Anaerobic Conversion of Saline Phenol-Containing Wastewater Under Thermophilic Conditions in a Membrane Bioreactor

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Closing water loops in chemical industries result in hot and highly saline residual streams, often characterized by high strength and the presence of refractory or toxic compounds. These streams are attractive for anaerobic technologies, provided the chemical compounds are biodegradable. However, under such harsh conditions, effective biomass immobilization is difficult, limiting the use of the commonly applied sludge bed reactors. In this study, we assessed the long-term phenol conversion capacity of a lab-scale anaerobic membrane bioreactor (AnMBR) operated at 55°C, and high salinity (18 gNa+ L−1). Over 388 days, bioreactor performance and microbial community dynamics were monitored using specific methanogenic activity (SMA) assays, phenol conversion rate assays, volatile fatty acids permeate characterization and Illumina MiSeq analysis of 16S rRNA gene sequences. Phenol accumulation to concentrations exceeding 600 mgPh L−1 in the reactor significantly reduced methanogenesis at different phases of operation, while applying a phenol volumetric loading rate of 0.12 gPh L−1 d−1. Stable AnMBR reactor performance could be attained by applying a sludge phenol loading rate of about 20 mgPh gVSS−1 d−1. In situ maximum phenol conversion rates of 21.3 mgPh gVSS−1 d−1 were achieved, whereas conversion rates of 32.8 mgPh gVSS−1 d−1 were assessed in ex situ batch tests at the end of the operation. The absence of caproate as intermediate inferred that the phenol conversion pathway likely occurred via carboxylation to benzoate. Strikingly, the hydrogenotrophic SMA of 0.34 gCOD-CH4 gVSS−1 d−1 of the AnMBR biomass significantly exceeded the acetotrophic SMA, which only reached 0.15 gCOD-CH4 gVSS−1 d−1. Our results indicated that during the course of the experiment, acetate conversion gradually changed from acetoclastic methanogenesis to acetate oxidation coupled to hydrogenotrophic methanogenesis. Correspondingly, hydrogenotrophic methanogens of the class Methanomicrobia, together with Synergistia, Thermotogae, and Clostridia classes, dominated the microbial community and were enriched during the three phases of operation, while the aceticlastic Methanosaeta species remarkably decreased. Our findings clearly showed that highly saline phenolic wastewaters could be satisfactorily...
INTRODUCTION

Phenols are major contaminants found in wastewaters of several chemical industries, which are often discharged at high temperatures (Busca et al., 2008; Rosenkranz et al., 2013; Wang et al., 2017). Additionally, closing water loops in the chemical sector often result in concentrated, highly saline wastewaters, which increases the complexity of the produced wastewater (Muñoz Sierra et al., 2017). Despite the existing physicochemical processes applied for phenol removal, i.e., membrane distillation, pervaporation, adsorption, extraction, nanofiltration, reverse osmosis, and oxidation processes (wet air, electrochemical, ozonation, UV/H2O2, Fenton) (Villegas et al., 2016; Raza et al., 2019); biological treatment processes are preferred due to its cost-effectiveness. In this regard, anaerobic treatment offers the advantages of minimal energy requirement, low sludge production, and the conversion of organic pollutants into energy-rich biogas. Under both saline and high-temperature conditions, effective biomass immobilization becomes cumbersome, constraining the application of anaerobic sludge bed systems to treat these wastewaters (Dereli et al., 2012; van Lier et al., 2015). Moreover, the phenol degrading capacity of methanogenic consortia is generally very low and is expected to develop only slowly. Therefore, combining anaerobic treatment with membrane assisted biomass separation is an attractive option when phenol conversion is required at high salinity, and thermophilic conditions (Lin et al., 2013; Muñoz Sierra et al., 2018b).

When chemical wastewaters are at high temperatures, direct thermophilic treatment becomes of interest because it reduces the need for cooling the wastewater. Particularly when process water reclamation is envisaged, maintaining the temperature reduces the overall energy requirement. Despite its potentials (van Lier, 2008), thus far, full-scale anaerobic membrane bioreactors (AnMBRs) are not applied for high-temperature chemical wastewater treatment (Duncan et al., 2017). Only a few previous studies have shown the potential of thermophilic conditions for treating phenolic compounds in continuous reactors (Wang et al., 2011; Ramakrishnan and Surampalli, 2013; Wang et al. (2011) compared UASB reactors under mesophilic and thermophilic conditions and concluded that thermophilic anaerobic digestion improves about 30% the degradation of phenolic compounds. Likewise, Ramakrishnan and Surampalli (2013) suggested that thermophilic conversion of phenolic wastewater in an anaerobic hybrid reactor is superior to mesophilic in terms of methane yield, effluent quality, and stability.

Conversely, other studies have also found drawbacks of treating phenol containing wastewater under thermophilic conditions. Fang et al. (2006) indicated that the phenol conversion rate at 55°C in a UASB reactor was significantly lower than under mesophilic conditions. Furthermore, Levén and Schnürer (2005) concluded that phenolics are mineralized to methane and carbon dioxide under mesophilic conditions, whereas under thermophilic conditions, only benzoic acid is degraded. Muñoz Sierra et al. (2018b) suggested that under mesophilic and hyper-mesophilic conditions (42–45°C), the phenol conversion capacity of an AnMBR at high salinity is more stable compared to thermophilic conditions. However, because the operation at 55°C in that study was carried out only during a short-term, it remains unclear whether or not a stable phenol degrading methanogenic consortium may develop. Therefore, this study aims to determine the maximum conversion capacity of a laboratory-scale AnMBR during a long-term operation at 55°C, treating phenol-containing wastewater at 18 gNa−. L−1. Moreover, the microbial community activity and structure in response to increasing phenol loading rates along with three phases of operation were evaluated.

