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Removal of nonylphenol ethoxylate surfactant in batch reactors: emphasis on methanogenic potential and microbial community characterization under optimized conditions

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ABSTRACT

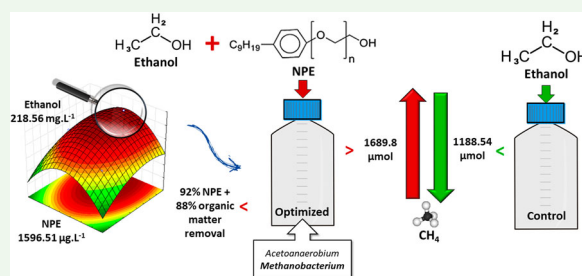
Nonylphenol ethoxylate (NPE) is an endocrine-disrupting chemical that has bioaccumulative, persistent and toxic characteristics in different environmental matrices and is difficult to remove in sewage treatment plants. In this study, the effects of the initial concentration of NPE ($0.2 \pm 0.03 - 3.0 \pm 0.02 \text{ mg. L}^{-1}$) and ethanol ($73.9 \pm 5.0 - 218.6 \pm 10.6 \text{ mg. L}^{-1}$) were investigated using factorial design. Assays were carried out in anaerobic batch reactors, using the Zinder basal medium, yeast extract (200 mg. L^{-1}), vitamin solution and sodium bicarbonate (10% v/v). The optimal conditions were $218.56 \text{ mg. L}^{-1}$ of ethanol and $1596.51 \text{ } \mu\text{g. L}^{-1}$ of NPE, with 92% and 88% of NPE and organic matter removal, respectively, and methane yield ($1689.8 \pm 59.6 \text{ mmol}$) after 450 h of operation. In this condition, bacteria potentially involved in the degradation of this surfactant were identified in greater relative abundance, such as *Acetoanaerobium* (1.68%), *Smithella* (1.52%), *Aminivibrio* (0.91%), *Petrimonas* (0.57%) and *Enterobacter* (0.47%), as well as archaea *Methanobacterium* and *Methanoregula*, mainly involved in hydrogenotrophic pathway.

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Non-ionic surfactant; co-substrates; *acetoanaerobium*; central composite rotational delineation; endocrine disruptor



1. Introduction

Nonylphenol ethoxylate (NPE) is a non-ionic surfactant and, due to its surface-active properties, it is widely used as a raw material in the formulation of industrial, agricultural, domestic use, personal care and cosmetics products [1, 2]. In this context, due to the numerous applications, since its first synthesis in 1940, NPE production has increased exponentially in recent decades (de Bruin et al., 2019; Fan et al., 2018). Worldwide, NPE production was estimated at 17.6 million tons in 2015 and an expected 24.19 million tons for 2022 [3].

Therefore, because NPE is produced on a large scale, it has been identified in various environmental matrices, such as soil [4], atmosphere [5], sediments [6, 7], surface

water [8, 9] and, mainly in effluents and sludge from wastewater treatment plants (WWTP) [10, 11, 12, 13, 14]. Thus, it is clear that in the environment, NPE is closely related to anthropogenic activities.

In different environments, NPE degradation occurs through the gradual loss of its ethoxylated groups, resulting in more toxic, persistent and bioaccumulative byproducts, such as nonylphenol monoethoxylate (NPE₁), nonylphenol diethoxylate (NPE₂) and 4-nonylphenol (4-NP) [10, 14]. It is noteworthy that 4-NP is the main by-product formed from NPE degradation [15].

The byproducts of the NPE degradation (NPE₁, NPE₂, 4-NP) are toxic, carcinogenic and estrogenic. In particular, 4-NP can mimic the female steroid hormone 17 β -estradiol, interfering with its synthesis, secretion,

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transport, binding and natural action, causing problems related to homeostasis, development, reproduction, maintaining female characteristics, maturation and functioning of accessory sexual organs [16, 17, 18]. Exposure to NPE and its byproducts can trigger several other problems, such as a decrease in sperm count, an increase in congenital malformations of the male reproductive tract, testicular cancer, respiratory problems, eye and skin conditions, feminization of aquatic organisms, a decrease in male fertility, a reduction in oxytocin secretion and cell cycle deregulation, therefore classified as an endocrine deregulator [19, 20]. In addition, phytotoxic effects were reported, such as a reduction in plant biomass, length and germination capacity, as NPE has the ability to resemble natural aleochemicals, also classified as a phytoendocrine disruptor [21, 22, 13, 23, 24]

Considering the current issue regarding NPE and its byproducts to the aquatic and terrestrial environment, as well as to humans, which have long and short-term harmful effects, the need to investigate the best conditions for removing this compound from treatment systems is clear. It is widely reported that NPE can be degraded by microbial consortia under aerobic conditions [25, 26, 27, 12]. On the other hand, under anaerobic conditions, NPE degradation can occur with lower efficiency and has been poorly documented, especially using anaerobic batch reactors.

Adding metabolic co-substrates can be an alternative to greater surfactant removal. Several authors [28, 29, 30, 31] reported that adding ethanol as a metabolic co-substrate favoured the removal efficiency of Linear Alkylbenzene Sulfonate (LAS), Linear Alcohol Ethoxylate (LAE) and 4-NP surfactants, respectively. Dornelles et al. [32] observed the removal of 78% ($76 \mu\text{g.L}^{-1}$) of 4-NP in an Anaerobic Fluidized Bed Reactor (AFBR), fed with synthetic substrate (586 mg carbon oxygen demand- COD L^{-1}) and a Hydraulic Detention Time (HDT) of 18 h. Under these conditions, biomass adapted to 4-NP and adding ethanol (200 mg.L^{-1}) was essential for greater surfactant removal.

Therefore, adding an easily biodegradable substrate, such as ethanol, can be used to obtain energy, production and cell maintenance, as an electron donor source for microbial metabolism, helping in the degradation of recalcitrant and toxic pollutants, such as NPE and its byproducts (4-NP) [29, 33, 34].

Given the high number of operational variables that can influence the degradation of these surfactants, adopting statistical methods is necessary, such as experimental design, a process by which certain factors are selected and varied in a controlled manner, to obtain effects on the response of interest, as well as the optimal ranges for NPE degradation and removal.

Factorial design, Plackett-Burman (PB) design and Central Composite Rotational Delineation (CCRD) are examples of experimental design possibilities [35].

Based on the above, the aim of this study was to evaluate the methanogenic potential of anaerobic sludge subjected to different concentrations of NPE and ethanol, as well as to obtain the optimal conditions for NPE degradation and organic matter removal in batch reactors, using CCRD, in addition to characterizing the microbial community of the reactors, through the massive sequencing of the 16S rRNA gene.

2. Material and methods

2.1. Nonylphenol ethoxylate

Nonylphenol Ethoxylate with 87% purity, CAS N° 27177-08-8, with molecular formula $\text{C}_9\text{H}_{19}\text{C}_6\text{H}_4(\text{OCH}_2\text{CH}_2)_n\text{OH}$ was obtained from *Destilaria Bauru*® LTDA (Catanduva/São Paulo/Brazil). This compound for commercial and industrial use was purchased in a viscous, colourless liquid state, with a cloud point at 54.50°C , a water/humidity ratio of 0.095% and a pH of 7.5 in its concentrated form. The stock/standard solution was prepared in Milli-Q Plus Ultra System ultra-purified water (Billerica, MA), at a final concentration of 500 mg.L^{-1} . The same chemical was used for NPE determination using High Performance Liquid Chromatography.

