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Effects of subinhibitory quinolone concentrations on functionality, microbial community composition, and abundance of antibiotic resistant bacteria and *qnrS* in activated sludge

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ABSTRACT

Wastewater treatment plants (WWTPs) are continuously exposed to sub-inhibitory concentrations of antibiotics that are thought to contribute to the spreading of antibiotic resistant bacteria and antibiotic resistance genes, which are eventually released to downstream environments through effluents. In order to understand the effects of sub-inhibitory concentrations of antibiotics on sludge microbiome and resistome, we spiked a conventional activated sludge (CAS) model system with ciprofloxacin, a common fluoroquinolone antibiotic, from 0.0001 mg/L (about twice the typical ciprofloxacin concentration observed in municipal wastewater) up to 0.1 mg/L (one order of magnitude below the clinical MIC for *Enterobacteriaceae*) for 151 days. The abundance of ciprofloxacin resistant bacteria and *qnrS*, a plasmid-associated gene that confers resistance to quinolones, in activated sludge and in effluents of control and spiked CAS reactors, showed no measurable effect of the antibiotic amendment. This was also true for the bacterial community structure and for indicators of WW treatment such as N removal efficiency. Surprisingly, temporal fluctuations in both reactors could explain the observed internal variability of these antibiotic resistance determinants better than the hypothesized antibiotic-driven selective pressure. Overall, this work shows that the core sludge microbiome in CAS systems is resilient to sub-inhibitory concentrations of ciprofloxacin at a functional, structural, and antibiotic resistance levels.

1. Introduction

Wastewater treatment plants (WWTPs) are considered as conside hot-spots for antibiotic resistance (AR), as they are continuously exposed to antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) of various origins, gathered with the feeding inflow [1]. The current understanding of the challenges embedded in the worldwide proliferation of AR highlighted the prominent role of the environment as a source and a receiver of ARB and ARGs in the intricate network of practices that link both human and the environment microbiomes [2]. WWTPs have been intensely studied over the last decade to infer the scopes of AR spread a development within these complex systems as well as in the environments downstream to them, such as surface water, groundwater, and agroecosystems, to name a few [3,4]. A highly diverse range of chemicals, including antimicrobial agents have been reported in WWTPs, although these mostly fall into a range of concentrations typically described as sub-inhibitory for many pathogens [5]. The effects of sub-inhibitory concentrations of antibiotics on single bacteria (as isolates or contextualized with an animal/human host infection) has been widely investigated [6–8]. It is commonly accepted that the establishment and maintenance of resistance genes in a bacterial population are largely dependent on direct antibiotic (or an equivalent chemical stressor) selection, including sub-inhibitory concentrations [9]. However, these effects in complex microbial communities remain

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unclear. Mixed together in complex biological processes, bacteria and residual antibiotics coexist in the sludge ecosystems of WWTPs, and It has often been hypothesised that the latter impose positive selective pressure towards ARB and ARGs, eventually underlining WWTPs not only as hot-spots of AR, but also as facilitators of a slow, yet continuous process of AR *de novo* onset. For instance, previous studies showed how sub-inhibitory concentrations of antibiotics, heavy-metals, and biocides, such as those found in WWTPs, can promote conjugal transfer of resistance plasmids [7,10]. Due to the central role of WWTPs as reservoirs of ARGs, it is important to understand how these mechanisms influence WWTP microbiomes, and particular attention should be given to the mobile resistome therein, because of its capacity to transfer ARGs such as that associated with plasmids [11–13]. In addition to the above, several studies suggest that residual concentrations of antibiotics in the influents may affect activated sludge, and specifically the nitrification process, which if impaired can affect the overall performance of a WWTP, resulting in reduced effluent quality.

