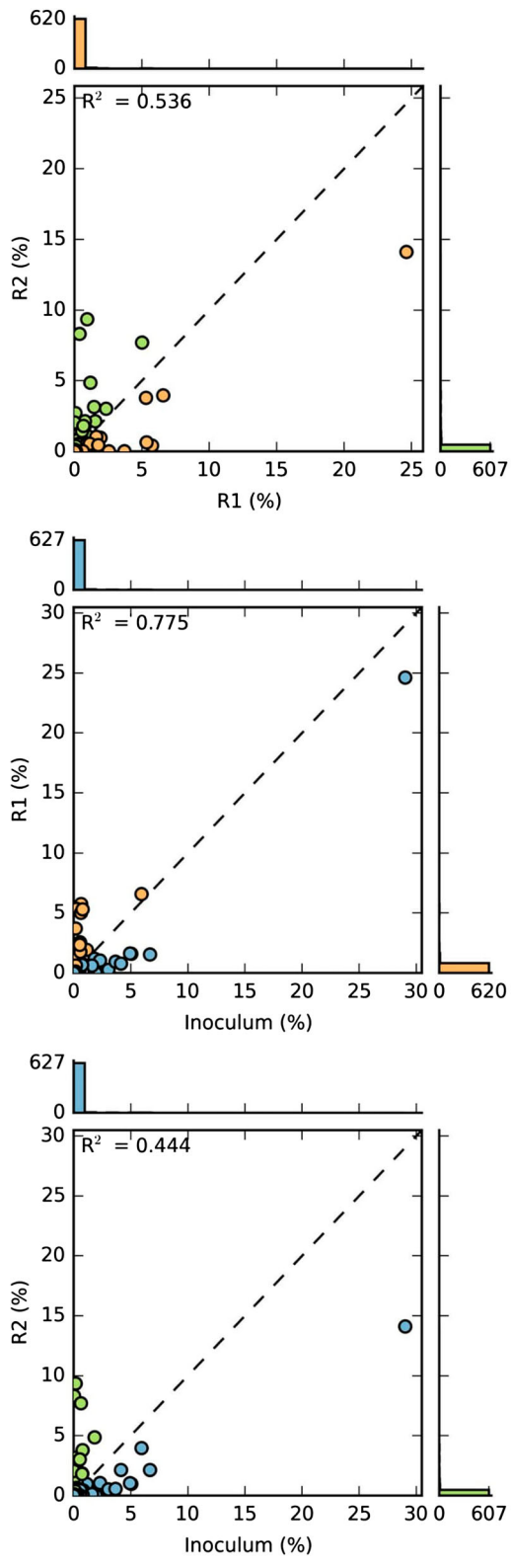

Figure 4. Scatter plot with histograms showing correlation in the taxonomic profiles among Inoculum, R1 and R2 samples at genus level using RDP database ($p < 0.05$).

belonging to the *Clostridium* genus (1.2% and 2.0% in R1 and R2, respectively; Figure 3b-c) have also been previously related to the fermentation and degradation of

aromatic compounds [59,60], potentially contributing to phenol degradation in both systems.

Scatter plot correlation graphs (Figure 4a-c) showed a low correlation between the microbial community established in R2 with the inoculum ($R^2 = 0.444$). This result was attributed to the application of sulfate-reducing conditions in R2, which stimulated the establishment of microorganisms related to the sulfur cycle (primarily the *Desulfonauticus* genus; Figure 3a). A higher correlation was observed between the microbial community from R1 and the inoculum ($R^2 = 0.775$), given the maintenance of predominant methanogenic conditions. However, important differences were still observed in the microbial community of both samples (R1 and inoculum), based on the predominance of specific genera in the inoculum (e.g. *Candidatus Cloacomonas, Clostridium, Ornithobacterium, Tannerela*, among others; Figure 3b), which presented lower relative abundances in R1. This pattern, in association with the intermediate correlation between R1 and R2 ($R^2 = 0.536$), resulted from the change in the nutritional composition of the substrates, i.e. poultry slaughterhouse wastewater (inoculum) and carbohydrate-rich wastewater (R1 and R2), which favored the establishment of genera related to carbohydrate fermentation, phenol degradation, and sulfate reduction (specifically in R2) in the bench-scale systems. The aforementioned correlations were further confirmed by analyzing the proportional Venn diagram (Figure S2; supplementary data section), which also indicated a closer similarity between the microbial populations established in R1 and the inoculum.

4. Conclusions

The anaerobic biodegradation of phenol from synthetic wastewaters simulating the soluble fraction of sugarcane vinasse was successfully achieved under methanogenic (R1) and simultaneous methanogenic/sulfidogenic (R2) conditions, confirming the potential for eliminating energy-intensive oxidative steps prior to the anaerobic process. High phenol removal efficiencies (>99%) were rapidly achieved under strictly methanogenic conditions (≈55 days). In turn, the application of sulfidogenic conditions required a longer biomass adaptation period (≈125 days) due to the dissolved sulfide concentrations, also leading to high phenol removal levels (97%). Molecular characterization of microbial populations showed that acetoclastic methanogens (*Methanosaeta*) were the major Archaea genera in the both reactors, although sulfidogenic conditions were apparently more restrictive for the establishment of these organisms. Dissimilatory sulfate-reduction in R2 was primarily performed by *Desulfonauticus* genus, which was most likely the main group maintaining low hydrogen partial pressures in the system. Phenol-degrading genera were also identified in both systems, including *Syntrophus* (R1), *Bacillus* (R1) and *Clostridium* (R1 and R2).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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