2.2. Synthetic substrate

In anaerobic batch reactors, Zinder basal medium was used, prepared in Milli-Q Plus Ultra System ultrapurified water (Billerica, MA), with the following composition: NH_4Cl (0.5 g.L^{-1}), K_2HPO_4 (0.4 g.L^{-1}), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.1 g.L^{-1}), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.05 g.L^{-1}), trace metal solution (10 mL^{-1}), vitamin solution (10 mL^{-1}) and 10 mL^{-1} of sodium bicarbonate solution (10%). The composition of the trace metal solution is as follows: nitrile acetic acid (4.5 g.L^{-1}), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.556 g.L^{-1}), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (0.086 g.L^{-1}), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.17 g.L^{-1}), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.21 g.L^{-1}), H_3BO_4 (0.19 g.L^{-1}), NiCl_2 (0.02 g.L^{-1}), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.01 g.L^{-1}) [36]. The composition of the vitamin solution is as follows (Dubourguier et al., 1987): biotin (0.002 g.L^{-1}), folic acid (0.002 g.L^{-1}), thiamin-HCl (0.005 g.L^{-1}), riboflavin (0.005 g.L^{-1}), nicotinic acid (0.005 g.L^{-1}), calcium pantothenate (0.005 g.L^{-1}), pyridoxine -HCl (0.010 g.L^{-1}), vitamin B12 (0.0001 g.L^{-1}), lipoic acid (0.005 g.L^{-1}).

Yeast extract (Kasvi, K25-611005) (200 mg.L^{-1}) was used to all reactors as a source of B-complex vitamins, nitrogen and carbon [37, 38]. Ethanol was added to all reactors in the range of 100 mg.L^{-1} – 200 mg.L^{-1} as described by [33].

2.3. Inoculum

In the batch reactor assays, an Upflow Anaerobic Sludge Blanket (UASB) Reactor and a poultry slaughterhouse wastewater treatment sludge blanket (Pereiras, São Paulo, Brazil) with $55.60 \pm 0.884 \text{ g.L}^{-1}$ of total solids (TS) were used as inoculum. Before inoculation, the anaerobic sludge was previously conditioned at 30°C for soluble organic matter consumption until the methane production stabilized (approximately 10 days) [32].

2.4. Kinetic batch assays

The assays carried out in batch reactors were prepared in triplicate in 1000 mL borosilicate flasks (Duran®) with 350 mL of reaction volume and 650 mL of headspace. The reactors were inoculated with 2 g.L^{-1} of total volatile solids (TVS). After inoculation, the reactors were submitted to a N_2 flow (100%) for 10 min, closed with a butyl lid and plastic screw and kept under stirring at 180 rpm at $30 \pm 1^\circ\text{C}$.

2.5. Central composite rotational delineation

The influence of the NPE and ethanol concentration was evaluated by the CCRD and analysis of the response surface. Therefore, 11 conditions were analyzed, as summarized in Table 1, besides a control assay without NPE. The NPE (X_1) and ethanol (X_2) concentrations were previously defined according to previous studies [39, 31, 40, 32, 33, 30], with $0.6\text{--}3.0 \text{ mg.L}^{-1}$ of NPE and $100\text{--}200 \text{ mg.L}^{-1}$ of ethanol.

2.6. Physico-chemical and chromatographic analyses

Samples were analyzed at the beginning and end of the reactor operation, such as the pH and COD, according to the Standard Methods for Examination of Water and Wastewater [41]. The determination of volatile fatty acids (VFA) and ethanol (EtOH) was performed in gas chromatography using liquid–liquid extraction (LLE) [42]. Methane was analyzed by gas chromatography (GC-2010) (Shimadzu, Japan), with a thermal conductivity detector and a Carboxen 1010 Plot column ($30\text{m} \times 0.53 \text{ mm}$). The carrier gas used was argon (12 mL min^{-1}), injector temperature of 220°C , column temperature of 130°C , detector temperature of 230°C and sample volume of $500 \mu\text{L}$.

NPE was determined by High Performance Liquid Chromatography (HPLC) (Shimadzu® Co., Kyoto Japan) equipped with an LC-10ADvp pump, FCV-10 ALvp valve, CTO-10Avp oven, RF10AXL fluorescence detector

whose wavelength was 220 nm (λ_{exc}) and 305 nm (λ_{em}) and SCL-10Avp controller unit and Class-VP software (Shimadzu®) for system control. A chromatographic column C-8 (Agilent®) was used with solid phase, and the chromatographic conditions used in this study were 0.8 mL.min^{-1} elution flow, oven temperature at 40°C , $100 \mu\text{L}$ injection volume, acetonitrile/water at a ratio of 90:10 (v/v) as the mobile phase.

Liquid samples collected from anaerobic batch reactors to determine NPE concentration were purified and concentrated using the Solid Phase Extraction (SPE) method with C18-ODS cartridges (Agilent Technologies®, Octadecyl Modified Silica 500 mg). Cartridge conditioning was performed with 2 mL of methanol, and then 2 mL of the sample containing NPE was added, elution with 1 mL of ethanol followed by 1 mL of ultra-purified water [29, 32, 43]

At the end of the reactor operation, biomass samples were taken to determine the adsorbed NPE and, later, to calculate the mass balance. The quantification of NPE adsorbed on biomass was based on the methodology described by [44]. The mass balance was determined as described by [45], according to Equations 1 and 2:

$$X_{\text{rem}} = X_{\text{influ}} - X_{\text{efflu}} \quad (1)$$

$$X_{\text{deg}} = X_{\text{influ}} - (X_{\text{efflu}} + X_{\text{ads}}) \quad (2)$$

Where:

X = NPE mass

X_{rem} = NPE mass removed by biological and physical processes

X_{influ} = NPE mass in the influent

X_{efflu} = NPE mass in the effluent

X_{deg} = NPE mass removed by biological processes

X_{ads} = NPE mass adsorbed in the sludge

2.7. Data analysis

The modified Gompertz model [46] was used to obtain the parameters of cumulative CH_4 production (H), maximum CH_4 production potential (P), maximum CH_4 production rate (R_m) and start-up time for CH_4 production (λ). The parameters were calculated using OriginPro® 8.1 software (OriginLab Corporation®), as described by [34].

$$H = P \exp \left\{ -\exp \left[\frac{R_m}{P} e (\lambda - t) + 1 \right] \right\} \quad (3)$$

where:

H = Cumulative CH_4 production (mmol)

P = Maximum CH_4 production potential (mmol)

R_m = Maximum CH_4 production rate (mmol.h^{-1})

λ = start-up time for CH_4 production (hours)

Table 1. Factorial Design Matrix (CCRD).

Condition	Coded Variables		Real Variables		
	Nonylphenol Ethoxylate (X_1)	Ethanol (X_2)	Nonylphenol Ethoxylate (mg.L^{-1})	Ethanol (mg.L^{-1})	NPE/EtOH (%)
1	0	-1.41	1.6 ± 0.03	73.9 ± 5.0	2.2
2	-1	1	0.6 ± 0.04	197.4 ± 6.4	0.30
3	1	-1	2.6 ± 0.05	95.1 ± 5.9	2.70
4	1	1	2.6 ± 0.05	197.4 ± 15.1	1.30
5	1.41	0	3.0 ± 0.02	146.2 ± 14.1	2.0
6	-1	-1	0.6 ± 0.02	95.1 ± 3.8	0.6
7	-1.41	0	0.2 ± 0.03	146.2 ± 26.4	0.1
8	0	1.41	1.6 ± 0.10	218.6 ± 10.6	0.7
9	0	0	1.6 ± 0.12	146.2 ± 16.1	1.10
10	0	0	1.6 ± 0.09	146.2 ± 18.3	1.10
11	0	0	1.6 ± 0.08	146.2 ± 11.8	1.10
Control	-	-	-	198.6 ± 10.4	-

The experimental validation was carried out from the CCRD assays to obtain the optimized condition with $1.6 \pm 0.10 \text{ mg.L}^{-1}$ of NPE and $218.6 \pm 10.6 \text{ mg.L}^{-1}$ of ethanol (EtOH).

t = Time (hours)

e = 2.718281828 (Euler number)

The response surface and parameter analysis (F Test and Analysis of Variance - ANOVA) were carried out using the Protimiza Experimental Design platform (Rodrigues and lemma, 2014). Non-significant coefficients ($p > 0.05$) were eliminated and the model parameters were recalculated. ANOVA was used to assess the significance of factors (NPE and ethanol) in the adjustment of the first-order polynomial equation. Finally, the response surface containing only the significant coefficients was obtained using the Statistica 10 software (StatSoftInc©, 2014, USA). The same factorial design matrix (Table 1) was used to obtain nonylphenol (Y_1) and organic matter (COD) (Y_2) removal responses.

