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Impact of graphene oxide addition on pharmaceuticals removal in anaerobic membrane bioreactor

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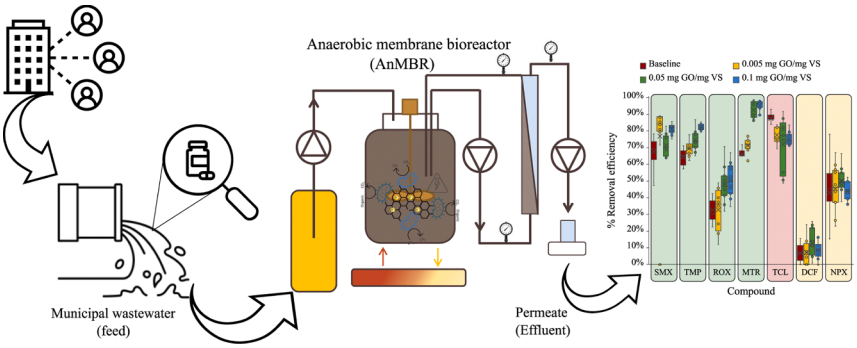
HIGHLIGHTS

- The removal of antibiotics was enhanced by the addition of graphene oxide.
- An enrichment in syntrophic bacteria and hydrogenotrophic methanogens was detected.
- Chemical oxygen demand removal was not compromised by graphene oxide addition.
- >80% removals of sulfamethoxazole, trimethoprim, and metronidazole in AnMBR.

GRAPHICAL ABSTRACT



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ABSTRACT

The addition of conductive materials to the anaerobic digestion bioreactor was suggested to enhance microbial activity. In the present work, an anaerobic membrane bioreactor treating municipal wastewater was operated for 385 days. The impact of different graphene oxide concentrations on the removal target pharmaceuticals and microbial community dynamics was investigated. The addition of graphene oxide did not impact the reactor stability, whereas the removals of antibiotics (e.g., trimethoprim and metronidazole) were enhanced. A shift in the microbial community was detected after the addition of 50–900 mg L^{-1} of graphene oxide, with the proliferation hydrogenotrophic methanogens. The proliferation of syntrophic microorganisms may indicate interactions via direct interspecific electron transfer. The obtained results suggest that the addition of graphene oxide at low mg L^{-1} concentrations to an anaerobic membrane bioreactor may be considered to improve the removal of antibiotics from municipal wastewater.

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1. Introduction

Anaerobic membrane bioreactor (AnMBR) has attracted significant attention as a potential technology for full-scale wastewater treatment (Oberoi et al., 2022). The use of membranes allows to decouple the hydraulic retention time and the sludge retention time, compensating for the low growth rates of anaerobic biomass. As a result, a high-quality effluent is obtained, with a chemical oxygen demand (COD) reduction>90% (Do and Stuckey, 2019; Robles et al., 2020). However, anaerobic processes suffer from long start-up times, low removal rates, and are susceptible to disruptions by organic overloading. These limitations are a consequence of the slow interspecific electron transfer (IET) between fermentative bacteria and methanogens. Any stagnation in IET causes the accumulation of volatile fatty acids (VFAs), thus acidifying the reactor and leading to process failure (Leng et al., 2018). The synergy between the microorganisms of anaerobic sludge is based on the exchange of electrons through IET, commonly by the production of formic acid and hydrogen, but also using an electrically conductive material through direct interspecies electron transfer (DIET) (Lin et al., 2017; Liu et al., 2020a). Synthropic bacteria can attach to the surface of the conductive materials and use them for electron exchange. Thus, the addition of carbon-based materials such as granular activated carbon (GAC), biochar, or graphene, was studied to enhance the electron flux among microorganisms in anaerobic digestion (Johnravindar et al., 2020; Muratçobanoğlu et al., 2021; Zhao et al., 2021). For example, addition of 30 g L^{-1} of GAC to batch reactors enhanced the total methane produced and methane production rate for 30 and 70%, respectively (Park et al., 2018). The dose of graphene-like materials, such as graphene oxide (GO), is typically in the order of mg L^{-1} (Colunga et al., 2015; Dong et al., 2019). Positive impact of GO on methane production was observed at 100 mg GO L^{-1} (Ponzelli et al., 2022a).

GO is an oxidized form of graphene with hydrophilic functional groups, i.e., hydroxyl, carbonyl, and carboxyl groups, which need to be (partially) reduced to ensure its electrical conductivity. Microorganisms can reduce the oxygen-containing functional groups of the non-conductive GO to produce the biologically reduced bio-rGO, forming a hydrogel that contains tightly linked microorganisms, extracellular polymeric substances and particles of the conductive material (Ponzelli et al., 2022b; Shen et al., 2018). Furthermore, GO addition to an up-flow sludge blanked reactor was reported to improve the removal of a range of organic pollutants, i.e., azo dyes and nitroaromatics (Colunga et al., 2015). Addition of GO to batch tests of waste-activated sludge enhanced the removal of pharmaceuticals (Casabella-Font et al., 2023). Nevertheless, further research is needed to gain insight into the long-term impact of GO addition on the microbial community and assess the potential of GO-enhanced anaerobic wastewater treatment in a continuously operated system.

