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Redox mediator, microaeration, and nitrate addition as engineering approaches to enhance the biotransformation of antibiotics in anaerobic reactors

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ABSTRACT

The present work assessed some engineering approaches, such as the addition of the redox mediator anthraquinone-2,6-disulfonate (AQDS) (50 and 100 μM), microaeration (1 mL air min^{-1}), and nitrate (100−400 mg L^{-1}), for enhancing the biotransformation of the antibiotics sulfamethoxazole (SMX) and trimethoprim (TMP) (200 μg L^{-1} each) in anaerobic reactors operated at a short hydraulic retention time (7.4 h). Initially, very low removal efficiencies (REs) of SMX and TMP were obtained under anaerobic conditions (~6%). After adding AQDS, the anaerobic biotransformation of these antibiotics significantly improved, with an increase of approximately 70% in the REs with 100 μM of AQDS. Microaeration also enhanced the biotransformation of SMX and TMP, especially when associated with AQDS, which provided REs above 70%, particularly for TMP (~91% with 1 mL air min^{-1} and 50 μM of AQDS). Concerning nitrate, the higher the added concentration, the higher the REs of the antibiotics (~86% with 400 mg L^{-1}). Therefore, all the assessed approaches were demonstrated to be very effective in improving the limited biotransformation of SMX and TMP in anaerobic reactors, ensuring REs comparable to those found in higher-cost wastewater treatment technologies, such as conventional activated sludge, membrane bioreactors, and hybrid processes.

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). Although increasing the HRT may improve the REs of some OMPs in such anaerobic systems (once the compound-biomass contact time increases), this is not a feasible option for domestic wastewater, especially in developing countries with low sanitation coverage, since larger treatment facilities are required, thus increasing capital expenditures (CAPEX) (Chernicharo et al., 2015; Harb et al., 2019).

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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 approaches, which may also be more cost-effective, 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). As humic substances, the most abundant organic fraction in the environment, are rich in quinone moieties, they can be used as a natural source of quinone-based redox mediators (Van der Zee and Cervantes, 2009). Nonetheless, the continuous addition of such substances to the influent may still represent a cost for the WWTPs. Therefore, the use of immobilized quinone-based redox mediators on different supporting materials (e.g., ferric oxide, activated carbon, biochar, etc.) in anaerobic reactors has been presented as a viable alternative for overcoming such economic limitation (Zhang et al., 2020; Cruz-Zavala et al., 2016).

Recent research has also demonstrated that microaeration (addition of less than 1 L O_2 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 reduction potential to that of oxygen (dos Santos et al., 2007), as several studies have reported that, under nitrate-reducing (or anoxic) conditions, OMPs are more effectively biotransformed than under anaerobic conditions (Alvarino et al., 2016; Inyang et al., 2016; Lakshminarasimman et al., 2018; Ogunlaja and Parker, 2018; Zhao et al., 2018). However, similarly to the redox mediators, the continuous dosage of nitrate to the WWTPs incur higher operational expenditures (OPEX), which may compromise the sustainability of the process.

Conversely, anaerobic reactors are usually followed by aerobic post-treatment systems (e.g., trickling filters, submerged aerated biofilters, activated sludge systems, etc.) for oxidation of ammonium into nitrate (nitrification) (Bressani-Ribeiro et al., 2018; Kassab et al., 2010). This sequential anaerobic-aerobic configuration has been commonly used in full-scale WWTPs, particularly in developing countries, as a cost-effective alternative to using exclusively the activated sludge system and variants as a wastewater treatment technology, in order to expand their (usually low) sanitation coverage, since it allows significant savings in CAPEX (20–50%) and OPEX (40–50%) (Bressani-Ribeiro et al., 2018; Chernicharo, 2006; Kassab et al., 2010). Consequently, in such anaerobic-aerobic WWTPs, if the nitrified effluent from the aerobic unit is recirculated back to the anaerobic reactors, it can be used as a nitrate source for the biotransformation of OMPs, making the process sustainable. Besides, nitrogen can also be eventually removed from the system, as nitrate can be reduced to nitrogen gas (denitrification).