MATERIALS AND METHODS

Experimental Set-Up and Operation

The experiments were performed by using a 6.5 L laboratory-scale AnMBR reactor, equipped with an ultra-filtration (UF) side-stream membrane module (Figure 1). A tubular polyvinylidene fluoride membrane (X-flow, Pentair, Netherlands) with 5.2 mm inner diameter, 0.64 m length and 30 nm nominal pore size was used. The reactor was equipped with feed, recycle, and effluent pumps with 4–20 mA variable speed (120U/ DV, 220 Du, Watson-Marlow, Netherlands), pH, and temperature sensors (Memosens, Endress & Hauser, Germany), and a biogas meter (Milligas Counter MGC-1 PMMA, Ritter, Germany). Transmembrane pressure (TMP) was measured by using three pressure sensors (AE Sensors ATM, Netherlands). The temperature of the jacketed reactor was controlled under thermophilic conditions by a thermostatic water bath (Tamson Instruments, Netherlands). The entire set-up was controlled by a programmable logic controller (PLC) connected to a PC with LabVIEW software (version 15.0.1f1, National Instruments, United States).

The AnMBR was operated at 55.0 ± 0.8°C for 388 days. During this time, the reactor was fed with synthetic phenolic wastewater with phenol concentration between 50 and 800 mgPhL−1 and a sodium concentration of 18 gNa−. L−1. The experiment was divided into three phases (I, II, and III). In all of the phases, the phenol concentration was increased step-wise to determine the maximum conversion capacity and the attainable phenol loading rate of the AnMBR. The applied total organic...
loading rates (OLRs) were between 2.0 and 4.0 gCOD L⁻¹ d⁻¹ during all phases (Table 1). The OLRs were calculated as OLR = influent COD (g L⁻¹) * Flow rate (L d⁻¹)/AnMBR volume (L). The applied flow rate was 1.0 L d⁻¹. An average solids retention time (SRT) of about 120 ± 13 days was maintained. The average SRT was calculated periodically as SRT = X (g VSS in the AnMBR) / X removed.net (gVSS d⁻¹), where X removed.net resulted from the biomass removed day⁻¹ (sampling for tests) and the biomass returned day⁻¹ (after some tests).

The AnMBR was completely mixed applying a turnover of 170 times d⁻¹. The membrane unit was operated at a cross-flow velocity of 0.65 m s⁻¹. A cyclic membrane filtration operation was carried out, consisting of 500 s filtration and 30 s backwash. Backwash was done by reversing the permeate pump flow. An operational flux of 4.0 L m⁻² h⁻¹ was applied as a result of the experimental settings. The permeate flow was controlled with the variable speed of the effluent and influent and pumps, and it was regularly double-checked manually. An average TMP of 177 ± 92 mbar and a total membrane filtration resistance of 9.77 × 10¹² [1/m] could be maintained during the AnMBR long-term operation.

Inoculum and Wastewater Composition
The reactor was initially inoculated with mesophilic anaerobic biomass obtained from a full-scale UASB reactor at 8 gNa⁺ L⁻¹ (Shell, Moerdijk, Netherlands) and subjected to hyper-mesophilic and thermophilic conditions at 16 gNa⁺ L⁻¹ before the start of the experiment. The synthetic phenolic wastewater consisted of phenol (0.1–0.8 gPh L⁻¹), acetate (10–20 g L⁻¹), yeast extract (2.0 g L⁻¹), NaCl, K₂HPO₄, KH₂PO₄, varying according

![Diag](image.png)

**FIGURE 1** | Schematic representation of the thermophilic AnMBR.

**TABLE 1** | Operational organic and phenol loading rates of the thermophilic AnMBR.

| Phase | Days | OLR [gCOD·L⁻¹·d⁻¹] | Phenol loading rate [gPh·L⁻¹·d⁻¹] | Phenol influent concentration [g·L⁻¹] |
|-------|------|-------------------|----------------------------------|---------------------------------|
| I     | 0–12 | 3.7               | 0.01                             | 0.05                            |
|       | 13–42| 3.7               | 0.02                             | 0.1                             |
|       | 43–69| 3.8               | 0.05                             | 0.3                             |
|       | 70–96| 3.9               | 0.09                             | 0.6                             |
|       | 97–108| 4.0            | 0.12                             | 0.8                             |
|       | 109–133| 2.3          | 0.12                             | 0.8                             |
|       | 134–155| 2.0            | 0.02                             | 0.1                             |
|       | 156–198| 2.1            | 0.03                             | 0.2                             |
|       | 199–219| 2.2            | 0.08                             | 0.5                             |
|       | 220–241| 2.3            | 0.11                             | 0.7                             |
|       | 242–262| 2.3            | 0.12                             | 0.8                             |
|       | 263–325| 2.0            | 0.01                             | 0.08                            |
|       | 326–332| 2.1            | 0.02                             | 0.15                            |
|       | 333–349| 2.1            | 0.04                             | 0.25                            |
|       | 350–388| 2.1            | 0.03                             | 0.2                             |
to the applied organic and phenol loading rates (Table 1), while maintaining 18 gNa\textsuperscript{+} L\textsuperscript{-1} and a K\textsuperscript{+}:Na\textsuperscript{+} ratio of 0.05. Macronutrients (9 mL L\textsuperscript{-1}), and micronutrients (4.5 mL L\textsuperscript{-1}) solutions were added. Macronutrients solution contained (in g L\textsuperscript{-1}): NH\textsubscript{4}Cl (170), CaCl\textsubscript{2}.2H\textsubscript{2}O (8), and MgSO\textsubscript{4}.7H\textsubscript{2}O (9); micronutrients solution contained (in g L\textsuperscript{-1}): FeCl\textsubscript{3}.6H\textsubscript{2}O (2), CoCl\textsubscript{2}.6H\textsubscript{2}O (2), MnCl\textsubscript{2}.4H\textsubscript{2}O (0.5), CuCl\textsubscript{2}.2H\textsubscript{2}O (0.03), ZnCl\textsubscript{2} (0.05), H\textsubscript{3}BO\textsubscript{3} (0.05), (NH\textsubscript{4})\textsubscript{6}Mo\textsubscript{7}O\textsubscript{24}.2H\textsubscript{2}O (0.09), Na\textsubscript{2}SeO\textsubscript{3} (0.1), NiCl\textsubscript{2}.6H\textsubscript{2}O (0.05), EDTA (1), Na\textsubscript{2}WO\textsubscript{4} (0.08). The chemical reagents were of analytical grade.