In this study, an AnMBR that treats municipal wastewater was operated for 385 days to investigate the impact of different GO concentrations (<1 g L^{-1}) on the overall AnMBR performance and removal of pharmaceuticals. The removal of seven pharmaceuticals commonly found in municipal wastewater, namely diclofenac (DCF), naproxen (NPX), roxithromycin (ROX), sulfamethoxazole (SMX), triclosan (TCL), trimethoprim (TMP) and metronidazole (MTR), was investigated. These pharmaceuticals are typically encountered in municipal wastewater in up to μg L^{-1} concentrations; for example, recent reviews on the occurrence of pharmaceuticals reported up to 13.4 μg L^{-1} of SMX, 6.95 μg L^{-1} of TMP, 0.6 μg L^{-1} of ROX, 2.3 μg L^{-1} of TCL, 20.0 μg L^{-1} of MTR, 108 μg L^{-1} of DCF, and 1370.0 μg L^{-1} of NPX present in municipal wastewater influents (AL Falahi et al., 2022; Khasawneh and Palaniandy, 2021; Singh et al., 2019; Wang et al., 2020). Conventional wastewater treatment achieved wide range in their degradation, e.g., the reported removal efficiencies of TMP and SMX are generally below 30%, over 40% for NPX, and > 70% for TCL and ROX, and ~ 10% for DCF (Angeles et al., 2020; Luo et al., 2014; Suarez et al., 2010; Zhu et al., 2021). Furthermore, changes in the microbial community during long-term exposure of AnMBR sludge to the GO were assessed. Finally, the impact of GO on methane production was assessed in separate, controlled experiments and compared with the freshly sampled (i.e., non-adapted) anaerobic sludge. To the best of the authors knowledge, this is the first study to investigate the impact of GO addition on the anaerobic microbial community, pharmaceutical removal, and overall performance of an AnMBR.

2. Materials and methods

2.1. Chemicals, wastewater, and sludge sources

Anaerobic sludge used as an inoculum was sampled from an anaerobic digestor of a municipal wastewater treatment plant (WWTP) in Girona, Spain, which treats primary and secondary sludge. Municipal wastewater used as substrate was collected after the fine screens (5 mm opening) pretreatment in the same WWTP. GO was purchased from Graphenea as a 4% w/w aqueous dispersion with a flake size < 10 μm. All reagents used for sample preparation and analysis were of analytical grade. Analytical standards of the target pharmaceuticals and their isotopically labeled compounds were provided by Sigma-Aldrich and Toronto Research Chemicals.

2.2. Anaerobic membrane bioreactor set-up and operation

An AnMBR with 6 L of working volume (10 L total volume) was operated for 385 days, keeping a constant hydraulic retention time (HRT) of 24 h. External membrane module with 0.125 m^2 polyvinylidene difluoride (PVDF) hollow fiber microfiltration (MF) membrane (0.4 μm nominal pore size, ZeeWeed10) was employed to extract the permeate. The membrane module was periodically cleaned (every 21 days), first with a solution of NaOCl (1 g L^{-1}) to remove the attached biomass, followed by washing with a solution of oxalic acid (0.5 g L^{-1}) to remove the colloidal material. The reactor was inoculated with 2.2 L of anaerobic sludge (13.57 ± 0.16 g VS L^{-1}) and filled with municipal wastewater to achieve a final concentration of 5 g of volatile solids (VS) L^{-1}. The mixed liquor was recirculated at a flowrate of 0.83 L min^{-1}, resulting in an ascendent velocity of 4.6 cm s^{-1} inside the membrane module. The head-space biogas was recirculated at 0.25 L min^{-1} inside the membrane module to increase the turbulence of the mixed liquor over the membrane and minimize fouling. The inoculation and operation of the AnMBR was performed according to the previous study (Ferrari et al., 2019).

The reactor was equipped with pH and redox potential probes (Crison) and a temperature sensor (Selecta). Temperature was controlled with a closed-loop control system using a hydrothermal bath, with a setpoint of 37 °C. Mixed liquor was continuously stirred at 40 rpm (RZR-1 Heidolph), and biomass was periodically removed from the system for VS and TS analysis. Sludge retention time (SRT) was > 250 days as the only withdrawal of biomass was done through sampling.

An influent tank with freshly sampled wastewater was prepared daily and kept in a refrigerated container at 4 °C, with continuous stirring (60 rpm) to keep the particulate matter in suspension. Target pharmaceuticals were spiked daily to the influent tank (stage *ii-v*) to a final concentration of 0.02 μM each. The concentration of pharmaceuticals in the feeding tank and effluent was monitored biweekly.

2.2.1. Graphene oxide dosing strategy

The AnMBR reactor was first operated without the addition of pharmaceuticals and GO, to establish a baseline (stage *i*, days 0–49). Next, addition of pharmaceuticals, each at 0.02 μM concentration, was performed daily to avoid their degradation before reaching the reactor, and continued until the end of the AnMBR operation (stage *ii*, days 50–175). In stage *iii*, GO was added to the reactor as a function of VS concentration in the mixed liquor to a final concentration of 0.005 g GO g^{-1} VS (days 176–238). After that period, the GO concentration was

