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DOI

[10.1016/j.cej.2022.140305](https://doi.org/10.1016/j.cej.2022.140305)

Publication date

2022

Document Version

Final published version

Published in

Chemical Engineering Journal

Citation (APA)

García Rea, V. S., Muñoz Sierra, J. D., El-Kalliny, A. S., Cerqueda-García, D., Lindeboom, R. E. F., Spanjers, H., & van Lier, J. B. (2022). Syntrophic acetate oxidation having a key role in thermophilic phenol conversion in anaerobic membrane bioreactor under saline conditions. *Chemical Engineering Journal*, 455, Article 140305. <https://doi.org/10.1016/j.cej.2022.140305>

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Syntrophic acetate oxidation having a key role in thermophilic phenol conversion in anaerobic membrane bioreactor under saline conditions

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ARTICLE INFO

Keywords:

Thermophilic
AnMBR
Phenol
SAO
Saline
Hydrogenotrophic

ABSTRACT

Phenol conversion under saline thermophilic anaerobic conditions requires the development and sustenance of a highly specialized microbial community. In the present research, an anaerobic membrane bioreactor (AnMBR) fed with an influent containing 0.5 g·L⁻¹ phenol and 6.5 gNa⁺·L⁻¹ was operated at 55 °C for 300 days. Phenol degradation was limited when phenol was the sole substrate. However, the phenol removal efficiency significantly ($p < 0.001$) increased to 80 % corresponding to a conversion rate of 29 mgPhenol·gVSS⁻¹·d⁻¹ when acetate (0.5 gCOD·L⁻¹) was simultaneously provided. Isotopic analysis using 1-¹³C labeled acetate and measuring ¹³CH₄ revealed that acetate was first oxidized to hydrogen and CO₂, prior to methanogenesis, resulting in an increased abundance of hydrogenotrophic methanogens. It is hypothesized that the latter is of crucial importance for achieving effective anaerobic oxidation of phenol and its metabolites. Remarkably, the phenol conversion rate in the membrane-associated biomass was three times higher than in the suspended biomass. The observed difference in the conversion rate could be explained by the presence of an increased abundance of hydrogenotrophic methanogens in the membrane-associated biomass confirmed by a microbial community analysis of Archaea. Benzoate was measured in the permeate suggesting that phenol degradation occurred via the benzoyl-CoA pathway. The results of the current study suggest that syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis, which results in the presence of an abundant electron sink, plays a key role in enhancing thermophilic phenol degradation. The obtained insights widen the application of anaerobic digestion to treat saline phenolic-rich wastewater at high temperatures.

Abbreviations: AD, Anaerobic digestion; AnMBR, Anaerobic membrane bioreactor; ANOVA, Analysis of variance; CES, Carbon and energy source; CGWW, Coal gasification wastewater; COD, Chemical oxygen demand; DNA, Deoxyribonucleic acid; ESEM, Environmental scanning electron microscope; GC, Gas chromatography; HM, Hydrogenotrophic methanogenesis; AFFT, Multiple alignment using fast Fourier transform; PCoA, Principal coordinate analysis; PH₂, Hydrogen partial pressure; PVDF, Polyvinylidene fluoride; qPCR, Real-time polymerase chain reaction; SAO, Syntrophic acetate oxidation; SAOB, Syntrophic acetate-oxidizing bacteria; sPhCR, Specific phenol conversion rate; sPhLR, Specific phenol loading rate; UASB, Upflow anaerobic sludge blanket reactor; rRNA, Ribosomal ribonucleic acid; PDB, Pee Dee Belemnite; VFA, Volatile fatty acids; ΔG_R⁰, Standard Gibbs free energy change; ΔG_R⁰¹, Gibbs free energy change under standard conditions but pH = 7; ΔG_R^{1,T}, Gibbs free energy change with corrections for temperature and concentrations/partial pressures; δ¹³C_{PDB}(CH₄)13C, delta in methane; γ, Degree of reduction.

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<https://doi.org/10.1016/j.cej.2022.140305>

Received 5 August 2022; Received in revised form 22 October 2022; Accepted 8 November 2022

Available online 12 November 2022

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1. Introduction

With the increasing focus on energy recovery from wastewater, thermophilic anaerobic digestion (AD) is of increasing industrial interest. The avoidance of cooling before and re-heating after biological treatment could lead to large energy savings when closing industrial water loops is targeted [1,2]. In addition, thermophilic AD offers several advantages over mesophilic digestion such as higher hydrolysis rates, better liquid-solids separation, improved mixing, and a higher decrease in pathogenic microorganisms [3]. However, several constraints such as poor effluent quality and the carry-over of active biomass, which tends to destabilize the process, have narrowed the potential for industrial application so far [4,5]. Besides, biomass retention or immobilization under thermophilic conditions is challenging to achieve, which may be aggravated by saline conditions [6,7].

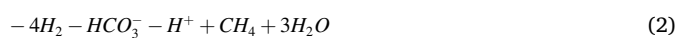
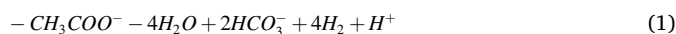
The vast majority of anaerobic industrial wastewater treatment systems are based on sludge bed technology, making use of granular sludge [8]. However, the treatment of wastewater with toxic or inhibitory compounds under prevailing saline thermophilic conditions will likely result in increased biomass decay rates which will constrain biomass granulation even more. Under such extreme conditions, the washout of viable microorganisms is likely to occur, ultimately leading to poor reactor performance [7]. Anaerobic membrane bioreactors, (AnMBR) have been proposed to overcome these problems [5,9,10] as the filtration process ensures that all the biomass, including specialized microbial groups, is retained inside the reactor.

Coal gasification wastewater (CGWW) is an example of a petrochemical industrial effluent typically characterized by temperatures > 45 °C, total dissolved solids concentrations ranging between 0.5 and 2.5 g·L⁻¹, phenol and aromatic compounds in concentrations ranging from 80 to 20,000 mg·L⁻¹, carboxylic acids from 200 to 600 mg·L⁻¹, pH values from 6.5 to 11.5, and alkalinity between 220 and 17,000 mg·L⁻¹ [11–16]. Because these streams are produced in large flows on a global scale, e.g. 1 ton of coke production generates approximately 1 m³ of coke oven wastewater [14], anaerobic conversion of phenolics at the prevalent process temperature would offer substantial energy recovery potential, while presenting a cost-effective solution for treating the wastewater and possibly providing reclaimed process water for reuse.

Research on the anaerobic degradation of phenol under thermophilic conditions is only scarcely documented [17–21] as most of the fundamental insights were obtained under mesophilic (nitrate-reducing) conditions [22–24]. For example, the benzoyl-CoA route has been described as the main degradation pathway in the anaerobic conversion of phenol into methane (Supplementary Material S1, Fig. S1 A) [25–27]. Under thermophilic conditions, however, an alternative degradation pathway via caproate as the main intermediate was proposed (Supplementary Material S2, Fig. S1 B) [20,28].

Previous research showed the feasibility of phenol degradation under saline and thermophilic conditions [29]. Nevertheless, there is a lack of understanding of the process, which is needed for guaranteeing process stability. More insights are required into the maximum attainable conversion rates, the thermophilic phenol degradation pathway, and the specialized microbial groups needed for the stable conversion of phenol. Recently, we showed that in AnMBRs under mesophilic and saline conditions, phenol degradation is enhanced by the dosage of additional carbon and energy sources (CES) such as acetate and butyrate. Both additional CES promoted the development of an enriched acetoclastic methanogenic population which attained maximum specific phenol conversion rates of 200 mgPh·gVSS⁻¹d⁻¹ [30]. Under thermophilic conditions, however, this dependency on acetoclastic methanogenesis would be remarkable, since at high temperatures syntrophic acetate oxidation (SAO) is more pronounced [3,4,31]. SAO is performed by syntrophic acetate-oxidizing bacteria (SAOB) that firstly oxidize acetate into HCO₃⁻, H₂, and H⁺ (Eq. (1)), followed by hydrogenotrophic methanogenesis (Eq. (2)), [31]. Under very extreme conditions, SAO might even be the sole pathway in acetate conversion, for example,

when temperatures higher than 75 °C are applied [4].



