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Anticancer drugs alter active nitrogen-cycling communities with effects on the nitrogen removal efficiency of a continuous-flow aerobic granular sludge system

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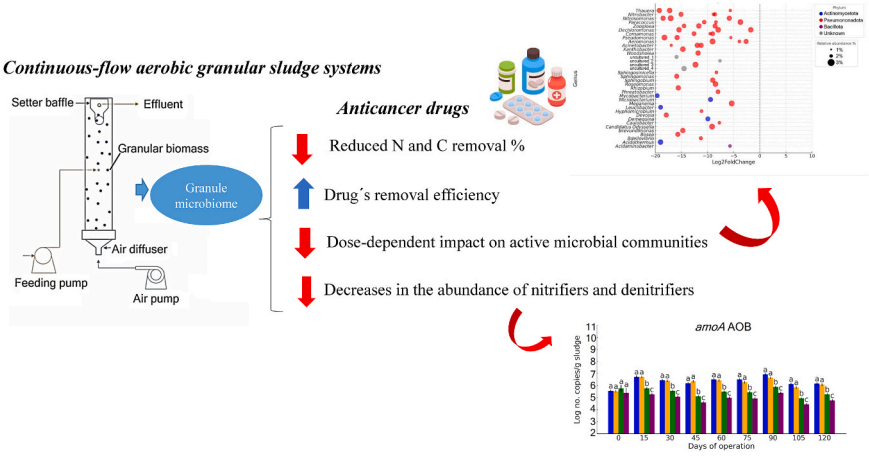
HIGHLIGHTS

- Anticancer drugs in aerobic granular sludge systems impact treatment performance.
- Organic matter and nitrogen removal efficiencies are reduced by the presence of drugs.
- Anticancer drugs are efficiently removed, but removal varies depending on the drug.
- Active nitrifiers and denitrifiers are reduced in a drug-dose-dependent manner.
- Anticancer drugs affect prokaryotic diversity and community composition.

GRAPHICAL ABSTRACT



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ABSTRACT

There is increasing awareness of the presence of anticancer drugs (ACDs) in wastewater. Nonetheless, how ACDs affect the performance of wastewater treatment systems and their microbial populations remains largely unclear. This study investigated the effects of three common ACDs (cyclophosphamide, tamoxifen, and methotrexate) at varying concentrations on physicochemical parameters and drug removal efficiency in an aerobic granular sludge (AGS) system operated in a continuous-flow reactor. Additionally, it examined the abundance of active microbial communities, including nitrifiers (*amoA* gene from ammonia-oxidizing bacteria and archaea) and denitrifiers (*napA*, narG, *nirK*, *nirS*, *nosZ* genes), as well as the biodiversity of active prokaryotic communities.

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The concentration level of ACDs determines variations in biomass density, granule integrity, and removal efficiencies of organic matter (OM) and total nitrogen. Both medium and high ACD concentrations negatively impact these physicochemical parameters. The findings revealed that AGS functioning within a continuous system could help remove ACDs, but removal efficiencies depended on the specific drug and concentration applied. At medium and high ACD concentrations a marked reduction in the abundance of active bacterial and archaeal communities, including nitrifiers and denitrifiers, was observed, alongside a decline in microbial diversity and a transformation in community composition. Specific bacterial genera, which are crucial for OM degradation, nitrification and denitrification were identified as particularly sensitive to anticancer drugs. Our findings highlight the need for monitoring and managing anticancer drugs in wastewater systems, as they can substantially alter treatment performance, nitrogen-cycling communities, and bacterial community composition.

1. Introduction

Cancer is one of the leading public health issues worldwide, resulting in approximately 10 million deaths and accounting for nearly one out of every six deaths in the year 2022 (WHO, 2022). As cancer incidence continues to rise, it is expected to reach an estimated 29.5 million new cases by 2040 (Yadav et al., 2021), the use of anticancer drugs (ACDs) has significantly increased (Franquet-Griell et al., 2017). These substances, designed to hinder DNA replication and cell division, are only partially metabolized by the human body and enter wastewater systems via excretion. Due to their high toxicity and persistence, ACDs are difficult to degrade biologically in wastewater (Ferrando-Climent et al., 2014; Li et al., 2021) and can travel through urban water systems, affecting rivers and groundwater (Castellano-Hinojosa et al., 2023a). Commonly detected ACDs in wastewater comprise tamoxifen (TMX), cyclophosphamide (CP), and methotrexate (MTX), with typical concentrations reaching hundreds to thousands of ng/L (Nassour et al., 2020; Castellano-Hinojosa et al., 2023a).

Conventional wastewater treatment plants (WWTPs) rely on activated sludge technology which appears not to be effective at removing ACDs (Franquet-Griell et al., 2017; Li et al., 2021; Yadav et al., 2021). Alternative methods, like membrane bioreactors (MBR), exhibit a wide range of removal efficiencies for anticancer drugs (20–90%), depending on the particular drug and its concentration (Yadav et al., 2021). Aerobic granular sludge (AGS) systems, an emerging technology, are being investigated for their potential to remove common contaminants and pharmaceuticals (Samaei et al., 2023; Castellano-Hinojosa et al., 2024a; Perez-Bou et al., 2024). In particular, continuous-flow AGS systems are noted for their energy efficiency and reduced footprint (Samaei et al., 2023), while efficiently removing organic matter (OM) and nitrogen (N) into a single bioreactor (Rosa-Masegosa et al., 2023). Recent research has demonstrated that continuous-flow aerobic granular sludge (AGS) systems can enhance the removal of anticancer drugs, with removal efficiencies varying between 53% and 100%, depending on the specific drug and its concentration (Castellano-Hinojosa et al., 2024a, 2024b). These investigations also indicated that ACDs could lead to a temporary decline in organic matter (OM) and nitrogen removal efficiencies when present at moderate to high concentrations. However, further research is necessary to assess the broader effects of ACDs on overall treatment performance and drug degradation in AGS systems.

Nitrification and denitrification are key processes for N removal in the granule microbiome of AGS systems (Rosa-Masegosa et al., 2023). Nitrification, which involves converting ammonium (NH_4^+) to nitrate (NO_3^-), is carried out by ammonia-oxidizing bacteria and archaea (AOB and AOA, respectively) containing the *amoA* gene and occurs in the outer layer of the granules (Rosa-Masegosa et al., 2023). Denitrification, the reduction of NO_3^- to dinitrogen gas (N_2), involves a series of genes: *napA* and *narG* encode nitrate reductases, *nirK* and *nirS* encode nitrite reductases, and *nosZ* encodes nitrous oxide reductase. Denitrification takes place in the inner layer of the granules (Rosa-Masegosa et al., 2023). Despite the critical roles that nitrifiers and denitrifiers play in AGS systems, the impact of ACDs on N-cycling communities remains largely unknown. This information may help understand variations in N removal efficiency when these substances are present in the wastewater and help optimize contentious-flow AGS systems. Other pharmaceutical substances, including antibiotics, have been shown to lower the abundance of nitrification and denitrification genes in aerobic granular sludge (AGS) systems, which in turn results in reduced nitrogen removal efficiency (Katipoglu-Yazan et al., 2016; Wang et al., 2020a; Muñoz-Palazon et al., 2021). Understanding how emerging contaminants such as ACDs may affect the abundance of specific N-cycling communities is important because this information can help elucidate potential distinct sensibilities of specific microbial groups to ACDs and whether they evolve over time and are controlled by the type of drug and/or concentration level. In addition, this information can help understand variations in specific N transformations within the granule microbiome and the impacts on the overall N removal efficiency of AGS systems.

