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Anticancer drugs impact the performance and prokaryotic microbiome of an aerobic granular sludge system operated in a sequential batch reactor

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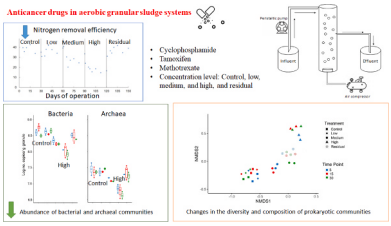
HIGHLIGHTS

- Anticancer drugs reduced nitrogen removal efficiency in aerobic granular systems.
- Aerobic granular systems can efficiently remove anticancer drugs.
- Anticancer drugs reduced prokaryotic abundance and diversity.
- Anticancer drugs altered prokaryotic community composition and reduced network complexity.
- Anticancer drugs reduced bacterial predicted functionality.

GRAPHICAL ABSTRACT



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ABSTRACT

Increased concerns exist about the presence of anticancer drugs in wastewater. However, knowledge of the impacts of anticancer drugs on the performance of the system and microbial communities during wastewater treatment processes is limited. We examined the effect of three anticancer drugs commonly detected in influents of wastewater treatment plants applied at three different concentration levels on the performance, efficiency of anticancer drug removal, and prokaryotic microbiome in an aerobic granular sludge system (AGS) operated in a sequential batch reactor (SBR). We showed that an AGS can efficiently remove anticancer drugs, with removal rates in the range of 53–100% depending on the type of drug and concentration level. Anticancer drugs significantly decreased the abundance of total bacterial and archaeal communities, an effect that was linked to reduced nitrogen removal efficiency. Anticancer drugs also reduced the diversity, altered the prokaryotic community composition, reduced network complexity, and induced a decrease of a wide range of predicted bacterial functions. Specific bacterial taxa responsive to the addition of anticancer drugs with known roles in nitrification and denitrification were identified. This study shows anticancer drugs should be monitored in the future as they can induce changes in the performance and microbiome of wastewater treatment technologies.

1. Introduction

Cancer is a significant global health concern responsible for approximately 10 million deaths and almost one out of every six deaths in 2022 according to the World Health Organization WHO [54]. As a result, there is a growing emphasis on investing in programs to prevent, detect, and treat cancer. Predictions indicate that over 29 million cancer cases will be reached by 2040 [56]. This increase in cancer incidence has

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led to a significant increase in the use of drugs that combat cancer [referred to as anticancer drugs (AD), antineoplastic drugs, or chemotherapy drugs] over the past two decades [21,4].

The AD prevent cellular proliferation by disrupting DNA synthesis which may have a variety of effects on certain organisms including cell death, genetic mutations, and teratogenicity [26]. These substances are not completely metabolized by the human organisms and are excreted in urine and feces, which ultimately end up in the wastewater system. Due to their high toxicity, AD can be persistent in wastewater and are difficult to biologically degrade [19,32]. A recent review study showed that AD can pass through the entire water system in urban areas, from hospitals and wastewater treatment plants (WWTPs) to rivers and groundwater [6]. Some of the most commonly detected AD in influents and effluents of WWTPs include cyclophosphamide (CP), tamoxifen (TMX), and methotrexate (MTX), with concentrations in the order of hundreds or thousands of nanograms per liter [42,6].

To date, most urban WWTPs have employed conventional activated-sludge technology. However, recent studies have shown that conventional biological methods of treating wastewater are not able to efficiently remove or degrade AD [21,32,56]. The membrane bioreactor (MBR) is the main biological technology that has been examined for removing AD from wastewater, with removal efficiencies ranging from 20% to 90% depending on the type of anticancer drug and treatment concentration [56]. New biological techniques such as aerobic granular sludge (AGS) systems are being explored for the removal of common contaminants (e.g., organic matter and nitrogen [N]) and emerging hazardous pollutants (e.g., pharmaceutical compounds such as antibiotics) [24,39,40,41,38]. This technology has gained attention due to its ability to save energy (20–50%) and reduce footprint (25–75%) [47] while removing organic matter and N in a single bioreactor [57]. The granular sludge is formed by the aggregation of prokaryotic and eukaryotic microorganisms embedded in a three-dimensional matrix held together by extracellular polymeric substances (EPS) which create a gradient of nutrients and oxygen from the outer to the inner layers of the granule [13,24]. AGS systems typically operate in a sequential batch reactor (SBR) with high-density biomass, low sludge volume, high resistance to toxic compounds, and high biomass retention, and can operate under high organic loads [45,52]. However, it is unknown whether an AGS system operated in an SBR could be used to remove AD, how efficient this technology could be for this purpose, and how the presence of substances at different concentrations may impact the overall system performance in terms of removal of organic matter and N.

Although numerous studies have shown that the presence of AD in urban wastewater can have negative effects on aquatic organisms, there is limited knowledge on how these substances may affect the abundance, diversity, and composition of microbial communities present in wastewater [5,7]. This is particularly important as microbes are the main drivers of C and N cycling in wastewater and alterations, for example, in the diversity and composition of prokaryotic communities can alter overall system performance in an AGS system [39]. Yet, it remains largely unknown how AD may impact the granule microbiome.

Therefore, the objective of this study was to examine the effect of three AD (CP, TMX, and MTX) commonly detected in influents of WWTPs applied at three different concentration levels (low, medium, and high) on the performance, efficiency of removing anticancer drugs from water and granules, and the abundance, diversity, and composition of prokaryotic (Bacteria and Archaea) communities in an AGS system operated in an SBR. We hypothesized that the application of AD at medium and high concentration levels would decrease the abundance and alter the diversity of prokaryotic communities, thus reducing the efficiency of C and N removal compared to control conditions without these substances. We expect an AGS system to favor the removal of AD due to the biomass in this system. We also hypothesized that AD would impact the abundance of predicted bacterial functions and network complexity (number of interactions between microorganisms) through alteration of the microbiome. We anticipate that the effects of AD on prokaryotic communities may last even a month after finishing adding these substances, showing the potential of these compounds to have residual effects on the granule microbiome.

2. Materials and methods

2.1. Bioreactor design, configuration, and operation

An AGS system operated in an SBR was used in this study. The bioreactor that was used has a height of 45 cm and a diameter of 9 cm, as illustrated in Fig. S1. It has an operational volume of 2.5 L, of which 50% was exchanged per cycle. The hydraulic retention time was 8 h and each cycle consisted of four stages: 3 min for adding synthetic water, 240 min of constant aeration, 3 min for decantation of the granules, and 4 min for discarding the effluent. Air was supplied to the bottom of the bioreactor through fine bubbles at a rate of 4 L min^{-1}. The bioreactor was kept at room temperature in the range of 15–20 °C during the experimental period.

The bioreactor was firstly inoculated with 1 L of mature granular biomass from a lab-scale AGS system operated in an SBR and inoculated with activated sludge from Los Vados WWTP (Granada, Spain). The bioreactor was fed from the top of the bioreactor using a peristaltic pump (Watson Marlow, UK) with synthetic wastewater simulating urban sewage with the following composition [14]: $CH_3COONa{\cdot}3\ H_2O$ 1.5 g L^{-1}, NH_4Cl 0.25 g L^{-1}, $MgSO_4{\cdot}7\ H_2O$ 0.1 g L^{-1}, K_2HPO_4 0.085 g L^{-1}, KCl 0.04 g L^{-1}, and KH_2PO_4 0.03 g L^{-1}. The bioreactor operated for a month in a steady-state phase until it reached stable conditions. Then, four consecutive treatments with different levels of anticancer drug concentration were run as follows: control without AD, and low, medium, and high concentration levels (Table 1). Each treatment had a duration of 30 operational days. Finally, the bioreactor was operated for an additional period of 30 days during which it was fed with synthetic wastewater without AD (control conditions) to investigate whether the performance and microbial community of the system would return to initial levels; this treatment was named “residual” (Table 1). CP, MTX, and TMX were selected for this study as they are the most studied and frequently reported AD in hospital effluents and wastewater influents and effluents [42,6]. The ranges of final concentration for each anticancer drug were taken from the literature based on those reported in wastewater influents [42,6] (Table 1). We selected three concentration levels that cover the high variability of the concentration ranges of these substances. The synthetic wastewater was inoculated with the corresponding anticancer drug concentration during the experimental period. All AD were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions containing AD were kept frozen at − 20 °C until use and prepared fresh weekly to prevent degradation of the AD and ensure their stability as previously recommended [18].

Table 1
List of treatments and concentration of cyclophosphamide (CP), tamoxifen (TMX), and methotrexate (MTX) that were used. Each treatment had a duration of 30 days and they were investigated in chronological order.

Treatment	Anticancer drug	Concentration (ng/L)
Control	None	-
Low	CP	60
	TMX	1.5
	MTX	40
Medium	CP	600
	TMX	15
	MTX	400
High	CP	6000
	TMX	150
	MTX	4000
Residual	No	-

2.2. Physicochemical determinations

Physicochemical analysis was carried out twice per week during the experimental period. The chemical oxygen demand (COD) was measured according to standard methods described by APHA [2] and the efficiency of its removal (%) calculated based on the concentration in the influent and effluent. Mixed liquor suspended solids (MLSS) in the bioreactor and suspended solids (SS) in the effluent were determined according to APHA [2]. The concentrations of acetate (CH_3—COO^-), ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) were analyzed using an ion chromatograph (Metrohm Ion Chromatograph, AG, Switzerland) in 0.22 μM-filtered samples. Acetate and nitrogen removal efficiency (%) was calculated as the difference in the concentration of CH_3—$COO-$ and $NH_4^+ + NO_2^- + NO_3^-$ between the influent and effluent, respectively. The dissolved oxygen in the bioreactor and pH in the effluent were measured using a Crison Oximeter and Crison pH meter, respectively. The dissolved oxygen was close to saturation during the experimental period in the range of 9.65–9.85 mg O_2 L^{-1}. The size of the granules was measured using a scale meter and the settling velocity of the granules was determined as described by Laguna et al. [31].

