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JOSÉ GILMAR DA SILVA DO NASCIMENTO

Engineering approaches for improving the removal of organic micropollutants in anaerobic reactors: microaeration, redox mediator, and nitrate addition

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2020

JOSÉ GILMAR DA SILVA DO NASCIMENTO

ENGINEERING APPROACHES FOR IMPROVING THE REMOVAL OF ORGANIC MICROPOLLUTANTS IN ANAEROBIC REACTORS: MICROAERATION, REDOX MEDIATOR, AND NITRATE ADDITION

Tese de doutorado apresentada ao Programa de Pós-Graduação em Engenharia Civil do Departamento de Engenharia Hidráulica e Ambiental da Universidade Federal do Ceará, como requisito parcial à obtenção do título de Doutor em Engenharia Civil. Área de concentração: Saneamento Ambiental.

Orientador: Prof. Dr. Paulo Igor Milen Firmino.
Coorientador: Prof. Dr. Marcos Erick Rodrigues da Silva.

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“Tudo que temos que decidir é o que fazer com o tempo que nos é dado.”

Gandalf

RESUMO

Nas últimas décadas, os micropoluentes orgânicos (MPOs), como produtos farmacêuticos, pesticidas, hormônios, produtos de higiene pessoal e outros, têm chamado muita atenção devido a seus possíveis impactos negativos nos ecossistemas e na saúde pública. Em geral, as estações de tratamento de águas residuais (ETARs) não são projetadas especificamente para remover MPOs, e, embora as eficiências de remoção (ERs) dependam do composto e da tecnologia de tratamento, elas geralmente são limitadas, principalmente em sistemas anaeróbios operados com curtos tempos de retenção hidráulica (TDHs < 10 h). Portanto, este trabalho avaliou, através de quatro estudos, algumas abordagens de engenharia, como adição de microaeração, mediador redox e nitrato, para aumentar as ERs de diferentes MPOs (hormônios, produtos farmacêuticos, bisfenol A e parabenos) em reatores anaeróbios de manta de lodo e fluxo ascendente (*upflow anerobic sludge blanket*, UASB) operados com um TDH de 7-8 h durante o tratamento de esgoto sintético contendo 200 $\mu g \cdot L^{-1}$ de cada composto. No primeiro estudo, o efeito do aumento das vazão de microaeração (1-6 mL de $ar \cdot min^{-1}$) na biotransformação de sete MPOs (estrona, 17β-estradiol, 17α-etinilestradiol, bisfenol A, diclofenaco, sulfametoxazol e trimetoprima) foi avaliada. A microaeração melhorou as ERs expressivamente, e os melhores resultados foram obtidos com 4 mL de $ar \cdot min^{-1}$ (~90%). No segundo estudo, o efeito do mediador redox antraquinona-2,6-dissulfonato (AQDS) (50 e 100 μM), associado ou não à microaeração (1 mL de $ar \cdot min^{-1}$), e de diferentes relações demanda química de oxigênio (DQO)/NO_3^- (2,5-10) nas ERs dos antibióticos sulfametoxazol e trimetoprima foi investigado. O AQDS acelerou significativamente a biotransformação anaeróbia dos antibióticos (um aumento de ~70% nas ERs com 100 μM de AQDS). A microaeração melhorou sua biotransformação, mesmo na presença de AQDS, quando as melhores ERs foram obtidas (> 70%), particularmente para a trimetoprima (~90% com 1 mL de ar · min-1 e 100 μM de AQDS). A adição de nitrato também mostrou resultados promissores, com os ERs mais altos (~85%) sendo alcançados na menor razão DQO/NO_3^- (2,5). No terceiro estudo, foi avaliado o efeito de diferentes vazões de microaeração (1-4 mL de $ar \cdot min^{-1}$) nas ERs de quatro parabenos (metilparabeno, etilparabeno, propilparabeno e butilparabeno). Baixas ERs (14-20%) foram alcançadas sob condições anaeróbias, mas a adição de apenas 1 mL de $ar \cdot min^{-1}$ impulsionou notavelmente a biotransformação de parabenos, garantindo ERs acima de 85% para todos os compostos. No quarto estudo, foram avaliados os efeitos individuais e combinados do mediador redox AQDS (50 e 100 μM) e da microaeração (1 mL de $ar \cdot min^{-1}$) na biotransformação dos mesmos parabenos. O AQDS melhorou consideravelmente as baixas ERs de todos os parabenos

(< 17%), mas teve um efeito mais significativo nos compostos com cadeias alquílicas mais longas (etilparabeno, propilparabeno e butilparabeno) (ERs > 90% com 100 μM de AQDS) do que no metilparabeno (ER de ~54% com 50 ou 100 μM de AQDS). Sob condições microaeróbias, o AQDS teve um impacto limitado na biotransformação dos parabenos de cadeia alquílica mais longa e até comprometeu o efeito do oxigênio na biotransformação do MeP, ou seja, o AQDS exerceu um efeito antagônico na microaeração. Finalmente, em todos os estudos, os reatores permaneceram notavelmente estáveis, com alta ER de DQO média (85-90%), sem acúmulo de ácidos graxos voláteis e valores de pH próximos à faixa neutra.

Palavras-chave: Micropoluentes. Microaeração. Mediador redox. Adição de nitrato.

ABSTRACT

In the last decades, organic micropollutants (OMPs), such as pharmaceuticals, pesticides, hormones, personal care products, and others, have drawn much attention due to their potential negative impacts on ecosystems and public health. In general, wastewater treatment plants (WWTPs) are not specifically designed to remove OMPs, and, although removal efficiencies (REs) depend on both the compound and the treatment technology, they are usually limited, particularly in anaerobic systems operated at short hydraulic retention times (HRTs < 10 h). Therefore, this work evaluated, through four studies, some engineering approaches, such as microaeration, redox mediator, and nitrate addition, to increase the REs of different OMPs (hormones, pharmaceuticals, bisphenol A, and parabens) in upflow anaerobic sludge blanket (UASB) reactors operated at an HRT of 7-8 h during the treatmenf ot synthetic wastewater containing 200 $\mu g \cdot L^{-1}$ of each compound. In the first study, the effect of increasing microaeration flow rates (1-6 mL $air \cdot min^{-1}$) on the biotransformation of seven OMPs (estrone, 17β-estradiol, 17α-ethinylestradiol, bisphenol A, diclofenac, sulfamethoxazole, and trimethoprim) was evaluated. Microaeration improved the REs expressively, and the best results were obtained with 4 mL $air \cdot min^{-1}$ (~90%). In the second study, the effect of the redox mediator anthraquinone-2,6-disulfonate (AQDS) (50 and 100 μM), associated or not with microaeration (1 mL $air \cdot min^{-1}$), and different chemical oxygen demand (COD)/NO_3^- ratios (2.5-10) on the REs of the antibiotics sulfamethoxazole and trimethoprim was investigated. AQDS accelerated the anaerobic biotransformation of the antibiotics significantly (an increase of ~70% in the REs with 100 μM of AQDS). Microaeration enhanced their biotransformation even in the presence of AQDS, when the best REs were obtained (> 70%), particularly for trimethoprim (~90% with 1 mL $air \cdot min^{-1}$ and 100 μM of AQDS). Nitrate addition also showed promising results, with the highest REs (~85%) being achieved at the lowest COD/NO_3^- ratio (2.5). In the third study, the effect of different microaeration flow rates (1-4 mL $air \cdot min^{-1}$) on the REs of four parabens (methylparaben, ethylparaben, propylparaben, and butylparaben) was assessed. Low REs (14-20%) were achieved under anaerobic conditions, but the addition of only of 1 mL $air \cdot min^{-1}$ boosted the biotransformation of parabens remarkably, ensuring REs above 85% for all compounds. In the fourth study, the individual and combined effects of the redox mediator AQDS (50 and 100 μM) and microaeration (1 mL $air \cdot min^{-1}$) on the biotransformation of the same parabens were evaluated. AQDS improved the low REs of all parabens (< 17%) considerably, but it had a more significant effect on the compounds with longer alkyl chains (ethylparaben, propylparaben, and butylparaben) (REs > 90% with 100 μM of AQDS) than on

methylparaben (RE of ~54% with 50 or 100 μM of AQDS). Under microaerobic conditions, AQDS had a limited impact on the biotransformation of the longer-alkyl chain parabens and even compromised the effect of oxygen on the biotransformation of MeP, i.e., AQDS exerted an antagonistic effect on microaeration. Finally, in all studies, the reactors remained remarkably stable, with high mean COD REs (85-90%), no accumulation of volatile fatty acids, and pH values close to the neutral range.

Keywords: Micropollutants. Microaeration. Redox mediator. Nitrate addition.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND ACRONYMS

AQDS	Anthraquinone-2,6-disulfonate
BPA	Bisphenol A
BuP	Butylparaben
COD	Chemical oxygen demand
DCF	Diclofenac
EtP	Ethylparaben
E1	Estrone
E2	17β-estradiol
EE2	17α-ethinylestradiol
GC	Gas Chromatography
HPLC	High performance liquid chromatography
HRT	Hydraulic detention time
MeP	Methylparaben
OMP	Organic micropollutants
PCR	Polymerase chain reaction
PrP	Propylparaben
PVC	Polyvinyl chloride
RE	Removal efficiency
SMX	Sulfamethoxazole
SPE	Solid phase extraction
TMP	Trimethoprim
UASB	Upflow anaerobic sludge blanket
VFA	Volatile fatty acids
WWTP	Wastewater treatment plant

CONTENTS

1 INTRODUCTION

In the last decades, organic micropollutants (OMPs), such as pharmaceuticals, pesticides, hormones, personal care products, and others, have drawn much attention due to their potential negative impacts on ecosystems and public health (e.g., development of antibiotic-resistant pathogens and reproductive disorder in animals and humans by endocrine disruptors) (Gogoi et al., 2018; Harb et al., 2019). The main sources of water pollution with such compounds are raw or treated domestic, agricultural, and some industrial wastewaters along with urban and rural runoff, in which OMPs may be found at concentrations ranging from ng to µg per liter (Gogoi et al., 2018; Luo et al., 2014).

The use of biological processes for the removal of OMPs from wastewater is extensively reported in the literature, but the efficiencies vary widely, depending on the technology and redox conditions used (Grandclément et al., 2017; Luo et al., 2014). Usually, anaerobic systems are less efficient than the aerobic ones for most OMPs (Alvarino et al., 2014; 2018; Harb et al., 2019), particularly when operated at short hydraulic retention times (HRTs) (< 10 h) (Brandt et al., 2013; Buarque et al., 2019; Vassalle et al., 2020). The upflow anaerobic sludge blanket (UASB) reactor is a consolidated wastewater treatment technology in developing countries, especially those with tropical climate, such as Brazil, Colombia, and India (Chernicharo et al., 2015). Thus, the use of approaches to enhance the removal of OMPs in such an anaerobic system treating domestic wastewater, specifically designed to be operated at short HRTs (usually between 6 and 8 h), is needed.

According to Harb et al. (2019), the compound-biomass contact time is an important factor for the anaerobic biotransformation of OMPs, i.e., longer contact times favors the process. The easiest way to increase the contact time is to use a longer HRT. However, there is a practical limit for low-strength wastewaters, such as domestic wastewater. Thus, other strategies has been recommended to increase the retention and, consequently, biotransformation of OMPs inside the reactors, such as the use of adsorbents (e.g., activated carbon) as supporting material (attached growth reactors) or highly selective membranes (anaerobic membrane bioreactors) (Harb et al., 2019). However, depending on the socioeconomics scenario of some regions of the aforementioned developing countries, these modifications may be technically and economically unfeasible. Thus, simpler and lower-cost approaches should be applied to these short-HRT anaerobic treatment systems for improving the removal of OMPs.

In this context, an alternative that may be relatively more cost-effective and easier to operate is to microaerate the anaerobic reactors, which consists of injecting small amounts

of oxygen into these systems to facilitate the initial degradation of recalcitrant compounds (e.g., aromatic hydrocarbons) by probable monooxygenase-producing microorganisms (Firmino et al., 2018; Fuchs, 2008; Siqueira et al., 2018). In fact, this technique was demonstrated to improve considerably the removal of OMPs in an anaerobic reactor without compromising its overall performance and stability (Buarque et al., 2019). However, this previous work investigated only one airflow rate (1 $mL \cdot min^{-1}$ at 27 °C and 1 atm, equivalent to 0.021 L $O_2 \cdot L^{-1}$ feed). Thus, the effect of the variation in the airflow rate on the removal/biotransformation of OMPs in anaerobic systems remains unknown.

Additionally, soluble quinone-based compounds, such as anthraquinone-2-sulfonate (AQS), anthraquinone-2,6-disulfonate (AQDS), and lawsone, and some vitamins, such as riboflavin (vitamin B_2), has been reported to have redox-mediating properties and accelerate the anaerobic biotransformation of several recalcitrant compounds (e.g., azo dyes, nitroaromatics, polyhalogenated pollutants, pharmaceuticals, etc.) (dos Santos et al., 2007; He et al., 2017; Van der Zee and Cervantes, 2009; Zhou et al., 2018). Another possibility could be using nitrate as an alternative terminal electron acceptor, which has a similar oxidation potential to oxygen (dos Santos et al., 2007), as several studies have reported that, under nitrate-reducing (or anoxic) conditions, OMPs are more effectively biotransformed (Alvarino et al., 2016; Inyang et al., 2016; Lakshminarasimman et al., 2018; Ogunlaja and Parker, 2018; Zhao et al., 2018).

However, as far as the author is concerned, there is no investigation into the application of microaeration, associated or not with redox mediators, to continuous-flow anaerobic reactors for improving the biotransformation of OMPs as well as into the effect of different COD/NO_3^- ratios on such process.

1.1 Objetives

1.1.1 General objective

To evaluate microaeration, AQDS, and nitrate addition as engineering approaches for improving the removal of organics micropollutants in anaerobic reactors.

1.1.2 Specific objectives

- To evaluate the effect of increasing microaeration flow rates on the removal/biotransformation of seven OMPs (three hormones, one xenoestrogen, and three pharmaceuticals) in a UASB reactor (**Chapter 2**).
- To assess some engineering approaches, such as the addition of AQDS, microaeration, and nitrate, for enhancing the biotransformation of sulfamethoxazole and trimethoprim in anaerobic reactors (**Chapter 3**).
- To analyse the microaeration as an effective strategy to boost the biotransformation of four parabens (methylparaben, ethylparaben, propylparaben, and butylparaben) in a short-HRT UASB reactor (**Chapter 4**).
- To investigate the individual and combined effects of the redox mediator AQDS and microaeration on the biotransformation of the parabens methylparaben, ethylparaben, propylparaben, and butylparaben in a short-HRT UASB reactor (**Chapter 5**).

2 MICROAERATION IMPROVES THE REMOVAL/BIOTRANSFORMATION OF ORGANIC MICROPOLLUTANTS IN ANAEROBIC WASTEWATER TREATMENT SYSTEMS

2.1 Introduction

In the last decades, organic micropollutants (OMPs), such as pharmaceuticals, pesticides, hormones, personal care products, and others, have drawn much attention due to their potential negative impacts on ecosystems and public health (e.g., development of antibiotic-resistant pathogens and reproductive disorder in animals and humans by endocrine disruptors) (Gogoi et al., 2018; Harb et al., 2019).

The main sources of water pollution with such compounds are raw or treated domestic, agricultural, and some industrial wastewaters along with urban and rural runoff, in which OMPs may be found at concentrations ranging from few ng to several µg per liter (Gogoi et al., 2018; Luo et al., 2014).

The use of biological processes for the removal of OMPs from wastewater is extensively reported in the literature, but the efficiencies vary widely, depending on the technology and redox conditions used (Grandclément et al., 2017; Luo et al., 2014). Usually, anaerobic systems are less efficient than the aerobic ones for most OMPs (Alvarino et al., 2014; 2018; Harb et al., 2019), particularly when operated at short hydraulic retention times (HRTs) (< 10 h) (Brandt et al., 2013; Buarque et al., 2019; Vassalle et al., 2020).

The upflow anaerobic sludge blanket (UASB) reactor is a consolidated wastewater treatment technology in developing countries, especially those with tropical climate, such as Brazil, Colombia, and India (Chernicharo et al., 2015). Thus, the use of approaches to enhance the removal of OMPs in such an anaerobic system treating domestic wastewater, specifically designed to be operated at short HRTs (usually between 6 and 8 h), is needed.

According to Harb et al. (2019), the compound-biomass contact time is an important factor for the anaerobic biotransformation of OMPs, i.e., longer contact times favors the process. The easiest way to increase the contact time is to use a longer HRT. However, there is a practical limit for low-strength wastewaters, such as domestic wastewater. Thus, other strategies has been recommended to increase the retention and, consequently, biotransformation of OMPs inside the reactors, such as the use of adsorbents (e.g., activated carbon) as supporting material (attached growth reactors) or highly selective membranes (anaerobic membrane bioreactors) (Harb et al., 2019). However, depending on the socioeconomics scenario of some

regions of the aforementioned developing countries, these modifications may be technically and economically unfeasible.

In this context, an alternative that may be relatively more cost-effective and easier to operate is to microaerate the anaerobic reactors, which consists of injecting small amounts of oxygen into these systems to facilitate the initial degradation of recalcitrant compounds (e.g., aromatic hydrocarbons) by probable monooxygenase-producing microorganisms (Firmino et al., 2018; Fuchs, 2008; Siqueira et al., 2018). In fact, this technique was demonstrated to improve considerably the removal of OMPs in an anaerobic reactor without compromising its overall performance and stability (Buarque et al., 2019). However, this previous work investigated only one airflow rate (1 $mL \cdot min^{-1}$ at 27 °C and 1 atm, equivalent to 0.021 L $O_2 \cdot L^{-1}$ feed). Thus, to the best of the author's knowledge, the effect of the variation in the airflow rate on the removal/biotransformation of OMPs in anaerobic systems remains unknown.

Hence, this work aimed to assess the effect of increasing microaeration flow rates on the removal/biotransformation of seven OMPs (three hormones, one xenoestrogen, and three pharmaceuticals) in a UASB reactor. Additionally, the operational stability of the system and the evolution of its microbial community under microaerobic conditions were evaluated.

2.2 Material and methods

2.2.1 Synthetic wastewater

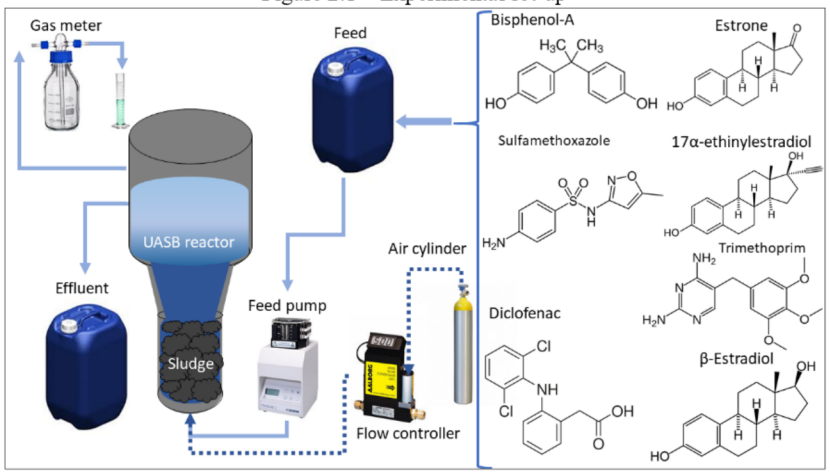
The synthetic wastewater consisted of an aqueous solution containing the OMPs (~200 $\mu g \cdot L^{-1}$ each) estrone (E1, natural hormone, 99%, Sigma-Aldrich, USA), 17β-estradiol (E2, natural hormone, 98%, Sigma-Aldrich, USA), 17α-ethinylestradiol (EE2, synthetic hormone, 100%, Sigma-Aldrich, USA), bisphenol A (BPA, xenoestrogen, 99%, Sigma-Aldrich, USA), diclofenac (DCF, anti-inflammatory, 98.5%, Sigma-Aldrich, USA), sulfamethoxazole (SMX, antibiotic, 99%, Sigma-Aldrich, USA), and trimethoprim (TMP, antibiotic, 98%, Sigma-Aldrich, USA), ethanol (1 g $COD \cdot L^{-1}$), basal medium (macro and micronutrients), prepared according to Firmino et al. (2010), and sodium bicarbonate (1 $g \cdot L^{-1}$), to maintain the pH near 7.0. The concentrations of OMPs were set according to those found in some local domestic wastewater (Vidal et al., 2020). All reagents were used as purchased without further purification.

2.2.2 Experimental set-up

The continuous-flow experiment was carried out in an upflow anaerobic sludge blanket (UASB) reactor with a working volume of 3.5 L and an HRT of 7.4 h. The reactor was inoculated with mesophilic anaerobic sludge (~50 g VSS·L^{-1}) from a UASB reactor of a domestic wastewater treatment plant (WWTP) located in Fortaleza, Ceará, Brazil.

The reactor was fed with the synthetic wastewater by a peristaltic pump (Minipuls 3, Gilson, USA) and operated at room temperature of approximately 28 °C. In some experimental periods, the reactor was microaerated at the feeding line through a needle with synthetic air (80% N_2:20% O_2, White Martins, Brazil), whose flow rate was controlled by a mass flow controller (GFC17, Aalborg, USA). The biogas produced was measured by a Mariotte flask containing a 3% sodium chloride solution at pH 2 (Figure 2.1).