To date, research on the impact of pharmaceutical products on microbial communities in AGS systems has mainly relied on DNA-based approaches. However, RNA-based assessments are considered more precise for studying microbiomes because the presence of relic DNA in the environment can distort the true abundance and diversity of microbial communities (Carini et al., 2016). Moreover, microbial species that exhibit high activity may be underrepresented or even overlooked in DNA-based analyses of microbial communities (Jousset et al., 2017). For this reason, we employed an RNA-based approach in our study. The objective of this research was to examine how three ACDs, frequently found in WWTPs, affect the absolute abundance of metabolically active microbial communities nitrifiers, and denitrifiers of a continuous-flow AGS system when applied at three varying concentration levels. Impacts on physicochemical parameters and ACD removal efficiency were also studied. In addition, variations in the diversity and composition of active prokaryotic communities were explored. Our hypothesis was that ACDs would reduce the abundance of nitrifiers and denitrifiers, leading to a negative impact on nitrogen removal efficiency when compared to a control without treatment. These effects were expected to be dependent on the concentration level of the drugs and may disappear after not adding these substances for a time. We also hypothesized that ACDs would induce changes in OM removal efficiency in the short term (e.g., a few weeks after the addition of the drugs) and affect the prokaryotic microbiome.

2. Materials and methods

2.1. Configuration and operation of the bioreactors

A novel continuous-flow AGS system was used in this study, which was comprehensively described by Rosa-Masegosa et al. (2023). A total of four bioreactors were used in the experiment. Each bioreactor (72 cm × 10 cm) had a total working volume of 6 L (see Supplementary Figs. S1A and B). The experiment was conducted with a hydraulic retention time of 8 h, and air was introduced at the base of the bioreactors at a flow rate of 6 L min^{-1}. A single compressor was used to provide consistent aeration across all bioreactors throughout the experiment. The main air supply line was connected to individual flow meters, allowing precise control of the airflow to each bioreactor. The temperature in the room was controlled, ranging between 18 °C and

22 °C. Initially, 1 L of granular biomass from a laboratory-scale AGS system at the Institute of Water Research, University of Granada (Spain), was used to inoculate each bioreactor. This granular biomass originated from activated sludge collected at the Los Vados WWTP (Granada, Spain). After inoculation, the bioreactors underwent a three-week acclimation period until stable operating conditions were reached. A synthetic medium resembling domestic wastewater was supplied to the bioreactors during the experiment [(De Kreuk et al., 2005); Supplementary Fig. S1C].

Four treatments were tested, each assigned to a separate bioreactor: control without ACDs (CT) and three concentrations of ACDs, classified as low (LW), medium (MD), and high (HG) concentration levels (Table 1). Each treatment was tested for 3 months. Subsequently, an additional month of operation followed, during which the bioreactors were supplied with a synthetic medium devoid of ACDs to check for a potential residual impact of the treatments on physicochemical parameters and microbial communities. The anticancer drugs (ACDs) used in this study—CP, MTX, and TMX—were selected due to their frequent detection in both influent and effluent of WWTPs (Nassour et al., 2020; Castellano-Hinojosa et al., 2023a), as well as their common combined application. The concentration of each drug was determined according to prior studies (Nassour et al., 2020; Castellano-Hinojosa et al., 2023a). To avoid degradation, new stock solutions of the ACDs were made each week throughout the experiment and kept at −20 °C until they were required.

2.2. Physicochemical analyses

Throughout the experimental phase, several physicochemical parameters were regularly monitored, including acetate (CH_3–COO^-), chemical oxygen demand (COD), ammonium (NH_4^+), nitrite (NO_2^-), NO_3^- levels following the protocol outlined by Castellano-Hinojosa et al. (2024a). Organic matter (OM) and nitrogen (N) removal efficiencies were calculated by comparing the concentrations of acetate and nitrogen species ($NH_4^+ + NO_2^- + NO_3^-$) in the influent and effluent, expressed as a percentage. Biomass concentrations in the reactors were determined by measuring mixed liquor suspended solids (MLSS) (APHA, 2012), while total suspended solids (SS) in the effluent were measured according to standard procedures (APHA, 2012). The size and settling velocity of the granules were assessed using the approach described by Castellano-Hinojosa et al. (2023b).

2.3. Quantification of anticancer drugs in water and granules

Duplicate granular samples (approximately 200 mL), as well as influent and effluent water samples (about 200 mL), were taken after 5, 15, 45, 60, 90, and 120 days of operation for each treatment and stored at −20 °C until analysis. The concentrations of the three selected drugs for this study were measured in the water and in the granular biomass according to the methods outlined by Castellano-Hinojosa et al. (2023b). Briefly, the granular samples were freeze-dried for approximately 10 days until they reached a stable weight. Then, 5 mL of extraction solvent (a 1:2 mixture of methanol and HPLC-grade water) was added, and the tube was vortexed for 1 min. The sample underwent ultrasonic extraction for 10 min, after which the tube was centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was then collected in a 16-mL glass test tube. Solid-phase extraction (SPE) with an Oasis HLB cartridge (200 mg, 6 mL, Waters Corporation, Milford, MA, USA) was used for sample pre-concentration both for water and granular samples, followed by nitrogen-assisted evaporation and reconstitution in 10:90 (v/v) methanol-water. Recovery rates were 72.4% ± 4.1%, 76.6% ± 3.1%, and 62.7% ± 3.2% for CP, MTX, and TMX, respectively, and they were determined as described by Ferrando-Climent et al. (2014). Chromatographic separation was carried out using an ultra-high-performance liquid chromatography system (1260 Infinity II, Agilent, USA) with a ZORBAX Eclipse Plus C18 column (Agilent, USA) and a 6470 triple quadrupole-QqQ mass spectrometer (Agilent, USA), following the method outlined by Castellano-Hinojosa et al. (2023b). The detection limit for all three drugs was set at 0.1 ppb.

Table 1
Treatments and anticancer drugs used in this study. Cyclophosphamide (CP), tamoxifen (TMX), and methotrexate (MTX).

