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Microbial diversity and metabolic inference of diclofenac removal in optimised batch heterotrophic-denitrifying conditions by means of factorial design

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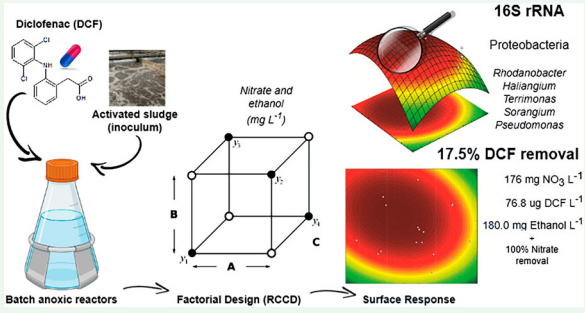
ABSTRACT

Using the Response Surface Methodology (RSM) and Rotational Central Composite Design (RCCD), this study evaluated the removal of DCF under denitrifying conditions, with ethanol as cosubstrate, in batch reactors, being 1 L Erlenmeyer flasks (330 mL of reactional volume) containing Dofing medium and kept under agitation at 130 rpm and incubated at mesophilic temperature (30 °C). It considered the individual and multiple effects of the variables: nitrate (130 - 230 mg NO_3^- L^{-1}), DCF (60–100 µg DCF L^{-1}) and ethanol (130 - 230 mg EtOH L^{-1}). The highest drug removal efficiency (17.5%) and total nitrate removal were obtained at 176.6 ± 4.3 mg NO_3 $^-L^{-1}$, 76.8 ± 3.7 µg DCF L^{-1}, and 180.0 ± 2.5 mg EtOH L^{-1}. Under such conditions, the addition of ethanol and nitrate was significant for the additional removal of diclofenac ($p > 0.05$). The prevalence of *Rhodanobacter*, *Haliangium* and *Terrimonas* in the inoculum biomass (activated sludge systems) was identified through the 16S rRNA gene sequencing. The potential of these genera to remove nitrate and degrade diclofenac was inferred, and the main enzymes potentially involved in this process were α-methylacyl-CoA racemase, long-chain fatty acid-CoA ligase, catalases and pseudoperoxidases.



Abbreviations: Analysis of Variance (ANOVA); Base-Pairs (bp); Chemical Oxygen Demand (COD); Confidence interval (IC); Denitrification rate (k); Diclofenac (DCF); Enzyme Comission number (EC); Ethanol (EtOH); Extracellular Polysaccharides (EPS); Flame Ionisation Detector (FID); Hydraulic Retention Time (HRT); Kyoto Encyclopaedia of Genes and Genomes (KEGG); Linear Alkylbenzene Sulphonate (LAS); National Centre for Biotechnology Information (NCBI); Nitro-diclofenac (NO-DCF); Non-Steroidal Snalgesic and Anti-Inflammatory Drugs (NSAIDs); Operational Taxonomic Units (OTUs); Organic Micropollutants (OMPs); Phosphate Buffered Saline (PBS); Polycyclic Aromatic Compounds (PAHs); Polymerase Chain Reaction (PCR); Polypropylene (PP); Polytetrafluoroethylene Polymer (PTFE); Propranolol (PRO); Quadratic terms (Q); Response Surface Methodology (RSM); Ribosomal Database Project (RDP); Rotational Central Composite Design (RCCD); Sulfamethoxazole (SMX); The Comprehensive Enzyme Information System (BRENDA); Total Solids (TS); Total Suspended Solids (TSS); Ultra Efficiency Chromatography Coupled to Mass Spectrometry (UPLC-MS/MS); Ultraviolet (UV); Volatile Fatty Acids (VFA); Volatile Suspended Solids (VSS); Volatile Total Solids (VTS); Wastewater Treatment Plants (WWTPs)

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Environmental implication

Besides Diclofenac is one of the most ubiquous analgesic and anti-inflammatory drugs in environmental matrices, due to its recalcitrance, its total removal during sewage treatment is still not possible. The continuous exposure to this type of micropollutant, even at low concentrations, may be potentially hazardous to living beings, especially in conjugated forms or in complex mixtures, causing synergistic effects of active ingredients on metabolic pathways, such as interference with endocrine systems, phytotoxicity and genotoxicity. These potential impacts to the environment and even to human health make it essential to develop new researches related to the removal of this drug.

Introduction

The emerging Organic Micropollutants (OMPs) are persistent natural or synthetic chemical compounds, which are potentially toxic even at concentrations of ng L^{-1} to µg L^{-1}, composed of commercial products such as sweeteners, pesticides, parabens and several medicines [1,2]. Because these compounds are produced worldwide on a large scale, they are frequently observed in aquatic environments, causing adverse impacts to the ecosystem balance and polluting environmental matrices [3].

Diclofenac, part of the non-steroidal analgesic and anti-inflammatory drugs (NSAIDs), is derived from phenylacetic acid, whose molecular formula is $C_{14}H_{11}Cl_2NO_2$ and molecular weight 318.0 g mol^{-1} [4]. This compound is considered one of the most commercialised analgesics in the world, and its annual consumption per capita is on average 15.0 g [5]. In addition, there is no mandatory prescription for its commercialisation, a factor that can further stimulate its large production scale and continuous consumption. The partial and parent structures of drugs can react or adsorb with other organic compounds and colloids, directly affecting biological activity. Therefore, studies related to removal technologies and optimisation of unit processes are fundamental. In wastewater treatment plants (WWTPs), chemical treatment technologies are widely applied in the removal of micropollutants, including oxidation methods such as Fenton, ozonation, photolysis, and advanced oxidative processes [4]. However, the operation and implementation costs of such practices are high, and complete removal is not obtained.

Since it occurs in environmental matrices, diclofenac is classified as moderately persistent and it is not completely removed in sewage treatment processes [6]. Therefore, this drug is recalcitrant and observed in sanitary sewage between 0.2–15.4 µg L^{-1} [5]. The added impacts, like toxicity to the fauna due to its persistence in the environment, make it essential to develop research related to the removal of this micropollutant [7,8].

The application of denitrifying bacteria in the degradation of toxic organic compounds is quite promising [9]. The route of degradation of aromatic compounds via denitrification offers higher energy yield and is more thermodynamically favourable than metabolisation by strict anaerobes, sulfate-reducing bacteria, and iron-reducing bacteria. Thus, the use of denitrifying bacteria for the degradation of recalcitrant compounds can be promising alternative [10,11].

In batch reactors with activated sludge inoculum under denitrifying conditions, ibuprofen removal was evaluated with nitrate and ethanol as nitrogen and organic cosubstrate sources, respectively [12]. The aforementioned authors obtained 97.5 ± 3.1% of maximum removal of ibuprofen (109.9 ± 1.6 µg L^{-1}) with 95.9 ± 5.0 mg L^{-1} of nitrate and 180.8 ± 11.0 mg L^{-1} of ethanol. Based on the inference of functional genes, there was a predominance of enzymes involved in denitrification, such as nitrate carriers and various reductases involved in the conversion of intermediates (*i.e.* nitrate, nitrite, nitric oxide and nitrous oxide reductases) were also predicted.

Denitrifying bacteria are anoxic, often related to the ability to remove sulphonamide antibiotics, such as sulfadiazine, sulfamethoxazole and sulfamethazine [13], in addition to nitrogenous drugs like fluoxetine [14] and paracetamol [15]. The use of electron-donor sources, such as ethanol as a cosubstrate, may favour microbial metabolism and enable greater biomass adaptation and biodegradation of micropollutants with complex molecular structures [16,17]. Cosubstrates can induce extracellular enzymatic production of the microbial consortium and therefore favour degradation of recalcitrant compounds [18,19]. Thus, the use of denitrifying biomass is an attractive alternative for diclofenac degradation. This pharmaceutical compound consists of two aromatic rings and an amine group, and can be cometabolized with ethanol via nitrate removal [10,20].

The use of electron-donor sources, such as ethanol as a cosubstrate, may favour microbial metabolism and enable greater biomass adaptation and biodegradation of micropollutants with complex molecular structures [12,13]. It is worth mentioning that ethanol is produced in large quantities in Brazil, The National Supply Company estimates that its production would achieve 24.8 billion litres of ethanol during the 2022/2023 harvest [21].

In contrast to the classic methods to assess the effect of different operating variables, known as 'one factor at a time', in which only one category is changed, and the others are kept constant, statistical planning methods can be used in order to optimise different nutritional conditions for the removal of a particular compound. The Rotational Central Composite Design (RCCD) is a statistical tool for the analysis of the optimal range in a process, as well as to evaluate the effects of the variables with the responses obtained in the experiments [22]. The optimisation of operating parameters in batch reactors allows not only the evaluation of the optimal conditions, but also facilitates the transition of the process to application in continuous reactors and scale-up in a more attractive way [23]. From the validation of the nutrient conditions in batch reactors, Pirete et al. [24] applied the concentration ranges of nitrate and ethanol in the continuous fluidised bed reactor to evaluate the removal of ibuprofen and diclofenac. The aforementioned authors observed greatest diclofenac and ibuprofen removal (52.9–55.8%, respectively) was found after 70 days of operation in acidogenic stage, with 18 ± 2 h of Hydraulic Retention Time (HRT) and 185.6 ± 29.9 µg L^{-1} of affluent diclofenac, 150 ± 44.4 µg L^{-1} of affluent ibuprofen and 89.7 ± 39.2 mg L^{-1} of ethanol. Andrade [25] achieved the removal of linear alkylbenzene sulphonate (LAS) greater than 90% in batch reactors via RCCD from the variables: nitrate (100–150 mg L^{-1}), ethanol (100–150 mg L^{-1}) and linear alkylbenzene sulphonate (LAS) (15.6–41.2 mg L^{-1}). According to these authors, the optimal condition for these variables was validated with greater removal efficiency and was consistent with the mathematical model predicted by planning. Thus, studies related to the degradation of contaminants via RCCD can enhance future research on the optimisation and validation of parameters dependent on the biological process.

Studies of effluent treatment processes and removal of emerging organic micropollutants are necessary, which start in smaller and laboratory scales, such as in batch reactors with synthetic effluents. This study confers in the analysis of the bioremediation process of environmental micropollutants, besides the analysis of possible metabolic routes for the degradation of diclofenac in the presence of co-substrate and nitrogen source.

