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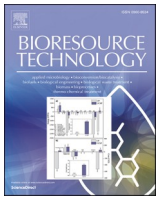

Novel insights into the impact of anticancer drugs on the performance and microbial communities of a continuous-flow aerobic granular sludge system

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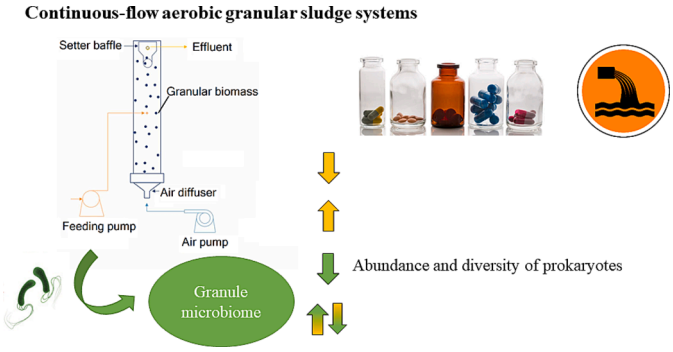
HIGHLIGHTS

- Study of anticancer drug effects in continuous-flow aerobic granular sludge systems.
- Impacts on treatment performance are temporary and dose-dependent.
- This type of aerobic granular systems can efficiently remove anticancer drugs.
- Anticancer drug impacts on the prokaryotic microbiome are dose-dependent.
- Specific microbial taxa are responsive to the application of the drugs.

GRAPHICAL ABSTRACT



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ABSTRACT

Anticancer drugs are frequently found in domestic wastewater, but knowledge of their impacts on wastewater treatment processes is limited. The effects of three levels of concentrations (low, medium, and high) of three anticancer drugs on physicochemical parameters and prokaryotic communities of a continuous-flow aerobic granular sludge (AGS) system were examined. Drugs at medium and high concentrations reduced the removal of total nitrogen and organic matter during the first 15 days of operation by approximately 15–20 % compared to a control, but these effects disappeared afterward. Removal efficiencies of drugs were in the range of 51.2–100 % depending on the concentration level. Drugs at medium and high concentrations reduced the abundance and diversity and altered the composition of prokaryotic communities. Specific taxa were linked to variations in performance parameters after the addition of the drugs. This study provides improved knowledge of the impacts of anticancer drugs in AGS systems operated in continuous-flow reactor.

1. Introduction

Cancer represents a substantial global public health challenge, and the World Health Organization (WHO) estimates that it accounted for almost one out of every six deaths in 2022 (WHO, 2022). Given the rising incidence of cancer, efforts are being made to invest in

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preventative, diagnostic, and therapeutic programs. Recent predictions suggest that by 2040 approximately 30 million new cases of cancer will be diagnosed (Yadav et al., 2021), necessitating the development and use of cancer-fighting drugs. These drugs, which are known as antineoplastic, anticancer, or chemotherapy drugs, have experienced a marked increase in usage over the last two decades in response to the growing prevalence of cancer diagnoses (Franquet-Griell et al., 2017).

Anticancer drugs are chemical compounds that are designed to disrupt the process of DNA synthesis. This inference has the potential to affect cellular growth, causing cell death, genetic alterations, and teratogenic effects in specific organisms (Huang et al., 2022). Notably, anticancer drugs are not completely metabolized within the human body, thus ending up in wastewater systems through urine and feces. The inherent high toxicity of these substances poses a considerable challenge to biodegradation processes, resulting in their persistence in water systems (Ferrando-Climent et al., 2014; Li et al., 2021). For instance, anticancer drugs have been found in the effluents of wastewater treatment plants (WWTPs), rivers, and groundwater (Nassour et al., 2020; Castellano-Hinojosa et al., 2023a). Among these drugs, tamoxifen (TMX), methotrexate (MTX), and cyclophosphamide (CP) are commonly found in influents and effluents of WWTPs, with concentrations ranging from almost zero to several thousands of ng L^{-1} (Nassour et al., 2020; Castellano-Hinojosa et al., 2023a).

Recent work showed that anticancer drugs cannot be completely removed from domestic wastewater by the conventional activated sludge (CAS) technology in WWTPs and other biological treatments such as the use of membrane bioreactors (MBRs). The removal efficiencies for these drugs range from 20 % to 80 %, depending on the level of concentration and type of anticancer drug (Franquet-Griell et al., 2017; Li et al., 2021; Yadav et al., 2021). The aerobic granular sludge (AGS) technology represents a biological system for wastewater treatment, offering advantages over CAS and MBRs (Nancharaiah et al., 2018). The AGS forms dense granules composed of microorganisms embedded in a polymer matrix due to hydrodynamic shear forces and continuous circular motion (Rollemberg et al., 2018). These dense granules provide several benefits, including improved biomass settleability (Winkler et al., 2013). Additionally, AGS promotes high biomass accumulation due to its compact structure, enhancing mass transfer and creating oxygen and nutrient gradients within the granules (Nancharaiah et al., 2018). This stratification leads to different metabolic activities within the granules, enabling the removal of organic matter (OM), nitrogen (N), and pharmaceuticals (Nancharaiah et al., 2018; Muñoz-Palazon et al., 2021). The AGS typically operates in a series of sequential batch reactors (SBRs) but AGS systems operating in a continuous-flow reactor (CFR) have been recently developed (Rosa-Masegosa et al., 2023). The constant flow of CFRs allows for the treatment of larger wastewater volumes and eliminates the need for storage systems (Xu et al., 2022). Yet, no studies have examined how the AGS technology operated in a CFR could be used to treat wastewater contaminated with anticancer drugs and whether the presence of these substances can impact treatment performance.

The prokaryotic microorganisms (Bacteria and Archaea) of the granule microbiome play a significant role in AGS systems as they contribute to granule formation and the removal of nutrients and other emerging contaminants (Xia et al., 2018). Pharmaceutical compounds such as antibiotics can impact the treatment performance of AGS by altering prokaryotic communities present in the granules (Muñoz-Palazón et al., 2021). In general, archaeal taxa are more resistant to antimicrobial agents compared to bacteria due to differences in cell wall (Khelaifia & Drancourt, 2012). Recently, strong linkages between the eukaryotic communities of the granule microbiome and the performance parameters in an AGS in SBR mode and treating wastewater contaminated with anticancer drugs were shown (Castellano-Hinojosa et al., 2023b). However, to date, no studies have examined how anticancer drugs may alter the prokaryotic communities of the granule microbiome using new AGS operated in a CFR and how these alterations may be linked to changes in treatment performance.