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

2.4. Biomass collection, RNA extraction, and cDNA synthesis

Granular biomass samples (50 mL) were collected in triplicate after 0, 15, 30, 45, 60, 75, 90, 105, and 120 days for each treatment. The samples were centrifuged at 13,000 rpm for 3 min at room temperature, and the resulting biomass was stored at −80 °C for future use. RNA was extracted using the FastRNA™ Pro Soil-Direct Kit (MP Biomedicals, Solon, OH, USA). RNA levels were determined using the Qubit™ RNA High Sensitivity Assay Kit (Thermo Fisher Scientific, USA). The RNA was then reverse transcribed into cDNA with the SuperScript™ IV First-Strand Synthesis System, which included the ezDNase™ enzyme and an RNase inhibitor (Thermo Scientific, USA), according to a previously established protocol (Castellano-Hinojosa et al., 2023c). The resulting cDNA concentration was measured using the Qubit™ DNA High Sensitivity Assay Kit (Thermo Scientific, USA) and subsequently stored at −80 °C for future analyses.

2.5. Quantification of total communities and N-cycling genes

The bacterial (16SB) and archaeal (16SA) communities were quantified, along with genes associated with nitrification (*amoA* AOB and *amoA* AOA) and denitrification (*napA*, *narG*, *nirK*, *nirS*, *nosZ*I, and *nosZ*II), using a QuantStudio 3 Real-Time PCR system (ThermoFisher, USA). The procedures for preparing the PCR reaction mixtures and standards followed the approach outlined in Castellano-Hinojosa et al. (2018). Details regarding the primers and PCR conditions can be found in Supplementary Table S1. The standard curves were highly linear ($R^2 > 0.991$), and amplification efficiencies varied between 83.3% and 99.2%. Amplifications were confirmed by analyzing melting curves and performing gel electrophoresis.

2.6. Sequencing analysis

The prokaryotic communities were sequenced with the Pro341F and Pro805R primers (Takahashi et al., 2014) at the Novogene Europe facility in Cambridge, UK. The resulting amplicon sequences were analyzed using QIIME2, based on the approach outlined by Castellano-Hinojosa and Strauss (2021). On average, 54,184 high-quality sequences per sample were obtained after processing. Alpha and beta diversity indices for these communities were calculated in R, employing the methods previously described by Castellano-Hinojosa et al. (2023c). Significant differences in the relative abundance of prokaryotic genera between treatments with ACDs and the control after 90 days were identified using DESeq2 analysis (Love et al., 2014) in R.

2.7. Statistical analysis

The data analysis was performed using R software, version 4.2.2 (http://www.rproject.org/). The physicochemical and microbial variables were assessed for normality using the Shapiro–Wilk test and for homoscedasticity using the Bartlett test. Pearson correlations of pairwise comparisons between treatments with ACDs (LW, MD, and HG) vs. the control for the N-cycling genes and TN removal % were calculated in R. Data were standardized and log-transformed before analysis. Statistical significance was assessed using Fisher's z-test.

3. Results and discussion

3.1. Impact of anticancer drugs on physicochemical parameters

The CT and LW treatments maintained relatively stable MLSS levels throughout the experiment, around 4200 mg/L (Fig. 1A). In contrast, the ME and HG treatments showed a significant decrease in MLSS concentration during the first 30 days of operation. MLSS concentration for the ME treatment stabilized at approximately 3700 mg/L, while HG stabilized at around 3100 mg/L, both lower than CT and LW treatments (Fig. 1A). The average granule size in the CT and LW treatments remained around 18 mm throughout the experiment (Fig. 1B). The ME treatment showed slightly smaller granules, averaging around 15 mm, while the HG treatment resulted in the smallest granules, around 13 mm (Fig. 1B). Settling velocity, indicative of granule density and integrity, was highest in the CT and LW treatments, averaging about 115 m/h (Fig. 1C). The ME treatment had lower settling velocities, around 100 m/h, and the HG treatment showed the lowest settling velocities, approximately 90 m/h (Fig. 1C). SS concentrations in the effluent were generally higher in the ME and HG treatments compared to CT and LW (Fig. 1D). The ME and HG treatments maintained SS levels of around 50 mg/L. LW treatment had levels of around 40 mg/L, and CT had the lowest levels, approximately 30 mg/L, showing some fluctuations (Fig. 1D).

We found that the MLSS levels for the ME and HG treatments stabilized at lower values than the CT and LW treatments, suggesting that medium and high concentrations of ACDs may reduce biomass density in AGS systems. This phenomenon aligns with previous findings that report decreases in MLSS levels under stress conditions induced by pharmaceutical compounds in wastewater treatment systems, including antibiotics (Muñoz-Palazon et al., 2021; Rosa-Masegosa et al., 2021), and ACDs (Castellano-Hinojosa et al., 2023b, 2024a). The observed reduction in granule size and settling velocity in the ME and HG treatments, particularly in the early stages of the experiment, further indicates a disruption in granule integrity and density, likely due to the cytotoxic nature of ACDs, which may impede microbial growth or cause cellular damage (Castellano-Hinojosa et al., 2023a, 2024a). The effluent SS concentrations were higher in the ME and HG treatments, suggesting an increase in fine suspended particles, likely due to the disintegration of granules. This effect was more pronounced in the HG treatment, indicating that higher concentrations of ACDs exacerbate the destabilization of granular sludge. This aligns with findings in studies examining the impact of other pharmaceuticals, such as antibiotics and ACDs in granular sludge systems, which similarly report an increase in effluent SS due to granule breakdown (Wan et al., 2018; Castellano-Hinojosa et al., 2024a).

The CT and LW treatments maintained high COD removal efficiencies throughout the experiment, consistently around 95–100% (Fig. 2A). The ME treatment initially showed high COD removal efficiency, similar to CT and LW, but experienced a decline, stabilizing at around 80–85%, after the initial 20 days of operation. The HG treatment exhibited the lowest COD removal efficiencies, dropping sharply to around 70% within the first 20 days and fluctuating slightly below this level for the remainder of the experimental period (Fig. 2A). Total N removal efficiency remained relatively stable for the CT and LW treatments, generally around 55–60% (Fig. 2B). The ME treatment showed a noticeable decline in TN removal %, stabilizing at approximately 45–50% after an initial drop. The HG treatment showed the lowest TN removal efficiency, decreasing to about 40% early in the experiment and maintaining this reduced level throughout the duration (Fig. 2B). Supplementary Fig. S2 shows that in the CT and LW treatments, both NH_4^+-N