Figure 2.1 – Experimental set-up



2.2.3 Experimental procedure

The experiment with the OMP-containing wastewater was carried out in seven periods (Table 2.1). Initially, the removal of the different OMPs was evaluated under anaerobic conditions (period I). Then, to investigate the effect of microaeration on the removal/biotransformation of such compounds, different flow rates of synthetic air (1, 2, 4, and 6 mL·min^{-1} at 28 °C and 1 atm, equivalent to 0.025, 0.051, 0.101, and 0.152 L O_2·L^{-1} feed,

respectively) were tested from period II to V. Subsequently, in period VI, to evaluate a likely adaptation of microbiota to microaerobic conditions, the microaeration flow rate was reduced to 1 mL·min^{-1} (28 °C and 1 atm). Finally, in period VII, to reinforce the oxygen effect and eliminate the hypothesis of microbiota adaptation to the OMPs throughout the experiment, the reactor was again operated under anaerobic conditions. The experimental periods were changed after verifying system stability.

Table 2.1 – Operational conditions of the reactor throughout the experiment

Period	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**
End of period (day)	106	155	263	290	318	355	372
Microaeration (mL·min^{-1})	-	1	2	4	6	1	-
Dose of oxygen (L O_2·L^{-1} feed)	-	0.025	0.051	0.101	0.152	0.025	-
HRT (h)	7.4	7.4	7.4	7.4	7.4	7.4	7.4
COD (g·L^{-1})	1.0	1.0	1.0	1.0	1.0	1.0	1.0
E1 (µg·L^{-1})	221	199	200	213	212	202	219
E2 (µg·L^{-1})	230	213	198	198	197	205	200
EE2 (µg·L^{-1})	213	223	196	204	216	202	196
BPA (µg·L^{-1})	208	210	206	211	202	207	212
DCF (µg·L^{-1})	232	203	201	204	205	200	217
SMX (µg·L^{-1})	203	205	200	209	207	205	216
TMP (µg·L^{-1})	230	205	196	207	210	207	214

BPA, bisphenol A; COD, chemical oxygen demand; DCF, diclofenac; E1, estrone; E2, 17β-estradiol; EE2, 17α-ethinylestradiol; HRT, hydraulic retention time; SMX, sulfamethoxazole; TMP, trimethoprim.

2.2.4 Chemical analysis

For the quantification of the OMPs, the samples (500 mL) were previously filtered (0.45 µm) and acidified with HCl (pH 2.5-3). Then, they were percolated through Strata-X® cartridges (500 mg, 6 mL) (Phenomenex®, USA) for the solid phase extraction (SPE) of the OMPs, which were eluted with HPLC/UV grade methanol (4 mL) (99.8%, Neon, Brazil) (Pessoa et al., 2014). The eluate (20 µL) was then analyzed by an LC-20A Prominence high-performance liquid chromatograph (HPLC) equipped with a Shim-pack CLC-ODS(M)® C18 column (4.6×150 mm, 5 µm) and a UV-Vis SPD-20A detector (258 nm) (Shimadzu Corporation, Japan). The elution was performed by mobile phase composed of HPLC/UV grade acetonitrile (99.9%, Sigma-Aldrich, Germany) and 0.1% HCl solution, based on Vidal et al. (2020), with the following gradient: 10% to 80% increase in acetonitrile in 10 min, returning to

10% in 4 min. The flow rate was initially 1.0 mL·min^{-1} and, after 5 min of run, it was increased to 2.0 mL·min^{-1}. The oven temperature was maintained at 35 °C throughout the run.

COD, alkalinity, and pH were determined according to (APHA, 2012). The volatile fatty acids (VFA) were determined by the Kapp titrimetric method (Buchauer, 1998). The biogas was characterized in terms of CH_4, CO_2, O_2, and N_2. CH_4 and CO_2 were quantified by gas chromatography with barrier-discharge ionization detection (GC-BID) (GC-2010 Plus, Shimadzu Corporation, Japan). The biogas sample (1.0 mL) was injected in split mode (split ratio of 30), and the chromatographic separation was performed on a GS-GasPro column (60 m, 0.32 mm I.D.) (Agilent Technologies, USA). The temperatures of the injector and the detector were 100 and 250 °C, respectively. The temperature of the oven started at 50 °C, was raised to 75 °C at 5 °C·min^{-1}, then to 105 °C at 8 °C·min^{-1}, and was finally maintained at 105 °C for 0.25 min (total run time of 9 min). Helium (White Martins, Brazil) was used as the carrier gas at a flow rate of 2.0 mL·min^{-1}. O_2 and N_2 were quantified by gas chromatography with thermal conductivity detection (GC-TCD) (GC-17A, Shimadzu Corporation, Japan). The biogas sample (1.0 mL) was injected in splitless mode, and the chromatographic separation was performed on a Mol Sieve 5A PLOT column (30 m, 0.32 mm I.D.) (Restek Corporation, USA). The temperatures of the injector, oven, and detector were 40, 35, and 230 °C, respectively. Helium (White Martins, Brazil) was used as the carrier gas at a flow of 7 mL·min^{-1}, and the run time was 5 min.

2.2.5 Microbiological analysis

To evaluate the dynamics of the microbial community under microaerobic conditions, the DNAs of the inoculum and sludge samples collected at the end of the periods II (1 mL air·min^{-1}), V (6 mL air·min^{-1}), and VI (1 mL air·min^{-1}) were extracted and then sequenced by an Illumina MiSeq Desktop Sequencer as detailed elsewhere (Rollemberg et al., 2019). The DNA was extracted using PowerSoil® DNA isolation kit (MoBio Laboratories Inc., USA) based on the manufacturer's instruction. The amplicon library of the 16S rRNA gene V4 region was prepared using the region-specific primers (515F/806R). After indexing, the PCR products were cleaned up using Agencourt AMPure XP—PCR purification beads (Beckman Coulter, Brea, CA, USA) based on the manufacturer's instruction and quantified using the dsDNA BR assay Kit (Invitrogen, Carlsbad, CA, USA) on a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). The libraries were sequenced using the 300-cycle MiSeq Reagent Kits v2 chemistry. The data obtained by the sequencing was analyzed with bioinformatics tools as

follows. All reads were trimmed using vsearch v2.8.1, with parameters -fastq_maxee 0.8 -fastq_trunclen 250. All reads were clustered into OTUs using QIIME script pick_open_reference_otus.py with 99% identity, using Greengenes 16S rRNA database (release 13_8). Some ecological indices, namely Chao1 (richness), inverse Simpson (diversity), and Bray-Curtis dissimilarity, were calculated by Mothur software. Based on the latter index, UPGMA (unweighted pair group method with arithmetic mean) cluster analysis at genus level was also performed.

2.2.6 Statistical analysis

The Mann-Whitney and Kruskal-Wallis non-parametric tests, which do not require a specific data distribution, were used to compare the performance of the reactor during the different experimental periods at a 95% confidence level.

2.3 Results and discussion

2.3.1 Removal of OMPs under anaerobic conditions

Under anaerobic conditions (period I), the removal efficiencies (REs) of all OMPs were considerably low (~20%) (Figure 2.2), with the lowest mean value achieved for BPA (Table 2.1). Actually, apart from this xenoestrogen, no significant difference was found among the REs of the other studied OMPs ($p = 0.440$).

These results are in agreement with those reported in previous studies (Buarque et al., 2019; Vassalle et al., 2020). Buarque et al. (2019), for instance, using a lab-scale mesophilic UASB reactor (28 °C) with a 7-h HRT to treat synthetic wastewater containing the same mixture of the OMPs used in the present investigation (~230 $\mu g \cdot L^{-1}$ of each compound) and ethanol (1 g $COD \cdot L^{-1}$) as co-substrate, obtained very low mean REs (4-9%). Vassalle et al. (2020), assessing the removal of OMPs (20-80 $ng \cdot L^{-1}$), such as BPA, DCF, E1, E2, EE2, and others, from domestic wastewater (~0.5 g $COD \cdot L^{-1}$) in a full-scale mesophilic UASB reactor (22 °C and HRT of 7 h), despite observing mean REs of hormones as high as 84%, also reported low mean REs of pharmaceuticals and xenoestrogens (< 22%), particularly of BPA (~1.5%). Therefore, even at concentrations much lower than those used in the current study, the REs of most OMPs were low, which shows the recalcitrance of such compounds under anaerobic conditions.

Figure 2.2 – Influent (■) and effluent (□) concentrations and removal efficiencies (REs) (○) of estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2), bisphenol A (BPA), diclofenac (DCF), sulfamethoxazole (SMX), and trimethoprim (TMP) throughout the experiment (continues)

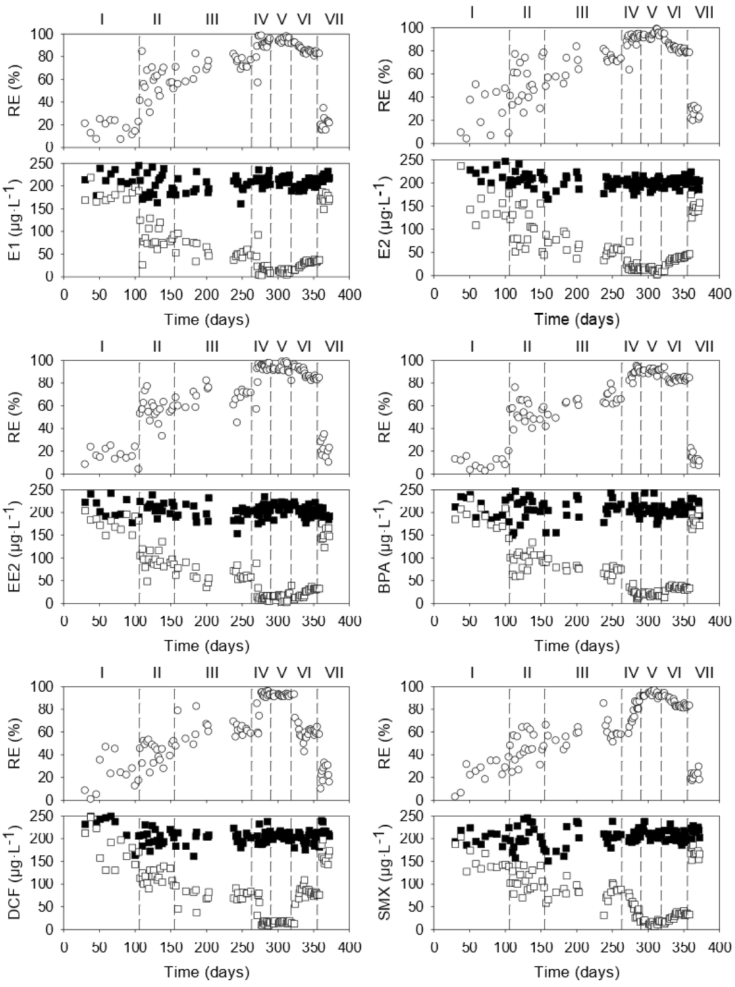
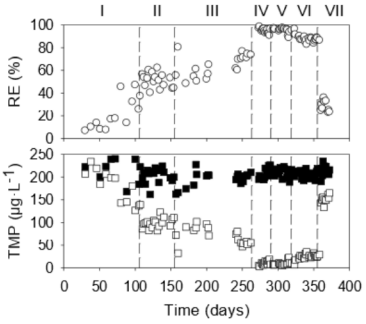


Figure 2.2 – Influent (■) and effluent (□) concentrations and removal efficiencies (REs) (○) of estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2), bisphenol A (BPA), diclofenac (DCF), sulfamethoxazole (SMX), and trimethoprim (TMP) throughout the experiment (conclusion)



In fact, according to the literature, despite some exceptions, such as the antibiotics SMX and TMP, OMPs are typically removed more efficiently under aerobic conditions than under anaerobic conditions (Alvarino et al., 2014; 2018; Brandt et al., 2013; Harb et al., 2019). However, in aerobic systems, adsorption on sludge may be a relevant removal mechanism, accounting for up to 30% of the influent load. On the other hand, in anaerobic systems, due to the long solid retention times (SRTs) (> 70 d), the aforementioned mechanism may be negligible in the long term (up to 3% of the influent load), as the sludge blanket tends to saturate very quickly (up to 1 week depending on the pollutant concentration). Therefore, although anaerobic systems are usually less efficient, biotransformation is their main removal mechanism of OMPs (Harb et al., 2019).

The recalcitrance of OMPs under anaerobic conditions is probably related to their (poly)aromatic structure, which confers to them high stability in oxygen-free environments (Aquino et al., 2013). Hence, in general, oxygen may play an essential role in the biotransformation of several OMPs, in which the hydroxylation reaction is reported as an important step (Chen et al., 2018; Jewell et al., 2016; Poirier-Larabie et al., 2016; Yu et al., 2013; Zhang et al., 2013). Although the hydroxylation of aromatic compounds (e.g., benzene) can also occur anaerobically, it is much more favorable in the presence of oxygen, which is used by oxygenases enzymes and inserted into the molecules as hydroxyl groups (Foght, 2008; Fuchs et al., 2011).

Furthermore, it is worth mentioning that the REs of OMPs in anaerobic systems depend not only on the physicochemical properties of the compounds but also on the operational parameters of the reactors, mainly the HRT, since it is directly related to the substrate-

microorganisms contact time (Harb et al., 2019). Therefore, anaerobic reactors with longer HRTs (19-24 h) tend to reach higher REs of some OMPs (e.g., SMX, TMP, and some hormones) than those obtained in the present work (Alvarino et al., 2014; 2019; Arias et al., 2018).

However, for low-strength wastewaters, such as domestic wastewater, using long HRTs is not viable (Harb et al., 2019). Additionally, the longer the HRT, the greater the reactor volume and, therefore, the investment costs (capital expenditures, CAPEX). Thus, other strategies to improve the removal/biotransformation of OMPs in anaerobic systems operated at short HRTs should be used. Besides increasing the retention of the compounds inside the reactor by using adsorbents or membranes (Harb et al., 2019), one of those strategies would be the injection of small amounts of oxygen (microaeration) into the anaerobic system to facilitate the initial biotransformation of OMPs without compromising its overall performance and stability (Buarque et al., 2019), especially methanogenesis, an important step for anaerobic biotransformation of OMPs (Gonzalez-Gil et al., 2018), as it will be discussed below.

2.3.2 Removal of OMPs under microaerobic conditions

In period II, with the application of microaeration (1 mL air·min^{-1}), although the fluctuation in the RE values has remained (Figure 2.2), the mean REs of all compounds were higher than 50%, except for DCF and SMX, which presented lower values ($p < 0.050$) (Table 2.2). Therefore, it was evident that the microaerobic conditions significantly favored the removal/biotransformation of OMPs compared to the anaerobic conditions (period I) ($p < 0.001$).

These results corroborate those by Buarque et al. (2019), who observed a similar remarkable increase in the mean REs of the same OMPs (from less than 10% to more than 50%) after microaerating a lab-scale UASB reactor (HRT of 7 h) at its feeding line, with 1 mL·min^{-1} of synthetic air (80% N_2:20% O_2) at 27 °C and 1 atm (0.021 L O_2·L^{-1} feed), in the presence of ethanol (1 g COD·L^{-1}) as co-substrate.

According to the literature, cometabolism is, most likely, the main process of biotransformation of OMPs, as their concentrations are very low, thus preventing the use of these compounds as a growth substrate. Actually, this process can occur due to the activity of some non-specific enzymes, mainly ammonia monooxygenase in nitrifying systems (Alvarino et al., 2018; Fernandez-Fontaina et al., 2016; Fischer and Majewsky, 2014). Therefore, in the present study, although nitrification has not happened (data not shown), the presence of small

amounts of oxygen may have stimulated the production of other non-specific monooxygenases enzymes able to catalyze the removal/biotransformation of OMPs.

It is worth mentioning that, under microaerobic conditions, oxygen is not used as the terminal electron acceptor as in aerobic respiration. It is only used by the monooxygenases for the initial biotransformation of the compounds through hydroxylation, and then their intermediates can be anaerobically degraded (Fuchs, 2008; Siqueira et al., 2018).

Concerning residual oxygen, less than 10% of the amount added to the system was found in the biogas (data not shown). Therefore, both the solubilization of oxygen and its consumption in the medium were efficient. Then, in periods III, IV, and V, the effect of increasing the microaeration flow rate on the removal of OMPs was evaluated.

With 2 mL air·min^{-1} (period III), the REs of all OMPs increased progressively over the period and tended to stabilize at the end of it (Figure 2.2), thus ensuring a significant improvement in their mean REs compared to period II ($p < 0.001$) (Table 2.2). However, the hormones (E1, E2, and EE2) were more easily removed than the other compounds ($p < 0.001$).

With the increase in the microaeration flow rate to 4 mL air·min^{-1} (period IV), the mean REs of all OMPs were considerably higher than those observed in period III (2 mL air·min^{-1}) ($p < 0.001$) (Table 2.2). The most significant increase was observed for the antibiotic TMP, which had an increase of 31% in its mean RE, reaching the highest value (95%) among all compounds evaluated. On the other hand, the lowest mean RE was observed for the other antibiotic, SMX (80%). However, it is worth mentioning that, noticeably for this compound, the REs increased gradually, reaching values above 90% only at the end of period IV (last seven days) (Figure 2.2).

The positive impact of the increase in the microaeration flow rate on the removal/biotransformation of the studied OMPs is evident, i.e., the greater availability of oxygen in the medium, most likely, enabled a higher enzymatic synthesis. Therefore, a more significant removal/biotransformation of the recalcitrant compounds was achieved. Additionally, the residual oxygen in the biogas remained low (~12% of the added amount) even at a fourfold higher airflow rate, thus indicating that mass transfer was not limited, and oxygen remained promptly available in the medium to be used.

In period V (6 mL air·min^{-1}), very high and stable REs were achieved (Figure 2.2), ensuring mean values above 90% for all OMPs (Table 2.2). Except for BPA, no statistically significant difference was observed among the mean REs of the compounds ($p = 0.118$). Therefore, with greater availability of oxygen, especially in periods IV and V, this difference decreased.

Table 2.2 – Mean influent and effluent concentrations and removal efficiencies (REs) of the organic micropollutants throughout the experiment

Period		**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**
Microaeration (mL·min^{-1})		**-**	**1**	**2**	**4**	**6**	**1**	**-**
	Influent (μg·L^{-1})	221 ± 20	199 ± 24	200 ± 17	213 ± 11	212 ± 10	202 ± 8	219 ± 8
E1	Effluent (μg·L^{-1})	183 ± 16	85 ± 25	56 ± 16	21 ± 21	12 ± 4	31 ± 6	172 ± 11
	RE (%)	17 ± 7	57 ± 13	72 ± 8	92 ± 5	94 ± 2	85 ± 3	21 ± 6
	Influent (μg·L^{-1})	230 ± 24	213 ± 17	198 ± 16	198 ± 10	197 ± 11	205 ± 8	200 ± 12
E2	Effluent (μg·L^{-1})	174 ± 20	101 ± 40	61 ± 17	21 ± 14	12 ± 5	38 ± 6	147 ± 13
	RE (%)	27 ± 18	53 ± 17	69 ± 10	90 ± 7	94 ± 3	82 ± 3	26 ± 5
	Influent (μg·L^{-1})	213 ± 24	223 ± 27	196 ± 17	204 ± 15	216 ± 8	202 ± 11	196 ± 12
EE2	Effluent (μg·L^{-1})	177 ± 17	96 ± 19	62 ± 14	18 ± 19	14 ± 10	25 ± 8	146 ± 20
	RE (%)	16 ± 6	57 ± 10	68 ± 8	92 ± 9	93 ± 5	87 ± 4	22 ± 8
	Influent (μg·L^{-1})	208 ± 22	210 ± 27	206 ± 25	211 ± 13	202 ± 16	207 ± 9	212 ± 18
BPA	Effluent (μg·L^{-1})	188 ± 24	94 ± 20	75 ± 12	23 ± 9	19 ± 4	34 ± 3	183 ± 13
	RE (%)	10 ± 5	55 ± 9	63 ± 9	89 ± 4	91 ± 2	83 ± 2	13 ± 5
	Influent (μg·L^{-1})	232 ± 27	203 ± 16	201 ± 14	204 ± 15	205 ± 10	200 ± 12	217 ± 12
DCF	Effluent (μg·L^{-1})	179 ± 37	116 ± 14	76 ± 15	24 ± 22	16 ± 2	82 ± 11	167 ± 19
	RE (%)	22 ± 15	43 ± 9	62 ± 9	88 ± 11	92 ± 1	59 ± 6	23 ± 7
	Influent (μg·L^{-1})	203 ± 15	205 ± 26	200 ± 25	209 ± 13	207 ± 8	205 ± 11	216 ± 9
SMX	Effluent (μg·L^{-1})	153 ± 24	109 ± 22	81 ± 19	40 ± 18	14 ± 4	31 ± 6	169 ± 11
	RE (%)	24 ± 11	46 ± 12	59 ± 9	80 ± 10	93 ± 2	85 ± 3	22 ± 4
	Influent (μg·L^{-1})	230 ± 32	205 ± 16	196 ± 16	207 ± 11	210 ± 11	207 ± 8	214 ± 13
TMP	Effluent (μg·L^{-1})	181 ± 35	101 ± 14	70 ± 19	10 ± 5	10 ± 5	26 ± 4	152 ± 9
	RE (%)	21 ± 14	51 ± 7	64 ± 10	95 ± 2	95 ± 3	88 ± 2	29 ± 5

BPA, bisphenol A; DCF, diclofenac; E1, estrone; E2, 17β-estradiol; EE2, 17α-ethinylestradiol; SMX, sulfamethoxazole; TMP, trimethoprim.

Compared to period IV (4 mL air·min^{-1}), the increase in the airflow rate to 6 mL·min-1 had a significant impact only on the removal of E2 ($p = 0.003$) and SMX ($p < 0.001$), with increases in the mean RE of 4% and 13%, respectively (Table 2.2). However, considering only the final data of period IV, when the REs were very stable (~92% for both E2 and SMX), this difference found between periods IV and V no longer exists ($p > 0.050$).

