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PHARMACEUTICALS EFFECT AND REMOVAL, AT ENVIRONMENTALLY
RELEVANT CONCENTRATIONS, FROM SEWAGE SLUDGE DURING
ANAEROBIC DIGESTION

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Abstract

This paper investigates the performance of AD in the presence of high-risk pharmaceuticals found in sewage sludge and its removal capacity. The digestion process of synthetic sewage sludge was observed in two 7L glass reactors (D1 and D2) at 38°C (OLR 1.3 gVS L⁻¹ d⁻¹ and HRT 43 d).

Environmentally relevant pharmaceuticals (clarithromycin, clotrimazole, erythromycin, fluoxetine, ibuprofen, sertraline, simvastatin and tamoxifen) were added in D2 at predicted environmental (sludge) conditions.

The results demonstrated that long-term presence of pharmaceuticals can affect AD and induce instability resulting in an accumulation of VFAs. This study showed a concurrent effect on AD microbial composition, increasing the percentage of Firmicutes (>70%) and decreasing the percentages of

Bacteroidetes and Euryarchaeota (<5%), which seems to be the cause of VFA accumulation and resultant the decrease in the biogas production.

However, it seems that anaerobic microorganisms offer enhanced the removal of the antibiotics clarithromycin and erythromycin over aerobic techniques.

Keywords

Anaerobic digestion; pharmaceuticals; synthetic sewage sludge; microbial composition; biological treatment.

1.

Introduction

In recent years there has been increasing scientific and regulatory attention towards emerging micro-pollutants such as pharmaceutical residues in the aquatic environment. Pharmaceutical residues (PR) can originate from agricultural activities, hospital effluents, industrial wastes and domestic wastes. They are typically found at very low concentrations in the environment and are unlikely to affect human health but could cause chronic exposure damage to aquatic organisms (Helwig et al., 2013).

Following human consumption, PR are generally excreted into the sewer and subsequently reach wastewater treatment plants (WWTPs), where they are not fully removed (Verlicchi and Zambello, 2015). Over the decades, the enormous demand for medicines used on a daily basis has led to a considerably increase in the influx of pharmaceutical residue into the sewage system and thus the environment. As a result, contamination of the environment through pharmaceutical residue is now considered a global problem through the potential long-term damage of ecosystems resulting from the associated increased release of pharmaceuticals.

Pharmaceuticals have been reported to cause various effects in the environment, the synthetic 17- α ethinylestradiol (EE2) found in oral contraceptives and the nonsteroidal anti-inflammatory drug diclofenac are perhaps the best known, with EE2 causing reproductive failure or sex change in fish (Nash et al., 2004). In terms of bacteria, most concern has focused on the role of released antibiotics and the development and maintenance of resistance mechanisms (Kümmerer, 2009).

Over 200 different pharmaceuticals have been reported in river waters globally and approximately 70 pharmaceuticals have been reported in UK environmental waters (Petrie et al., 2014). Although many pharmaceuticals have relatively short half-lives in the environment, they are termed “pseudopersistent” because they are continuously infused into aquatic systems via WWTW effluents (Hernando et al., 2006).

In the WWTP, the solids are commonly separated through sludge sedimentation in primary and secondary clarifiers. It is assumed that about 11 million tons of sewage sludge dry matter per year are generated in the EU, and in the future, that is expected to increase to 15 million tons. According to estimates, almost 50% of sewage sludge produced in EU countries is used for agricultural purposes while about 12% is composted and used in the fertilization of forests and gardens (Fijalkowski, 2019). Sewage sludge has a very high tendency to absorb many potential contaminants from wastewater and therefore, substances not degraded into simple compounds in the treatment process at WWTP will mostly be adsorbed on sewage sludge (Fijalkowski, 2019). There have been several studies on the occurrence of pharmaceutical residues in sewage sludge (Malmborg and Magnér, 2015). This sewage sludge needs a

stabilization, usually by anaerobic digestion (AD). This biological process affects the PRs to different extents and many are still detectable in the digested sludge (Malmborg and Magnér, 2015). Due to the complexity of the AD process a range of factors can affect its performance, including feedstock characteristics and the presence of these PR compounds.

Other researchers have evaluated the effect of pharmaceuticals on the AD process; however, the focus has been on the effect of antibiotics, frequently tested individually, and in particular those frequently associated with the veterinary market (Cheng et al., 2018; Mitchell et al., 2013). Consequently, the behaviour of emerging contaminants in sewage sludge during the AD process requires further research. Therefore, this paper investigates the effect of eight different PR (clarithromycin, clotrimazole, erythromycin, fluoxetine, ibuprofen, sertraline, simvastatin, and tamoxifen) – selected based on consumption patterns, ecotoxicity, therapeutic class, regulatory relevance, their strong potential to bind to sludge particles, and availability of an analytical method in the laboratory – on anaerobic digestion process and microbial composition. Therefore, the aims of this study were to investigate the biogas production and microbial composition with and without the presence of these pharmaceuticals and to determine the ability of these communities to remove these micro-pollutants.