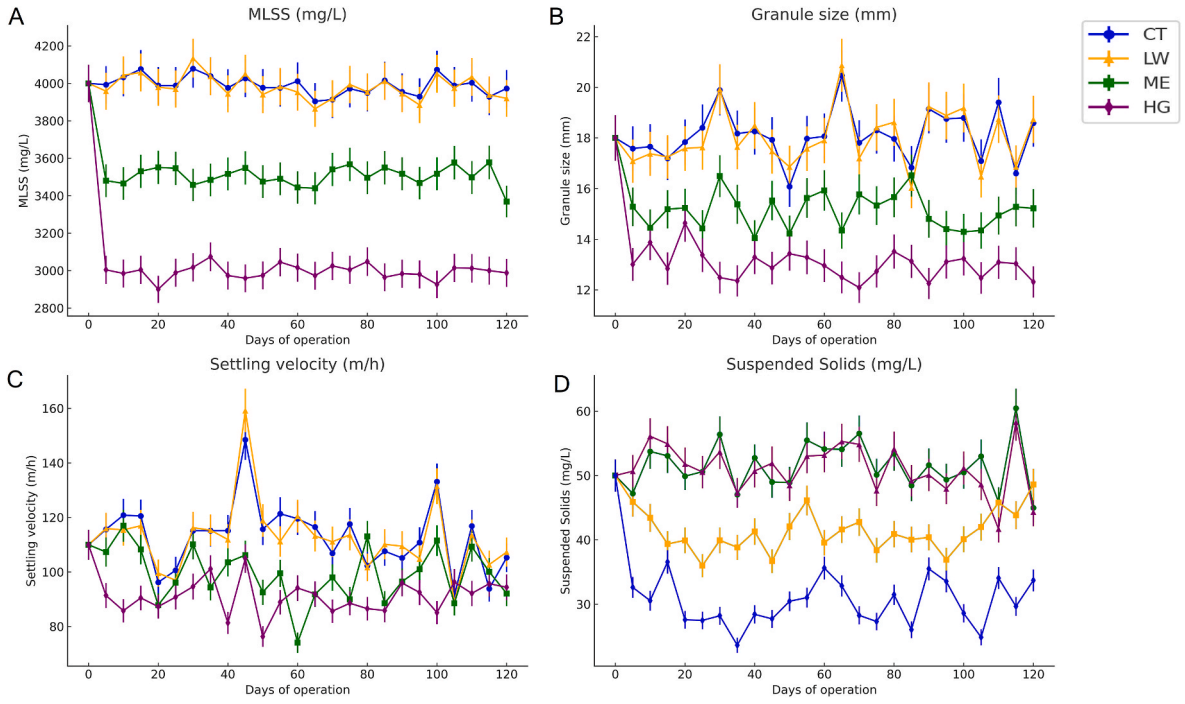

Fig. 1. Average MLSS (A), granule size (B), setting velocity of the granules (C) and suspended solids (D) during the experimental period. Treatments consisted of a control without anticancer drugs (CT) and three concentration levels of anticancer drugs classified as low (LW), medium (ME), and high (HG). MLSS, mixed liquor suspended solids.

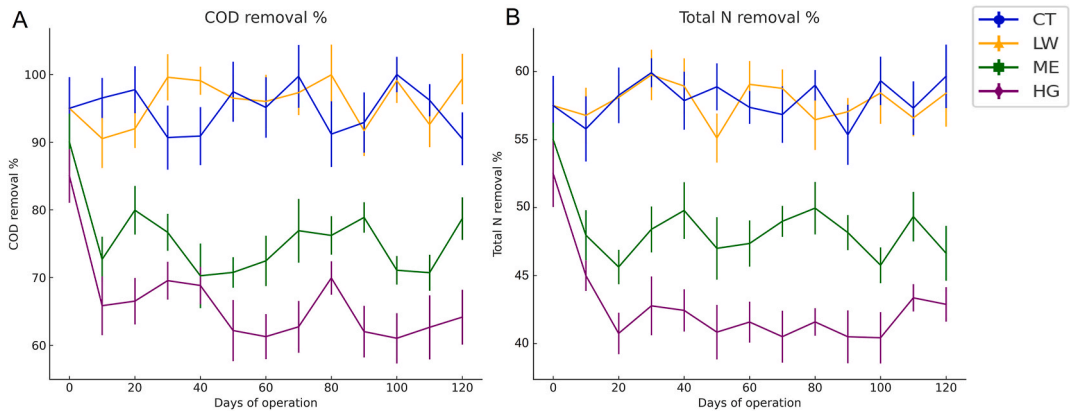

Fig. 2. Average COD removal %(A) and TN removal % (B) during the experimental period. Treatments consisted of a control without anticancer drugs (CT) and three concentration levels of anticancer drugs classified as low (LW), medium (ME), and high (HG). COD, chemical oxygen demand; OM, organic matter, TN, total nitrogen.

and NO_3^--N concentrations remained relatively stable during the experimental period, indicating consistent nitrification and denitrification processes. In contrast, the ME treatment led to slightly increased NH_4^+-N and NO_3^--N levels, suggesting minor detrimental impacts on N-cycling transformations. The HG treatment showed the most significant impact, with elevated NH_4^+-N and NO_3^--N concentrations, reflecting a considerable reduction in the system's efficiency in removing and converting these N forms.

The impaired COD and TN removal efficiencies observed in the ME and HG treatments suggest that the presence of ACDs at these levels can reduce the treatment efficiency of the AGS system. These changes can be attributed to the adverse effects of ACDs on the microbial communities responsible for OM degradation and N cycling (see section 3.3). The reduced efficiency, especially in nitrification and denitrification processes, suggests a susceptibility of nitrifying and denitrifying microorganisms to these compounds, as also supported by qPCR data (see section 3.3). This is consistent with the literature indicating that pharmaceuticals can hinder the activities of specific microbial groups (e.g., nitrifiers and denitrifiers) involved in N removal (Muñoz-Palazon et al., 2021). It is interesting to note that OM and TN removal efficiencies in the ME and HG treatments did not recover to CT or LW levels after not adding the drugs for 30 days. These results suggest a residual effect of ACDs on treatment performance that can last for at least 30 days. Whether removal efficiencies could eventually be recovered should be explored in future studies. Overall, our results underscore the potential challenges posed by ACDs in wastewater treatment systems, particularly those employing AGS technology. The findings highlight the need for further investigation into the mechanisms by which these drugs impact microbial processes and the potential for treatment optimization to mitigate their effects.

3.2. *Efficiency of anticancer drug removal*

The heat map in Fig. 3 illustrates the removal efficiencies of the three ACDs across treatments and time points. Complete removal (100%) of CP was observed consistently in the LW treatment across all time points. The ME treatment exhibited a gradual increase in CP removal efficiency from 65.1% at day 5–72.3% at day 90. The HG treatment showed lower CP removal efficiencies, ranging from 51.5% to 55.8%. TMX was completely removed (100%) in all treatments at all time points. In the LW treatment, MTX was removed with high efficiency, reaching up to 88.8% by day 90. The ME treatment maintained similarly high MTX