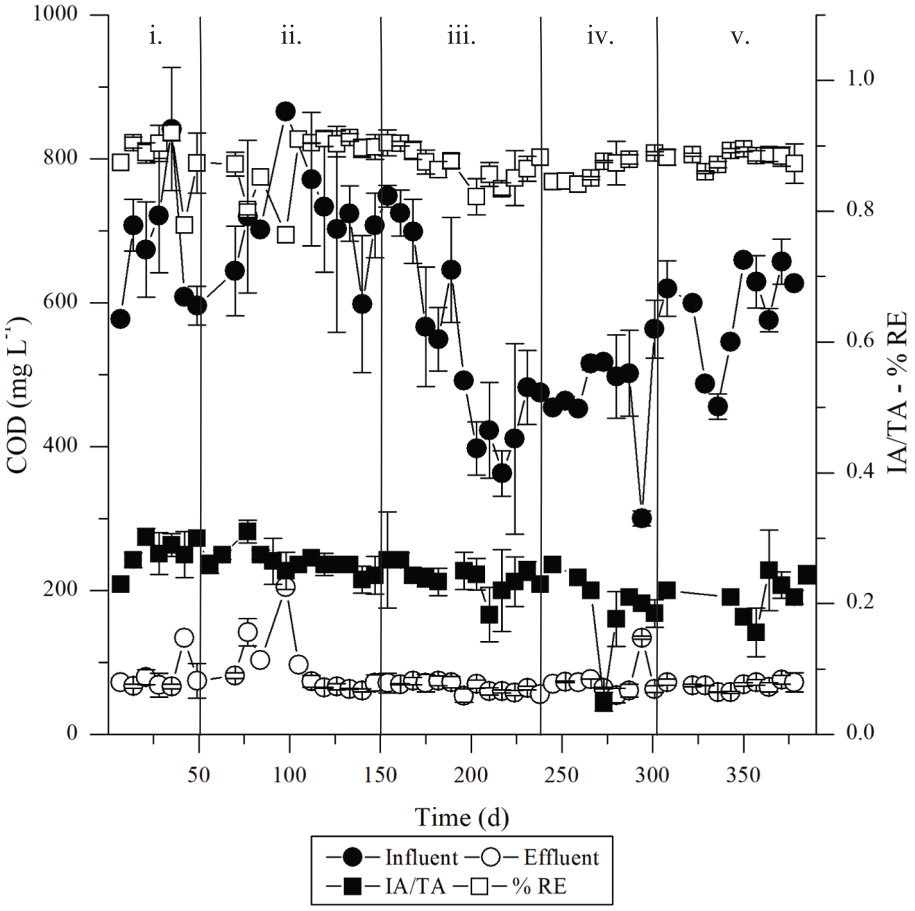

Fig. 1. Weekly average for influent and permeate COD (mg L-1) with standard deviation, COD removal efficiency (%), and intermediate (IA) /total alkalinity (TA) ratio separated in five different stages according to GO concentration: i. baseline, ii. pharmaceutical addition (0 g GO g-1 VS), iii. 0.005 g GO g-1 VS, iv. 0.05 g GO g-1 VS, and v. 0.1 g GO g-1 VS (in steps of 0.05 g GO g-1 VS).

increased up to 0.05 g GO g^{-1} VS in stage *iv* (days 239–301). Finally, during stage *v* (days 302–385), GO was added weekly at 0.005 g GO g^{-1} VS, until reaching a final concentration of 0.1 g GO g^{-1} VS. After each GO addition, the recirculation and permeation of the mixed liquor were halted for 8 h to ensure that the added GO was biologically reduced and incorporated into the sludge. In the previous study, rapid biological reduction of GO to bioRGO was observed within the first 8 h (Ponzelli et al., 2022b).

2.3. Analytical methods

VFAs (i.e., acetic, propionic, isobutyric, and N-butyric acid) were analyzed with gas chromatography (Trace GC Ultra ThermoFisher Scientific). Volatile solids, total solids (TS), and biochemical oxygen demand (BOD) were analyzed following standard methods (APHA 2017). Total and partial alkalinity was measured by pH titration (endpoints 5.75 and 4.3) using a 0.1 N solution of HCl.

Genomic DNA was extracted from the sludge using FastDNA™ SPIN Kit for soils (MP Biomedicals; Santa Ana, CA). High-throughput sequencing of 16S rRNA genes was then performed using the MiSeq platform (Illumina Inc., San Diego, CA). Different samples were analyzed in duplicate from days 1, 28, 119, 168, 231, 322, and 371, representing different stages of AnMBR operation. Bioinformatic analyses were conducted using the MOTHUR software package (Schloss et al., 2009). Data was deposited in the NCBI BioProject database under the access number PRJNA970669. The metabolic preferences of the archaeal community were defined according to the literature.

The analysis of pharmaceuticals in AnMBR influent and permeate was performed biweekly in duplicate. The samples were filtrated through 0.22 μm PVDF filters and concentrated by solid phase extraction (SPE) using Oasis HLB (200 mg, 6 mL) cartridges (Waters Corporation, USA), according to the previously developed method (Gros et al., 2019). Samples were analyzed using an Acquity Ultra-High Performance Liquid chromatography (UHPLC) system (Waters Corporation, MA, USA) in tandem with a 5500 QTRAP hybrid quadrupole-linear ion trap tandem mass spectrometer (AB Sciex, Foster City, USA). Matrix interferences were corrected using isotopically labeled standards: diclofenac-d4, ronidazole-d4, ibuprofen-d3, sulfamethoxazole-d4, triclosan-d3, and trimethoprim-d3.

2.4. Biochemical methane potential tests

2.4.1. Impact of the graphene oxide addition on the production of methane and removal of pharmaceuticals

Biochemical methane potential (BMP) tests were carried out in 600 mL (400 mL working volume) glass bottles using an automatic BMP system (Gas Endeavour, Bioprocess Control, Lund, Sweden) for the online methane monitoring. The experiments were conducted in triplicate using microcrystalline cellulose (Sigma-Aldrich) as substrate, with an inoculum/substrate ratio (I/S) of 2, following the procedures described by Zahedi et al. (2018). Mixed liquor sampled from the AnMBR reactor during stage *v* (i.e., adapted to GO), and fresh sludge from Girona's WWTP anaerobic digester were used as anaerobic inoculums. AnMBR sludge contained 0.1 g GO g^{-1} VS of inoculum, and GO was added to freshly sampled (i.e., non-adapted) sludge (Control_GO) to the same concentration of 0.1 g GO g^{-1} VS. Specific methane production (SMP)

Table 1
Summary of the measured parameters for each stage of AnMBR operation, expressed as the mean of six replicates with the standard deviation. COD and VFAs are expressed in mg L^{-1}. VS and TS are expressed in g kg^{-1}.

Stage	*i*	*ii*	*iii*	*iv*	*v*
Influent COD, mg L^{-1}	691 ± 89	709 ± 110	460 ± 102	489 ± 78	594 ± 63
Effluent COD, mg L^{-1}	75 ± 19	79 ± 29	64 ± 9	71 ± 22	69 ± 7
COD Removal, %	89 ± 4	89 ± 4	85 ± 3	84 ± 9	88 ± 1
IA/TA, -	0.28 ± 0.03	0.26 ± 0.03	0.23 ± 0.04	0.18 ± 0.07	0.21 ± 0.04
VS, g kg^{-1}	3.6 ± 0.7	5.4 ± 0.5	5.1 ± 0.4	6.7 ± 0.9	9.7 ± 0.7
TS, g kg^{-1}	5.5 ± 1.2	8.5 ± 1.0	8.1 ± 1.1	9.7 ± 1.2	13.2 ± 1.1
VS/TS, %	65 ± 3	64 ± 5	64 ± 5	69 ± 1	73 ± 3
Acetic acid, mg L^{-1}	6.3 ± 8.1	3.4 ± 6.5	<LOD	<LOD	2.9 ± 3.7
Propionic acid, mg L^{-1}	2.2 ± 4.4	1.3 ± 3.4	<LOD	<LOD	0.7 ± 1.2
Isobutyric acid, mg L^{-1}	1.7 ± 2.7	1.1 ± 2.2	<LOD	<LOD	0.6 ± 0.4
N-butyric acid, mg L^{-1}	1.6 ± 2.8	1.1 ± 2.0	<LOD	<LOD	0.6 ± 0.4