Here, we examined the effects of different concentration levels of three anticancer drugs commonly detected in domestic wastewater (CP, TMX, and MTX) on treatment performance (removal of OM and N, and concentration of granular biomass), drug removal efficiency, and the diversity composition, and abundance of bacterial and archaeal communities in an AGS operated in a CFR. We hypothesized that the concentration level of the drugs may control variations in the removal of these substances and in the impact of drugs on nutrient removal. We also hypothesized that a group of specific microorganisms may adapt to the different concentrations of anticancer drugs, thus leading to alterations in the abundance, diversity, and composition of the prokaryotic granule microbiome.

2. Materials and methods

2.1. Experimental setup

Four AGS systems operated in a CFR were used in this study (see supplementary materials). The bioreactors (height of 72 cm and diameter of 10 cm) had an operational volume of 6 L (see supplementary materials). A complete description of the novel AGS technology used in this study was recently provided by Rosa-Masegosa et al. (2023). A hydraulic retention time of 8 h was used. Air was supplied through fine bubbles at a rate of 4.5 L min^{-1} from the bottom of the bioreactors. All bioreactors were connected to a single compressor to ensure aeration was similar in all of them during the experimental period. The bioreactors were kept at a controlled room temperature in the range of 19–21 °C.

The bioreactors were inoculated with 1 L of granular biomass taken from a lab-scale AGS operated in an SBR for 3 months in the Institute of Water Research (Granada, Spain). The granular biomass was made from activated sludge from the WWTP of “Los Vados” (Granada, Spain). The bioreactors operated for 2 weeks until they reached stable conditions; they were fed with synthetic medium (see supplementary materials) simulating domestic wastewater during the experiment (De Kreuk et al., 2005). Four treatments were assayed for 3 months, with one treatment per bioreactor: control without anticancer drugs (CT) and three concentration levels of drugs (low, LW; medium, MD; and high, HG) (Table 1). The bioreactors were operated for an additional month, during which they were fed with synthetic medium without anticancer drugs. The anticancer drugs CP, MTX, and TMX were used in this experiment because they are commonly detected in influents and effluents of WWTPs (Nassour et al., 2020; Castellano-Hinojosa et al., 2023a). The concentration levels for each anticancer drug were selected from those reported previously (Nassour et al., 2020; Castellano-

Table 1
List of treatments assayed in this study. Cyclophosphamide (CP), tamoxifen (TMX), and methotrexate (MTX) applied at different concentration levels were used.

Concentration level	Acronym	Anticancer drug	Concentration (ng/L)
Control	CT	None	–
Low	LW	CP	60
		TMX	1.5
		MTX	40
Medium	MD	CP	600
		TMX	15
		MTX	400
High	HG	CP	6000
		TMX	150
		MTX	4000

Hinojosa et al., 2023a). Fresh stock solutions of the drugs were made weekly during the experiment to reduce degradation and kept in the freezer at −20 °C until use.

2.2. Physicochemical analyses

The physicochemical parameters chemical oxygen demand (COD), concentrations of ammonium, nitrate, and nitrite (NH_4^+, NO_3^-, and NO_2^-, respectively), and acetate (CH_3-COO^-) in the influent and effluent were analyzed twice per week during the experimental period as described by Castellano-Hinojosa et al., (2023b). The concentration of NO_2^- was below the detection limit of 0.1 ppm during the experiment. The OM removal efficiency (%) was calculated as the difference in the concentration of acetate between the influent and effluent. Changes in biomass concentration in the reactors were quantified by measuring mixed liquor suspended solids (MLSS) (APHA, 2022). The total suspended solids (SS) in the effluent were measured (APHA, 2022). The setting velocity and size of the granules were measured as reported by Castellano-Hinojosa et al., (2023b).

2.3. Anticancer drug quantification

Granules (approximately 200 mL) and water (approximately 200 mL) from the bioreactors were collected in duplicate after 5, 15, 45, 60, 90, and 120 days of operation and stored at −20 °C until use. The concentrations of CP, MTX, and TMX in the granules and water were determined as recently described in Castellano-Hinojosa et al., (2023b). Briefly, samples were pre-concentrated using solid-phase extraction (SPE) and the Oasis HLB (200 mg, 6 mL, Waters Corporation – Milford, MA, USA) cartridge (Ferrando-Climent et al., 2014). A nitrogen stream was used to evaporate the final extracts, which were then reconstituted with 250 μL of methanol–water (10:90, v/v). Standard curves containing six different concentrations of each of the anticancer drugs were prepared in the synthetic medium to estimate the possible matrix effect during the pre-concentration step (Castellano-Hinojosa et al., 2023b). The recovery efficiencies (%) for CP, MTX, and TMX were 68.1 % ± 5.2 %, 77.1 % ± 2.5 %, and 51.8 % ± 2.8 %, respectively, calculated as described earlier (Ferrando-Climent et al., 2014).

2.4. UHPLC-QqLit method

Chromatographic separation was performed using an ultra-high-performance liquid (UHPLC; model 1260 Infinity II) chromatography system (Agilent, USA) equipped with a ZORBAX Eclipse Plus C18 column (Agilent, USA) and a 6470 triple quadrupole-QqQ (Agilent, USA), as reported by Castellano-Hinojosa et al., (2023b). The detection limit was of 0.1 ppb for the three drugs.

2.5. Collection of biomass and DNA extraction and quantification

Granules were collected in duplicate after 5, 15, 45, 90, and 120 days of operation. Samples were centrifuged at 10,000 rpm at 20 °C for 3 min, and the pelleted biomass was kept at −20 °C until use. The FastDNA SPINK Kit for Soil (MP Biomedicals, Solon, OH, USA) was selected for DNA extraction. The DNA was quantified using the Qubit™ DNA High Sensitivity Assay Kit (Thermo Scientific, USA) and stored at −20 °C until use.

2.6. Quantification, sequencing, and analysis of prokaryotic communities

The absolute abundances of bacterial and archaeal (16SB and16SA, respectively) communities were estimated by quantitative PCR (qPCR). The PCR reactions and standards were prepared and checked as described in Castellano-Hinojosa et al. (2018).

The prokaryotic (Bacteria and Archaea) community was sequenced using the Pro341F and Pro805R primers (Takahashi et al., 2014) in Novogene Europe facilities (Cambridge, United Kingdom). The amplicon sequences were processed using QIIME2, as described earlier (Castellano-Hinojosa & Strauss, 2021). On average, 38,694 high-quality sequences per sample were obtained after the analysis of the sequences. Raw sequences can be found in the NBCI under BioProject PRJNA1033055.