In our present work, we investigated the simultaneous conversion of phenol and acetate under saline (6.5 g Na⁺·L⁻¹) and thermophilic (55 °C) conditions using an AnMBR. By applying membrane filtration, complete biomass retention is achieved, which promoted the development of the specialized microbial community needed for the process. The degradation pathway of acetate was determined by a ¹³C isotopic analysis and insight into the phenol degradation route was obtained by the measurement of different intermediates. The phenol removal efficiency and conversion rates of the suspended and membrane-associated biomass were determined. Analyses of the microbial community for getting insight into the role of acetate as an additional substrate were conducted and related to the bioconversion. Additionally, a thermodynamic and stoichiometric analysis of a strict anaerobic microorganism growing on phenol was performed. The thermodynamics and stoichiometry analysis allowed to show and understand the syntrophic association for phenol degradation expected in the reactor's biomass and its effects on the thermodynamic feasibility of the degradation process.

2. Materials and methods

2.1. Anaerobic membrane bioreactor

The setup consisted of an AnMBR (7.0 L total volume, 6.5 L working volume) connected to a 130 mL external ultrafiltration module with a tubular inside-out PVDF membrane (X-Flow compact 33, Pentair, The Netherlands) as previously reported [30,32]. The membrane module was operated with a cross-flow velocity of 0.8 ± 0.1 m·s⁻¹ and a constant flux of 4.0 LMH (Q = 1 L/d). The inoculum biomass was obtained from a full-scale UASB reactor treating petrochemical wastewater (Shell Moerdijk, The Netherlands), and it was acclimated and used in previous experiments [29]. The reactor was mixed thoroughly by biomass recirculation at a rate of 200 d⁻¹. During the reactor operation, the VSS concentration was kept at 1.90 ± 0.28 gVSS·L⁻¹, so the specific phenol loading rate was attained at a lower phenol concentration in the influent. Membrane filtration was operated with a cycle of 500 s of filtration, 20 s of backwashing (same flow as filtration), and 5 s idle.

2.2. Feeding solution during the different reactor operation stages

For the experiment, the reactor operation was divided into four main stages (Table 1). Micronutrients [18 mL·L⁻¹] & macronutrients [9 mL·L⁻¹] solutions, and phosphate buffer solutions A [13.0 mL·L⁻¹] and B [19.9 mL·L⁻¹], respectively, were added to the feeding solution. The composition of the solutions is reported in [30]. NaCl in the feed was adjusted during the operation stages to maintain the sodium concentration in the reactor at 6.5 g·L⁻¹.

2.3. Chemical and physicochemical analysis

COD and phenol concentrations were determined as previously reported [30]. An extended-run-time protocol (23 min) was applied for the

Table 1
Feeding composition during the anaerobic membrane bioreactor operation.

Stages	Operation day	Yeast [g·L ⁻¹] extract	Phenol [g·L ⁻¹ /gCOD·L ⁻¹]	Acetate [gCOD·L ⁻¹]
A	0–45	1.2	0.5/1.2	0
B	46–82	1.0, 0.5, 0.4	0.5/1.2	0
C	83–241	0.4	0.5/1.2	0.7
D	242–344	0.4	0.5/1.2	1.4

determination of VFA, ethanol, propanol, butanol, cyclohexanol, cyclohexanone, and benzoate as reported in [32].

2.4. Syntrophic acetate oxidation activity in the AnMBR biomass

For determining the SAO activity of the AnMBR biomass, a test was designed by modifying an existing protocol [33]. On day 304 of reactor operation, 1.6 % (w/w) of the total acetate in the feed solution was dosed as $1\text{-}^{13}\text{C}$ -labeled sodium acetate ($^{12}\text{CH}_3^{13}\text{COO}^-$) (Merck, Germany). The acetate isotope was dosed for twenty days. During this period, biogas samples of approximately 24 mL were collected from the reactor's gas line and stored in 2 (12 mL) Labco exetainer vials (Labco, Germany). Samples were outsourced for analysis (Isolab, The Netherlands). The carbon isotope ratio of methane was analyzed with an Agilent 6890 N GC (Agilent Technologies, Santa Clara, US) interfaced to a Finigan Delta S IRMS (Thermo Scientific, Bremen, Germany) using a Finigan GC-C II interface. The GC was equipped with a 12 m, 0.32 mm molsieve column (Agilent) and an injection valve. Samples were calibrated against a calibration standard. Results are reported in promille vs Pee Dee Belemnite (PDB) [34].

2.5. DNA extraction and microbial community dynamics study

Biomass from the reactor was sampled during the various operational stages. At the end of the reactor operation, the membrane module was sectioned and additional samples from the upper, middle, and lower parts of the membrane biofilm were taken. DNA extraction and storage were performed as previously reported [30].

2.6. Targeted amplicon libraries construction

Library construction and sequencing were performed at the Roy J. Carver Biotechnology Center, the University of Illinois at Urbana-Champaign. Approximately 1 ng of DNA was used for amplification with the bacterial 16 s V3-F357-V4-R805 and archaeal 349F-806R primers in the Fluidigm Access Array. The barcoded amplicons generated from each sample were harvested, transferred to a 96 well plate, quantified on a Qubit fluorometer, and the average size of the amplicons was determined on a Fragment Analyzer (AATI, IA). All amplicons were pooled in equimolar concentration, size selected on a 2 % agarose Ex-gel (ThermoFisher) to remove primer dimers and extracted from the isolated gel slice with a Qiagen gel extraction kit (Qiagen, Hilden, Germany). The cleaned size selected product was quantitated and run on a Fragment Analyzer again to confirm appropriate profile and for determination of average size. The pool was diluted to 5 nM and further quantitated by qPCR on a CFX Connect Real-Time qPCR system (Biorad, Hercules, CA) for maximization of the number of clusters in the flow cell.

2.7. DNA sequencing

The pool was denatured and spiked with 20 % non-indexed PhiX V3 control library provided by Illumina and loaded onto the MiSeq V2 flow cell at a concentration of 8 pM for cluster formation and sequencing. The libraries were sequenced from both ends of the molecules to a total read length of 250nt from each end. The run generated.bcl files which were converted into demultiplexed fastq files using bcl2fastq 2.20 (Illumina, CA).

2.8. Statistics and bioinformatics for the analysis of sequences

Statistical analyses (One way ANOVA, planned contrasts, Kruskal-Wallis test, and Wilcoxon robust ANOVA) were conducted in R [35].

The paired-end reads (2×250) were processed as detailed in [30]. An alignment was performed with the MAFFT algorithm. After masking positional conservations and gap filtering, a phylogeny was built with the FastTree algorithm. The feature table and phylogeny were exported

to the R environment, and the statistical analyses were performed with the phyloseq, ggplot2, and vegan packages [36]. A principal coordinate analysis (PCoA) was conducted with the weighted unifrac distance. The sequences were deposited in the SRA (NCBI) database under the accession number PRJNA671743.