Four foremost open questions concerning AR in WWTPs are: (i) do residual (below MIC) antibiotic compounds contribute to AR augmentation in WWTPs, (ii) do ARB have selective advantages in WWTPs relative to their non-resistant counterparts, and (iii) do residual antibiotic concentrations affect the biological process of WWTPs? Accordingly, the present study aimed at testing the hypothesis that subinhibitory concentrations of ciprofloxacin, a common fluoroquinolone antibiotic, affect WWTP sludge microbiomes. To that end, we used a lab-scale conventional activated sludge (CAS) system bioreactor as a model bioreactor. Activated sludge is one of the most commonly applied biological step in wastewater treatment for C and N removal, and several studies showed lab-scale bioreactors to constitute efficient and relevant models to address the objectives of this study [14, 15]. This model system was fed with synthetic wastewater (to reduce degrees of freedom), exposed to stepwise-increasing subinhibitory doses (0.0001, 0.001, 0.01, 0.1 mg/L) of ciprofloxacin and operated in parallel with a control system (with no antibiotic spiking). Complementary culture-based and molecular analyses were adopted to determine: (i) abundance of targeted ARB and a plasmid-mediated quinolone-resistance (PMQR) gene (*qnrS*) in sludge and effluents, (ii) bacterial community composition in activated sludge, and (iii) functional resilience of nitrification and of denitrification in the activated sludge. Ciprofloxacin was chosen as a model antibiotic because of the following properties: (i) it has broad spectrum of activity against both Gram-positive and Gram-negative bacteria [16], (ii) fluoroquinolones are commonly present in wastewater streams at sub-inhibitory concentrations (in the range of 0.00001 to 0.03 mg/L) [17,18], which have been shown to facilitate selection for resistance in *Pseudomonas aeruginosa* biofilms in *in-vitro* tests [19], and (iii) fluoroquinolones strongly adsorb to sludge and are hardly-biodegradable [20].

Our study is novel relative to the previous mentioned works given (i) the use of a state of the art model CAS reactor that mimics the full structure and functionality of a real CAS system, (ii) the wide range of antibiotic concentrations explored, including realistic concentrations commonly observed in WWTPs, (iii) the evaluation of functional parameters such as nitrification, denitrification and organic removal, emulating real systems, and (iv) specific evaluation of quinolone antibiotics, known to persist in CAS sludge and effluents. The presented results complement previous studies by filling knowledge gaps and providing new ecological insights into the dynamics of antibiotic resistance in sludge.

2. Materials and methods

2.1. Experimental design

The experiment incorporated two lab-scale CAS systems, (described in paragraph 2.2) operated in parallel for 151 days. The bioreactors were initially inoculated with activated sludge from a full-scale municipal WWTP and operated for 52 days to ensure stable and comparable biocoenoses, operation and performance. Subsequently, one bioreactor was spiked with an initial dose of 0.0001 mg/L ciprofloxacin. This concentration resembles about 2 times what was previously described to be a typical ciprofloxacin concentration in WWTP influents [21,22]. In order to test a wide range of sub-inhibitory concentrations, doses were subsequently increased by ten-fold increments to 0.1 mg/L every three weeks (except for 0.001 mg/L, which was operated for 4 weeks, details in paragraph 2.2.) (Table 1). The control bioreactor was operated with no antibiotic spiking but otherwise received identical treatment. Samples for monitoring ARB and ARGs abundance were collected weekly, while those used for 16S rRNA gene Illumina amplicon sequencing were collected at the end of each cycle (Supplementary Material, Table S1 and S2) before increasing the antibiotic concentration.

2.2. Operational conditions of bioreactors

The activated sludge used as an inoculum was collected from the biological treatment step in a municipal WWTP of 7250 population equivalents in Burgerland, Austria, implementing full nitrification and denitrification together with chemical P precipitation. The inoculated lab-scale systems were behrotest KLD 4 N/SR (behr Labor-Technik) WWTPs (Fig. 1). Initial stability as well as comparability between the two bioreactors were assessed based on comparing nitrification and denitrification performance as well as microscopic observations of the respective activated sludge.

The reactors that mimic CAS systems, were comprised of three chambers (see Fig. 1): a 4 L anoxic chamber (A) for pre-denitrification, a 4 L aerobic chamber (B) for nitrification, and a 2 L secondary clarifier (C). Aeration was automatically controlled via oxygen probe to ensure an oxygen concentration of 2 mg/L in the aerobic chamber. For denitrification, an internal recirculation (from the aerobic to the anoxic bioreactor) was set as 300 % and external recirculation (from the bottom of clarifier to anoxic chamber) as 150 % of the influent flow. Sludge in both, the anoxic and the aerobic chambers, was continuously mixed by agitators. Systems were operated in continuous flow, with constant synthetic wastewater inflow into the pre-denitrification bioreactor set at 0.6 L/h. Excess sludge was collected daily. Hydraulic retention time was 17.5 h and SRT was 21 d. Efficient nitrification and denitrification processes require a minimal SRT (sludge retention time) of 14 days. Therefore, to achieve nitrification and denitrification ≥80 %, the bioreactors were operated with SRT of three weeks, which was also presumed to contribute to the longer exposure to the increasing doses of ciprofloxacin in the test system. The last samples from the control reactor were taken in the 19th week for culture-based analyses and on the 20th week for PCR-based analyses. After week 19, the control reactor was not sampled due to overgrowth of filamentous bacteria, which hindered the reactors performance. We decided to operate the antibiotic amended reactor for 2 weeks more to prolong the observations assessing the effect of the highest ciprofloxacin concentration on the activated sludge biocenosis. Temperature in the bioreactors was monitored constantly on-line. Due to increase of temperature in week 12 in the bioreactors (to 25 °C) due to increasing ambient temperatures, cooling of the bioreactors was applied from the proceeding weeks. The double-walled vessels allowed control of temperature through the glass coat

Table 1
Description of antibiotic spiking time intervals over time in the test system.