2.8. Taxonomic characterization and functional inference

Biomass samples from optimized anaerobic batch reactors ($1.6 \pm 0.10 \text{ mg.L}^{-1}$ of NPE and $218.6 \pm 10.6 \text{ mg.L}^{-1}$ of ethanol) and control (without NPE) were collected at the end of the operation (after approximately 180 h) and washed with phosphate-buffered saline (PBS; 8% NaCl, 0.2% KCl, 1.44% Na_2HPO_4 , 0.24% KH_2PO_4) [47]. DNA extraction was performed using the phenolchloroform method (Sakamoto et al., 2019), and the extraction product was quantified ($\text{ng}/\mu\text{l}$) and purified (ratio 260/280 nm, rate ≥ 1.8) in an ND-2000 spectrophotometer (Thermo Fisher Scientific, USA), and DNA fragments were verified by agarose gel electrophoresis.

Amplification of the 16S rRNA gene was performed using a Polymerase Chain Reaction (PCR) with the 341F/806R primer set (Bacteria Domain) of the 16S rRNA gene, variable region V3-V4, and the U519F/806R primer set (Archaea domain) of the 16S rRNA gene, variable region V4. Sequencing was performed via the Illumina - MiSeq platform following the manufacturer's

guidelines. The sequences were submitted to the NCBI database under number PRJNA717379.

Functional profile inference was performed using the Piphillin pipeline (<http://secondgenome.com/Piphillin/>) [48] for the Kyoto Encyclopedia of Genes and Genomes (KEGG) database of October 2018, with 97% identity cutoff. This metagenomic inference tool is based on the functional content of microorganism genes based on known information from the genome of the closest organisms taxonomically [49].

The KO (KEGG Orthology) of interest were filtered through basic R language commands (<https://www.R-project.org/>) and analyzed through the ggplot2 package [50]. KO of interest for the methanogenic pathway were filtered out according to [51]. Metabolic maps were inferred based on the KEGG Mapper - Reconstruct Pathway tool [52]. The EC numbers (Enzyme Commission Numbers) referring to each KO were analyzed using The Comprehensive Enzyme Information System (BRENDA) platform (<https://www.brenda-enzymes.org/>).

3. Results and discussion

3.1. Optimization of NPE and organic matter removal

Factorial design, using the CCRD, was carried out to investigate the individual and synergistic effects of the NPE concentration (X_1) and EtOH (X_2) on the response of NPE removal (Y_1), as well as of organic matter removal (Y_2). Table 2 summarizes the average results of each assay.

According to the statistical analysis of the factorial design, the optimal levels for the two factors studied (NPE and ethanol concentrations) were observed, as well as their effects on NPE and organic matter removal. By applying multiple linear regression with the experimental data from the factorial design

Table 2. Variables, responses and kinetic parameters of the CCRD assays in batch reactors.

Condition	Response Variables		Kinetic Parameters (CH ₄)				Other Parameters				Mass Balance				
	Removal (Y ₁) (%)	COD Removal (Y ₂) (%)	P (mmol)	R _m (mmol h ⁻¹)	λ (h)	R ²	Initial COD (mg.L ⁻¹)	Final COD (mg.L ⁻¹)	mLCH ₄ g ⁻¹ COD _{rem}	Initial pH	Final pH	Initial NPE (mg.L ⁻¹)	Removal (%)	Adsorption (%)	Degradation (%)
1	75 ± 1.4	51 ± 2.3	471.9 ± 19.2	5.5 ± 0.4	86.5 ± 2.8	0.98	233.3 ± 3.1	140.4 ± 4.5	510.5 ± 5.2	7.48 ± 0.05	7.15 ± 0.18	1.6 ± 0.03	75 ± 1.4	29 ± 0.2	46 ± 1.0
2	61 ± 6.2	68 ± 9.1	1337.1 ± 19.1	19.9 ± 2.5	89.6 ± 1.1	0.99	531.3 ± 34.4	172.7 ± 60.8	374.7 ± 3.8	7.47 ± 0.12	7.12 ± 0.22	0.6 ± 0.04	61 ± 6.2	26 ± 3.2	35 ± 3.0
3	85 ± 1.9	56 ± 8.9	493.3 ± 4.4	25.3 ± 0.7	111.0 ± 0.7	0.99	361.6 ± 11.6	160.7 ± 37.7	246.7 ± 1.7	7.47 ± 0.10	7.10 ± 0.15	2.6 ± 0.05	85 ± 1.9	39 ± 9.7	46 ± 3.1
4	87 ± 1.3	75 ± 7.1	1110.2 ± 37.1	29.3 ± 0.9	107.9 ± 2.9	0.98	630.0 ± 35.0	154.0 ± 36.4	234.4 ± 3.6	7.52 ± 0.02	7.24 ± 0.20	2.6 ± 0.05	87 ± 1.3	38 ± 2.5	49 ± 2.6
5	86 ± 2.9	63 ± 7.8	758.2 ± 2.5	18.5 ± 1.6	144.6 ± 2.1	0.98	520.3 ± 61.0	191.4 ± 41.2	231.6 ± 0.8	7.50 ± 0.01	7.18 ± 0.22	3.0 ± 0.02	86 ± 2.9	39 ± 0.3	47 ± 8.3
6	51 ± 14.0	51 ± 5.4	617.1 ± 35.2	16.9 ± 1.3	69.0 ± 1.9	0.98	369.4 ± 12.1	181.4 ± 24.0	329.8 ± 1.0	7.49 ± 0.03	7.15 ± 0.14	0.6 ± 0.02	51 ± 4.0	26 ± 2.5	25 ± 5.6
7	29 ± 15.9	73 ± 6.3	1147.6 ± 31.9	15.9 ± 0.4	45.9 ± 3.3	0.98	327.4 ± 35.7	90.0 ± 10.2	385.8 ± 1.5	7.50 ± 0.09	7.12 ± 0.18	0.2 ± 0.03	29 ± 5.9	12 ± 6.5	17 ± 1.1
8	92 ± 1.9	88 ± 2.1	1689.8 ± 59.6	27.1 ± 1.4	89.5 ± 2.8	0.98	650.6 ± 30.1	76.7 ± 9.9	395.2 ± 0.7	7.49 ± 0.07	7.17 ± 0.11	1.6 ± 0.10	92 ± 1.9	35 ± 4.4	57 ± 1.2
9	87 ± 1.1	82 ± 2.2	1546.7 ± 53.2	26 ± 2.6	127.6 ± 2.9	0.99	560 ± 20.0	98 ± 10.0	336.4 ± 2.5	7.51 ± 0.05	7.21 ± 0.20	1.6 ± 0.12	87 ± 1.1	32 ± 4.7	55 ± 2.3
10	85 ± 5.3	84 ± 1.4	1462.7 ± 24.3	24 ± 1.8	128.5 ± 1.6	0.99	534.5 ± 19.9	87.3 ± 4.3	328.7 ± 3.0	7.51 ± 0.10	7.20 ± 0.22	1.6 ± 0.09	85 ± 5.3	35 ± 6.2	50 ± 3.5
11	86 ± 4.7	84 ± 0.6	1474.7 ± 19.1	25.2 ± 1.5	130.2 ± 1.3	0.99	541.3 ± 21.6	84.7 ± 4.5	324.5 ± 2.8	7.50 ± 0.06	7.20 ± 0.19	1.6 ± 0.08	86 ± 4.7	35 ± 7.5	51 ± 4.8
Control	–	86 ± 8.1	1290.4 ± 4.90	19.3 ± 0.5	47.0 ± 3.5	0.99	554.6 ± 14.3	75.1 ± 2.5	270.4 ± 1.5	7.50 ± 0.07	7.20 ± 0.20	–	–	–	–

Maximum CH₄ production potential (P), maximum CH₄ production rate (R_m), start-up time for CH₄ production (λ), Chemical oxygen demand removed (COD_{rem}) and R² of the modified Gompertz model. The errors presented after the means (±) correspond to the standard deviation calculated for each condition in triplicate.