However, to the best of the authors' knowledge, 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. Additionally, although previous studies assessed the effect of nitrate on the biotransformation of these compounds (mostly in batch assays), no investigation into the impact of different COD/NO_3^- ratios on the biotransformation of OMPs in continuous-flow anaerobic reactors could be found in the literature. 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).

2. Material and methods

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) at approximately 200 μg L^{-1} each (according to the concentrations found in some local domestic wastewater) (Vidal et al., 2020), 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, 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 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.

2.2. Experimental procedure

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 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).

Table 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.

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.

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 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 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 L^{-1}, and, in period VI, the anaerobic conditions were reestablished.

2.3. Chemical analysis

For the quantification of SMX and TMP (at least five times a week), 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). 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 with the following gradient: increase from 10% to 80% 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, pH, volatile fatty acids (VFA), nitrate, and nitrite were analyzed three times a week. COD, alkalinity, and pH were determined according to (APHA, 2012). The 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 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 three times a week 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.

Table 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 L^{-1})	1.0	1.0	1.0	1.0	1.1	1.1
SMX (μg L^{-1})	205	194	194	213	204	209
TMP (μg L^{-1})	206	189	202	215	205	209
NO_3^- (g 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.

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. Results and discussion

3.1. Effect of the redox mediator and microaeration on the anaerobic biotransformation of antibiotics

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, with mean values close to only 6% (Table 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 μg L^{-1} each), including SMX and TMP, in the presence of ethanol (1 g COD L^{-1}) as a primary substrate.

Concerning the removal mechanisms, although adsorption on sludge may play a relevant role in the removal of OMPs in aerobic systems, this mechanism is reported to be negligible in anaerobic systems. As their solid retention times are usually long (> 70 d), the sludge blanket tends to saturate very quickly (up to 1 week depending on the pollutant

Table 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.

concentration) (Harb et al., 2019). Therefore, biotransformation is the main removal mechanism of OMPs in such systems. However, 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 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 NO— bond in the isoxazole ring of SMX (Fig. 1a) (Alvarino et al., 2016; Jia et al., 2017; Mohatt et al., 2011) and cleavage of the OC— bond in the methoxy functional group (*O*-demethylation) mainly at C-4 position of TMP (Fig. 1b) (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, in the current study, with the addition of 50 and 100 μM of AQDS in periods II and III, respectively, there were subsequent significant increases in the mean REs of these antibiotics from approximately 6% (period I) to values close to 50% (period II) and 75% (period III) ($p < 0.001$) (Table 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 μg 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 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 (Fig. 1). However, it is worth mentioning that the biotransformation products were not analyzed in the present study. Therefore, further investigation is necessary to confirm the suggested initial biotransformation pathways for these antibiotics in the presence of AQDS.