The alpha and beta diversity indices of the prokaryotic community were calculated as described earlier by Castellano-Hinojosa et al. (2021), using the R software v. 4.2.2. The DESeq2 analysis (Love et al. 2014) in R was used to identify prokaryotic genera with relative abundance significantly different among treatments at different operational days.

2.7. Statistical analysis

Data analysis was run in the R software v. 4.2.2. Measured physicochemical and microbial abundance variables were examined by conducting the Shapiro–Wilk test to evaluate normality and the Bartlett test to assess homoscedasticity. A redundancy analysis (RDA) was run to investigate the relationships between the absolute abundances of 16SB and 16SA communities and changes in the performance parameters (% COD removal, %OM removal, %TN removal, MLSS, and SS). Data were log-transformed. The RDA was run in the CANOCO 4.5 software, using the Monte Carlo permutation test (Leps & Smilauer, 2003). Correlation coefficients of Pearson were calculated between the relative abundances of the differentially abundant prokaryotic genera and each of the performance parameters (% COD removal, % OM removal, % TN removal, MLSS, and SS), using the vegan package in R.

3. Results and discussion

3.1. Effects of anticancer drugs on granular biomass

Treatment with MD and HG rapidly reduced the MLSS concentration compared to treatment with CT until day 30 of operation, after which the MLSS concentrations remained, without variation, in the range of 3,780–3,520 mg L^{-1} and 3,400–3,210 mg L^{-1}, respectively (Fig. 1A). In the MD treatment, the MLSS concentration returned to CT levels after not adding the drugs for 30 days at the end of the experimental period, whereas in the HG treatment, it remained lower (Fig. 1A). No differences in the MLSS concentration were observed during the experimental period between the LW and CT treatments, with values from 4,200–4,500 mg L^{-1} (Fig. 1A). The application of MD and HG gradually reduced the size (Fig. 1B) and setting velocity (Fig. 1C) of the granules compared to CT application, particularly during the first 30 days of operation. Granule size and setting velocity returned to CT levels after not adding the drugs for 30 days in the MD treatment but remained lower in the HG treatment (Fig. 1B and C). Treatment with MD and HG resulted in gradual increases in the SS concentration in the effluents compared to CT during the first 30 days of operation to gradually diminish afterwards towards the end of the experiment (Fig. 1D). These increases in SS in the effluent were greater in HG and did not reach CT levels, even after not adding the drugs for 30 days (Fig. 1D). Treatment with LW had no impact on the size and setting velocity of the granules and SS in the effluent compared to CT (Fig. 1B – D).

Our results show that anticancer drugs present at medium and high concentrations can have negative impacts on the concentration of granular biomass and alter the setting velocity and size of the granules in an AGS system operated in a CFR. These effects occurred rapidly during the first 30 days of operation and remained until the end of the experimental period but did not increase with time, suggesting that the microbial community can adapt to the external stress induced by the anticancer drugs. The size of the granules in this study was similar to that in other AGS systems operated in SBR and CFR (Muñoz-Palazón et al., 2021; Rosa-Masegosa et al., 2023) (see supplementary materials). The dose of anticancer drugs appears to control the variations in MLSS as

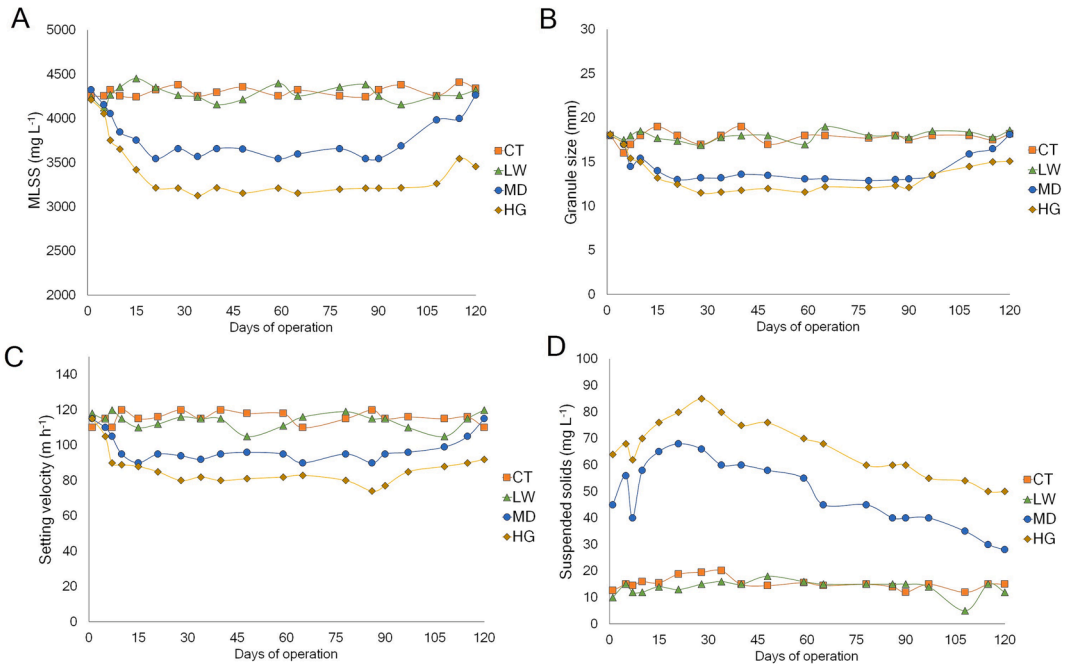

Fig. 1. Average MLSS (A), granule size (B), setting velocity of the granules (C) and SS (D) during the experimental period. Treatments are defined in Table 1. MLSS, mixed liquor suspended solids; SS, suspended solids.

well as the setting velocity and size of the granules since these alterations increased with greater drug concentrations. Interestingly, the negative impacts of MD treatment on MLSS, setting velocity of granules, and granule size disappeared after not adding the drugs for 30 days, showing that the plasticity of the AGS technology operated in a CFR had returned to control conditions. However, the ability to recover to control conditions appears to be driven by the drug concentration present in the wastewater since recoveries of MLSS as well as setting velocity and granule size were not observed in the HG treatment after not adding the drugs for 30 days.