2.9. Stoichiometry and thermodynamics of anaerobic growth on phenol under thermophilic conditions

The redox reactions used for the stoichiometric and thermodynamic analysis of anaerobic growth on phenol under thermophilic conditions were calculated according to Kleerebezem and van Loosdrecht (2010) [37]. The Gibbs energy dissipation method was used for the calculation of the Gibbs free energy change of the reactions (ΔG_R) corrected for the expected products and reagents concentrations and process temperature ($\Delta G_R^{1,55^\circ\text{C}}$) [37]. Through the coupling of the catabolism and anabolism, the overall metabolic equations of anaerobic growth on phenol were derived and syntrophic relationships visualized. To perform the required calculations the following foreseen concentrations and partial pressures expected in the AnMBR were used: phenol 0.53 mmol (50 mgPh·L⁻¹), acetate 0.017 mmol (1.0 mg·L⁻¹), carbon dioxide 0.4 atm, hydrogen 1×10^{-4} atm, ammonium 1 mmol (18 mg·L⁻¹), and H^+ 1×10^{-7} M.

3. Results and discussion

3.1. AnMBR operation for phenol degradation and its simultaneous conversion with acetate

An AnMBR under saline thermophilic conditions was operated for 344 days to measure phenol degradation as the sole COD source and the simultaneous degradation of phenol with acetate in a reactor that ensures full biomass retention.

During stage A, phenol and yeast extract were the main COD sources. During the first 20 days, the phenol removal efficiency decreased to a minimum of 38.2 %, which corresponded to a specific phenol conversion rate (sPhCR) of 11 mgPh·gVSS⁻¹d⁻¹ (day 19) and a phenol concentration in the permeate of 293 mgPh·L⁻¹ (Fig. 1). However, phenol degradation was recovered on day 38. The average (± 95 % confidence interval) phenol removal efficiency and sPhCR during stage A were 58.9 ± 4.4 % and 26.8 ± 2.6 mgPh·gVSS⁻¹d⁻¹, respectively. Previous studies reported that yeast extract promotes the degradation of phenol [26] and phenolic compounds under anaerobic conditions [38,39]. Yeast extract is a source of COD, as well as macro- and micronutrients, vitamins, and amino acids, which are considered essential for anaerobic bacteria and archaea [40]. The exact mechanism of how yeast extract may promote phenol degradation is, however, completely unknown.

At the start of stage B, the yeast extract concentration in the feeding solution was decreased from 1.2 to 0.4 gCOD/L, which resulted in a strong decrease in the phenol removal efficiency and sPhCR. Concomitantly, the phenol concentration in the permeate increased to a maximum concentration of 310 mgPh·L⁻¹ on day 82, corresponding to a removal efficiency of 38 %. The average phenol removal efficiency and sPhCR during stage B were 55.2 ± 4.8 % and 23.4 ± 2.0 mgPh·gVSS⁻¹d⁻¹.

To study the simultaneous conversion of phenol and acetate, on day 83 (beginning of Stage C, Table 1) 30.4 % of the influent COD [0.7 gCOD/L] was provided by the acetate. Phenol comprised the other 52.2 % of the influent COD and yeast extract the remaining 17.4 %. As a result, the phenol removal efficiency and sPhCR increased, reaching an average of 65.0 ± 2.4 % and 29.2 ± 1.2 mgPh·gVSS⁻¹d⁻¹, respectively. In the last stage (D), when phenol COD was 38.0 % of the influent COD, the removal efficiency and sPhCR remained stable, with an average of 65.9 ± 1.3 % and 29.2 ± 1.2 mgPh·gVSS⁻¹d⁻¹, respectively. Based on a Kruskal-Wallis test, we determined that the phenol removal efficiency was significantly different when acetate was dosed $H(3) = 21.9$, $p < 0.001$. Focused comparisons of the mean ranks showed that phenol

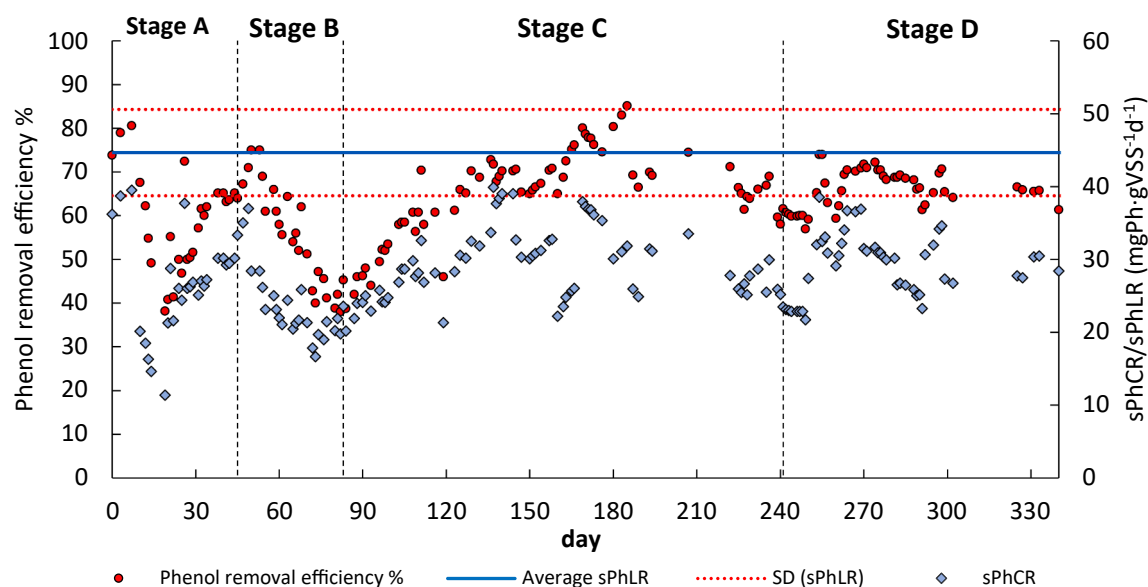


Fig. 1. Phenol removal efficiency, specific phenol loading rate (sPhLR), and specific phenol conversion rate (sPhCR) of the AnMBR treating model phenolic and high salinity wastewater under thermophilic conditions. The red dotted lines show the interval for the standard deviation of the sPhLR. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

removal efficiency was not significantly different ($p < 0.05$) between stages A and B (difference = 11), nor between stages C and D (difference = 0.5). Nonetheless, among the stages with (C & D) and without acetate (B), there were significant differences. A one-way ANOVA analysis showed that there was a significant effect of the dosage of acetate on the sPhCR as well, $F(3, 167) = 9.86$, $p < 0.001$, indicating that with the dosage of acetate the sPhCR was different. After the ANOVA, a comparison between the stages by planned contrasts was made, and we determined that the sPhCR with acetate was significantly higher $t(167) = -4.9$, $p < 0.001$ (one-tailed) than the operation without acetate. The phenol loading rate [$\text{mgPh} \cdot \text{gVSS}^{-1} \cdot \text{d}^{-1}$] during the reactor operation was maintained at $44.7 \pm 0.9 \text{ mgPh} \cdot \text{gVSS}^{-1} \cdot \text{d}^{-1}$. A Wilcoxon robust ANOVA test showed that there was no significant difference in the mean sPhLR values across the four stages $F_t(52, 27) = 1.7$, $p = 0.17$.