2. Methods and Materials

2.1 Synthetic sewage sludge

The feedstock used in the AD process was a synthetic sewage sludge developed by the authors to simulate the constituents of typical wastewater prior to treatment whilst providing steady process conditions. This substrate

contained 1.07 g proteins; 1.53 g polysaccharides and 0.88 g lipids per 100 mL wet sludge, comparable to an ideal level of proteins (1.15 g); polysaccharides (1.55 g) and lipids (0.80 g), derived from previous studies (Higgins et al., 1982; Metcalf and Eddy, 2003; Riffat et al., 2013), based on the values in Table 1. The total solids (TS) and volatile solids (VS) contents of the synthetic sludge were 32.7 and 31.1 % respectively; the pH was 6.4; the total ammoniacal nitrogen (TAN) was 149 mg L⁻¹ and the chemical oxygen demand (COD) was 57.7 gO₂ L⁻¹.

Table 1 Composition of organic synthetic sludge.

Product	Synthetic sludge (g in 100mL)	Dry matter (%)	Protein (g in 100g)	Polysaccharide (g in 100g)	Lipid (g in 100g)
Lactose Free baby formula (Aptamil) ^a	2.65	97	10.3	57.1	27.3
Meat Extract ^b	0.97	94	100	0	0
Organic Wheat Bran ^c	0.41	97	13.5	26.8	5.5

^a Aptamil Lactose Free DATACARD. <https://eln.nutricia.co.uk/our-products/aptamil-range/specialist-milks/aptamil-lactose-free-from-birth>.

^b Sigma-Aldrich Meat extract for microbiology. <https://www.sigmaaldrich.com/catalog/product/sial/70164?lang=en®ion=GB>.

^c Tree of Life Organic Wheat Bran. http://www.open.treeoflife.co.uk/index.php?route=product/product&product_id=4624.

2.2 Experimental set-up.

The digestion experiments were performed in 2 glass tank reactors (Bell-Flo Spinner Flasks, Bellco Glass, USA) of volume 6 L (D1 and D2), which were placed on a Bell-ennium magnetic stirrer (Bellco Glass, operating at 22 rpm). The temperature was maintained at 37°C using a thermostatic bath with recirculation (Lauda RA12, LAUDA-Brinkmann, USA) and each reactor was connected to a gas flowmeter (Ritter MilliGas counter MGC-1) to monitor biogas production.

Eight different PR were selected based on consumption patterns, ecotoxicity, therapeutic class, regulatory relevance, their strong potential to bind to sludge particles, and availability of an analytical method in the laboratory, and added to the synthetic sewage sludge of D2 at the following concentrations: clarithromycin $95 \mu\text{g L}^{-1}$, clotrimazole $104 \mu\text{g L}^{-1}$, erythromycin $60 \mu\text{g L}^{-1}$, fluoxetine $132 \mu\text{g L}^{-1}$, ibuprofen $544 \mu\text{g L}^{-1}$, sertraline $62 \mu\text{g L}^{-1}$, simvastatin $878 \mu\text{g L}^{-1}$, and tamoxifen $19 \mu\text{g L}^{-1}$. This selection was due to occurrence in 250 most prescribed by NHS Scotland and having a potential to bind to sludge based on $K_d > 500 \text{ L kg}^{-1}$ (Ternes and Joss, 2006) and/or $\log K_{ow} > 2.5$ (Rogers, 1996). Three additional compounds (clarithromycin, erythromycin and ibuprofen) were added to the list as they were prescribed in >one tonne per annum and were frequently reported in sewage sludge (Verlicchi and Zambello, 2015).

The concentrations dosed in the current study were based on the predicted environmental conditions (PEC) in the sludge, calculated following the equation $\text{PEC}_{\text{sludge}} = C_{\text{water}} \times K_{d\text{sludge}}$ (Verlicchi and Zambello, 2015) where C_{water} corresponds to PEC in water. The PEC in water value was obtained for raw sewage entering a typical Scottish WWTP of 5000 population equivalent using annual compound consumption data.