The decreases in the size and setting velocity of the granules observed after adding the anticancer drugs at MD and HG concentrations levels were accompanied by increases in SS in the effluent. This indicates that anticancer drugs may alter granule formation by inducing the formation of smaller and less compact granules and/or increasing the disintegration of granular sludge. Similar results were recently observed in an AGS system in SBR mode and treating anticancer drugs (Castellano-Hinojosa et al., 2023b). Other studies reported more condense granules in AGS systems operated in an SBR but treating antibiotics (Wan et al., 2018; Muñoz-Palazón et al., 2021). Whether differences in the action mode, chemical structure of the drugs, and/or degradation rates between anticancer drugs and antibiotics may explain these differences should be further explored.

Because anticancer drugs are frequently found in domestic wastewater (Castellano-Hinojosa et al., 2023a), our results suggest that increased attention should be paid to the presence and concentration of these substances as they may impact treatment performance by temporary altering the granular biomass.

3.2. Effects of anticancer drugs on OM and N removal

Treatment with MD and HG rapidly reduced the COD (Fig. 2A) and OM (Fig. 2B) removal compared to treatment with CT during the first 10 days of operation, but the values gradually increased afterwards to reach CT levels after approximately 60 days of operation. Treatment with LW had no impact on COD and OM removal compared to treatment with CT throughout the experimental period. The application of MD and HG reduced TN removal (Fig. 2C) compared to CT application, particularly during the first 15 days of operation. Subsequently, TN removal gradually increased to reach CT levels after approximately 90 days of operation. Temporal variations in the content of NH_4^+-N and NO_3^--N are presented in the supplementary material. Although overall microbial activity was not measured in this study (e.g., via enzyme activities or RNA analyses), our results suggest that N-cycling processes are negatively affected by the concentration of anticancer drugs (Fig. 2C) and that they recover after not adding these substances.

Based on our results, anticancer drugs present at medium and high concentrations can temporarily reduce OM and TN removal in an AGS system operated in a CFR. These gradual decreases are likely due to reduced microbial abundances (see Section 3.4.) and MLSS concentrations as well as increased cell death immediately after treatment. Because the treatment performance in terms of OM and TN removal was totally recovered after 60 days of operation, our results suggest that microorganisms take some time to adapt to the medium and high concentrations of anticancer drugs, followed by efficient nutrient removal. Notably, whilst OM and TN removal totally recovered to control levels in the MD and HG treatments, MLSS, size and setting velocity of the granules remained lower during the first 90 days of operation. Together, these results show that nutrients can efficiently be removed when sufficient granular biomass is present in the system under the stress conditions induced by different anticancer drug concentration levels. The temporal changes in the NH_4^+ and NO_3^- concentrations suggest that the alteration in TN removal after drug application was likely due to reduced nitrification. This agrees with previous studies on the impacts of antibiotics on nitrifiers in AGS in SBR mode (Muñoz-Palazón et al., 2021). Whether nitrifiers may be more sensitive to anticancer drugs compared to denitrifiers should be further explored.

No significant impacts of medium and high concentrations of anticancer drugs on OM removal were observed in AGS systems in SBR mode but TN removal was temporary reduced (Castellano-Hinojosa et al., 2023b). It is possible that differences in technology configuration, environmental conditions (e.g., temperature), and/or original microbial community composition may explain the temporal reduction in OM removal observed in this study as the composition of the synthetic medium and the COD concentration of the influent were similar among studies. Other studies reported that antibiotics had no impact on OM

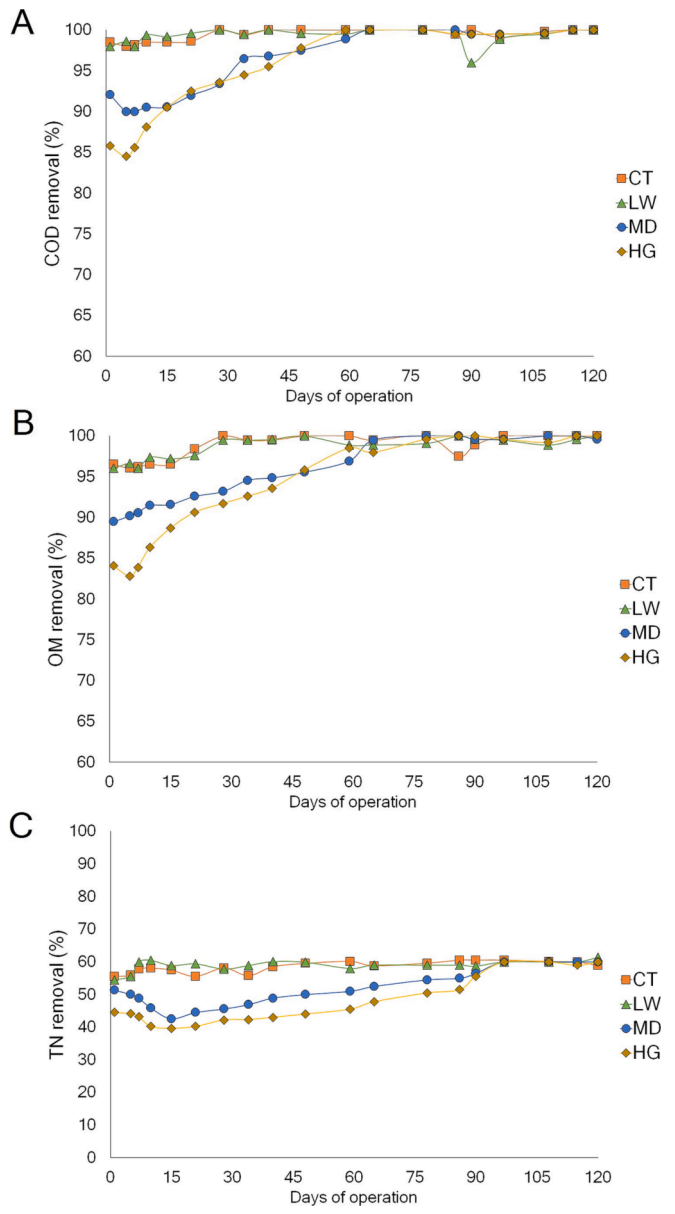

Fig. 2. Average COD (A), OM (B), and TN (C) removal % (A). Treatments are defined in Table 1. COD, carbon oxygen demand; OM, organic matter, TN, total nitrogen.

removal using AGS systems in SBR operational mode (Muñoz-Palazón et al., 2021). Future studies on the effects of anticancer drugs on treatment performance under different COD concentrations may help better understand the impact of anticancer drugs on nutrient removal in AGS systems operated in a CFR.