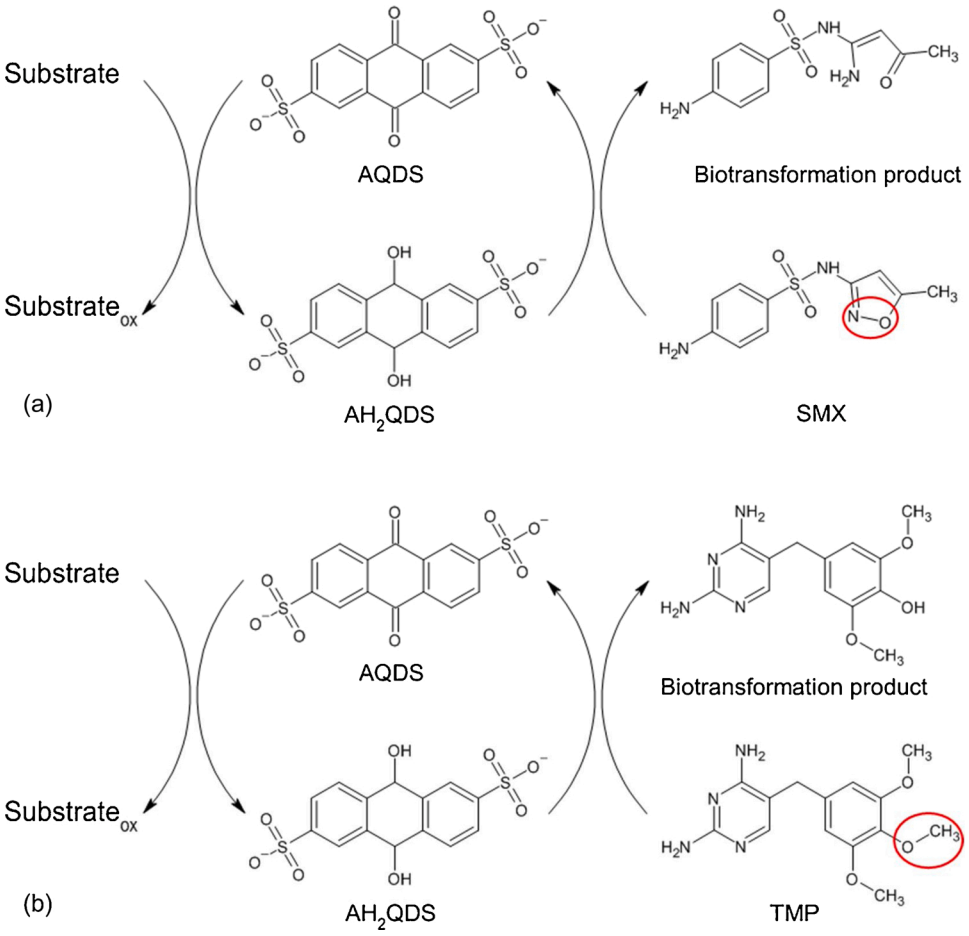

Fig. 1. Suggested initial reductive biotransformation of sulfamethoxazole (SMX) (a) and trimethoprim (TMP) (b) in the presence of the redox mediator anthraquinone-2,6-disultonate (AQDS). Anthrahydroquinone-2,6-disulfonate (AH_2QDS) is the reduced form of AQDS. The bonds most likely cleaved during the process are indicated by red ellipses (Jia et al., 2019; Mohatt et al., 2011) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

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, 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). However, from day 83 of operation (i.e., second half of period IV), there was an increase in the REs of SMX (due to a likely adaptation), resulting in a mean value of approximately 80.6% (considering the data from day 83 to day 92), which is significantly higher than that of period III (74%) ($p < 0.001$). Therefore, actually, microaeration also improved the biotransformation of SMX, although it was more noticeable for TMP.

In period V, the airflow rate was kept at 1 mL 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 period IV (~7%) ($p < 0.001$). Nonetheless, compared to period II, when the same concentration of AQDS (50 μM) was used without microaeration, the mean REs of both antibiotics in period V were much higher ($P < 0.001$) (Table 3). Thus, the impact of microaeration on the biotransformation of SMX and TMP was more significant when the reactor was supplemented with a lower concentration of redox mediator. However, it is worth mentioning that the mean RE of SMX in period V (50 μM of AQDS and 1 mL air min^{-1}) was lower than that obtained in period III (100 μM of AQDS without microaeration) ($p = 0.026$) (Table 3). Therefore, for this antibiotic, using solely the redox mediator at a higher concentration seems more advantageous than combining microaeration with a lower concentration of AQDS.

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 (which remained below −200 mV), 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 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).

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), emphasizing the role of AQDS in their biotransformation. Nonetheless, the REs of this period were still much higher than those of period I (anaerobic). 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). 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 (mean ammonium RE of 6.6% and absence of nitrate and nitrite in the effluent during the entire experiment), 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, reaching mean values similar to those observed in period I (< 10%) ($p > 0.050$) (Table 2). 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.

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

As observed in the previous experiment (section 3.1), without nitrate addition (period I), low mean REs of SMX and TMP were found (< 15%) (Table 4). Then, with the introduction of nitrate (100 mg L^{-1}) as an alternative electron acceptor (COD/NO_3^- ratio of 10.0) (period II), the REs of antibiotics increased considerably ($p < 0.001$). 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 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

Table 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 (μg L^{-1})	205 ± 15	194 ± 17	194 ± 15	213 ± 10	204 ± 21	209 ± 9
	Effluent (μg 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 (μg L^{-1})	206 ± 10	189 ± 11	202 ± 15	215 ± 13	205 ± 13	209 ± 10
	Effluent (μg 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.

aerobic conditions (Alvarino et al., 2016; Inyang et al., 2016; Lakshminarasimman et al., 2018; Ogunlaja and Parker, 2018; Zhao et al., 2018) because the reduction 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 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 $NO_3^-L^{-1}$), although to a lesser extent than in the previous periods, the REs increased again, reaching mean values higher than 85% for both OMPs (Table 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.