2.3. Quantification of anticancer drugs in water and granules

Water (100 mL) from the effluent and granules (300 mL) from the bioreactor were collected in duplicate after 5, 15, and 30 days of operation for each the control, low, medium, high, and residual treatments, and kept at − 20 °C until use in bottles which were pre-rinsed with Milli-Q water. Quantification of AD was performed within a month after collecting the samples to avoid some degradation of these substances, as recommended by Ferrando-Climent et al. [18]. The quantification of AD in water and granules was developed as described in full detail by Ferrando-Climent et al. [18] and Gallardo-Altamirano et al. [22], respectively. The chelating agent ethylenediaminetetraacetic acid (EDTA) was added to all samples to a final concentration of 0.1% (g solute g solution^{-1}), as it is reported to improve the extraction of AD [19]. Samples were pre-concentrated by solid phase extraction (SPE) as described by Ferrando-Climent et al. [19] using an Oasis HLB cartridge (200 mg, 6 mL, Waters Corporation, Milford, MA, USA). The final extract was evaporated under gentle nitrogen stream and reconstituted with 250 μL of methanol–water (10: 90, v/v). Working solution mixtures were also prepared in the sample matrix (synthetic wastewater) supplemented with 0.1% EDTA using four different concentrations of CP (0.06, 0.6, 2.4, and 6 ng mL^{-1}), MTX (0.04, 0.4, 1.6, and 4 ng mL^{-1}), and TMX (0.001, 0.01, 0.03, and 0.1 ng mL^{-1}) that cover the range of concentrations in this study (Table 1), to estimate the possible matrix effect during the pre-concentration method through an HLB cartridge and for internal calibration purposes. As for the samples, the final extracts were evaporated using nitrogen and reconstituted with 250 μL of methanol–water (10: 90, v/v). Recovery efficiencies (%) were calculated as described by Ferrando-Climent et al. [18] and were 45.2 ± 1.2%, 75.3 ± 1.5%, and 35.6 ± 1.4% for CP, MTX, and TMX, respectively. All reagents necessary for pre-treatment of the samples were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the solutions were stored at − 20 °C until use.

2.4. UHPLC-QqLit method

Chromatographic separation was carried out with an ultra-high performance liquid chromatography system (model UHPLC 1260 Infinity II; Agilent, USA) equipped with a ZORBAX Eclipse Plus C18 column (50 mm × 3 mm i.d. 1.8 μm particle size; Agilent, USA) under positive electrospray ionization (PI). The UHPLC instrument was coupled to a 6470 triple quadrupole-QqQ (Agilent, USA) with a quaternary UHPLC pump – 1260 Infinity II Flexible Pump G7104C. A multiple reactive monitoring mode (MRM) was used to record, acquire, and process all transitions using Agilent MassHunter software. The instrumental detection limit was of 0.1 ppb for all AD.

2.5. Biomass collection and DNA extraction

Granular biomass (50 mL) was collected in duplicate after 5, 15, and 30 days of operation for each of the control, low, medium, high, and residual treatments. Samples were centrifuged at 3000 rpm at room temperature for 7 min and the pelleted biomass kept at − 20 °C until use. DNA was extracted using a FastDNA SPINK Kit for Soil (MP Biomedicals, Solon, OH, USA) and quantified using NanoDrop (Fisher Scientific, USA).

2.6. Quantification of prokaryotic communities

The absolute abundance of total bacterial (16SB) and archaeal (16SA) communities was measured using a QuantStudio 3 Real-Time PCR system (Thermo Fisher, USA). The methods used to prepare the PCR reaction mixtures and standards were based on a study by Castellano-Hinojosa et al. [9]. The specific techniques and parameters used in the PCR process are listed in Table S1. The qPCR standard curves had good linearity ($R^2 > 0.998$), and amplification efficiencies (in the range of 87.3–100%) in all assays. The quality of the PCR amplifications was checked by melting curve analysis and agarose gel electrophoresis.

2.7. Microbial community analysis

Amplicon sequencing was conducted by Novogene Europe (Cambridge, United Kingdom) using the primer pairs Pro341F and Pro805R that amplify the V3–V4 region of the 16S rRNA of prokaryotes (Bacteria + Archaea) in a MiSeq sequencer (Illumina, Inc., San Diego, CA, USA) as described by Takahashi et al. [49]. Sequence reads were analyzed in QIIME2 following the procedure described by Castellano-Hinojosa et al. [8]. The final dataset consisted of an average of 47,564 sequences per sample. Raw sequence data are available in the NCBI's Sequence Read Archive under BioProject PNR1234.

The analysis of sequence reads was done as described in detail by Castellano-Hinojosa et al. [8]. Briefly, the R packages "vegan" v2.5–2 [44] and "Phyloseq" v1.24.0 [36] were used to conduct alpha (number of ASVs, and Shannon and Inverse Simpson indices) and beta diversity analyses on log-normalized data to prevent errors caused by rarefaction [37]. The beta diversity analysis included an analysis of unweighted UniFrac distances using non-metric multidimensional scaling (NMDS). Significant changes in the composition of the prokaryotic community between treatments and time points were also evaluated by permutational analysis of variance (PERMANOVA). Differentially abundant bacterial and archaeal ASVs between treatments were identified using the "DESeq2" package in R [34].

2.8. Predicted functional traits of the bacterial community

The PICRUSt2 tool [17] was used to predict bacterial functions as described by Castellano-Hinojosa et al. [9]. Significant variations in the mean relative abundance of predicted bacterial KEGG pathways [29] between treatments and time points were determined using Welch's t-test with the Benjamini–Hochberg FDR procedure [3].

2.9. Construction of co-occurrence networks

The impact of the different concentration levels of AD on the organization and potential ecological interactions of the prokaryotic community organization compared to the control was assayed using co-occurrence networks based on Spearman correlation as described in detail by Castellano-Hinojosa et al. [9]. The igraph package [12] was used to infer properties of the networks such as the number of nodes, and edges, mean degree, and density. Networks showing significant associations were constructed and visualized using Cytoscape v3.9.0 software

[48].

2.10. Statistical analysis

The analysis of data was performed using R software version 4.0.5 (http://www.rproject.org/). The Shapiro–Wilk test and Bartlett's test were used to check if variables met the normality and homoscedasticity assumptions required for analysis of variance (ANOVA), respectively. One-way and two-way ANOVA comparisons of means and post hoc (Tukey) tests were used for comparisons between treatments and/or time points and p-values ≤ 0.05 were considered significant. Redundancy analysis (RDA) was performed to assess the association between the total abundance of 16SB and 16SA communities and the physicochemical parameters (COD, efficiency of COD removal, acetate, and N,