This is a novel research, once the removal of diclofenac was evaluated in batch reactors. Besides the microbial characterisation by sequencing the 16S rRNA gene of the inoculum from the activated sludge system, the biomass of the reactors at the end of each operation. From the analysis and interpretation of the molecular biology results, it was possible to associate the identified microbial population with the diclofenac degradation potential under the different operational condition.

Thus, diclofenac removal was evaluated in batch reactors under denitrifying conditions by means of RCCD, considering the statistical effects of the variables: nitrate (130 - 230 mg L^{-1}), diclofenac (60 - 100 µg L^{-1}) and ethanol (130 - 230 mg L^{-1}) concentrations. In addition, microbial characterisation of optimised and control conditions was performed via massive sequencing of the 16S rRNA gene (*Illumina* platform), as well as inference of the main enzyme coding genes involved in the diclofenac degradation process.

2. Material and methods

2.1 Inoculum

Biomass of activated sludge applied to sewage treatment at the factory of Volkswagen Brazil (São Carlos, São Paulo, Brazil) was used as an inoculum in batch reactors. This inoculum was selected due to positive results in previous studies [16], some of them also using Dolfing medium, the same medium described below in section '2.3 Culture Medium' [25], showing that the sludge provides a set of bacterial consortia with high biodegradability potential, being widely related to nitrogen removal.

Aerobic sludge was collected in a 10 L plastic bottle and kept under 4 °C. The initial composition of the inoculum was 16.3 ± 2.2 g L^{-1} of total solids (TS). In all batch reactors, 1.0 g L^{-1} of inoculum volatile total solids (VTS) was added.

2.2 Diclofenac

The formulation of the Diclofenac (DCF) used to prepare the solutions was purchased commercially in a handling pharmacy. The stock solution was prepared in methanol (99.9%) at a final concentration of 5.0 g L^{-1}. The physicochemical properties of diclofenac are presented in Table A.1 of the supplementary material.

2.3 Culture medium

The nutritional medium used in batch reactors was proposed by Dolfing et al. (1990), composed of 0.3 g L^{-1} KH_2PO_4, 1.2 g L^{-1} Na_2HPO_4, 0.1 g L^{-1} NH_4Cl, 0.1 g L^{-1} $MgSO_4.7H_2O$. In addition, 1 mL of the micronutrient solution [26] is composed of 0.1 g L^{-1} $FeSO_4.7H_2O$, 0.1 g L^{-1} $MnSO_4.H_2O$, 0.2 g L^{-1} $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.2 g L^{-1} $Na_2B_4O_7.10H_2O$, 0.2 g L^{-1}, $Co(NO_3)_2.6H_2O$ and 0.2 g L^{-1} $CuCl_2.2H_2O$. Sodium nitrate was used as nitrate source

in the experimental reactors, thus the standard solutions of nitrate in the form of $NaNO_3$ (99%) were prepared at 3.0 g L^{-1} and ethanol (99%) at 1.0 g L^{-1}.

Control reactors were carried out to evaluate the influence of nitrate (NO_3^-), ethanol (EtOH) and diclofenac (DCF), namely: (R_I) 150 mg NO_3^- L^{-1}; (R_{II}) 150 mg $NO_3^-L^{-1}$ and 150 mg EtOH L^{-1}; (R_{III}) 150 mg $NO_3^-L^{-1}$ and 90 µg DCF L^{-1}; (R_{IV}) 150 mg NO_3^- L^{-1}, 90 µg DCF L^{-1} and 150 mg EtOH L^{-1}.

Batch reactors were performed in triplicates, in Erlenmeyer flasks of 1 L total volume and 330 mL of reactional volume, and 1.0 gSTV L^{-1} (35 mL) of inoculum and 295 mL of culture medium and micronutrient solution were added. Nitrate, ethanol and diclofenac were added according to the predicted concentrations for each condition listed in Table 1. All reactors were subjected to an argon atmosphere (100%) for 10 min, closed with a butyl cap and plastic screw.

During batch operation, the reactors were kept under agitation at 130 rpm and incubated at mesophilic temperature (30 °C). Samples were collected at one hour intervals during the total removal of nitrate (10–12 h).

2.4 Experimental design

The response surface was performed via Rotational Central Composite Design (RCCD), which considered three independent variables and two levels composed of 14 reactors, and three repetitions of the central point. The variables evaluated via RCCD were the following: nitrate (x_1), diclofenac (x_2), and ethanol (x_3) concentrations. The setup of this design and the data analysis were performed using the Statistica 10 software (StatSoftInc, 2014, USA).

The concentrations of the variables evaluated were previously defined at 130, 180 and 230 mg L^{-1} for nitrate and ethanol [25,27,28] and 60, 80 and 100 µg $L^{-1}$for ibuprofen [29]. Table 1 summarises the values of the coefficients adopted in the RCCD and the coded data.

The quadratic polynomial equation was used to describe the removal of DCF ($Y_{response}$) according to the independent variables (nitrate, ethanol and diclofenac) as presented in Equation 1, with the statistical calculation of the effects among the variables at a confidence level of 0.05. The Analysis of Variance (ANOVA) was performed to evaluate the significance of the independent variables by obtaining the value of $F_{calculated}$ and comparing it with $F_{tabulated}$.

$$Y = \beta 0 + \beta 1X1 + \beta 2X2 + \beta 3X3 + \beta 11X12 + \beta 22X22 + \beta 33X32 + X1X2 + X2X3 + X1X3 \quad (1)$$

Where: Y = predicted response;

β_0, β_1, β_2 and β_3 = linear coefficients;

β_{11}, β_{22}, β_{33} = quadratic coefficients;

X_1, X_2, X_3 = independent variables.

The validation of the optimal conditions predicted via response surface was performed after the results obtained via RCCD were analysed. For this, the condition 176.6 ± 4.3 mg NO_3^- L^{-1}, 76.8 ± 3.7 µg DCF L^{-1} and 180 ± 2.5 mg EtOH L^{-1} was evaluated.

2.6 Data analysis

The effects of nitrate, ethanol and diclofenac initial concentrations were evaluated based on the removal efficiency of diclofenac at the end of each batch

Table 1. Experimental matrix of RCCD reactors and optimised condition.

	Coded variables			Real values		
Reactor	Nitrate (X_1)	Diclofenac (X_2)	Ethanol (X_3)	Nitrate (mg L^{-1})	Diclofenac (µg L^{-1})	Ethanol (mg L^{-1})
1	−1	−1	−1	124.3 ± 1.6	73 ± 8.1	133.3 ± 16.7
2	1	−1	−1	227.8 ± 5.5	78 ± 3.6	132.8 ± 9.1
3	−1	1	−1	128.4 ± 5.1	101 ± 13.6	138.5 ± 8.1
4	1	1	−1	200.5 ± 5.9	104 ± 5.6	158.2 ± 4.5
5	−1	−1	1	131.2 ± 7.1	75 ± 2.5	243 ± 10.2
6	1	−1	1	226.3 ± 7.3	76 ± 2.7	223.5 ± 12.4
7	−1	1	1	135.2 ± 2.1	100 ± 4.7	230 ± 6.0
8	1	1	1	225.8 ± 7.9	112 ± 2.8	247.5 ± 7.8
9	−1.68	0	0	95.9 ± 2.1	101.5 ± 0.7	162.4 ± 12.5
10	1.68	0	0	250 ± 10.2	96 ± 1.1	160.0 ± 6.9
11	0	−1.68	0	176.6 ± 3.7	57 ± 2.0	158.8 ± 5.3
12	0	1.68	0	184.2 ± 2.4	146.3 ± 22.3	203.0 ± 7.8
13	0	0	−1.68	197.7 ± 5.4	95 ± 8.6	100.5 ± 25.3
14	0	0	1.68	179.0 ± 2.5	96 ± 5.2	278.9 ± 13.2
15	0	0	0	180.5 ± 8.2	78 ± 2.3	183.5 ± 4.8
16	0	0	0	186.7 ± 4.9	80.7 ± 4.1	178.8 ± 9.2
17	0	0	0	180.5 ± 8.2	73.3 ± 3.6	183.5 ± 28.7
Optimised	0	0	0	176.6 ± 5.2	76.8 ± 3.7	180 ± 2.5

reactor (Equation 2),

$$\text{Removal DCF (\%)} = \frac{\text{DCF initial-DCF final}}{\text{DCF initial}} \text{ x } 100 \quad (2)$$

Where:

$DCF_{initial}$ = initial concentration of diclofenac (μg L^{-1})

DCF_{final} = final diclofenac concentration (μg L^{-1})

The triplicate results were averaged for each condition using OriginPro 9.0 software and the nitrate removal data were fitted to the single-phase exponential decay equation 'Fit Exponential Decay' (ExpDec 1 model) (Equation 3).

$$y = y_0 + A_1\, e^{-x/t1} \quad (3)$$

Where:

y = Final nitrate concentration (mg L^{-1})

y_0 = Initial concentration of nitrate (mg L^{-1})

A = Amplitude

t = time of decay (h)

2.7 Diclofenac adsorption reactors

Diclofenac was extracted from the biomass of batch reactors at the end of operation, according to the validated methodology described by Gago-Ferrero et al. [30]. The mass balance was performed according to Equations 3 and 4.

$$X_{rem} = X_{initial} - X_{final} \quad (4)$$

Where:

X_{rem} = removed diclofenac mass (μg);

Xi_{nicial} = diclofenac mass added at the beginning of batches (μg);

X_{final} = diclofenac mass at the end of batch operation (μg);

$$X_{rem} = X_{initial} - (X_{final} + X_{ads}) \quad (5)$$

Where:

X_{rem} = diclofenac mass removed by biological process (μg);

$X_{inicial}$ = diclofenac mass added at the beginning of batches (μg);

X_{final} = diclofenac mass at the end of batch operation (μg);

X_{ads} = diclofenac mass adsorbed in batch sludge (μg);

2.8 Analytical and chromatographic methods

Nitrate concentrations were quantified by ultraviolet (UV) spectrophotometry at 220 and 275 nm wavelengths [31]. The concentrations of volatile fatty acids (VFA) and solvents (n-butanol, methanol, and ethanol) were determined by gas chromatography model GC-2010 (Shimadzu Scientific Instruments, Columbia, MD, USA), equipped with a flame ionisation detector (FID) and HP-INNOWAX column (30 m long, 0.2 mm internal diameter and 0.2 μm film thickness) according to the methodology developed and protocoled by Adorno et al. [32]. Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) were performed according to APHA/AWWA/WEF [33].