Alvarino et al. (2016) also observed an increase in the RE of SMX (272 μg L^{-1}) from 10% to 60% in a fed-batch reactor inoculated with activated sludge when the nitrate concentration was raised from 22.1–221.4 mg L^{-1} in the presence of acetate (50 mg COD L^{-1}). According to Rodríguez-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.

When the COD/NO_3^- ratio was raised back to 10.0 (100 mg NO_3^- L^{-1}) (period V), the REs decreased immediately and were highly unstable, reaching mean values similar to those of period II ($p > 0.050$) (Table 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 ($p > 0.070$). 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.

3.3. Operational stability

In both experiments (sections 3.1 and 3.2), 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 5 and 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 the methane production, particularly when the reactor was operated at lower COD/NO_3^- ratios (periods III and IV) (Table 6). It is worth mentioning that, although the methane content in the biogas also decreased when the reactor was microaerated (periods IV, V and VI) (Table 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 the methane production was not impaired. In addition, in both experiments, the methane content in the biogas remained higher than 55% (Tables 5 and 6). Therefore, the biogas can still be used in combined heat and power plants, since a minimum methane content of only 40% is required (Haubrichs and Widmann, 2006).

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 6). Furthermore, only in period IV, with the addition of 400 mg NO_3^- L^{-1} (COD/NO_3^- ratio of 2.5), nitrite was detected in the effluent (27 mg L^{-1}). Therefore, except in this period, the removed nitrate was most likely completely reduced to nitrogen gas (complete denitrification).

Table 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 L^{-1})	1045 ± 52	1011 ± 41	1032 ± 65	1039 ± 51	1061 ± 54	1078 ± 63
Effluent COD (mg 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 L^{-1})	–	99 ± 7	213 ± 8	400 ± 13	112 ± 9	–
Effluent NO_3^- (mg 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 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 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. General considerations on the engineering approaches

Comparing the three approaches (addition of AQDS, microaeration,

Table 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.

and nitrate), when assessed individually, the addition of nitrate ensured the highest REs of SMX and TMP (> 85%), but 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, as mentioned before, 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 the most indicated, since REs of SMX and TMP close to 75% were 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 OPEX. 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 may be necessary to achieve higher REs of OMPs, 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 (e.g., SMX) may not justify the additional costs involved.

Finally, all three 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 (Grandclément et al., 2017).

4. Conclusions

All the assessed approaches (addition of AQDS, microaeration, and nitrate) were demonstrated to be very effective for improving the limited biotransformation of SMX and TMP in anaerobic reactors. The REs increased from approximately 6% to more than 90% (depending on the antibiotic and the approach), being comparable to those found in higher-cost wastewater treatment technologies. For AQDS and nitrate, the best results were achieved with the highest concentrations (100 μM of AQDS and 400 mg NO_3^- L^{-1}). Consequently, the requirement for continuous dosage of chemicals may be an economic limitation. Therefore, using immobilized redox mediators and nitrified effluents as nitrate source is recommended. Regarding microaeration, airflow rates above 1 mL min^{-1} may be necessary to achieve higher REs of SMX and TMP. However, it can dilute further the biogas, preventing its use as an energy source. Finally, although microaeration had a synergistic effect with the redox mediator, this combined approach should be carefully assessed, as a small increase in the REs of some OMPs (e.g., SMX) may not justify the additional costs involved.

CRediT authorship contribution statement

José Gilmar da Silva do Nascimento: Methodology, Formal analysis, Investigation, Writing - original draft. **Maria Helena Peres de Araújo:** Investigation. **André Bezerra dos Santos:** Resources, Writing - review & editing, Project administration, Funding acquisition. **Marcos Erick Rodrigues da Silva:** Conceptualization, Formal analysis, Writing - review & editing, Supervision. **Paulo Igor Milen Firmino:** Conceptualization, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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