**Volatile Fatty Acids**

Prior analysis, 10 mL of the AnMBR permeate samples was filtrated over 0.45 μm filter paper. The filtrated liquid was diluted with pentanol (300 mg L\textsuperscript{-1}). 10 μL of formic acid (purity > 99%) was added into the final 1.5 mL vials. Volatile fatty acids (VFAs) were measured by gas chromatography (GC) using an Agilent 19091F-112, 25 m × 320 μm × 0.5 μm column and an FID detector (Agilent 7890A, United States). The sample injection volume was 1 μL. Helium was used as carrier gas with a total flow rate of 67 mL/min and a split ratio of 25:1. The GC oven temperature was programmed to increase from 80 to 180°C in 10.5 min. The temperatures of the injector and detector were 80 and 240°C, respectively.

**Permeate Characterization**

Phenol concentrations were measured using high-performance liquid chromatography HPLC LC-20AT (Shimadzu, Japan) equipped with a 4.6 mm reversed-phase C18 column (Phenomenex, Netherlands) and a UV detector at a wavelength of 280 nm. The mobile phase used was 25% (v/v) acetonitrile at a flow rate of 0.95 mL min\textsuperscript{-1}. The column oven was set at 30°C. Fast phenol measurements were also carried out by Merck – Spectroquant Phenol cell kits by using a spectrophotometer NOVA60 (Merck, Germany). Hach Lange kits were used to measure chemical oxygen demand (COD). The COD was measured using a VIS – spectrophotometer (DR3900, Hach Lange, Germany) making proper dilutions to minimize interference by high chloroform concentrations, without compromising the accuracy of the measurement.

**Anaerobic Phenol Conversion Rates**

Batch tests were conducted in triplicate at the end of phase II to assess the phenol conversion recovery after AnMBR performance perturbation. The volatile suspended solid (VSS) were analyzed according to standard protocols using the lowest possible sample volume (APHA, 2005). Biomass samples were taken with a 150 mL syringe and transferred to 500 mL Schott glass bottles. The bottles were filled to a volume of 400 mL with AnMBR biomass (0.68 g VSS), and a medium containing acetate (3.1−4.6 g L\textsuperscript{-1}), phenol (60−109 mgPh L\textsuperscript{-1}), 6 mL L\textsuperscript{-1} macro- and 6 mL L\textsuperscript{-1} micronutrients solution, 10 mM phosphate buffer solution, and 18 gNa\textsuperscript{+} L\textsuperscript{-1}. Three consecutive feedings of the medium were applied. Initial COD and phenol concentrations varied between 3.4−5.1 gCOD L\textsuperscript{-1} and 60−109 mgPh L\textsuperscript{-1}, respectively. Temperature and mixing were controlled in an orbital incubator shaker (New Brunswick Biological Shakers Innova 44/44R, United States) at 55°C and 120 rpm respectively. Periodically, liquid samples were taken, and phenol and COD concentrations were measured. Phenol conversion rates [mgPh gVSS\textsuperscript{-1} d\textsuperscript{-1}] were calculated by using the slope of the phenol concentration vs time curve in each bottle. After the batch tests were finished, the supernatant was removed and biomass was returned to the AnMBR. Similarly, at the end of the AnMBR operation at day 388, biomass samples were taken, and phenol conversion batch tests were carried out with initial phenol concentrations of 40 and 60 mgPh L\textsuperscript{-1}.

**Specific Acetotrophic and Hydrogenotrophic Methanogenic Activity**

Specific acetotrophic methanogenic activity (SMA) tests were performed in triplicate using an automated methane potential test system (AMPTS, Bioprocess Control, Sweden). All the SMA tests were carried out at 55°C, following the method described by Spanjers and Vanrollehem (2016).

For the hydrogenotrophic methanogenic activity, 250 mL Schott glass bottles were filled with biomass (0.57 g VSS) and medium (6 and 0.6 mL L\textsuperscript{-1} macro- and micro-nutrients solution, respectively) and 10 mM phosphate buffer solution at pH 7.0 to a liquid volume of 200 mL. The gas-phase of the bottles was exchanged by using a gas exchange board (G.R Instruments B.V, Netherlands) with a gas mixture of 80% CO\textsubscript{2} and 20% H\textsubscript{2} to an end pressure of 0.5 bar during 5 continuous automated cycles to ensure the complete absence of oxygen. Bottles were incubated in an orbital shaker (New Brunswick Biological Shakers Innova 44/44R, United States) at 55°C and 120 rpm for 10 days, and biogas production was calculated using the exact headspace volume and the drop in headspace pressure versus time. The headspace pressure was measured as described by Coates et al. (1996) using a pressure transducer. The methane content of the biogas was analyzed by using a gas chromatograph 7890A GC (Agilent Technologies, United States) equipped with a front thermal conductivity detector (TCD). The temperature of the oven was 45°C for 6 min, then 25°C/min to 100°C. The temperatures of the front inlet, and a front detector were both 200°C.