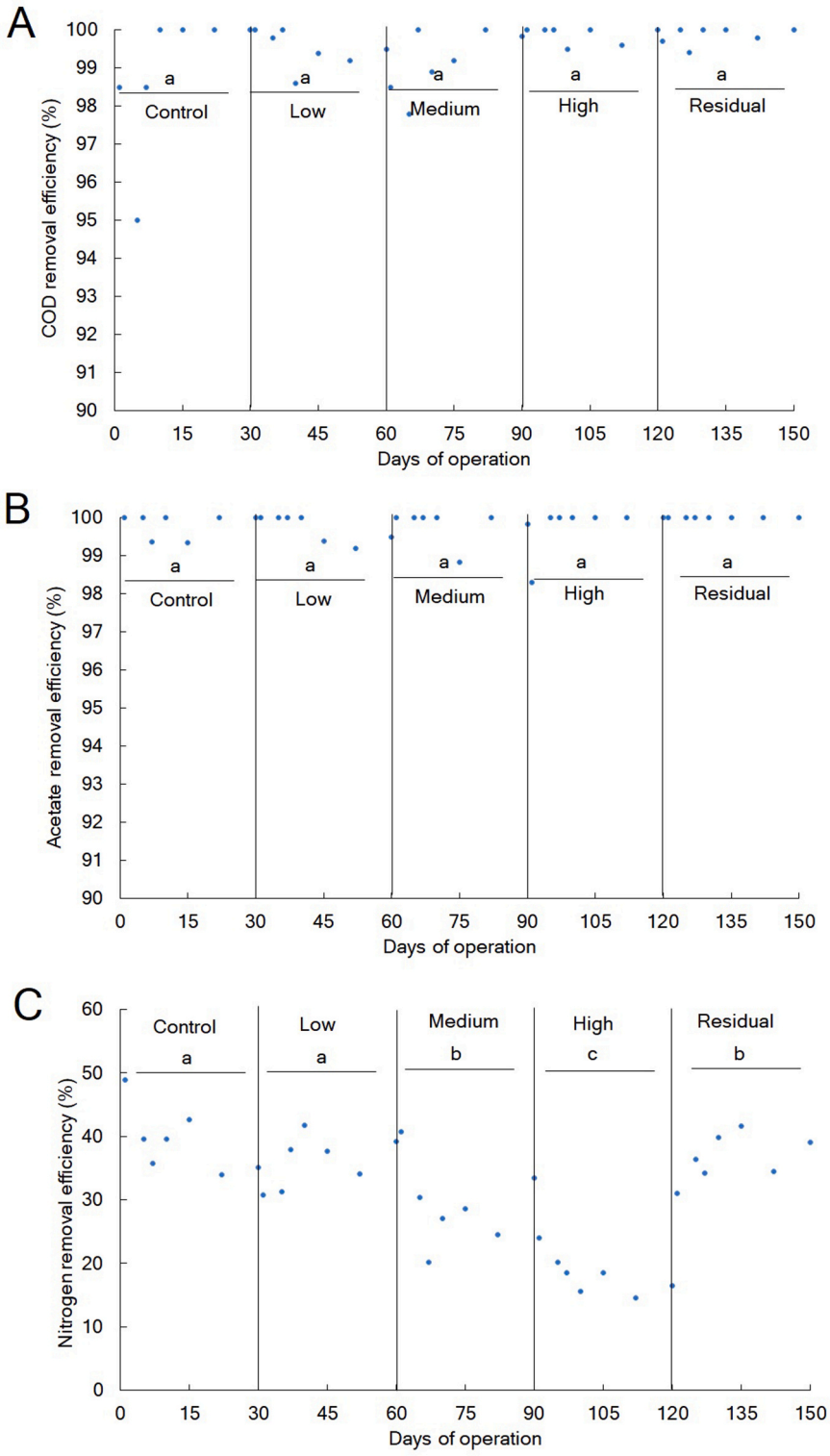

Fig. 1. Average COD (A), acetate (B), and nitrogen (C) removal efficiencies during the experimental period. Different letters above the bars indicate significant differences between treatments (Tukey's HSD, $p \leq 0.05$). COD, carbon oxygen demand.

MLSS, SS, granule size, settling velocity, and pH) using Canoco 5.0 software. Pearson's correlation coefficients between vectors representing biotic and abiotic variables in the RDA plots were calculated.

3. Results

3.1. Effect of anticancer drugs on physicochemical parameters

There were no significant differences in the efficiency of removing COD (Fig. 1A) and acetate (Fig. 1B) between treatments over the experimental period. The application of medium and high treatments significantly reduced the efficiency of N removal compared to the control and low treatments (Fig. 1C). Compared to the rest of the treatments, the lowest N removal efficiency was detected after the application of AD at the high rate (Fig. 1C). A recovery in the N removal efficiency was detected in the residual treatment and values were similar to those detected in the control (Fig. 1C). Variations in the concentration of NH_4^+-N, NO_2^--N, and NO_3^--N in the effluent over the experimental period are presented in Fig. S2.

The MLSS concentration (Fig. 2A), granular size (Fig. 2B), and settling velocity of the granules (Fig. 2C) gradually and significantly decreased with increased concentration levels of AD, particularly with the medium and high treatments. Although the MLSS concentration, (Fig. 2A), granular size (Fig. 2B), and settling velocity of the granules (Fig. 2C) were lowest after application of the high treatment, values increased afterwards in the residual treatment to reach levels similar to those detected in the medium treatment. The concentration of SS in the effluent increased with a greater concentration of AD and was highest in the high treatment (Fig. 2D). No significant differences in the SS concentration were observed between the residual and control treatments (Fig. 2D).

3.2. Concentration of anticancer drugs in water and granules

An analysis of the concentration of CP, TMX, and MTX in the influent of the plant confirmed these substances were applied at the expected concentration over the experimental period and no differences in their concentrations were observed between time points for each treatment (Table 2). CP, TMX, and MTX were not detected in the effluent and granules when they were applied at the low concentration level. With the exception of TMX in the effluent from the medium treatment, the three AD were detected in the effluent and granules from the medium and high treatments (Table 2). CP and MTX were detected in the effluent and the granules after 5 days of operation in the residual treatment but no AD were detected afterwards. Regardless of the treatment, CP and MTX were detected at greater concentrations in the effluent compared to the granules. However, the concentration of TMX was significantly greater in the granules compared to the effluents for the medium and high treatments (Table 2).

The anticancer drug removal efficiencies were dependent on the type and concentration level of each substance and were in the order MTX > TMX > CP during the experimental period, and varied between 53% and 100% (Table S2).

3.3. Abundance of prokaryotic communities and its relationship to physicochemical parameters

The total abundance of the bacterial and archaeal 16 S gene decreased significantly with increased concentration levels of AD (Fig. 3). The abundance of 16SB and 16SA communities was lowest in the high treatment, but values increased afterwards in the residual treatment to reach levels similar to those detected in the control (Fig. 3).

An RDA together with Pearson correlation coefficients showed that the total abundances of 16SB and 16SA were positively and negatively correlated ($r > 0.75$; $p \leq 0.01$), respectively, with MLSS concentration, and COD, acetate, and N removal efficiencies (Fig. 4; Table S3). A significant positive correlation ($r = 0.80$; $p \leq 0.01$) was found between granule size and settling velocity (Fig. 4; Table S3). The RDA also showed that control and low treatments grouped with 16SB, 16SA, MLSS, and efficiency of removal of COD, acetate, and N while time points from the medium, high, and residual treatments formed separated groups (Fig. 4).

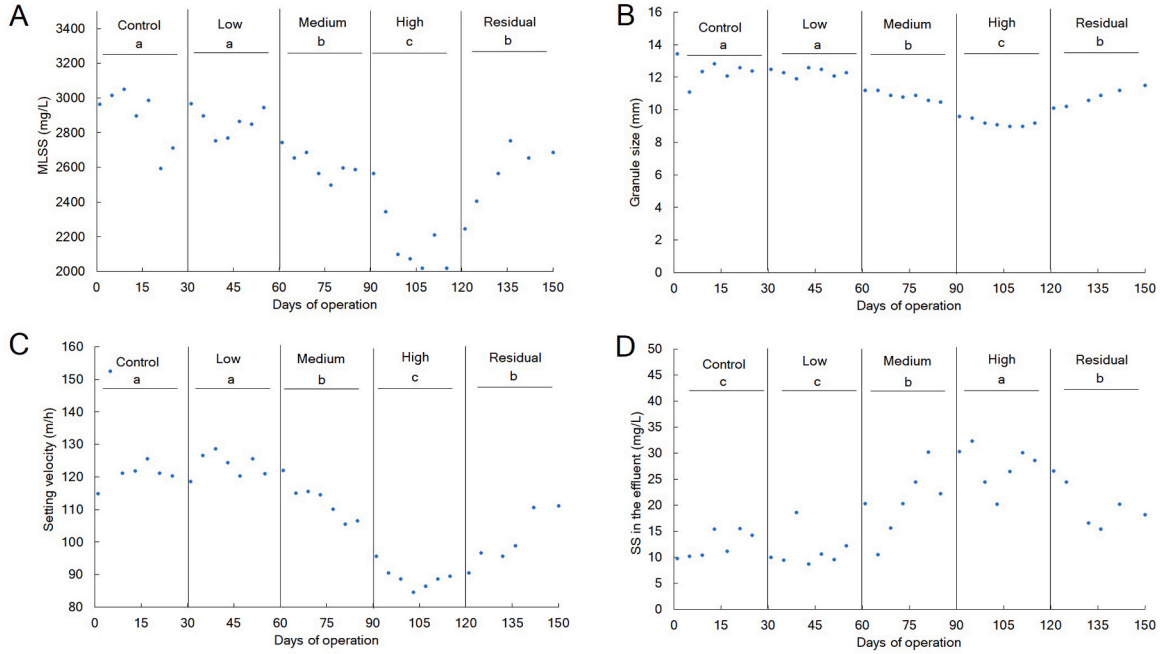

Fig. 2. Average concentration of MLSS (A), granule size (B), setting velocity of granules (C), and concentration of suspended solids in the effluent (D) during the experimental period. Different letters above the bars indicate significant differences between treatments (Tukey's HSD, $p \leq 0.05$). MLSS, mixed liquor suspended solids; SS, suspended solids.

Table 2
Concentration (ng/L) of cyclophosphamide (CY), tamoxifen (TMX), and methotrexate (MTX) in the influent, effluent, and granules at different time points for each treatment. For each row and anticancer drug, values followed by the same lowercase letters are not statistically different among influent, effluent, and granules. Significant differences were determined by two-way ANOVA and Tukey's HSD tests ($p \leq 0.05$). *n.d: not detected. Values are expressed as mean with standard error (n = 2). Recovery efficiencies (%) were considered to calculate the final concentration values (see Section 2.3).