*Chemical oxygen demand (COD); IA/TA (intermediate, total alkalinity); volatile (VS) and total solids (TS);
**The detection (LOD) and quantification (LOQ) limits were: acetic acid = 0.99 and 3.29 mg L^{-1}, propionic acid = 0.28 and 0.94, isobutyric acid = 0.47 and 1.56, N-butyric acid = 0.48 and 1.60, respectively.
***The number of samples for each stage was, respectively: 21, 33, 35, 27, and 25.

values in mL CH_4 g^{-1} $VS_{substrate}$ (at normal conditions, P = 1 atm and T = 273 K) were presented as means with their standard deviations. Inoculum blank tests (without substrate) were prepared to determine the endogenous methane production. Methane produced by the endogenous metabolism (inoculum) was subtracted from the methane produced in the BMP tests.

To evaluate the potential of GO-adapted AnMBR (0.1 g GO g^{-1} VS) sludge to degrade the target pharmaceuticals, separate tests were performed at the end of the AnMBR operation (day 386) with TMP and MTR, which were selected as their removal in the AnMBR was the most affected by the GO addition. The impact of GO addition on the removal of TMP and MTR was studied in separate tests at an initial concentration of 1 μM each, using the GO-adapted AnMBR sludge. GO was added to GO-adapted sludge to a final concentration 0.15 g GO g^{-1} VS (AnMBR_GO). The methodology used was the same as previously described for BMP tests.

2.4.2. Biochemical methane potential test modeling: Specific methane production and pharmaceuticals removal

The data on the methane production kinetics was fitted to the Gompertz equation model (**Eq. (1)**). Kinetic parameters were estimated with iteration using a solver function in Excel MS, setting the sum of squared errors of the model data against the experimental specific methane potential data as an objective function (Ware and Power, 2017).

$$M(t) = M_{\infty} \cdot e\left\{ -e \cdot \left[\frac{\mu_{max} \cdot e}{M_{\infty}} \cdot (\lambda - t) + 1 \right] \right\} \quad (1)$$

where $M(t)$ was the cumulative daily methane production (mL CH_4 g^{-1} VS), M_{∞} was the maximum biochemical methane potential (mL CH_4 g^{-1} VS), μ_{max} was the maximum methane production rate (mL CH_4 g^{-1} VS·d^{-1}), λ was the lag phase (d), and t was the time (d).

The removal of TMP and MTR was fitted to a first-order kinetic equation (**Eq. (2)**), where k (d^{-1}) was estimated with iteration using a solver function in Excel MS. ANOVA test was done using Minitab 17 Statistical Software (State College, PA: Minitab, Inc.) to check for the statistically significant differences in the experimental results.

$$C(t) = C_0 \cdot e^{-k \cdot t} \quad (2)$$

Table 2
Values of alpha diversity of taxonomic characterization on different operational periods. Samples were analyzed in duplicate and presented as the mean and standard deviation.

Sample	N. of OTUs		Chaol richness estimator		Shannon diversity index	
Day	Archaea	Bacteria	Archaea	Bacteria	Archaea	Bacteria
1	494 ± 6	2541 ± 24	939 ± 92	6142 ± 123	1.67 ± 0.01	5.54 ± 0.01
28	380 ± 26	2116 ± 35	772 ± 29	5183 ± 466	1.72 ± 0.03	4.82 ± 0.02
119	344 ± 9	1798 ± 37	779 ± 3	4352 ± 62	1.62 ± 0.02	4.13 ± 0.04
168	312 ± 28	1986 ± 152	602 ± 43	4823 ± 99	1.61 ± 0.02	4.44 ± 0.08
231	379 ± 38	2268 ± 93	697 ± 4	5920 ± 183	1.78 ± 0.03	4.56 ± 0.05
322	388 ± 57	1678 ± 27	675 ± 105	3751 ± 91	1.74 ± 0.02	4.13 ± 0.13
371	324 ± 13	2489 ± 458	575 ± 25	7081 ± 2245	1.56 ± 0.01	4.59 ± 0.11

*Operational taxonomic units (OTUs);
**The results are expressed as the mean with standard deviation of a duplicate. Archaea and bacteria community were sequenced separately.

3. Results and discussion

3.1. Impact of the addition of graphene oxide on the anaerobic membrane bioreactor operational performance

Fig. 1 shows the mean (weekly) COD values with standard deviations, obtained from the daily measured COD values for the influent (feed) and effluent (permeate), as well as the weekly COD removal percentages. The weekly average COD removal achieved was>84% for all operational conditions, and in line with the previously reported performance of an AnMBR treating municipal wastewater (Robles et al., 2020). The AnMBR stability was monitored by at-line analysis of the ratio of intermediate (IA) and total alkalinity (IA/TA), a commonly employed parameter to determine the correct operation of the anaerobic digestion process. IA is given as the difference between the total and partial alkalinity. IA/TA values lower than 0.3 indicate a correct performance of the anaerobic digestion process, i.e., without the accumulation of VFAs that would cause an irreversible reactor failure (Ferrari et al., 2019). At the beginning of the baseline operation, the performance of AnMBR was somewhat unstable, with IA/TA values > 0.3. The concentrations of VFAs in the permeate were the highest of all periods during the baseline operation (Table 1), with an acetic acid accumulating to up to 20.68 ± 1.83 mg L^{-1} at peak points. Except for one peak accumulation on day 120 (stage *ii*), with an IA/TA value of 0.32, the operation of AnMBR with the addition of GO was stable, with the permeate COD concentration of ~ 80 mg L^{-1} and VFA concentrations close or below the limit of detection (Table 1).