Days after inoculation of bioreactors	Weeks after inoculation of bioreactors	Ciprofloxacin final concentration in the test system (mg/L)
0 - 52	1 - 8	0
53 - 73	8 - 11	0.0001
74 – 102	11 - 15	0.001
103 - 122	15 - 18	0.01
123 - 151	18 - 22	0.1

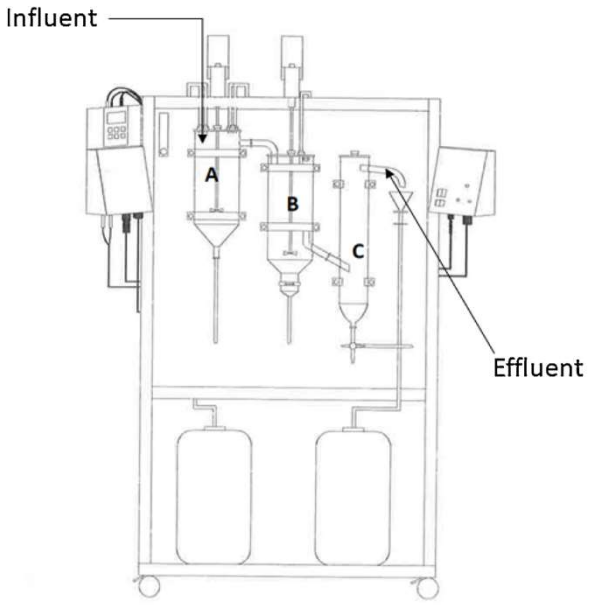

Fig. 1. Schematic diagram of the activated sludge system bioreactors. A denitrification chamber (anoxic conditions); B nitrification chamber (aerobic conditions); C clarifier. A detailed scheme of the systems can be found in Supplementary Material (Figure S1 and S2).

resulting in heat exchange with the bioreactors and stabilization of the temperature at 18 °C. Due to these changes, it was decided not to include week 12 in the final data analysis and prolong the spiking of the test system with 0.001 mg/L ciprofloxacin for an additional week.

The bioreactors were fed with synthetic wastewater to ensure lack of external and uncontrolled input of ARB and ARGs as well as to reduce degrees of freedom by providing a constant and stable supply of nutrients (C/N/P & trace elements) within the 51 weeks of operation. Wastewater was prepared twice a week according to ISO norm 11,733 (details in Supplementary Material, Table S3). The wastewater contained approximately: 470 mg/L COD, 40 mg/L NH_4^+-N, and 3 mg/L PO_4^{3-}-P. The test system was spiked with increasing ciprofloxacin concentrations by pumping appropriate amounts of antibiotic stock solution alongside the influent to the anoxic chamber. Stock solution was prepared and dosed to ensure targeted ciprofloxacin concentration in the bioreactor influent. The antibiotic stock solution and the chambers of the test bioreactor were covered with aluminium foil to prevent photodegradation of ciprofloxacin, and the control system was covered as well to ensure comparability of conditions.

2.3. Analytical methods

Samples of influent wastewater and effluent from clarifier were collected twice a week for analysis of COD (chemical oxygen demand, DIN 38409−43 und DIN ISO 15,705), PO_4^{3-}-P (DIN EN ISO 6878), NH_4^+-N (DIN EN ISO 11,732), NO_x-N (DIN EN ISO 13,395), NO_2–N (DIN EN ISO 13,395), TP (total phosphorus, DIN EN ISO 6878), and TN (total nitrogen, DIN EN ISO 11905−1). Samples of activated sludge in aerobic chamber and excess sludge were collected twice a week for TS (total solids, DIN 38409−1) and oTS (organic total solids, DIN EN 12,879) analyses.