3.3. Efficiency of anticancer drug removal

The concentrations of the three anticancer drugs in the influent, effluent, and granules after 5, 15, 45, 60, 90, and 120 days of operation are shown in the supplementary material. Regardless of the time point and treatment, a greater concentration of CP and MTX were detected in the granules compared to the effluents. Whilst CP and MTX were detected in the effluent and granules after not adding drugs for 30 days in the HG treatments, only CP was detected in granules in the MD treatment after this period (see supplementary materials). The CP, TMX, and MTX drugs were totally removed in the LW treatment (Table 2), whereas only TMX was totally removed in the MD and HG treatments.

Table 2
Removal efficiency of cyclophosphamide (CP), tamoxifen (TMX), and methotrexate (MTX). Efficiency removal % was calculated as follows: [concentration in the influent, ng/L – (concentration in the effluent, ng/L + granules × MLSS, ng/L)]/concentration in the influent, ng/L ×100. For each column, values followed by the same letter are not significant different among according to ANOVA and Tukey's HSD tests ($p \leq 0.05$). Values are expressed as mean with standard error (n = 2). MLSS, mixed liquor suspended solids.

	Time point	CP	TMX	MTX
LW	5	100a	100a	100a
	15	100a	100a	100a
	45	100a	100a	100a
	60	100a	100a	100a
	90	100a	100a	100a
MD	5	65.1±3.1b	100a	85.6±3.1b
	15	67.4±3.6b	100a	87.9±3.8b
	45	69.0±3.5b	100a	88.7±3.4b
	60	70.1±3.2b	100a	88.8±4.2b
	90	72.3±3.0b	100a	88.7±3.5b
HG	5	51.5±3.8c	100a	63.6±3.0c
	15	53.9±3.3c	100a	65.6±2.9c
	45	55.2±2.8c	100a	66.6±4.1c
	60	55.7±3.0c	100a	67.1±3.6c
	90	55.8±2.1c	100a	67.2±3.3c

The CP and MTX removal efficiencies were significantly greater in MD (in the range of 65.1 %–72.3 % and 85.6 %–88.7 %, respectively) compared to the HG (in the range of 51.5 %–55.8 % and 63.6 %–67.2 %, respectively) treatment and gradually increased over time (Table 2).

Our results show that AGS systems in a CFR mode can help remove anticancer drugs under different concentrations. These results have important environmental implications as these types of drugs pass through WWTPs almost unaltered (Castellano-Hinojosa et al., 2023a). Recently, it was demonstrated that AGS systems operated in an SBR cannot completely degrade TMX at high levels (Castellano-Hinojosa et al., 2023b). Therefore, the results of this study show that the continuous flow may induce a greater removal of specific anticancer drugs, most likely because of constant aeration and/or greater MLSS concentration, favoring the removal of anticancer drugs in CFRs. Previous work has shown CP removal efficiencies ranging from 20 % to 100 % using medium concentration levels and the MBR technology (Yadav et al., 2021). Our results showed that continuous-flow AGS systems can have similar CP removal efficiencies even at high concentration levels. Yet, additional studies on anticancer drug removal optimization (e.g., at different HRTs) using the AGS technology and different drugs are needed.

In previous studies, CP and MTX were more persistent in wastewater compared to TMX (Ferrando-Climent et al., 2014; Castellano-Hinojosa et al., 2023a). This is in line with our results showing a greater prevalence of CP and MTX in granules and water over time compared to TMX. The CP and MTX drugs are known to have high toxicity, persistence [e.g., half-live and log K_{ow} (octanol/water partition coefficient), and ubiquity in wastewater and natural environments (Ferrando-Climent et al., 2014; Castellano-Hinojosa et al., 2023a). Our results also indicate that CP and MTX may accumulate in granules more than in water, suggesting a type-dependent sorption to granular biomass. These results also suggest that anticancer drugs can be removed by absorption and biodegradation in AGS systems but the main removal mechanism may be anticancer-drug specific. Future studies on the biodegradation of anticancer drugs should consider both biomass and water to fully optimize the removal of these substances, as previously observed with antibiotics in AGS systems (Muñoz-Palazon et al., 2021; Cheng et al., 2023).

3.4. Impacts of anticancer drugs on the abundance of the bacterial and archaeal communities and their relationship with performance parameters

Treatment with MD and HG significantly decreased the absolute abundances of 16SB and 16SA communities after 5, 15, 45, and 90 days of operation (Fig. 3A and B). These decreases were significantly greater in the HG treatment compared to MD. In the MD treatment, the absolute abundances of the 16SB and 16SA communities returned to CT levels after 30 days without adding the drugs (Fig. 3A and B). The RDA results indicate that the absolute abundances of 16SB and 16SA communities were significantly and positively correlated with the % removal of OM, COD, and TN as well as granule size (r >0.75; Fig. 3C; see supplementary materials). A strong negative correlation was observed between the 16SB and 16SA gene abundances and SS in the effluent (r > −0.91; Fig. 3C; see supplementary materials). Granule size and the setting velocity of the granules were positively correlated among them (r = 0.80; Fig. 3C; see supplementary materials).