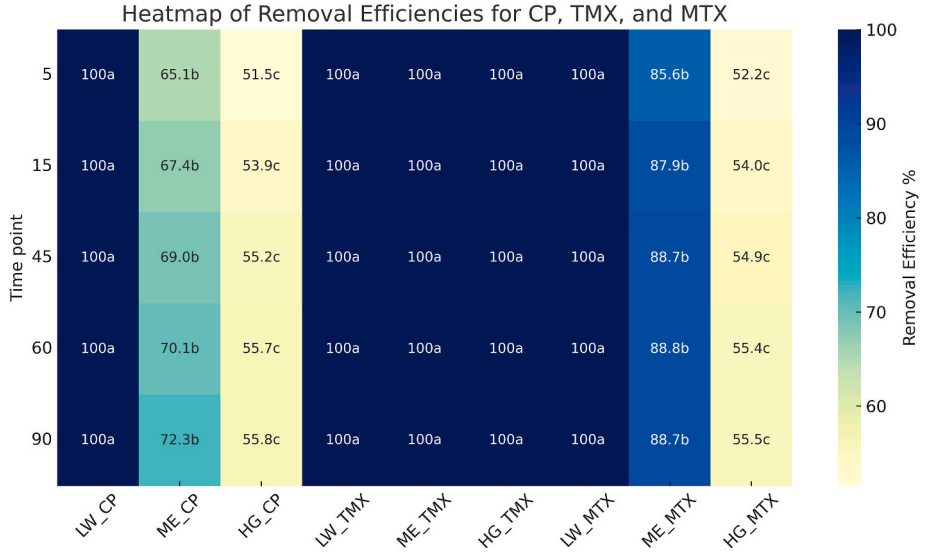

Fig. 3. Heat map showing variations in the removal efficiency of cyclophosphamide (CP), tamoxifen (TMX), and methotrexate (MTX) for the treatments with anticancer drugs. Three concentration levels of anticancer drugs classified as low (LW), medium (MD), and high (HG) were tested. Efficiency removal % 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 time point, values followed by the same letter are not significant different among treatments according to ANOVA and Tukey's HSD tests ($p \leq 0.05$). Values are expressed as mean with standard error. MLSS, mixed liquor suspended solids.

removal efficiencies, ranging from 85.6% to 88.7% throughout the study. However, in the HG treatment, MTX removal efficiencies were lower, ranging from 52.2% to 55.5%, suggesting that higher drug concentrations may impede removal efficiency. No ACDs were detected in the granular biomass nor the effluent after 120 days of operation for any of the treatments.

Our findings indicate that AGS systems in continuous-flow reactors are capable of removing anticancer drugs; however, the effectiveness of removal varied based on the specific type of drug and its concentration. This finding has significant environmental implications, as these pharmaceuticals are known to pass through conventional wastewater treatment plants (WWTPs) with minimal degradation (Castellano-Hinojosa et al., 2023a). The varying removal efficiencies observed in this study across treatments suggest that further optimization, possibly involving adjustments to the hydraulic retention times and other operational parameters (e.g., aeration), could enhance the treatment of more persistent compounds (Yadav et al., 2021). Together, our results point toward AGS technology as a suitable approach to remove ACDs with removal efficiencies even greater than those reported in other biological technologies such as MBR (Yadav et al., 2021).

Nevertheless, while our results demonstrate significant removal of ACDs, it is important to acknowledge that complete mineralization was

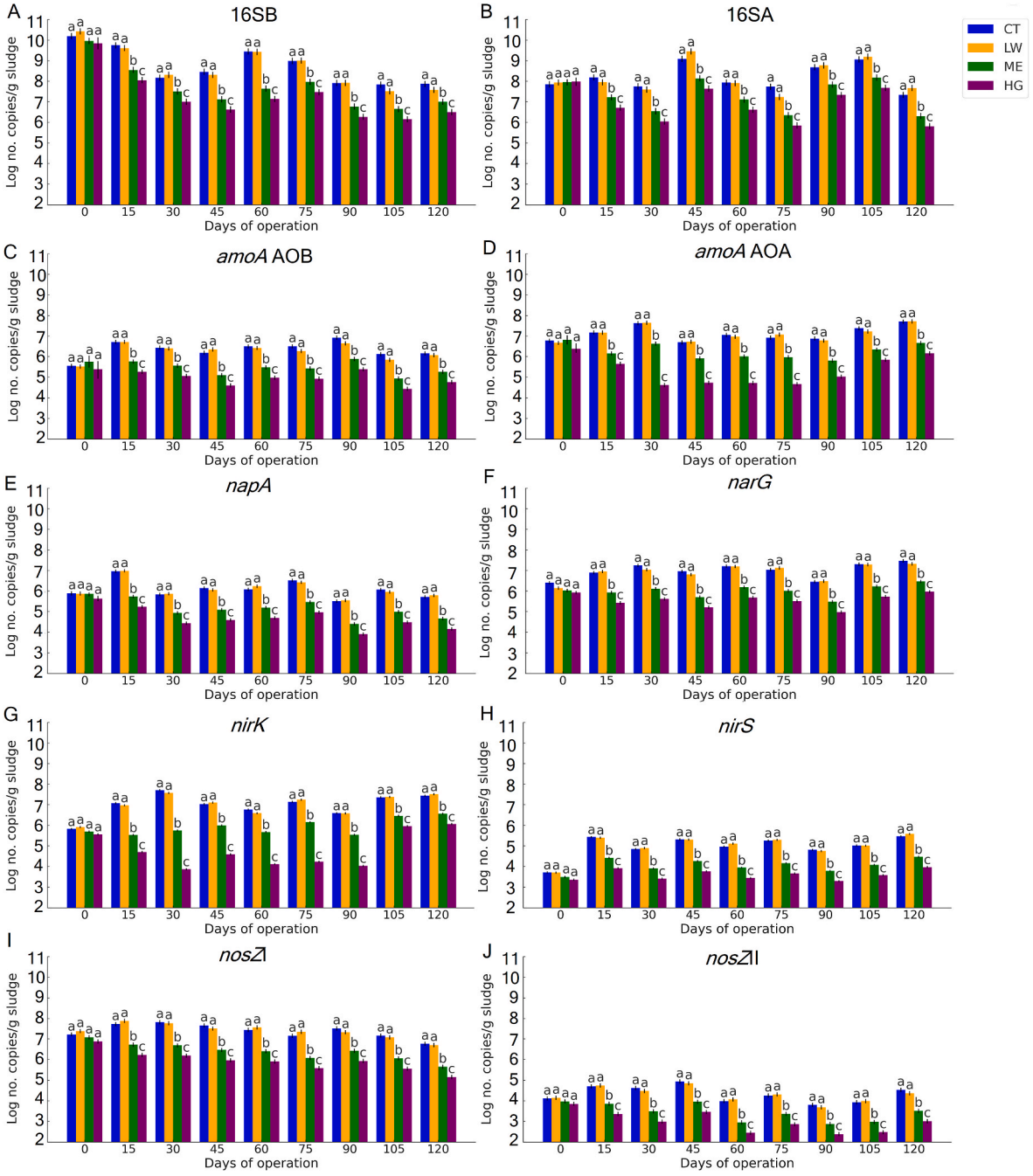

Fig. 4. Changes in the absolute abundance of total bacterial (16SB, A) and archaeal (16SA, B), ammonia oxidizing bacteria (*amoA* AOB, C), ammonia oxidizing archaea (*amoA* AOA, D), *napA* (E), *narG* (F), *nirK* (G), *nirS* (H), *nosZ*I (I) and *nosZ*II (J) communities in the granule microbiome during the experimental period. Treatments consisted of a control without anticancer drugs (CT) and three concentration levels of anticancer drugs classified as low (LW), medium (ME), and high (HG). For each time point, different letters above the bars indicate significant differences between treatments (Tukey's HSD, $p < 0.05$). Values are expressed as mean with standard error.

not directly assessed in this study. The potential formation of toxic transformation products (TPs) during biodegradation remains a concern, as previous studies have reported that some ACDs can degrade into persistent or even more toxic intermediates (Fatta-Kassinos et al., 2011). Future research should incorporate targeted analysis of potential TPs using advanced analytical techniques such as high-resolution mass spectrometry to confirm whether complete mineralization occurs or if harmful byproducts persist in the effluent.