The monitoring of organic matter in the liquid phase regarding Chemical Oxygen Demand (COD) was performed according to the procedures described in APHA/AWWA/WEF [33]. The concentrations of carbon sources added in synthetic medium: ethanol and diclofenac were determined by chromatography model GC 2010 (Shimadzu Scientific Instruments, Columbia, MD, USA) and Ultra Efficiency Chromatography Coupled to Mass Spectrometry (UPLC-MS/MS) [29] respectively.

Diclofenac was quantified according to the methodology described by Campanha et al. [29], based on Ultra Efficiency Chromatography Coupled to Mass Spectrometry (UPLC-MS/MS) with Acquity UPLC BEH C18 reversed phase column; flow rate of 0.45 ml min^{-1}, temperature of 40 °C and at detection limit of 0.08 ng L^{-1}. The samples were previously filtered on hydrophilic polytetrafluoroethylene polymer (PTFE) membranes of 0.22 μm porosity with polypropylene (PP) pre-filter. The chromatographic conditions were as follows: Acquity UPLC BEH C18 reverse phase column, flow rate of 0.4 mL min^{-1}, temperature of 40 °C and detection limit of 0.08 ng L^{-1}.

2.9 Molecular biology analysis

Biomass samples from control R_{II} (149.7 ± 14.1 mg NO_3^- L^{-1}, 150 ± 3.6 mg EtOH L^{-1}) and optimised reactors (176.6 ± 4.3 mg NO_3^- L^{-1}, 76.8 ± 3.7 μg DCF L^{-1} and 180 ± 2.5 mg EtOH L^{-1}) were stored in Falcon tubes (15 mL) at the end of the batch reactors for microbial characterisation. After centrifugation at 5,000 rpm for 5 min, the supernatant was discarded and the biomass pellets formed were washed with phosphate buffered saline (PBS; NaCl 8%, KCl 0.2%, Na_2HPO_4 1.44%, KH_2PO_4 0.24%) [34], again centrifuged and the supernatant discarded, and the wet biomass (0.5 g) was stored at −20 °C.

Genomic DNA was extracted from the samples using the FastDNA™ SPIN Kit for Soil DNA Extraction (MP Biomedicals) according to the manufacturer's recommendations. DNA concentration and purity were evaluated using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA), with the absorbance ratio (A260/A280) in the range of 1.8–2.0 which is indicative of nucleic acid purity. The size of the DNA fragment

(> 10,000 bp) was verified by electrophoresis in 0.8% agarose gel.

The sequencing of the 16S rRNA gene was performed by GenomeDX - Advanced Genetics (Rio de Janeiro, Brazil). Amplification of the 16S rRNA gene (Region V3 + V4; Bacteria Domain) via polymerase chain reaction (PCR) was performed using the primer sets 341F and 806R [35], via Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Sequencing libraries were generated via DNA UltraNBNext® Kit for *Illumina* (New England Biolabs, Ipswich, USA), following the manufacturer's recommendations. Sequences were assembled and filtered (QPhred ≥ 33) to remove sequences containing low quality bases, and chimaeras were detected (UCHIME algorithm) and excluded. Sequence analysis was performed by Uparse software (v7.0.1001, http://drive5.com/uparse/), where sequences with similarity ≥97% were assigned to the same Operational Taxonomic Units (OTUs). Taxonomic classification of OTUs was performed with the Ribosomal Database Project (RDP) classifier (http://rdp.cme.msu.edu/). Sequences were submitted to the National Centre for Biotechnology Information (NCBI) database under accession number PRJNA76708.

The potential metabolic pathways involved in the diclofenac degradation process were defined based on the study by Granatto et al. [36]. In addition, the prediction of potential genes encoding enzymes involved in such pathways was performed using the Tax4Fun2 tool [37] in R language, based on the Kyoto Encyclopaedia of Genes and Genomes (KEGG) (97% identity cutoff) and on The Comprehensive Enzyme Information System (BRENDA) database (https:// www.brenda-enzymes.org/).

3. Results and discussion

3.1 Control reactors

Control reactors (R_I, R_{II}, R_{III} and R_{IV}) were carried out to verify potential removal of nitrate and organic matter under denitrifying conditions.

The highest organic matter removal efficiency values were observed in the reactors with ethanol, such as R_{II} (55.1 ± 1.5%) and R_{IV} (67.4 ± 1.1%), with initial concentration of 361 ± 3.6 mg COD L^{-1} and 370 ± 5.0 mg COD L^{-1}, respectively. In relation to the reactors without addition of organic cosubstrate, the organic matter removal efficiencies were 44.6 ± 4.9% and 51.3 ± 1.5% in R_I and R_{III} to 13.4 ± 1.7 mg COD L^{-1} and 19.3 ± 0.2 mg COD L^{-1} initial concentration, respectively. In addition, the organic matter removal efficiency of the control reactors was not significant ($p > 0.05$); however, greater removal of organic matter was observed in the ethanol condition (150.0 ± 12.5 mg EtOH L^{-1}) compared to the other reactors (without ethanol) (Table 2). Thus, ethanol as a biodegradable electron donor allowed the biomass to better adapt and remove organic matter from the drug.

Based on these results, it could be inferred that in the presence of nitrate, the initial concentration of ethanol interfered with the removal of organic matter. Similar results were observed by Torresi et al. [38], who evidenced cometabolism in the removal of organic matter in batch reactors with the addition of 70 mg NO_3^- L^{-1}, 40 mg EtOH L^{-1} and 2 µg DCF L^{-1}. According to these authors, the addition of ethanol as an external carbon source contributed to 60% of organic matter removal, while in conditions without the cosubstrates, the removal was 30%.

The average rate of nitrate removal values observed were 0.1 h^{-1} for R_I (150 mg NO_3^- L^{-1}); 0.1 h^{-1} for R_{II} (150 mg NO_3^- L^{-1} + 150 mg EtOH L^{-1}); 0.1 h^{-1} for R_{III} (150 mg NO_3^- L^{-1} + 90 µg DCF L^{-1}) and 0.2 h^{-1} for R_{IV} (150 mg NO_3^- L^{-1} + 90 µg DCF L^{-1} + 150 mg EtOH L^{-1}) (Figure 1 and Table 2). The heterotrophic denitrification reactions were in accordance with that proposed by Madigan et al. [39] and Stams [40], with Equation 6 being equivalent to the conversion of nitrate to molecular nitrogen, followed by Equation 7, with ethanol hydrolysis.

Equation 8 is proposed for denitrification with ethanol, resulting in the formation of molecular nitrogen and the mineralisation of the organic compound. Thus, Equation 6 represents the conversion of nitrogen under autotrophic conditions, as may have occurred in reactor R_I; while Equation 8 represents denitrification with ethanol assimilation, predominant in reactors R_{II} and R_{IV}. In addition, only in reactors R_{II} and R_{IV} was the complete removal of nitrate observed, inferring that denitrification possibly occurred only in the presence of ethanol as an electron donor.

Thus, based on the control reactors R_{II} and R_{IV}, it was observed that ethanol favoured denitrification via nitrate

Table 2. Kinetic coefficients and removal of organic matter in the control reactors. The errors presented (±) correspond to the standard deviation calculated for each condition in triplicate.

Reactors	Initial COD	Removal (%)	Kinetic coefficients k (h^{-1})	R^2	Cr*
R_I (NO_3^-)	13.4 ± 1.7	44.6 ± 4.9	0.1	97	89.3
R_{II} (NO_3^-/EtOH)	361.0 ± 3.6	55.1 ± 0.6	0.1	99	0
R_{III} (NO_3^-/DCF)	19.3 ± 0.2	51.3 ± 1.6	0.1	92	80.0
R_{IV} (NO_3^-/DCF/EtOH)	370.0 ± 5.0	67.4 ± 1.1	0.2	95	0

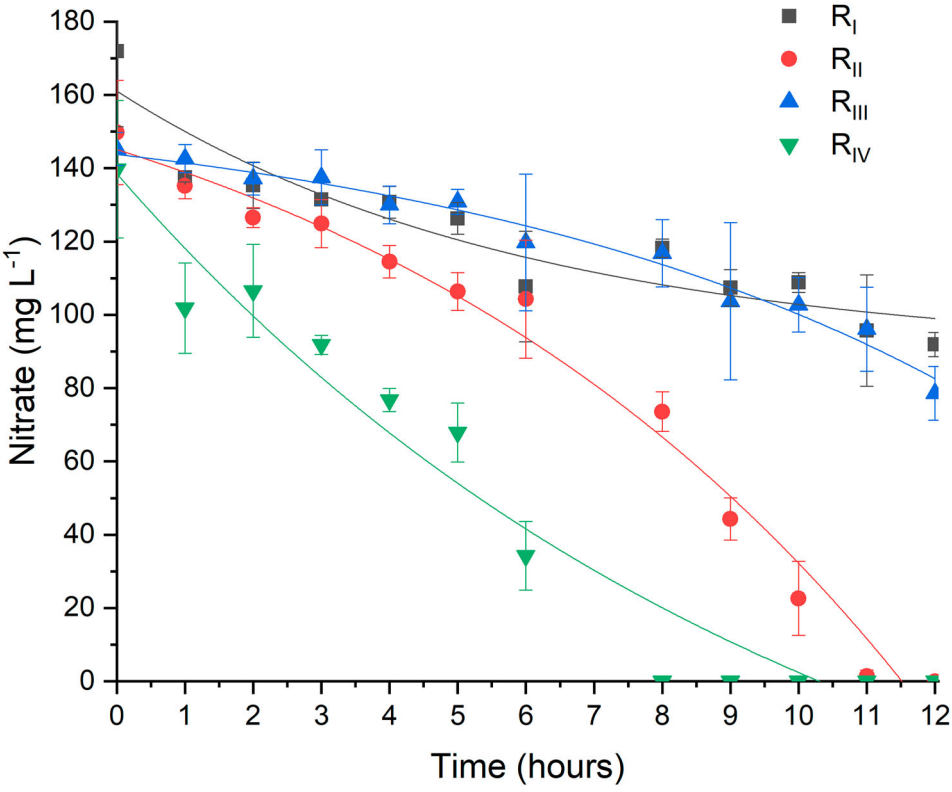

Figure 1. Temporal decay of nitrate concentration.

removal, as well as the removal of the nitrogenous compound. Similarly, Horová et al. [41] related the denitrifying potential of the inoculum from activated sludge biomass to the addition of different sources of organic carbon (ethanol, methanol, and acetate) from 1.5 g NO_3^- L^{-1}, and its effects were evaluated in batch reactors. The authors obtained total denitrification with ethanol (447 mg COD L^{-1}) and a faster rate of nitrate removal compared to the other exogenous carbon sources.