(Table 3), empirical models were obtained for the NPE removal - Y₁ (Equation 4) and organic matter removal - Y₂ (Equation 5) variables as a function of NPE concentration (X₁) and EtOH concentration (X₂).

$$Y_1 = 84.94 + 17.58X_1 - 13.79X_1^2 + 4.51X_2 \quad (4)$$

$$Y_2 = 82.00 + 4.68X_1 - 13.81X_1^2 + 11.04X_2 - 6.06X_2^2 \quad (5)$$

where:

Y₁ – The value of the dependent variable (NPE removal)

Y₂ – The value of the dependent variable (organic matter removal - COD)

X₁ –NPE concentration

X₂ –EtOH concentration

The linear and quadratic effect of NPE, as well as the linear effect of EtOH were considered significant in the regression coefficient analysis ($p < 0.05$); while the interaction between the variables (EtOH and NPE) and the quadratic effect of EtOH did not affect the response (NPE removal) ($p > 0.05$). Regarding organic matter removal, the linear and quadratic effect of the initial concentrations of NPE and EtOH were statistically significant ($p < 0.05$). On the other hand, the interaction between effects (X₁,X₂) did not affect this response (Table 4).

It can be observed for the NPE concentration and EtOH concentration factors that there were significant effects on the responses of NPE removal and organic matter removal. Regarding the NPE removal response (Y₁), it was observed that the linear and quadratic effects of the NPE concentration are, in this order, the most significant. Regarding the organic matter removal response (Y₂), the quadratic effect of the NPE concentration was the most important followed by the linear effect of the EtOH concentration.

In the empirical quadratic models to examine the goodness-of-fit obtained for the two response variables (Y₁ and Y₂), a statistically significant regression was observed, with p -value < 0.0001 , confirming that the models obtained with the factorial design data represent the CCRD (Table 4).

From the ANOVA, F_{calc} values of the two responses were obtained, higher than the tabulated values (level of 90%). For NPE removal, F_{calc} = 91.82 and F_{tab} = 4.347 were obtained, while for organic matter removal, F_{calc} = 56.2 and F_{tab} = 5.192 were obtained. According to [53], a model is considered highly predictive when the F_{calc} value is greater than ten times the F_{tab}, a result that was observed for the two studied response variables (NPE and organic matter removal).

Table 3. Regression Coefficients for NPE and organic matter removal.

Response	Factors	Regression Coefficient	Standard Error	t calculated	p-value (Prob > F)
NPE Removal (%)^a	Mean	84.94	1.56	54.35	< 0.0001*
	X₁ (L)	17.58	1.32	13.36	0.0001*
	X₁ (Q)	-13.79	1.50	-9.22	0.0002*
	X₂ (L)	4.51	1.32	3.43	0.0126*
	X₂ (Q)	-1.13	1.62	-0.70	0.5173
	X₁.X₂	-2.00	1.92	-1.04	0.3453
Organic matter removal (%)^b	Mean	82.00	1.83	44.89	< 0.0001*
	X₁ (L)	4.68	1.12	4.19	0.0058*
	X₁ (Q)	-13.81	1.33	-10.37	< 0.0001*
	X₂ (L)	11.04	1.12	9.87	0.0001*
	X₂ (Q)	-6.06	1.33	-4.55	0.0039*
	X₁.X₂	0.50	1.72	0.29	0.7828

a: $R^2 = 0.9752$, $p < 0.05$, Pure Error = 9.24; *Significant Error at 95% confidence.

b: $R^2 = 0.9745$, $p < 0.05$, Pure Error = 8.45.

From the empirical models, the response surfaces and contour curves for NPE removal and organic matter removal were obtained (Figure 1).

In linear terms and within the analyzed ranges of NPE (0.214–2.978 mg.L⁻¹) and EtOH (73.93–218.56 mg.L⁻¹), it was observed that the increase in the NPE and EtOH concentrations positively interfered in the NPE and organic matter removal. Higher NPE and organic matter (COD) removal efficiency, i.e. $92 \pm 1.9\%$ and $88 \pm 2.1\%$, respectively, were observed with 1.6 ± 0.10 mg.L⁻¹ of NPE and 218.6 ± 10.6 mg.L⁻¹ of EtOH (Table 2). In the same condition, the highest NPE degradation was also observed ($57 \pm 1.2\%$). It is noteworthy that degradation was the main route involved in NPE removal (Table 2). [11] also found that 50% of the NPE byproduct removal (4-NP) was by degradation and 32% by adsorption in nine different sewage treatment systems.

In addition, it was found that when the NPE concentration was increased, the percentage of adsorption in the anaerobic sludge increased (Table 2). [54] observed NPE adsorption in soil and sediment under the same operational conditions of this study (pH = 7.0 and T = 25°C) with three NPE concentrations (10, 20 and 30 mg.L⁻¹). The authors found that with increasing NPE concentration there was greater adsorption in both adsorbents, following a pseudo-second order kinetic model.

The increase in NPE and organic matter removal in the presence of EtOH (73.93– 218.56 mg.L⁻¹) may be

related to metabolism induction as EtOH is an easily degradable carbon source that can be used to obtain energy, cell production and maintenance, as an electron donor source for microbial metabolism and helps in recalcitrant compound degradation, such as LAS [31] and drugs such as triclosan and propranolol [45]. [55] reported that easily degraded compounds, such as short-chain alcohols (i.e. EtOH), can induce the disassimilative metabolism of sulfate-reducing bacteria (SRB) and favour organic matter removal.

Finally, the assay validation in anaerobic batch reactors was carried out to confirm the optimal conditions predicted by the model. Therefore, 218.56 mg.L⁻¹ of EtOH and 1.596 mg.L⁻¹ of NPE were used for the two response variables (NPE and organic matter removal). Under these conditions, 90.5% of NPE removal and 87% of organic matter removal were obtained, that is, there was a good correlation with the predicted values of 92% and 88%, respectively.

3.2. Methanogenic potential

The average values of cumulative methane production and other kinetic parameters obtained based on the cumulative methane production curve are summarized in Table 2.

Based on the kinetic analyses, it can be observed that when there is an increase in the EtOH concentration, it

Table 4. Analysis of Variance (ANOVA) for NPE and organic matter removal.