Overall, our results show that the AGS systems consistently achieved complete or near-complete removal of ACDs when they are present at low concentration levels. The consistently high removal rates for TMX across all treatments suggest a favorable profile for its degradation in the systems used, aligning with previous findings that indicate TMX's lower persistence in wastewater environments compared to CP and MTX (Ferrando-Climent et al., 2014; Castellano-Hinojosa et al., 2023a). Lower removal efficiencies for CP and MTX were observed in the ME and HG treatments compared to LW, particularly under higher concentration levels. This variability is consistent with earlier research indicating that certain drugs may exhibit higher persistence and toxicity and highlight the need for tailored treatment strategies that account for the specific physicochemical properties of each drug, such as their half-lives and octanol/water partition coefficients (log Kow) (Ferrando-Climent et al., 2014). It is interesting to note that removal efficiencies for the three ACDs evaluated in this study were greater compared to those observed in AGS systems operations in sequential batch reactor with the same drugs and concentration levels (Castellano-Hinojosa et al., 2023b). This could be due to greater MLSS concentration and/or constant aeration in the AGS operated in the continuous-flow reactor used in this study, which may favor more efficient ACD degradation.

3.3. Impact of anticancer drugs on the abundance of active bacterial and archaeal communities, nitrifiers and denitrifiers

Variations in the absolute abundance of active bacterial and archaeal communities, nitrifiers and denitrifiers in the granule microbiome across treatments and time points are shown in Fig. 4. Regardless of the time point, the use of the ME and HG treatments significantly reduced the abundance of 16SB and 16SA communities compared to CT and LW, with the HG treatment causing the most pronounced decreases throughout the experiment. The abundance of *amoA* AOB communities decreased significantly in the ME and HG treatments compared to CT and LW, with the HG treatment showing the greatest reductions across all time points. The *amoA* AOA communities followed a similar pattern, with lower abundances observed in the ME and HG treatments compared to CT and LW, particularly notable from 30 days onward. The genes associated with denitrification processes (*napA*, *narG*, *nirK*, *nirS*, *nosZ*I, and *nosZ*II) were adversely affected by the application of the ME and HG levels of ACDs. Generally, the HG treatment resulted in the most substantial reductions in gene abundance. The ME treatment also caused significant decreases, though to a lesser extent. No significant recoveries in the abundance of bacterial and archaeal communities, nitrifiers and denitrifiers were observed in the ME and HG treatments after not adding ACDs for 30 days.

Pearson correlations of pairwise comparisons between treatments with ACDs (LW, ME, and HG) vs. the control for the N-cycling genes and TN removal % are presented in Supplementary Fig. S3. The LW treatment showed no significant correlation with gene abundance or TN removal efficiency, indicating minimal impact compared to CT. In contrast, the ME treatment exhibited moderate to strong negative (in the range of -0.62 to -0.86) and significant correlations ($p < 0.01$) for all nitrification and denitrification genes and TN removal compared to CT. The HG treatment showed an even stronger negative (in the range of -0.91 to -0.95) and significant ($p < 0.001$) effect across all measured parameters compared to CT.

Our results showed that increasing concentrations of ACDs lead to significant disruptions in N-cycling microbial communities and nitrification and denitrification processes, with the highest concentration causing the most substantial reductions. The observed decrease in the abundance of the *amoA* AOA and *amoA* AOB genes in the ME and HG treatments, particularly under high drug concentrations, suggests a substantial inhibition of ammonia oxidation capacity. This inhibition could be attributed to the cytotoxic nature of ACDs, which may disrupt cellular function or metabolic pathways critical for nitrifying organisms (Lopez et al., 2021; Pashaei et al., 2022). Such disruptions can lead to an accumulation of NH_4^+ and reduced TN removal efficiency, as indicated by the changes in these physicochemical parameters (Fig. 2; Supplementary Fig. S2), thereby altering the N balance within the system. Our study also found significant reductions in the abundance of denitrification genes, particularly in the HG treatment. This reduction indicates a compromised denitrification capacity, leading to alterations in the reduction of NO_3^- (Supplementary Fig. S2). The accumulation of NO_3^- can be detrimental, as shown by the observed decrease in TN removal efficiency in Fig. 2. Although NO_2^- content was below the detection limit in this study, the reductions in the abundance of *napA* and *narG* genes in the ME and HG treatments suggest that ACDs may also favor the accumulation of NO_2^- due to partial reduction of NO_3^- via denitrification. In addition, reductions in the abundance of *nirK*, *nirS*, *nosZ*I, and *nosZ*II suggest that ACDs may induce a reduction in complete denitrification in the granule microbiome, thus favoring the emission of the greenhouse gas nitrous oxide. It is interesting to note that we observed no distinct sensibilities among specific N-cycling communities to ACDs, suggesting that these substances have broad spectrum impacts on microorganisms involved in N transformations in AGS systems. To our knowledge, this is the first report about ACD effects on active nitrifiers and denitrifiers in AGS system, though previous work showed that other pharmaceutical products, such as antibiotics, can temporally reduce the abundance of nitrification and denitrification genes (Katipoglu-Yazan et al., 2016; Wang et al., 2020a,b; Muñoz-Palazon et al., 2021).

The dose-dependent response of nitrifiers and denitrifiers observed across treatments, with higher concentrations of ACDs leading to greater gene abundance reductions, underscores the sensitivity of N-cycling communities to these emerging contaminants. The lack of recovery in the abundance of active microbial communities after the cessation of ACD addition for 30 days suggests long-term or possibly irreversible impacts on microbial ecology within the AGS system. The significant correlations between ACD concentration and the abundance of nitrification and denitrification genes emphasize the need for careful management and monitoring of pharmaceutical contaminants in wastewater treatment systems.