The MF membrane module of the AnMBR enabled to maintain the solid content of the mixed liquor, expressed as a ratio of VS and TS, at 64–65% in the first three stages (Table 1). After the addition of higher GO concentrations, VS/TS percentage was increased from 64 to 65% (stage *iii*) to 69 ± 1% (stage *iv*) and 73 ± 3% (stage *v*). The cost of the addition of GO to an AnMBR in the concentration range studied was estimated as 600–1400 € per m3 of the AnMBR reactor, considering the current price of GO (https://www.abalonyx.no). Nevertheless, further research is needed to evaluate whether the added GO is degraded by the microorganisms, and if a repeated addition of GO would be required. (Ahmad Farid and Andou, 2022).

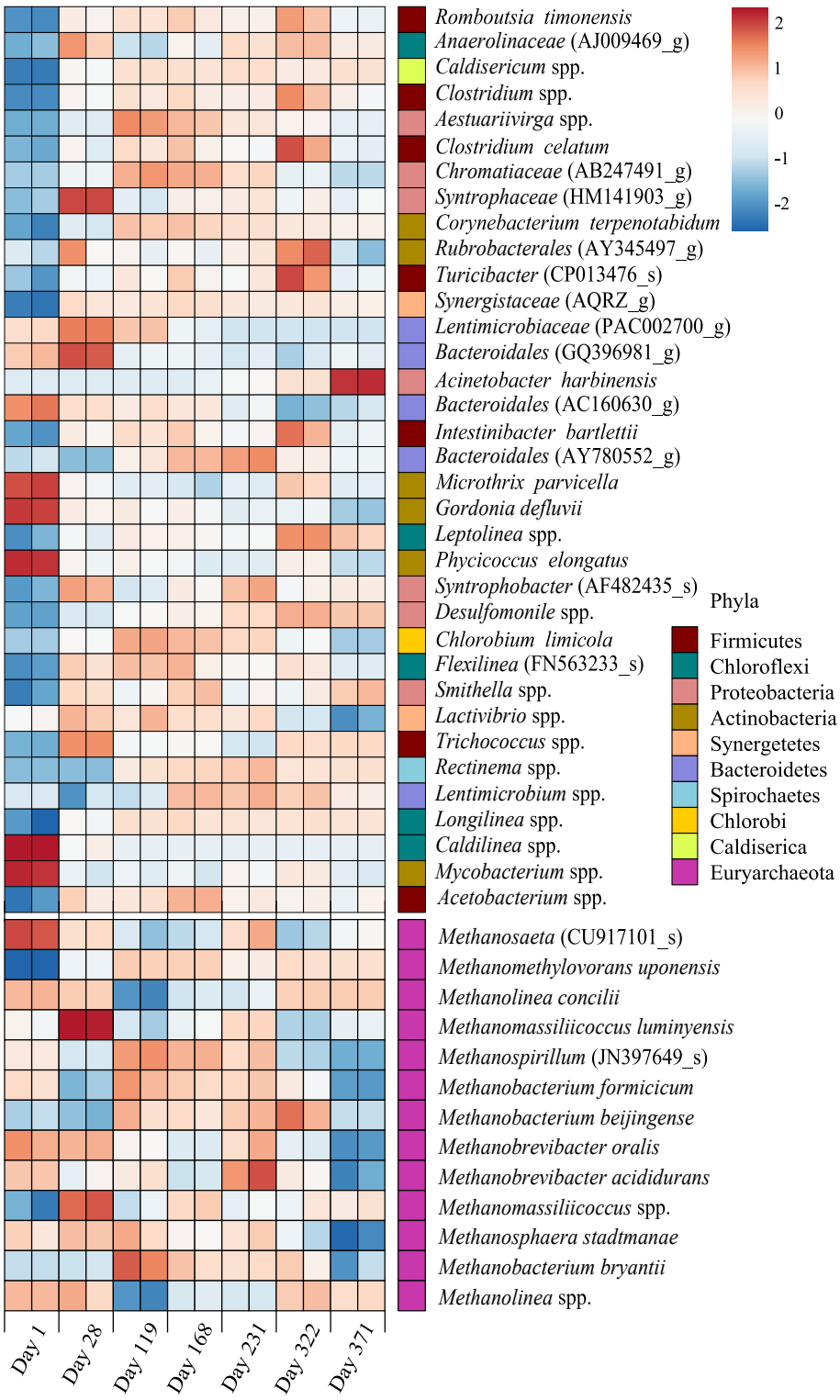

Fig. 2. Microbial community changes represented by the most present operational taxonomic units (OTUs) in relative abundance for archaea and bacteria domains, classified in the different phylum.

3.1.1. Impact of graphene oxide addition on the microbial community

The microbial community of the AnMBR was characterized in each operational period, to gain insight into the impact of GO addition on the long-term changes in the bacterial and archaeal community. The number of bacterial operational taxonomic units (OTUs) remained stable during all operational periods, but a significant ($p < 0.05$) decrease was detected for archaeal OTUs after comparing the initial community with all the other samples of stage *ii* to *v* During the treatment, similar Shannon diversity index and Chaol richness estimator were observed for archaea and bacteria (Table 2).Variations detected for bacteria and archaea OTUs are represented in Fig. 2, according to their relative abundance. During the AnMBR operation, twelve more OTUs for bacteria appeared as prevalent, whereas they were only marginally present in the initial sludge inoculum. Chloroflexi and Firmicutes were the