2.4. Culture-based methods

Nutrient Agar was chosen for testing the culturable fraction of the sludge (heterotrophs), while Reasoner's 2A agar, conventionally used for microbiological testing of potable waters, was chosen to investigate slow growers in the effluents [23]. Effluent (0.2 L) and activated sludge (0.1 L) samples were homogenized with an ULTRA TURRAX® T25 basic homogenizer for 2 min at 9500 rpm to break up and disperse activated sludge flocks without destroying bacterial cells. Serial dilutions of the homogenized effluent in 0.85 % (w/v) NaCl solution were filtered (2 mL) in duplicate through 0.45 µm filters (cellulose acetate, Pall Life Sciences, USA), and placed on R2A (VWR Chemicals, Belgium) plates supplemented with and without ciprofloxacin (0, 0.001, 0.01, 0.1, 1, 4 mg/L). Colonies were enumerated after 5 days of incubation at 37 °C. For sludge samples, 50 µL from serial dilutions of homogenized samples were plated on Nutrient Agar (NA; Sigma-Aldrich, Germany) supplemented with and without ciprofloxacin (0, 0.001, 0.01, 0.1, 1, 4 mg/L). Plates were incubated for 24 h at 37 °C and colonies enumerated. Dilutions containing 20–80 colonies per plate were used to calculate bacterial concentrations. Selective media targeting *Escherichia coli* and coliforms were also initially used in the analysis. Due to their known high die-off rate in wastewater treatment systems and lack of substantial regrowth as well as no inoculation via inflow in bioreactors, *Escherichia coli* was only detected on Chromogenic Coliform agar (VWR Chemicals, Belgium) at the beginning of the experimental phase with decreasing quantities till complete disappearance in week 5 and therefore were excluded from subsequent data analysis (data not shown).

The range of ciprofloxacin concentrations supplemented to agar media overlapped those in the test system (0.001, 0.01, 0.1 mg/L), and further included concentrations considered as breakpoint for susceptible and resistant *Enterobacteriaceae* (1 and 4 mg/L) according to [24]. For both media used, at each sampling date and for all the five tested antibiotic concentrations therein, the percentage of resistant bacteria was calculated as the ratio of the average abundance (CFUs/mL) of antibiotic resistant bacteria (enumerated from each antibiotic containing plate) relative to the total abundance (CFUs/mL enumerated on the same medium without antibiotic).

2.5. Culture-independent methods

Following DNA extraction from effluents and sludge samples (section 2.5.1), real-time qPCR (section 2.5.2), conventional PCR, and 16S rRNA Illumina amplicon sequencing (section 2.5.3) were conducted to quantify or detect the *qnrS* gene, and to evaluate changes in the bacterial community structure (described below).

In both, the test and control systems, in order to support the qPCR analyses, the proportion of *qnrS* positive isolates was determined among sub samples of heterotrophs in sludge plated on NA supplemented with 0.1 mg/L of ciprofloxacin by colony-PCR. Randomly selected colonies (30) were isolated by re-streaking and incubating in the same conditions, at the beginning of the spike-in experiment (week 8) and at the endpoint (week 19 for the control, week 22 for the test system) for both systems. *qnrS* positive isolates were determined by gel electrophoresis of PCR products; the same primer set and positive and negative control used in qPCR were used with the conventional PCR.

2.5.1. DNA extraction

Effluent samples (150 mL) were filtered through 0.45 µm filters (cellulose acetate, Pall Life Sciences, USA). DNA was extracted from biomass captured on the filters by E.Z.N.A. Water DNA Kit (Omega Biotek) according to manufacturer instructions. Homogenized activated sludge samples (50 mL) were centrifuged (4500 × *g* at 4 °C for 15 min) and 0.3 *g* of wet pellet used for DNA extraction with a PowerSoil® DNA Isolation Kit (Mo Bio laboratories) according to manufacturer instructions.

2.5.2. Real-time qPCR

The *qnrS* gene copy number in effluents and sludge samples was quantified by quantitative real-time PCR, together with the 16S rRNA gene, which was used to evaluate bacterial abundance. The relative abundance of *qnrS* was then normalized relative to the total bacterial abundance. Reagents, reaction setup, primers, and cycling conditions

are described in R. B. M. Marano et al., [25].

All of the herein reported procedures for qPCR analyses were conducted in accordance with Bustin et al. (2009), including an inhibitors test. The limit of quantification (LOQ) was defined considering the minimum copy number quantifiable by the qPCR procedure (10 considering the lowest point of the standard curve) times volumes of DNA extraction, divided by the sample volume, eventually accounting for 20 copies per ml of sludge and, 6.67 copies per mL of effluent.

2.5.3. Microbial community analysis by 16S rRNA gene amplicon sequencing

DNA extracted from sludge collected at the end of each antibiotic spiking period and additionally in the test bioreactor after a week of spiking with 0.1 mg/L ciprofloxacin, and after the temperature adjustment (from control bioreactor) (Table 2) was sent to 16S rRNA amplicon sequencing by Illumina technology (Microsynth Austria GmbH). The Nextera two-step PCR amplification was conducted using the primer set 341 F and 802R for Illumina (V3-V4 region of 16S rRNA), and sequencing followed with Illumina MiSeq technology with a depth of 1 million reads.