In addition, Ciudad et al. [42] observed that the occurrence of heterotrophic denitrification depends directly on the availability of organic matter, requiring the addition of an external carbon source in case of insufficient organic substrate in the reaction medium. According to the authors, the addition of easily biodegradable organic matter results in higher denitrification rates, corroborating the results obtained in this research.

$$N - NO_3^- \rightarrow N - NO_2^- \rightarrow N - NO \rightarrow N - N_2O \rightarrow N - N_2 \quad (6)$$

$$CH_3CH_2OH + H_2O \rightarrow C_2H_3O_2^- + H^+2H_2 \quad (7)$$

$$12NO_3^- + 5CH_3CH_2OH \rightarrow 6N_2 + 10CO_2 + 12OH^- + 9H_2O \quad (8)$$

R_I = 150 mg NO_3^- L^{-1}; R_{II} = 150 mg NO_3^- L^{-1} and 150 mg EtOH L^{-1}; R_{III} = 150 mg NO_3^- L^{-1} and 90 µg DCF L^{-1}; R_{IV} = 150 mg NO_3^- L^{-1}; 90 µg DCF L^{-1} and 150 mg EtOH L^{-1}. The errors presented (±) correspond to the standard deviation calculated for each condition in triplicate.

In contrast, there was no complete denitrification in the presence of diclofenac in R_{III} (150 mg NO_3^- L^{-1} + 90 µg DCF L^{-1}). Perhaps the concentration of the drug was insufficient for denitrification, as corroborated by Ozdemir et al. [9], who obtained nitrate accumulation during 10h of batch reactors operation with 100 µg DCF $L^{-1}$and 100 mg NO_3^- L^{-1}. These conditions were similar to the present study with 150 mg NO_3^- L^{-1} and 90 µg DCF L^{-1}. According to the mentioned authors, low concentration of diclofenac did not favour total denitrification via nitrate.

Regarding the kinetic parameters, for reactor R_{III} (150 mg NO_3^- L^{-1} + 90 µg DCF L^{-1}), from 19.1 mg COD L^{-1} initial, it was found that the constant of the denitrification rate (0.1 h^{-1}) was similar to that obtained for reactor R_I, only containing nitrate (150 mg L^{-1}), with 0.1 h^{-1} and 7.7 ± 0.36 mg COD L^{-1} initial, and for reactor R_{II} (150 mg NO_3^- L^{-1} + 100 mg EtOH L^{-1}), containing 361.0 ± 3.6 mg COD L^{-1} initial. However, for the condition R_{IV}, the denitrification rate (0.2 h^{-1}) was higher, probably favoured by ethanol and diclofenac, promoting the complete removal of nitrate in less time and at a higher rate.

In reactor R_{III} (85 ± 3.5 μg DCF L^{-1} and 150.0 ± 6.5 mg NO_3^- L^{-1}) 9.6 ± 0.9% of DCF removal efficiency was observed. In this reactor, the main source of organic matter was related to the addition of DCF, and the removal of organic matter was 51.2 ± 0.3% for the initial concentration of 19.1 mg COD L^{-1}. Under such conditions, the removal of nitrate was 43.1 ± 1.2% for initial concentration of 145.2 ± 4.4 mg NO_3^- L^{-1}. Thus, diclofenac was degraded under denitrifying condition with nitrate as electron receptor.

In reactor R_{IV} (80.0 ± 0.7 μg DCF L^{-1} + 150.0 mg NO_3^- L^{-1} + 150.0 mg EtOH L^{-1}) the DCF removal efficiency observed was 12.3 ± 5.0%, in addition to 67.4 ± 1.1% COD removal from 370 ± 5.0 mg COD L^{-1} initial. In this condition, the ethanol may have favoured the removal of the drug; in addition, nitrate removal was 100% (initial 150 mg L^{-1}) during 8 h of experiment. In the conditions without added organic matter (R_I), the nitrate removal efficiency was 38.8 ± 3.2% for 150.3 ± 16.6 mg NO_3^- L^{-1} initial (Table 3). In all control reactors, no VFA were observed, which is a common result in denitrifying conditions [43].

Regarding the diclofenac adsorbed on the activated sludge biomass, 0.08 μg DCF L^{-1} were observed. Through the mass balance in reactors R_{II} and R_{IV}, the adsorption observed was 1.4% and 1.9%, respectively. This showed that adsorption was not the main route of diclofenac removal, as observed by Granatto et al. [44], who obtained 1.7% adsorption of propranolol (PRO) in the biomass of batch reactors with addition of ethanol. PRO and DCF have an aromatic ring in their chemical structures and octanol–water partition coefficient higher than 3.0 (log Kow DCF = 3.9; log Kow PRO = 3.5).

The adsorption of diclofenac in the biomass was quantified at the end of the reactor operation, between 0.2–0.6 μg DCF L^{-1}. Since the maximum diclofenac removal efficiency was 23.6 ± 8.6% for initial concentration of 180.50 ± 8.2 mg NO_3^- L^{-1}, 73.3 ± 3.6 μg DCF L^{-1} and 183.5 ± 28.7 mg EtOH L^{-1}. Thus, adsorption was not the main route of diclofenac removal.

Table 3. Nitrate, diclofenac and ethanol removal in control reactors. The errors presented (±) correspond to the standard deviation calculated for each condition in triplicate.

Reactors	R_I	R_{II}	R_{III}	R_{IV}
Nitrate (NO_3^-)				
Initial (mg L^{-1})	171.9 ± 20.6	149.7 ± 14.1	145.2 ± 4.5	139.7 ± 18.7
Final (mg L^{-1})	91.9 ± 3.3	0	78.5 ± 7.3	0
Removal (%)	46.5 ± 2.6	100	45.9 ± 5.3	100
Diclofenac (DCF)				
Initial (μg L^{-1})	-	-	85 ± 3.5	80 ± 0.7
Final (μg L^{-1})	-	-	77 ± 2.0	70 ± 2.3
Removal (%)	-	-	9.65 ± 0.9	12.3 ± 5.0
Adsorption (%)	-	-	1.0	1.5
Ethanol (EtOH)				
Initial (mg L^{-1})	-	150 ± 5.6	-	160 ± 12.3
Final (mg L^{-1})	-	73.6 ± 5.6	-	75.3 ± 12.5
Removal (%)	-	50.2 ± 7.8	-	65.6 ± 2.3

3.2 RCCD Reactor

In all RCCD experimental conditions, it was possible to observe the removal of up to 23.6% of diclofenac and total nitrate (Table A.2) from the central point ranges with initial concentrations of 180.5 ± 8.2 mg NO_3^- L^{-1}, 78.0 ± 2.6 μg DCF L^{-1} and 183.5 ± 28.7 mg EtOH L^{-1}. Although higher concentrations of nitrate (> 230 mg NO_3^- L^{-1}) and ethanol (> 230 mg EtOH L^{-1}) did not favour drug removal, it was observed that these variables had a significant impact on drug removal ($p > 0.05$).

Under the condition with the lowest nitrate concentration (95.1 ± 2.1 mg NO_3^- L^{-1}) with 162.4 ± 12.5 mg EtOH L^{-1} and 101.5 ± 0.7 μg DCF L^{-1}, 5.9% diclofenac removal and 100% nitrate removal were obtained. Under such conditions, it was inferred that the lower concentration of nitrate was averse to drug removal. Similarly, Kassotaki et al. [45] obtained maximum removal of sulfamethoxazole (SMX) (60%) under anoxic condition in batch reactors, starting at 1.0 g NO_3^- L^{-1} and 1.3 g COD L^{-1}. It is worth noting that SMX is structurally like DCF, due to the presence of the amine grouping and two aromatic rings.

The nitrate removal was complete under all conditions, even in reactors containing maximum concentrations of diclofenac (146.3 ± 22.3 μg DCF L^{-1}). Thus, the higher concentration of the drug did not affect nitrate removal, and denitrification occurred via assimilation of diclofenac as an electron donor. However, the consumption of nitrate was favoured by ethanol in the batches, as corroborated in the control reactors, in which nitrate was partially removed only with diclofenac, making the addition of organic cosubstrate necessary for complete denitrification. Thus, it is possible to infer that for the conditions analysed, diclofenac and ethanol were electron donors in the denitrification, and that ethanol contributed to the maximum removal of nitrate.

The results obtained in this study were similar to that of Torresi et al. [38], who obtained complete nitrate removal (70 ± 3 mg L^{-1}) in 24 h, even at maximum concentration (2.0 μg L^{-1}) of the drugs sulfamethoxazole, carbamazepine and sulfametizole. The authors operated batches under denitrifying conditions with 70 ± 3 mg NO_3^- L^{-1} and 239 ± 2 mg COD L^{-1} methanol as a sole cosubstrate.

Ethanol concentrations in the initial and final phases of the experiment, as well as the removal efficiency obtained in each reactor are summarised in Figure 2 and Table A.2. At lower ethanol concentrations (100.5 ± 25.3 mg L^{-1}), diclofenac removal (95.0 ± 8.6 mg L^{-1}) and nitrate removal (197.7 ± 5.4 mg L^{-1}) were 1.7 ± 0.5% and 100%, respectively. Moreover, in such conditions, a significant effect ($p > 0.05$) was observed in the removal of diclofenac, thus it can be inferred that ethanol favoured the removal of the drug. It is probable that the use of biodegradable organic and recalcitrant compound by cometabolism occurred for the denitrifying biomass. The highest ethanol removal efficiency (77.8%) was observed in the condition containing 135.2 ± 2.1 mg NO_3^- L^{-1}, 100 ± 4.72 µg DCF L^{-1} and 230 ± 6.03 mg EtOH L^{-1}. Under the reactor with 230 ± 6.0 mg EtOH L^{-1}, 25.2 ± 2.4% removal of diclofenac was obtained from 100 ± 4.7 µg L^{-1} and 100% of nitrate removal (135.2 ± 2.1 mg L^{-1}) with ethanol removal efficiency of 77.85 ± 85%. In addition, no accumulation of organic acids was observed in all RCCD conditions. Finally, based on the RCCD reactors, it can be concluded that the presence of the cosubstrate (EtOH) favoured the removal of diclofenac.