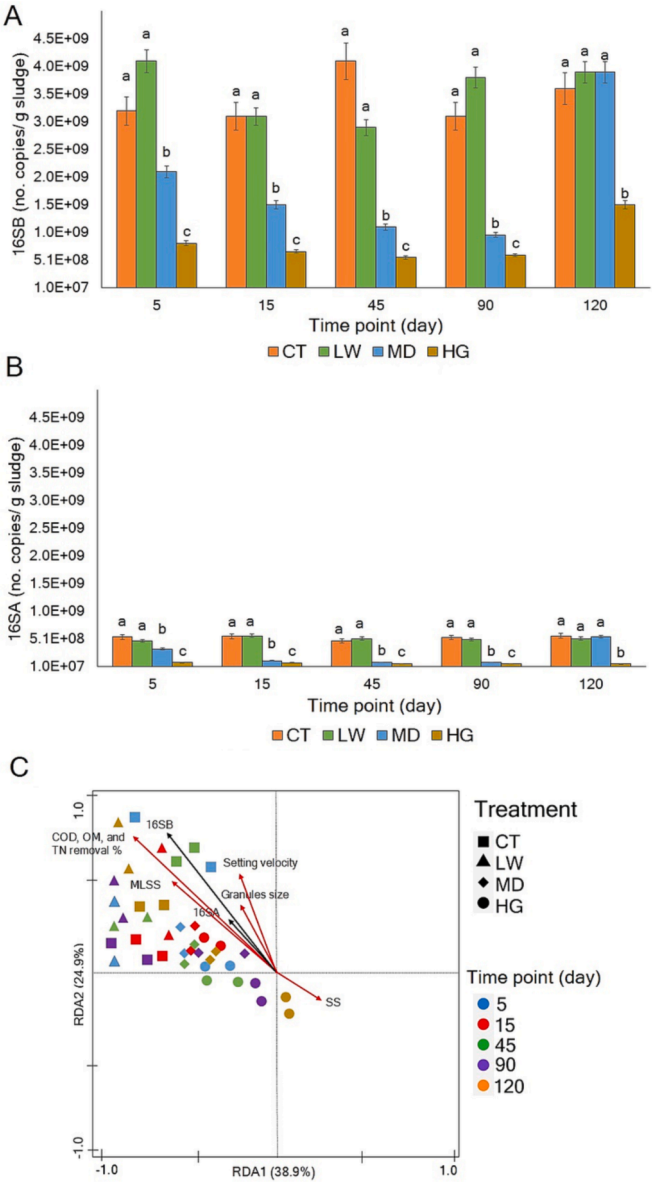

Fig. 3. Changes in the absolute abundance of the bacterial (16SB, A) and archaeal (16SA, B) communities in the granule microbiome during the experimental period. Treatments are defined in Table 1. Different letters above the bars indicate significant differences between treatments (Tukey's HSD, $p < 0.05$). Values are expressed as mean with standard error. C. RDA plot of correlation between physicochemical parameters and the absolute abundance of the bacterial (16SB) and archaeal (16SA) communities during the experimental period. Treatments are defined in Table 1. Red solid arrows indicate physicochemical parameters, and black arrows indicate total abundances of target genes. COD, chemical oxygen demand; OM, organic matter, TN, total nitrogen; MLSS, mixed liquor suspended solids; SS, suspended solids.

We found that anticancer drugs present at medium and high concentration levels can reduce the absolute abundances of prokaryotic communities of the granule microbiome, an effect that occurred rapidly after treatment application and did not increase over time. This is the first study showing the impacts of anticancer drugs on the abundances of microbial communities using a continuous-flow AGS technology. The alterations of 16SB and 16SA microbial communities were closely related to variations in different performance parameters. For example, decreases in the abundances of 16SB and 16SA after MD and HG application were directly related to decreases in % removal of OM, COD, and TN, MLSS concentration, and granule size. Although the microorganisms of the granule microbiome are the key drivers of treatment performance in AGS systems, our findings indicate a dose-dependent effect of anticancer drugs on treatment parameters through changes in the absolute abundances of archaeal and bacterial communities. Interestingly, the abundance of these communities could rapidly recover after not adding drugs for 30 days, as shown in the MD treatment. However, this recovery may take longer in the HG treatment.

3.5. Impacts of anticancer drugs on the diversity and composition of prokaryotic communities

Regardless of the time point, treatment with MD and HG significantly reduced all indices of alpha diversity of the prokaryotic community (Fig. 4A). These decreases were significantly greater in the HG treatment compared to MD and increased over time (Fig. 4A). In the MD treatment, the values of alpha diversity returned to the CT level after not adding the drugs for 30 days, whereas in the HG treatment, they remained significantly lower (Fig. 4A). Treatment with LW had no significant impact on alpha diversity compared to CT. Both treatment and time point had a significant impact on the beta diversity of the prokaryotic community, according to the NMDS and PERMANOVA results (Fig. 4B). Beta diversity was significantly different among MD, and HG, and the CT treatment throughout the experimental period ($p < 0.01$). Regardless of the time point, no significant differences in the prokaryotic community composition were observed between CT and LW treatments ($p > 0.05$). The prokaryotic community of the granule microbiome was dominated by the phyla Proteobacteria (82.5 %) and Actinobacteria (10.1 %) (see supplementary materials). Gradual increases in the relative abundances of taxa belonging to the phyla Parcubacteria and Firmicutes were observed in the MD and HG treatments compared to CT over time (see supplementary materials). It is interesting to note that all archaeal phyla had less than 1 % of relative abundance in the prokaryotic granule microbiome, thus showing that Bacteria was the dominant and more diverse prokaryotic domain.

Medium and high concentrations of anticancer drugs reduced the diversity and induced changes in the composition of the prokaryotic microbiome of the granule in an AGS system operated in a CFR. Variations in alpha diversity values showed that anticancer drugs do not only reduce the number of taxa but also result in a less diverse community. It is important to note that anticancer drugs are designed to inhibit DNA synthesis, and therefore, a decrease in alpha diversity was expected. In line with this, previous studies have shown that anticancer drugs can negatively impact different aquatic communities in natural environments (Negreira et al., 2014; Castellano-Hinojosa et al., 2023a). The recovery of alpha diversity in the MD treatment after not adding the drugs for 30 days points to the plasticity of the granule microbiome, facilitating recovery after external stress induced by anticancer drugs. The changes in the composition of prokaryotic communities suggest that the impacts of these substances on the granule microbiome are broad