Treatment	Time point	Influent			Effluent			Granules		
		CP (ng/L)	TMX (ng/L)	MTX (ng/L)	CP (ng/L)	TMX (ng/L)	MTX (ng/L)	CP (ng/g)	TMX (ng/g)	MTX (ng/g)
Low	5	58.1 ± 2.8a	1.4 ± 0.2a	39.2 ± 0.8a	3.5 ± 0.9b	n.d.*	n.d.	1.0 ± 0.4c	n.d.	n.d.
	15	60.2 ± 1.9a	1.5 ± 0.4a	39.2 ± 0.6a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	30	59.2 ± 1.7a	1.4 ± 0.2a	39.1 ± 0.5a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Medium	5	598.9 ± 1.9a	15.1 ± 0.4a	399.5 ± 9.0a	180.2 ± 8.9b	n.d.	80.6 ± 5.4b	40.2 ± 1.5c	7.1 ± 1.9b	32.2 ± 4.5c
	15	595.6 ± 1.8a	14.9 ± 0.5a	397.9 ± 9.1a	130.1 ± 7.8b	n.d.	73.2 ± 6.6b	42.2 ± 0.9c	6.3 ± 2.5b	30.1 ± 5.9c
	30	597.6 ± 1.9a	14.7 ± 0.5a	400.6 ± 9.6a	128.9 ± 5.5b	n.d.	70.2 ± 7.3b	42.6 ± 1.2c	5.5 ± 2.3b	30.6 ± 5.1c
High	5	5997.9 ± 8.3a	150.9 ± 8.8a	4001.2 ± 9.4a	1888.7 ± 7.9b	7.4 ± 2.4c	496.4 ± 9.4b	481.6 ± 9.6c	19.1 ± 4.9b	160.9 ± 8.2c
	15	5998.3 ± 8.5a	148.9 ± 8.9a	4000.8 ± 8.5a	1854.2 ± 8.1b	5.2 ± 1.9c	420.4 ± 8.2b	440.6 ± 9.9c	16.3 ± 5.5b	150.6 ± 5.9c
	30	5999.7 ± 8.9a	150.1 ± 7.9a	3998.9 ± 8.9a	1350.8 ± 9.8b	3.2 ± 1.4c	200.7 ± 9.6b	437.8 ± 8.1c	15.5 ± 5.3b	149.5 ± 6.5c
Residual	5	-	-	-	30.2 ± 2.0b	n.d.	20.2 ± 1.1b	11.2 ± 1.5c	n.d.	3.2 ± 0.5c
	15	-	-	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	30	-	-	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

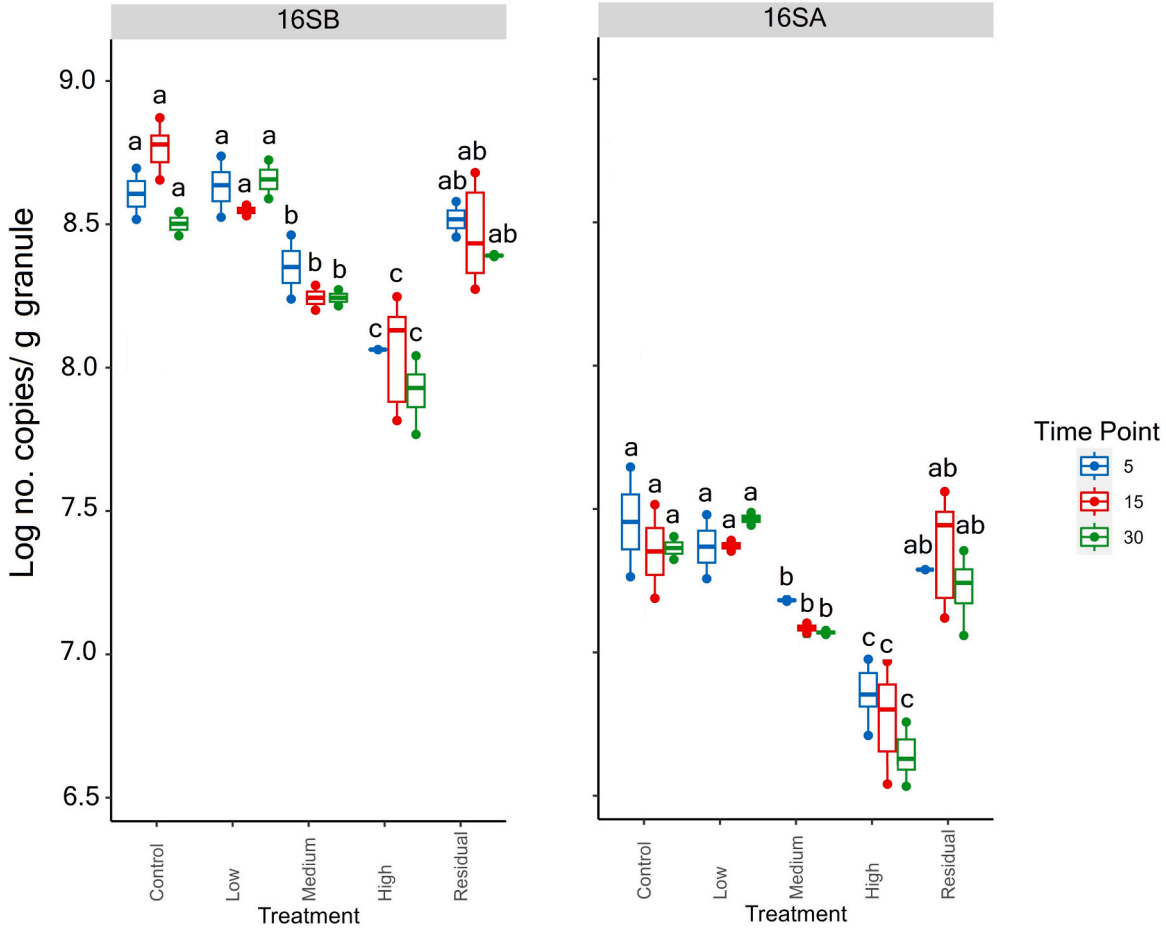

Fig. 3. Total abundance of the 16SB (A) and 16SA (B) communities during the experimental period. Different letters above the bars indicate significant differences between treatments (Tukey's HSD, $p < 0.05$). Values are expressed as mean with standard error.

3.4. Effect of anticancer drugs on the diversity and composition of the prokaryotic microbiome

The application of AD at any concentration level had no significant effect on the number of ASVs compared to the control during the experimental period (Fig. 5). However, significant decreases and increases in the values of the Shannon and Simpson indices, respectively, were observed after the application of medium, and particularly, high concentrations of AD (Fig. 5). No significant differences in the values of Shannon and Simpson indices were observed between the residual and medium treatments (Fig. 5).

NMDS analyses on unweighted UniFrac distances together with

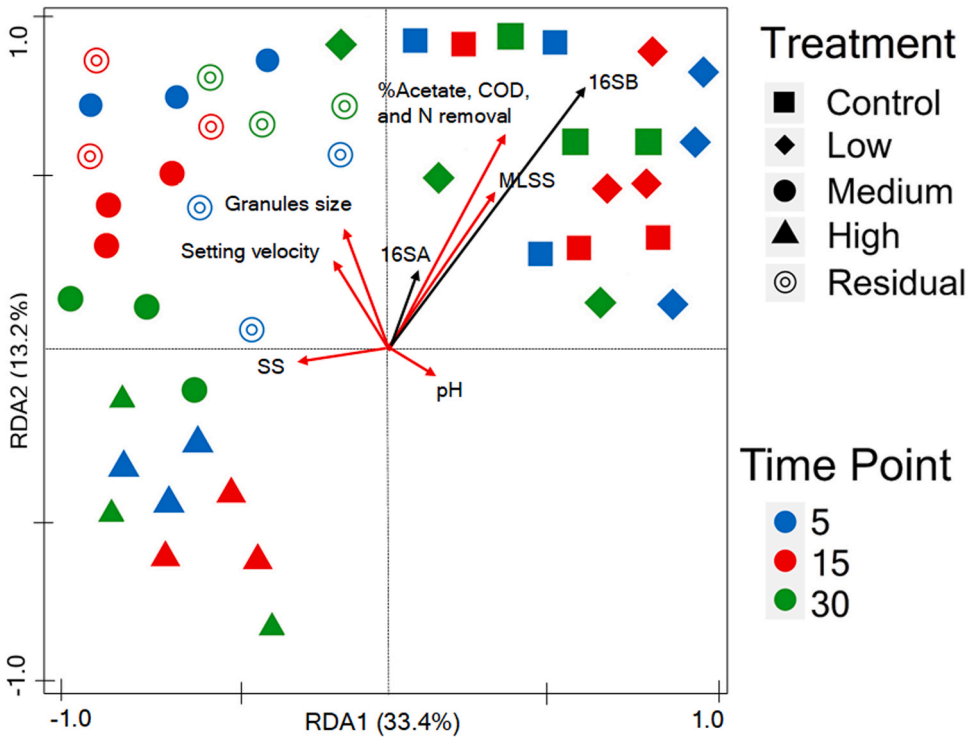

Fig. 4. RDA plots of correlation between physicochemical parameters and the total abundance of bacterial (16SB) and archaeal (16SA) communities during the experimental period. Red solid arrows indicate physicochemical parameters, and blue arrows indicate total abundances of target genes.