Cometabolism with substrate supplementation for biomass is one alternative to improve diclofenac degradation [4]. Organic cosubstrate is readily available electron-donor sources, which may favour microbial metabolism and enable greater biomass adaptation and biodegradation of micropollutants, by inducing extracellular enzymatic production, supplying ATP in the system, favouring the degradation of recalcitrant xenobiotics [34].

Based on the control reactors R_{II} (149.7 ± 14.1 mg NO_3^- L^{-1} + 150 ± 5.6 + 90 mg EtOH L^{-1}) and R_{II} (139.7 ± 18.7 mg NO_3^- L^{-1} + 80.0 ± 0.7 µgDCF L^{-1}), it was observed that ethanol favoured denitrification via nitrate removal. This emphasises the importance of organic cossubstrates in bioremediation studies, moreover, can come from the organic composition of the wastewater and sanitary domestic.

The maximum diclofenac removal efficiency was 23.6 ± 8.6% for initial concentration of 180.50 ± 8.2 mg NO_3^- L^{-1}, 73.3 ± 3.6 µg DCF L^{-1} and 183.5 ± 28.7 mg EtOH L^{-1}. Ooi et al. [11] evaluated the removal of diclofenac in batch reactors containing hospital wastewater. The authors found 20 ± 3% removal of diclofenac for initial concentrations of 20 µg DCF L^{-1}, 20 mg NO_3^- L^{-1} and 100 mg EtOH L^{-1}. Furthermore, Ozdemir et al. [9] obtained 2% removal of diclofenac (100 µg L^{-1}) in batch reactors with activated sludge inoculum and synthetic medium with 140 mg L^{-1} nitrate, 36 mg L^{-1} yeast extract and 720 mg L^{-1} glucose. Like the present study, the authors observed 100% nitrate removal in 6 days of operation. Thus, the drug removal efficiencies observed by Ooi et al. [11] and Ozdemir et al. [9] were lower compared to those observed in the present study. However, denitrification via nitrate removal occurred regardless of the initial concentration of diclofenac, like that observed by the aforementioned authors.

In the present study, although nitrate removal was complete in all conditions analysed (95.1 ± 2.1 a 227.8 ± 5.5 mg NO_3^- L^{-1}), it is important to note that the highest diclofenac removal efficiency (25.2 ± 2.4%) was observed in the condition with 135.2 ± 2.1 mg NO_3^- L^{-1} and 230 ± 6.0 mg EtOH L^{-1}. At lower concentrations of

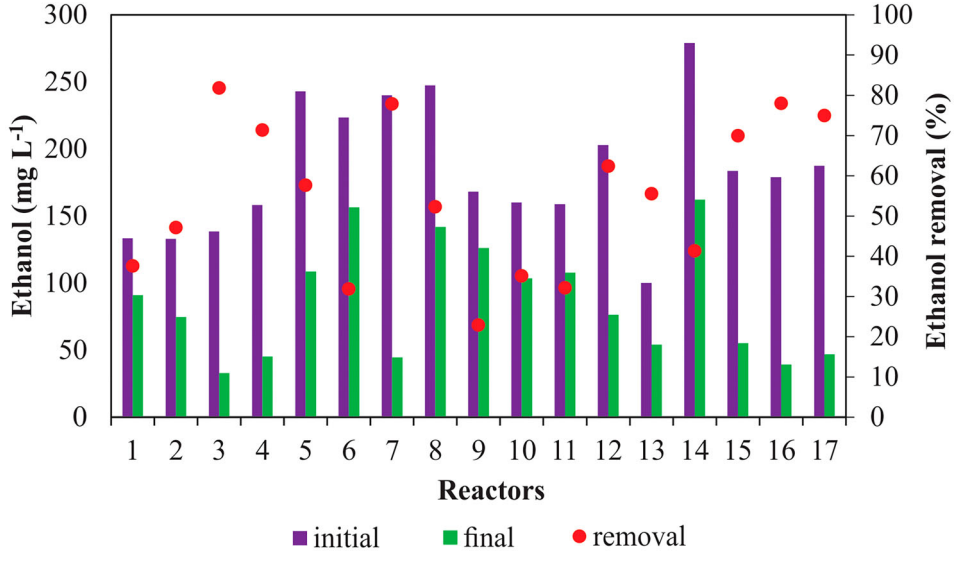

Figure 2. Ethanol removal in RCCD reactor.

ethanol and diclofenac, Arias et al. [10] evaluated this drug removal under denitrifying conditions in batch reactors with nitrification biomass as inoculum. In reactors containing 80 mg EtOH L^{-1}, 10 µg DCF L^{-1} and 130 mg NO_3^- L^{-1}, the authors obtained 15% diclofenac removal efficiency and 100% nitrate removal over 6 h of operation. Thus, with the operation of the reactors via RCCD, it was observed that diclofenac was removed under denitrifying conditions from ethanol and diclofenac as carbon sources. The denitrification via nitrate removal occurred from the assimilation of diclofenac as an electron donor, however, this process was favoured by ethanol, in the cometabolic process.

According to the statistical analysis of the experimental planning, the effects of diclofenac removal were verified for the independent variables (NO_3^-, DCF and EtOH), with a level of 93.45%. The complete adjusted equation was obtained (Equation 9) after applying a multiple linear regression model to the diclofenac removal reactors, ($Y_{response}$), where Y is the independent variable (diclofenac removal, %), x_1 is the initial concentration of nitrate (mg L^{-1}), x_2 is the initial concentration of diclofenac (µg L^{-1}) and x_3 is the initial concentration of ethanol (mg L^{-1}). The regression coefficients of factors interactions (NO_3^-, DCF and EtOH) (Table 4) were obtained with coded matrix.

$$Y = 22.30 - 2.59X_1 + 2.09X_3 - 6.55X_1^2 - 4.36X_2^2 - 5.95X_3^2 - 4.34X_1X_2 \quad (9)$$

Analysis of variance (ANOVA) was calculated in order to evaluate the effects of the interactions between nitrate, diclofenac and ethanol on drug removal (DCF). This enabled to select the best conditions for the predicted response - $Y_{response}$ (diclofenac removal). Table 5 showed that the *p*-value of the regression coefficient (98%) was significant at the 0.05% level, with good fit between the experimental and predicted values. Furthermore, the $F_{calculated}$ coefficient (10.72) was higher than the $F_{tabulated}$ (2.72), allowing to conclude that the obtained model (Equation 9) is appropriate to evaluate the removal of diclofenac, considering the difference of the initial concentration of nitrate, diclofenac, and ethanol.

It was observed that linear and quadratic terms of the variables nitrate (x_1), diclofenac (x_2) and ethanol (x_3), as well as the interaction of the variable nitrate with the other factors (diclofenac and ethanol) were statistically significant at 5% significance level ($p < 0.05$). This showed that the response variable was more sensitive to the variation of nitrate than ethanol. Based on the regression model (Equation 9), it is also noted that the lower concentration of nitrate (x_1), relative to the higher linear concentration of diclofenac and ethanol, contributed to higher diclofenac removal ($Y_{response}$).

The surface response (Figure 3) was elaborated based on the quadratic model obtained via Equation 9. This analysis allowed to obtain the maximum values of DCF removal based on the differences of the initial concentration of 95.1–250.0 mg NO_3^- L^{-1}, 57.0–146.3 µg DCF L^{-1} and from 100.5–278.9 mg EtOH L^{-1}. The optimal ranges observed were 180 mg NO_3^- L^{-1} and 180 mg EtOH L^{-1}, resulting in 23.6% diclofenac removal (80 µg L^{-1}).

It can be observed that for ethanol concentration in the range between 80–140 mg EtOH L^{-1}, the removal efficiency of diclofenac ($Y_{response}$) is more sensitive to differences in the initial nitrate concentration. However, when ethanol concentration is higher (180-240 mg L^{-1}), diclofenac removal is greater (12% – 20%) and less susceptible to differences in the initial nitrate concentration. Thus, for the conditions presented, the presence of ethanol as a cosubstrate may have favoured the removal of diclofenac under denitrifying conditions. In addition, at a lower concentration of this compound (100.5 ± 25.3 mg EtOH L^{-1}) in 95 ± 8.6 µg DCF L^{-1} and 197.7 ± 5.4 mg NO_3^- L^{-1}, diclofenac removal (1.7 ± 0.5%) was lower than the other reactors.

The adsorption of diclofenac in the biomass was quantified at the end of the reactor operation, between 0.2 and 0.6 µg DCF L^{-1}. The percentage

Table 4. Regression coefficients of experimental design.

Factors	Regression coefficients	Coef - IC	Coef + IC	IC	*p*-value	Conclusion*
Average	22.31	17.46	27.13	4.83	0.0000	s
x_1 (L)	−2.59	−4.86	−0.32	2.27	0.0266	s
x_1 (Q)	−6.55	−9.06	−4.05	2.50	0.0004	s
x_2 (L)	1.91	−0.35	4.18	2.27	0.0780	ns
x_2 (Q)	−4.36	−6.86	−1.85	2.50	0.0037	s
x_2 (L)	3.09	0.82	5.36	2.27	0.0125	s
x_3 (Q)	−5.95	−8.46	−3.45	2.50	0.0006	s
$x_1 \times x_2$	−4.34	−7.30	−1.36	2.96	0.0090	s
$x_1 \times x_3$	−1.89	−4.83	1.10	2.96	0.1681	ns
$x_2 \times x_3$	1.04	−1.93	4.00	2.96	0.4202	ns

IC = Confidence interval. Pure error = 2.88. *Significant effect at 95% confidence. R^2 = 0.98; $p < 0.05$. L = linear terms; Q = quadratic terms; x_1 = NO_3^- initial concentration (mg L^{-1}); x_2 = DCF initial concentration (µg L^{-1}); x_3 = EtOH initial concentration (mg L^{-1}); s = significant; ns = not significant.

Table 5. ANOVA of diclofenac removal obtained after RCCD reactors.