2.6. Data processing

The 16S amplicon sequencing reads, obtained for samples listed in Table 2, were analysed for their quality (trimming) and merged (forward and reverse reads) with use of the public server at usegalaxy.org (version 20.0, [26]). Clustering and OTU (operational taxonomic unit) annotation was done with SILVAngs (version: 1.9.5/1.4.3). Data was further analysed using the Rhea pipeline [27] in R software (R Core Team 2014, Version 3.6.2) with adjusted scripts for normalization of input tables, calculation of alpha-diversity and taxonomic relative abundances. Beta diversity analyses were done in R software with use of vegan package. Dissimilarity between test and control reactors was assessed by generating a non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis distances calculated from the OTU presence and abundances for each sample and constructing a dendrogram (based on Bray-Curtis distance matrix) to visualize clustering of the data. To evaluate the statistical significance of dissimilarities between data groups, PERMANOVA (Permutational Multivariate Analysis Of Variance) was run by comparing the centroids and dispersion of the groups of data. Following, a principal coordinate analysis (PCoA) plots were generated to visualize the dispersion of data groups.

Bacteria counts, *qnrS* relative abundance, taxonomy, alpha diversity, nitrification and denitrification efficiency, and physicochemical parameters such as temperature were analysed statistically with the non-parametric Kruskal-Wallis Rank Sum Test, followed by non-parametric Mann-Whitney Test to identify significantly different groups ($\alpha = 0.05$). Spearman correlation analyses were conducted to compare the test and the control systems trends for both *qnrS* distribution and the percentage of bacteria resistant to ciprofloxacin at 0.1 μg/mL in the culture media for sludge and effluents at $\alpha = 0.05$ (Supplementary Material).

Table 2
Sampling of bioreactors (sludge) for 16S rRNA gene amplicon sequencing.

Day (week) of experiment	Sampling timepoint	Sample for NGS from the control bioreactor	Sample for NGS from the test bioreactor
52 (8)	T0	x	x
73 (11)	T1	x	x
95 (14)	T2	x	–
102 (15)	T3	x	x
122 (18)	T4	x	x
129 (19)	T5	x	x
144 (21)	T6	–	x

3. Results

The effects of ciprofloxacin at different sub-inhibitory concentrations on the activated sludge biocoenosis were evaluated by monitoring the following parameters: (i) functional stability, by carbon removal, nitrification and denitrification efficiency; (ii) abundance of targeted bacteria resistant to sub-inhibitory concentrations of ciprofloxacin, as CFU/mL); (iii) abundance of a target quinolone resistance gene associated with a plasmid-mediated resistance mechanisms (i.e. by qPCR of *qnrS*); (iv) changes in phylogenetic composition of sludge microbiomes.

3.1. Bioreactors operation and nitrogen removal

Inoculation of lab-scale bioreactors fed with synthetic media with well-adapted activated sludge from full-scale WWTPs causes the biocenosis to undergo a large transition to adapt to the new conditions [28]. In the present study, bioreactors were initially operated for three SRTs allowing initial adaptation to operational conditions and synthetic wastewater according to recommended practices [29].

Both systems were operated as pre-denitrification CAS bioreactors with C and N removal. In the test system, the average COD removal was 92.7 % (±3.50 %), NH_4^+-N removal was 99.3 % (±1.0 %), NO_2^--N and NO_3^--N removal was 75.1 % (±10.3 %), Supplementary Material, Table S4 and S5. For the control system, the values were 91.4 % (±9.4 %), 96.3 % (±7.8 %), and 77.9 % (±12.3 %), respectively. In both systems, COD, NH_4^+-N, NO_2^--N and NO_3^--N removal (Fig. 2) was comparably high and stable over the experimental process and no differences between antibiotic spiking periods were observed. The statistical analyses showed no significant differences between COD an N removal in the control and test WWTPs.

3.2. Ciprofloxacin resistant bacteria in sludge and effluents

At low ciprofloxacin concentrations in the plates (0.001 mg/L), the CFU/mL counts were comparable to those on plates without ciprofloxacin, for both the test and control bioreactors, throughout the whole experiment (week 8 to week 22) with only minor fluctuations (Fig. 3a-b; Supplementary Figure S5; Table S7, S9). In contrast, from week 8 to week 22, in both systems, a variable fraction of bacteria were resistant to ciprofloxacin concentrations of 0.01, 0.1, and 1 mg/L, while no bacteria grew at the highest tested ciprofloxacin concentration (4 mg/L) (Fig. 3a-b; Supplementary Figure S5), with the exception of weeks 8–10 where sporadic colonies were reported, accounting for 0.02–1 % of the total enumerated bacteria (Table S7, S9).