predominant phyla in all the investigated conditions, representing>50% of the bacterial community. In a previous study, addition of GO to the anaerobic digestion process of waste-activated sludge enhanced the growth of *Rombustia* spp. (Firmicutes) (Casabella-Font et al., 2023). In the AnMBR, the abundance of *Rombustia* spp., and other bacteria belonging to Firmicutes phylum (i.e., *Clostridium* spp. and *Turicibacter* spp.), first increased to reach a maximum relative abundance at day 322 (beginning of stage *v*, stepwise dosing), but decreased by the end of this stage (Fig. 2). Thus, even though the addition of GO favored the microorganisms belonging to Firmicutes phylum, more frequent addition of GO in stage *v* had a negative impact on their growth. The relative abundances Proteobacteria phylum was ~ 20% in all stages of AnMBR operation. The *Syntrophaceae* family was represented by *Smithella* spp. and an other unclassified genus, and it is known that the growth of these bacteria is promoted by the syntrophic interactions, which may be achieved via DIET (Kuever, 2014). The maximum abundance of the *Syntrophaceae* family was detected on day 28 (stage *i*, without GO), and further decreased by day 119 (stage *ii*). After the addition of GO, *Syntrophaceae* augmented its relative abundance between the end of stage *iii* (day 231) and the beginning of stage *v* (day 322). The relative abundance of the Bacteroidetes phylum decreased from 14 ± 1% on day 28 to ~ 2% by the end of the AnMBR operation. Actinobacteria was the most present phylum in the initial inoculum with the relative abundance of 36 ± 2%, which was decreased to 5 ± 1% (day 28), and remained stable for the rest of the AnMBR operation.

The archaeal community in the AnMBR, responsible for methane production in anaerobic digestion, belonged to Euryarchaeota phylum. The changes detected during the AnMBR operation are depicted in Fig. 2. At the beginning of the experiment, the sludge used as inoculum contained 68 ± 1% of autoclastic methanogens, microorganisms that use fatty acids as substrate, and 32 ± 1% of hydrogenotrophic methanogens that can use carbon dioxide as a carbon source to be reduced to methane. After 28 days of operation, enrichment of the hydrogenotrophic community was detected, reaching values of ~ 40%, mainly because of the proliferation of *Methanomassiliicoccus luminyensis,* likely due to the adaptation of the inoculum to municipal wastewater. From stage *ii* (day 119) onwards, the autoclastic methanogens represented ~ 40% of the methanogenic community. The other fraction was composed of hydrogenotrophic (~25%) and methylotrophic (~35%) methanogens. Previous studies reported an enrichment of the hydrogenotrophic methanogens during the anaerobic digestion of cattle manure with the addition of rGO and magnetite, which led to DIET enhancement (Muratçobanoğlu et al., 2021; Zhong et al., 2022). The proliferation of the methylotrophic archaea was justified by the substrate used in the present study, collected during the primary treatment, and also reported in the previous study on anaerobic municipal wastewater treatment (Tian et al., 2017). Furthermore, the growth rate of this species using methylamines as substrate can be doubled in the presence of hydrogen. The proliferation of *Methanomethylovorans uponensis* can be explained by their better use of electrons due to the enhanced electrical conductivity of the bioRGO-sludge gel-like structure (Cha et al., 2013; Jeon et al., 2009). Moreover, previous studies reported the promotion of the hydrogenotrophic archaea by the addition of different conductive materials, such as magnetite and manganese oxide, to the anaerobic digestion of waste-activated sludge and synthetic wastewater. The metabolic pathway used by the hydrogenotrophic archaea to produce methane involves the coenzyme F420, and the overexpression of that coenzyme was described as DIET indicator in graphene-amended electroactive reactors (Liu et al., 2020a; Rotaru et al., 2014; Wang et al., 2019; Zhong et al., 2022).

3.2. Impact of graphene oxide addition on the production of methane

To assess the methane production of the AnMBR sludge adapted to GO, mixed liquor from the AnMBR after 385 days of operation was used as inoculum for BMP tests and compared with the freshly sampled anaerobic sludge (Control) from the same anaerobic digester used for inoculum sampling, with (Control_GO) and without (Control) the added GO. Fig. 3 shows the cumulative daily SMP values, whereas the Gompertz model parameters are summarized in Table 3. AnMBR sludge exhibited ~ 15% lower SMP and ~ 55% lower μ_{max} compared with the fresh sludge (Control), likely due to the microbial population shift in the AnMBR that was treating municipal wastewater. However, the graphene-adapted sludge from the AnMBR presented a higher μ_{max} (62.2 ± 3.8 mL CH_4 g^{-1} VS d^{-1}) and SMP (M_∞) (350.1 ± 3.8 mL CH_4 g^{-1} VS) compared with the freshly sampled anaerobic sludge amended with the same concentration of GO (0.1 g GO g^{-1} VS). These values of SMP and μ_{max} of AnMBR sludge represented 26% and 33% increase compared with the Control_GO, respectively.

In terms of the lag phase (λ) in the methane production, the longest lag-phase (2.2 ± 0.3 day) was detected for the Control_GO sludge, likely caused by the initial biological reduction of GO. Once the GO was incorporated into the AnMBR sludge (1.4 ± 0.2 d), no significant differences were detected with the Control (1.3 ± 0.1 d) sludge. The slower rate of methane production in the AnMBR sludge (28.4 ± 4.1 mL CH_4 g^{-1} VS d^{-1}) compared with the Control sludge (62.2 ± 3.8 mL CH_4 g^{-1}

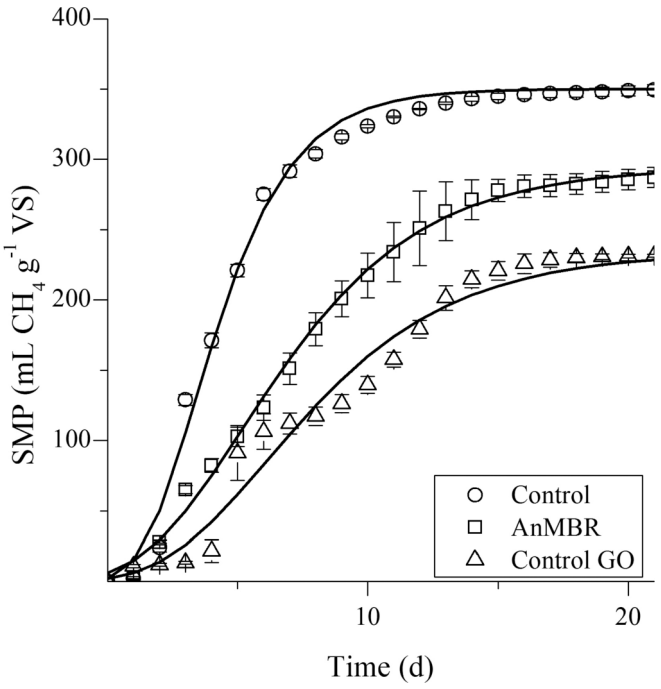

Fig. 3. Experimental methane production expressed as specific methane potential (SMP) with standard deviation and Gompertz model (average) for the different conditions. Comparison among non-adapted sludge and sludge adapted (AnMBR).