**Microbial Community Analysis**

Biomass samples were taken from the AnMBR on days 88, 241, and 376 to evaluate the microbial community. The DNA extraction was performed from 0.5 g of biomass by using the DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany). Agarose gel electrophoresis and Qubit3.0 DNA detection (Qubit dsDNA HS Assay Kit, Life Technologies, United States) were used for quality and quantity control of the DNA. The amplification of the 16S rRNA gene (V3–V4 region) was performed and followed by high throughput sequencing using the MiSeq Illumina platform (BaseClear, Leiden, Netherlands) using the primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAAATCC-3′). The Illumina fastq reads (2 x 250) were processed in the QHIME2 pipeline (2018.7) (Bolyen et al., 2019). Reads were quality filtered, denoised, and the amplicon sequences variants (ASVs) were resolved with
the DADA2 plugin (Callahan et al., 2016), removing chimeras with the “consensus” method. The taxonomic classification of the representative sequences of ASVs was performed with the “classify-consensus-vsearch” plugin (Rognes et al., 2016) using the SILVA (132) database as a reference. The representative sequences were aligned with the MAFFT algorithm (Katoh and Standley, 2013), and a phylogenetic tree was constructed with FastTree (Price et al., 2010). The feature table and tree were exported to the R environment. Differential abundance analyses between reactor operation phases were performed with the DESeq2 library (Love et al., 2014). The abundance and the tree were visualized with the phyloseq library (McMurdie and Holmes, 2013). ASVs with differential abundances within the operational phases were analyzed with BLAST against the reseq RNA database to identify the closest related species. The sequences reported in this paper have been deposited at ENA under the study accession number PRJEB38467.

RESULTS

Thermophilic AnMBR Performance

Influent and effluent phenol and COD concentrations during the long-term thermophilic AnMBR operation are shown in Figure 2. During days 0–96 in phase I, the effluent COD concentrations were in the range of 2.0–10.0 gCOD L$^{-1}$, and the corresponding removal efficiencies were between 59.0 and 92.3% (Figure 2B) at an average OLR of 3.8 gCOD L$^{-1}$ d$^{-1}$. The increase in the phenol loading rate (Table 1) from 0.01 to 0.09 gPh L$^{-1}$ d$^{-1}$ concomitantly increased the phenol removal efficiency from 54 to 95% (Figure 2A). At the end of phase I, the phenol concentration in the reactor reached about 738 mgPh L$^{-1}$ (Figure 2A).

On day 199 in phase II, the phenol loading rate was increased from 0.03 gPh L$^{-1}$ d$^{-1}$ (6.5 mgPh gVSS$^{-1}$ d$^{-1}$) to 0.08 gPh L$^{-1}$ d$^{-1}$ (17.4 mgPh gVSS$^{-1}$ d$^{-1}$) which resulted in an increase in converted phenol from 188 to 349 mgPh L$^{-1}$. Concomitantly, the phenol removal efficiency dropped to 24% (Figure 2A) and the COD removal efficiency to 32% (Figure 2B). However, on day 240 in phase II, the phenol removal efficiency increased to 88%, while applying a phenol loading rate of 0.11 gPh L$^{-1}$ d$^{-1}$ (18.4 mgPh gVSS$^{-1}$ d$^{-1}$). When the phenol loading rate was further increased to 0.12 gPh L$^{-1}$ d$^{-1}$ (20.1 mgPh gVSS$^{-1}$ d$^{-1}$) the reactor performance again deteriorated, resulting in a decrease in both the COD and phenol removal efficiencies to 31 and 23%, respectively. By decreasing the phenol loading rate back to 0.01 gPh L$^{-1}$ d$^{-1}$ (2.2 mgPh gVSS$^{-1}$ d$^{-1}$), the COD and phenol removal efficiencies were gradually recovered during phase III.

Volatile Fatty Acids (VFAs) Spectrum

Throughout the entire thermophilic operation, VFAs were detected in the AnMBR permeate (Figure 3), indicating a limiting methanogenic conversion capacity. The effluent COD mainly consisted of acetate (0.02–9.6 g L$^{-1}$), which peaked at almost 9.6 g L$^{-1}$ between 133 and 144 days when phenol accumulation occurred. Concomitantly, the butyrate concentration increased to 616 mg L$^{-1}$ in this period. In phase II, high concentrations of acetate (5.2 g L$^{-1}$) and to a lesser extent butyrate (295 mg L$^{-1}$) were found at day 260 when phenol again accumulated after an increase in the phenol loading rate. Propionate was most of the time present in the reactor effluent with an average concentration of 129 ± 57 mg L$^{-1}$. The valerate concentration increased to 254 and 156 mg L$^{-1}$ when the reactor performance deteriorated in phases I and II, respectively. In phase III, on day 350, an increase in all VFAs was observed when reactor phenol concentration increased to 124 mgPh L$^{-1}$.

Conversion Rates and Methanogenic Activity

Specific Methanogenic Activity and Phenol Conversion Rates

SMA tests using acetate as the substrate showed a drop in the methanogenic activity of the phenol-degrading biomass in phase III.
I, meanwhile, an increase in the specific phenol conversion rates was observed (Table 2). During phase II the lowest observed SMA was 0.04 ± 0.02 gCOD-CH_{4} gVSS^{-1} d^{-1}, following phenol accumulation at the end of phase I. In phase III when the influent phenol concentration was decreased to 0.2 gPh, the SMA of the phenol-degrading biomass increased to 0.13 gCOD-CH_{4} gVSS^{-1} d^{-1}, which was similar to the value observed at the beginning of phase I.

Table 2 also shows the calculated maximum in-reactor phenol conversion rates in the different phases. At the end of phase I, the phenol conversion rate had increased from an initial value of 1.3 to 21.3 ± 0.2 mgPh gVSS^{-1} d^{-1}. During the recovery periods of perturbation, i.e., days 156–198, the phenol conversion rate was 5.9 ± 0.3 mgPh gVSS^{-1} d^{-1}. From days 220 to 262, the phenol loading rate increased until an average of 16.9 ± 0.6 mgPh gVSS^{-1} d^{-1}. In phase III, the phenol conversion rate decreased to the range 2.6 ± 0.1–7.6 ± 1.6 mgPh gVSS^{-1} d^{-1}.