Therefore, saturation in the removal/biotransformation capacity of OMPs in the microaerobic system may have likely occurred due to biochemical limitations and not to a mass transfer problem (oxygen solubilization), since the residual oxygen detected in the biogas was only 17% of the amount provided by microaeration. Hence, in general, among the microaeration flow rates tested in the present study, the most relevant results of removal of the evaluated OMPs were obtained with 4 mL air·min^{-1} (period IV) because, above this flow rate, the efficiency increase was negligible.

According to Siqueira et al. (2018), increasing the availability of oxygen in a microaerobic system do not always improve the removal of a compound because oxygen may diffuse more deeply into the anaerobic sludge granule and inhibit obligate anaerobic microorganisms that live in the granule core, namely acetogenic bacteria and methanogenic archaea. Consequently, as methanogenesis plays an important role in the biotransformation of some OMPs during anaerobic digestion (Gonzalez-Gil et al., 2018), the inhibition of this step may hinder the removal process of these compounds.

In period VI, with the decrease in the microaeration flow rate to 1 mL·min^{-1}, as expected, all OMPs had their REs reduced (Figure 2.2). However, except for DCF, which presented a mean RE below 60%, the mean REs of the other compounds remained considerably high (> 80%) (Table 2.2). Surprisingly, compared to period II, in which the same airflow was applied, the REs of all compounds were much higher ($p < 0.001$). For instance, the increase in the mean RE of the hormones E1, E2, and EE2 was almost 30%, and that of SMX and TMP, approximately 40% (Table 2.2). Therefore, it is likely that the enzymes (probably monooxygenases) increasingly produced throughout the periods II to V remained present and active even when a lower microaeration flow rate was applied, which guaranteed higher REs than in period II. However, further studies (e.g., long-term operation with enzyme quantification by real-time PCR) are necessary to verify if the REs would reach the same values as in period II due to a decrease in the amount of enzymes in the long term, caused by the lesser availability of oxygen.

Finally, in period VII, the reactor was again operated under anaerobic conditions as in period I. Consequently, the REs of OMPs decreased significantly ($p < 0.001$) (Figure 2.2),

achieving a similar performance as in period I ($p > 0.050$) (Table 2.2). Therefore, these results strongly reinforce the key role of oxygen, even at low concentrations (microaeration), in the removal/biotransformation of OMPs, i.e., the enhanced reactor performance was not a result of a mere adaptation of the microbiota to the compounds throughout the experiment.

2.3.3 Operational stability of the system

In period I, under anaerobic conditions, the system was stable and showed a high mean COD RE (89%) and a methane content in the biogas above 75% (Table 2.3). Therefore, apparently, the OMPs did not affect the methanogenic microorganisms. In period II, with the addition of microaeration (1 mL air·min^{-1}), despite the statistic difference ($p = 0.003$), the mean COD RE remained high and very close to that of period I (only 1% lower). Conversely, no significant change in the methane content was observed (Table 2.3). Therefore, as observed in the aforementioned investigation by Buarque et al. (2019), the addition of 1 mL air·min^{-1} did not cause a significant impact on the reactor performance either in terms of organic matter removal or methane dilution/molar production.

In periods III, IV, and V, the microaeration flow rates were elevated to 2, 4, and 6 mL air·min-1, respectively. COD removal remained quite stable, and no significant differences were found among these periods ($p = 0.099$) (Table 2.3). On the other hand, the methane content sharply decreased (from 57% to 22%) throughout them, with values well below those obtained in periods I and II (~78%) (Table 2.3). However, no significant change in methane productivity was observed ($p = 0.464$) (data not shown). Therefore, the increase in airflow rates did not compromised methanogenesis, and the decrease in the methane content resulted from biogas dilution by the high nitrogen content (80%) of microaeration source (synthetic air). When the biogas is intended to be used in combined heat and power plants, a minimum methane content of 40% is required (Haubrichs and Widmann, 2006). Hence, the use of pure oxygen instead of air as the microaeration source should be considered, as dilution problem can be effectively mitigated.

In periods VI and VII, the reactor was operated under the same operational conditions as in periods II and I, respectively, and there were no significant differences in both organic matter removal and methane content ($p > 0.050$) (Table 2.3). Finally, the reactor remained stable during the whole experiment, as there was no significant variation in pH and accumulation of volatile fatty acids (Table 2.3).

Table 2.3 – Parameters of operational stability of the reactor throughout the experiment

Period	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**
Microaeration ($mL \cdot min^{-1}$)	**-**	**1**	**2**	**4**	**6**	**1**	**-**
Influent COD ($mg \cdot L^{-1}$)	1017 ± 73	1041 ± 60	1053 ± 84	1016 ± 82	1011 ± 42	1020 ± 63	998 ± 30
Effluent COD ($mg \cdot L^{-1}$)	109 ± 49	124 ± 13	106 ± 19	118 ± 25	113 ± 17	114 ± 13	130 ± 17
COD RE (%)	89 ± 5	88 ± 2	90 ± 2	88 ± 3	89 ± 2	89 ± 2	87 ± 2
Biogas production ($L \cdot d^{-1}$)	1.8 ± 0.4	3.8 ± 0.2	4.9 ± 0.1	7.3 ± 0.3	11.0 ± 0.2	4.0 ± 0.1	2.1 ± 0.2
CH_4 in the biogas (%)	77 ± 4	78 ± 3	57 ± 3	33 ± 3	22 ± 2	81 ± 1	82 ± 2
pH	7.3 ± 0.1	7.2 ± 0.1	7.1 ± 0.2	7.2 ± 0.2	7.1 ± 0.1	6.9 ± 0.3	7.2 ± 0.2
TA ($mg \cdot L^{-1}$)	641 ± 31	640 ± 50	656 ± 56	670 ± 34	655 ± 27	684 ± 33	723 ± 45
VFA ($mg \cdot L^{-1}$)	409 ± 72	310 ± 62	282 ± 36	285 ± 30	298 ± 31	301 ± 28	310 ± 62
VFA/TA	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1

COD, chemical oxygen demand; RE, removal efficiency; TA, total alkalinity; VFA, volatile fatty acids.

2.3.4 Dynamics of the microbial community of the system

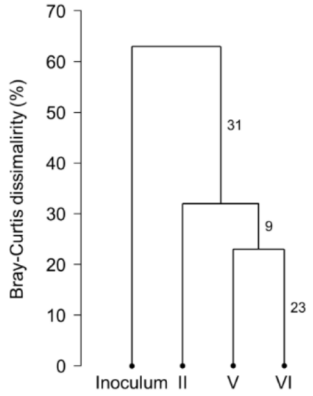
Comparing the sludge sample from period II (1 mL air·min^{-1}) with the inoculum (anaerobic), the ecological indices Chao1 and inverse Simpson indicate that both microbial richness and diversity increased expressively (Table 2.4). Accordingly, not only the amount of observed species, represented by the number of operational taxonomic units (OTUs), increased but also the community became more even, i.e., without dominance of only some species. In fact, the microbial community in period II was very different from that of the inoculum at genus level (63% dissimilarity) (Figure 2.3). Therefore, as also observed by Buarque et al. (2019) and Firmino et al. (2018), microaeration at a flow rate as low as 1 mL air·min^{-1} may have played a key role in increasing the microbial diversity, thus probably stimulating the growth of monooxygenases-producing species.

Table 2.4 – Ecological indices of richness (Chao1) and diversity (inverse Simpson) for the inoculum and samples collected at the end of periods II (1 mL air·min^{-1}), V (6 mL air·min^{-1}), and VI (1 mL air·min^{-1})

Sample	OTUs[a]	Chao1	Inverse Simpson
Inoculum	1196	2042	7.68
II	1782	3413	16.67
V	1117	1995	13.43
VI	1961	3319	14.43

[a]Number of operational taxonomic units.

Figure 2.3 – UPGMA cluster analysis at genus level based on Bray-Curtis dissimilarity index for the inoculum and samples collected at the end of periods II (1 mL air·min^{-1}), V (6 mL air·min^{-1}), and VI (1 mL air·min^{-1})



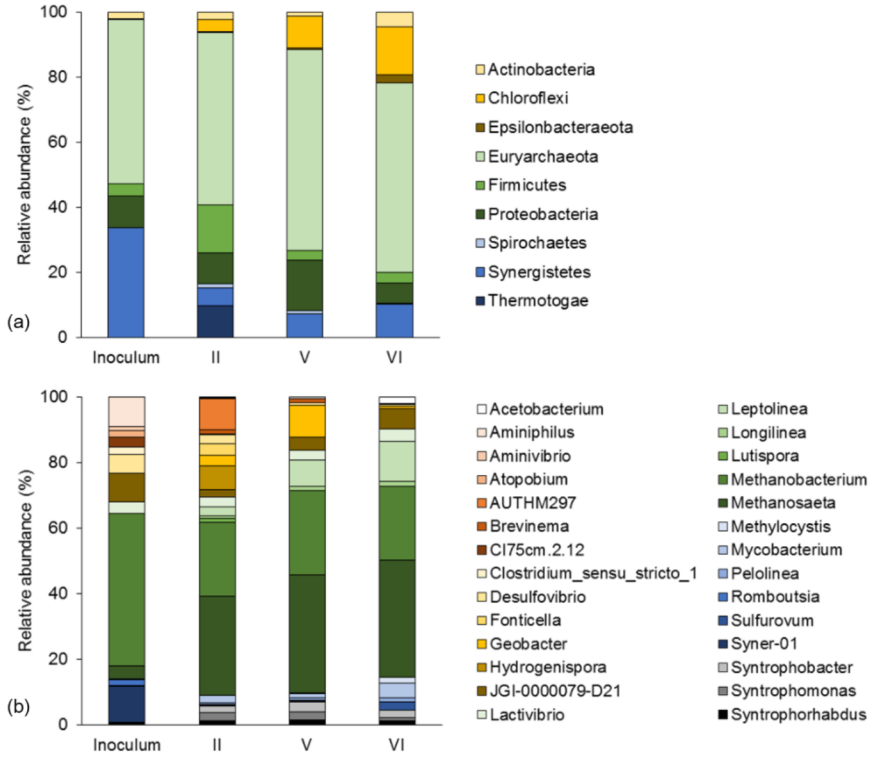
On the other hand, increasing the airflow rate to 6 mL·min^{-1} (period V) negatively affected the community richness (41.5% decrease), reaching a value close to that of the inoculum. However, the diversity/evenness remained rather similar (less than 20% lower than in period II) (Table 2.4). As a result, the sample of period V was much more similar to that of period II than to the inoculum (Figure 2.3). Therefore, the higher availability of oxygen in the medium may have imposed a greater selection pressure on the microbiota, impairing the survival of less aerotolerant species in the outer zones of the anaerobic sludge granule.

When the microaeration flow rate was reduced back to 1 mL air·min^{-1} (period VI), the microbial richness increased again, presenting a Chao1 index quite similar to that obtained in period II, when the same airflow rate was used. Moreover, although the number of OTUs increased, the community evenness was maintained (comparable inverse Simpson index) (Table 2.4). Nevertheless, at genus level, the sample of period VI was more similar to that of period V (6 mL air·min^{-1}) than to that of period II (1 mL air·min^{-1}) (Figure 2.3). Therefore, due to continued exposure to microaerobic conditions, a gradual evolution of the microbiota seemed to occur over time, which may explain why the removal of OMPs in period VI was better than in period II (Table 2.2).

It is worth mentioning that methanogenesis was not compromised throughout the experiment because microaeration did not harm the archaeal community. Actually, the relative abundance of the phylum Euryarchaeota remained above 50% and even increased when a higher airflow rate was applied (period V) (Figure 2.4a). At genus level, compared to the inoculum, there was a remarkable increase in the relative abundance of *Methanosaeta* (exclusively acetoclastic methanogens) (Figure 2.4b). Probably, it resulted from the used carbon source (ethanol), which is converted into acetate and hydrogen by syntrophic acetogenic bacteria, whose found genera (*Syntrophomonas, Syntrophobacter*, and *Sytrophorhabdus*) kept their relative abundance rather constant in the microaerated periods (Figure 2.4b).

The maintenance of the methanogenic activity under microaerobic conditions is possible due to the layered structure of granular sludge, in which obligate anaerobes (e.g., acetogenic bacteria and methanogenic archaea) are mostly found in the inner layers (core of the granule) and protected by facultative species that grow in the outer layers (Baloch et al., 2008; Picioreanu et al., 2005).

Figure 2.4 – Microbial diversity at phylum (a) and genus (b) levels of the inoculum and samples collected at the end of periods II (1 mL air·min^{-1}), V (6 mL air·min^{-1}), and VI (1 mL air·min^{-1})



Hence, as acetoclastic methanogenesis may be involved in the cometabolic biotransformation of some OMPs (e.g., DCF and BPA) due to the activity of the acetate kinase enzyme (Gonzalez-Gil et al., 2017), the preservation of the archaeal community, as well as of the acetogenic bacteria, may have also played a role in the removal of the tested compounds. Furthermore, although syntrophy between acetogens and methanogens has been hardly reported to be directly related to anaerobic biotransformation of OMPs (Carneiro et al., 2020; Wolfson et al., 2018), the importance of this ecological relationship for anaerobic degradation of aromatic compounds is widely accepted (Gieg et al., 2014; Qiu et al., 2008). For instance, *Syntrophorhabdus aromaticivorans* is one of these species able to degrade syntrophically phenol to acetate (Qiu et al., 2008).

Concerning other genera that seemed to be positively affected by microaeration, *Geobacter* and *Leptolinea* were the most evident, reaching relative abundances of 9.5% and 8.1% in period V (6 mL air·min^{-1}), respectively, i.e., the most abundant bacterial genera (Figure 2.4b). Despite being classified as obligate anaerobes, some *Geobacter* and *Leptolinea* species can tolerate low oxygen concentrations and even grow under microaerobic conditions (Lovley et al., 2011; Ward et al., 2015). However, whereas Leptolinea was even more abundant (12.2%) at the end of the long microaerobic term (249 days), unexpectedly, *Geobacter* practically vanished (0.7%) after the reduction in the microaeration flow rate to 1 mL air·min^{-1} (period VI) (Figure 2.4b).

The genus *Longilinea*, which is very similar to *Leptolinea*, since they belong to the same family of strictly anaerobic bacteria (Anaerolineaceae) (Yamada et al., 2007), despite keeping relative abundance below 2% in the microaerobic periods, presented the same increasing tendency over time as *Leptolinea* (Figure 2.4b).

To the best of the author's knowledge, the aforementioned genera have not been associated with the biotransformation of OMPs. However, several *Geobacter* species can degrade aromatic compounds independently or with syntrophic partners, mainly *Methanosaeta* species, under anaerobic conditions (Lovley et al., 2011). Additionally, *Longilinea* was associated with aromatic ring cleavage in the presence of oxygen, probably by oxygenases (Zhu et al., 2018). In contrast, no studies on degradation of aromatics by the genus *Leptolinea* were found. Nevertheless, due to its high similarity to *Longilinea*, *Leptolinea* may have the same ability. Therefore, as some *Geobacter*, *Leptolinea*, and *Longilinea* species are microaerophilic, they may be capable of producing oxygenases that could have cometabolized the OMPs studied in the current work.

Finally, other genera that also drew attention for having become more abundant throughout the microaerobic term were *Methylocystis* and *Mycobacterium* (1.7% and 4.5% in period VI, respectively) (Figure 2.4b). *Mycobacterium* is an aerobic genus that contains some dioxygenase-producing strains able to degrade polycyclic aromatic hydrocarbons (Guo et al., 2010). *Methylocystis* species are aerobic methanotrophs, but some of them can grow under microaerobic conditions (Vecherskaya et al., 2009). Additionally, methane monooxygenase, the key enzyme for methane oxidation by methanotrophic bacteria, was demonstrated to degrade cometabolically SMX and benzotriazole (Benner et al., 2015). Hence, both genera may have participated in the biotransformation of the tested compounds, particularly in hydroxylation reactions.

Considering that these five supposed microaerophilic genera were someway involved in the biotransformation of OMPs, especially in the first steps, the sum of their relative abundances were much higher in period VI (20.7%) than in period II (9.1%), although the same airflow rate was used (1 mL air·min^{-1}). Therefore, this could justify the better removal of OMPs in period VI, as there was some microbial enrichment throughout the microaerobic periods.

2.4 Conclusions

Microaeration was demonstrated to be an effective strategy to improve the limited removal/biotransformation of the evaluated OMPs in short-HRT anaerobic wastewater treatment systems.

The rise in the airflow rate (1-6 mL·min^{-1}, i.e., 0.025-0.152 L O_2·L^{-1} feed) considerably increased the REs of all OMPs. However, there seems to be a saturation limit for the biochemical reactions. Then, the best results were obtained with 4 mL air·min^{-1} (0.101 L O_2·L^{-1} feed) (~90%).

The long-term exposure to microaerobic conditions (249 days) led the microbiota to a gradual evolution. Consequently, there was some enrichment with species potentially associated with the biotransformation of OMPs, which may explain the better performance at the end of the microaerobic term even with the lowest airflow rate tested.

3 REDOX MEDIATOR, MICROAERATION, AND NITRATE ADDITION AS ENGINEERING APPROACHES TO ENHANCE THE BIOTRANSFORMATION OF ANTIBIOTICS IN ANAEROBIC REACTORS*

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3.1 Introduction

One of the main sources of water contamination with antibiotics and other organic micropollutants (OMPs) (e.g., hormones and other pharmaceuticals) is domestic wastewater, since these compounds can enter the municipal wastewater collection system through excreta or their inadequate disposal in the toilet (Jewell et al., 2016). Therefore, the increasing world consumption of antibiotics over the years is directly related to the occurrence of such OMPs in water environments, representing an emerging environmental and public health concern, since it can favor the development of antibiotic-resistance genes in bacteria (Felis et al., 2020; Thiebault, 2020).

Sulfamethoxazole (SMX) and trimethoprim (TMP), which are usually consumed in association (co-trimoxazole), are the fourth most used antibiotics in the world (Thiebault, 2020) and belong to the list of essential medicines of World Health Organization (WHO), as they are indicated for lower urinary tract infections, acute invasive diarrhea/bacterial dysentery, prevention of HIV-related opportunistic infections, pneumocystosis, and toxoplasmosis (WHO, 2019). Consequently, both SMX and TMP are frequently found in wastewater as well as in other environmental matrices (Felis et al., 2020; Luo et al., 2014; Thiebault, 2020).

In general, wastewater treatment plants (WWTPs) are not specifically designed to remove OMPs, and, although removal efficiencies (REs) depend on both the compound and the treatment technology, they are usually limited (Grandclément et al., 2017; Jewell et al., 2016), especially in anaerobic systems operated at short hydraulic retention times (HRTs) (< 10 h) (Brandt et al., 2013; Buarque et al., 2019; Vassalle et al., 2020). Therefore, some upgrades to WWTPs have been proposed in the last years to improve the removal of OMPs, such as using hybrid processes (a combination of biofilm with suspended biomass) or adding advanced processes (e.g., ozonation, UV oxidation, and adsorption on activated carbon) (Grandclément et al., 2017; Jewell et al., 2016). However, these options may not be adequate to the

socioeconomic reality of many developing countries, such as India and those in Latin America, where upflow anaerobic sludge blanket (UASB) reactors, a more cost-effective technology, are widely used for domestic wastewater treatment (HRT of 6-8 h) (Chernicharo et al., 2015). Thus, simpler and lower-cost approaches should be applied to these short-HRT anaerobic treatment systems for improving the removal of OMPs.

According to the literature, the biotransformation of OMPs and other compounds, such as sulfonated reactive azo dyes, nitroaromatics, halogenated aliphatics, halogenated aromatics, and metalloids, is usually quite slow (dos Santos et al., 2007; Harb et al., 2019; Lakshminarasimman et al., 2018; Van der Zee and Cervantes, 2009). However, soluble quinone-based compounds, such as anthraquinone-2-sulfonate (AQS), anthraquinone-2,6-disulfonate (AQDS) and lawsone, and some vitamins, such as riboflavin, can act as redox mediators and accelerate the anaerobic biotransformation of these pollutants (dos Santos et al., 2007; He et al., 2017; Van der Zee and Cervantes, 2009; Zhou et al., 2018).

Recent research has also demonstrated that microaeration (addition of less than 1 L $O_2 \cdot L^{-1}$ feed), a consolidated technology for hydrogen sulfide removal in anaerobic reactors (Krayzelova et al., 2015), is an effective strategy to enhance the biotransformation of recalcitrant compounds in these systems, such as BTEX (benzene, toluene, ethylbenzene, and xylenes) (Firmino et al., 2018; Siqueira et al., 2018) and even OMPs (Buarque et al., 2019). Additionally, another possibility could be using nitrate as an alternative terminal electron acceptor, which has a similar oxidation potential to oxygen (dos Santos et al., 2007), as several studies have reported that, under nitrate-reducing (or anoxic) conditions, OMPs are more effectively biotransformed (Alvarino et al., 2016; Inyang et al., 2016; Lakshminarasimman et al., 2018; Ogunlaja and Parker, 2018; Zhao et al., 2018).

However, as far as the author is concerned, there is no investigation into the application of redox mediators, associated or not with microaeration, to continuous-flow anaerobic reactors for improving the biotransformation of OMPs as well as into the effect of different COD/NO_3^- ratios on such process. Therefore, the present work assessed some engineering approaches, such as the addition of AQDS (50 and 100 μM), microaeration (1 mL air·min^{-1}), and nitrate (100-400 mg·L^{-1}), for enhancing the biotransformation of SMX and TMP (200 μg·L^{-1} each) in anaerobic reactors operated at a short HRT (7.4 h).