At 0.1 mg/L of ciprofloxacin in NA plates, the percentages of

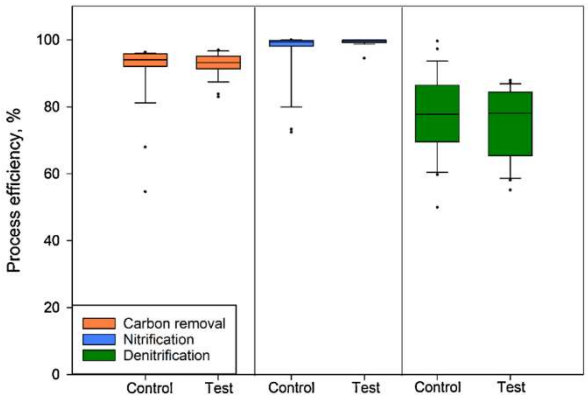

Fig. 2. COD, NH_4^+-N, NO_2^--N and NO_3^--N removal in the control and test bioreactors. Data were collected twice a week during the experimental phase (weeks 8 to 22). Whiskers indicate 10th and 90th percentile with outliers as dots, boxes – 25th to 75th percentile with marked median value. For control bioreactor, n = 25, for test, n = 27, Supplementary Material, Table S4 and S5.

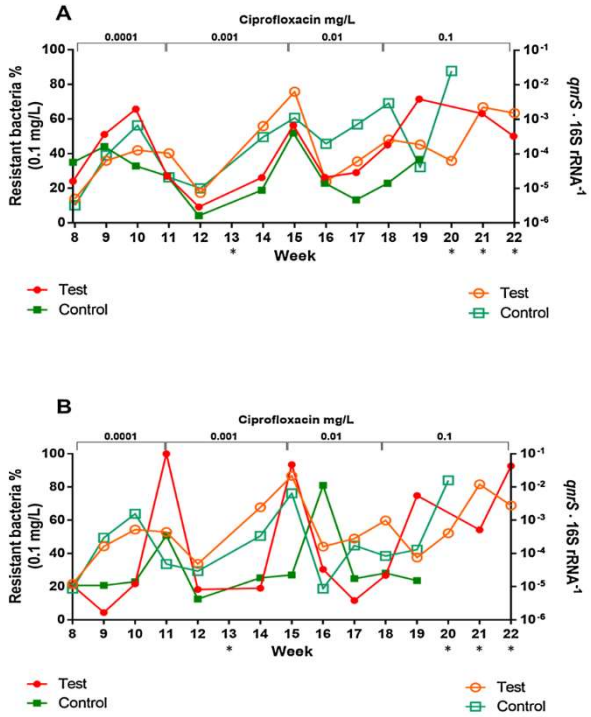

Fig. 3. Distribution of ciprofloxacin-resistant bacteria and the qnrS gene copy number in sludge (A) and effluents (B) from test and control bioreactors over time. A: sludge on NA supplemented with 0.1 mg/L ciprofloxacin. B: effluents on R2A supplemented with 0.1 μg/mL ciprofloxacin. All graphs showing the percentage of bacteria resistant to ciprofloxacin (left y axes) are overlapped with the related distributions of the qnrS gene normalized to 16S rRNA in the tested matrixes (right y axis). Origin of x axis refers to the first week of spiking of the test system with 0.0001 mg/L of ciprofloxacin. Asterisks indicate not available samplings (week 13) and not available samplings for the control system (weeks 20-22) due to hindered operational conditions (filamentous bulking). Spearman correlations between control and test systems: qnrS in effluents, $r = 0.65$, $p < 0.05$; qnrS in sludge, $r = 0.49$, $p > 0.05$; percentage of sludge bacteria resistant to ciprofloxacin at 0.1 μg/mL, $r = 0.61$, $p < 0.05$; percentage of effluent bacteria resistant to ciprofloxacin at 0.1 μg/mL, $r = 0.63$, $p < 0.05$.

resistant bacteria in both systems varied from 4% to 70 % over time (Fig. 3a), displaying higher amplitude of fluctuations than cultures amended with 0.01 and 1 mg/L ciprofloxacin (Supplementary Fig. 5a-b). Noticeably, the oscillating patterns observed in the test and in the control systems were comparable, both showing a period of about three weeks. The difference in the concentration of bacteria resistant to 0.1 mg/L of ciprofloxacin between them was statistically insignificant ($p > 0.05$).