The results obtained during the continuous operation suggested that the biomass of the AnMBR was not able to degrade phenol under saline thermophilic conditions when phenol was the sole COD source. Limited phenol degradation could be attributed to a reduced presence of a phenol-degrading microbial population or an inhibitory effect on such population. An inhibitory process due to phenol concentration will be very unlikely as it has been shown that inhibition is expected at higher concentrations ($>600 \text{ mgPh} \cdot \text{L}^{-1}$) than those prevailing in the reactor [29,30]. Product accumulation or reduced product consumption may affect as well, for example, in the case of the intermediates acetate and/or H_2 . Another possibility for limited phenol degradation is an impaired or decreased methanogenic population. Such a population will be insufficiently capable of scavenging the reducing equivalents generated during phenol degradation steps, implying a cessation of the AD biochemical steps. Under the prevailing reactor conditions, SAO may be the major acetate degradation pathway, whereas hydrogenotrophic methanogenesis could be the main methane generation process. An impairment in the SAO activity might then lead to substrate shortage for the hydrogenotrophic methanogens. On the other hand, cessation of hydrogenotrophic methanogenesis will halt SAO and phenol degradation as the electrons generated in both processes will have no way to be disposed of. The addition of acetate [$0.7 \text{ gCOD} \cdot \text{L}^{-1}$ in the feed] significantly enhanced the sPhCR and phenol removal efficiency. Albeit, doubling the concentration of acetate [$1.4 \text{ gCOD} \cdot \text{L}^{-1}$ in the feed] did neither increase the removal efficiency nor the sPhCR.

Fang *et al.* (2006) reported thermophilic degradation of phenol as the

sole COD source under thermophilic methanogenic conditions in a UASB reactor (2.8 L) operating at a volumetric phenol loading rate of $38 \text{ mgPh} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$ [20]. Nonetheless, neither the specific conversion rate nor the VSS concentration were reported. Sreekanth *et al.* 2009, reported the degradation of four different phenolic compounds in UASB reactors (7 L) under thermophilic conditions using glucose [$1.07 \text{ gCOD} \cdot \text{L}^{-1}$] in the feed as an additional COD source, but the mechanism by which glucose increased the degradation was not explained [41]. In previous studies, the effect of a temperature shift from 35°C to 55°C on phenol degradation in an AnMBR at $16 \text{ gNa}^+ \cdot \text{L}^{-1}$ was researched. Acetate [$19.5 \text{ gCOD} \cdot \text{L}^{-1}$] was provided in the feed as an additional COD source and an sPhCR of $1.7 \text{ mgPh} \cdot \text{gVSS}^{-1} \cdot \text{d}^{-1}$ was determined [42]. In addition, in a long-term study (338 days), we examined the phenol conversion at 55°C and $18 \text{ gNa}^+ \cdot \text{L}^{-1}$ with acetate ($10 - 20 \text{ g} \cdot \text{L}^{-1}$) as an additional COD source, and we found an sPhCR of $21 \text{ mgPh} \cdot \text{gVSS}^{-1} \cdot \text{d}^{-1}$ [29]. Although, neither the effect of acetate nor the mechanism in which it affected the process were elucidated. Remarkably, the sPhCR of $29.2 \pm 1.2 \text{ mgPh} \cdot \text{gVSS}^{-1} \cdot \text{d}^{-1}$ that we found in our present study is the highest reported under saline thermophilic conditions. It should be noted that in previous research, experiments were conducted at Na^+ concentrations of $16 - 18 \text{ g} \cdot \text{L}^{-1}$ [29,42]. Such Na^+ concentrations in combination with high temperature could have resulted in the observed low phenol conversion rates despite the presence of acetate [29]. The maximum sPhCR found in our present research, however, is almost 7 times lower than the one reported for AnMBR biomass under saline ($8.0 \text{ g} \cdot \text{Na}^+ \cdot \text{L}^{-1}$) and mesophilic (35°C) conditions [30].

Previously, it was hypothesized that an abundant and active acetoclastic methanogenic sub-population is a prerequisite for effective anaerobic phenol degradation in an AnMBR under mesophilic and saline conditions [30]. However, very likely, under thermophilic and saline conditions, the presence of SAO coupled to hydrogenotrophic methanogenesis will be more pronounced, while acetoclastic methanogenesis may play a less important role.

3.2. Syntrophic acetate oxidation determination by ^{13}C isotope labeling in the continuous operation

To assess whether the acetate was mainly converted via acetoclastic methanogenesis or SAO in the reactor, the $\delta^{13}\text{C}_{\text{PDB}}(\text{CH}_4)$ in the biogas of the AnMBR was determined. The symbol δ expresses the abundance of

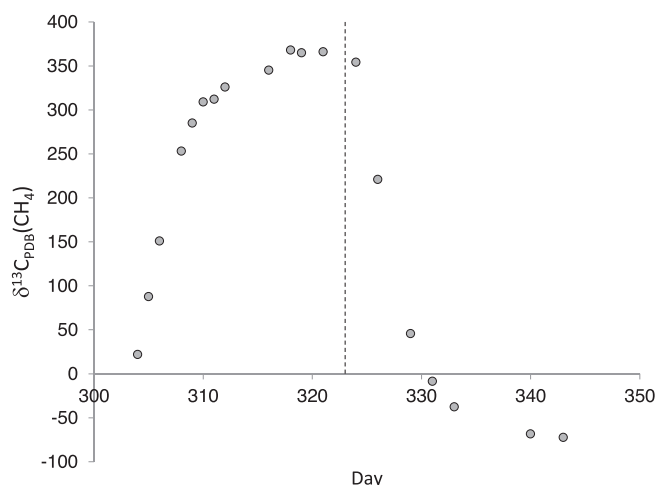


Fig. 2. $\delta^{13}\text{C}_{\text{PDB}}(\text{CH}_4)$ in the methane of the AnMBR. After the dosage of ^{13}C -1 labeled acetate, there was an increase in the $\delta^{13}\text{C}_{\text{PDB}}(\text{CH}_4)$, which indicates that methane was enriched with the ^{13}C isotope. Such isotope enrichment suggests syntrophic acetate oxidation coupled to hydrogenotrophic activity. Once the dosage of the ^{13}C -1 labeled acetate stopped, the $\delta^{13}\text{C}_{\text{PDB}}(\text{CH}_4)$ decreased indicating, therefore, a decrease in the ^{13}C isotope content in methane.

isotope 13 of carbon in a sample, relative to the abundance of that same isotope in an arbitrarily designated reference material or isotopic standard [34]. The experiment resulted in an increase in the $\delta^{13}\text{C}_{\text{PDB}}(\text{CH}_4)$ in the reactor. After 24 h that the ^{13}C -labeled acetate was fed (day 304), the $\delta^{13}\text{C}_{\text{PDB}}(\text{CH}_4)$ rose from 22 to a maximum value of 368 (Fig. 2) on day 319. On day 324, and after 24 h of removing the ^{13}C -labeled acetate from the feed solution, the $\delta^{13}\text{C}_{\text{PDB}}(\text{CH}_4)$ started to decrease. The $\delta^{13}\text{C}_{\text{PDB}}(\text{CH}_4)$ reached a value of -72 on day 342 which corresponded to 40 days after the initial dosage of the ^{13}C -labeled acetate. The observed peak in the labeled $^{13}\text{CH}_4$ while feeding ^{13}C -labeled acetate ($^{12}\text{CH}_3^{13}\text{COO}^-$), showed that the acetate was firstly anaerobically oxidized to HCO_3^- and then converted into methane by the hydrogenotrophic methanogens (Eqs. (1) and (2)).