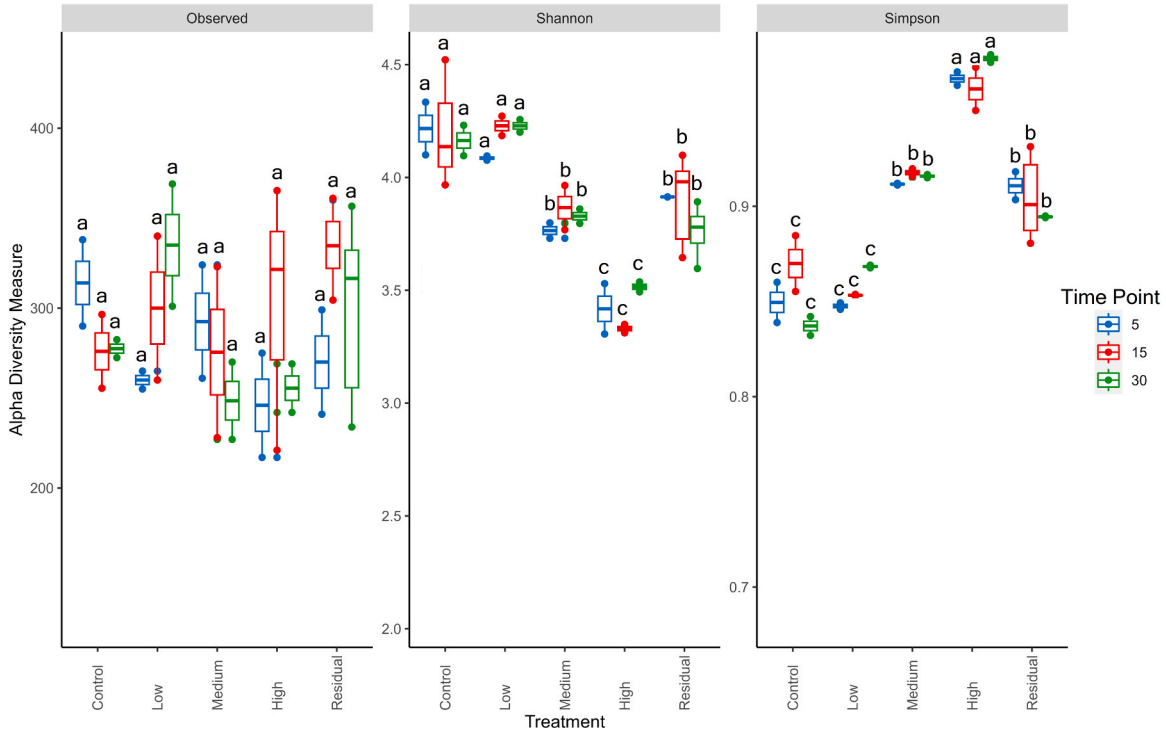

Fig. 5. Number of ASVs, and values of Shannon and inverse Simpson diversity indices for the prokaryotic communities during the experimental period. Different letters above the bars indicate significant differences between treatments and time points (Tukey's HSD, $p \leq 0.05$). Values are expressed as mean with standard error.

PERMANOVA showed significant differences in the composition of the prokaryotic community between treatments ($p \leq 0.001$) and time points ($p \leq 0.01$) (Fig. 6). No significant differences in beta diversity were detected between the control and low treatments ($p \geq 0.05$) or between the medium and residual treatments ($p \geq 0.05$) (Fig. 6).

On average, Proteobacteria (80.2%) and Actinobacteria (15.9%) were the most abundant prokaryotic phyla across treatments (Fig. S3A). Alphaproteobacteria and Actinobacteria were the most abundant classes in the granules (Fig. S3B). In general, Rhodobacteraceae, Methylobacteraceae, and Corynebacteraceae were the dominant prokaryotic

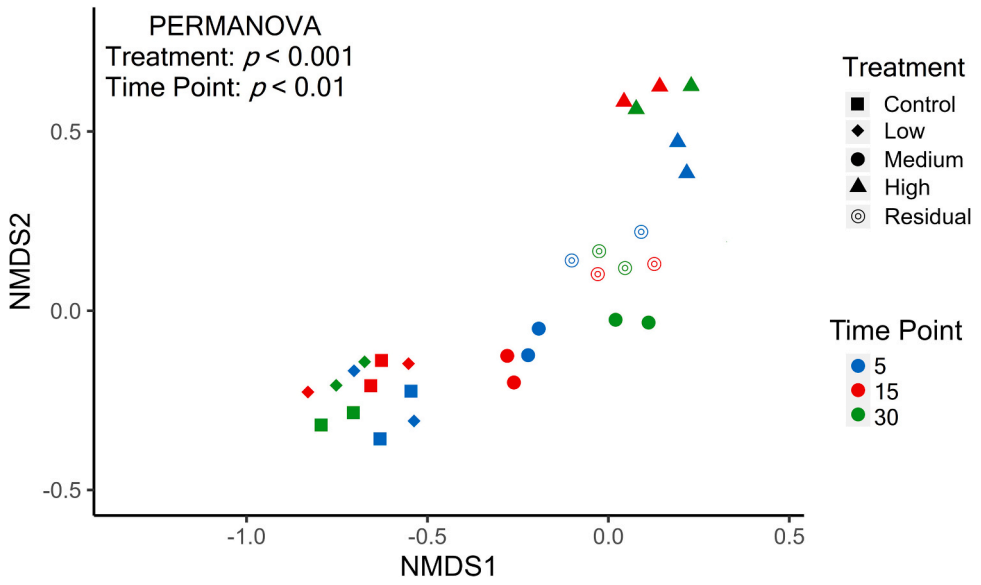

Fig. 6. Non-metric multidimensional scaling (NMDS) plots on unweighted UniFrac distances for the prokaryotic community during the experimental period. Differences in community composition between treatments and time points were tested by permutational analysis of variance (PERMANOVA), and p values ≤ 0.01 were considered significant.

families in the granule microbiome but their relative abundances varied with time and treatments (Fig. S3C). A total of 13 different bacterial genera with more than 1% relative abundance were detected across treatments and time points but their relative abundance differed with time (Fig. S3D).

3.5. Differentially abundant prokaryotic taxa between treatments

A total of 10 prokaryotic ASVs significantly enriched and depleted between treatments were identified at the genus taxonomic level (Fig. 7). The application of AD at medium and/or high concentration levels caused significant decreases in the relative abundance of ASVs belonging to the bacterial genera *Bosea*, *Brevundimonas*, *Meganema*, *Devosia*, and *Leucobacter* compared to the control (Fig. 7). However, the relative abundances of the bacterial genera *Corynebacterium*, *Microbacterium*, *Rhizobium*, *Sphingosinicella*, and *Xanthobacter* were significantly enriched in the medium and high treatments compared to the control (Fig. 7). The negative and positive impacts of AD on the relative abundance of *Meganema*, and *Sphingosinicella*, *Rhizobium*, and *Microbacterium*, respectively, tended to disappear after not adding drugs for a month in the residual treatment (Fig. 7).

3.6. Effect of anticancer drugs on predicted bacterial functions and microbial network complexity

The use of AD at high concentration levels significantly reduced the relative abundance of nine KEGG bacterial pathways (N, methane, and sulfur metabolisms, C fixation in prokaryotes, bacterial secretion system, DNA replication, oxidative phosphorylation, ABC transporters, and cell cycle) compared to the control treatment (Fig. S4). Significant differences in the relative abundance of the above-mentioned predicted pathways between treatments disappeared afterwards in the residual treatment and values were similar to those in the control treatment (Fig. S4).

Co-occurrence networks of the prokaryotic community were constructed to explore the co-occurrence patterns of microbes as a response

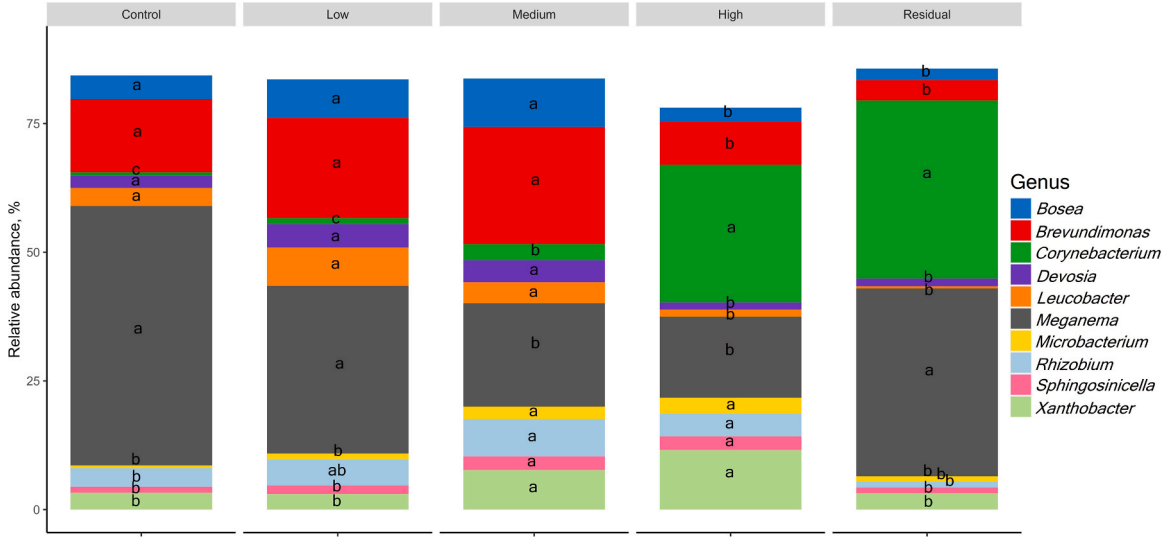

Fig. 7. Differential abundance prokaryotic genera across treatments and time points according to DESeq2 analysis ($p \leq 0.01$).

to the different concentration levels of AD during the experimental period (Fig. S5). The density and number of edges and nodes in the prokaryotic networks gradually and significantly decreased after the application of medium, and particularly, high concentrations of AD ($p \leq 0.05$; Fig. S5; Table S4). Network complexity increased afterwards in the residual treatment but to levels similar to those detected in the medium treatment (Fig. S5; Table S4).