The digesters were initially charged with 6L of inoculum, a methanogenically active biomass from a mesophilic anaerobic digestion treatment plant which handles around 30,000 tonnes of food waste (Deerdykes AD plant, Cumbernauld, Scotland). The TS and VS contents of the inoculum were 2.4 and 1.6 % respectively; the pH was 8.0 and the COD was $18.3 \text{ gO}_2 \text{ L}^{-1}$. Thereafter, they were fed once a day with synthetic sewage sludge (Table 1), except at

weekends. The substrate was introduced by an in-feed located at the top of the digester. Prior to introducing the feeding substrate, the corresponding amount of digestate was removed from the digester. In both reactors, previous to the pharmaceutical dosing into D2, a slow, gradual acclimatization strategy was used to enable the stability of the systems to be assessed, where the loading rate was increased and the hydraulic retention time (HRT) decreased as the systems reached stability. The stability was verified by periodic determination of the following control parameters: alkalinity, biogas production and pH. The initial organic loading rate (OLR) was $0.24 \text{ gVS L}^{-1} \text{ d}^{-1}$ and HRT was 72 days. The OLR was increased and the HRT decreased throughout the experiment, so that the final loading rate was $1.3 \text{ gVS L}^{-1} \text{ d}^{-1}$ and the HRT was 43 days (data not shown). This OLR and HRT was maintained for 43 days (Start-up) to ensure the systems' stability, before adding pharmaceuticals into D2's synthetic sludge feedstock. Both digesters were fed and monitored for 4 more cycles of 43 days. Biogas production and pH was monitored on a daily basis and alkalinity was measured every 2 – 3 days in each digester during the experiment. Samples of the digestate were collected once per week and stored at $-20 \text{ }^{\circ}\text{C}$ for PR and once per month microbial genomic analysis. Gas samples from sample bags were collected using a SGE gas tight syringe fitted with a Luer lock valve. Approximately 20 cm^3 was collected and stored until analysis.

2.3 Physico-chemical analyses

The pH, TS and VS were measured according to the standard methods outlined by the APHA (2012) and the COD was determined using the Palintest COD/2000 method. Total ammoniacal nitrogen (TAN) was extracted by steam distillation and back titrated to pH 4.7 using 0.2 N hydrochloric acid.

The alkalinity was measured by a method proposed by Hills and Jenkins (1989), in which a two-stage titration is carried out with 0.1N HCl, first to 5.75 and subsequently to 4.3. Considering these two pH endpoints, three alkalinity measurement parameters were defined: total alkalinity (TA), measured to pH 4.3, partial alkalinity (BA), associated with bicarbonate alkalinity and measured to pH 5.75, and intermediate alkalinity (IA), associated with the concentration of volatile fatty acids (VFAs) and estimated as the difference between TA and BA. Changes in the VFA content in the process can be monitored by changes in alkalinity during the experiment (Hill and Jenkins, 1989).

The composition of the collected gas was measured using a Shimadzu GC-2014 gas chromatograph fitted with a Hayes Sep Q80/100 column (3.0m x 0.33mm i.d.) operating at a constant temperature of 60°C. The injection volume was 2mL and the run time period was 4 minutes. Two detectors were used: flame ionization detector (FID) operating at 200°C to detect hydrocarbons (methane), and electron capture detector (ECD) operating at 300°C to detect CO₂. The CH₄ and CO₂ concentrations in the sample gases were measured as the peak area at 1.3 minutes for methane and at 2.3 minutes for carbon dioxide.

2.4 Pharmaceutical analysis.

The mass spectrometer used for the pharmaceuticals analysis was a Thermo Scientific Q-exactive Orbitrap mass spectrometer, fitted with a Dionex Ultimate 3000 RS pump, Dionex Ultimate 3000 RS autosampler (temperature controlled at 10°C) and Dionex Ultimate 3000 RS column compartment (temperature controlled at 30°C). The operating software was Chromeleon, Xcalibur and Tracefinder. In both positive and negative ionisation mode the electrospray conditions were sheath gas 45 arbitrary units, auxiliary gas 10 arbitrary units,

capillary and auxiliary gas temperature 300°C. The spray voltage was set at 3.5 kV and -4.5 kV in positive and negative mode respectively. The pharmaceuticals were detected in positive or negative mode using parallel reaction monitoring by using the transitions described in “Supplementary information”.

The mobile phase for the LC separation was A = acetonitrile, B = 10mmol ammonium formate in water adjusted to pH 3.5 with formic acid) The gradient LC conditions were: 99%B for 1 minute then up to 70% B over 1 minute. Maintained at 70% B for 5 minutes then up to 1% B over a further 1 minute. The gradient was maintained at 1% B for a further 3 minutes, back to 99% B over 1 minute and re-equilibrated with 99% B for 8 minutes. The flow was 0.2 mL min⁻¹ and the injection volume was 10 µL.