3.3. Phenol degradation intermediates

To assess whether phenol degradation occurred via the benzoyl-CoA or caproate pathway, the permeate was additionally analyzed for the presence of benzoate and the expected different metabolites that may occur during the caproate degradation pathway (Section 2.3). Only benzoate was detected at a concentration up to $220 \text{ mg}\cdot\text{L}^{-1}$, which suggested that phenol degradation in the AnMBR under saline thermophilic conditions occurred via the benzoyl-CoA pathway [23]. Fang et al. (2006) proposed that during thermophilic degradation of phenol, caproate, and cyclohexanone may occur as intermediate compounds [20]. However, we were not able to detect caproate nor cyclohexanone during the reactor operation, while benzoate was detected, in accordance with what has been reported by Hoyos-Hernandez et al. (2014) for thermophilic phenol degradation [19]. Our current results, therefore, strongly indicate that phenol degradation under saline thermophilic anaerobic conditions followed the benzoyl-CoA pathway. Hence, similar to that followed under mesophilic conditions [43]. In this regard, some syntrophic aromatic compound degraders have been reported to excrete benzoate besides acetate [44,45]. This process is reported to be conducted through benzoate thiolation (BamY) and transporter (BtrABCX) complexes to alleviate thermodynamic inhibition through decreased H_2 production [45]. However, such studies considered mesophilic microorganisms (e.g., *Syntrophorhabdus*) as a model; therefore, it is not clear whether the same process will be valid for phenol-degrading microorganisms under thermophilic conditions.

Acetate was found in the permeate during the first two stages when

phenol was the main COD source. However, concentrations were low with an average of $29.3 \pm 29.1 \text{ mg}\cdot\text{L}^{-1}$ and $31.9 \pm 24.7 \text{ mg}\cdot\text{L}^{-1}$ and maximum values of $97 \text{ mg}\cdot\text{L}^{-1}$ and $73 \text{ mg}\cdot\text{L}^{-1}$ in Stages A and B, respectively. Acetate was rarely detected during the next operational periods. The accumulation of acetate during the first two stages might be related to a decrease in the SAO and/or hydrogenotrophic activity, which corroborates the decrease in phenol removal observed during both stages.

3.4. Contribution of the membrane-attached biomass to phenol conversion

To examine the contribution of the membrane-attached biomass to the observed phenol conversion, phenol, benzoate, and acetate in the reactor's bulk liquid and permeate were measured during stage D (Fig. 3). For phenol, there was a significant reduction of 38 % in the permeate concentration ($269.3 \pm 26.8 \text{ mg}\cdot\text{L}^{-1}$) in comparison to the reactor's bulk liquid concentration ($166.0 \pm 22.6 \text{ mg}\cdot\text{L}^{-1}$), $t(21) = -26.9$, $p < 0.001$. For acetate, the concentration in the reactor's bulk liquid was higher ($34.5 \pm 28.4 \text{ mg}\cdot\text{L}^{-1}$) than in the permeate ($1.5 \pm 4.9 \text{ mg}\cdot\text{L}^{-1}$). Whereas for benzoate, there was an increase in the permeate by a factor of 3.8, from $20.1 \text{ mg}\cdot\text{L}^{-1}$ in the reactor's bulk liquid to $75.5 \text{ mg}\cdot\text{L}^{-1}$ in the permeate. Our results indicated that during the passage of the liquid through the membrane-attached biomass a further decrease in the phenol concentration occurred. Similarly, an increase in the benzoate concentration was observed, while acetate was consumed by the membrane-attached biomass. If phenol degradation indeed followed the benzoyl-CoA pathway, the increased benzoate concentration measured may indicate a thermodynamic constraint in the benzoyl-CoA degradation due to high H_2 concentration [43]. It should be noted that the observed differences in the concentrations of phenol, benzoate, and acetate over the membrane might be due to the relatively low bulk VSS concentration. Therefore, resulting in a relatively high contribution of the membrane-attached biomass to the overall conversion. Despite the low operational flux (4.0 LMH) and the applied filtration cycle which considered a short backwash time, apparently, a specialized microbial community developed on the membrane surface. The biofilm formed by such microbial community likely contributed to the general decrease in the membrane permeability from $13.1 \pm 1.8 \text{ LMH}\cdot\text{bar}^{-1}$ in Stage A to 8.4 ± 1.2 in Stage D, having values of $6.5 \text{ LMH}\cdot\text{bar}^{-1}$ during the last

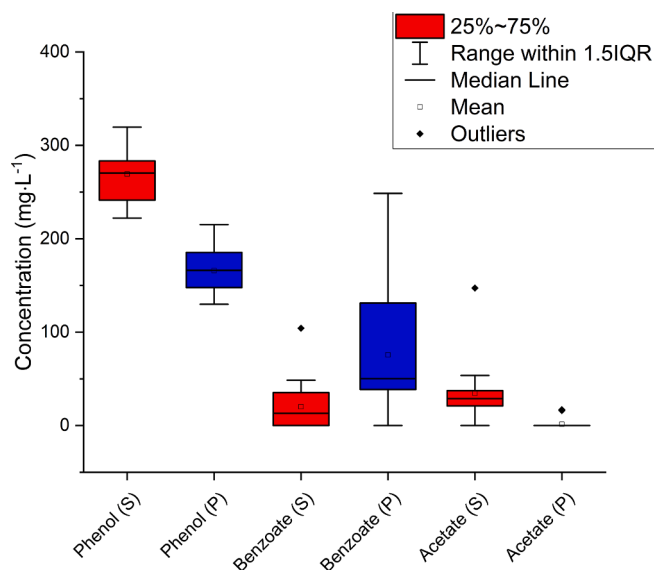


Fig. 3. Phenol, benzoate, and acetate concentration in the reactor's bulk liquid and permeate of the AnMBR. The phenol and VFAs had a higher concentration inside the reactor when compared to the permeate. For benzoate, the concentration in the permeate was higher than the one in the reactor's bulk liquid. $n = 18$. (S) = supernatant and (P) = permeate.

days of operation (Supplementary Material S2).

3.5. Membrane analysis

After determining the contribution of the membrane-attached biomass to the phenol degradation and membrane permeability loss, the membrane was examined by environmental scanning electron microscopy (ESEM) and by optical microscopy to measure the dry and wet biomass layer thickness (Supplementary Material S3, Fig. S3). Based on the calculated average conversion rate of $29.1 \text{ mgPh gVSS}^{-1} \text{ d}^{-1}$ in the reactor and assuming the density of the biomass in the biomass layer of the membrane as 1 g mL^{-1} , we calculated a required thickness of $337 \mu\text{m}$ for achieving the observed conversion. The maximum measured thickness of the dried biofilm was $39.2 \mu\text{m}$, while the wet layer was $106 \mu\text{m}$. Apparently, the specific phenol conversion rate of the membrane-attached biomass was about 3.2 times higher than that observed for the reactor's bulk liquid biomass. Possibly, environmental conditions inside the biomass layer on the membrane were more advantageous for phenol conversion or, as previously hypothesized, (Section 3.4) a specialized phenol-degrading community developed in such membrane-attached biomass.

3.6. Microbial community dynamics

To examine the microbial community dynamics along the different stages of the reactor operation, and to determine whether there was a development of specialized microbial populations in the bulk liquid and the membrane-attached biomass (Fig. 4), the relative abundances of bacteria and archaea at different time instants were analyzed.