Response	Variation source	Sum of Squares	Degrees of freedom	Squared mean	F _{calc}	F _{tab}	p - value
NPE removal (%)	Regression	3810.0	3	1270.0	91.8	4.34	0.00001
	Residual	96.9	7	13.84			
	Goodness-of-fit	94.9	5	19	19.0	19.29	0.05082
	Pure Error	2.0	2	1.0			
	Total	3906.9	10				
	Regression	2252.7	4	563.2	56.2	5.19	0.00007
Organic matter removal (%)	Residual	60.1	6	10.0			
	Goodness-of-fit	58.1	4	14.5	14.5	19.24	0.06548
	Pure Error	2.0	2	1.0			
	Total	2312.7	10				

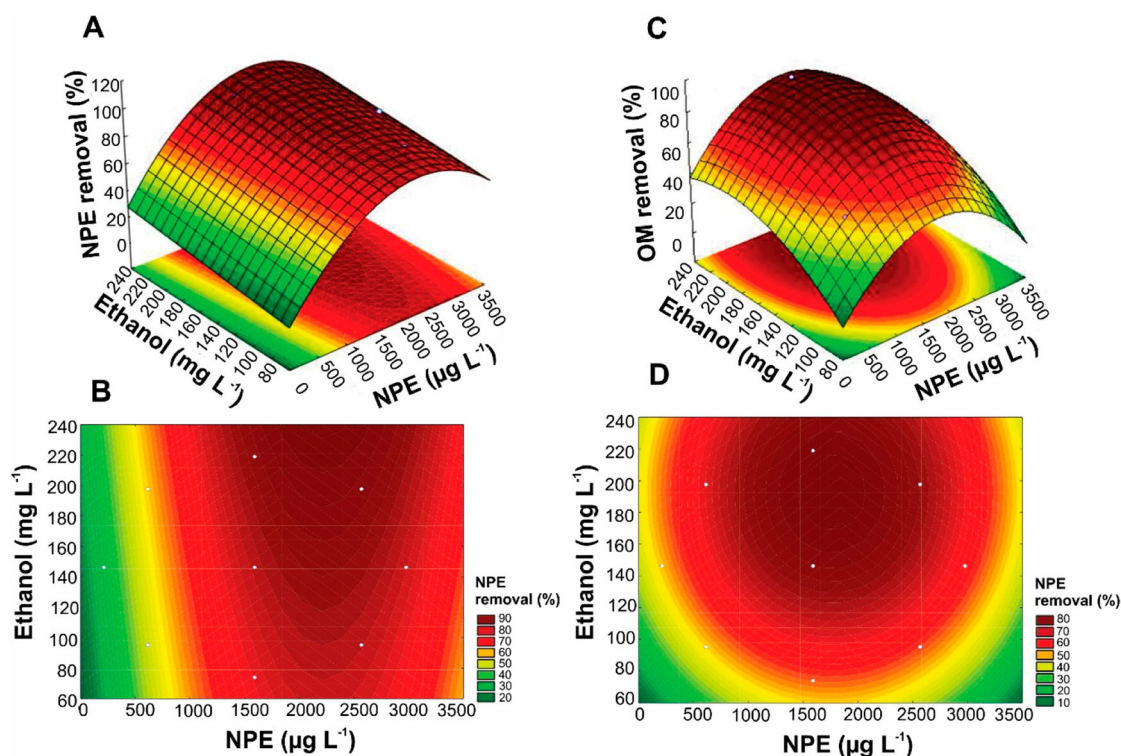


Figure 1. Response and contour surface for NPE (A) and organic matter (B) removal as a function of EtOH and NPE concentration.

resulted in a higher P value, i.e. 471.9 ± 19.2 mmol of CH_4 for 73.9 mg.L^{-1} of EtOH (Condition 1) and 1689.8 ± 59.6 mmol CH_4 to 218.6 mg.L^{-1} EtOH (Condition 8). For the same conditions, an increase in R_m was also observed, from $5.5 \pm 0.4 \text{ mmol h}^{-1}$ (Condition 1) to $26 \pm 2.6 \text{ mmol h}^{-1}$ (Condition 8).

In the ethanol route, acetic acid is formed directly from its oxidation and the amount of energy that can be conserved by the acetoclastic methanogenic archaea does not depend on the H_2 concentration in the medium. For these archaea, acetate is activated by acetyl-CoA which is cleaved into methyl and carbonyl, which are

further reduced to methane and oxidized to CO_2 [56]. In this context, VFA concentrations were observed below the detection limit (2 mg HAc L^{-1}) for all conditions at the end of the reactor operation. From this possibility, the use of acetic acid by homoacetogenic bacteria and methanogenic archaea can be inferred, which corroborates the greater methanogenic potential obtained with the use of EtOH. The VFA data are available in Table 15.

Furthermore, the higher methane production associated with a higher EtOH concentration may be related to SRB, which, in the absence of sulfate, can carry out incomplete fermentation of organic substrates. Under conditions of strict anaerobiosis, such bacteria can use a wide range of substrates, including volatile organic acids, aromatic acids, hydrogen, methanol, ethanol, glycerol sugars, amino acids and phenolic compounds such as NPE [57]. In the present study, the genera *Syntrophobacter*, *Geobacter*, *Petrimonas*, *Desulfobulbus* and *Desulfovibrio* were identified in the optimized condition and control assay (without NPE). These genera may use EtOH in fermentation processes, with concomitant production of acetic acid, CO_2 and H_2 , which favours the cometabolism of acetoclastic and hydrogenotrophic methanogenic archaea [31].

Regarding the NPE concentration, it was observed that, maintaining the EtOH concentration constant and increasing the NPE concentration, there was no significant decrease ($p < 0.05$) of p , as can be observed for

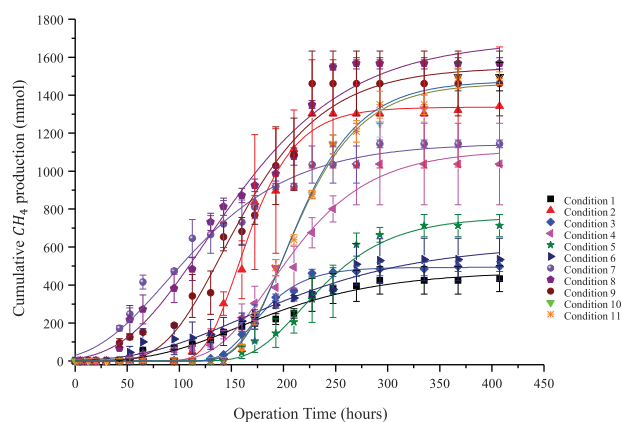


Figure 2. Cumulative methane production and adjustment to the modified Gompertz model for different batch reactor tests.

Condition 2 (0.62 mg.L⁻¹ of NPE and 197.4 mg.L⁻¹ of EtOH) and Condition 4 (2.6 mg.L⁻¹ of NPE and 197.4 mg.L⁻¹ of EtOH); Condition 3 (2.6 mg.L⁻¹ of NPE and 95.1 mg.L⁻¹ of EtOH) and Condition 6 (0.6 mg.L⁻¹ of NPE and 95.1 mg.L⁻¹ of EtOH); Condition 5 (3.0 mg.L⁻¹ of NPE and 146.3 mg.L⁻¹ of EtOH) and Condition 7 (0.2 mg.L⁻¹ of NPE and 146.2 mg.L⁻¹ of EtOH).

Under conditions 2 and 4, the EtOH concentration was constant (197.4 mg.L⁻¹), while that of NPE was respectively 0.62 and 2.6 mg.L⁻¹. For a lower NPE concentration, a higher P (1337.1 ± 19.0 mmol of CH₄) was observed compared to a higher NPE concentration (1110.20 ± 37.1 mmol of CH₄). Likewise, for conditions 3 and 6, with 95.10 mg.L⁻¹ of EtOH and NPE from 0.60 mg.L⁻¹–2.6 mg.L⁻¹, respectively, it was found that for lower NPE concentration (Condition 6), higher P (617.0 ± 35.2 mmol of CH₄) compared to Condition 3, with a higher NPE concentration (493.3 ± 4.4 mmol of CH₄) (Figure 3).