Due to the nature of the samples a pre-treatment to remove proteins was required. One mL of the collected sample was added to a 50 mL centrifuge tube to which 5 mL of acetonitrile was subsequently added. The tubes were mixed for 5 mins using a vortex mixer, afterwards the samples were centrifuged at 2500 rpm for 10 min and the supernatant collected. This supernatant was filtered through a polyvinylidene difluoride 0.45µm syringe filters and transferred to 1.5mL vials for LMCS analysis.

Pharmaceutical recoveries from the samples using the extraction and quantitation method previously described were determined by spiking the artificial sludge with 100x concentrations of the selected pharmaceuticals. This was done in triplicate and the recovery calculated by comparing the concentration in matrix with the concentration in solvent. The acetonitrile-based extraction procedure was designed to be a general “catch all” approach and

thus it was expected that some pharmaceuticals would be recovered better than others. This was the case, with the mean percentage recovery ranging from 19 to 122%. Due to the variation in the percentage recovery and matrix issues affecting recovery, all subsequent calculation and analysis was based on 'as measured' data with only the overall trends in pharmaceutical levels being considered.

2.5 DNA extraction

For the next generation sequencing analysis, 12 samples from D1 and D2 were collected approximately monthly to cover the whole digestion process. Triplicate samples (15mL), previously stored at -20°C, were centrifuged at 2500 rpm for 10 minutes, the supernatant was discarded and the collected pellet used for DNA extraction. Total DNA was extracted from each sample pellet using DNeasy® PowerSoil® Kit (Qiagen, Germany) according to the manufacturer's protocol. The concentration and quality of the extracted DNA was determined using a NanoDrop spectrophotometer.

Extracted DNA were amplified by polymerase chain reaction using the primer set 341F (5'-CCT ACG GGN GGC WGC AG-3') and 785R (5'-GAC TAC HVG GGT ATC TAA TCC-3') from IDT (UK). Each reaction contained 5.0µL DreamTaq Green PCR Master Mix (Thermo Fisher Scientific); 1.0µL primer mix (1µM); 3.0µL nuclease free water and 1.0µL sample DNA (20ng µL⁻¹).

2.6 Metagenomic sequencing and taxonomic analysis

The next-generation sequencing was carried out on the PCR amplification products using an Illumina MiSeq instrument with 2 x 300 base-pair paired-end reads at LCG Biotech (Germany). Universal primers of 16S rRNA genes were used to amplify the hypervariable regions (V3-V5). Reads were demultiplexed at

LGC and basic statistics. All reads were reported as fastq along with fastqc files showing phred values > 20. Reads and metadata have been submitted to the SRA.

Data processing, quality trimming, taxonomic classification, diversity (plugIns) in QIIME2-2018.8 (<https://qiime2.org>; <https://peerj.com/preprints/27295/>) (Bolyen et al., 2019). Demultiplexed reads were imported using the Fastq manifest protocol (<https://docs.qiime2.org/2019.1/tutorials/importing/>) prior to joining and quality selection using DADA2 (settings p-trim-left 10 bp and p-trim-right 240) (Callahan et al., 2016). Taxonomy was assigned to features using a classifier trained with the QIIME2 plugin (Bokulich et al., 2018) using the Greengenes data base (v13.8) set at 99% sequence identity (McDonald et al., 2012).

Diversity of the microbial communities were assessed using several methods available in the q2-diversity plugin (see <https://forum.qiime2.org/t/alpha-and-beta-diversity-explanations-and-commands/2282> for original citations). Results were obtained using the ARCHIE-WeSt High Performance Computer (www.archie-west.ac.uk) based at the University of Strathclyde.

3. Results and discussion

3.1 Effect of pharmaceuticals on AD performance.

During the start-up cycle, with an OLR of 1.3 gVS L⁻¹ d⁻¹ and an HRT of 42 days, the biogas production reached 0.55 L gCOD_{added}⁻¹ d⁻¹ and 1.01 L gVS_{added}⁻¹ d⁻¹ in both reactors D1 and D2 (Fig 1).