**Ex situ Phenol Conversion Rate After Reactor Perturbation**

After reactor perturbation at the end of phase II, the phenol conversion rate was assessed in a batch test. Three different feedings were applied with different initial phenol concentrations (see Table 3). After the first feed, the phenol conversion rate was calculated as 4.0 ± 1.4 mgPh gVSS^{-1} d^{-1} after 7 days of incubation. The phenol conversion rate increased to 9.6 ± 2.6 and 10.5 ± 3.3 mgPh gVSS^{-1} d^{-1} after the second and third consecutive feeding respectively.

**Specific Methanogenic Activity and Phenol Conversion Rates at the End of the Long-Term Operation**

Since low acetate-fed SMA values at the end of the long-term thermophilic operation period were observed of 0.13 ± 0.10 gCOD-CH_{4} gVSS^{-1} d^{-1}, SMA tests were performed with both acetate and hydrogen as electron donor. Likewise, the phenol conversion rate was measured in batch test with an initial phenol concentration of 40 and 60 mgPh L^{-1} (Table 4). The acetate-fed SMA obtained was 0.15 ± 0.04 gCOD-CH_{4} gVSS^{-1} d^{-1} while the hydrogenotrophic methanogenic activity was 0.34 ± 0.08 gCOD-CH_{4} gVSS^{-1} d^{-1}. A maximum

![VFAs concentrations during the AnMBR long-term operation at 55°C and 18 gNa⁺ L⁻¹. Acetate (left y-axis), Propionate, Butyrate, and Valerate (right y-axis). The decreased of VFAs concentration values on day 79 is due to a stop of feeding.](image-url)

**FIGURE 3**

| Table 2 | SMA (acetate as the substrate) and phenol conversion rates at different phases of the AnMBR operation under thermophilic conditions. |
|---------|---------------------------------------------------------------|
| Phase | Days | SMA [gCOD-CH_{4} gVSS^{-1} d^{-1}] | AnMBR phenol conversion rate [mgPh gVSS^{-1} d^{-1}] |
| I | 0–12 | 0.13 ± 0.05 | 1.3 ± 0.6 |
| | 43–69 | 0.08 ± 0.05 | 8.4 ± 0.3 |
| | 100–133 | N.D.* | 21.3 ± 0.2 |
| II | 156–198 | 0.04 ± 0.02 | 5.9 ± 0.3 |
| | 220–241 | N.D.* | 16.0 ± 0.6 |
| | 242–262 | N.D.* | 16.9 ± 0.6 |
| III | 326–332 | 0.09 ± 0.07 | 2.6 ± 0.1 |
| | 333–349 | N.D. | 7.6 ± 1.6 |
| | 350–388 | 0.13 ± 0.10 | 6.3 ± 0.2 |

*N.D., not determined.

**Table 3** | Phenol conversion rate during three consecutive feedings in the batch test. |
|-----------------|---------------------------------|---------------------------------|-----------------|
| Feed | Phenol conversion rate [mgPh gVSS^{-1} d^{-1}] | Initial phenol concentration [mgPh L^{-1}] | Initial COD concentration (phenol+acetate) [gCOD L^{-1}] |
| 1st | 4.0 ± 1.4 | 109 ± 12 | 5.1 ± 0.3 |
| 2nd | 9.6 ± 2.6 | 60 ± 3 | 3.4 ± 0.0 |
| 3rd | 10.5 ± 3.3 | 89 ± 25 | 3.6 ± 0.1 |
phenol conversion rate of $32.8 \pm 0.5 \text{mgPhL}^{-1}\text{d}^{-1}$ was found, applying an initial phenol concentration of $60 \text{mgPhL}^{-1}$.

### Microbial Community Structure Analysis

The microbial community analysis revealed a total of 141 ASVs with differential abundance across samples. **Figure 4** shows the genera from both bacteria and archaea domains with main population changes in relative abundance at the different phases of the thermophilic AnMBR at $18 \text{gNa}^+\text{L}^{-1}$. *Petrotoga* (Thermotogae class) was enriched along with the long-term operation up to 21.1% in phase III. *Thermovirga* (Synergisitia class) increased from 8.0 to 14.1% from phase I to II and then decreased to 7.95% relative abundance in phase III. *Acetomicrobium* also belonging to phylum Synergistetes

| SMA                  | Phenol conversion rates [mgPh gVSS$^{-1}$ d$^{-1}$] |
|----------------------|----------------------------------------------------|
| Acetate 0.15 ± 0.04  | 29.2 ± 0.1                                         |
| H$_2$/CO$_2$ 0.34 ± 0.08 | 32.8 ± 0.5                                         |

**TABLE 4** | Acetate-fed and hydrogen-fed specific methanogenic activities, as well as phenol conversion rates assessed at two different initial phenol concentrations.

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**FIGURE 4** | Heatmap of the genera from bacteria and archaea domains in the thermophilic AnMBR that were positive after differential abundance analysis (DESeq2) among the three phases of reactor operation. The color scale ranges from 0 to 22% relative abundance.