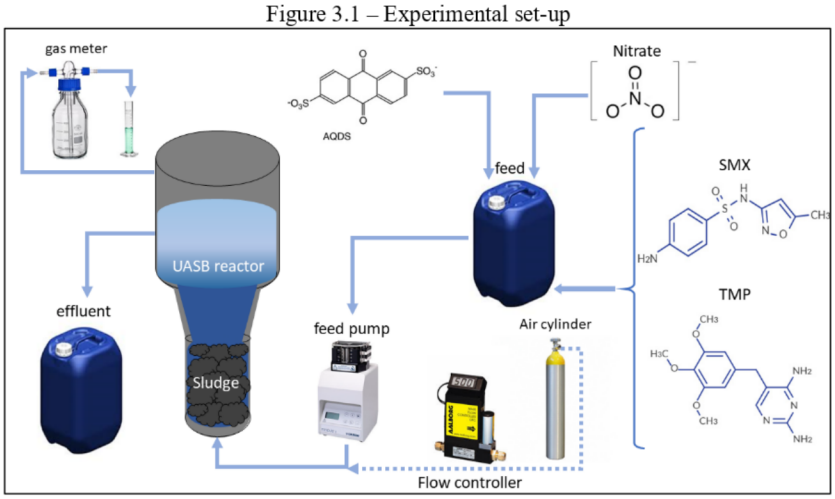
3.2 Material and methods

3.2.1 Experimental set-up

Two lab-scale UASB reactors (working volume of 3.5 L), inoculated with anaerobic sludge (~50 g VSS·L^{-1}) from a mesophilic UASB reactor treating domestic wastewater (Fortaleza, Ceará, Brazil), were operated, in parallel, at an HRT of 7.4 h and room temperature of approximately 28 °C. The reactors were fed with synthetic wastewater containing the antibiotics sulfamethoxazole (SMX, 99%, Sigma-Aldrich, USA) and trimethoprim (TMP, 98%, Sigma-Aldrich, USA) (~200 µg·L^{-1} each), ethanol (~1 g COD·L^{-1}) as a primary carbon source, nutrients (Firmino et al., 2010), and sodium bicarbonate (1 g·L^{-1}) as a buffer to keep the pH close to 7.0. The concentrations of the antibiotics were set according to those found in some local domestic wastewater (Vidal et al., 2020).

In some experimental periods, one of the reactors was supplemented with the redox mediator AQDS (98%, Sigma-Aldrich, USA) and/or microaerated with synthetic air (80% N_2:20% O_2, White Martins, Brazil) at the feeding line through a needle by a mass flow controller (GFC17, Aalborg, USA), whereas the other reactor was supplemented with sodium nitrate (98%, Dinâmica Química, Brazil). The biogas produced was measured by a Mariotte flask containing a 3% sodium chloride solution at pH 2 (Figure 3.1).

Figure 3.1 – Experimental set-up



3.2.2 Experimental procedure

3.2.2.1 Effect of the redox mediator and microaeration on the anaerobic biotransformation of antibiotics

The individual and combined effects of the redox mediator AQDS and microaeration on the anaerobic removal of the antibiotics SMX and TMP were assessed throughout a seven-period experiment (Table 3.1). In period I, the reactor was fed only with the antibiotic-containing wastewater and operated under anaerobic conditions. Then, in periods II and III, it was supplemented with AQDS at 50 and 100 µM, respectively. Subsequently, in periods IV and V, the reactor remained supplemented with the redox mediator (100 and 50 µM, respectively), but it was also microaerated at a flow rate of 1 mL air·min^{-1} at 28 °C and 1 atm (equivalent to 0.025 L O_2·L^{-1} feed) (microaerobic conditions). Afterwards, in period VI, AQDS supplementation was interrupted, whereas microaeration was maintained. Finally, in period VII, to evidence the effect of the operational changes throughout the experiment and exclude the hypothesis of microbiota adaptation to the antibiotics over time, the anaerobic conditions were reestablished. The experimental periods were changed after verifying system stability.

Table 3.1 – Operational conditions of the reactor throughout the experiment with anthraquinone-2,6-disulfonate (AQDS) and microaeration

Period	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**
End of period (day)	22	54	70	92	117	140	152
HRT (h)	7.4	7.4	7.4	7.4	7.4	7.4	7.4
COD (g·L^{-1})	1.0	1.0	1.0	1.0	1.0	1.0	1.0
SMX (µg·L^{-1})	194	205	207	216	196	199	219
TMP (µg·L^{-1})	214	207	198	202	216	202	213
AQDS (µM)	-	50	100	100	50	-	-
Microaeration (mL·min^{-1})	-	-	-	1	1	1	-
Dose of oxygen (L O_2·L^{-1} feed)	-	-	-	0.025	0.025	0.025	-

COD, chemical oxygen demand; HRT, hydraulic retention time; SMX, sulfamethoxazole; TMP, trimethoprim.

3.2.2.2 Effect of COD/NO_3^- ratio on the anaerobic biotransformation of antibiotics

The effect of the use of nitrate as an alternative terminal electron acceptor on the anaerobic removal of the antibiotics SMX and TMP was assessed throughout a six-period experiment (Table 3.2). In period I, the reactor was fed only with the antibiotic-containing

wastewater and operated under anaerobic conditions. Afterwards, from period II to IV, it was supplemented with increasing nitrate concentrations (100, 200, and 400 $mg \cdot L^{-1}$, respectively), which equaled to COD/NO_3^- ratios of 10.0, 5.0, and 2.5 (denitrifying conditions). Finally, to reinforce the role of the nitrate concentration and exclude the hypothesis of microbiota adaption to the antibiotics over time, in period V, the nitrate concentration was decreased to 100 $mg \cdot L^{-1}$, and, in period VI, the anaerobic conditions were reestablished. The experimental periods were changed after verifying system stability.

Table 3.2 – Operational conditions of the reactor throughout the experiment with nitrate

Periods	**I**	**II**	**III**	**IV**	**V**	**VI**
End of period (day)	16	38	63	86	108	122
HRT (h)	7.4	7.4	7.4	7.4	7.4	7.4
COD ($g \cdot L^{-1}$)	1.0	1.0	1.0	1.0	1.1	1.1
SMX ($\mu g \cdot L^{-1}$)	205	194	194	213	204	209
TMP ($\mu g \cdot L^{-1}$)	206	189	202	215	205	209
NO_3^- ($g \cdot L^{-1}$)	-	0.1	0.2	0.4	0.1	-
COD/NO_3^-	-	10.0	5.0	2.5	10.0	-

COD, chemical oxygen demand; HRT, hydraulic retention time; SMX, sulfamethoxazole; TMP, trimethoprim.

3.2.3 Chemical analysis

For the quantification of SMX and TMP, the samples (500 mL) were previously filtered (0.45 µm) and acidified with HCl (pH 2.5-3). Then, they were percolated through Strata-X® cartridges (500 mg, 6 mL) (Phenomenex®, USA) for the solid phase extraction (SPE) of the antibiotics, which were eluted with HPLC/UV grade methanol (4 mL) (99.8%, Neon, Brazil) (Pessoa et al., 2014). The eluate (20 µL) was then analyzed by an LC-20A Prominence high-performance liquid chromatograph (HPLC) equipped with a Shim-pack CLC-ODS(M)® C18 column (4.6×150 mm, 5 µm) and a UV-Vis SPD-20A detector (258 nm) (Shimadzu Corporation, Japan). The elution was performed by mobile phase composed of HPLC/UV grade acetonitrile (99.9%, Sigma-Aldrich, Germany) and 0.1% HCl solution, based on Vidal et al. (2020), with the following gradient: 10% to 80% increase in acetonitrile in 10 min, returning to 10% in 4 min. The flow rate was initially 1.0 $mL \cdot min^{-1}$ and, after 5 min of run, it was increased to 2.0 $mL \cdot min^{-1}$. The oven temperature was maintained at 35 °C throughout the run.

COD, alkalinity, and pH were determined according to (APHA, 2012). The volatile fatty acids (VFA) were determined by the Kapp titrimetric method (Buchauer, 1998). Nitrate and nitrite were quantified by a Dionex™ ICS-1100 ion chromatograph equipped with a

Dionex™ IonPac™ AG23 pre-column (2×50 mm), a Dionex™ IonPac™ AS23 column (2×250 mm), and a Dionex™ AERS™ 500 suppressor (2 mm) (Thermo Scientific, USA). 5 µL of the filtered sample (0.45 µm) were injected and then eluted by an aqueous solution containing 4.5 mM sodium carbonate and 0.8 mM sodium bicarbonate at a constant flow of 0.25 $mL \cdot min^{-1}$. The oven temperature was 30 °C, the applied current was 7 mA, and the running time was 30 min.

The biogas was characterized in terms of CH_4, CO_2, O_2, and N_2. CH_4 and CO_2 were quantified by gas chromatography with barrier-discharge ionization detection (GC-BID) (GC-2010 Plus, Shimadzu Corporation, Japan). The biogas sample (1.0 mL) was injected in split mode (split ratio of 30), and the chromatographic separation was performed on a GS-GasPro column (60 m, 0.32 mm I.D.) (Agilent Technologies, USA). The temperatures of the injector and the detector were 100 and 250 °C, respectively. The temperature of the oven started at 50 °C, was raised to 75 °C at 5 $°C \cdot min^{-1}$, then to 105 °C at 8 $°C \cdot min^{-1}$, and was finally maintained at 105 °C for 0.25 min (total run time of 9 min). Helium (White Martins, Brazil) was used as the carrier gas at a flow rate of 2.0 $mL \cdot min^{-1}$. O_2 and N_2 were quantified by gas chromatography with thermal conductivity detection (GC-TCD) (GC-17A, Shimadzu Corporation, Japan). The biogas sample (1.0 mL) was injected in splitless mode, and the chromatographic separation was performed on a Mol Sieve 5A PLOT column (30 m, 0.32 mm I.D.) (Restek Corporation, USA). The temperatures of the injector, oven, and detector were 40, 35, and 230 °C, respectively. Helium (White Martins, Brazil) was used as the carrier gas at a flow of 7 $mL \cdot min^{-1}$, and the run time was 5 min.

3.2.4 Statistical analysis

The Mann-Whitney and Kruskal-Wallis non-parametric tests, which do not require a specific data distribution, were used to compare the performance of the reactors during the different experimental periods at a 5% significance level.

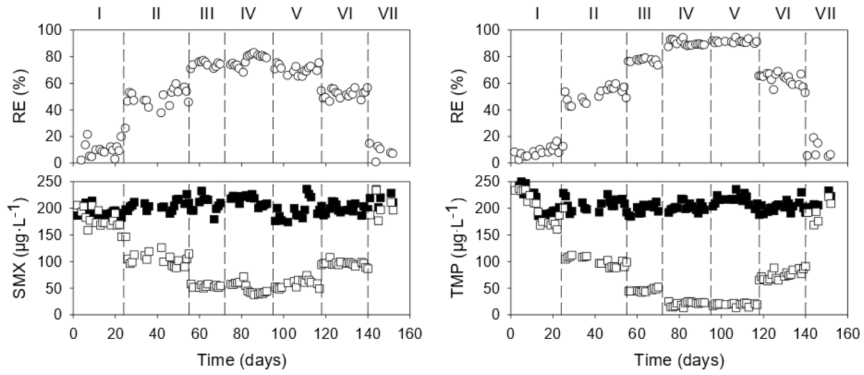
3.3. Results and discussion

3.3.1 Effect of the redox mediator and microaeration on the anaerobic biotransformation of antibiotics

3.3.1.1 Redox mediator

In period I, when the reactor was operated under anaerobic conditions without AQDS addition, the REs of SMX and TMP were very low (Figure 3.2), with mean values close to only 6% (Table 3.3). These results are comparable to those by Buarque et al. (2019), who registered mean REs below 10% in a UASB reactor (28 °C and HRT of 7 h) treating synthetic wastewater containing a mixture of seven OMPs (~230 $\mu g \cdot L^{-1}$ each), including SMX and TMP, in the presence of ethanol (1 g $COD \cdot L^{-1}$) as a primary substrate.

Figure 3.2 – Influent (■) and effluent (□) concentration and removal efficiencies (REs) (○) of the antibiotics sulfamethoxazole (SMX) and trimethoprim (TMP) throughout the experiment with anthraquinone-2,6-disulfonate (AQDS) and microaeration



According to Harb et al. (2019), the anaerobic biotransformation of OMPs is usually very slow, thus requiring longer reaction times. Hence, long-HRT anaerobic systems may be more successful at removing these compounds. In fact, some authors reported high REs of SMX and TMP (> 80%) in UASB reactors operated at HRTs ranging between 19 and 24 h (Alvarino et al., 2014; 2019; Arias et al., 2018). However, using long HRTs (> 12 h) for low-strength wastewaters (e.g., domestic wastewater) is not practical, since it incurs higher capital expenditures (CAPEX) (larger-volume reactors) (Chernicharo et al., 2015; Harb et al., 2019).

Therefore, other strategies are needed to enhance anaerobic biotransformation of OMPs in UASB reactors designed for domestic wastewater, whose HRT usually ranges from 6 to 8 h (Chernicharo et al., 2015).

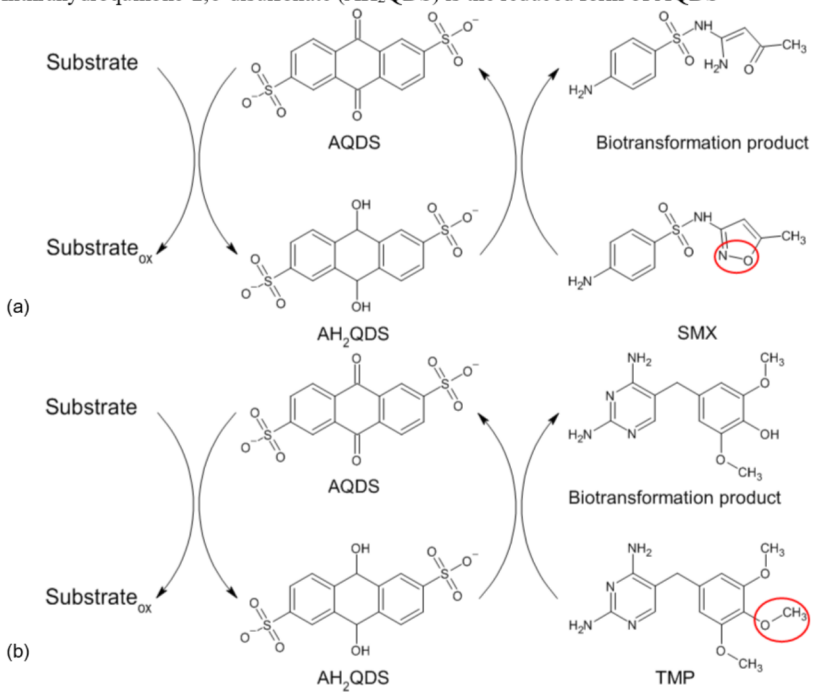
Under anaerobic conditions, the initial biotransformation of SMX and TMP is reported to occur through reductive reactions: cleavage of the N–O bond in the isoxazole ring of SMX (Figure 3.3a) (Alvarino et al., 2016; Jia et al., 2017; Mohatt et al., 2011) and cleavage of the O–C bond in the methoxy functional group (*O*-demethylation) mainly at C-4 position of TMP (Figure 3.3b) (Jia et al., 2019; Liang et al., 2019). Accordingly, since quinone-based compounds (e.g., AQDS, AQS, and lawsone) can act as redox mediators and accelerate the reductive biotransformation of several pollutants (e.g., azo dyes, nitroaromatics, and polyhalogenated compounds) (dos Santos et al., 2007; Van der Zee and Cervantes, 2009), applying them to short-HRT anaerobic reactors may be an effective strategy to enhance the biotransformation of SMX and TMP.

In fact, with the addition of 50 and 100 μM of AQDS in periods II and III, respectively, there were subsequent significant increases in the REs of these antibiotics ($p < 0.001$) (Figure 3.2), reaching mean values close to 50% (period II) and 75% (period III) (Table 3.3). Therefore, as AQDS had a remarkable positive effect on the anaerobic biotransformation of SMX and TMP, it may have overcome a likely electron transfer limitation. These results agree with those by He et al. (2017), who carried out anaerobic batch tests with synthetic wastewater containing 10 $\mu g \cdot L^{-1}$ of SMX and found that only 10 μM of AQDS accelerated the biotransformation of this antibiotic. However, differently from the present study, no significant difference was observed when higher concentrations of AQDS (100 and 1000 μM) were applied. On the other hand, Zhou et al. (2018), in anaerobic batch assays with *Shewanella oneidensis* MR-1, observed a 1.4-fold higher biotransformation rate of SMX (~10 $mg \cdot L^{-1}$) when the concentration of AQDS was increased from 200 to 500 μM. Thus, apparently, the impact of the concentration of the redox mediator may depend on the concentration of the target pollutant.

According to the literature, the reductive biotransformation of pollutants in the presence of a redox mediator occurs in two distinct steps. Firstly, this compound is biologically reduced during the oxidation of organic substrates (e.g., sugars, alcohols, fatty acids). Then, it is chemically reoxidized during the reduction of the target pollutant (electron acceptor) (dos Santos et al., 2007; Van der Zee and Cervantes, 2009). In fact, both the cleavage of the isoxazole ring and *O*-demethylation reaction were reported to occur abiotically (Bradley et al., 2006; Mohatt et al., 2011). Thus, after AQDS was biologically reduced to anthrahydroquinone-2,6-

disulfonate (AH_2QDS), it most likely transferred the electrons to SMX and TMP through a purely chemical reaction (Figure 3.3).

Figure 3.3 – Initial reductive biotransformation of sulfamethoxazole (SMX) (a) and trimethoprim (TMP) (b) in the presence of the redox mediator anthraquinone-2,6-disultonate (AQDS). The bonds cleaved during the process are indicated by red ellipses. Anthrahydroquinone-2,6-disulfonate (AH_2QDS) is the reduced form of AQDS



3.3.1.2 Redox mediator and microaeration

In period IV, a microaeration flow rate of 1 mL air·min^{-1} (0.025 L O_2·L^{-1} feed) was added to the reactor, which remained supplemented with 100 µM of AQDS. Consequently, there was a significant increase in the biotransformation of TMP ($p < 0.001$), reaching a mean RE close to 90%. In contrast, considering the whole period, the effect on the biotransformation of SMX was not significant ($p = 0.064$), with an increase of only 3% in the mean RE (Table 3.3). However, from the half of this period, there was an apparent increase in the REs of SMX (mean of ~80.6%) (Figure 3.2). Therefore, actually, microaeration also improved the

biotransformation of SMX ($p < 0.001$), although the values of RE were still far below those of TMP.

In period V, the airflow rate was kept at 1 $mL \cdot min^{-1}$, and the concentration of AQDS was reduced to 50 μM. Whereas the mean RE of TMP remained nearly 90%, that of SMX decreased significantly compared to the previous period (~7%) ($p < 0.001$) (Table 3.3). Nonetheless, even with a lower concentration of AQDS, the REs of both antibiotics remained very high, especially compared to period II (Table 3.3), when the same concentration of AQDS was used without microaeration.

Reductive biotransformation of pollutants is usually hindered under aerobic conditions, even in the presence of redox mediators, because oxygen is a much more effective electron acceptor (dos Santos et al., 2007). Moreover, some microorganisms can use the reduced redox mediator as an electron donor in aerobic respiration (Van der Zee and Cervantes, 2009). Therefore, introducing oxygen into anaerobic reactors could compromise such a biotransformation process. However, in the current investigation, microaeration did not hamper the effect of AQDS on the biotransformation of SMX and TMP. Actually, it had a synergistic effect with the redox mediator. Thus, the small amount of oxygen added was not sufficient to increase the low oxidation-reduction potential (ORP) of the medium (data not shown), ensuring the role of AQDS in the reduction of the chemical bonds. Similarly, Barros et al. (2018) did not observe a negative effect either on the reduction of the azo dye Reactive Red 2 facilitated by AQDS when their UASB reactor (HRT of 24 h) was microaerated at a flow rate of 1 mL air·min^{-1} (0.095 L $O_2 \cdot L^{-1}$ feed at 28 °C and 1 atm). On the other hand, differently from the present study, microaeration did not have a positive effect either.

Under microaerobic conditions, instead of acting as a final electron acceptor, oxygen is only used by monooxygenase-producing microorganisms to hydroxylate organic compounds, facilitating their subsequent anaerobic biotransformation (Fuchs, 2008). In fact, microaeration has been previously demonstrated to enhance the anaerobic biotransformation of several OMPs, including SMX and TMP (Buarque et al., 2019). According to the literature, oxygen is also involved in the cometabolic biotransformation of these antibiotics (Fischer and Majewsky, 2014; Jewell et al., 2016). Therefore, microaeration may have promoted a biotransformation pathway parallel to the aforementioned reductive processes, justifying the increase in the REs of SMX and TMP in periods IV and V (microaerobic) compared to periods II and III (anaerobic).

Table 3.3 – Mean influent and effluent concentrations and removal efficiencies (REs) of the antibiotics throughout the experiment with anthraquinone-2,6-disulfonate (AQDS) and microaeration

Period		**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**
AQDS (μM)		**-**	**50**	**100**	**100**	**50**	**-**	**-**
Microaeration (mL·min^{-1})		**-**	**-**	**-**	**1**	**1**	**1**	**-**
SMX	Influent (μg·L^{-1})	194 ± 8	205 ± 9	207 ± 14	216 ± 8	196 ± 19	199 ± 8	219 ± 12
	Effluent (μg·L^{-1})	181 ± 15	103 ± 11	54 ± 3	49 ± 10	58 ± 7	96 ± 4	201 ± 20
	RE (%)	6.2 ± 7.8	49.7 ± 6.0	74.0 ± 8.5	77.1 ± 4.4	70.2 ± 3.3	51.8 ± 3.1	8.7 ± 4.9
TMP	Influent (μg·L^{-1})	214 ± 25	207 ± 11	198 ± 9	202 ± 8	216 ± 10	202 ± 10	213 ± 12
	Effluent (μg·L^{-1})	201 ± 29	100 ± 9	46 ± 3	21 ± 4	19 ± 3	75 ± 9	193 ± 20
	RE (%)	6.2 ± 5.6	51.4 ± 5.6	76.7 ± 8.2	89.8 ± 2.1	91.1 ± 1.2	62.6 ± 4.6	9.3 ± 6.0

SMX, sulfamethoxazole; TMP, trimethoprim.