For Bacteria, the results showed a high relative abundance of microorganisms from the class Gammaproteobacteria (22.6 % A, 36.0 % B, 27.3 % C, 22.7 % D, and 0.9 % in the membrane-attached biomass), with *Morganella* sp., *Providencia* sp. and *Tepidiphilus* sp. being the most

abundant genera. However, none of these microorganisms have been reported as phenol degraders or have been found in phenol-degrading reactors under thermophilic conditions. The class Thermotogae showed high relative abundance during the first two stages (25.9 % A, 24.3 % B, 8.4 % C, 6.6 % D, and 0.4 % in the membrane-attached biomass) and consisted mainly of *Petrotoga* sp., *Mesotoga* sp. and *Deffluviitoga*. The microorganisms of this class have been reported in reactors treating phenolic wastewater under mesophilic [46], thermophilic [17], and saline thermophilic conditions [29]. Moreover, some of these microorganisms are thermophiles, halophiles, and hydrogen-producing [47]. Class Synergistia (9.3 – 18.3 %) had high relative abundances as well (Fig. 4 A & B), mainly represented by *Acetomicrobium* sp., an acetate-forming microorganism reported in thermophilic digesters [48]. *Acetomicrobium* sp. was found during all four stages with relative abundances of 8.5 % (A), 17.0 % (B), 8.9 % (C), 7.4 % (D) and 3.5 % in the membrane-attached biomass. The class Clostridia increased from 2.9 % in stage B to 7.9 % and 17.2 % in stages C and D respectively, and was also present in the membrane-attached biomass (29.2 %). Members of this class have been reported as SAOB such as *Syntrophaceticus* sp. [31,48] with relative abundances of 2.4 % (A), 0.2 % (B), 2.6 % (C), 4.8 % (D) and 24.4 % in the membrane-attached biomass. *Syntrophaceticus* sp. was the most abundant genus during the last two stages, where acetate was fed as an additional COD source.

Regarding possible phenol degraders, we mainly found microorganisms from the class Clostridia that are commonly reported in anaerobic reactors degrading phenol. Members of the class Clostridia such as *Clostridium sensu stricto* and *Pelotomaculum* sp. [17] have been suggested to have phenol degradation activity [17,49–51]. However, their relative abundance during the stages was low (≈ 6.0 %) which could explain the relatively low phenol removal efficiency. Overall, and opposite to what has been reported for phenol degradation in AnMBRs and batch reactors under mesophilic conditions, at 55°C we did not find a high abundance of known specific phenol degraders, such as

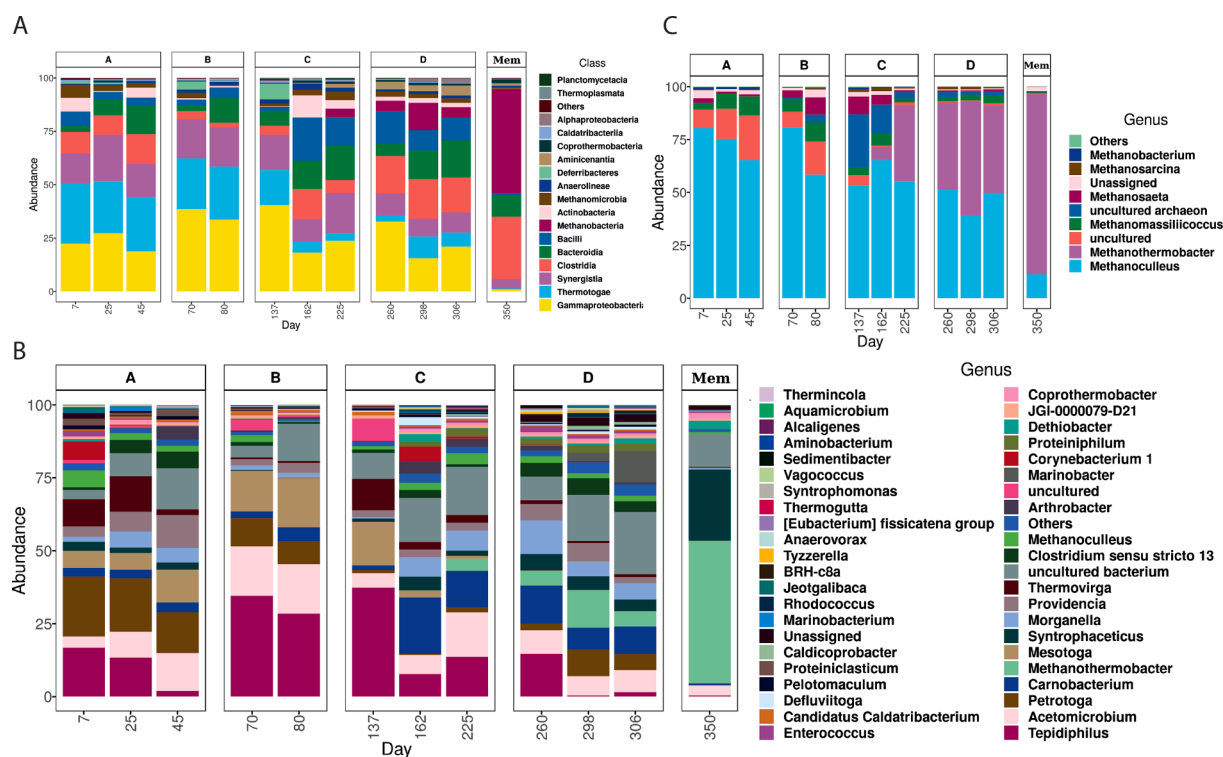


Fig. 4. Relative abundance of Bacteria (A and B) and Archaea (C) in the AnMBR biomass during the different reactor operation stages (A-D) and in the membrane-attached biomass (Mem). For Bacteria, the class and genus taxonomic ranks are shown whereas for Archaea just genus. In A, it is noticeable the increase in the clostridia class for the stages C and D, with genus as *Syntrophaceticus* and *Clostridium sensu stricto* reported as SAOB and phenol degrader bacterium, respectively. Archaea microorganisms were mainly hydrogenotrophic and represented most of the community in the membrane (Mem).

Syntrophorhabdus sp. [30,32,46]. Possibly, thermophilic phenol degraders are not described yet, but these results may also explain the observed lower phenol removal efficiencies and sPhCR in comparison to AnMBRs under mesophilic conditions [30].

For Archaea, the microbial community analysis showed that the predominant microorganisms were hydrogenotrophic methanogens. Their relative abundance in stage A was 3.4 % which previously decreased to 1.4 % in stage B. After the addition of acetate, an increase in the abundance was observed to 3.9 % in stage C, and 9.7 % in stage D. Remarkably, the hydrogenotrophic methanogens represented 49.6 % of the microorganisms in the membrane-attached biomass (Fig. 4 A & C). A detailed analysis in the Archaea domain showed that the microbial community profile had a marked presence in the reactor's biomass of *Methanoculleus* sp. (73.7 % in A, 69.6 % in B, 57.9 % in C, 46.8 % in D and 22.7 % in the membrane-attached biomass) from the class Methanomicrobia. Likewise, *Methanothermobacter* sp. (class Methanobacteria) was found during the stage D and in the membrane-attached biomass (end of the experiment) with relative abundances of 45.2 % and 73.5 %, respectively. The distinct presence of hydrogenotrophic methanogens over acetoclastic ones strongly suggests the predominant presence of HM. Hydrogenotrophic methanogens played a crucial role in the ultimate COD conversion to CH₄, which was confirmed by the results of the 1-¹³C labeled acetate experiment. After dosing the 1-¹³C labeled acetate to the AnMBR, the δ¹³C_{PDB}(CH₄) increased during days 304–321, indicating an increased content of ¹³C in the methane (Section 3.2). The later is in accordance with the high relative abundance of the hydrogenotrophic methanogens *Methanoculleus* sp. (49.7 %) and *Methanothermobacter* sp. (41.3 %), and the presence of bacteria such as *Syntrophaceticus* sp. (3.9 %), described as SAOB [52]. It should be realized that anaerobic biomass under thermophilic conditions have relatively high growth and decay rates meaning that unfed periods or periods with low loading rates may result in relatively high decay of the hydrogenotrophic methanogenic sub-population. For the same reason, feeding a reactor with solely phenol is very difficult to operate under high temperatures and saline conditions.