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**Arthrobacter** 0.08  | 0.06  
**Rhodococcus** 1.5  | 1.05  
**Corynebacterium** 3.47  | 2.15  
**ADurb.Bin120** 6.63  | 6.63  
**Dethiobacter** 0.74  | 0.74  
**Moorella** 0.07  | 0.07  
**Proteiniclasticum** 0.68  | 0.31  
**[Eubacterium] fissicatenia group** 0.11  | 0.11  
**Lachnolostridium** 0.02  | 0.02  
**Tyzzerella** 0.14  | 0.14  
**Candidatus Desulfurospira** 0.06  | 0.06  
**Pelotomaculum** 1.23  | 1.23  
**Syntrophaceticus** 1.43  | 4.81  
**Syntrophomonas** 0.07  | 0.07  
**Gariella** 0.05  | 0.05  
**Anaerofustis** 0.39  | 0.27  
**Paramaeobacter** 0.05  | 0.05  
**Soehngenia** 0.18  | 0.27  
**Tepidimicrobium** 2.17  | 2.28  
**Anaerovorax** 0.3  | 0.3  
**Anoxytrospira** 0.17  | 0.17  
**Caldiclospira** 4.73  | 1.11  
**Syntrophobacter** 0.13  | 0.52  
**Morganella** 1.75  | 1.75  
**Providencia** 3.65  | 3.65  
**Tepidiphilus** 3.39  | 3.39  
**Alcaligenes** 0.07  | 0.07  
**Marinobacterium** 0.18  | 0.18  
**uncultured organism** 9.15  | 9.15  
**Methanobacterium** 0.01  | 0.01  
**Methanoecoccus** 0.18  | 0.18  
**Methanolinea** 6.06  | 6.06  
**Methanoseta** 1.97  | 1.97  
**Thermomicrobium** 7.95  | 7.95  
**Acetococcus** 3.93  | 3.93  
**JGI-000000079-D2** 0.87  | 0.87  
**SC103** 0.03  | 0.03  
**PETrotoga** 0.26  | 0.26  
**Mesotoga** 5.52  | 5.52  
**Unassigned** 0.99  | 0.99  
**Uncultured** 1.11  | 1.11  
**Uncultured** 3.36  | 3.36  
**uncultured organism** 0.18  | 0.18  
**uncultured candidate division WS6 bacterium** 0.19  | 0.19  

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**FIGURE 4** | Heatmap of the genera from bacteria and archaea domains in the thermophilic AnMBR that were positive after differential abundance analysis (DESeq2) among the three phases of reactor operation. The color scale ranges from 0 to 22% relative abundance.
reported inhibition of phenol conversion at a concentration of 600 mgPh L$^{-1}$, and biogas production nearly stopped when phenol reached 895 mgPh L$^{-1}$. In order to reduce the phenol concentration in the AnMBR, the phenol loading rate was decreased to 0.02 gPh L$^{-1}$ d$^{-1}$ (3.8 mgPh gVSS$^{-1}$ d$^{-1}$) on day 133 to prevent further intoxication, while the OLR was reduced to 2.0 gCOD L$^{-1}$ d$^{-1}$. In phase II, the reactor performance again deteriorated. Consequently, biogas production almost ceased on day 260 when the reactor phenol concentration reached 616 mgPh L$^{-1}$, inhibiting the methanogenic consortium. Surprisingly in phase III, by applying a phenol loading rate of 0.04 gPh L$^{-1}$ d$^{-1}$ (9.3 mgPh gVSS$^{-1}$ d$^{-1}$) on day 352, phenol and COD removal efficiencies decreased to 50 and 80%, respectively. The latter indicates an increased sensitivity of the biomass to phenol compared to phase I.

The observed maximum phenol conversion rate of 21.3 ± 0.2 mgPh gVSS$^{-1}$ d$^{-1}$ in the AnMBR at the phenol loading rate of 0.12 gPh L$^{-1}$ d$^{-1}$ (22.6 mgPh gVSS$^{-1}$ d$^{-1}$) at 55$^\circ$C and 18 gNa$^+$ L$^{-1}$ was substantially higher than the observed phenol conversion rate of 1.7 mgPh gVSS$^{-1}$ d$^{-1}$ at a loading rate of 0.02 gPh L$^{-1}$ d$^{-1}$ (3.9 mgPh gVSS$^{-1}$ d$^{-1}$), which was observed in our previous work after shifting an AnMBR operation from 35 to 55$^\circ$C at a sodium concentration of 16 gNa$^+$ L$^{-1}$ (Muñoz Sierra et al., 2018b). However, it is lower than the 81.3 mgPh gVSS$^{-1}$ d$^{-1}$ found by others (Wang et al., 2011). It should be noted that in our present work, the AnMBR was exposed to more extreme conditions, combining high phenol concentrations, high temperature, and high salinity (18 gNa$^+$ L$^{-1}$) compared to the previous thermophilic studies (see Table 5). Still, our observed phenol conversion rates can be considered low when compared to the 52.7–489 mgPh gVSS$^{-1}$ d$^{-1}$ that was achieved with thermophilic phenol degrading methanogenic enriched consortia (Chen et al., 2008).

Volatile fatty acid concentrations in the permeate, mainly acetate, indicated a limiting methanogenic conversion capacity for the OLR applied. The high observed acetate concentration reaching 9.6 g L$^{-1}$ (Figure 3) when phenol accumulation occurred, could have caused a secondary inhibitory effect to the anaerobic microorganisms such as propionate-oxidizing bacteria (Van Lier et al., 1993). Despite the peaks of 620 and 300 mg L$^{-1}$ of butyrate during the phenol accumulation events, all VFA concentrations (propionate, butyrate, and valerate) remained at a relatively low level, commonly observed in anaerobic reactors under anaerobic thermophilic conditions (Van Lier et al., 1993). Caproate always remained below detection level throughout the entire thermophilic AnMBR operation. Previous studies inferred that at 55$^\circ$C, phenol-degrading biomass might degrade phenol via $\alpha$-caproate (Evans and Fuchs, 1988; Fang et al., 2006). On the other hand, Hoyos-Hernandez et al. (2014) have demonstrated that thermophilic phenol degradation is possible via the benzoate conversion route, similar to mesophilic conditions, contrasting these previous studies. Even though benzoate was not determined analytically in the AnMBR, the absence of caproate in any of the VFA analyses strongly suggests that the prevailing phenol conversion pathway was likely via benzoate carboxylation at 55$^\circ$C. Following the proposed pathway, benzoate is subsequently de-aromatized to form cyclohexane carboxylic acid, which is
cleaved to heptanoate, degraded further through β-oxidation to form valerate, propionate, and acetate, or directly to propionate and butyrate, which are further degraded to acetate (Liang and Fang, 2010).