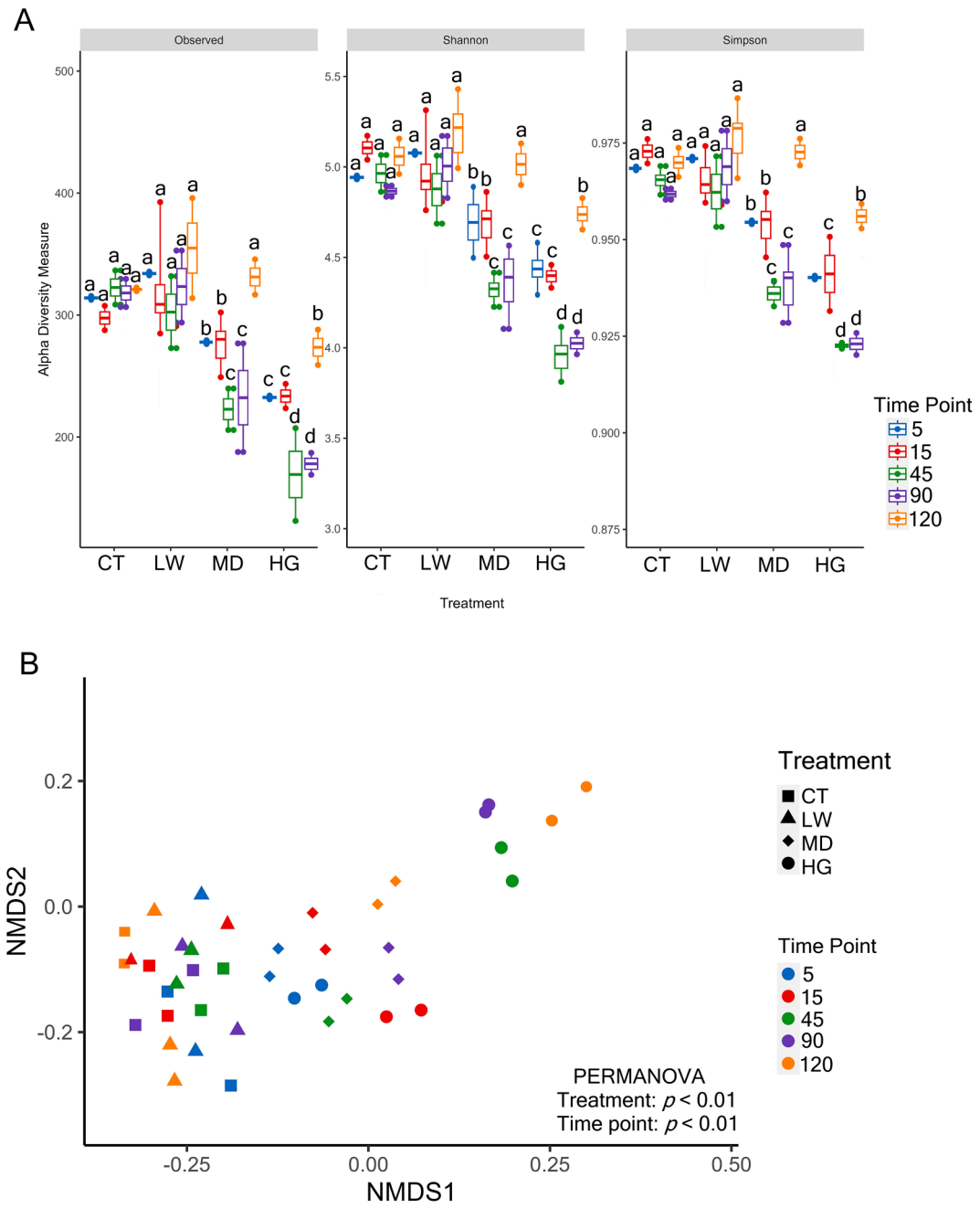

Fig. 4. A. Number of ASVs, and values of Shannon and inverse Simpson diversity indices for the prokaryotic community during the experimental period. Different letters above the bars indicate significant differences between treatments and time points (Tukey's HSD, $p \leq 0.05$). Values are expressed as mean with standard error. B. Non-metric multidimensional scaling (NMDS) plots on unweighted UniFrac distances for the prokaryotic community during the experimental period. Differences in community composition between treatments and time points were tested by permutational analysis of variance (PERMANOVA), and p values ≤ 0.01 were considered significant. Treatments are defined in Table 1.

and vary over time. Overall, our results have important bioengineering implications as they show that the presence of anticancer drugs in wastewater can directly impact the diversity and composition of prokaryotic communities, which play critical roles in biological wastewater treatment. Our data suggest a dose-dependent effect of these type of pharmaceutical drugs on the granule microbiome, highlighting the importance of controlling temporal variations in the concentrations of anticancer drugs.

3.6. Genera differing in their abundances among treatments and their relationships with performance parameters

Differentially abundant genera were detected among LW, MD, and HG treatments compared to CT, providing additional insights into the effects of anticancer drugs on the granule microbiome. No prokaryotic genera with different abundances between LW and CT treatments were observed throughout the experimental period. A total of 13 and 22 genera were significantly depleted in MD and HG treatments, respectively, compared to CT (Fig. 5). The genera *Bdellovibrio*, *Meganema*, and *Roseomonas* were enriched in MD and HG treatments compared to CT (Fig. 5). Further, the genus *Caulabacter* was enriched in HG compared to

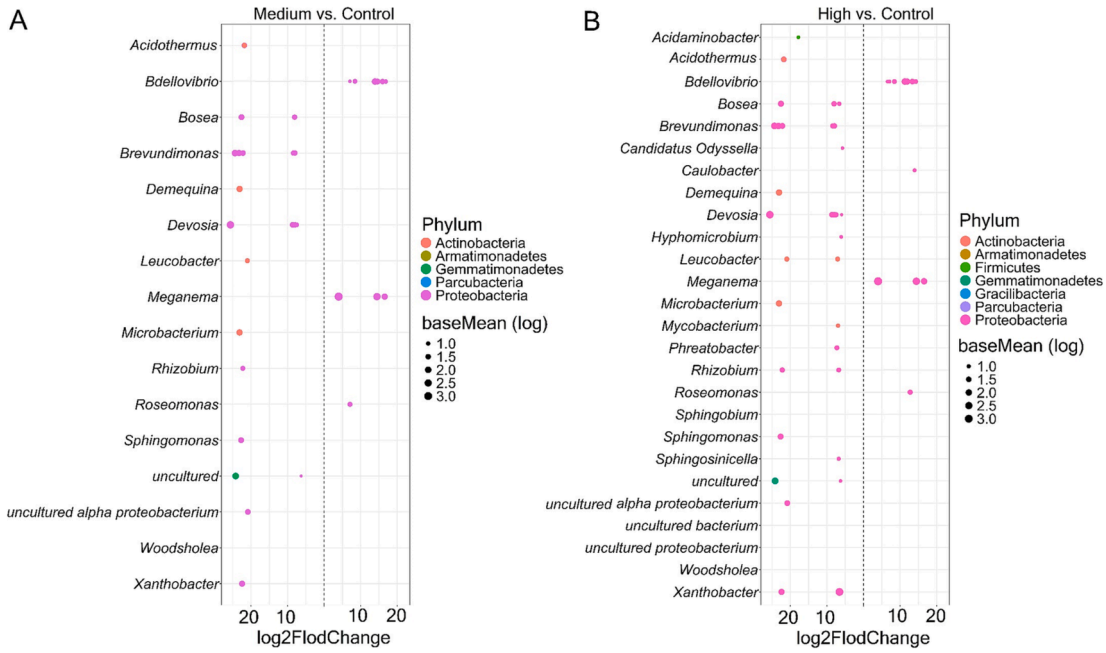

Fig. 5. Differential abundance ASVs at the genus taxonomic level between medium and control (A) and high and control (B) treatments. Treatments are defined in Table 1. The fold change is shown on the X axis and genera are listed on the Y axis. Each colored dot represents an ASV that was identified by DESeq2 analysis ($p \leq 0.05$).