The prevalence of hydrogenotrophic microorganisms was consistent with our hypothesis that acetate degradation was related to a high SAO activity, explaining the δ¹³C_{PDB}(CH₄) observations after the dosage of the labeled acetate.

Fig. 5 shows the PCoA of the microbial communities during the different stages of the reactor operation, and the community that developed in the membrane-attached biomass. Although between stages B and C there is an overlap (days 70 (B), 80 (B), and 137(C)), there is a difference between the microbial communities in stages A and D.

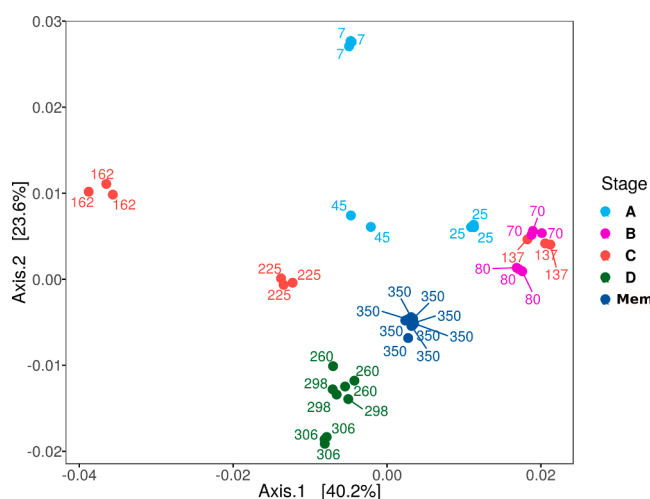


Fig. 5. Principal coordinate analysis of the microbial communities during the different reactor operation stages (A-D) and in the membrane (Mem).

Remarkably, the community developed on the membrane was clearly different from the community in the AnMBR bulk biomass.

3.7. Stoichiometry and thermodynamics of anaerobic growth on phenol under thermophilic conditions: effects of hydrogen partial pressure and syntrophic relationships

A thermodynamic state analysis of environmental systems is a tool that provides a better insight into the microbial processes and related metabolism [37]. It allows the identification of the stoichiometry of the overall redox reaction in the system, the (sub)reactions occurring, and the effects of physicochemical variables (such as temperature) or microbial syntrophic interactions on the thermodynamic feasibility of these reactions [37].

The stoichiometry of the catabolic (Eq. 5), anabolic (Eq. 8), and metabolic reactions (Eq. 9) of anaerobic growth on phenol are presented in Table 2. The reactions consider a microorganism capable of complete oxidation of phenol into acetate [44].

At 55 °C the increase in the hydrogen partial pressure (PH₂) has a strong effect on the thermodynamics of both catabolic and metabolic reactions increasing the ΔG_R^{1,55 °C} mainly due to the presence of H₂ as a reaction product (Eq. 5 and Eq. 9) (Fig. 6 A). Thus, the decrease in the PH₂ will increase the thermodynamic feasibility of both catabolic and metabolic phenol conversion reactions. Therefore, implying the requirement of a syntrophic relationship between the phenol degraders with hydrogen consumers, namely, the hydrogenotrophic methanogens (Fig. 6 B).

In agreement with previous work [30], it was initially considered that a robust and active acetoclastic methanogenic population could enhance phenol degradation by maintaining the concentration of acetate low. Our current results show that under thermophilic conditions we should modify this hypothesis to the need of a robust and active hydrogenotrophic methanogenic population which acts as an effective scavenger of reducing equivalents (electron sink). The presence of such hydrogenotrophic population mainly depends on an active SAOB population able to maintain a low acetate concentration while ensuring hydrogen production (Fig. 6 B).

Some research studies have proposed that supplementation with hydrogen may enhance phenol degradation by increasing the conversion of phenol to (4-hydroxy)benzoate [28,53]. However, it is unlikely that

Table 2

Stoichiometry of anaerobic growth on phenol. The catabolic (Eq. 5), anabolic (Eq. 8), and metabolic (Eq. 9) reactions are shown.

	Reaction	Stoichiometry	Eq. No
Catabolism	Oxidation: phenol to acetate	$-C_6H_5OH - 5H_2O + 3C_2H_3O_2^- + 7H^+ + 4e^-$	Eq. 3
	Reduction: H ⁺ respiration	$-e^- - H^+ + 0.5H_2$	Eq. 4
	Overall catabolic reaction	$-C_6H_5OH - 5H_2O + 3C_2H_3O_2^- + 2H_2 + 3H^+$	Eq. 5
Anabolism	Oxidation: phenol to biomass	$-0.17C_6H_5OH - 0.33H_2O - 0.2NH_4^+ + CH_{1.8}O_{0.5}N_{0.2} + 0.67H^+ + 0.47e^-$	Eq. 6
	Reduction: H ⁺ respiration	$-e^- - H^+ + 0.5H_2$	Eq. 7
	Overall anabolic reaction	$-0.17C_6H_5OH - 0.33H_2O - 0.20NH_4^+ + CH_{1.8}O_{0.5}N_{0.2} + 0.24H_2 + 0.2H^+$	Eq. 8
Metabolism of anaerobic growth on phenol		$-2.31C_6H_5OH - 11.07H_2O - 0.20NH_4^+ + CH_{1.8}O_{0.5}N_{0.2} + 6.44C_2H_3O_2^- + 4.53H_2 + 6.64H^+$	Eq. 9