AD transforms sludge organic solids to biogas via the following biochemical reaction in an anaerobic condition (Eq.(1)).

where, $x = 1/8 (4c + h - 2o - 3n - 2s)$ and $y = 1/4(4c + h - 2o + 3n + 3s)$ (Raheem et al., 2018). Biogas composition (CH_4 , CO_2) in this study was constant during this period, reaching concentrations between 46- 51% and 29-30% of CH_4 and CO_2 respectively in both reactors and trace amounts of other gases (e.g. hydrogen, hydrogen sulfide and nitrogen) not quantified. Therefore, the methane yield achieved was around $505 \text{ mL CH}_4 \text{ gVS}_{\text{added}}^{-1}$. In comparison, these values are at the higher than the end of biogas yield from AD of sewage sludge reported in other studies ($157\text{--}495 \text{ mL CH}_4 \text{ gVS}_{\text{added}}^{-1}$) (Filer et al., 2019; Raheem et al., 2018), which may be due to the use of synthetic sludge free of inhibiting substances as well as a results of the reported high variabilities in biogas composition and productions as consequence of the differences in the mixing, OLR, reactor size, feedstock composition, etc.

During the first cycle of pharmaceuticals dosing in D2, biogas production remained relatively constant in the two reactors, with an average of 0.5 and $0.61 \text{ L gCOD}_{\text{added}}^{-1} \text{ d}^{-1}$ in D1 and D2 respectively. However, the biogas production in D2 started to decrease during cycle 2 to values of around $0.1 \text{ L gCOD}_{\text{added}}^{-1} \text{ d}^{-1}$, coinciding with instability in the alkalinity values (Fig. 1, Table 2).

FIGURE 1

Table 2 Average values of the main characteristics of the effluent of D2 during the start-up phase and cycle 4.

		Start-up phase			Cycle 4		
		n	mean	SD	n	mean	SD
Biogas	$\text{L gCOD}_{\text{added}}^{-1} \text{ d}^{-1}$	11	0.60	0.07	27	0.09	0.06
pH		12	7.45	0.06	27	5.62	0.10
COD	g L^{-1}	6	10.65	1.81	12	45.63	5.51
Total Alkalinity (TA)	$\text{mg CaCO}_3 \text{ L}^{-1}$	13	6390	419	27	3370	199
Partial Alkalinity (BA)	$\text{mg CaCO}_3 \text{ L}^{-1}$	13	5219	335	27	117	139
Intermediate Alkalinity (IA)	$\text{mg CaCO}_3 \text{ L}^{-1}$	13	1171	124	27	3254	188

IA / TA		13	0.18	0.01	27	0.97	0.04
TAN	mg L ⁻¹	6	1401	19	9	1220	23
Clotrimazole	µg L ⁻¹				15	92.65	20.36
Clarithromycin	µg L ⁻¹				15	50.86	9.86
Erythromycin	µg L ⁻¹				15	1.58	1.84
Fluoxetine	µg L ⁻¹				15	87.21	21.60
Ibuprofen	µg L ⁻¹				15	452.50	51.43
Setraline	µg L ⁻¹				15	50.40	8.52
Simvastatin	µg L ⁻¹				15	163.10	12.01
Tamoxifen	µg L ⁻¹				15	12.86	3.25

Alkalinity is the most suitable factor for detecting any inhibition in AD system process, particularly the Intermediate Alkalinity (IA) / Total Alkalinity (TA) ratio, thus the purpose of studying alkalinity dynamics was to predict any inhibition caused by accumulation of VFAs in anaerobic digestion. Palacios-Ruiz et al. (2008) suggested that the recommended limit of IA/TA ratio is 0.3 – 0.4 in order to prevent acidification due to accumulation of VFA and to avoid digester instability.

In D1 (where no pharmaceuticals were added) the IA/TA ratio remained constant at values of around 0.2 during the 4 cycles, ensuring equilibrium of the system (Fig. 2). However, in D2, during cycle 2 the IA increased consistently, thereby increasing the IA/TA ratio up to a limit of 0.9, indicative of an imbalance in the system, probably due to accumulation of VFAs (Hill and Jenkins, 1989). This increase in the IA/TA ratio coincided with the relative decrease in biogas production (Fig. 1). It is suggested that an increase of VFAs concentration inhibits the growth of microorganisms and therefore the biogas production (Fitamo et al., 2017).

FIGURE 2

These results also coincide with a decrease in the pH in D2 (Fig 3), where it dropped dramatically during the third cycle to an average of 5.7. The growth of

anaerobic microorganisms and especially methanogenic bacteria are affected by the medium pH. Optimal pH range for methanogenic microorganisms has been reported to be close to neutral (pH 6.3 to 7.8) (Cuetos et al., 2008). A decrease in pH may be a result of accumulation of VFAs in the reactor, indicating that the methane producing pathway had broken down.

The abrupt drop of the biogas production and pH values in D2 coincided with an increase in COD, as a result of a low degradation rates achieved by the anaerobic microorganisms and therefore a COD accumulation in the digester (Fig. 3).

FIGURE 3

In combination, the observations on biogas production, alkalinity, pH and COD removal indicate a clear system inhibition in the digester D2 due to the dosing of complex pharmaceuticals.