Conversion Rates and Methanogenic Activity

The assessed SMA values found are similar to those reported elsewhere for phenol degrading biomass under thermophilic conditions, i.e., 0.1 gCOD-CH₄·gVSS⁻¹·d⁻¹ (Fang et al., 2006).

The phenol conversion rate assessed in ex situ batch tests increased to 10.5 ± 3.3 mgPh·gVSS⁻¹·d⁻¹, inferring a 62% recovery of the conversion capacity after about 17 days of incubation. Interestingly, also Chen et al. (2008) found a maximum phenol conversion rate in the AnMBR incubation. The ex situ assessed phenol conversion rate at the end of the batch tests was about 61% of the AnMBR conversion rate before phenol accumulation occurred (see Table 3). Apparently, the phenol-degrading biomass could recover from the phenol shocks after a relatively short recovery period, while being exposed to low phenol concentrations in the reactor (<100 mgPh·L⁻¹). The observed maximum phenol conversion rate of 32.8 ± 0.5 mgPh·gVSS⁻¹·d⁻¹ in batch test at the end of the experiment is higher than the maximum observed phenol conversion rate in the AnMBR during phase I, which likely can be attributed to long-term adaptation of the biomass to phenol after three phases. Comparing our present results with the different studies summarized in Table 5, which are performed in a broad range of temperatures, the observed maximum phenol conversion rates are comparable to those obtained under both mesophilic (Rosenkranz et al., 2013; Franchi et al., 2020) and psychrophilic (Collins et al., 2005; Scully et al., 2006) conditions, using other anaerobic high-rate reactors configurations. Nonetheless, it should be recalled that our present results were obtained under extreme salinity conditions applying sodium concentrations of 18 gNa⁺·L⁻¹.

The acetate-fed SMA obtained at the end of the long-term operation was 0.15 ± 0.04 gCOD-CH₄·gVSS⁻¹·d⁻¹ similar to what was observed at the beginning of phase I and at the end of phase III. Remarkably, the hydrogenotrophic methanogenic activity was a factor 2.3 higher (0.34 ± 0.08 gCOD-CH₄·gVSS⁻¹·d⁻¹), which made us hypothesize that methanogenesis of acetate proceeded via syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis, rather than aceticlastic methanogenesis. Of interest is the relatively high hydrogenotrophic SMA, which indicates that acetate is possibly syntrophically methanised via oxidation to hydrogen and carbon dioxide. Note that syntrophic acetate oxidation is energetically more favorable at elevated temperature and high acetate concentration and is more often observed as the dominant pathway in a large number of thermophilic anaerobic reactors (van Lier, 1996; Westerholm et al., 2016; Li et al., 2020). Moreover, hydrogenotrophic methanogenesis is commonly observed at elevated salt concentrations (De Vrieze et al., 2016).

Based on our present results, follow-up research in a thermophilic AnMBR should reveal the minimum acetate

### Table 5: Anaerobic phenol conversion in continuous flow reactor systems operated at different temperatures.

| Temperature [°C] | Reactor [Volume L] | Operation time [d] | Substrate | Phenol [mgPh·L⁻¹] | OLR [gCOD·L⁻¹·d⁻¹] | Specific phenol conversion rate [mgPh·gVSS⁻¹·d⁻¹] | Removal [%] | References |
|------------------|-------------------|--------------------|-----------|-------------------|-----------------|---------------------------------|-------------|------------|
| 15–18 | EGSB-AF [3.5] | 415 | Phenol, ethanol, butyrate, propionate and acetate | 400–1200 | 5–10 | 15.4–88.9 (ex situ) | >80 (as COD) | Collins et al., 2005 |
| 9.5–15 | EGSB-AF [3.5] | 673 | Phenol, ethanol, butyrate, propionate and acetate | 500–1000 | 1–2 (COD₄) | Up to 68, 43–137 (15°C, ex situ) | 50–98 | Scully et al., 2008 |
| 26 | UASB [2.8] | 512 | Phenol | 1260 | 6 | N.A | 33–100 | Fang et al., 2004 |
| 37 | ASBR [5] | 281 | Phenol/glucose | 120–1200 | N.D | 11–27 | >90 | Rosenkranz et al., 2013 |
| 37 | ASBR [5] | 200 | Phenol | 120–1200 | N.D | 11–31 | >90 | Franchi et al., 2020 |
| 35–55 | AnMBR [6.5] | 270 | Phenol, acetate (high salinity) | 100–500 | 1.26–4.35 | 1.2–3.5 | 40–100 | Muñoz Sierra et al., 2018b |
| 55 | UASB [2.8] | 224 | Phenol | 630 | 0.9 | N.A | 59–99 | Fang et al., 2006 |
| 55 | UASB [1] | 303 | Phenol, phenolics, CGWW | 300–500 (total phenols) | 1.5–2.5 | 30.5–81.3 (ex situ) | 50–60 (total phenols) | <95 | Wang et al., 2011 |
| 55 | AnMBR [6.5] | 388 | Phenol, acetate (high salinity) | 50–800 | 2–4 | 21.3–32.8 (ex situ) | This study |

N.A, not available; N.D, not determined; CGWW, coal gasification wastewater.
concentration that is required to enhance phenol conversion and to avoid high VFA concentrations in the permeate. In such research, concentrations in the range of 0.3–1.0 gCOD L\(^{-1}\) acetate, and 500–800 mgPh L\(^{-1}\) phenol are recommended. Applying similar salinity conditions, we propose an OLR of 2.0–2.2 gCOD L\(^{-1}\) d\(^{-1}\) and phenol loading rates of 15–20 mgPh gVSS\(^{-1}\) d\(^{-1}\) in order to maximize the phenol conversion capacity, without compromising the methanogenic activity.