Table 3
Maximum specific methane potential (M_∞), maximum specific methane production rate (μ_{max}), lag phase(λ) and coefficient of determination (R^2) with standard deviation (triplicate) for Gompertz kinetic model.

Name	M_∞ (mLCH_4 g^{-1} VS)	μ_{max} (mLCH_4 g^{-1} VS d^{-1})	λ (d)	Coefficient of determination (R^2)
Control	350.1 ± 3.8	62.2 ± 3.8	1.3 ± 0.1	0.994 ± 0.001
AnMBR	294.5 ± 8.9	28.4 ± 4.1	1.4 ± 0.2	0.997 ± 0.001
Control_GO	234.1 ± 0.8	21.5 ± 0.6	2.2 ± 0.3	0.983 ± 0.006

*Maximum specific methane potential (M_∞); maximum specific methane production rate (μ_{max}); lag-phase (λ).

Table 4
Summary of the removal efficiencies (means with their standard deviation) expressed in %, obtained for each target pharmaceutical in AnMBR in each operational stage.

Compound	Stage *ii*	Stage *iii*	Stage *vi*	Stage *v*
[2] DCF	7 ± 5	7 ± 8	13 ± 8	9 ± 5
[2] NPX	43 ± 20	46 ± 13	50 ± 7	44 ± 6
[1] ROX	33 ± 9	33 ± 13	48 ± 11	51 ± 10
[1] SMX	68 ± 9	77 ± 25	72 ± 6	81 ± 3
[3] TCL	88 ± 4	78 ± 5	72 ± 16	75 ± 4
[1] TMP	64 ± 5	70 ± 4	75 ± 6	82 ± 2
[1] MTR	66 ± 4	71 ± 4	93 ± 5	96 ± 2

*diclofenac (DCF); naproxen (NPX); roxithromycin (ROX); sulfamethoxazole (SMX); triclosan (TCL); trimethoprim (TMP); metronidazole (MTR).
**The number of samples for each stage was, respectively: 14, 19, 21, 15, and 17.
***Superscript represents the classification group of each compound.

VS d^{-1}) may be due to the presence of methylotrophic methanogens (~35%) in the AnMBR sludge. Methylotrophic methanogens only use methylamines as a carbon source to produce methane (Naphtali et al., 2022), and this substrate was not available in the BMP tests.

The obtained data indicate that although the initial exposure of anaerobic sludge to GO inhibits the production of methane, long-term adaptation of sludge to the presence of GO leads to a partial recovery of the methane production capacity. In the initial addition of GO, anaerobic microorganisms use the electrons available in the organic substrate to reduce GO to bioRGO, instead of producing methane, thus resulting in a prolonged lag phase (lag phase of 2.2 ± 0.3 days for Control_GO). However, the exposure of sludge to GO leads to its adaptation, and the lag phases observed for both freshly sampled anaerobic sludge (Control) and AnMBR sludge are ~ 1.3 days. The lower values of methane production kinetic parameters for AnMBR sludge compared with the Control can be explained by the microbial community shift in the AnMBR sludge treating municipal wastewater, with an enhanced presence of microorganisms requiring a specific carbon source that was not available in the BMP tests.

3.3. Impact of graphene oxide addition on the removal of pharmaceuticals

Removal efficiencies obtained during each stage of the AnMBR operation are summarized in Table 4. Depending on the observed impact of the GO addition on the removal of pharmaceuticals, they were classified into three categories: (1) enhanced removal at higher GO concentrations, (2) no change in the removal efficiency after the addition of GO, and (3) decreased removal efficiency at higher GO concentrations.

MTR, SMX, TMP, and ROX were classified into group (1) as the removals of these antibiotics were increased with the addition of GO at any concentration. MTR had the highest removal efficiency among all the compounds analyzed, reaching 93 ± 5 and 96 ± 2% removal in stages *iv* and *v*, respectively, which represents an increment of + 30% compared to stage *ii* (i.e., no GO added).

The increase in GO concentration led to a progressive enhancement in the removal of TMP and SMX from 64 to 66% (stage *ii*) to e.g., 72 ± 6 and 75 ± 6% (stage *iv*), and reaching 82 ± 2% and 81 ± 3% (stage *v*). A previous study reported an enhancement in the removal of these antibiotics with the addition of particulate activated carbon to a lab-scale AnMBR, leading to an increase in SMX removal efficiency from 68% to ~ 90% (Xiao et al., 2017). Both SMX and TMP are hydrophilic pollutants with very low tendency to absorb onto the sludge (Monsalvo et al., 2014; Wijekoon et al., 2015). Also, previous studies identified syntrophic bacteria, *Syntrophaceae* and *Clostridium* spp., and methanogens *Methanomethylovorans* spp., and *Methanobacterium* spp. as microorganisms that play a key role in the anaerobic biotransformation of SMX (Cetecioglu et al., 2016; Harb et al., 2021; Oberoi et al., 2022). As