3.3.1.3 Microaeration

In period VI, the reactor continued to be operated under microaerobic conditions (1 mL air·min^{-1}), however without AQDS. Then, the removal of both antibiotics decreased significantly ($p < 0.001$) (Table 3.3), emphasizing the role of AQDS in their biotransformation. Nonetheless, the REs of this period were still much higher than those of period I (anaerobic) (Figure 3.2). Actually, the mean RE of SMX was similar to that of period II (anaerobic with 50 μM of AQDS) ($p = 0.159$), whereas that of TMP was even better ($p < 0.001$) (Table 3.3). Thus, microaeration seems to have a much more significant effect on the biotransformation of these antibiotics than what was evidenced in the presence of the redox mediator (periods IV and V), especially for TMP. Similar results were obtained in a previous work, in which the injection of 1 mL air·min^{-1} (0.021 L O_2·L^{-1} feed at 27 °C and 1 atm) into a 3.7-L UASB reactor (HRT of 7 h) fed with synthetic OMP-containing wastewater. The mean REs of the OMPs (~230 μg·L^{-1} each), including SMX and TMP, increased from less than 10% to approximately 55% (Buarque et al., 2019).

Under aerobic conditions, biotransformation of OMPs is usually catalyzed by oxygenase enzymes. Since these compounds are present at very low concentrations, it is unlikely they are used as a primary carbon and energy source by microorganisms. Therefore, the most probable hypothesis is that OMPs are biotransformed through cometabolic pathways by non-specific enzymes, especially ammonia monooxygenase (AMO) (Fischer and Majewsky, 2014; Fernandez-Fontaina et al., 2016). However, in the present study, the concentration of dissolved oxygen (< 0.1 mg·L^{-1}) was insufficient to promote nitrification (data not shown), i.e., to stimulate the synthesis/activation of AMO. Therefore, microaeration may have stimulated the synthesis of other monooxygenases with low specificity, which could have hydroxylated both SMX and TMP.

In period VII, the system was operated under the same anaerobic conditions as in period I. A decrease in the REs was immediately found (Figure 3.2), reaching mean values similar to those observed in period I (< 10%) ($p > 0.050$) (Table 3.3). Therefore, the differences among the periods with respect to the biotransformation of SMX and TMP in the UASB reactor were due to the operational conditions imposed to it rather than microbial adaptation over time.

3.3.2 Effect of COD/NO_3^- ratio on the anaerobic biotransformation of antibiotics

As observed in the previous experiment (section 3.2.2.1), without nitrate addition (period I), low mean REs of SMX and TMP were found (< 15%) (Table 3.4). Then, with the introduction of nitrate (100 $mg \cdot L^{-1}$) as an alternative electron acceptor (COD/NO_3^- ratio of 10.0) (period II), the REs of antibiotics increased considerably ($p < 0.001$) (Figure 3.4). Whereas this increase was almost immediate for SMX, it was gradual for TMP, with a slight decrease at the end of the period. Consequently, TMP achieved a 7% lower mean RE than that of SMX (Table 3.4), even though there was no statistical difference ($p = 0.056$).

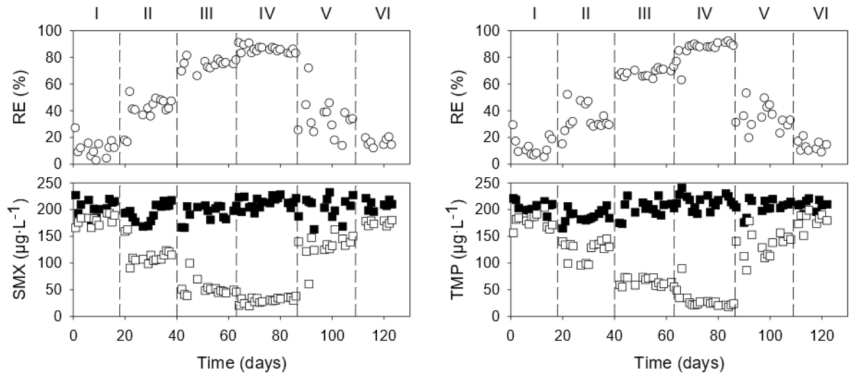
Previous batch assays with activated sludge have shown that biotransformation rates of SMX and TMP are remarkably higher under nitrate-reducing (anoxic) conditions than under anaerobic conditions and sometimes comparable to or even higher than those found under aerobic conditions (Alvarino et al., 2016; Inyang et al., 2016; Lakshminarasimman et al., 2018; Ogunlaja and Parker, 2018; Zhao et al., 2018) because the oxidation potentials of nitrate and oxygen are very similar (dos Santos et al., 2007).

In period III, doubling the concentration of nitrate (COD/NO_3^- ratio of 5.0), the mean REs of both antibiotics increased almost 35% (Table 3.4). However, the difference between these OMPs became statistically evident ($p < 0.001$), which suggests that TMP is slightly more recalcitrant than SMX under anoxic conditions. In period IV, at a COD/NO_3^- ratio of 2.5 (400 mg $NO_3^- \cdot L^{-1}$), although to a lesser extent than in the previous periods, the REs increased again, reaching mean values higher than 85% for both OMPs (Table 3.4). Thus, with a higher nitrate concentration, the difference between the removals of SMX and TMP was negligible. In addition, a high stability in the efficiency values was also observed in this period (Figure 3.4).

Alvarino et al. (2016) also observed an increase in the RE of SMX (272 $\mu g \cdot L^{-1}$) from 10 to 60% in a fed-batch reactor inoculated with activated sludge when the nitrate concentration was raised from 22.1 to 221.4 $mg \cdot L^{-1}$ in the presence of acetate (50 mg $COD \cdot L^{-1}$). According to Rodriguez-Escales and Sanchez-Vila (2016), the biotransformation of SMX under nitrate-reducing conditions is also a cometabolic process, in which SMX is abiotically converted into 4-nitro-SMX and desamino-SMX in the presence of nitrite, an intermediate of denitrification. Therefore, the higher the added nitrate concentration, the greater the nitrite production, increasing the reaction rate. It is worth mentioning that, although no information on biotransformation intermediates of TMP under anoxic conditions was found in the literature,

nitrosation and deamination reactions may also occur with this antibiotic, as it contains amino functional groups.

Figure 3.4 – Influent (■) and effluent (□) concentration and removal efficiencies (REs) (○) of the antibiotics sulfamethoxazole (SMX) and trimethoprim (TMP) throughout the experiment with nitrate



When the COD/NO_3^- ratio was raised back to 10.0 (100 mg $NO_3^-{\cdot}L^{-1}$) (period V), the REs decreased immediately and were highly unstable (Figure 3.4), reaching mean values similar to those of period II ($p > 0.050$) (Table 3.4). Finally, in period VI, the system was operated again without nitrate addition, i.e., under the same conditions as in period I. Consequently, the REs of SMX and TMP decreased again and were similar to those obtained in period I ($p > 0.070$) (Figure 3.4). Thus, these results reinforce the effect of the nitrate concentration on the biotransformation of these antibiotics, rejecting any hypothesis of microbial adaptation to these OMPs over time.

Finally, the results found in the current experiment have practical engineering applications, since anaerobic reactors are usually followed by aerobic post-treatment systems for conversion of ammonium into nitrate, which can be reduced to nitrogen gas when the nitrified effluent is recirculated back to the anaerobic reactors (Kassab et al., 2010). Thus, the recirculation of nitrate-containing effluents may also enhance the overall removal of OMPs in wastewater treatment plants, preventing or at least decreasing the continuous dosage of nitrate, which represents an additional operational cost.

Table 3.4 – Mean influent and effluent concentrations and removal efficiencies (REs) of the antibiotics throughout the experiment with nitrate

Period		**I**	**II**	**III**	**IV**	**V**	**VI**
COD/NO_3^-		**-**	**10.0**	**5.0**	**2.5**	**10.0**	**-**
SMX	Influent ($\mu g \cdot L^{-1}$)	205 ± 15	194 ± 17	194 ± 15	213 ± 10	204 ± 21	209 ± 9
	Effluent ($\mu g \cdot L^{-1}$)	180 ± 10	116 ± 20	49 ± 7	30 ± 5	132 ± 23	175 ± 4
	RE (%)	11.8 ± 6.6	40.1 ± 10.5	74.6 ± 3.8	85.8 ± 2.5	34.6 ± 14.2	16.2 ± 2.9
TMP	Influent ($\mu g \cdot L^{-1}$)	206 ± 10	189 ± 11	202 ± 15	215 ± 13	205 ± 13	209 ± 10
	Effluent ($\mu g \cdot L^{-1}$)	179 ± 13	125 ± 18	64 ± 7	30 ± 17	133 ± 24	181 ± 14
	RE (%)	13.0 ± 7.2	33.5 ± 10.0	68.2 ± 2.8	86.2 ± 6.7	35.3 ± 9.4	13.5 ± 4.0

COD, chemical oxygen demand; SMX, sulfamethoxazole; TMP, trimethoprim.

3.3.3 Operational stability

In both experiments (sections 3.2.2.1 and 3.2.2.2), the mean COD REs were very high in all periods (near 90%) because ethanol (primary substrate) is easily degraded under anaerobic conditions. Additionally, the pH remained within the neutral range, and no accumulation of VFA was evidenced (Tables 3.5 and 3.6). Therefore, the introduction of AQDS, air, or nitrate did not affect organic matter conversion. However, under denitrifying conditions, part of the electrons produced during substrate oxidation was deviated from methanogenesis to denitrification, leading to a decrease in methane production, particularly when the reactor was operated at lower COD/NO_3^- ratios (periods III and IV) (Table 3.6). It is worth mentioning that, although methane content in the biogas also decreased when the reactor was microaerated (periods IV, V and VI) (Table 3.5), it was only a consequence of biogas dilution by air (oxygen source), whose N_2 content is very high (80%). Thus, there was no inhibition of methanogenesis, since methane production was not impaired.

Regarding nitrate removal, mean efficiencies above 90% were obtained in all periods, although there was a slight decreasing tendency when higher nitrate concentrations were added (Table 3.6). Furthermore, only in period IV, with the addition of 400 mg $NO_3^- \cdot L^{-1}$ (COD/NO_3^- ratio of 2.5), nitrite was detected in the effluent (27 mg$\cdot L^{-1}$). Therefore, except in this period, the removed nitrate was most likely completely reduced to nitrogen gas (complete denitrification).

Table 3.5 – Parameters of operational stability of the reactor throughout the experiment with anthraquinone-2,6-disulfonate (AQDS) and microaeration

Period	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**
AQDS (μM)	**-**	**50**	**100**	**100**	**50**	**-**	**-**
Microaeration (mL·min^{-1})	**-**	**-**	**-**	**1**	**1**	**1**	**-**
Influent COD (mg·L^{-1})	1017 ± 9	1042 ± 67	1001 ± 60	1042 ± 68	1031 ± 70	1019 ± 63	1005 ± 48
Effluent COD (mg·L^{-1})	108 ± 5	96 ± 14	106 ± 7	112 ± 10	104 ± 11	100 ± 16	116 ± 8
COD RE (%)	89.4 ± 1.0	90.8 ± 1.1	89.4 ± 1.0	89.3 ± 0.6	89.9 ± 0.9	90.1 ± 2.1	88.5 ± 0.7
Biogas production (L·d^{-1})	1.9 ± 0.2	1.9 ± 0.2	1.8 ± 0.1	3.1 ± 0.1	3.0 ± 0.2	2.9 ± 0.2	1.8 ± 0.1
CH_4 in the biogas (%)	78.0 ± 2.5	77.3 ± 2.5	81.7 ± 2.0	58.0 ± 3.1	59.0 ± 3.3	62.0 ± 2.3	78.3 ± 1.6
pH	7.2 ± 0.3	7.3 ± 0.2	7.3 ± 0.1	7.2 ± 0.2	7.2 ± 0.2	7.2 ± 0.3	7.1 ± 0.1
VFA (mg·L^{-1})	405 ± 74	414 ± 88	440 ± 72	427 ± 79	424 ± 55	412 ± 75	432 ± 60
VFA/TA	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1

COD, chemical oxygen demand; RE, removal efficiency; TA, total alkalinity; VFA, volatile fatty acids.

Table 3.6 – Parameters of operational stability of the reactor throughout the experiment with nitrate

Period	**I**	**II**	**III**	**IV**	**V**	**VI**
COD/NO_3^-	**-**	**10.0**	**5.0**	**2.5**	**10.0**	**-**
Influent COD ($mg \cdot L^{-1}$)	1045 ± 52	1011 ± 41	1032 ± 65	1039 ± 51	1061 ± 54	1078 ± 63
Effluent COD ($mg \cdot L^{-1}$)	115 ± 5	111 ± 11	118 ± 6	122 ± 5	111 ± 10	117 ± 9
COD RE (%)	89.0 ± 0.5	89.0 ± 1.0	88.5 ± 0.8	88.2 ± 0.8	89.5 ± 1.0	89.2 ± 0.8
Influent NO_3^- ($mg \cdot L^{-1}$)	-	99 ± 7	213 ± 8	400 ± 13	112 ± 9	-
Effluent NO_3^- ($mg \cdot L^{-1}$)	-	4 ± 1	18 ± 6	36 ± 26	7 ± 4	-
NO_3^- RE (%)	-	95.7 ± 1.1	91.6 ± 2.6	90.9 ± 6.5	93.9 ± 3.5	-
Biogas production ($L \cdot d^{-1}$)	1.7 ± 0.2	2.0 ± 0.2	1.9 ± 0.1	1.9 ± 0.1	1.8 ± 0.1	2.3 ± 0.1
CH_4 in the biogas (%)	73.9 ± 1.9	73.9 ± 2.5	61.7 ± 1.9	57.1 ± 3.5	70.7 ± 0.8	79.0 ± 2.8
N_2 in the biogas (%)	13.4 ± 1.9	19.1 ± 3.0	29.1 ± 2.3	36.3 ± 3.1	23.2 ± 0.6	11.1 ± 1.9
pH	7.0 ± 0.1	7.1 ± 0.2	7.1 ± 0.2	7.2 ± 0.1	7.3 ± 0.1	7.1 ± 0.2
VFA ($mg \cdot L^{-1}$)	313 ± 76	311 ± 43	359 ± 45	327 ± 32	386 ± 56	377 ± 75
VFA/TA	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1

COD, chemical oxygen demand; RE, removal efficiency; TA, total alkalinity; VFA, volatile fatty acids.

3.4 Conclusions

AQDS accelerated the anaerobic biotransformation of SMX and TMP significantly (an increase of ~70% in the REs with 100 μM of AQDS), thus probably overcoming an electron transfer limitation.

Microaeration enhanced the biotransformation of these antibiotics even in the presence of AQDS, when the best REs were obtained (> 70%), particularly for TMP (~90% with 1 mL air·min^{-1} and 100 μM of AQDS).

Nitrate addition also showed promising results, with the highest REs of SMX and TMP (~85%) being achieved at the lowest COD/NO_3^- ratio (2.5).

Therefore, the assessed lower-cost approaches ensured REs of SMX and TMP in the UASB reactors comparable to those found in higher-cost wastewater treatment technologies, such as conventional activated sludge, membrane bioreactors, and hybrid processes.

4 CAN MICROAERATION BOOST THE BIOTRANSFORMATION OF PARABENS IN HIGH-RATE ANAEROBIC SYSTEMS?*

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4.1 Introduction

The increasing presence of organic micropollutants (OMPs) in the environment is a result of the excessive use of synthetic organic compounds, namely pesticides, personal care/cosmetic products (PCPs), industrial chemicals, food additives and detergents, and some naturally occurring substances, such as estrogens (Goswami et al., 2018). Among these compounds, parabens, i.e., esters of p-hydroxybenzoic acid, are widely used in food, pharmaceuticals, and PCPs because they have excellent preservative properties, preventing the growth of Gram-positive bacteria, yeast, and mold (Haman et al., 2015; Ma et al., 2018). The parabens usually found on product labels are methylparaben (MeP), ethylparaben (EtP), propylparaben (PrP), butylparaben (BuP), isobutylparaben (iBuP), and benzylparaben (BeP) (Gałwa-Widera, 2019).

As an emerging group of endocrine-disrupting chemicals (EDCs), parabens have attracted growing attention due to their potential long-term effects on human health and aquatic organisms (Li et al., 2015). For instance, these compounds, even at very low concentrations, were reported to be associated with the incidence of breast cancer (Giulivo et al., 2016). Thus, the occurrence of parabens in water and wastewater is an emerging concern, and it is necessary to remove them from wastewater before their release into water bodies.

These compounds can be efficiently removed (> 80%) in high-cost wastewater treatment systems, such as activated sludge and its variants (Ashfaq et al., 2017; Karthikraj et al., 2017; Ma et al., 2018; Wang and Kannan, 2016). However, there are very few studies on the removal of parabens in continuous-flow anaerobic systems (Hernández Leal et al., 2010; Londoño and Peñuela, 2015), such as upflow anaerobic sludge blanket (UASB) reactors, a more cost-effective option for domestic wastewater treatment, widely used in warm-climate developing countries (e.g., Brazil, Colombia, and India) (Chernicharo et al., 2015). Additionally, although parabens can be anaerobically biotransformed, the reaction is very slow (Wu et al., 2017). Consequently, their removal may be limited in UASB reactors designed for

domestic wastewater treatment, which usually are operated at short hydraulic retention times (HRTs) (6-8 h) (Chernicharo et al., 2015).

Therefore, some relatively simple and inexpensive strategies should be implemented to overcome this limitation, such as the injection of small amounts of oxygen into these systems (microaeration). In fact, despite being initially proposed for sulfide removal (Krayzelova et al., 2015), this technique has been recently reported to enhance considerably the biotransformation of recalcitrant compounds in high-rate anaerobic reactors, such as monoaromatic hydrocarbons (benzene, toluene, ethylbenzene and xylenes, i.e., BTEX) (Firmino et al., 2018; Siqueira et al., 2018) and even OMPs (hormones, pharmaceuticals, and bisphenol A) (Buarque et al., 2019). However, to the best of the author's knowledge, there is no investigation into the removal of parabens under microaerobic conditions.

Hence, the main objective of the present study was to demonstrate microaeration as an effective strategy to boost the biotransformation of four parabens (MeP, EtP, PrP, and BuP) in a short-HRT (8 h) UASB reactor. Moreover, the effect of different airflow rates (1-4 $mL \cdot min^{-1}$) was also assessed from an engineering and microbiological perspective.

4.2 Material and methods

4.2.1 Synthetic wastewater

The synthetic wastewater consisted of an aqueous solution containing MeP, EtP, PrP, and BuP (~200 $\mu g \cdot L^{-1}$ each) (> 99%, Sigma-Aldrich, USA), ethanol (1 g $COD \cdot L^{-1}$), basal medium (macro and micronutrients), prepared according to Firmino et al. (2010), and sodium bicarbonate (1 $g \cdot L^{-1}$), to maintain the pH near 7.0.

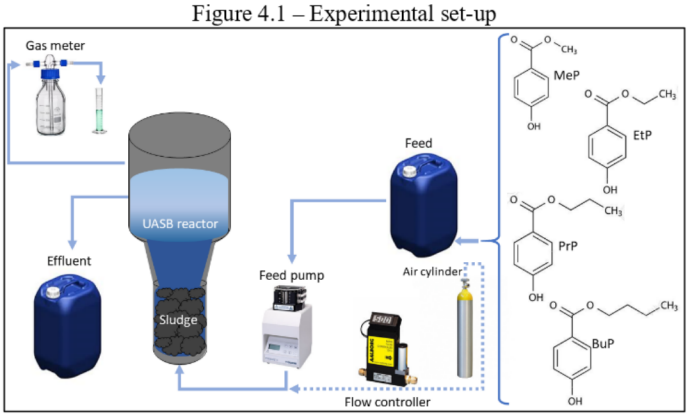
4.2.2 Experimental set-up

The experiment was performed in a UASB reactor (working volume of 3.5 L) inoculated with anaerobic sludge (~50 g $VSS \cdot L^{-1}$) from a mesophilic UASB reactor of a domestic wastewater treatment plant (WWTP) (Fortaleza, Ceará, Brazil) and operated at an HRT of 8 h and room temperature of approximately 28 °C.

The reactor was fed with the synthetic paraben-containing wastewater by a peristaltic pump (Minipuls 3, Gilson, USA) and, in some experimental periods, was microaerated with synthetic air (80% N_2:20% O_2, White Martins, Brazil) at its feeding line

through a needle by a mass flow controller (GFC17, Aalborg, USA). The biogas produced was measured by a Mariotte flask containing a 3% sodium chloride solution at pH 2 (Figure 4.1).

Figure 4.1 – Experimental set-up



4.2.3 Experimental procedure

In period I, the removal of parabens was assessed under anaerobic conditions. Subsequently, from period II to IV, to investigate their removal under microaerobic conditions, the reactor was microaerated at increasing flow rates (1, 2, and 4 mL air·min^{-1} at 28 °C and 1 atm, equivalent to 0.027, 0.055, and 0.110 L O_2·L^{-1} feed, respectively). Afterwards, in period V, to evaluate a likely adaptation of microbiota to microaerobic conditions, the microaeration flow rate was reduced to 1 mL·min^{-1} (28 °C and 1 atm). Finally, in period VI, to reinforce the oxygen effect and eliminate the hypothesis of microbiota adaptation to the parabens throughout the experiment, the reactor was again operated under anaerobic conditions. The experimental periods were changed after verifying system stability. The operational parameters of the reactor in each period are shown in Table 4.1.