4. Discussion

This study shows that the presence of emerging contaminants such as AD in wastewater can impact the performance of the system and the prokaryotic microbiome of an AGS system operated in an SBR, an effect that is dependent on the concentration of AD. We showed that AGS can help efficiently remove CP, TMX, and MTX, with removal rates in the range of 53–100% depending on the type of anticancer drug and concentration level. Our study shows that, under concentration levels commonly reported in influents of WWTPs, AD significantly decreased the abundance of total bacterial and archaeal communities which was linked to reduced efficiency of N removal. The AD also reduced the diversity, altered the prokaryotic community composition, reduced network complexity, and induced a decrease of a wide range of predicted bacterial functions, particularly when they were applied at medium and high concentration levels. We identified specific bacterial taxa responsive to the addition of AD with known roles in nitrification and denitrification processes. It was reported that the detrimental impact of AD on the performance of the system and microbial communities can last for a month after not adding these substances, suggesting a residual impact of these substances in an AGS system operated in an SBR.

The application of AD at medium and particularly at high concentrations resulted in significantly lower N removal efficiencies, which was linked to decreased abundance of total bacterial and archaeal communities compared to the control and low application rates. However, AD had no significant impact on organic matter removal. Although both bacterial and archaeal communities were strongly positively linked to the removal of organic matter and N, our results show that those involved in N cycling may be more sensitive to AD. This can be explained considering that a small proportion of the total microbial community is often formed by N-cycling microorganisms which carry out processes such as nitrification and denitrification. Because our data showed successful denitrification during the whole experimental period, it is possible ammonia oxidizers were primarily affected by the AD. On the other hand, organic components from wastewater are easily oxidized by a wide range of microorganisms which includes not only prokaryotic but also eukaryotic microorganisms. Our results agree with those of [40] who reported that antibiotics decrease N removal efficiency in an AGS system operated in an SBR. We observed that the detrimental impact of AD on N removal rates appears to be temporal and dependent on the concentration of AD. Because the concentration levels tested in this study were similar to those reported in influents of real domestic WWTPs [6], the results suggest the presence of AD in wastewater should be monitored carefully in the future as it can directly impact wastewater treatment performance and microbial communities, particularly those involved in N removal.

It was observed that AD can have negative impacts on MLSS concentration and granule size in an AGS system operated in an SBR but these effects are dependent on the concentration level of these substances. Previous studies have also reported decreases in granular sludge concentration in AGS treating antibiotics [40]. Decreases in granule size in this study were linked to decreased granule settling velocity, thus showing that AD induce the formation of smaller and less condense granules. This was further supported by detecting a greater concentration of SS in the effluents with an increased concentration of AD. These results are in contrast with those of Wan et al. and Muñoz-Palazón [39] who reported that the presence of antibiotics induces EPS production and stronger aggregation of cells in AGS. Differences in the chemical structure, mode of action, and/or environmental persistence between antibiotics and AD may explain these contrasting results. Our results suggest the existence of a close link between the presence of AD in wastewater and simultaneous changes in MLSS, granule size, settling velocity, and SS. Of note, it was observed that variations in all these physicochemical parameters do not recover to control levels after not adding AD for a month, thus showing the presence of AD at medium and high concentration levels may have temporal impacts on system performance.

This study shows that an AGS system operated in an SBR can efficiently remove CP, MTX, and TMX with removal rates in the range of 53–100% depending on the type of anticancer drug and concentration level. To our knowledge, this is the first study on the feasibility of AGS as a novel biological treatment to remove AD from wastewater. MBR have previously been shown to be able to remove AD but efficiencies are reported to vary largely between $< 20\%$ and 100% for CP [46,30,15]. Recent studies have reported that TMX and CP can pass unaltered through WWTPs [19,6,5], agreeing with our observation that these two substances were detected after 5 days of operation in the residual treatment. However, we showed that an AGS system operated in an SBR can efficiently remove CP and TMX with removal efficiencies of 100% at low concentration levels and in the range of 53–85% at medium and high concentration levels. It is interesting to note that we found AD may differentially accumulate in the effluent and granules. This may be due to differences in the molecular structure between different types of AD that may impact absorption rates to granules and subsequent biodegradation [1]. Overall, a gradual increase of anticancer drug removal was observed with time for TMX and MTX, likely related to increased adsorption and biodegradation by AGS biomass as previously reported for the treatment of pharmaceutical compounds [40]. Yet, it is unknown whether AD can penetrate granular sludge and where degradation occurs (e.g., on the surface or in the interior of the granule).

The presence of medium and particularly high concentrations of AD reduced prokaryotic diversity and altered prokaryotic community composition. Variations in the Shannon and Simpson indices after the application of AD showed the prokaryotic community was dominated by a less diverse group of taxa with an increased anticancer drug concentration. These results can be explained considering that AD such as CP, TMX, and MTX are designed to disrupt DNA synthesis and therefore are expected to have broad-spectrum impacts on non-target microorganisms such as those in the granule microbiome in AGS. Although previous studies have shown AD can have important toxicological effects on aquatic microorganisms [6,43,11], this is, to our knowledge, the first report of microbiome impacts of these substances in AGS. Interestingly, it was observed that the microbial diversity and community composition did not recover to original values after not adding AD for a month, thus highlighting the potential residual impact of these substances on the prokaryotic microbiome. Understanding the impact of AD on prokaryotic microorganisms is important as microbial communities play a key role in wastewater treatment processes [6]. Of note, we found archaeal communities were poorly represented in the granule microbiome which agrees with previous observations in AGS systems used to treat pharmaceutical compounds [40]. Additional studies using different and more specific primer pairs for Archaea may help better characterize this domain in AGS.

The application of AD provoked significant changes in the abundance of specific prokaryotic taxa. *Bosea*, *Brevundimonas*, *Devosia*, *Meganema*, and *Leucobacter* were depleted with an increased anticancer drug concentration while *Corynebacterium*, *Microbacterium*, *Rhizobium*, *Sphingosinicella*, and *Xanthobacter* were enriched. *Bosea* has been related to increased degradation of pharmaceutical products such as ciprofloxacin [58]. Decreases in the abundance of the genera *Brevundimonas* and *Devosia* have been correlated with decreased ammonia oxidation in MBR and other technologies [16,27], and wetlands [51]. Suppression of *Leucobacter* has been related to decreased denitrification across different wastewater treatment technologies [28,55]. *Meganema* is a filamentous

bacterium whose proliferation in AGS has been related to decreased granule stability and treatment performance [33]. Proliferation of *Corynebacterium*, *Microbacterium*, *Rhizobium*, and *Xanthobacter* has been associated with increased nitrification and denitrification during wastewater treatment processes [10,25,35,50,53]. Members of *Sphingosinicella* have been found in wastewater and are known to have exceptional capacity to degrade synthetic β-peptides [23].

The AD decreased prokaryotic network complexity when they were applied at medium and high concentration levels and altered the abundance of predicted bacterial functions primarily at high concentration levels. To our knowledge, this is the first study examining the impacts of AD on network complexity and microbial functions. Previous studies have shown that chemical substances such as fungicides and nematicides can reduce microbial network complexity in soil, which can impact the response of these communities to external perturbations [9,20]. Here, decreases in network complexity together with decreases in microbial diversity, and changes in microbial community composition after the application of AD, suggest these substances induced a decrease or even a loss of some microbial functions, particularly under high concentration levels. This was further supported by identifying nine bacterial KEGG pathways assigned to different biogeochemical cycles (including carbon, sulfur, methane, and N) and general metabolic pathways whose relative abundances were significantly reduced. Overall, these results suggest AD can have broad-spectrum impacts on prokaryotic functionality.

5. Conclusions

This study shows, for the first time, that the presence of AD in wastewater can alter the performance and prokaryotic microbiome of an AGS system operated in an SBR. We show that the combined application of three commonly reported AD at medium and high concentrations similar to those found in influents of real domestic WWTPs can significantly reduce N removal efficiency, an effect that is closely linked to decreased abundance of total bacterial and archaeal communities. We show that the use of an AGS system operated in an SBR can be a reliable biological alternative to efficiently remove AD from wastewater. Our study contributes to putting in the spotlight that AD can reduce the diversity, alter the prokaryotic community composition, reduce network complexity, and induce a decrease of a wide range of predicted bacterial functions. By identifying sensitive taxa responsive to the application of AD, we show that these substances impact the relative abundance of bacterial taxa involved in nitrification and denitrification processes. The AD can have residual impacts on the performance of the system and prokaryotic microbiome of an AGS operated in an SBR that may last for at least one month. Future studies should explore the effect of AD on the abundance of N-cycling communities in more detail as well as variations in the diversity of other microbial groups (e.g., eukaryotes) involved in granule formation and removal of organic matter and N in AGS. Studies on the impact of varying operational conditions (e.g., hydraulic retention time) may help further understand how AGS technology can be optimized to favor efficient removal of AD.

Environmental implication

Increased concerns exist about the presence of anticancer drugs in wastewater. However, knowledge of the impacts of these substances on the performance of the plants and microbial communities during wastewater treatment processes is limited. Anticancer drugs are broad-spectrum by design and understanding their impacts during wastewater treatment processes is critical for helping design management strategies. We provide a detailed analysis of the impact of anticancer drugs on the performance and prokaryotic microbiome of an aerobic granular sludge system operated in a sequential batch reactor. Anticancer drugs are chemical compounds regulated by national and international agencies, and therefore considered “hazardous material.”