CT (Fig. 5). It is interesting to note that only bacterial genera were affected by the anticancer drugs whereas no archaeal genera showed significant changes in its relative abundance after the addition of these substances. This could be due to archaeal taxa being more sensitive to the anticancer drugs compared to bacteria (Khelaifia & Drancourt, 2012), and/or the low diversity of archaeal communities in the granule microbiome in this study.

A Pearson correlation showed significant associations between all differentially abundant genera and different performance parameters (Table 3). For example, 12 genera, including *Acidothermus*, *Bosea*, *Brevundimonas*, *Demequina*, *Devosia*, *Sphingobium*, *Woodsholea*, and others, were significantly and positively correlated (r >0.72) with COD and OM removal (Table 3). Previous studies have shown that the members of these genera are involved in OM removal in biological treatment processes including AGS systems (He et al., 2020; Dai et al., 2021). Other genera such as *Bosea*, *Demequina*, *Hypomicrobium*, *Phreatobacter*, and *Rhizobium* were positively correlated (r >0.78) with TN removal and are key denitrifiers in AGS systems (Bucci et al., 2022; Zhang et al., 2022; Guo et al., 2023). Strong positive correlations (r >0.77) were detected between the relative abundances of *Acidaminobacter*, *Hypomicrobium*, *Leucobacter*, and *Sphingomonas* and the concentration of MLSS, and setting velocity and size of the granules (Table 3). These genera are involved in granule formation in AGS systems (Chen et al., 2018; Gonzalez-Martinez et al., 2017; Guo et al., 2023; Liu et al., 2023). The

Table 3
Pearson's product-moment correlation coefficients between the differentially abundant genera displayed in Fig. 5 and the physicochemical parameters measured during the experimental period. Correlations between the gene abundances and abiotic variables are presented. Significant correlations ($p \leq 0.05$) are boldfaced. COD, chemical oxygen demand; OM, organic matter, TN, total nitrogen; MLSS, mixed liquor suspended solids; SS, suspended solids.

Domain	Genus	%COD removal	%OM removal	%TN removal	MLSS	SS	Granule size	Setting velocity
Bacteria	*Acidaminobacter*	0.33	0.21	0.85	**0.84**	0.29	**0.80**	**0.90**
	Acidothermus	**0.75**	**0.80**	0.25	0.16	0.54	0.54	0.45
	Bdellovibrio	−0.15	−0.25	−0.33	−0.15	**0.85**	−0.15	−0.25
	Bosea	**0.78**	**0.80**	**0.78**	0.15	0.20	−0.24	−0.33
	Brevundimonas	**0.90**	**0.85**	**0.21**	**0.80**	−0.25	0.09	0.11
	Candidatus Odysella	**0.77**	**0.81**	0.15	−0.33	−0.11	−0.05	0.14
	Caulobacter	0.34	0.54	0.22	**0.90**	**0.75**	−0.56	0.58
	Demequina	**0.77**	**0.88**	**0.80**	−0.15	0.34	0.08	0.06
	Devosia	**0.72**	**0.75**	**0.89**	−0.22	0.27	0.25	0.18
	Hyphomicrobium	0.45	0.50	**0.90**	**0.77**	−0.19	**0.87**	**0.88**
	Leucobacter	0.33	0.22	0.15	**0.88**	−0.11	**0.86**	**0.90**
	Meganema	−0.28	−0.36	−0.45	−0.65	**0.88**	−0.32	−0.54
	Microbacterium	**0.88**	**0.95**	0.22	**0.77**	−0.41	−0.33	−0.22
	Mycobacterium	**0.95**	**0.95**	0.33	0.22	−0.21	−0.10	−0.09
	Phreatobacter	0.45	0.40	**0.95**	0.15	0.16	0.18	0.36
	Rhizobium	0.33	0.11	**0.76**	−0.10	−0.06	0.15	0.11
	Roseomonas	−0.15	0.22	−0.33	−0.55	**0.90**	−0.40	−0.29
	Sphingobium	**0.80**	**0.85**	0.65	0.54	−0.25	−0.17	−0.16
	Sphingomonas	0.30	0.29	0.42	**0.85**	−0.35	**0.80**	**0.85**
	Sphingosinicella	**0.77**	**0.80**	0.31	0.15	0.16	0.06	0.03
	Woodsholea	**0.80**	**0.78**	**0.90**	0.22	0.15	0.10	0.06
	Xanthobacter	0.22	0.15	0.32	**0.90**	−0.35	**0.80**	**0.77**
	uncultured	**0.84**	**0.95**	0.33	0.45	0.33	0.15	0.35

genera *Bdellovibrio*, *Caulobacter*, *Meganema*, and *Roseomonas* were strongly positively related to SS in the effluent (r >0.75) and have previously been related to the degradation of granule biomass in AGS systems (Chen et al., 2018; Feng et al., 2017; Liu et al., 2020).

Based on our results, the changes induced by the medium and high concentrations of anticancer drugs in the relative abundances of specific taxa directly impacted treatment performance. We identified specific depleted taxa with known key roles in C and N removal (mainly denitrification) and granule formation, which aligns well with the reductions in the absolute abundance of prokaryotic communities, and suggests biological activity was temporarily reduced after the addition of anticancer drugs. Of note, we found no microbial taxa known to be involved in nitrification were affected by the presence of anticancer drugs, thus suggesting that nitrifiers may not be sensitive to these drugs in AGS operated in CFR. This is important as nitrification is a key process in AGS systems. The taxa enriched in the MD and HG treatments were associated with an increased level of SS in the effluent, which again suggests than investigating the effects of anticancer drugs on the prokaryotic microbiome can help understand variations in treatment performance in AGS systems operated in a CFR.

4. Conclusions

This study shows that medium and high concentration levels of anticancer drugs present in domestic wastewater can alter treatment performance and the granule microbiome of a continuous-flow aerobic granular sludge system. These impacts mainly occur during the first 15 days of operation. Removal efficiencies of anticancer drugs were in the range of 51.2–100 %, showing that this technology facilitates the removal of drugs. The effects of such drugs on the granule microbiome were dose-dependent, with medium and high concentration levels reducing the abundance and diversity of prokaryotic communities, altering their community composition. This study has important environmental and bioengineering implications.

CRediT authorship contribution statement

Antonio Castellano-Hinojosa: Conceptualization, Investigation, Supervision, Validation, Visualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Manuel J. Gallardo-Altamirano:** Conceptualization, Investigation, Methodology, Supervision, Data curation, Writing – review & editing. **Alejandro González-Martínez:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Jesús González-López:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.130195.

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