Table 4.1 – Operational parameters of the reactor throughout the experiment

Period	I	II	III	IV	V	VI
End of period (day)	46	96	148	179	228	263
Microaeration ($mL \cdot min^{-1}$)	-	1	2	4	1	-
Dose of oxygen ($L\ O_2 \cdot L^{-1}$ feed)	-	0.027	0.055	0.110	0.027	-
HRT (h)	8.0	8.0	8.0	8.0	8.0	8.0
COD ($g \cdot L^{-1}$)	1.0	1.1	1.0	1.1	1.1	1.0
MeP ($\mu g \cdot L^{-1}$)	210	202	202	205	224	199
EtP ($\mu g \cdot L^{-1}$)	222	210	204	210	222	195
PrP ($\mu g \cdot L^{-1}$)	202	206	199	197	219	197
BuP ($\mu g \cdot L^{-1}$)	203	186	206	205	197	204

BuP, buthylparaben; COD, chemical oxygen demand; EtP, ethylparaben; HRT, hydraulic retention time; MeP, methylparaben; PrP, propylparaben.

4.2.4 Chemical analysis

The parabens were determined by an LC-20A Prominence high-performance liquid chromatograph (HPLC) equipped with a Shim-pack CLC-ODS(M)® C18 column (4.6×150 mm, 5 µm) and a UV-Vis SPD-20A detector (215 nm) (Shimadzu Corporation, Japan). Firstly, 500 mL of pre-filtered samples (0.45 µm) were percolated through Oasis HLB cartridges (3 cc, 60 mg, 30 µm) (Waters Corporation, USA) for solid-phase extraction of parabens, which were then eluted by 4 mL of HPLC/UV grade methanol (99.8%, Neon, Brazil) (Pessoa et al., 2014). Afterwards, 10 µL of this methanolic solution was injected in the HPLC, based on Vidal et al. (2020), and eluted by a mobile phase composed of ultrapure water and HPLC/UV grade acetonitrile (99.9%, Sigma-Aldrich, Germany) at a constant flow of 1.2 $mL \cdot min^{-1}$, using the following gradient: increase from 10% to 60% of acetonitrile in 8 min, followed by 10% reduction in 4 min. The temperature of the oven was maintained at 35 °C throughout the run.

COD, alkalinity, and pH were determined according to APHA (2012). The volatile fatty acids (VFA) were determined by the Kapp titrimetric method (Buchauer, 1998). The levels of CH_4 and CO_2 in the biogas were determined by gas chromatography with barrier-discharge ionization detection (GC-BID) (GC-2010 Plus, Shimadzu Corporation, Japan). The biogas sample (1.0 mL) was injected in split mode (split ratio of 30), and the chromatographic separation was performed on a GS-GasPro column (60 m, 0.32 mm I.D.) (Agilent Technologies, USA). The temperatures of the injector and the detector were 100 and 250 °C, respectively. The temperature of the oven started at 50 °C, was raised to 75 °C at 5 $°C \cdot min^{-1}$, then to 105 °C at 8 $°C \cdot min^{-1}$, and was finally maintained at 105 °C for 0.25 min (total run time of 9 min). Helium (White Martins, Brazil) was used as the carrier gas at a flow rate of 2.0 $mL \cdot min^{-1}$. The levels

of O_2 and N_2 in the biogas were determined by gas chromatography with thermal conductivity detection (GC-TCD) (GC-17A, Shimadzu Corporation, Japan). The biogas sample (1.0 mL) was injected in splitless mode, and the chromatographic separation was performed on a Mol Sieve 5A PLOT column (30 m, 0.32 mm I.D.) (Restek Corporation, USA). The temperatures of the injector, oven, and detector were 40, 35, and 230 °C, respectively. Helium (White Martins, Brazil) was used as the carrier gas at a flow of 7 $mL \cdot min^{-1}$, and the run time was 5 min.

4.2.5 Microbiological analysis

To evaluate the dynamics of the microbial community under microaerobic conditions, the DNAs of the inoculum and sludge samples collected at the end of the periods I (anaerobic), II (1 mL $air \cdot min^{-1}$), IV (4 mL $air \cdot min^{-1}$), and V (1 mL $air \cdot min^{-1}$) were extracted and then sequenced by an Illumina MiSeq Desktop Sequencer as detailed elsewhere (Rollemberg et al., 2019). The DNA was extracted using PowerSoil® DNA isolation kit (MoBio Laboratories Inc., USA) based on the manufacturer's instruction. The amplicon library of the 16S rRNA gene V4 region was prepared using the region-specific primers (515F/806R). After indexing, the PCR products were cleaned up using Agencourt AMPure XP—PCR purification beads (Beckman Coulter, Brea, CA, USA) based on the manufacturer's instruction and quantified using the dsDNA BR assay Kit (Invitrogen, Carlsbad, CA, USA) on a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). The libraries were sequenced using the 300-cycle MiSeq Reagent Kits v2 chemistry. The data obtained by the sequencing was analyzed with bioinformatics tools as follows. All reads were trimmed using vsearch v2.8.1, with parameters -fastq_maxee 0.8 -fastq_trunclen 250. All reads were clustered into OTUs using QIIME script pick_open_reference_otus.py with 99% identity, using Greengenes 16S rRNA database (release 13_8). Some ecological indices, namely Chao1 (richness), inverse Simpson (diversity), and Bray-Curtis dissimilarity, were calculated by Mothur software. Based on the latter index, UPGMA (unweighted pair group method with arithmetic mean) cluster analysis at genus level was also performed.

4.2.6 Statistical analysis

The Mann-Whitney and Kruskal-Wallis non-parametric tests, which do not require a specific data distribution, were used to compare the performance of the reactor during the different experimental periods at a 95% confidence level.

4.3 Results and discussion

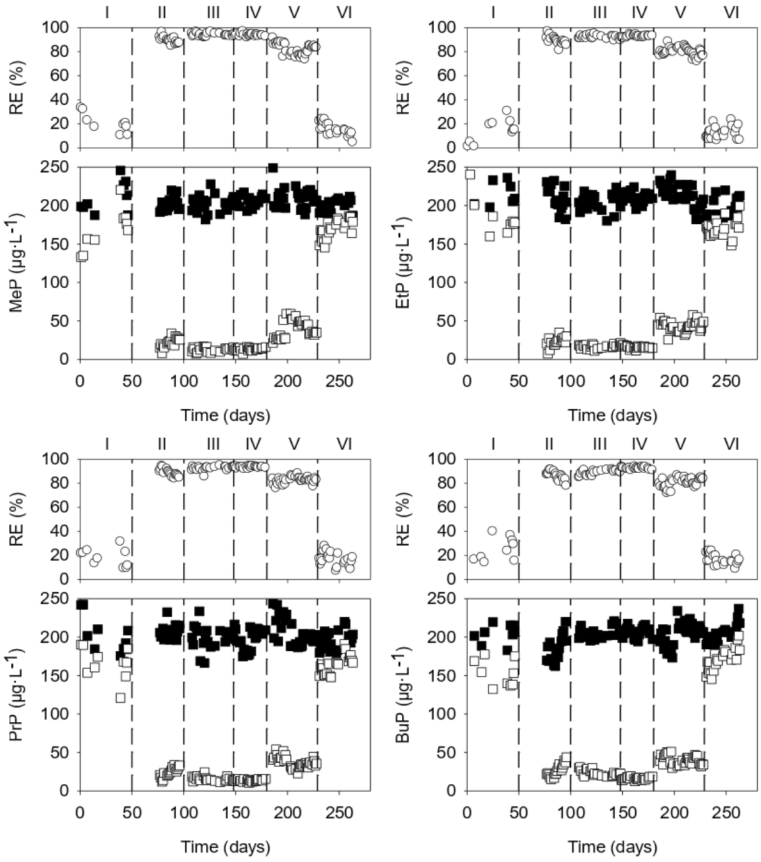
4.3.1 Removal of parabens under anaerobic conditions

In period I, under anaerobic conditions, the removal efficiencies (REs) of all parabens fluctuated significantly (Figure 4.2), and the mean values were quite low (≤ 20%), particularly for EtP (14%) (Table 4.2). Nevertheless, there was no statistical difference among the REs of these compounds ($p = 0.158$).

According to Wu et al. (2017), although parabens can be anaerobically degraded, the reaction is very slow even in the presence of alternative terminal electron acceptors (SO_4^{2-}, Fe^{3+}, and NO_3^-), with REs of 43.2-70.1% for MeP (1-10 $mg \cdot L^{-1}$) and 94.1-97.8% for PrP (1-10 $mg \cdot L^{-1}$) being achieved only after 72 h and 48 h, respectively, in batch assays with activated sludge. Probably, the recalcitrance of parabens under such conditions is related to their ester functional group (–COOR), as electron-withdrawing groups tends to hamper anaerobic biotransformation (Wijekoon et al., 2015). Therefore, in short-HRT anaerobic systems (< 10 h), such as the UASB reactors designed for domestic wastewater treatment, whose usual HRT ranges from 6 to 8 h (Chernicharo et al., 2015), the removal of parabens may be limited.

In fact, in anaerobic reactors operated at longer HRTs (> 10 h), higher REs of parabens were reached. For instance, Londoño and Peñuela (2015) found REs of MeP above 80% in an anaerobic expanded granular sludge bed (EGSB) reactor fed with synthetic wastewater containing different concentrations of this compound (300-1000 $\mu g \cdot L^{-1}$) and glucose (~1 g $COD \cdot L^{-1}$) when operated at a long HRT (26-27 h). Additionally, Hernández Leal et al. (2010) evaluated a UASB reactor operated at an HRT of 12 h for the treatment of gray water (830 mg $COD \cdot L^{-1}$) containing some xenobiotics, including PrP (2.9 $\mu g \cdot L^{-1}$) and BuP (0.9 $\mu g \cdot L^{-1}$), and found mean REs of approximately 75% and 67%, respectively.

Figure 4.2 – Influent (■) and effluent (□) concentrations and removal efficiencies (REs) (○) of methylparaben (MeP), ethylparaben (EtP), propylparaben (PrP), and butylparaben (BuP) throughout the experiment



4.3.2 Removal of parabens under microaerobic conditions

In period II, with the airflow rate of 1 mL·min^{-1} (0.027 L O_2·L^{-1} feed), the mean REs of all evaluated compounds were above 85% (Table 4.2). Therefore, it was evident that the addition of small amounts of oxygen to the reactor significantly favored the removal of parabens ($p < 0.001$). Interestingly, even at a very low airflow rate, there was no apparent limitation on the gas-liquid mass transfer, since a low oxygen content was found in the biogas (< 15% of the added amount) (data not shown), i.e., oxygen seemed to be effectively solubilized in the liquid and promptly used by microaerophilic or facultative microorganisms.

Although there are no reports on the effect of microaeration on the removal of parabens in anaerobic reactors, previous studies have shown that this technique can significantly improve the biotransformation of recalcitrant compounds, such as BTEX (Firmino et al., 2018; Siqueira et al., 2018) and OMPs (Buarque et al., 2019). For example, Siqueira et al. (2018) found that microaeration at a flow rate of 1 mL air·min^{-1} (0.14 L O_2·L^{-1} feed at 27 °C and 1 atm) increased the REs of BTEX, mainly for benzene (from 55% to 84%), in a UASB reactor (HRT of 24 h) fed with water contaminated with ethanol (1 g COD·L^{-1}) and these monoaromatics (4-5 mg·L^{-1} each). Similarly, Buarque et al. (2019) also observed an increase in the REs of seven OMPs (~230 µg·L^{-1} each) from less than 10% to more than 50% in a UASB reactor (HRT of 7 h) treating synthetic wastewater (~1 g COD·L^{-1}) after being microaerated at 1 mL air·min^{-1} (0.021 L O_2·L^{-1} feed at 27 °C and 1 atm).

Table 4.2 – Mean removal efficiencies of the parabens throughout the experiment

Period	**I**	**II**	**III**	**IV**	**V**	**VI**
Microaeration (mL·min)	**-**	**1**	**2**	**4**	**1**	**-**
MeP (%)	20.2 (8.1)	89.2 (2.8)	93.6 (1.4)	93.7 (1.1)	81.7 (4.8)	15.5 (4.6)
EtP (%)	14.0 (9.6)	89.0 (3.6)	91.8 (1.4)	92.5 (0.9)	79.6 (3.6)	12.4 (5.1)
PrP (%)	18.0 (7.3)	88.3 (3.1)	92.0 (1.9)	93.1 (0.7)	82.1 (2.7)	16.5 (5.5)
BuP (%)	20.0 (13.9)	85.5 (3.8)	88.5 (2.0)	92.2 (0.9)	80.3 (3.8)	16.1 (4.3)

BuP, buthylparaben; EtP, ethylparaben; MeP, methylparaben; PrP, propylparaben.
The standard deviation is shown in parentheses.

Under both aerobic and anaerobic conditions, the initial degradation of esters can take place through the hydrolysis of the ester bond by esterases or lipases, producing a carboxylic acid and an alcohol (Ghattas et al., 2017; Valkova et al., 2001). Specifically for parabens, the generated carboxylic acid is p-hydroxybenzoic acid (Wang et al., 2018), which can be further degraded through different pathways depending on the redox condition. Accordingly, under aerobic conditions, p-hydroxybenzoate (the conjugate base of the aforementioned acid) is converted into protocatechuate and then cleaved, both reactions catalyzed by oxygenases (β-ketoadipate pathway), whereas, under anaerobic conditions, p-hydroxybenzoate is converted into benzoyl-CoA, the central intermediate of anaerobic degradation of aromatic compounds (Fuchs et al., 2011).

According to the literature, the aerobic degradation of aromatic compounds is faster than the anaerobic one because oxygen is a much more favorable terminal electron acceptor (Ghattas et al., 2017; Weelink et al., 2010). However, under microaerobic conditions, oxygen is not used as the terminal electron acceptor. Actually, oxygenase-producing microorganisms only use oxygen for the hydroxylation of the aromatic ring, facilitating its cleavage and further anaerobic degradation, i.e., a hybrid pathway (Fuchs, 2008). Therefore, in the current investigation, the addition of small amounts of oxygen to the UASB reactor may have stimulated the activity of oxygenase enzymes, favoring the biotransformation of parabens, probably though hydroxylation.

In period III, the microaeration flow rate was increased to 2 mL air·min^{-1} (0.055 L O_2·L^{-1} feed), and the mean REs of parabens were higher than 90%, except for BuP (Table 4.2). Although the difference between the mean REs obtained in periods II and III was not so high (2.7-4.5% depending on the compound) (Table 4.2), it was still statistically significant for all parabens ($p < 0.050$). Moreover, the efficiency values in period III were more stable than in period II (Figure 4.2). Thus, the increase in the airflow rate from 1 to 2 mL·min^{-1} had indeed a positive effect on the biotransformation of parabens. In period IV, when the microaeration flow rate was 4 mL air·min^{-1} (0.110 L O_2·L^{-1} feed), the mean REs of all parabens were higher than 90% and slightly above the values observed in period III (Table 4.2). However, this increase was significant only for PrP ($p = 0.013$) and BuP ($p < 0.001$). Therefore, a saturation in the biotransformation capacity of parabens in the microaerobic system may have occurred. Since the mass transfer remained effective (residual oxygen in the biogas < 27% of the added amount), this saturation is likely related to biochemical limitations rather than a lack of oxygen in the medium. These results agree with those by Siqueira et al. (2018), who also observed a positive correlation between the microaeration flow rate and the REs of BTEX and a saturation in the removal capacity of their UASB reactor when the airflow rate was raised from 1 to 2 mL·min^{-1} (from 0.14 to 0.27 L O_2·L^{-1} feed).

In period V, when the airflow rate was reduced back to 1 mL·min^{-1} (0.027 L O_2·L^{-1} feed), as expected, the mean REs of all parabens decreased (11-13%). Surprisingly, the values were even lower than those obtained in period II ($p < 0.001$), when the same airflow rate was used (Table 4.2). Nevertheless, they remained close to 80%. Finally, in period VI, microaeration was interrupted to reestablish the anaerobic conditions. Consequently, the REs of parabens were similar to those obtained at the beginning of the experiment, when the reactor was also operated under anaerobic conditions (period I). Therefore, these results reinforce the importance of

oxygen availability to the biotransformation of parabens and exclude the hypothesis of microbiota adaptation to these compounds throughout the experiment.

Lastly, it is important to highlight that the high REs of parabens (> 90%) reached in the present work, with a more cost-effective system (microaerated UASB reactor), are comparable to those found in high-cost wastewater treatment systems, such as activated sludge and its variants (Ashfaq et al., 2017; Karthikraj et al., 2017; Ma et al., 2018; Wang and Kannan, 2016).

4.3.3 Operational stability of the system

During the entire experiment, the reactor remained remarkably stable, with high mean COD REs (85-90%), no accumulation of VFA, and pH values close to the neutral range (Table 4.3). Therefore, neither the parabens nor microaeration impaired organic matter removal. Regarding the methane content in the biogas, the higher the airflow rate, the lower this content (Table 4.3). However, this reduction in the methane content is only a consequence of biogas dilution with nitrogen from the synthetic air (80%) injected into the system, i.e., microaeration did not harm methanogenesis. However, a very low methane content, as observed in period IV (Table 4.3), may be a problem if the biogas is intended to be used in combined heat and power plants, which require a minimum limit of 40% (Haubrichs and Widmann, 2006). Therefore, for situations in which a greater availability of oxygen is necessary to ensure high REs of the target pollutants, the use of pure oxygen as the microaeration source is a way of overcoming the biogas dilution problem.

Table 4.3 – Parameters of operational stability of the reactor throughout the experiment

Period	**I**	**II**	**III**	**IV**	**V**	**VI**
Microaeration (mL·min)	-	1	2	4	1	-
COD removal (%)	87.7 (1.6)	89.9 (0.8)	87.7 (1.6)	90.1 (1.0)	89.4 (0.9)	86.6 (1.9)
Biogas production ($L \cdot d^{-1}$)	2.1 (0.1)	3.4 (0.3)	6.7 (0.2)	10.5 (0.2)	3.6 (0.1)	2.2 (0.2)
CH_4 in the biogas (%)	81 (2)	65 (2)	46 (6)	21 (5)	79 (2)	85 (2)
pH	7.2 (0.4)	7.1 (0.4)	7.1 (0.3)	7.0 (0.4)	7.2 (0.2)	7.4 (0.3)
VFA ($mg \cdot L^{-1}$)	260 (27)	186 (54)	233 (42)	214 (40)	247 (25)	237 (85)
VFA/TA	0.4 (0.1)	0.3 (0.1)	0.4 (0.1)	0.3 (0.1)	0.4 (0.1)	0.4 (0.1)

COD, chemical oxygen demand; TA, total alkalinity; VFA, volatile fatty acids.
The standard deviation is shown in parentheses.

4.3.4 Dynamics of the microbial community of the system

Apparently, in period I, the exposure to a different substrate (ethanol + parabens) increased the richness and diversity of the microbiota when compared to the inoculum (Table 4.4), anaerobic sludge from a full-scale UASB reactor that treats domestic wastewater. From period II to IV, when the reactor was continuously microaerated at increasing flow rates (from 1 to 4 mL air·min^{-1}), both ecological attributes continued to increase, reaching their maximum values at the end of the period IV (Table 4.4), when the highest airflow rate was applied to the system. Therefore, microaeration seemed to exert a significant selection pressure on the microbial community, favoring the growth of different species, probably those microaerophilic or facultative able to synthesize oxygenase enzymes, which may have played a role in the biotransformation of parabens. In agreement with the present results, Buarque et al. (2019) and Firmino et al. (2018) also observed an increase in the richness and diversity of the microbiota of their UASB reactors when operated under microaerobic conditions.

In period V, when the airflow rate was set back to 1 $mL \cdot min^{-1}$, the number of observed species (or OTUs, operational taxonomic units) decreased remarkably, reaching a value even lower than that observed in period I (anaerobic) (Table 4.4). Probably, the lower availability of oxygen may have impaired the survival of some microaerophilic microorganisms that grew throughout the previous microaerobic periods. Nevertheless, the inverse Simpson

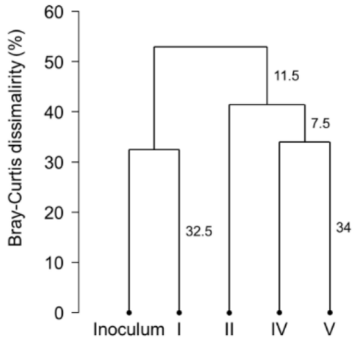
index remained high (Table 4.4), i.e., the microbial community maintained a high diversity/evenness (no dominance of specific groups). In fact, the sample of this period is more similar to that of period IV (4 mL air·min^{-1}) than that of period II (Figure 4.3), when the reactor was also microaerated at 1 mL air·min^{-1}.

Table 4.4 – Ecological indices of richness (Chao1) and diversity (inverse Simpson) for the inoculum and samples collected at the end of periods I (anaerobic), II (1 mL air·min^{-1}), IV (4 mL air·min^{-1}), and V (1 mL air·min^{-1})

Sample	OTUs[a]	Chao1	Inverse Simpson
Inoculum	1196	2042	7.68
I	1622	2695	8.67
II	1869	3235	9.85
IV	2188	4225	30.43
V	1419	2466	22.09

[a]Number of operational taxonomic units.