CRediT authorship contribution statement

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

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.133674.

References

[1] Amorim, C.L., Moreira, I.S., Ribeiro, A.R., Santos, L.H.M.L.M., Delerue-Matos, C., Tiritan, M.E., et al., 2016. Treatment of a simulated wastewater amended with a chiral pharmaceuticals mixture by an aerobic granular sludge sequencing batch reactor. Int Biodeterior Biodegradation 115, 277–285. https://doi.org/10.1016/j.ibiod.2016.09.009.

[2] APHA, 2012, Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington DC, USA.

[3] Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B 57, 289–300. https://doi.org/10.1111/J.2517-6161.1995.TB02031.X.

[4] Besse, J.P., Latour, J.F., Garric, J., 2012. Anticancer drugs in surface waters. What can we say about the occurrence and environmental significance of cytotoxic, cytostatic and endocrine therapy drugs? Environ Int 39, 73–86. https://doi.org/10.1016/J.ENVINT.2011.10.002.

[5] Castellano-Hinojosa, A., Gallardo-Altamirano, M.J., González-López, J., González-Martínez, A., 2023. Anticancer drugs drive changes in the performance, abundance, diversity, and composition of eukaryotic communities of an aerobic granular sludge system. Chemosphere 345, 140374. https://doi.org/10.1016/j.chemosphere.2023.140374.

[6] Castellano-Hinojosa, A., Gallardo-Altamirano, M.J., González-López, J., González-Martínez, A., 2023. Anticancer drugs in wastewater and natural environments: A review on their occurrence, environmental persistence, treatment, and ecological risks. J Hazard Mater 447, 130818. https://doi.org/10.1016/J.JHAZMAT.2023.130818.

[7] Castellano-Hinojosa, A., Gallardo-Altamirano, M.J., González-López, J., González-Martínez, A., 2024. Novel insights into the impact of anticancer drugs on the performance and microbial communities of a continuous-flow aerobic granular sludge system. Bioresour Technol 394, 130195. https://doi.org/10.1016/j.biortech.2023.130195.

[8] Castellano-Hinojosa, A., Meyering, B., Nuzzo, A., Strauss, S.L., Albrecht, U., 2021. Effect of plant biostimulants on root and plant health and the rhizosphere microbiome of citrus trees in huanglongbing-endemic conditions. Trees - Struct Funct 35, 1525–1539. https://doi.org/10.1007/S00468-021-02133-8/FIGURES/2.

[9] Castellano-Hinojosa, A., Noling, J.W., Bui, H.X., Desaeger, J.A., Strauss, S.L., 2022. Effect of fumigants and non-fumigants on nematode and weed control, crop yield, and soil microbial diversity and predicted functionality in a strawberry production system. Sci Total Environ 852, 158285. https://doi.org/10.1016/J.SCITOTENV.2022.158285.