Total ammoniacal nitrogen (TAN) can play a significant aspect in influencing the stability of AD system processes (Chen et al., 2008). However, in the current study, these values stayed constant in both digesters reaching average concentrations of 1.3 g L^{-1} and therefore, it did not seem to be the cause of D2's inhibition.

Based on the obtained results, it seems that the accumulation and long term presence of pharmaceuticals could affect the AD process and induce instability resulting on an accumulation of VFAs in the anaerobic reactor, causing a decrease in the biogas production and the pH values and an increase in the COD concentration in the digester.

This inhibition could be caused by the presence of pharmaceuticals such as clarithromycin and erythromycin based on results reported in previous studies,

such as Huang et al. (2020) who stated that 60 – 1000 mg of clarithromycin per kg of total suspended solids promoted the accumulation of VFA by waste activated sludge fermentation. Furthermore, Cetecioglu et al. (2015) observed at low doses of erythromycin (1–100 mg L⁻¹) that the stoichiometry between substrate removal and methane generation was disturbed: Methanogenic activity was significantly reduced, despite total removal of the VFA mixture, suggesting partial substrate utilization due to enzymatic blockage. Therefore, it seems that these antibiotics, alone or in association with other pharmaceuticals, could have an effect on the anaerobic digestion system.

There are not previous studies regarding the effect of the other studied pharmaceuticals in AD systems and biogas production except for ibuprofen, where concentrations of 100, 500 and 1000 µg L⁻¹ did not have an effect on COD removal during AD (Jia et al., 2020).

3.2 Effect of pharmaceuticals on the microbial compositions of the AD reactors

Following the 16S rRNA sequencing, from the 12 samples collected over time in the two reactors, 749,761 operational taxonomic units (OTU's) were recovered, 91.7% were classified as bacteria and 8.3% as Archaea. These OTU's were assigned to 30 phylums which accounted for 99.9% of the collected OTUs, to 72 orders (accounting for 98.9% OTU's), 86 families (90.6%), 64 genera (75.6%) but only 11 species (0.4%).

FIGURE 4

Four phyla (Bacteroidetes, Chloroflexi, Euryarchaeota and Firmicutes) accounted for over 90% of the total OTU's in both reactors. Firmicutes predominated in both reactors accounting initially for nearly fifty percent. Once

dosing of the pharmaceutical mixtures commenced in D2, the percentage of Firmicutes OTU's increased with time reaching >70% in the end of the third cycle and during the fourth cycle. Both Bacteroidetes and Euryarchaeota appeared to be affected by pharmaceutical exposure, in the control reactor these phyla were present generally >20% and around 10% respectively, whereas in the reactor dosed with pharmaceuticals both phyla were <5% of the OTU's from the third cycle onwards. Chloroflexi was the only phylum found in consistent levels in both reactors. This facultative anaerobic bacterium has been shown to have strong resistance to certain antibiotics such as chlortetracycline and oxytetracycline (Chen et al., 2020).

According to Fitamo et al. (2017), Firmicutes have been reported to utilize a wide range of substrates including cellulose, proteins and pectin, therefore, the accumulation of organic matter in D2 onwards supplied additional substrate for microbes, thus enriching the abundance of this phyla. Furthermore, the dominance of Firmicutes in D2 onwards is consistent with the accumulation of VFA as a previous study has reported that this phylum can degrade refractory cellulose into short-chain acids during the AD process, such as acetate or propionate (Carballa et al., 2007). These results are contrary to the work of Chen et al. (2020), who stated that Firmicutes dominated the bacterial community during stable process performance at low organic loading rate, and in contrast, the onset of organic overloading raised the relative abundance of Bacteroidetes. This study concludes that in addition to the significant negative correlation between the relative abundance of Firmicutes and Bacteroidetes, populations of Firmicutes and Bacteroidetes were found to be linked to process

parameters including organic loading rate, volatile fatty acids concentration, and methane production.

Xu et al. (2019) stated that microbes in the AD system experienced selective pressure from various competitors in the AD process, which led to some specific groups being abundant and many more being scarce. This could explain the abrupt decrease in Bacteroidetes due to the abrupt increase of Firmicutes in D2. Also, Aydin (2016) found that after exposure to a mixture of erythromycin and tetracycline ($> 100 \mu\text{g L}^{-1}$) the microbial composition of the AD reactor was affected with Acinetobacter, Bacteroidetes and Proteobacteria all negatively affected by these antibiotics. Moreover, Jia et al. (2020) observed that the relative abundance of Bacteroidetes groups decreased with increasing ibuprofen concentration $> 100 \mu\text{g L}^{-1}$.