**Microbial Community Structure**

*Petrotoga* was enriched to 21.1% during the AnMBR long-term operation. This bacteria has been enriched under anaerobic thermophilic conditions from an oil reservoir containing mostly halophilic species (Dellagnezze et al., 2016). Similarly, high salinity conditions will enrich for salt-tolerant and halophilic *Thermovirga* and *Clostridium* species (Muñoz Sierra et al., 2018a). *Acetomicrobium hydrogeniformans* sp., which also increased during phase III, is found in oil production water and is capable of producing hydrogen. Some species required NaCl for growth (Cook et al., 2018). In the case of the Clostridia class, the genera found *Syntrophaceticus* are known for their capability of syntrophic acetate oxidation, and *Pelotomaculum* play an important role in the conversion of phenol and benzoate under methanogenic conditions (Chen et al., 2008).

The abundance of bacteria of the classes Actinobacteria, Bacilli, Synergistia, Gammaproteobacteria, and Thermotogae increased in phase III. Microorganisms corresponding to these classes have been found in thermophilic saline environments. Clostridia class remained in a comparable relative abundance along with the phases, but with changes at genus level. Especially, microorganisms that belong to this class have been reported as the most essential fermentative bacteria and syntrophic phenol degraders as *Pelotomaculum* (Chen et al., 2009; Muñoz Sierra et al., 2019).

Our results showed a high relative abundance of uncultured microorganisms in the AnMBR. Bacteria belonging to the class JS1, and the candidate phylum Atribacteria (see Supplementary Figure 1) were dominant during phases I (9.15%) and II (9.41%). Microorganisms belonging to Atribacteria, mostly have been found in deep-sea methane-rich sediments (Carr et al., 2015). Lee et al. (2018) suggested a fermentative role of these microorganisms, capable of using various substrates, and syntrophic acetate oxidation coupled with hydrogen scavenging methanogens. Recently, the first culturable representative strain of this phylum was isolated and it was confirmed that it plays a role in hydrogenogenic fermentative metabolism (Katayama et al., 2019). In our case, the highest species similarity found was *Bacillus alkalitolerans strain T3-209*, with only 83%.

In the archaea domain, the microbial dynamics indicated a clear switch from acetotrophic to hydrogenotrophic methanogens in the class Methanomicrobia. Both the microbial community structure and the observation that at the end of the experiment the biomass hydrogenotrophic methanogenic activity was substantially higher than the acetate-fed methanogenic activity, support our hypothesis that acetate conversion switched from aceticlastic methanogenesis to acetate oxidation coupled to hydrogenotrophic methanogenesis.

**Perspectives and Further Research**

The observed AnMBR performance perturbation following phenol accumulation indicated that the cultivated thermophilic phenol-degrading biomass was dependent on the presence of active methanogens. A drop in the hydrogenotrophic methanogenic activity may have led to the observed reduced phenol conversion capacity. It should be noted that the entire experiment was performed under high salinity (18 gNa\(^+\) L\(^{-1}\)) conditions. Further research will focus on the role of syntrophic acetate oxidation and phenol degradation intermediates (e.g., benzoate). Our present study showed that highly saline phenolic wastewaters indeed could be treated in a thermophilic AnMBR. However, the achievable phenol conversion capacity was restricted to 20 mgPh gVSS\(^{-1}\) d\(^{-1}\), determining an applicable phenol loading rate of about 0.12 gPh L\(^{-1}\) d\(^{-1}\). Although thermophilic operation will bring operational energy benefits when treating high-temperature industrial wastewaters with the target of process water reuse, the phenol conversion capacity of the reactor will be lower than when opting for mesophilic operation.

**CONCLUSION**

Maximum COD and phenol removal efficiencies of about 95% were achieved during the long-term thermophilic AnMBR operation at 18 gNa\(^+\) L\(^{-1}\). However, severe perturbations occurred following relatively small increments in the phenol loading rate from 0.01 to 0.12 gPh L\(^{-1}\) d\(^{-1}\). Moreover, by exceeding a sludge phenol loading rate of 20 mgPh gVSS\(^{-1}\) d\(^{-1}\) the system immediately responded in phenol build-up to concentrations higher than 200 mgPh L\(^{-1}\) leading to significant deterioration of methanogenesis. The observed maximum phenol conversion rates were 21.3 ± 0.2 and 32.8 ± 0.5 mgPh gVSS\(^{-1}\) d\(^{-1}\) in the AnMBR, and in *ex situ* batch test at the end of the reactor operation, respectively. The absence of caproate in the VFAs spectrum inferred that the phenol conversion pathway was likely via benzoate carboxylation. The assessed hydrogenotrophic SMA was a factor 2.3 higher than the acetate-fed SMA. Correspondingly, microbial population dynamics indicated that hydrogenotrophic methanogens were enriched during the long-term operation and Clostridia class was dominant. Overall, thermophilic AnMBR operation under high salinity seemed to be susceptible to sudden increase in phenol loading rate or phenol shocks, indicating that the specific phenol conversion capacity under the studied conditions was limiting the treatment process.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ ena, PRJEB38467.
AUTHOR CONTRIBUTIONS

JM designed the experiment. JM and VG performed the experiments, contributed with the reactor’s maintenance, and carried out analytical methods. JM analyzed the data and wrote the manuscript. DC-G did the Bioinformatics analysis. HS and JvL provided feedback that helped shape the research and analysis, and critically revised the manuscript. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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