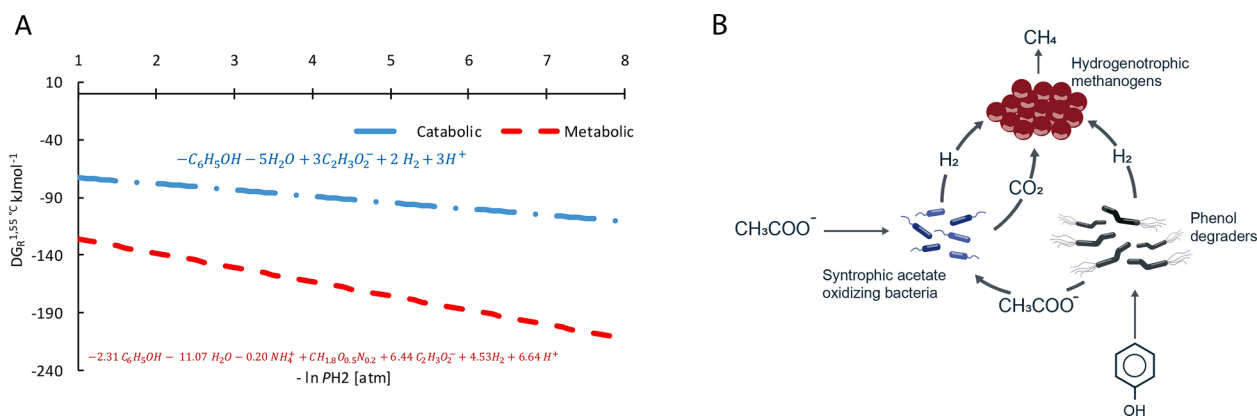


Fig. 6. Effect of the changes in the hydrogen partial pressure (PH_2) on the $\Delta G^{1.55^\circ C}$ (correction for temperature and reagents concentration) of the catabolic (blue) and metabolic reactions (red) of phenol degradation under thermophilic anaerobic conditions. The metabolic equation considers a microorganism capable of the anaerobic degradation of phenol to acetate and hydrogen (A). Scheme for the proposed syntrophic association for phenol degradation under thermophilic anaerobic conditions between phenol degraders, syntrophic acetate oxidizing bacteria (SAOB) and hydrogenotrophic methanogens. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hydrogen is used during this oxidative step, as the mechanism for converting phenol into benzoyl-CoA requires a carboxylation, thiolation, and finally a dehydroxylation [43]. Dependence on hydrogen for such a process will be unlikely unless the 4-hydroxybenzoyl-CoA reductase, which catalyzes the reductive dehydroxylation from 4-hydroxybenzoyl-CoA to benzoyl-CoA could obtain its reducing equivalents (H) from H_2 . Similar mechanisms may be speculated for the downstream reductive steps, e.g., ring splitting by the action of the benzoyl-CoA reductase to yield cyclohex-1,5-dienecarbonyl-CoA, a process that has been suggested to be mediated by the electron-donor ferredoxin [43]. Nevertheless, genomic analyses performed in a mesophilic phenol degrading community have shown that for energy conservation and electron flow, such microorganism has an electron-confurcating system which is used to produce H_2 while regenerating NAD^+ and oxidized ferredoxin (Fd_{ox}) [43]. More research is needed to determine the effect of hydrogen supplementation on the phenol degradation process and its biochemical implications.

4. Conclusions

It was shown that during the operation of an AnMBR under saline thermophilic anaerobic conditions, the addition of acetate as an extra COD source significantly increased ($p < 0.001$) the phenol removal efficiency and the specific phenol conversion rate up to 65 % and 29 mgPh.gVSS⁻¹d⁻¹, respectively.

Syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis seemed to be the main pathway for acetate conversion and methane production, whereas benzoate measurement in the reactor's bulk liquid and permeate suggests that phenol degradation in the AnMBR under saline thermophilic anaerobic conditions follows the benzoyl-CoA pathway. The membrane-attached biomass exhibited an sPhCR of approximately three times higher than the reactor's bulk biomass.

The microbial community dynamics showed a high relative abundance of hydrogenotrophic methanogens in the suspended and the membrane-attached biomass; furthermore, the microbial community corresponding to the membrane-attached biomass had a high abundance of SAOB.

Finally, the stoichiometric and thermodynamic analyses support our hypothesis for the need of an abundant and active hydrogenotrophic methanogenic sub-population in the AnMBR acting as an electron sink and therefore increasing the sPhCR by avoiding (bio)energetical constraints.

5. Outlook, recommendations, and further applications

The present study showed that the treatment of thermophilic saline phenolic wastewater is possible provided a complete and robust methanogenic ecosystem is ensured. The main problems faced by anaerobic sludge-bed reactors when treating these types of effluents are related to biomass degranulation and its subsequent washout. These problems are, however, mitigated by coupling the bioreactor with a membrane filtration unit, which guarantees the retention of all specialized microbial populations needed for the degradation of phenol, namely phenol degraders and methanogens. Under saline thermophilic conditions, however, and as highlighted by the isotopic $^{13}CH_{14}$ analysis, the methanogenic population, will mainly comprise hydrogenotrophic instead of acetoclastic archaea. Therefore, providing the hydrogenotrophic population with reducing equivalents from an easily degradable COD source, for example, hydrogen coming from SAO, will support their development. In this regard, other specific microbial populations, such as SAOB, will then play a more pronounced role in the conversion process. Results from the AnMBR operation, explained by the decrease in thermodynamic constraints, indeed showed higher phenol removal efficiency when simultaneously degraded with acetate. The development of a biofilm layer on the reactor's membrane, enriched with SAOB and hydrogenotrophic methanogens in addition to phenol degraders, supported our hypothesis on the required presence of syntrophic consortia for achieving enhanced phenol degradation.

Future research is needed to address the limitations of the present study and expand the knowledge regarding the degradation of toxic and inhibitory compounds under saline and thermophilic conditions. In our present work, we limited the simultaneous degradation to a compound that might be expected in chemical wastewater. However, other COD sources, such as carbohydrates (mono-, di-, and complex saccharides) may have as well an enhancing positive effect on the degradation of phenolic or other aromatic molecules, either when targeting the degradation of one compound or even in compound mixtures. The effect of hydrogen and CO_2 supplementation in the phenol conversion process under thermophilic conditions is another possibility. Recently, experiments on anaerobic biochemical conversions using high-pressure reactors have been conducted [54] allowing the application of different H_2 partial pressures. High-pressure reactors can be used to investigate whether an increased PH_2 would lead to accelerated conversion rates, for example, by enhancing the reductive aromatic ring splitting, e.g., converting benzoate to alkyl products. Although our present results indicate that the degradation pathway of phenol under thermophilic conditions is similar to that under mesophilic conditions and follows the

benzoyl-CoA route, more insight into the molecular, biochemical, and microbiological aspects is indispensable for fully understanding the conversion process. In this regard, it remains uncertain why the molecular biology analyses did not reveal the presence of a sound phenol-degrading microorganism such as *Syntrophorhabdus* sp. Thus far, no detailed studies were performed in which the thermophilic phenol degradation pathway is completely described as it has been done for mesophilic conditions [43]. Moreover, the membrane-attached biomass results indicate that conversion rates might be improved by the development of additional biofilm layers inside the reactor, for example by adding carrier materials such as those used in moving bed bioreactors. Finally, the presence of high salinity (and changes herein) adds another constraint to the conversion process and microbial consortia that needs to be fully understood. For instance, higher salinities will imply higher energy requirements for maintenance leading to additional stress when treating phenolic compounds.

In the current situation and considering a near future in which energy is becoming more expensive, the gained knowledge offers useful applications. In our present work, we used acetate as an additional COD source, which will be questionable at full-scale due to the involved costs. Though, the codegradation of phenolic streams under thermophilic conditions with acetate-rich waste streams may be a possibility. Acetate is commonly found in many chemical wastewater streams such as rubber vulcanization wastewater, having temperatures exceeding 40 °C [55]. In addition, acetate is the central intermediate, or precursor, for methanogenesis, meaning that any additional COD will generate acetate. The potential for simultaneous digestion of industrial streams will be the highest at industrial parks, where wastewater streams are generally cooled to the mesophilic temperature range. Particularly for phenol conversion, and possibly other toxic and inhibitory compounds, nevertheless, higher efficiencies and (specific) loading and conversion rates are expected under mesophilic conditions. However, when recovery of hot process water is anticipated, the thermophilic digestion route might be considered as most beneficial.

Funding

This work was supported by the Dutch Technology Foundation (STW, Project No 13348), which is part of the Netherlands Organisation for Scientific Research (NWO), partly funded by the Dutch Ministry of Economic Affairs. This research was co-sponsored by Evides Industriewater and Paques B.V.

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.

Data availability

Data will be made available on request.

Acknowledgments

The authors thank Mohammed Jafar and Armand Middeldorp for the assistance in the lab work and analyses, Ensiyeh Taheri for helping with the reactor operation, Arjan Thijssen for the support using the SEM, and Flor Arminda Garcia Rea for the help in the graphical design. Amer S.El-Kalliny thanks the postdoc scholarship received from the Egyptian Cultural Affairs & Mission Sector (Egyptian government). Victor Servando Garcia Rea thanks all the Mexican people who through the Mexican Council of Science and Technology (CONACyT) granted him the PhD scholarship no. 410669. Likewise, Victor Servando Garcia Rea thanks Pamela Cerón C., Magela Odriozola A., Alexander Hendriks, and Xuedong Zhang for their support and fruitful academic discussions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cej.2022.140305>.

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