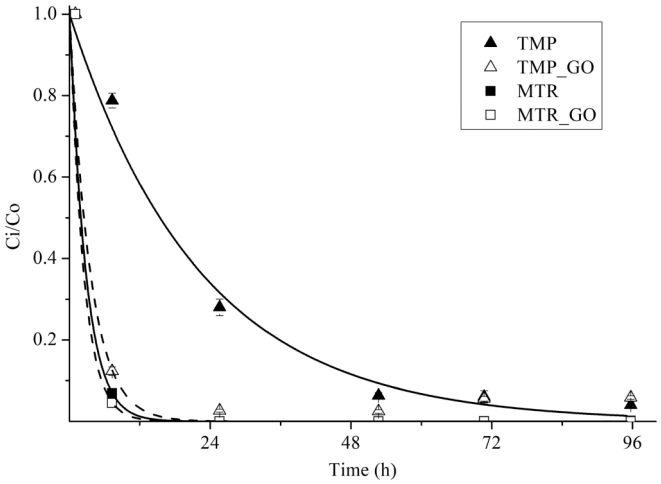

Fig. 4. Kinetic plots for trimethoprim (TMP) and metronidazole (MTR) with and without GO. The lines represent the first-order kinetic model. Straight for conditions without extra addition of GO and dashed for GO amended sludge.

explained above (section 3.1), these microorganisms also proliferated with the addition of GO, suggesting their prominent role in the biotransformation of SMX in AnMBR. ROX had the lowest removal efficiencies from group (1), with ~ 33% removal obtained in stage *ii* (no GO added) and *iii* (0.005 g GO g^{-1} VS). This is in accordance with the previous study in which the removal of ROX in anaerobic batch tests was < 35%, and was assigned to its biotransformation (Gonzalez-Gil et al., 2018). ROX removal was enhanced to 48 and 51% after increasing the GO concentration in stages *iv* and *v*, respectively.

In terms of group (2), the addition of GO did not impact the AnMBR removal efficiencies of DCF and NPX (Table 4). DCF is a very persistent compound with < 15% removal during the entire AnMBR operational period. This is in agreement with the literature data, where in general DCF removal efficiencies in anaerobic treatment are below 20% (Liu et al., 2020b; Monsalvo et al., 2014; Song et al., 2018; Wijekoon et al., 2015; Xiao et al., 2017) (Table 4). Some authors suggested that the low removals (i.e., <20%) observed for DCF in anaerobic processes are associated to its sorption onto the sludge. (Monsalvo et al., 2014; Wijekoon et al., 2015), which may explain its constantremoval efficiency throughout the different stages of AnMBR operation. The removal efficiencies of NPX were 43–50%, somewhat below the values reported in the literature for AnMBR, and unaffected by the presence of GO. NPX is a hydrophilic compound with very limited adsorption on the anaerobic sludge (Monsalvo et al., 2014; Wijekoon et al., 2015). The main removal mechanism of NPX in anaerobic systems is biotransformation (Carballa et al., 2007).

Triclosan (TCL) was the only pharmaceutical belonging to group (3), exhibiting a decrease in the removal efficiency after the addition of GO from 88% (stage *ii*) to 72–78% (stages *iii-v*). Nevertheless, the observed removal efficiencies are still in the upper range of the reported values for TCL removal in the AnMBR and anaerobic digestion processes reported

Table 5
Kinetic rate (k) and coefficient of determination (R^2) with standard deviation for first order kinetic model.

Name	k (d^{-1})	Coefficient of determination (R^2)
TMP [A]	0.04 ± 0.00	0.992 ± 0.002
TMP_GO [B]	0.29 ± 0.01	0.997 ± 0.000
MTR [C]	0.34 ± 0.11	0.999 ± 0.001
MTR_GO [C]	0.43 ± 0.04	0.999 ± 0.001

*trimethoprim (TMP); graphene oxide (GO); metronidazole (MTR).
**Each condition was studied in triplicate.
***Superscript represents the result of statistical analysis. Same letters indicate p-values > 0.05.

in literature (22–93%) (Liu et al., 2020a; Monsalvo et al., 2014; Song et al., 2016, 2018; Xiao et al., 2017). Previous studies reported low tendency of TCL to absorb onto the anaerobic sludge, being biodegradation its main removal mechanism (Wijekoon et al., 2015). The reduction in the removal efficiency may be related to the microbial community changes induced by the addition of GO to AnMBR.

MTR and TMP exhibited the largest increase in the AnMBR removal efficiencies with the addition of GO. To gain further insight into the impact of GO on their biotransformation, additional batch tests were performed with the AnMBR sludge, with and without added GO (Fig. 4). The first-order kinetic model (**Eq. (2)**) was fitted to the experimental data, achieving R^2 values>0.99 (Table 5). Significant differences were observed between the removal rates of TMP, with the removal rate constant of 0.04 ± 0.01 d^{-1} in the AnMBR sludge increased to 0.29 ± 0.01 d^{-1} after adding GO. Previous study reported biotransformation as the principal removal mechanism of TMP, in both presence and absence of GO, with no adsorption onto the sludge or GO nanosheets (Ponzelli et al., 2022b). In the case of MTR, no significant differences were observed between the removal rates obtained in the AnMBR_GO and GO-adapted sludge from AnMBR (Table 5). Anaerobic biotransformation of MTR is preceded by its absorption by the microorganism and was previously related to the presence of Clostridium spp. (Zhao et al., 2022). The addition of GO enhanced the abundance of Clostridium spp. in AnMBR (Fig. 2). This can explain the rapid removal of MTR observed in the conducted batch tests with the AnMBR sludge.

4. Conclusions

A microbial community shift was detected during the operational period of the AnMBR after the addition of GO. The proliferation of hydrogenotrophic methanogens suggests the promotion of syntrophic interactions via DIET. The addition of GO enhanced the removal of the target antibiotics SMX, TMP, ROX, and MTR. The removal of non-steroidal anti-inflammatory drugs DCF and NPX was not affected by the addition of GO, whereas the removal of TCL was somewhat lowered in the presence of GO. The addition of GO to AnMBR may be used to enhance the removal of antibiotics and lower their discharge into the environment.

CRediT authorship contribution statement

Oriol Casabella-Font: Methodology, Investigation, Writing – original draft. **Michele Ponzelli:** Methodology, Investigation. **Melina Papapanou:** Investigation. **Jose Luis Balcazar:** Formal analysis, Writing – review & editing. **Maite Pijuan:** Methodology, Writing – review & editing, Funding acquisition. **Jelena Radjenovic:** Methodology, Writing – review & editing, Funding acquisition.

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.

Data availability

Data will be made available on request.

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