Variation source	Quadratic sum	Degrees of freedom	Square mean	F_{calc}	F_{tab} (95%)
Regression	1086.1	6	181.02	10.722	2.98
Residual	168.83	10	16.88		
Lack of Adjustment	165.95	8	20.74	14.406	19.35
Pure error	2.88	2	1.44		
Total	1254.9	16			

distribution parameters of diclofenac adsorbed, removed and recovered in the liquid fraction are summarised in Figure 4 and Table A.2. It is noteworthy that the higher initial concentration of diclofenac did not indicate an increase in the adsorbed fraction (reactor 12; 146.3 µg DCF L^{-1}), and the adsorption was 0.3 µg from 48.2 µg DCF added, in which no significant difference was observed compared to the other reactors. Similar results were observed by Plósz et al. [46] in the biodegradation of the drugs diclofenac and carbamazepine, in batches inoculated with biomass from activated sludge, whose adsorption was 1.0%, close to the values observed in this study (1% to 2%).

Moreover, Yan et al. [47] investigated the diclofenac adsorption in three different inoculum matrices from sanitary sewage treatment systems: concentrated sludge (581.1 g Kg^{-1}), sedimentation effluent (312.4 g Kg^{-1}) and suspended particles (143.3 g Kg^{-1}). These authors concluded that the highest diclofenac adsorption occurred in concentrated sludge, followed by

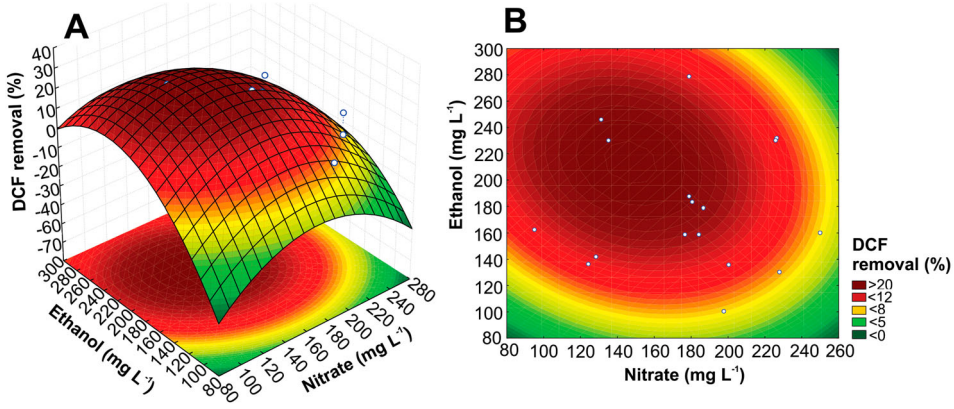

Figure 3. Surface response (A) and contour (B) of diclofenac removal as a function of nitrate, diclofenac and ethanol concentrations.

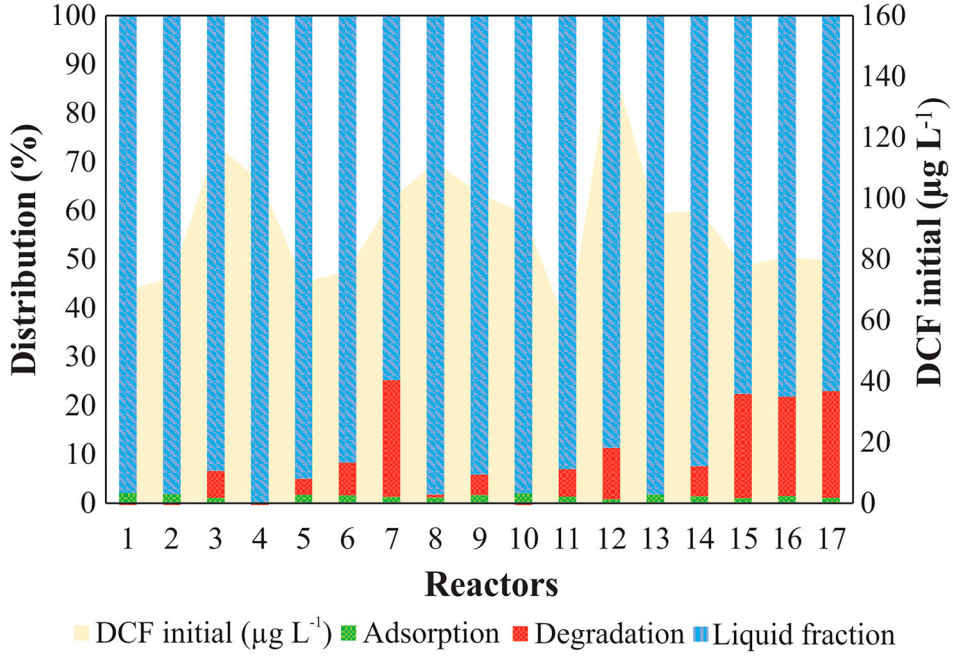

Figure 4. Mass balance of diclofenac in batch reactors of RCCD reactor.

sedimentation tank effluent, and diluted sludge in suspended particles in the effluent. Thus, since a higher concentration of solids in the sludge interferes directly in the sorption capacity of organic compounds, the low concentration of adsorbed diclofenac observed in this study may be related to the low concentration of solids in the inoculum (1.0 gSSV L^{-1}).

3.3 Nitrate removal

The denitrification rate (k) was correlated with the initial nitrate and diclofenac concentrations, which showed random kinetic parameters in all conditions applied to the RCCD (Figure 5). Based on these considerations, it can be inferred that increasing the concentrations of nitrate, diclofenac and ethanol (independent variables) did not interfere in the kinetics of nitrate removal (Table 5).

Similarly, Suarez et al. [48] observed in batch reactors, with denitrification tank inoculum, that the ranges between 10–40 µg DCF L^{-1} in synthetic medium with 500 mg EtOH L^{-1} and 100 mg NO_3^- L^{-1} did not affect the nitrate removal rate. The results presented by these authors corroborate those observed in the present study, in the range between 95.11 ± 2.1 and 227.8 ± 5.5 mg NO_3^- L^{-1}.

Thus, it was observed that the optimum conditions for diclofenac removal (22.4 ± 2.5%) occurred in the reactors with the following conditions: 183.5 ± 28.7 mg EtOH L^{-1}, 180.5 ± 8.2 mg NO_3^- L^{-1} and 80.0 ± 2.6 µg DCF L^{-1}.

3.4 Validation reactor

The validation of the experimental planning model was performed in order to verify the optimal ranges predicted by multiple regression (optimised condition), which evaluated the removal of diclofenac in batch reactors with 76.8 ± 3.7 µg DCF L^{-1}, 180 mg NO_3^- L^{-1} and 180 mg EtOH L^{-1}. Under these conditions, 17.5% diclofenac removal and 100% nitrate removal were observed, obtaining correlation between the predicted value and the experimental value of 18.7% and 17.5%, respectively.

In the optimised condition, nitrate removal was complete under 16 h of operation, with a velocity (k) of 0.096 h^{-1}, at an average time of 7.24 h (Figure A.1).

3.5 Microbial community characterisation

Microbial community characterisation was carried out for control reactor R_{II} (149.7 ± 14.1 mg NO_3^- L^{-1} and 150 ± 3.6 mg EtOH L^{-1}) and RCCD optimised conditions

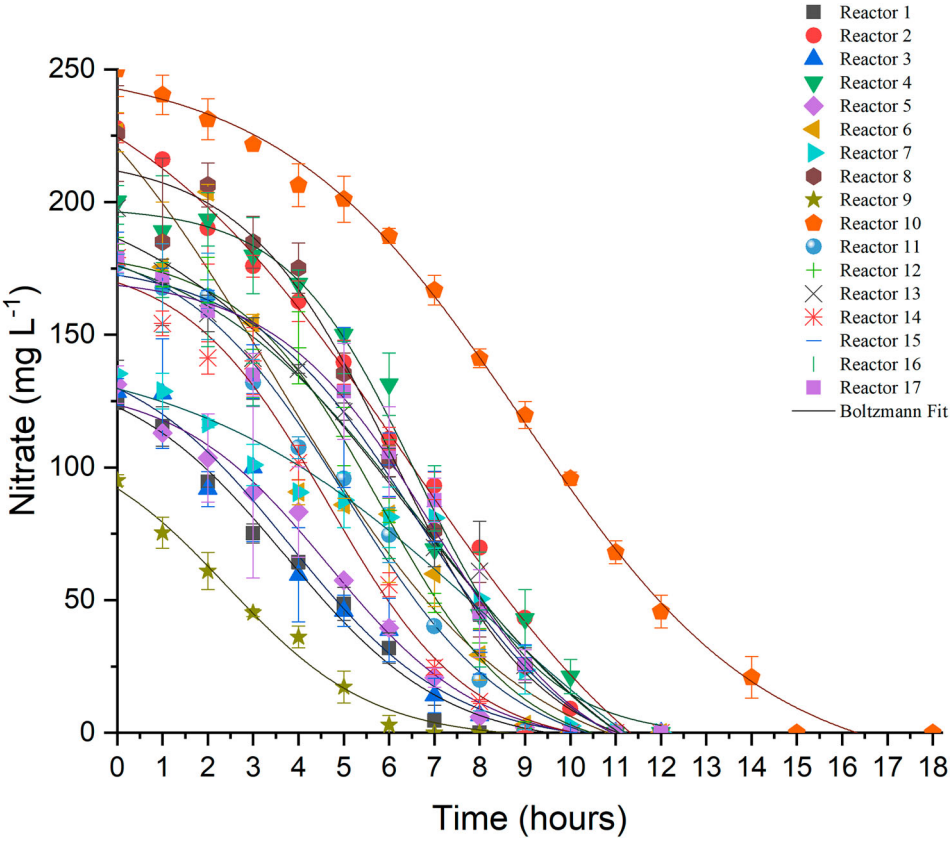

Figure 5. NO_3^- removal in RCCD reactors. The errors presented (±) correspond to the standard deviation calculated for each condition in triplicate.

(176.6 ± 4.3 mg NO_3^- L^{-1}, 76.8 ± 3.7 µg DCF L^{-1} and 180 ± 2.5 mg EtOH L^{-1}). The sequencing parameters, as well as the diversity indices for the Bacteria domain, are summarised in Table A.3.

The Shannon diversity index values of the control reactor (5.31) and optimised condition (5.30) were close, while the relative richness Chao-1 was higher in the control reactor compared to the optimised reactor. Probably, the addition of diclofenac influenced the microbial community richness.

In both analysed conditions, 26 phyla were identified, and the highest relative abundance was observed for Proteobacteria (59.8 - 69.1%), Acidobacteria (19.3 - 10.2%), Bacteroidetes (9.7 - 10.6%) and Actinobacteria (2.0 - 3.3%) for the control and optimised reactor, respectively (Figure 6A). The phyla Saccharibacteria, Gemmatimonadetes, Verrucomicrobia, Spirochaetes, Chlamydiae and Chloroflexi accounted for less than 4% in both reactor conditions.