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

The application of ME and HG treatments led to a notable reduction in the number of ASVs, as well as in the Shannon and Simpson indices for the prokaryotic community, when compared to CT and LW (Fig. 5A). The reduction was more pronounced in the HG treatment compared to ME (Fig. 5A). In contrast, the LW treatment did not cause any significant changes in alpha diversity compared to CT. Notably, in the ME and HG treatments, alpha diversity indices did not recover after a 30-day period without drug addition, and they did not reach CT levels (Fig. 5A). Treatment and time point significantly influenced the prokaryotic community composition (Fig. 5B). The ME and HG treatments showed distinct shifts in community structure compared to CT and LW, with the HG treatment showing the most pronounced differences. However, the LW treatment did not result in significant compositional changes compared to the CT, suggesting a minimal impact of low drug concentrations on the prokaryotic community structure. Supplementary Fig. S4 displays the relative abundance of prokaryotic phyla across different treatments and time points during the experimental period. The granule microbiome was predominantly composed of the phylum *Pseudomonadota,* which consistently showed high relative abundance across all

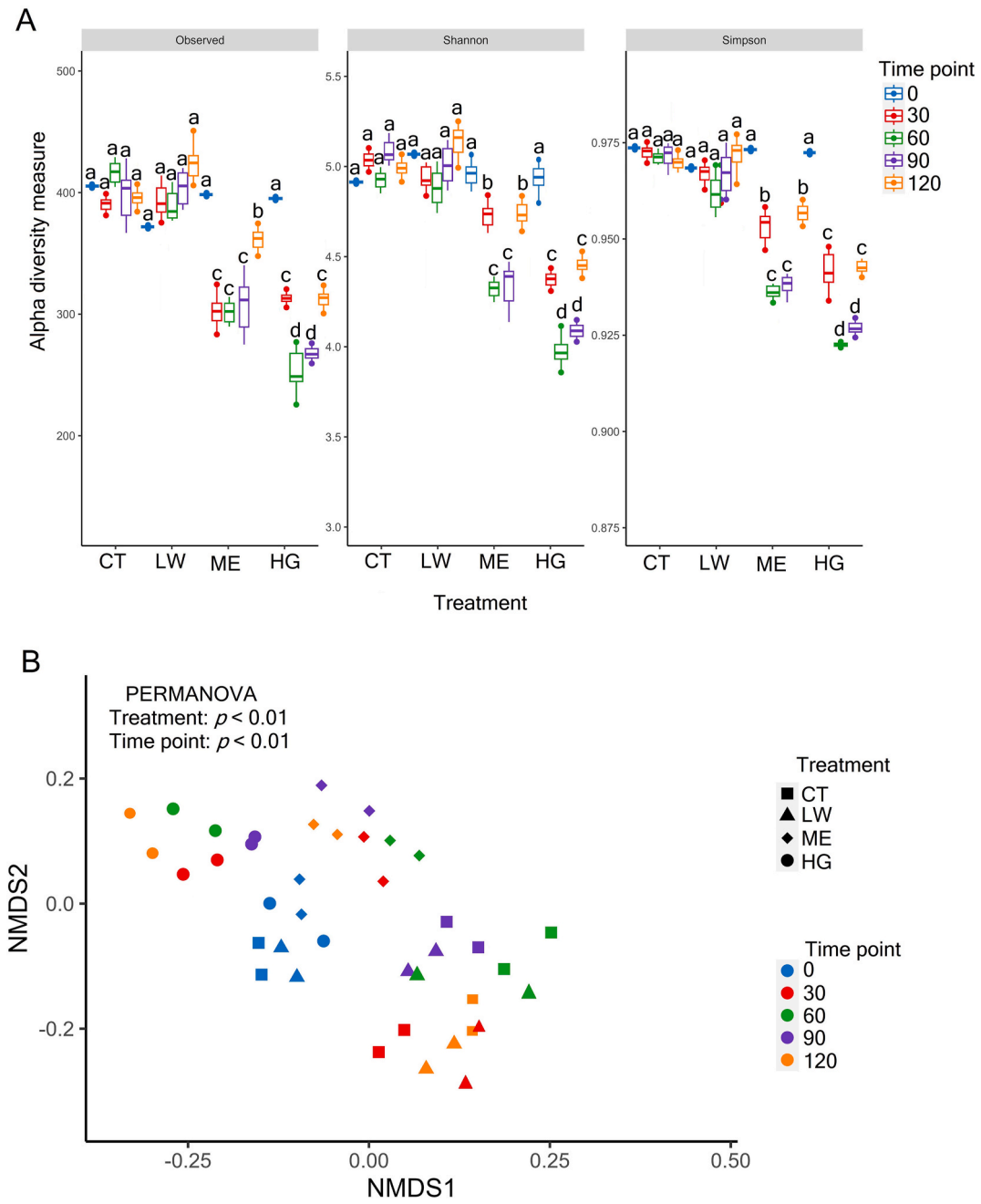

Fig. 5. A. Number of ASVs, and values of Shannon and inverse Simpson diversity indices for the prokaryotic community during the experimental period. Different letters above the bars indicate significant differences between treatments and time points (Tukey's HSD, $p \leq 0.05$). Values are expressed 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 consisted of a control without anticancer drugs (CT) and three concentration levels of anticancer drugs classified as low (LW), medium (ME), and high (HG).

treatments and time points. The ME and HG treatments showed gradual increases in the relative abundances of phyla such as *Parcubacteria* and *Bacillota* over time, particularly noticeable at the 60 days and 120 days of operation. These changes indicate a shift in the microbial community structure under higher concentrations of ACDs. Notably, the archaeal phyla remained below 1% relative abundance throughout the study, underscoring the dominance of bacterial taxa in the prokaryotic granule microbiome.

Our results showed that medium and high concentrations of ACDs can reduce not only the number of taxa but also the overall diversity of metabolically active prokaryotic communities within the granule microbiome. This could be due to ACDs disrupting cellular processes, leading to increased cell death and/or microbial adaptations to the stress induced by these substances, as shown in previous studies in AGS systems amended with antibiotics (Liu et al., 2019; Shi et al., 2021) and ACDs (Castellano-Hinojosa et al., 2024a, 2024b). Notably, the alpha diversity did not fully recover even after a 30-day cessation of the addition of ACDs, highlighting a sustained impact on the microbial community diversity and functionality. This finding underscores the resilience yet vulnerability of the granule microbiome to ACD exposure, as it shows partial recovery without reaching CT levels.

The distinct shifts observed in community composition under ME

and HG treatments, particularly the increase in phyla such as *Parcubacteria* and *Bacillota,* indicate that the microbial community composition is sensitive to ACD concentrations. These changes were most pronounced in the HG treatment, suggesting a dose-dependent response where higher ACD levels exacerbate the shift in prokaryotic community composition. In contrast, the LW treatment did not significantly alter the community composition or diversity, implying that lower concentrations may have a minimal impact on prokaryotic communities, which aligns well with minimal impacts on physicochemical parameters (see section 3.1). Overall, our findings have important implications for understanding the ecological effects of ACDs in wastewater treatment systems and their effects on treatment performance. The significant influence of treatment and time point on community composition highlights the need for careful management of ACD concentration levels in such systems to preserve microbial diversity and function. The observed reduction in microbial diversity and the shift in community composition under higher ACD concentrations strongly suggest that ACDs may affect microbial functions in ways that extend beyond nitrogen cycling. These changes may involve the suppression of critical metabolic pathways, such as organic matter degradation, and the alteration of microbial interactions crucial for the stability of the microbial community.