Figure 4.3 – UPGMA cluster analysis at genus level based on Bray-Curtis dissimilarity index for the inoculum and samples collected at the end of periods I (anaerobic), II (1 mL air·min^{-1}), IV (4 mL air·min^{-1}), and V (1 mL air·min^{-1})

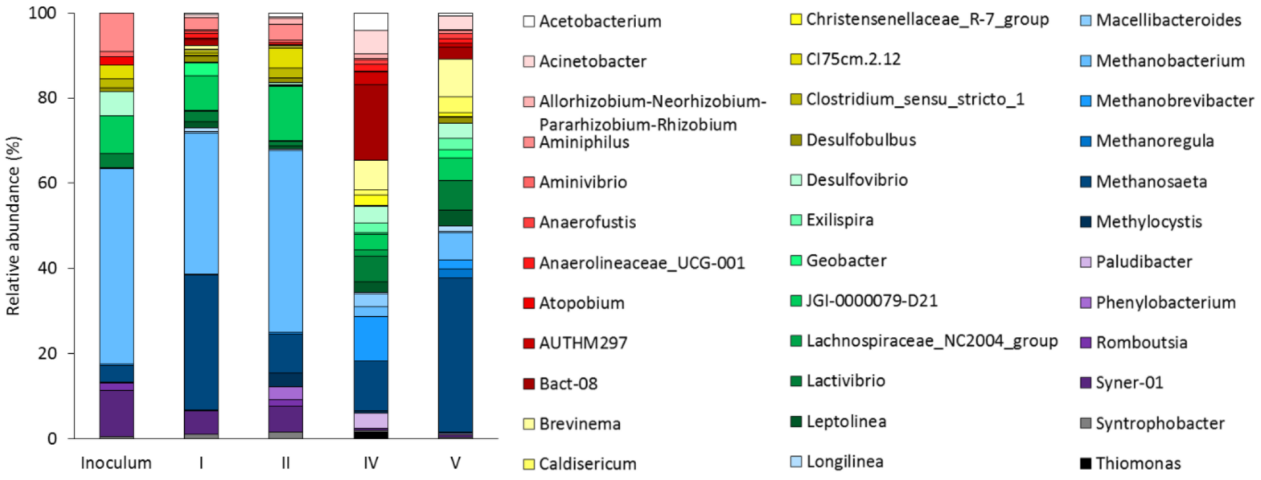


Concerning the archaeal community, its relative abundance remained above 45% except in period IV, when it decreased to approximately 25%. Although the granular structure of the anaerobic sludge tends to protect these strictly anaerobic microorganisms from oxygen in the granule core (Baloch et al., 2008; Picioreanu et al., 2005), an airflow rate as high as 4 mL·min^{-1} may have allowed a deeper diffusion of this gas into the sludge granule, inhibiting some less oxygen-resistant archaeal species. Nevertheless, even in this period, the methanogenic activity was not compromised (section 4.3.3). This is an important observation

because, in anaerobic consortia, the degradation of aromatic compounds is hardly performed by a single species, but most likely through syntrophic relationships between fermentative bacteria and methanogenic archaea (Gieg et al., 2014).

With respect to the bacterial community, the relative abundance of some genera seemed to increase under microaerobic conditions, namely *Acetobacterium*, *Acinetobacter*, *Brevinema*, *Caldisericum*, *Desulfovibrio*, *Exilispira*, *Lactivibrio*, *Leptolinea*, and *Longilinea* (Figure 4.4). However, just a few have been reported to be directly related to the degradation of aromatic compounds, such as *Acinetobacter* (Jung and Park, 2015) and *Longilinea* (Zhu et al., 2018), and nitrogen-based heterocyclic compounds, such as *Caldisericum* (Shi et al., 2019).

Figure 4.4 – Microbial diversity at genus level of the inoculum and samples collected at the end of periods I (anaerobic), II (1 mL air·min^{-1}), IV (4 mL air·min^{-1}), and V (1 mL air·min^{-1})



4.4 Conclusions

Low mean REs (14-20%) were achieved under anaerobic conditions, but the addition of only of 1 mL air·min^{-1} (0.027 L O_2·L^{-1} feed) remarkably boosted the biotransformation of parabens, ensuring mean REs above 85% for all compounds. In contrast, the increase in the airflow rate had a minor impact on the process, and an apparent saturation in the removal capacity was observed, noticeably from 2 to 4 mL air·min^{-1}.

The reactor presented high stability throughout the experiment, and microaeration did not impair the organic matter removal and methanogenesis. However, high airflow rates can dilute biogas, compromising its use as a fuel in combined heat and power units.

The microaerobic conditions increased both richness and diversity of the microbiota of the reactor, likely favoring the growth of oxygenase-producing microorganisms, which may have played a role in the biotransformation of parabens.

Finally, the high REs of parabens reached in the microaerated reactor, a more cost-effective technology, are comparable to those found in high-cost wastewater treatment systems, such as activated sludge and its variants.

5 OVERCOMING THE LIMITATION ON THE ANAEROBIC BIOTRANSFORMATION OF PARABENS: INSIGHTS INTO BOTH INDIVIDUAL AND COMBINED EFFECTS OF ANTHRAQUINONE-2,6-DISULFONATE AND MICROAERATION

5.1 Introduction

Parabens are esters of p-hydroxybenzoic acid with an alkyl (methyl, ethyl, propyl, butyl, or heptyl) or benzyl group. They are widely used in food, pharmaceuticals, and personal care products (PCPs) because they have excellent preservative properties, preventing the growth of Gram-positive bacteria, yeast, and mold (Haman et al., 2015; Ma et al., 2018). Methylparaben (MeP), ethylparaben (EtP), propylparaben (PrP), and butylparaben (BuP) are the most used and, therefore, the most commonly found parabens in aquatic environments (Gałwa-Widera, 2019). These organic micropollutants (OMPs) are reported to cause problems in the environment and human health because they have endocrine-disrupting properties (Giulivo et al., 2016; Li et al., 2015). Therefore, the removal of parabens from wastewaters (the main water pollution source) before its discharge into water bodies is important to prevent the occurrence of such endocrine-disrupting chemicals (EDCs) in the environment and their associated impacts.

Although parabens can be efficiently removed (> 80%) in activated sludge wastewater treatment plants (WWTPs) (Ashfaq et al., 2017; Karthikraj et al., 2017; Ma et al., 2018; Wang and Kannan, 2016), there is still a shortage of information on the removal of these OMPs in high-rate anaerobic reactors, such as upflow anaerobic sludge blanket (UASB) reactors and its variants (Hernández Leal et al., 2010; Londoño and Peñuela, 2015). Moreover, these systems are frequently used by several developing countries, such as Brazil, India, and others in Latin America, for being an efficient and more economical wastewater treatment technology (Chernicharo et al., 2015). However, similarly to other OMPs (Harb et al., 2019; Lakshminarasimman et al., 2018), parabens are slowly biotransformed under anaerobic conditions (Wu et al., 2017). Therefore, unless long hydraulic retention times (HRTs) (> 10 h) are used, anaerobic reactors may present low removal efficiencies of these compounds. Hence, as UASB reactors designed for domestic wastewater treatment are usually operated at short HRTs (6-8 h) (Chernicharo et al., 2015), some relatively simple and economical approaches should be proposed to ensure similar removal efficiencies to those obtained with the aforementioned high-cost technology (activated sludge and variants).

For instance, quinone-based compounds, such as anthraquinone-2-sulfonate (AQS) and anthraquinone-2,6-disulfonate (AQDS), and some vitamins, such as riboflavin (vitamin B2), has been reported to have redox-mediating properties and accelerate the anaerobic biotransformation of several recalcitrant compounds (e.g., azo dyes, nitroaromatics, polyhalogenated pollutants, pharmaceuticals, etc.) (dos Santos et al., 2007; He et al., 2017; Van der Zee and Cervantes, 2009; Zhou et al., 2018). In addition, the introduction of oxygen at low concentrations (up to 1 L $O_2 \cdot L^{-1}$ feed) into anaerobic systems, i.e., microaeration, has also been shown to boost the biotransformation of some of these pollutants, such as BTEX (benzene, toluene, ethylbenzene, and xylenes) (Firmino et al., 2018; Siqueira et al., 2018), hormones, pharmaceuticals, and bisphenol A (Buarque et al., 2019).

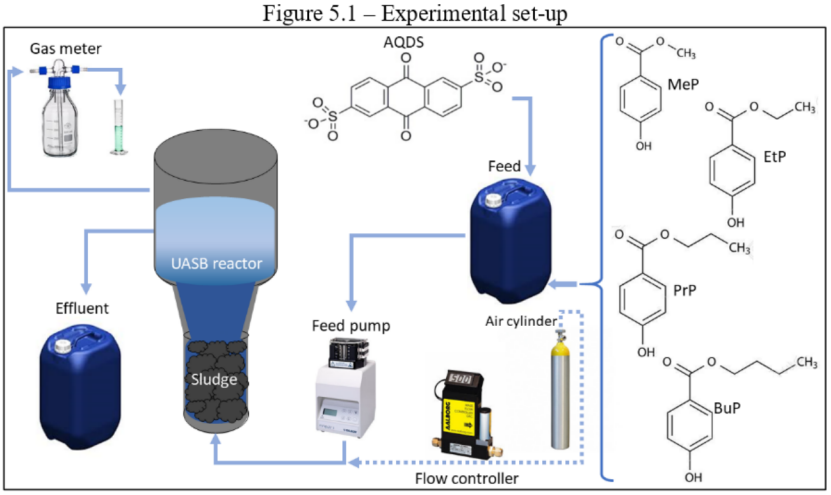
However, as far as the author is concerned, there is no investigation into the impact of redox mediators on the biotransformation of parabens under anaerobic or microaerobic conditions. Therefore, this work aimed to evaluate the individual and combined effects of the redox mediator AQDS (50 and 100 μM) and microaeration (1 mL air·min^{-1}) on the biotransformation of the parabens MeP, EtP, PrP, and BuP (200 μg·L^{-1} each) in a short-HRT (8 h) UASB reactor.

5.2 Material and methods

5.2.1 Experimental set-up

A lab-scale UASB reactor (working volume of 3.5 L), inoculated with anaerobic sludge (~50 g VSS·L^{-1}) from a mesophilic UASB reactor treating domestic wastewater (Fortaleza, Ceará, Brazil), was operated at an HRT of 8 h and room temperature of approximately 28 °C. It was fed with synthetic wastewater containing the parabens MeP, EtP, PrP, and BuP (~200 μg·L^{-1} each) (> 99%, Sigma-Aldrich, USA), ethanol (~1 g COD·L^{-1}) as a primary carbon source, nutrients (Firmino et al., 2010), and sodium bicarbonate (1 g·L^{-1}) as a buffer to keep the pH close to 7.0. In some experimental periods, the reactor was supplemented with the redox mediator AQDS (98%, Sigma-Aldrich, USA) and/or microaerated with synthetic air (80% N_2:20% O_2, White Martins, Brazil) at the feeding line through a needle by a mass flow controller (GFC17, Aalborg, USA). The biogas produced was measured by a Mariotte flask containing a 3% sodium chloride solution at pH 2 (Figure 5.1).

Figure 5.1 – Experimental set-up



5.2.2 Experimental procedure

The individual and combined effects of the redox mediator AQDS and microaeration on the anaerobic removal of parabens were assessed throughout an eight-period experiment (Table 5.1). In period I, the reactor was fed only with the paraben-containing wastewater and operated under anaerobic conditions. Then, in periods II and III, it was supplemented with AQDS at 50 and 100 µM, respectively. Subsequently, in periods IV and V, the reactor remained supplemented with the redox mediator (100 and 50 µM, respectively), but it was also microaerated at a flow rate of 1 mL air·min^{-1} at 28 °C and 1 atm (equivalent to 0.027 L O_2·L^{-1} feed) (microaerobic conditions). Afterwards, in period VI, AQDS supplementation was interrupted, whereas microaeration was maintained, and, in period VII, to evidence the effect of the operational changes throughout the experiment and exclude the hypothesis of microbiota adaptation to the antibiotics over time, the anaerobic conditions were reestablished. Finally, in period VIII, the possible formation of MeP as an intermediate of the biotransformation of the other parabens (EtP, PrP, and BuP) was assessed. For this, the reactor, operated under anaerobic conditions, was fed with paraben-free wastewater for 15 days to ensure the removal of any residual of parabens from it (absence in the effluent). Then, it was supplemented only with EtP, PrP, and BuP (200 µg·L^{-1} each) and AQDS (100 µM). The experimental periods were changed after verifying system stability.

Table 5.1 – Operational parameters of the reactor throughout the experiment

Period	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
End of period (day)	37	57	79	104	127	149	156	181
HRT (h)	8	8	8	8	8	8	8	8
COD ($g \cdot L^{-1}$)	1.0	1.0	1.0	1.1	1.0	1.0	1.1	1.0
MeP ($\mu g \cdot L^{-1}$)	199	198	214	210	207	209	207	-
EtP ($\mu g \cdot L^{-1}$)	196	208	215	213	206	205	197	207
PrP ($\mu g \cdot L^{-1}$)	197	206	193	203	206	207	206	206
BuP ($\mu g \cdot L^{-1}$)	204	206	222	205	203	210	202	208
AQDS (μM)	-	50	100	100	50	-	-	100
Microaeration[a] ($mL \cdot min^{-1}$)	-	-	-	1	1	1	-	

BuP, buthylparaben; COD, chemical oxygen demand; EtP, ethylparaben; HRT, hydraulic retention time; MeP, methylparaben; PrP, propylparaben.
[a]1 $mL \cdot min^{-1}$ is equivalent to a dose of oxygen of 0.027 L $O_2 \cdot L^{-1}$ feed.

5.2.3 Chemical analysis

The parabens were determined by an LC-20A Prominence high-performance liquid chromatograph (HPLC) equipped with a Shim-pack CLC-ODS(M)® C18 column (4.6×150 mm, 5 µm) and a UV-Vis SPD-20A detector (215 nm) (Shimadzu Corporation, Japan). Firstly, 500 mL of pre-filtered samples (0.45 µm) were percolated through Oasis HLB cartridges (3 cc, 60 mg, 30 µm) (Waters Corporation, USA) for solid-phase extraction of parabens, which were then eluted by 4 mL of HPLC/UV grade methanol (99.8%, Neon, Brazil) (Pessoa et al., 2014). Afterwards, 10 µL of this methanolic solution was injected in the HPLC, based on Vidal et al. (2020), and eluted by a mobile phase composed of ultrapure water and HPLC/UV grade acetonitrile (99.9%, Sigma-Aldrich, Germany) at a constant flow of 1.2 $mL \cdot min^{-1}$, using the following gradient: increase from 10% to 60% of acetonitrile in 8 min, followed by 10% reduction in 4 min. The temperature of the oven was maintained at 35 °C throughout the run.

COD, alkalinity, and pH were determined according to APHA (2012). The volatile fatty acids (VFA) were determined by the Kapp titrimetric method (Buchauer, 1998). The levels of CH_4 and CO_2 in the biogas were determined by gas chromatography with barrier-discharge ionization detection (GC-BID) (GC-2010 Plus, Shimadzu Corporation, Japan). The biogas sample (1.0 mL) was injected in split mode (split ratio of 30), and the chromatographic separation was performed on a GS-GasPro column (60 m, 0.32 mm I.D.) (Agilent Technologies, USA). The temperatures of the injector and the detector were 100 and 250 °C, respectively. The temperature of the oven started at 50 °C, was raised to 75 °C at 5 $°C \cdot min^{-1}$, then to 105 °C at 8 $°C \cdot min^{-1}$, and was finally maintained at 105 °C for 0.25 min (total run time of 9 min). Helium

(White Martins, Brazil) was used as the carrier gas at a flow rate of 2.0 $mL \cdot min^{-1}$. The levels of O_2 and N_2 in the biogas were determined by gas chromatography with thermal conductivity detection (GC-TCD) (GC-17A, Shimadzu Corporation, Japan). The biogas sample (1.0 mL) was injected in splitless mode, and the chromatographic separation was performed on a Mol Sieve 5A PLOT column (30 m, 0.32 mm I.D.) (Restek Corporation, USA). The temperatures of the injector, oven, and detector were 40, 35, and 230 °C, respectively. Helium (White Martins, Brazil) was used as the carrier gas at a flow of 7 $mL \cdot min^{-1}$, and the run time was 5 min.

5.2.4 Statistical analysis

The Mann-Whitney and Kruskal-Wallis non-parametric tests, which do not require a specific data distribution, were used to compare the performance of the reactor during the different experimental periods at a 5% significance level.

5.3 Results and discussion

5.3.1 Effect of AQDS on the anaerobic biotransformation of parabens

In period I, when the reactor was operated under anaerobic conditions without AQDS addition, the mean REs of all parabens were lower than 17%, with no statistical difference among them ($p = 0.086$) (Table 5.2). In contrast, Londoño and Peñuela (2015) observed REs of MeP (300-1000 $\mu g \cdot L^{-1}$) higher than 80% in an anaerobic expanded granular sludge bed (EGSB) reactor (HRT of 26-27 h) fed with synthetic wastewater containing glucose (~1 g $COD \cdot L^{-1}$) as a primary carbon source. Probably, these higher REs were obtained due to the long HRT used in that investigation, as the anaerobic biotransformation of parabens is reported to be very slow, thus requiring a long reaction time (Wu et al., 2017).

Quinone-based compounds (e.g., AQS and AQDS) have been widely reported to enhance the reductive biotransformation of several pollutants (e.g., azo dyes, nitroaromatics, and halogenated compounds) under anaerobic conditions, i.e., they act as redox mediators, accelerating the electron transfer from an electron donor, such as an organic substrate (e.g., sugars, alcohols, fatty acids), to the pollutant, which is the terminal electron acceptor (dos Santos et al., 2007; Van der Zee and Cervantes, 2009). Additionally, they can also facilitate the oxidation of some recalcitrant compounds (e.g., benzene, toluene, and phenols), acting as

electron acceptors (Cervantes et al., 2000; 2001; 2008; 2011). However, they can be subsequently regenerated to their oxidized form by donating the accepted electrons to different terminal electron acceptors, such as nitrate, fumarate, arsenate, selenite, perchlorate, and metal oxides (Cervantes et al., 2001; Van der Zee and Cervantes, 2009; Lovley et al., 1999), i.e., also acting as redox mediators. Even under methanogenic conditions, quinones can play a redox-mediating role because they accelerate the interspecies electron transfer (IET) between syntrophic partners in a process defined as quinone-mediated IET (QUIET) (Smith et al., 2015). Thus, using AQDS may boost the biotransformation of parabens in anaerobic reactors operated at short HRTs (< 10 h).

Table 5.2 – Mean removal efficiencies of the parabens throughout the experiment

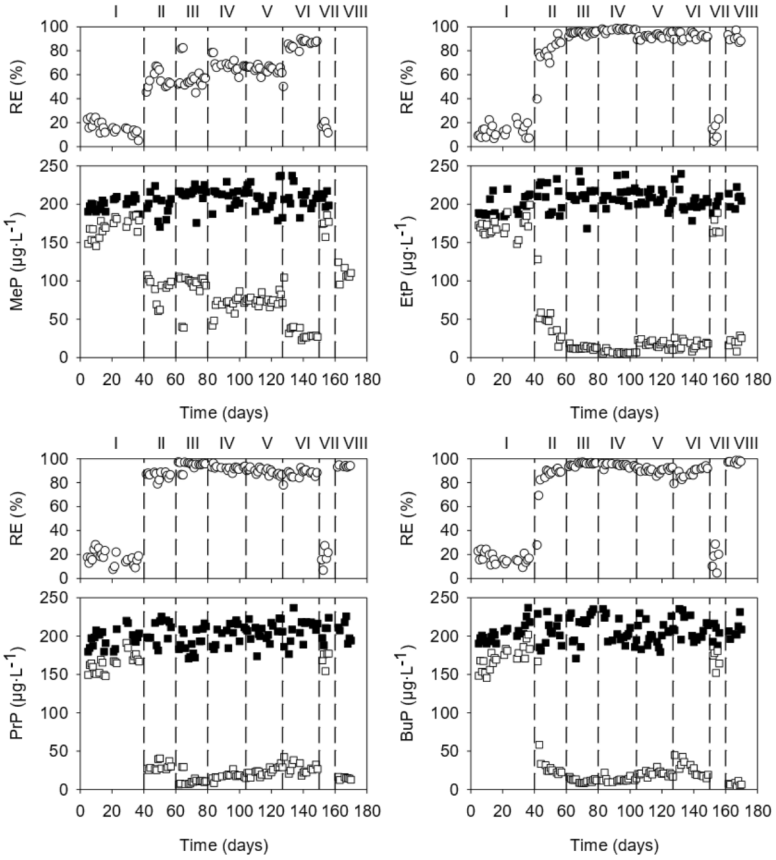
Period	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
AQDS (μM)	**-**	**50**	**100**	**100**	**50**	**-**	**-**	**100**
Microaeration (mL·min)	**-**	**-**	**-**	**1**	**1**	**1**	**-**	**-**
MeP (%)	15.0 (5.1)	55.2 (7.1)	53.6 (3.9)	67.6 (5.2)	63.8 (2.6)	85.2 (3.0)	15.9 (3.5)	-
EtP (%)	12.7 (5.1)	80.3 (7.1)	94.0 (1.2)	96.8 (1.1)	91.6 (2.0)	91.6 (2.6)	12.8 (7.4)	90.2 (3.4)
PrP (%)	16.5 (5.5)	85.3 (3.0)	93.9 (3.3)	91.6 (1.8)	88.5 (2.4)	86.5 (3.6)	16.8 (7.6)	93.0 (0.7)
BuP (%)	16.1 (4.3)	85.8 (6.3)	94.9 (1.3)	93.7 (1.4)	89.7 (2.0)	87.4 (4.3)	15.7 (9.3)	96.6 (1.1)

BuP, buthylparaben; EtP, ethylparaben; MeP, methylparaben; PrP, propylparaben.
The standard deviation is shown in parentheses.

Definitely, in period II, the addition of 50 μM of AQDS caused an immediate increase in the REs of all parabens ($p < 0.001$) (Figure 5.2), in which the highest mean values were obtained for PrP and BuP (~85%). However, the mean RE of MeP (~55%) was far lower than those of the other compounds ($p < 0.001$) (Table 5.2). In period III, with the increase in the concentration of AQDS to 100 μM, the mean REs of EtP, PrP, and BuP, although to a lesser extent, increased again ($p < 0.001$). On the other hand, even with a higher concentration of AQDS, the biotransformation of MeP did not improve, and its mean RE remained similar to that of period II ($p = 0.898$) (Table 5.2). Therefore, AQDS had a more significant effect on the parabens with longer alkyl chains (EtP, PrP, and BuP) than on MeP. In fact, the literature reports that the biotransformation rate of MeP is much slower than the other parabens, noticeably under

anaerobic conditions (Wu et al., 2017; Lu et al., 2018). Thus, even in the presence of a redox mediator, this limitation could not be completely overcome.