Similarly to what observed with sludge bacteria, fluctuations in the percentage of ciprofloxacin-resistant bacteria were also observed in the effluents, at ciprofloxacin concentrations of 0.001–1 mg/L (Fig. 3b; Supplementary Fig. 5c-d). The relative abundance of resistant bacteria was always greater than 50 % in both systems when plating on 0.001 mg/L ciprofloxacin, and spanned from 40 % to 100 % in the test system, and 20%–100 % in the control system, when plating on 0.01 mg/L ciprofloxacin. Larger fluctuations (as amplitude) were observed when plating on 0.1 and 1 mg/L ciprofloxacin. In effluents, bacteria resistant to ciprofloxacin in concentration 1 mg/L reached 48 % at week 15 for the test system, corresponding to similar peak observed on NA for sludge plated on 1 mg/L ciprofloxacin, while in the control system the highest percentage of resistant bacteria under the same conditions was 24 % at week 16. No bacterial growth was observed after 5 days of incubation with 4 mg/L of antibiotic in the culture medium (Table S8).

3.3. *qnrS* gene abundance in sludge and effluents

The relative abundance of *qnrS* (expressed as copies per 16S rRNA) was determined weekly in sampled sludge and effluents from both test and control systems. Efficiency in qPCR was found to be 100 ± 5 % and R^2 values greater than 0.99, and had specificity confirmed by melting curve analyses. *qnrS* was consistently quantifiable in both systems, and values in sludge were always within the same order of magnitude of the corresponding sampling values in effluent samples at each sampling date. The relative abundance of *qnrS* fluctuated over time, spanning from below 10^{-5} copies/16S rRNA to above 10^{-2}copies/16S rRNA following overlapping temporal patterns observed for ciprofloxacin-resistant bacteria quantified on plates (Fig. 3a-b). The differences in *qnrS* abundance between bioreactors were not statistically significant ($p > 0.05$).

The proportion of *qnrS* positives among random isolates on NA supplemented with 0.1 mg/L of ciprofloxacin was analysed from sludge samples at the beginning and the end of the experiment. The reported proportions of *qnrS* positives among the isolates were 30/30 and 28/30 at the beginning and the end respectively (i.e. 6% variation in proportion) for the test system and 17/30 and 23/30 at the beginning and the end respectively (i.e. 16 % variation in proportion) for the control system. This analysis was conducted to support the hypothesis that the observed variations of *qnrS* in qPCR stemmed out of variations of the *qnrS* hosts' proportion, rather than in changes in the variation of the *qnrS* determinants among the hosts; for this reason, two extreme time points were chosen, i.e. beginning and end of the spiking.

3.4. *Bacterial community analysis*

Sludge samples collected from the control and the test bioreactors at chosen dates (Table 2) were analysed for bacterial community composition by 16S rRNA gene amplicon sequencing. The raw sequences were uploaded into NCBI (Sequence Read Archive, SRA) database under the project with accession number: PRJNA664738. The most abundant phylum was Proteobacteria (average 52.4 ± 5.7 % and 57.2 ± 5.7 % in the control and the test bioreactor, respectively), followed by Bacteroidetes (28.7 ± 5.7 % and 27.1 ± 5.1 %), Chloroflexi (6.9 ± 3.1 % and 4.6 ± 2.6 %), Planctomycetes (3.3 ± 1.8 % and 2.9 ± 1.9 %), Patescibacteria (2.3 ± 2.1 % and 2.4 ± 1.8 %), Nitrospirae (2.4 ± 1.0 % and 2.2 ± 1.2 %), Verrucomicrobia (1.1 ± 0.7 % and 1.1 ± 0.6 %) and Acidobacteria (0.9 ± 0.6 % and 0.8 ± 0.6 %). The relative abundance of Proteobacteria in the control system was relatively stable over the whole experiment (with an average of 52.4 %, Table S24). In the test system the abundance of Proteobacteria during T0 to T4 (weeks 8 – 18) was relatively stable with an average of 53 % relative abundance, comparable to the control system, but between T5 and T6 (weeks 18–22), an increase in relative abundance of Proteobacteria to 66 % was observed. These sampling points correspond to the first and last weeks of spiking of the test system with 0.1 mg/L ciprofloxacin.