As with the Bacteroidetes, the effect of the addition of pharmaceuticals on the Euryarchaeota became observable at the end of the third cycle. Euryarchaeota dominates the archaeal kingdom ($> 99\%$), and are a well-known group participating in methane generation (Xu et al., 2019). The abrupt decrease of this phylum during the third cycle coincides with the VFA accumulation and therefore, it seems to be the cause of the decrease in the biogas production. According to Xu et al. (2019), long term antibiotics pressure affected archaeal composition, leading to a VFA accumulation and reduction in methane production.

The AD reactor was exposed to a mixture of eight pharmaceuticals representing seven drug classes and it was expected that the antibiotics would influence the microbial composition. For example, mechanism investigations described by Huang et al. (2020) showed that clarithromycin had negative effect on the

processes of hydrolysis, acidogenesis, acetogenesis, homo-acetogenesis and methanogenesis, but the greatest inhibitory effect, with time, was seen with acetogenesis and methanogenesis and this was the main reason for the lower CH₄ production rate in the CLA-added reactors. However, the non-antibiotics have also been reported to demonstrate antibacterial effects; frequently these compounds have been evaluated for repurposing against clinically important bacteria and their effects are described in terms of minimum inhibitory concentrations (MIC). MIC describes the concentration at which total inhibition of an overnight culture occurs, but detrimental effects will be seen at lower concentrations.

Ko et al. (2017) examined 16 published studies and simvastatin to have antibacterial effect against a wide spectrum of Gram-positive and Gram-negative bacteria. This statin was more effective against Gram-positive (MIC Streptococci of 7.8 mg L⁻¹) than Gram-negative (MIC Acinetobacter of 32 mg L⁻¹) bacteria. MIC values for sertraline and fluoxetine starting at 4mg L⁻¹ have been reported by Kalayci and Sahin (2014). Obad et al. (2015) detailed the antibacterial properties of ibuprofen against Gram-positive bacteria and Al-Janabi (2010) observed a similar effect against Gram-negative bacteria but the MIC values were in g L⁻¹ levels.

Tamoxifen has been reported to disrupt membranes of Gram-positive bacteria with a MIC against Staphylococcus aureus of around 4mg L⁻¹ (Levinson, 2017). The impact of pharmaceuticals in mixtures are rarely considered, especially the influence of non-antibiotics on the performance of antibiotics. The limited evidence obtained in this study suggests that the non-antibiotic compounds examined may have increased the influence of the two antibiotics used. Ayaz et

al. (2015) found that the antibacterial activities of the antibiotics examined were significantly increased with the addition of sertraline (100 mg L^{-1}) against *S. aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Ibuprofen was demonstrated to act synergistically with two antibiotics (cefuroxime or chloramphenicol) against *S. aureus* isolates (Chan et al., 2017), as was tamoxifen which displayed synergistic activity in combination with polymyxin B against *P. aeruginosa*, *Actinobacter baumannii* and *Klebsiella pneumoniae* isolates (Hussein et al., 2017).

This study has demonstrated that when combined with a synthetic sewage sludge substrate a mixture of eight pharmaceuticals can have a serious impact on AD performance, seemingly affecting all stages. However, it is not known if it is the parent compound, subsequent metabolites or combinations that are having the effect.

3.3. Fate of pharmaceuticals during semi-continuous AD

The fate of PRs during D2 digestion was monitored by analysis of their concentration in the liquid and the solid phase, therefore including those absorbed into the anaerobic sludge. These concentrations were compared to those predicted in the digestate based on the mass-flow of these PRs (in the synthetic sewage sludge substrate) and assuming no effect of AD (Fig 5).

FIGURE 5

Removal rates differed significantly depending on the pharmaceutical studied. On the one hand, the concentration of some compounds like clotrimazole, fluoxetine, ibuprofen and tamoxifen increased during the cycles coinciding with the accumulated predicted values in the digester, suggesting that these compounds were very persistent and their removal was non-existent. On the

other hand, concentrations of compounds like the clarithromycin, erythromycin, simvastatin or sertraline remained lower than the predicted valued.

Clarithromycin is a macromolecular antibiotic with a stable structure, which is generally not readily biodegradable and highly persistent in a variety of environmental matrices (Baumann et al., 2015). However, in the present study, clarithromycin seemed to be removed completely during the first 2 cycles and its concentration increased in the remaining 2 cycles, coinciding with the system's inhibition. Therefore, it seems that anaerobic microorganisms might have an effect on this compound's removal. When the digester was performing adequately, before the microorganisms where inhibited, a complete removal of clarithromycin was observed. These removal rate seems to be higher than the ones reported in previous studies, with degradation rates between 0 and 63%, where they state that this compound was refractory and stable, and difficult to be degraded in a short time (Huang et al., 2020). According to Göbel et al., (2007), higher degradation rate could be expected with longer solids retention time (of 60–80 day).