Finally, under conditions 5 and 7, for 146.2 mg.L⁻¹ of EtOH and 0.20 mg.L⁻¹ (Condition 7) to 3.0 mg.L⁻¹

(Condition 5) of NPE, the highest P value was observed for the lowest NPE concentration (1147.7 ± 31.9 mmol CH₄) (Condition 7) compared to the condition of higher NPE concentration (758.20 ± 25.00 mmol CH₄) (Condition 5). Thus, for the same EtOH concentration, the addition of NPE did not significantly interfere ($p < 0.05$) in the CH₄ production (Figure 2). However, it was observed that the higher the NPE concentration (0.214–2.978 mg.L⁻¹) for the same EtOH concentration, the greater the λ , that is, the longer the period preceding the maximum point of methane production. This result may be related to an increase in the biomass adaptation time, which reduces the substrate conversion rate into methane. [32] observed a similar result, in which the addition of 4-NP, regardless of the co-substrate used (fumarate, EtOH or methanol) in anaerobic batch reactors, increased the start-up time of CH₄ production compared to the control condition (without 4-NP). Regarding the condition containing EtOH (200 mg.L⁻¹) and 4-NP (288.97 ± 96.49 mg.L⁻¹), the aforementioned authors observed λ of 122 ± 10.3 h, higher than the control condition (without 4-NP and λ of 17.4 ± 5 h).

Condition 1–1596.51 mg.L⁻¹ NPE and 73.93 mg.L⁻¹ of EtOH; **Condition 2**–619.529 mg.L⁻¹ NPE and 197.38 mg.L⁻¹ of EtOH; **Condition 3**–2573.50 mg.L⁻¹ NPE and 95.11 mg.L⁻¹ of EtOH; **Condition 4**–2573.50 mg.L⁻¹ NPE and 197.38 mg.L⁻¹ of EtOH; **Condition 5**–2978.18 mg.L⁻¹ NPE and 146.25 mg.L⁻¹ of EtOH; **Condition 6**–619.529 mg.L⁻¹ NPE and 95.11 mg.L⁻¹ of EtOH; **Condition 7**–214.85 mg.L⁻¹ NPE and 146.25 mg.L⁻¹ of EtOH; **Condition 8**–1596.51 mg.L⁻¹ NPE and 218.56 mg.L⁻¹ of EtOH; **Condition 9**–1596.51 mg.L⁻¹ NPE and 146.25 mg.L⁻¹ of EtOH. **Condition 10**–1596.51 mg.L⁻¹ NPE and 146.25 mg.L⁻¹ of EtOH. **Condition 11**–1596.51 mg.L⁻¹ NPE and 146.25 mg.L⁻¹ of EtOH. The errors presented after the means (±) correspond to the standard deviation calculated for each condition in triplicate. **Control** – 198.6 mg.L⁻¹ of EtOH.

From the statistical analysis of the results obtained via CCRD, the optimized condition for NPE removal was obtained, of 218.56 mg.L⁻¹ of EtOH and 1596.51 mg.L⁻¹ of NPE, with 92% of this surfactant removal. In order to estimate the main organisms, as well as infer the main metabolic pathways involved in this process, a comparative analysis of the microbial community of the optimized condition with the control condition was carried out, that is, without NPE.

3.3. Characterization of the microbial community

In order to evaluate the effects of adding NPE on the microbial community, 20 genera were selected that

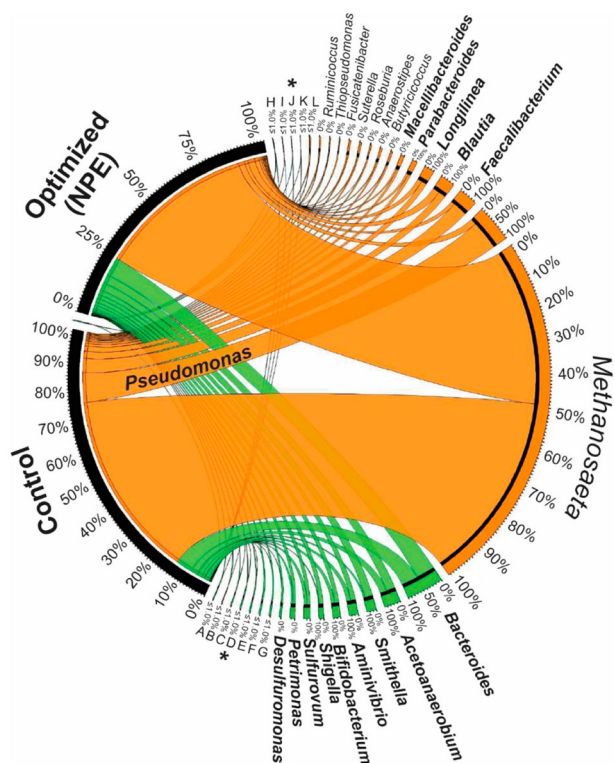


Figure 3. Comparison between the relative abundances of the most abundant genera observed in the control condition (without NPE, in orange) and in the optimized condition (with NPE, in green). Note: (*) = genera with low relative abundance ($\leq 0.01\%$) in the optimized condition, *Methanobacterium*, *Syntrophus*, *Syntrophorhabdus*, *Syntrophomonas*, *Sphingomonas*, *Geobacter* and *Methanoregula* (A–G, respectively) and for the control condition, *Guggenheimella*, *Paludibacter*, *Thauera*, *Proteocatella* and *Subdoligranulum* (H–L, respectively).

were more abundant under optimized (with NPE) and control (without NPE) conditions. The comparison between the most abundant genera in each condition is summarized in Figure 3. In addition, the relative abundance of all genera observed is available in the **Supplementary Material 2**.

After analyzing the main genera observed under control and optimized conditions, a reduction in the relative abundance of organisms similar to *Pseudomonas* (5.06–1.16%), *Faecalibacterium* (2.52–0.70%), *Longilinea* (0.91–0.84%), *Parabacteroides* (0.46–0.40%), *Macellibacteroides* (0.35–0.30%), *Butyricicoccus* (0.23–0.13%), *Anaerostipes* (0.22–0.17%), *Roseburia* (0.19–0.05%) and *Thiopseudomonas* (0.16–0.01%) was observed for the Bacteria domain, and *Methanosaeta* (32.63–30.28%) for the Archaea Domain.

Bacteroides were the genus observed with the highest relative abundance under the optimized condition (3.82%) compared to the control condition (2.84%). This genus is composed of Gram-negative organisms, strict anaerobes and fermentatives, which can degrade simple carbon sources, such as EtOH, the co-substrate used in the present study, with concomitant production of acetic, propionic and succinic acids [58]. This genus has been observed in high relative abundance (between 7.34% to 31.0%) in methanogenic inoculum of an Expanded Granular Sludge Bed (EGSB) reactor for the treatment of anionic surfactant LAS, similar to that used in the present study [59, 60].

Several bacterial genera previously related to the NPE degradation potential were observed in low relative abundance (<1.0%), such as *Aminivibrio*, *Petrimonas*,