Microorganisms that belong to the Proteobacteria phylum are facultative anaerobes, chemoautotrophs or heterotrophs [49]. Furthermore, representatives of this phylum have been related to the removal of recalcitrant compounds and long-chain alkanes by the denitrifying pathway [50,51]. In free oxygen respiration, nitrate is the main electron acceptor that confers a series of transformations in the generation of benzoyl-CoA as a common intermediate [50].

After the formation of benzoyl-CoA, the aromatic ring is reduced, followed by β-oxidation of the carbon chain, cleavage of the aromatic ring and mineralisation via glutaryl-CoA. The microorganisms that participate in these reactions belong mostly to subclasses β- and γ-Proteobacteria [50] and use complex organochlorine compounds. Higher relative abundance of Gammaproteobacteria (40.14%) belonging to the phylum Proteobacteria (Figure 6A) was observed in the optimised condition (176.6 ± 4.3 mg NO_3^- L^{-1}, 76.8 ±

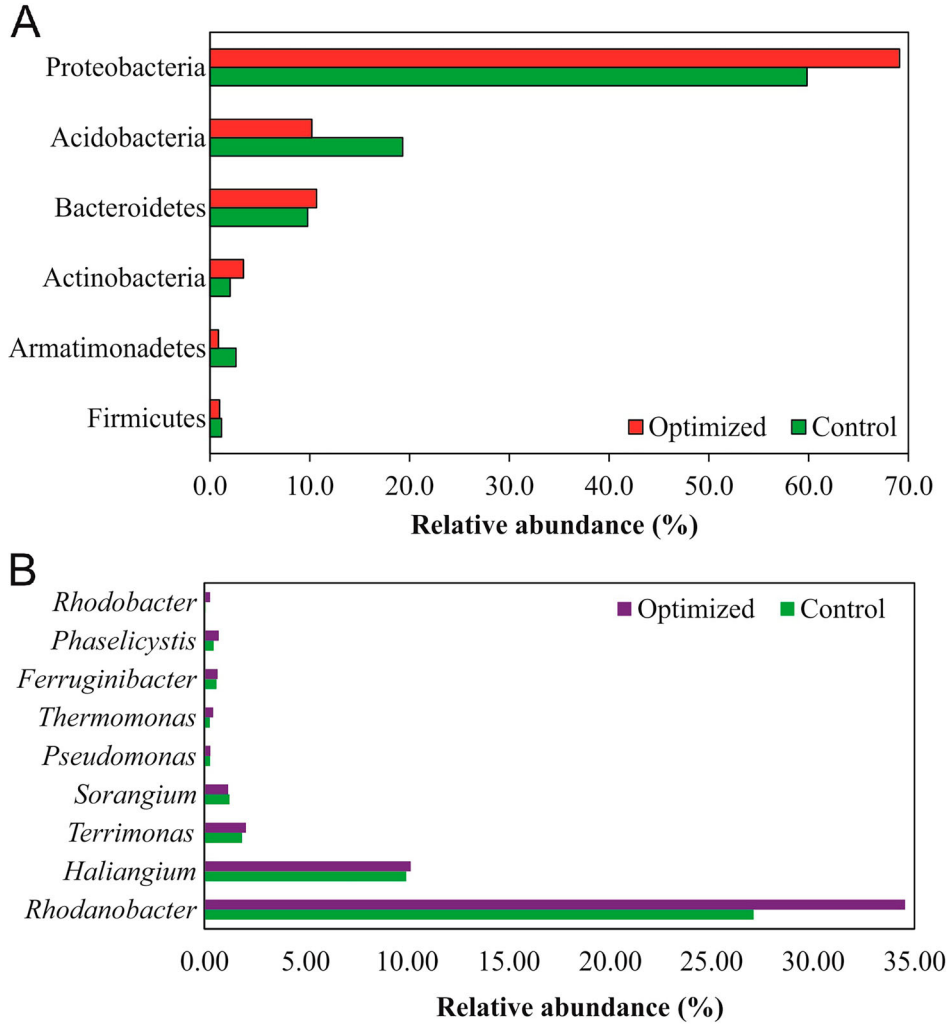

Figure 6. Relative abundance of phyla (A) and genera (B) observed in control and optimised reactors.

3.7 µg DCF L^{-1} and 180 ± 2.5 mg EtOH L^{-1}) under control reactor (149.7 ± 14.1 mg NO_3^- L^{-1}, 150 ± 3.6 mg EtOH L^{-1}), these bacteria may have been favoured by the addition of diclofenac.

Bacteria belonging to the phylum Acidobacteria are heterotrophs and can reduce nitrate under anoxic conditions [52]. Microorganisms belonging to this phylum consume organic acids, such as acetic acid, and also ethanol as carbon and energy source [52]. However, higher relative abundance values were observed for the control reactor (21%) compared to the optimised one (11%). Thus, it can be assumed that diclofenac did not favour organisms in this phylum. The third phylum with the highest relative abundance in both conditions was Bacteroidetes (9.7 - 10.6%), whose representatives can degrade high molecular weight compounds, such as proteins and carbohydrates [53].

Members of the Xanthomonadaceae family were identified with 29.25% and 37.8%, respectively in the control and optimised reactors. These microorganisms are denitrifying and have a chemorganotrophic metabolism, which may have contributed to the higher relative abundance observed in both conditions. Furthermore, the families Blastocatellaceae (18.63% and 9.52%), Haliangiceae (9.96% and 9.52), Comamonadaceae (4.68 and 4.38%), Chitinophagaceae (4.01 and 4.09%) and Saprospiraceae (3.62 and 4.14%) were identified in the control and optimised reactors, respectively. The families Fimbriimonadaceae, Polyangiaceae, Sphingomonadaceae, Gemmatimonadaceae, Hyphomonadaceae and Cytophagaceae were observed with relative abundance ≤3% in both conditions evaluated.

Among the major genera identified (Figure 6B), the highest relative abundance was observed for *Rhodanobacter* (27.0 and 34.5%), *Haliangium* (9.9 and 10.1%) and *Terrimonas* (1.8 and 2.0%) in the control and optimised reactors, respectively. The genera *Sorangium, Ferruginibacter, Phaselicystis, Woodsholea, Pseudomonas* and *Thermomonas* were identified at relative abundance ≤1% in both conditions.

Rhodanobacter was identified as the most abundant genus (27.08 - 34.53%) in both conditions. This genus includes Gram-negative, aerobic and chemorganotrophic bacteria, and uses nitrate or oxygen as an electron receptor [51,54]. In addition, *Rhodanobacter* have been commonly identified in activated sludge systems, due to their ability to promote denitrification under facultative conditions and degradation of aromatic compounds, such as polycyclic aromatic compounds (PAHs) [55], anionic surfactants [56–58], phenols, toluene and benzoate [51,59–61], and also VFA [62] and anti-inflammatory non-steroidal drugs, such as diclofenac and ibuprofen [61,63].

Navrozidou et al. [63] identified 20% relative abundance of *Rhodanobacter* in immobilised biofilms with activated sludge inoculum for the degradation of diclofenac in sanitary sewage, from 400 mg L^{-1} of the drug in a fixed-bed reactor under continuous operation. The authors reported the denitrifying capacity of *Rhodanobacter* under anoxic conditions with high concentrations of DCF (mg L^{-1} range). In addition, the aforementioned authors related the higher relative abundance of this genus to its ability to use recalcitrant compounds as carbon sources, in addition to nitrate as an electron receptor.

In this study, it was observed that the relative abundance of *Rhodanobacter* was higher in the optimised reactor (176.6 ± 4.3 mg NO_3^- L^{-1}, 76.8 ± 3.7 µg DCF L^{-1} and 180 mg EtOH L^{-1}) when compared to the control reactor (150 mg NO_3^- L^{-1} + 150 mg EtOH L^{-1}). As observed by Navrozidou et al. [63], the concentration of diclofenac in the optimised sample (80 µg L^{-1}) may have favoured the relative abundance of this genus. Also, according to Cai et al. [64], *Rhodanobacter* play a major role in denitrification in bioprocesses applied to remove drugs, such as ciprofloxacin ($C_{17}H_{18}FN_3O_3$), a compound with more than ten carbons and aromatic rings, similar to diclofenac molecular structure ($C_{14}H_{11}Cl_2NO_2$), making it possible to be used as substrate for the enzyme α-methylacyl-CoA racemase [EC: 5.1.99.4], inferred in the present study, as better detailed further in this section.

Regarding the kinetic parameters of the analysed reactors, it was found that the denitrification rate (k) in the optimised condition (0.096 h^{-1}) was higher than that of the control condition (0.0034 h^{-1}), thus, it can be inferred that the higher relative abundance of *Rhodanobacter* may have corroborated the higher value of this kinetic coefficient in the metabolic reaction, in addition to contributing to denitrification.

The genus *Haliangium* was identified as one of the most abundant in both conditions evaluated (10%). This genus includes heterotrophic denitrifying bacteria, which use nitrogen or oxygen as electron acceptor and assimilate acetic acid as a substrate under anaerobic conditions [65]. Thus, in this study the relative abundance of this genus may have been favoured by the availability of NO_3^- (150 mg L^{-1}) as electron receptor, and EtOH (150 mg L^{-1}) as an easily degradable carbon source and electron donor in both samples.

The genus *Terrimonas* was identified with a relative abundance of 2% in both conditions. Representatives of this genus are aerobic, Gram-negative, organotrophic bacteria that reduce nitrate under anoxic conditions. In addition, such bacteria can metabolise hexoses and disaccharides [51] associated with oxidation of recalcitrant

aromatic hydrocarbons, such as surfactants [57] and kerosene [66]. Zhao et al. [67] obtained relative abundance of 12% of *Terrimonas* in an anammox reactor and observed that such bacteria promoted nitrogen removal through biosynthesis of hydrophobic extracellular polysaccharides (EPS) and biofilm aggregation of the anammox microbial consortium. Similarly, representatives of the genus *Sorangium*, identified in the present study with relative abundance of 1.1% in the control reactor and 1.2% in the optimised reactor, can degrade aromatic compounds through decarboxylation of the aromatic ring [51].