Erythromycin was removed completely both in the first two cycles and the last ones, suggesting that this compound is highly degradable under the incubation conditions and that the anaerobic microorganisms might not have had a significant effect on these compounds' removal. The degradation pattern of erythromycin was similar as reported by Feng et al (2017) during the anaerobic digestion of pig manure. In contrast, poor removals of this compound has been reported for aerobic treatments such as advanced membrane bioreactor and conventional activated sludge (Göbel et al., 2007). Therefore, it seems that anaerobic digestion conditions improve the removal of erythromycin.

The high persistence of clotrimazole in the samples coincides with the results obtained in other studies which observed a strong sorption of this compound into the sludge and did not show any degradation (Fijalkowski, 2019). Similar results were reported for fluoxetine, where no or very little degradation of this compound were observed during anaerobic digestion (Suanon et al., 2017). The persistence pattern of ibuprofen observed in this study is both supported by previous findings where a medium-low biotransformation was observed (Gonzalez-Gil et al., 2016) and differ with others, where an almost complete removal of this compound was reported during AD (Samaras et al., 2014). Regarding tamoxifen, Wang et al. (2018) reported a complete removal of this compound through the adsorption of the sludge instead of through biodegradation. In the current study, PR concentrations were analysed as a sum of their presence in both liquid and solid phase, which might explain constant concentration and lack of removal observed for this compound during the cycles.

Similar trends were observed in concentrations of sertraline and simvastatin during the experiment, where values in the digestate tended to remain slightly lower than the predicted values for most of the experiment, regardless of the system's inhibition. In the case of simvastatin, this is a pro-drug with complex metabolism, involving acid/lactone inter-conversion. The acid/lactone inter-conversion is minimised between pH 4 and 5 (Nováková et al., 2009), however pH above 6 facilitates the conversion of the lactone to the acid, not only is tenivastatin the main metabolite, but it is also the main degradation product at pH 6 and above. Therefore, in the current study, the removal of simvastatin might be caused by a degradation of this compound, since the pH stayed above

5.5 values during all of the trial. Similar conclusions can be reached for sertraline removal, though, this compound seemed to be slightly more affected by the pH drop in the digester after day 100 of the trial.

Therefore, based on the obtained results, it seems that anaerobic microorganisms enhanced the removal of the antibiotic clarithromycin during anaerobic digestion. Removals of other PRs were also observed, such as erythromycin, that was completely degraded during this study; however, it cannot be concluded to be a consequence of anaerobic microorganisms.

5. Conclusions

Anaerobic microorganisms provide a means of enhanced removal of the antibiotics clarithromycin and erythromycin compared to aerobic treatment. However, other environmentally relevant pharmaceuticals (clotrimazole, fluoxetine, ibuprofen, sertraline, simvastatin and tamoxifen) showed poor removal and affected long-term process instability resulting in an accumulation of VFAs and eventual process collapse. This appears to be correlated to changes in microbial composition, increasing the presence of Firmicutes and decreasing Bacteroidetes and Euryarchaeota, thus impacting process parameters including VFA and methane production. Especially the abrupt decrease of Euryarchaeota coincided with the VFA accumulation and therefore, it seems to be the main cause of the decrease in the biogas production.

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Figure captions

Fig 1. Changes in biogas production in D1 (without the addition of pharmaceuticals) and D2 (dosing a mixture of pharmaceuticals after the start-up cycle) under mesophilic AD conditions (each cycle equivalent to the HRT of 43 days).

Fig 2. Changes in the total alkalinity (TA), partial alkalinity (BA), intermediate alkalinity (IA) and IA/TA ratio in D1 and D2 during the semi-continuous anaerobic digestion process (each cycle equivalent to the HRT of 43 days).

Fig 3. Variations in pH and COD during mesophilic anaerobic digestion in reactors D1 and D2, without and with pharmaceuticals respectively (each cycle equivalent to the HRT of 43 days).

Fig 4. Phyla present in excess of 2% of the OTU's in D1 (without the addition of pharmaceuticals) and D2 (dosing a mixture of pharmaceuticals after the start-up cycle) at the end of each cycle (each cycle equivalent to the HRT of 43 days).

Fig 5. Concentrations ($\mu\text{g L}^{-1}$) of pharmaceuticals in D2 effluent (measured and no-effect prediction). Each point represents mean value from three replicate determinations with standard deviation.