[10] Chen, X., Zhang, Q., Zhu, Y., Zhao, T., 2021. Response of wastewater treatment performance, microbial composition and functional genes to different C/N ratios and carrier types in MBBR inoculated with heterotrophic nitrification-aerobic denitrification bacteria. Bioresour Technol 336, 125339. https://doi.org/10.1016/j.biortech.2021.125339.
[11] Cristóvão, M.B., Janssens, R., Yadav, A., Pandey, S., Luis, P., Van der Bruggen, B., et al., 2020. Predicted concentrations of anticancer drugs in the aquatic environment: What should we monitor and where should we treat? J Hazard Mater 392, 122330. https://doi.org/10.1016/J.JHAZMAT.2020.122330.
[12] Csárdi, G., Nepusz, T., 2006. The igraph software package for complex network research. InterJ Comp Syst 1695, 1–9.
[13] de Graaff, D.R., Felz, S., Neu, T.R., Pronk, M., van Loosdrecht, M.C.M., Lin, Y., 2019. Sialic acids in the extracellular polymeric substances of seawater-adapted aerobic granular sludge. Water Res 155, 343–351. https://doi.org/10.1016/J.WATRES.2019.02.040.
[14] De Kreuk, M.K., Pronk, M., Van Loosdrecht, M.C.M., 2005. Formation of aerobic granules and conversion processes in an aerobic granular sludge reactor at moderate and low temperatures. Water Res 39, 4476–4484. https://doi.org/10.1016/J.WATRES.2005.08.031.
[15] Delgado, L.F., Faucet-Marquis, V., Pfohl-Leszkowicz, A., Dorandeu, C., Marion, B., Schetrite, S., et al., 2011. Cytotoxicity micropollutant removal in a crossflow membrane bioreactor. Bioresour Technol 102, 4395–4401. https://doi.org/10.1016/J.BIORTECH.2010.12.107.
[16] Dong, K., Feng, X., Wang, W., Chen, Y., Hu, W., Li, G., Wang, D., 2021. Simultaneous partial nitrification and denitrification maintained in membrane bioreactor for nitrogen removal and hydrogen autotrophic denitrification for further treatment. membranes 11, 911. ⟨https://www.mdpi.com/2077-0375/11/12/911⟩.
[17] Douglas, G.M., Maffei, V.J., Zaneveld, J.R., Yurgel, S.N., Brown, J.R., Taylor, C.M., et al., 2020. M.G.I. PICRUSt2 for prediction of metagenome functions. Nat. Biotechnol. 386 (38), 685–688. https://doi.org/10.1038/s41587-020-0548-6.
[18] Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló, D., 2013. Development of a UPLC-MS/MS method for the determination of ten anticancer drugs in hospital and urban wastewaters, and its application for the screening of human metabolites assisted by information-dependent acquisition tool (IDA) in sewage samples. Anal Bioanal Chem 405, 5937–5952. https://doi.org/10.1007/S00216-013-6794-4/TABLES/5.
[19] Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló, D., 2014. Incidence of anticancer drugs in an aquatic urban system: From hospital effluents through urban wastewater to natural environment. Environ Pollut 193, 216–223. https://doi.org/10.1016/J.ENVPOL.2014.07.002.
[20] Fournier, B., Pereira Dos Santos, S., Gustavsen, J.A., Imfeld, G., Lamy, F., Mitchell, E.A.D., et al., 2020. Impact of a synthetic fungicide (fosetyl-Al and propamocarb-hydrochloride) and a biopesticide (Clonostachys rosea) on soil bacterial, fungal, and protist communities. Sci Total Environ 738, 139635. https://doi.org/10.1016/J.SCITOTENV.2020.139635.
[21] Franquet-Griell, H., Gómez-Canela, C., Ventura, F., Lacorte, S., 2017. Anticancer drugs: Consumption trends in Spain, prediction of environmental concentrations and potential risks. Environ Pollut 229, 505–515. https://doi.org/10.1016/J.ENVPOL.2017.06.011.
[22] Gallardo-Altamirano, M.J., Maza-Márquez, P., Montemurro, N., Pérez, S., Rodelas, B., Osorio, F., Pozo, C., 2021. Insights into the removal of pharmaceutically active compounds from sewage sludge by two-stage mesophilic anaerobic digestion. Sci Total Environ 789, 147869. https://doi.org/10.1016/J.SCITOTENV.2021.147869.
[23] Geueke, B., Busse, H.-J., Fleischmann, T., Kämpfer, P., Kohler, H.-P., 2007. Description of *Sphingosinicella xenopeptidilytica* sp. nov., a β-peptide-degrading species, and emended descriptions of the genus *Sphingosinicella* and the species *Sphingosinicella microcystinivorans*. Int J Syst Evol Microbiol 57, 107–113. https://doi.org/10.1099/ijs.0.64509-0.
[24] Gonzalez-Martinez, A., Muñoz-Palazon, B., Maza-Márquez, P., Rodriguez-Sanchez, A., Gonzalez-Lopez, J., Vahala, R., 2018. Performance and microbial community structure of a polar Arctic Circle aerobic granular sludge system operating at low temperature. Bioresour Technol 256, 22–29. https://doi.org/10.1016/J.BIORTECH.2018.01.147.
[25] Hu, T.-L., Kung, K.T., 2000. Study of heterotrophic nitrifying bacteria from wastewater treatment systems treating acrylonitrile, butadiene and styrene resin wastewater. Water Sci Technolo 42, 315–321. https://doi.org/10.2166/wst.2000.0397.
[26] Huang, J., Liu, W., Kang, W., He, Y., Yang, R., Mou, X., Zhao, W., 2022. Effects of microbiota on anticancer drugs: Current knowledge and potential applications. eBioMedicine 83, 104197. https://doi.org/10.1016/J.EBIOM.2022.104197.
[27] Iannacone, F., Di Capua, F., Granata, F., Gargano, R., Esposito, G., 2020. Simultaneous nitrification, denitrification and phosphorus removal in a continuous-flow moving bed biofilm reactor alternating microaerobic and aerobic conditions. Bioresour Technol 310, 123453. https://doi.org/10.1016/j.biortech.2020.123453.
[28] Ito, T., Miyazato, N., Hatamoto, M., Fuchigami, S., Yamaguchi, T., Watanabe, Y., 2019. Diversity and abundance of denitrifying bacteria in a simultaneously nitrifying and denitrifying rotating biological contactor treating real wastewater at low temperatures. H2Open J 2, 58–70. https://doi.org/10.2166/h2oj.2019.021.
[29] Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., Morishima, K., 2017. KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res 45, D353–D361. https://doi.org/10.1093/NAR/GKW1092.
[30] Kovalova, L., Siegrist, H., Von Gunten, U., Eugster, J., Hagenbuch, M., Wittmer, A., et al., 2013. Elimination of micropollutants during post-treatment of hospital wastewater with powdered activated carbon, ozone and UV. Environ Sci Technol 47, 7899–7908. https://doi.org/10.1021/es400708w.
[31] Laguna, A., Ouattara, A., Gonzalez, R.O., Baron, O., Famá, G., El Mamouni, R., Guiot, S., Monroy, O., Macarie, H., 1999. A Simple and Low Cost Technique for Determining the Granulometry of Upflow Anaerobic Sludge Blanket Reactor Sludge. Water Sci Technol 40, 1–8. https://doi.org/10.2166/WST.1999.0371.
[32] Li, D., Chen, H., Liu, H., Schlenk, D., Mu, J., Lacorte, S., Ying, G.G., Xie, L., 2021. Anticancer drugs in the aquatic ecosystem: Environmental occurrence, ecotoxicological effect and risk assessment. Environ Int 153, 106543. https://doi.org/10.1016/J.ENVINT.2021.106543.
[33] Liu, Y., Guo, L., Ren, X., Zhao, Y., Jin, C., Gao, M., Ji, J., She, Z., 2022. Effect of magnetic field intensity on aerobic granulation and partial nitrification-denitrification performance. Process Saf Environ Prot 160, 859–867. https://doi.org/10.1016/j.psep.2022.02.065.
[34] Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15, 1–21. https://doi.org/10.1186/S13059-014-0550-8/FIGURES/9.
[35] Macêdo, W.V., Santos, C.E.D., Guerrero, R.B.S., Sakamoto, I.K., Amorim, de, Azevero, E.L.C., Damianovic, E.B., MHRZ, 2019. Establishing simultaneous nitrification and denitrification under continuous aeration for the treatment of multi-electrolytes saline wastewater. Bioresour Technol 288, 121529. https://doi.org/10.1016/j.biortech.2019.121529.
[36] McMurdie, P.J., Holmes, S., 2013. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One 8, e61217. https://doi.org/10.1371/JOURNAL.PONE.0061217.
[37] McMurdie, P.J., Holmes, S., 2014. Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible. PLOS Comput Biol 10, e1003531. https://doi.org/10.1371/JOURNAL.PCBI.1003531.
[38] Muñoz-Palazon, B., Gorrasi, S., Rosa-Masegosa, A., Pasqualetti, M., Braconcini, M., Fenice, M., 2023. Treatment of High-Polyphenol-Content Waters Using Biotechnological Approaches: The Latest Update. Molecules 28, 314. https://doi.org/10.3390/molecules28010314.
[39] Muñoz-Palazon, B., Rodriguez-Sanchez, A., Hurtado-Martinez, M., Santana, F., Gonzalez-Lopez, J., Mack, L., Gonzalez-Martinez, A., 2020. Polar Arctic Circle biomass enhances performance and stability of aerobic granular sludge systems operated under different temperatures. Bioresour Technol 300, 122650. https://doi.org/10.1016/J.BIORTECH.2019.122650.
[40] Muñoz-Palazon, B., Rosa-Masegosa, A., Hurtado-Martinez, M., Rodriguez-Sanchez, A., Link, A., Vilchez-Vargas, R., Gonzalez-Martinez, A., Lopez, J.G., 2021. Total and metabolically active microbial community of aerobic granular sludge systems operated in sequential batch reactors: Effect of pharmaceutical compounds. Toxics 9. https://doi.org/10.3390/TOXICS9050093/S1.
[41] Muñoz-Palazon, B., Hurtado-Martinez, M., Rosa-Masegosa, A., Vilchez-Vargas, R., Link, A., Gorrasi, S., Gonzalez-Lopez, J., Gonzalez-Martinez, A., 2023. Simultaneous removal of nitrate and pesticides from contaminated groundwater using aerobic granular biomass technology. J Environ Chem Eng 11, 109527. https://doi.org/10.1016/j.jece.2023.109527.
[42] Nassour, C., Barton, S.J., Nabhani-Gebara, S., Saab, Y., Barker, J., 2020. Occurrence of anticancer drugs in the aquatic environment: a systematic review. Environ Sci Pollut Res 27, 1339–1347. https://doi.org/10.1007/S11356-019-07045-2/FIGURES/3.
[43] Negreira, N., de Alda, M.L., Barceló, D., 2014. Cytostatic drugs and metabolites in municipal and hospital wastewaters in Spain: Filtration, occurrence, and environmental risk. Sci Total Environ 497, 68–77. https://doi.org/10.1016/J.SCITOTENV.2014.07.101.
[44] Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2016. Vegan: community ecology package. ⟨https://github.com/vegandevs/vegan⟩ (accessed April 2022).
[45] Rosa-Masegosa, A., Muñoz-Palazon, B., Gorrasi, S., Fenice, M., Gonzalez-Martinez, A., Gonzalez-Lopez, J., 2023. Description of new single-chamber continuous-flow reactors of aerobic granular sludge: Technical and biological study. J Environ Chem Eng 11, 109938. https://doi.org/10.1016/j.jece.2023.109938.
[46] Seira, J., Sablayrolles, C., Montréjaud-Vignoles, M., Albasi, C., Joannis-Cassan, C., 2016. Elimination of an anticancer drug (cyclophosphamide) by a membrane bioreactor: Comprehensive study of mechanisms. Biochem Eng J. 114–155. https://doi.org/10.1016/J.BEJ.2016.07.001.
[47] Sguanci, S., Lubello, C., Caffaz, S., Lotti, T., 2019. Long-term stability of aerobic granular sludge for the treatment of very low-strength real domestic wastewater. J Clean Prod 222, 882–890. https://doi.org/10.1016/J.JCLEPRO.2019.03.061.
[48] Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., Ideker, T., 2003. Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. Genome Res 13, 2498–2504. https://doi.org/10.1101/GR.1239303.
[49] Takahashi, S., Tomita, J., Nishioka, K., Hisada, T., Nishijima, M., 2014. Development of a Prokaryotic Universal Primer for Simultaneous Analysis of Bacteria and Archaea Using Next-Generation Sequencing. PLoS One 9, e105592. https://doi.org/10.1371/JOURNAL.PONE.0105592.
[50] Tanikawa, D., Yamashita, S., Kataoka, T., Sonaka, H., Hirakata, Y., Hatamoto, M., Yamagucho, T., 2019. Non-aerated single-stage nitrogen removal using a down-flow hanging sponge reactor as post-treatment for nitrogen-rich wastewater treatment. Chemosphere 233, 645–651. https://doi.org/10.1016/j.chemosphere.2019.06.012.
[51] Tao, Z., Jing, Z., Tao, M., Kong, Y., Guan, L., Jia, Q., 2023. A novel filter-type constructed wetland for secondary effluent treatment: Performance and its

microbial mechanism. Bioresour Technol 380, 129075. https://doi.org/10.1016/j.biortech.2023.129075.

[52] Tiwari, S.S., Iorhemen, O.T., Tay, J.H., 2019. Aerobic granular sludge and naphthenic acids treatment by varying initial concentrations and supplemental carbon concentrations. J Hazard Mater 362, 348–357. https://doi.org/10.1016/J.JHAZMAT.2018.09.043.

[53] Wei, B., Luo, X., Ma, W., Lv, P., 2022. Biological nitrogen removal and metabolic characteristics of a novel cold-resistant heterotrophic nitrification and aerobic denitrification *Rhizobium* sp. WS7. Bioresour Technol 362, 127756. https://doi.org/10.1016/j.biortech.2022.127756.

[54] WHO, 2022. Cancer. ⟨https://www.who.int/news-room/fact-sheets/detail/cancer⟩ (accessed 19 November 2022).

[55] Wu, H., Zhang, Q., Chen, X., Zhu, Y., Yuan, C., Zhang, C., Zhao, T., 2021. Efficiency and microbial diversity of aeration solid-phase denitrification process bioaugmented with HN-AD bacteria for the treatment of low C/N wastewater. Environ Res 202, 11786. https://doi.org/10.1016/j.envres.2021.111786.

[56] Yadav, A., Rene, E.R., Mandal, M.K., Dubey, K.K., 2021. Threat and sustainable technological solution for antineoplastic drugs pollution: Review on a persisting global issue. Chemosphere 263. https://doi.org/10.1016/J.CHEMOSPHERE.2020.128285.

[57] Yan, L., Zhang, S., Hao, G., Zhang, X., Ren, Y., Wen, Y., Guo, Y., Zhang, Y., 2016. Simultaneous nitrification and denitrification by EPSs in aerobic granular sludge enhanced nitrogen removal of ammonium-nitrogen-rich wastewater. Bioresour Technol 202, 101–106. https://doi.org/10.1016/J.BIORTECH.2015.11.088.

[58] Zhang, L., Yue, Q., Yang, K., Zhao, P., Gao, B., 2018. Analysis of extracellular polymeric substances (EPS) and ciprofloxacin-degrading microbial community in the combined Fe-C micro-electrolysis-UBAF process for the elimination of high-level ciprofloxacin. Chemosphere 193, 645–654.