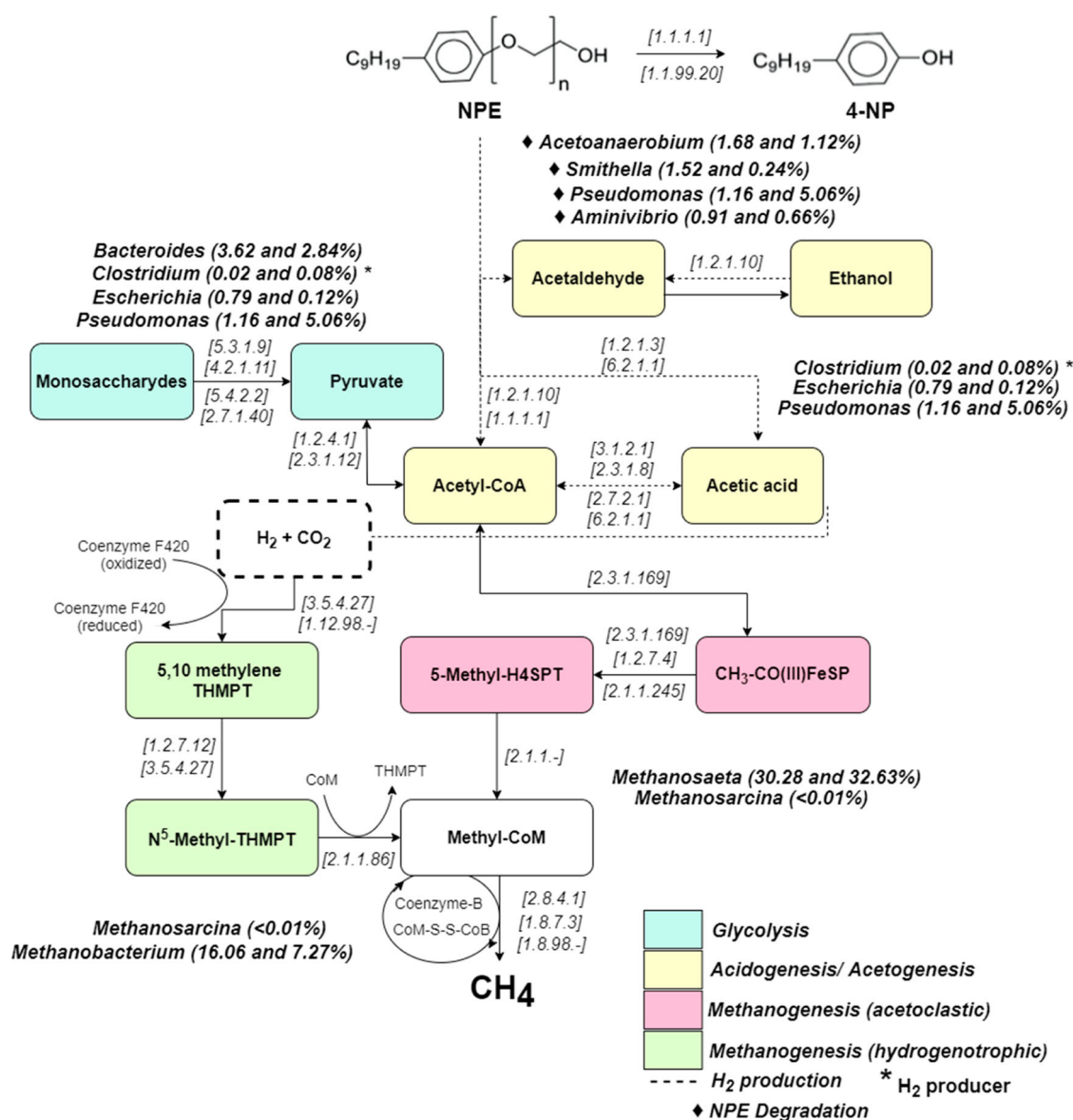


Figure 4. Possible NPE degradation pathways under the optimized condition in batch reactors containing EtOH based on the metabolic inferences of the KO found in the study.

Enterobacter, *Syntrophus*, *Syntrophorhabdus*, *Sphingomonas*, *Thiobacillus*, *Guggenheimella*, *Paludibacter*, *Thauera*, *Novosphingobium* and *Lactobacillus* [61] (Figure 2S). Despite their low relative abundance, it is noteworthy that these genera may have contributed to the NPE degradation, as highlighted by [62], who indicated the importance of less abundant groups in consortia, both due to syntrophy and due to cell–cell interaction and metabolite exchange.

The genera *Acetoanaerobium* (1.68%), *Smithella* (1.52%), *Aminivibrio* (0.91%), *Petrimonas* (0.57%) and *Enterobacter* (0.47%) were identified with greater relative abundance in the optimized condition compared to the control condition (1.12%; 0.24%; 0.66%; 0.30% and 0.41%, respectively). The genera mentioned are composed of Gram-negative and anaerobic organisms, producing acetic, propionic and isobutyric acids, H₂ and CO₂ as main metabolites [57]. In addition, these genera can act in toxic degradation metabolic pathways, such as LAS or NPE, and in the formation of assimilable byproducts by methanogenic archaea [63, 33], especially related to syntrophy with the hydrogenotrophic methanogenic archaea, such as *Methanobacterium* [33, 31, 64] observed in higher relative abundances in the reactors under optimized conditions (16.06%) compared to the control condition (7.34%).

Furthermore, for *Sphingomonas* (0.10%), *Thauera* (0.03%), *Thiobacillus* (0.06%), *Novosphingobium* (0.02%), *Paludibacter* (0.04%) and *Guggenheimella* (0.05%), despite its lower relative abundance, an increase in their relative abundance was identified for the optimized condition compared to the control condition (0.03%; 0.01%; 0.02%, < 0.01%, 0.02% and 0.01%, respectively). These genera have already been described in the literature as related to NPE degradation and its byproducts, as well as the synthesis of short-chain fatty acids due to NPE degradation [61, 65, 66, 67, 68]. Moreover, these organisms can use the aromatic ring of the NPE or LAS molecule as a source of carbon and energy [29, 68], which explains their higher relative abundance under optimized conditions, containing NPE.

Concerning genera related to the sulfur cycle, such as *Sulfurovum*, *Desulfuromonas* and *Geobacter*, an increase in relative abundance was identified for the optimized condition (0.63%, 0.29% and 0.10%, respectively), compared to the condition control (0.51%, 0.05% and 0.01%, respectively). These organisms are strict anaerobes and degrade volatile organic acids, alcohols, and aromatic compounds [69]. Thus, it can be inferred that the presence of organic acids, EtOH, NPE and, probably, the trace metal solution from the Zinder basal medium, which contained sulfur compounds, favoured the prevalence of these organisms in the optimized condition (with NPE). Furthermore, these genera have been

associated with the degradation of other anionic surfactants, such as LAS and LAE [33, 34, 31].

Representatives of the genus *Syntrophus* were identified in the present study with a relative abundance ranging from 0.07% (control condition) to 0.22% (optimized condition). These organisms are strict anaerobes and have metabolic pathways for aromatic compound degradation [70]. Bacteria of this genus have been associated with the ability to express the enzyme alcohol dehydrogenase, favoured by the presence of EtOH, a co-substrate used in the present study (218.56 mg.L⁻¹), which may have favoured the NPE removal [71, 31] identified an increase in relative abundance for the representatives of the genus *Syntrophus* in the presence of EtOH, LAE and LAS.

Syntrophorhabdus, observed in relative abundance of 0.06% under the control condition and 0.12% under the optimized condition, is a genus composed of strict anaerobic bacteria, which do not have motility and do not use sulfur and iron compounds as final electron acceptors [72]. These bacteria can degrade aromatic compounds, such as those contained in NPE or LAS molecules, in syntrophy with methanogenic hydrogenotrophic archaea, such as *Methanobacterium*, identified with higher abundance under the optimized condition (16.06%) compared to the control condition (7.34%).

In addition to *Methanobacterium* and *Methanomassiliicoccus* were other genera of the Archaea Domain observed with a higher relative abundance under the optimized condition (0.26%) compared to the control condition (0.21%). These genera are represented by hydrogenotrophic methanogenic archaea [57], indicating possible syntrophy between *Smithella*, *Aminivibrio*, *Syntrophorhabdus*, *Syntrophomonas*, *Petrimonas*, *Methanobacterium* and *Methanomassiliicoccus* for NPE removal. In addition to NPE, these bacterial genera were associated with the removal of other surfactants (LAS and LAE) and organic matter, as reported by [29, 33, 31], and the importance of the aforementioned genera for surfactant removal can be inferred.