3.5. Impacts of anticancer drugs on specific active prokaryotic genera

Genera with differential abundance were identified between the ME and HG treatments when compared to the CT treatment after 90 days of operation, underscoring the influence of ACDs on the granule microbiome (Fig. 6). In contrast, no notable shifts in active prokaryotic genera were found between the LW and CT treatments over the course of the experiment. In the ME treatment, a total of 13 genera were significantly depleted, including genera such as *Xanthobacter*, *Woodsholea*, *Sphingomonas*, and *Rhizobium*, which are known for their roles in N_2 fixation, bioremediation, and degradation of organic pollutants (Cydzik-Kwiatkowska et al., 2017; Gómez-Basurto et al., 2019; Wang et al., 2020b; Jiang et al., 2021). The HG treatment showed a more pronounced effect, with 22 genera significantly depleted. Among these, key genera such as *Nitrosomonas*, *Paracoccus*, *Zoogloea*, *Pseudomonas*, and *Acinetobacter* were notably affected. *Nitrosomonas* and *Paracoccus* are crucial for N cycling, particularly in nitrification and denitrification processes, which are vital for the stability and efficiency of wastewater treatment systems. The significant reduction in these genera could indicate a potential disruption in N cycling, leading to reduced treatment efficiency and increased risk of nitrogenous waste accumulation in line with the observed changes in physicochemical parameters in this study (see section 3.1).

Decreases in the relative abundance of *Zoogloea*, *Pseudomonas*, and *Acinetobacter* genera were also observed in the HG treatment compared to CT. These genera are known for their roles in biofilm formation, biodegradation, and resistance to environmental stressors (Yang et al., 2021; Liu et al., 2023; Liang et al., 2024), which suggest a potential decline in the resilience and structural integrity of the microbial community following the addition of ACDs at high concentration levels. This could make the system more vulnerable to environmental perturbations and contribute to explaining the significant decreases in OM and TN removal efficiencies in the HG treatment. The lack of significant changes in prokaryotic genera between the LW and CT treatments indicates that lower concentrations of ACDs may not have a noticeable impact on the microbial community structure, suggesting a threshold effect where only higher concentrations of ACDs lead to significant microbial shifts. While the primary focus of this study was on nitrogen-cycling microorganisms, the observed reductions in genera responsible for organic matter degradation, such as *Pseudomonas* and *Acinetobacter*, suggest that ACDs may have broader inhibitory effects on microbial processes, possibly extending to the degradation of other pollutants. This highlights the potential for anticancer drugs to disrupt additional microbial metabolic pathways beyond nitrogen cycling.

A potential increase in the abundance of specific functional microorganisms involved in the biodegradation of ACDs was not directly observed in our study. Although some genera, such as *Pseudomonas* and *Acinetobacter*, are known for their roles in organic pollutant degradation, their numbers also declined under high ACD concentrations, indicating a possible detrimental effect on these communities. The observed reductions in these genera suggest that medium and high concentration of ACDs may exert inhibitory effects on microbial consortia responsible for drug degradation rather than selecting for microbial taxa capable of metabolizing these compounds. Together, our findings highlight the importance of monitoring and managing ACDs in wastewater treatment systems to prevent adverse impacts on microbial communities and the associated ecological processes. Further studies should focus on understanding the mechanisms by which ACDs affect specific microbial taxa and explore potential mitigation strategies to protect the functional integrity of microbial communities in AGS systems.

4. Conclusions

This study shows that the presence and concentration of ACDs significantly impact physicochemical parameters, the abundance of metabolically active nitrifiers and denitrifiers, and the diversity and composition of prokaryotic communities of continuous-flow AGS systems. We show that this technology can help remove ACDs, but removal efficiencies were dependent on the type of ACD and its concentration level. The concentration level of ACDs determines variations in biomass density, granule integrity, and removal efficiencies of OM and N, with medium and particularly high concentrations having a detrimental

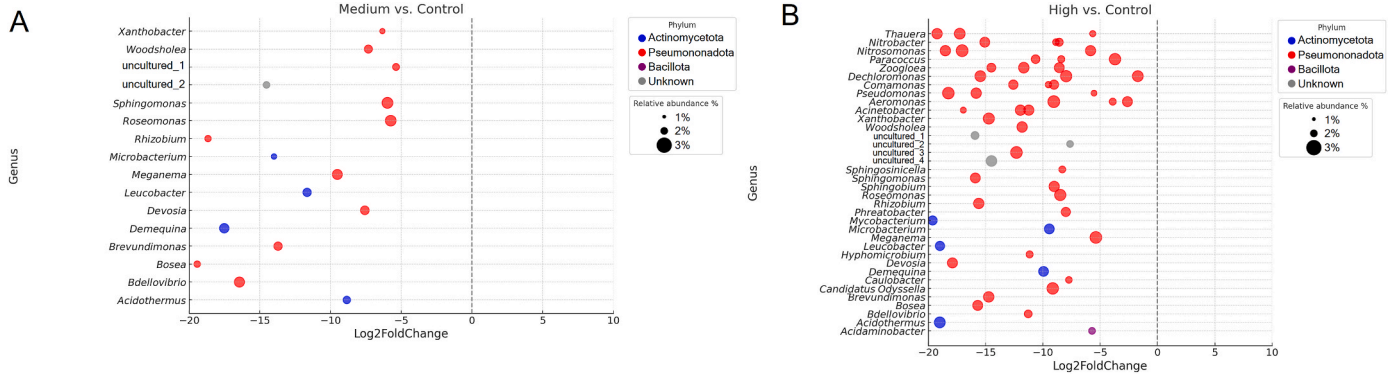

Fig. 6. Differential abundance ASVs at the genus taxonomic level between medium (ME) and control (CT) (A) and high (HG) and CT (B) treatments after 90 days of operation. Treatments are defined in Table 1. The fold change is shown on the X axis and genera are listed on the Y axis. Each colored dot represents an ASV that was identified by DESeq2 analysis ($p \leq 0.05$).

effect on these physicochemical parameters. Microbial analyses revealed a dose-dependent reduction in the abundance of total bacterial and archaeal communities, nitrifiers, denitrifiers, and overall microbial diversity and community composition when ACDs were present at medium and high concentration levels. These findings emphasize the need for careful management of ACD levels in wastewater systems to mitigate their adverse effects on active microbial communities and treatment performance. Overall, our study underscores the potential ecological risks posed by ACDs in wastewater treatment environments and suggests the necessity for further research on optimizing treatment strategies to enhance the resilience of AGS systems to pharmaceutical contaminants.

CRediT authorship contribution statement

Antonio Castellano-Hinojosa: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Manuel J. Gallardo-Altamirano:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Nicoly Dal Santo Svierzoski:** Writing – review & editing, Methodology, Investigation. **Clementina Pozo:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Jesús González-López:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Alejandro González-Martínez:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2025.144279.

Data availability

Data will be made available on request.

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