Figure 5.2 – Influent (■) and effluent (□) concentrations and removal efficiencies (REs) (○) of methylparaben (MeP), ethylparaben (EtP), propylparaben (PrP), and butylparaben (BuP) throughout the experiment



Concerning the biotransformation pathways of parabens under anaerobic conditions, although no specific studies on it were found, literature reports that the anaerobic degradation of esters may initiate through the cleavage of the ester bond by hydrolytic enzymes (e.g., esterases or lipases), yielding an alcohol and a carboxylic acid (Ghattas et al., 2017), which, in the case of parabens, it is p-hydroxybenzoic acid (Wang et al., 2018). Then, p-hydroxybenzoate (the conjugate base of p-hydroxybenzoic acid) is converted into benzoyl-

CoA, the central intermediate of anaerobic degradation of aromatic compounds (Fuchs et al., 2011). Therefore, it is not clear in which specific steps of the biotransformation pathway the redox mediator may have participated and why it had a lower effect on MeP. Hence, further investigation is necessary to understand the biochemistry of this process.

5.3.2 Effect of AQDS and microaeration on the anaerobic biotransformation of parabens

In period IV, the reactor remained supplemented with 100 μM of AQDS and was microaerated at a flow rate of 1 mL air·min^{-1} (0.027 L O_2·L^{-1} feed). There was a significant increase in the mean REs of EtP and, particularly, MeP (14% increase) ($p < 0.001$). In contrast, the mean REs of PrP and BuP decreased slightly ($p < 0.050$) when compared to period III, although they remained very high (> 90%) (Table 5.2). In period V, reducing the concentration of AQDS to 50 μM and maintaining the same microaeration flow rate as in the previous period, there was a small drop in the REs of all parabens ($p < 0.050$) (Figure 5.1). Therefore, the decrease in the concentration of AQDS had a negative impact on the biotransformation of parabens. In addition, comparing the periods II and V, the positive effect of microaeration on the removal of all parabens was evident with a lower concentration of AQDS (50 μM) ($p < 0.050$), especially for MeP and EtP (Table 5.2).

The positive effect of microaeration on the biotransformation of parabens is likely associated with the activity of oxygenase enzymes, which catalyze hydroxylation reactions, facilitating the subsequent anaerobic degradation of these compounds as suggested by previous studies on the biotransformation of BTEX (Firmino et al., 2018; Siqueira et al., 2018) and OMPs (Buarque et al., 2019) in microaerated UASB reactors. In fact, under aerobic conditions, after the hydrolysis of parabens (Valkova et al., 2001), oxygenase-producing microorganisms convert p-hydroxybenzoate into protocatechuate and then cleave the aromatic ring, which is further degraded through the β-ketoadipate pathway (Fuchs et al., 2011). However, it is worth mentioning that, under microaerobic conditions, there is not sufficient oxygen to be used as the terminal electron acceptor as in a typical aerobic pathway, i.e., oxygen is only used by oxygenases to cleave the aromatic compounds, which are further degraded anaerobically (Fuchs, 2008).

5.3.3 Effect of microaeration on the anaerobic biotransformation of parabens

In period VI, the reactor remained operated under microaerobic conditions (1 mL air·min^{-1}), but without AQDS supplementation. Compared to period V, there was no significant difference in the mean REs of EtP ($p = 0.984$), PrP ($p = 0.092$) and BuP ($p = 0.147$) (Table 5.2). However, surprisingly, the mean RE of MeP increased expressively from 64% to 85% ($p < 0.001$). Therefore, under microaerobic conditions, AQDS had a limited impact on the biotransformation of the longer-alkyl chain parabens and even compromised the effect of oxygen on the biotransformation of MeP. A possible reason for this behavior is that the reduced redox mediator (anthrahydroquinone-2,6-disulfonate, AH_2QDS) might have transferred electrons to a large fraction of the dosed oxygen, which has a high oxidation potential (dos Santos et al., 2007), converting it into H_2O and thus preventing its effective use in the hydroxylation reactions catalyzed by the oxygenases, i.e., AQDS exerted an antagonistic effect on microaeration.

In period VII, the system was operated again under anaerobic conditions as in period I. Consequently, the REs of parabens decreased immediately (Figure 5.2), reaching mean values similar to those of that period ($p > 0.050$) (Table 5.2). Thus, the hypothesis of microbiota adaptation to parabens over time can be eliminated, i.e., the different responses of the system were related to the operational conditions imposed on it throughout the experiment. These results also emphasize the importance of microaeration to the biotransformation of parabens, which ensured high mean REs (> 85%) in period VI (Table 5.2). Similarly, Siqueira et al. (2018) also observed a remarkable increase in the REs of BTEX (4-5 mg·L^{-1} each), especially for benzene (from 55% to 84%), when they microaerated a UASB reactor (HRT of 24 h) at a flow rate of 1 mL air·min^{-1} (0.14 L O_2·L^{-1} feed at 27 °C and 1 atm).

5.3.4 Evaluation of MeP as a likely intermediate of the anaerobic biotransformation of parabens in the presence of AQDS

As the REs of MeP did not increase as much as those of the other parabens in the presence of AQDS (periods II and III) even at a higher concentration (100 μM), the hypothesis that this compound may be an intermediate of the anaerobic biotransformation of the longer-alkyl chain parabens was suggested. In fact, MeP has been reported as an intermediate of the biotransformation of these other parabens under aerobic conditions (activated sludge), but only in the presence of methanol due to a transesterification reaction (Lu et al., 2018; Wang et al.,

2018). However, in the current investigation, ethanol was used as the primary substrate. Thus, in this case, the expected product of the transesterification is EtP (Wang et al., 2018). Nonetheless, since no accumulation of EtP was evidenced, the hypothesis of MeP as a likely intermediate of the anaerobic biotransformation of parabens was evaluated in period VIII, when the reactor was fed with the synthetic wastewater supplemented only with EtP, PrP, and BuP (200 $\mu g \cdot L^{-1}$ each) and AQDS (100 µM). As a result, surprisingly, MeP was detected in the effluent at a mean concentration of 109 $\mu g \cdot L^{-1}$, which was even higher than the concentrations of periods II (89 $\mu g \cdot L^{-1}$, $p = 0.005$) and III (91 $\mu g \cdot L^{-1}$, $p = 0.004$) (Figure 5.2). Therefore, the low REs found for MeP in periods II (50 µM of AQDS) and III (100 µM of AQDS) might have been a consequence of a transient accumulation due to the biotransformation of the longer-alkyl chain parabens. However, since the formation of MeP in the absence of methanol is an unusual reaction (never reported in the literature to the best of the author's knowledge), further investigations into the anaerobic biotransformation pathway of parabens should be carried out to confirm this hypothesis.

5.3.5 Operational stability

The reactor presented high operational stability in all experimental periods, with mean COD REs ranging from 89% to 92%, no accumulation of VFA, and pH close to 7 (Table 5.3). Therefore, the application of either AQDS or microaeration did not affect positively or negatively the anaerobic digestion process. Regarding the methane content in the biogas, despite the decrease in the microaerobic periods (IV, V, and VI) (Table 5.3), it was only a consequence of biogas dilution by air (oxygen source), whose N_2 content is very high (80%). Therefore, there was no apparent inhibition of methanogenesis by the small amount of oxygen added to the reactor.

Table 5.3 – Parameters of operational stability of the reactor throughout the experiment

Period	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
AQDS (μM)		**50**	**100**	**100**	**50**	**-**	**-**	**100**
Microaeration (mL·min)	**-**	**-**	**-**	**1**	**1**	**1**	**-**	**-**
COD removal (%)	89.5 (1.9)	90.2 (1.9)	91.2 (1)	91.5 (1.2)	89.3 (1.3)	89.3 (1.4)	89.8 (1.4)	89.5 (1.9)
Biogas production ($L \cdot d^{-1}$)	2.1 (0.2)	2.3 (0.2)	2.3 (0.8)	3.5 (0.2)	3.4 (0.2)	3.2 (0.1)	1.7 (0.2)	2.1 (0.2)
CH_4 in the biogas (%)	79 (3)	80 (3)	80 (4)	61 (8)	58 (11)	62 (7)	77 (2)	79 (3)
pH	6.7 (0.2)	7.5 (0.2)	7.1 (0.3)	7.2 (0.2)	7.0 (0.3)	7.2 (0.2)	7.3 (0.1)	6.7 (0.2)
VFA ($mg \cdot L^{-1}$)	294 (55)	254 (88)	252 (45)	223 (68)	414 (45)	386 (71)	288 (48)	294 (55)
VFA/TA	0.6 (0.1)	0.6 (0.2)	0.6 (0.1)	0.6 (0.2)	0.6 (0.2)	0.6 (0.3)	0.4 (0.1)	0.6 (0.1)

COD, chemical oxygen demand; TA, total alkalinity; VFA, volatile fatty acids.
The standard deviation is shown in parentheses.

5.4 Conclusions

AQDS improved expressively the low REs of all parabens (< 17%), but it had a more significant effect on the compounds with longer alkyl chains (EtP, PrP, and BuP) (REs > 90% with 100 μM of AQDS) than on MeP (RE of ~54% with 50 or 100 μM of AQDS).

Under microaerobic conditions, AQDS had a limited impact on the biotransformation of the longer-alkyl chain parabens and even compromised the effect of oxygen on the biotransformation of MeP, i.e., AQDS exerted an antagonistic effect on microaeration.

In the absence of AQDS, microaeration ensured high REs for all compounds (> 85%), similar to those found in high-cost treatment systems (e.g., activated sludge). Therefore, it seems to be the best approach for boosting the removal of parabens in short-HRT high-rate anaerobic reactors.

Finally, MeP might be a likely intermediate of the anaerobic biotransformation of the longer-alkyl chain parabens in the presence of AQDS, which could justify the lower REs found for MeP under such conditions.

6 FINAL CONSIDERATIONS

Comparing the three approaches (addition of AQDS, microaeration, and nitrate), when assessed individually, the addition of nitrate ensured REs of OMPs above 85% only when the highest nitrate concentration (400 mg·L^{-1}) was used. Consequently, it may compromise the economic sustainability of the process due to the required continuous dosage of chemicals (nitrate salts). On the other hand, this approach may be a viable option for existing sequential anaerobic-aerobic treatment systems, as the nitrified effluent from the aerobic treatment unit can be used as a nitrate source in the anaerobic reactor, overcoming such economic limitation. However, it is worth mentioning that the content of inorganic nitrogen (ammonium) in raw domestic wastewater typically ranges from 20 to 75 mg N·L^{-1}, with mean values near 45 mg N·L^{-1} for moderately concentrated wastewater (Chen et al., 2020). Therefore, domestic wastewater hardly ever will have sufficient influent ammonium to achieve the highest nitrate concentration tested in the present work (400 mg NO_3^-·L^{-1} equals to 90.3 mg N·L^{-1}). Accordingly, if the amount of recirculated nitrate is not sufficient to provide such high REs, this approach may be associated with the other two approaches (redox mediator and/or microaeration) evaluated in the present study. Furthermore, despite this apparent limitation for domestic wastewater, the results demonstrate the potential use of this approach for other OMP-containing wastewaters that present higher nitrogen concentrations, such as livestock wastewaters (Hu et al., 2020).

For scenarios in which anaerobic reactors are used as the sole secondary treatment unit in the WWTPs, the addition of AQDS seems to be very promising, since high REs of OMPs (> 75%) could be achieved with 100 μM of this redox mediator. However, similarly to the addition of nitrate, this approach also requires continuous dosage of chemicals. Although the demand for AQDS is much lower than that for nitrate, it may still cause a considerable increase in operational costs. Therefore, the use of immobilized quinone-based redox mediators on supporting materials (e.g., ferric oxide, activated carbon, biochar, etc.) in anaerobic reactors is recommended. However, for systems that are already in operation, it may be a drawback, as significant interventions may be required.

Regarding microaeration, it is the simplest approach to be implemented in already functioning anaerobic reactors, since the small amounts of air can be directly injected into the feeding line. However, higher airflow rates (4-6 mL·min^{-1}) may be necessary to achieve higher REs of OMPs (> 90%), which can decrease further the methane content in the biogas due to the dilution with N_2 of the air added to the system. Thus, it is a disadvantage of this approach when

the biogas is intended to be used as an energy source. An alternative to minimize this issue is the use of pure oxygen instead of air. However, its economic viability should be evaluated because the purchase of pure oxygen may incur higher costs. Another option to increase the REs of is the association of microaeration with the addition of AQDS. However, this combined approach should be carefully assessed, as a small increase in the REs of some OMPs may not justify the additional costs involved.

Finally, all three approaches ensured REs of OMPs in the UASB reactors comparable to those found in higher-cost wastewater treatment technologies, such as conventional activated sludge, membrane bioreactors, and hybrid processes (Grandclément et al., 2017).

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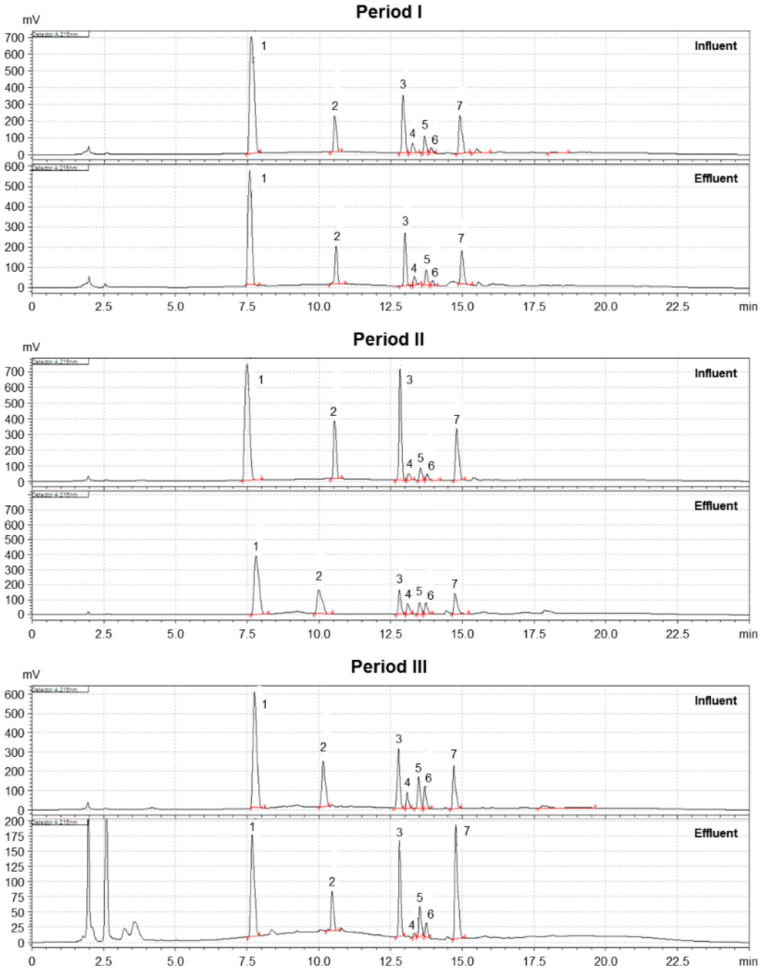
APPENDIX

Figure A.1 – Typical HLPC chromatograms in periods I to VII. Peaks: (1) trimethoprim, (2) sulfamethoxazole, (3) bisphenol A, (4) 17β-estradiol, (5) estrone, (6) 17α-ethinylestradiol, (7) diclofenac



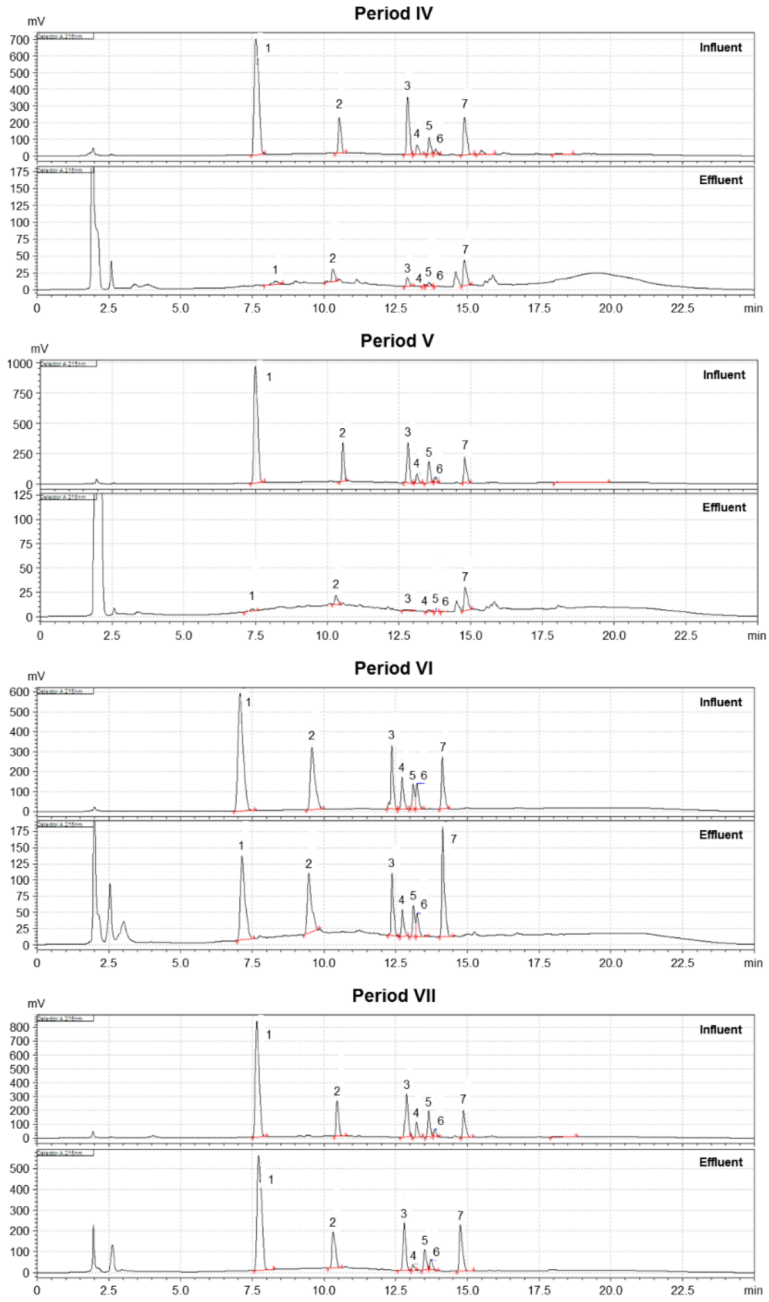

Period IV
mV
Influent
Effluent
1
2
3
4
5
6
7
min
Period V
mV
Influent
Effluent
Period VI
mV
Influent
Effluent
Period VII
mV
Influent
Effluent

Figure A.2 – Experimental set-up used in the experiment of chapter 2

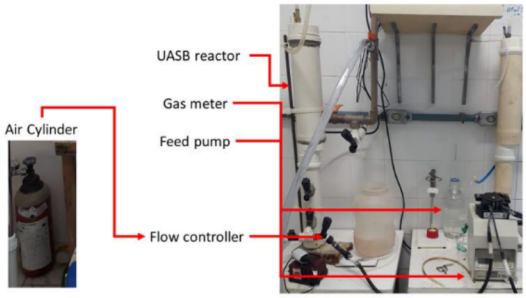


Figure A.3 – Experimental set-up used in the experiment of chapter 3

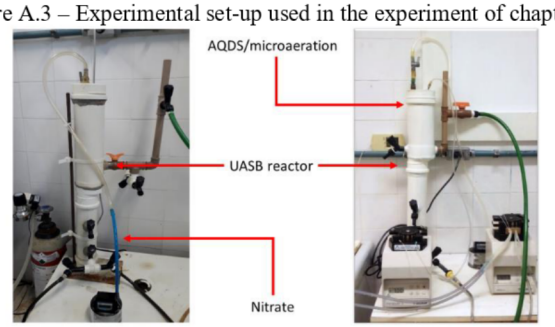


Figure A.4 – Experimental set-up used in the experiments of chapters 4 and 5

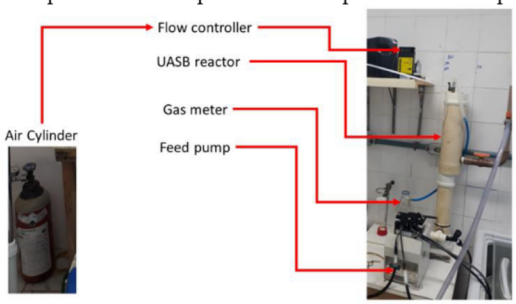


Figure A.5 – Detail of the microaeration application point

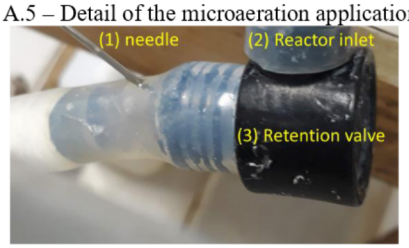


Table A.1 – Recovery efficiency (%) of organic micropollutants (OMPs)

Compound	Recovery %
E1	105,0
E2	76,0
EE2	74,0
BPA	107,5
DCF	104,1
SMX	70,4
TMP	96,1

Table A.2 – Recovery efficiency (%) of the parabens

Compound	Recovery %
MeP	90,2
EtP	89,8
PrP	94,0
BuP	94,7