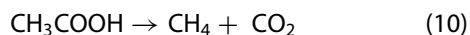
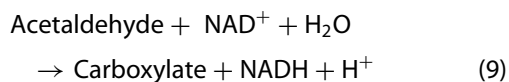
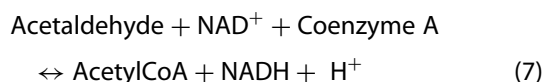
From the characterization data of the microbial community, it was possible to infer the main metabolic pathways and genera involved in the NPE degradation process and in the conversion of EtOH into methane, based on the functional content of microorganism genes and known information from the genome of the closest taxonomic organisms [49]. It was considered that the main genera involved in NPE degradation were *Acetoanaerobium*, *Smithella*, *Pseudomonas* and *Aminivibrio*, while *Bacteroides*, *Clostridium*, *Escherichia* and *Pseudomonas* were related to the degradation of monosaccharides and the conversion of EtOH to acetaldehyde and/or acetic acid. Furthermore, *Methanosarcina* and *Methanobacterium* were the archaea related to hydrogenotrophic

methanogenesis, while *Methanosarcina* and *Methanosaeta* could be related to acetoclastic methanogenesis.

3.4. Functional profile inference

By predicting the functional profiles of the metabarcoding data (16S rRNA), it was possible to obtain 1587 KO for the sample in the optimized condition (containing NPE) and 1551 KO for the sample under the control condition (without NPE). The main metabolic pathways observed for the optimized and control conditions are summarized in Figure 1S, as the energy metabolism pathways, where methane metabolism (2.30 and 2.20%, respectively) and oxidative phosphorylation stand out (1.68 and 1.64%, respectively). For methane metabolism (Figure 2S), it can be observed that all KO observed in the control sample were also observed in the NPE-containing assay. From this result, it can be inferred that microorganisms that contain genes encoding enzymes related to methane production were favoured after exposure of the biomass to NPE, suggesting a possible metabolic adaptation of the biomass to this xenobiotic.

As EtOH cannot be readily assimilated by methanogenic archaea, its conversion into acetic acid becomes essential. EtOH added to the mineral medium was possibly converted by fermentative bacteria to acetic acid through the enzymes alcohol dehydrogenase acetaldehyde dehydrogenase (K00132 and K04072), catalyzing the reaction to acetaldehyde and this in turn to acetyl CoA (Equations 6 and 7, respectively). Acetyl CoA, through the enzyme acetyl-CoA hydrolase (K01067), is then converted into acetic acid (Equation 8), is assimilated by methanogenic archaea, and favours methane production. Alternatively, acetaldehyde can be directly converted to carboxylic acid, such as acetic acid, by the enzyme aldehyde dehydrogenase (K00128) (Equation 9), which can be used for methane production by acetoclastic archaea (Equation 10) [73]. Thus, it can be inferred that EtOH was favourable for the anaerobic degradation of recalcitrant toxic compounds, such as NPE.



The main bacterial genera related to EtOH conversion to acetic acid were *Clostridium* (0.02% to 0.08%), *Escherichia* (0.79% to 0.12%) and *Pseudomonas* (1.16% to 5.06%), for conditions containing NPE and control, respectively [74, 75, 76]. It should be noted that NPE recalcitrance increases with the decrease in the number of ethoxylate groups [10, 77], resulting in more toxic, persistent and bioaccumulative metabolites, such as 4-NP and NPE₁. 4-NP can be used as a substrate for the reaction catalyzed by the enzyme alcohol sulfo-transferase, in the steroid hormone biosynthesis, and is, therefore, considered an endocrine disruptor [18]. However, there are few reports in the literature on the anaerobic biodegradation of 4-NP, and its metabolic pathway for degradation is still poorly understood.

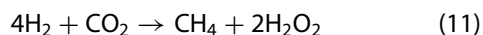
Regarding KO related to methanogenesis, it is noteworthy that K00402, referring to the methyl-coenzyme M reductase subunit gamma (mcrG), was observed in greater proportions under optimized conditions (0.090%) compared to the control condition (0.050%). This transferase is involved in catalysis of the final step of methanogenesis, in which the biological methane production actually occurs. Regarding acetoclastic methanogenesis, in both analyzed samples some of the main KO related to this pathway were observed, in proportions of approximately 0.041%. K00193 is related to the synthesis of methyl-Co(III) corrinoid Fe-S protein (CH₃-CO(III)FeSP) from Co(I) corrinoid Fe-S protein and Acetyl-CoA. CH₃-CO(III)FeSP is then converted into the Co(I) corrinoid Fe-S protein and 5-methyltetrahydrosarcinapterin (5-Methyl-H4SPT) by enzymes related to acetyl-CoA decarbonylase/synthase (K00194 and K00197).

Other KOs related to enzymes involved in methanogenesis observed in smaller proportions in both samples were phosphate acetyltransferase (K13788), heterodisulfide reductase (K03388 and K03389), methenyltetrahydromethanopterin cyclohydrolase (K01499) and acetate kinase (K00925). The acetate kinase enzyme is closely related to the production of methane from acetic acid, and is commonly related to *Methanosaeta*, observed in a relative abundance of 30.28 and 32.63% in the optimized and control conditions, respectively.

Regarding the KO related to hydrogenotrophic methanogenesis, K14128 stands out, referring to the oxidoreductase activity of the enzyme F₄₂₀-non-reducing hydrogenase small subunit (mvhG), which is interconnected with the hydrogenotrophic methanogenesis in the step of reducing coenzyme F₄₂₀ using H₂.

Another enzymatic group related to methanogenesis observed in this study was transferases K00577, related

to tetrahydromethanopterin S-methyltransferase subunit A (mtrA), which is part of the archaeon *Methanobacterium thermoautotrophicum* multienzyme complex, comprising eight subunits and involved in the formation of methane from reduction of CO₂ using H₂ (Equation 10), especially related to the biosynthesis of Methyl-CoM, by hydrogenotrophic archaea. These transferases corroborate the high relative abundance of the genus *Methanobacterium* in this study, whose values were 16.06% under the optimized condition and 7.27% under the control condition.



From the results obtained, it can be inferred that there was a predominance of the hydrogenotrophic pathway, followed by the acetoclastic pathway. Macedo et al. [51] observed a predominance of the hydrogenotrophic pathway in a 19.8 L Fluidized Bed Reactor (FBR) containing LAS (6.3–24.7 mg.L⁻¹) and EtOH(50, 100, 200 mg.L⁻¹) as a co-substrate.

Possible metabolic pathways involved in the conversion of EtOH into methane in batch reactors inoculated with or without NPE (optimized and control conditions, respectively) are included in Figure 4. All KO involved in the process were observed in both samples, but in different proportions depending on operating conditions.

The numbers between squared brackets represent the EC (Enzyme Commission Numbers) referring to the main KO inferred in the samples. The values between brackets refer to the relative abundance of the main genera observed in the samples under the optimized condition (with NPE) and control condition (without NPE), respectively.

4. Conclusions

The optimal range for NPE removal (92%) and organic matter (88%) occurred between 218.56 mg.L⁻¹ of EtOH and 1596.51 µg.L⁻¹ of NPE. The NPE impacted microbial diversity in anaerobic batch reactors, and the main genera possibly related to NPE removal were *Acetonaerobium* (1.68%), *Smithella* (1.52%), *Aminivibrio* (0.91%), *Petrimonas* (0.57%) and *Enterobacter* (0.47%). Regarding methanogenesis, the archaeal genera identified in the highest relative abundance were *Methanobacterium*, *Bathyarchaeota* and *Methanomassiliicoccus*.

After inferring the main metabolic routes involved in the degradation of NPE and EtOH, the importance of genes encoding enzymes such as the methyl-coenzyme M reductase subunit gamma (mcrG), responsible for the mineralization of byproducts generated throughout the process, as well as the acetaldehyde/acetyl-CoA and

alkanol dehydrogenase, which catalyze reactions involving EtOH metabolic compounds from NPE, favouring the surfactant removal under anaerobic conditions.

The results obtained regarding the optimal conditions established in relation to EtOH as a metabolic co-substrate may support future research on the removal and degradation of nonylphenol ethoxylate from anaerobic reactors, especially regarding the application of continuous reactors for wastewater treatment contaminated with this or other surfactants.

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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