Thermomonas are Gram-negative and filamentous, moderately thermophilic and of chemolithotrophic metabolism. In addition, they can grow in an anaerobic medium in the presence of nitrate as electron receptor [51]. In this study, the relative abundance of this genus in the control condition (0.3%) was similar in the optimised condition (0.4%), implying that the presence of DCF did not interfere in the abundance of this genus; however, the presence of nitrate was essential for its metabolism.

Pseudomonas was identified in both reactors with a relative abundance of less than 1%. This genus assimilates nitrate as electron receptor in anoxic respiration. The microorganisms belonging to this genus have metabolic flexibility, which implies the use of various sources of carbon and nitrogen [51,54]. In this study, this genus may have contributed to the total removal of nitrate in both analysed conditions, due to the similarity of the relative abundance for the control reactor (0.28%) and the optimised condition (0.3%).

Ferruginobacter was identified at a relative abundance of 0.6% and 0.7% in the control and optimised reactors, respectively. This genus includes Gram-negative bacteria, which degrade aromatic compounds under aerobic conditions such as naphthylphosphate and nitro-phenyl-galactopyranoside [51,54]. Relative abundance of 0.04% and 0.07% of *Phaselicystis* was observed in the control and optimised reactors, respectively. This genus includes myxotrophic bacteria, associated with the ability to desulfonate under anaerobic conditions [68].

Diclofenac is an acetylsalicylic acid that consists of acetate, two chlorinated groups and two aromatic rings in an amine grouping that can be used by microorganisms as carbon or nitrogen source [10]. The degradation of diclofenac under denitrifying conditions can occur from the assimilation of nitrate as an electron receptor to the nitrogen group of the diclofenac molecule, which results in the formation of nitro-DCF (NO-DCF) as an intermediate nitro-derived compound. This nitration reaction is considered an irreversible process, culminating in the formation of NO_2-DCF [69].

Furthermore, Chiron et al. [70] observed another denitrifying route that can culminate in the biodegradation of diclofenac through codenitrification, a restricted process under anoxic conditions, where nitrate removal and formation of nitrous oxide occurs, followed by nitrogen trioxide, leading to N-nitrosation of the DCF amine. From the rearrangement of N-nitrosamines to nitrous-DCF and in the presence of nitrite, NO-DCF is converted to NO_2-DCF, and oxidised, generating a carbon radical that is neutralised by nitrite. The addition of ethanol as cosubstrate is noteworthy, since under denitrifying conditions, ethanol and also fumarate can be used as electron donors, keeping sufficient ATP in the system through the addition of cosubstrates, favouring the phosphorylation of diclofenac [18,36,71].

Another possible degradation route of diclofenac refers to the ω-oxidation of the terminal methyl group, followed by the β-oxidation of the carboxyl group of the molecular structure of the organochlorine compound [72]. There is also the oxidative cleavage of the carbon units that cause the opening of the para-acetyl-CoA ring and acidic intermediates, and its oxidation to CO_2. This mechanism is similar in other aromatic compounds, such as the surfactant LAS [73]. *Pseudomonas*, *Clostridium* and *Syntrophobacter* are some of the main genera associated with this step in anaerobic environments, especially for ω/β-oxidation, desulfonation and cleavage of aromatic rings [71,74]

Diclofenac ($C_{14}H_{11}C_{l2}NO_2$) is a compound with more than ten carbons, which, in addition to its aromatic rings, can be used as substrate for the enzyme α-methylacyl-CoA racemase [EC: 5.1.99.4]. Furthermore, it is worth mentioning the importance of catalase [EC: 1.11.1.6], which acts as a pseudoperoxidase [EC: 1.11.1.7] in several organic substances, with great affinity for ethanol as a hydrogen donor, forming acetaldehyde through this reaction. The addition of Coenzyme A during the degradation of DCF occurs due to the action of the long-chain-fatty-acid-CoA ligase enzyme [EC: 6.2.1.3], through the oxidation of an octane, such as phenylacetate ($C_8H_8O_2$), which can occur by the reactions described in Equations 10 and 11.

$$\text{ATP} + \text{long chain fatty acid} + \text{CoA} \rightarrow \text{AMP} + \text{diphosphate} + \text{acyl CoA} \quad (10)$$

$$\text{ATP} + \text{(R)ibuprofen} + \text{CoA} \rightarrow \text{AMP} + \text{diphosphate} + \text{(R)ibuprofenoyl CoA} \quad (11)$$

In studies conducted by Jewell et al. [75], hydroxylation and decarboxylation reactions were selected as the main transformation routes of the DCF molecule in activated sludge. In the drug molecule, the chlorinated

aromatic ring can be hydroxylated, generating 4'OHDCF. Next, this intermediate metabolite is decarboxylated, followed by conjugation of the carboxyl group of the 4'-OHDCF to the hydroxyl, forming an amino group and phenylacetic acid [72].

The possible degradation mechanisms of diclofenac are summarised in Figure 7, as well as the possible genera involved in each transformation process of the molecule.

4. Conclusion

The RCCD experimental planning enabled to study the optimisation of diclofenac degradation in batch reactors. This statistical tool demonstrated that ethanol and nitrate concentrations were significant for the maximum removal of diclofenac, and the highest drug removal efficiency (17.5%) and total nitrate removal were obtained at the optimal conditions of 176.6 ± 4.3 mg NO_3^- L^{-1}, 76.8 ± 3.7 µg DCF L^{-1}, and 180.0 ± 2.5 mg EtOH L^{-1}.

The statistical analysis of the effects between the independent variables (nitrate and ethanol) allowed to conclude that nitrate (linear and negative quadratic effect) represented the greatest magnitude of impact on d removal, followed by ethanol concentration (linear and positive quadratic effect). Nitrate was completely removed under all conditions evaluated (>90%), and it can be inferred that denitrification occurred with the assimilation of diclofenac as an electron donor, although ethanol favoured the nitrate removal pathway, as verified in the control reactors.

The denitrifying bacteria belonging to the genera *Rhodanobacter, Haliangium*, *Terrimonas* and *Sorangium* were identified in the optimised condition with the highest relative abundance, and these genera are

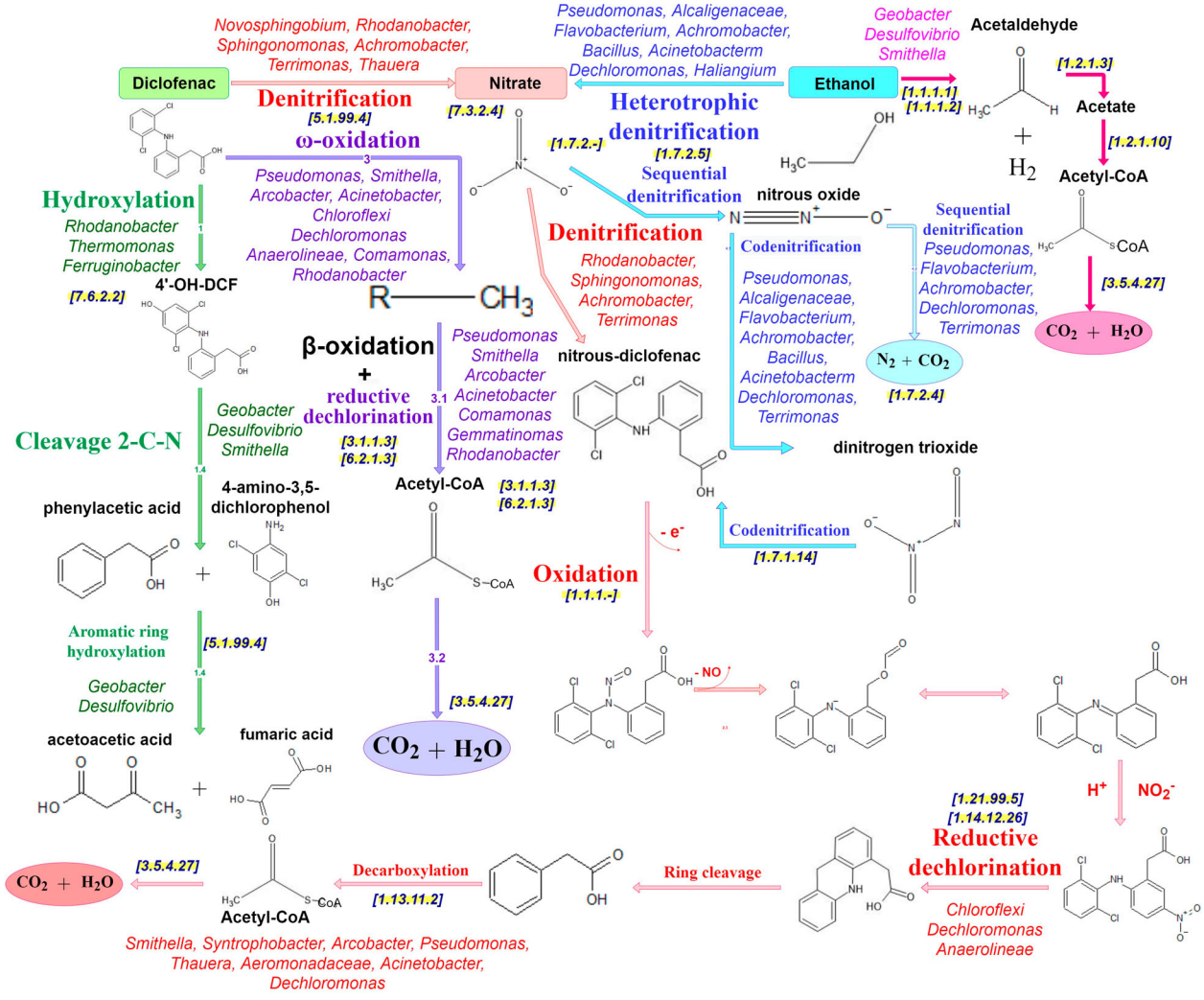

Figure 7. Possible metabolic pathways involved in reactors applied to diclofenac degradation, containing ethanol and nitrate. The EC numbers presented in square brackets, referring to the enzymes potentially involved in each reaction, were inferred by the Tax4Fun2 package.

related to the nitrogen cycle and degradation of aromatic compounds, confirming the adaptation of these microorganisms in the experimental conditions of this study, being the main enzymes potentially involved in this process were α-methylacyl-CoA racemase, long-chain fatty acid-CoA ligase, catalases and pseudoperoxidases.

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Disclosure statement

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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/or] its supplementary materials.

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