Alphaproteobacteria was the predominant proteobacterial class in both bioreactors, and its relative abundance increased in the test bioreactor spiked with 0.1 mg/L ciprofloxacin (Table S24). This increase was mainly attributed to the increased occurrence of the *Caulobacteraceae* family, *Brevundimonas* spp. ($p = 0.014$) (Table S24) *Rhodobacter* spp., family *Rhodobacteraceae* (Table S12, Supplementary Material), and in the relative abundance of the genus *Zoogloea* (Table S12, Supplementary Material); (Supplementary Material, Fig. S6 and S7). The predominant bacterial Families in the activated sludge samples from both systems are shown in Figs. 4 and 5, respectively. The relative abundances of *Nitrospira* spp. (nitrite-oxidizing bacteria) and *Nitrosomonas* spp (ammonia-oxidizing bacteria) were stable over the whole experimental period in both bioreactors (Table S21 and S22, Supplementary Material) except from last sampling data point in the control reactor where a decrease of *Nitrospira* spp. abundance was observed. No effect attributable to the antibiotic spiking in the test bioreactor was observed

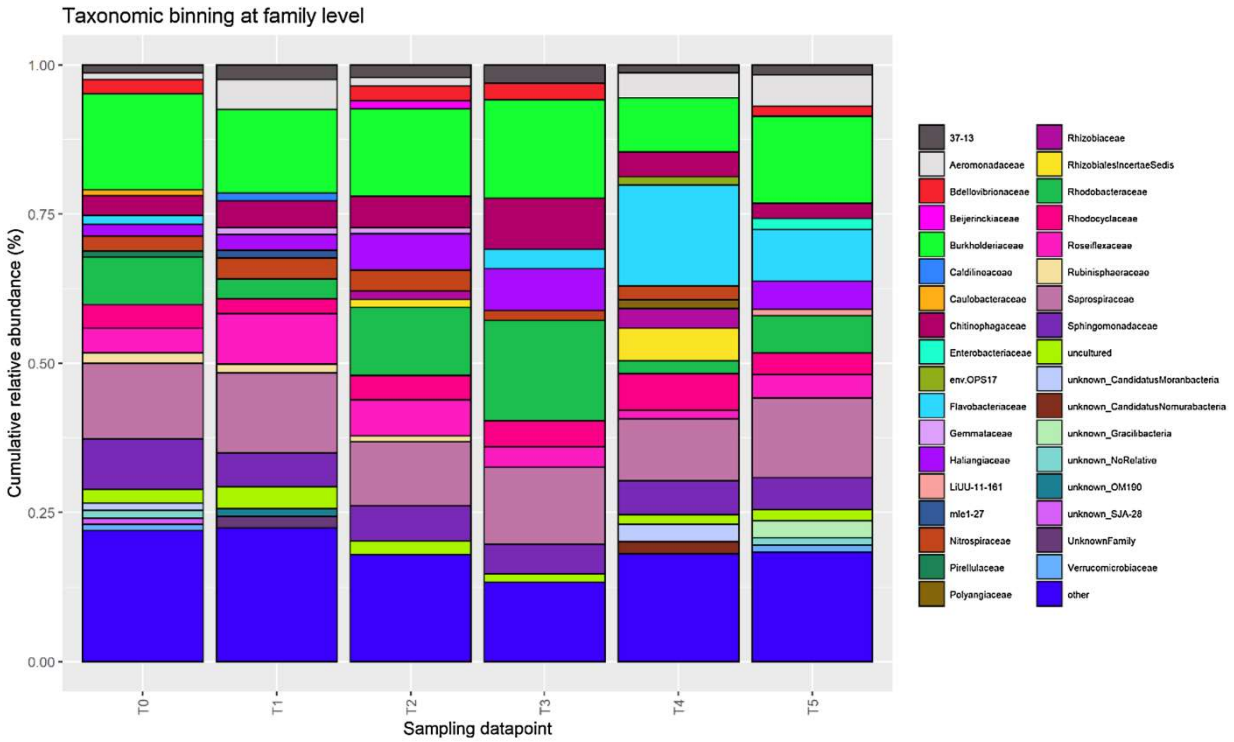

Fig. 4. Relative abundance of taxonomic groups at Family level in the control reactor. Sampling timepoints as in scheme in Table 2 (T0 – day before first spiking of test system, T1 – the last day of spiking of test system with 0.0001 mg/L ciprofloxacin, T2 – a day after temperature change in bioreactors, T3 – the last day of spiking of test system with 0.001 mg/L ciprofloxacin, T4 – the last day of spiking of test system with 0.01 mg/L ciprofloxacin, T5 – the last day of the first week of test system spiking with 0.1 mg/L ciprofloxacin; control system shut down).

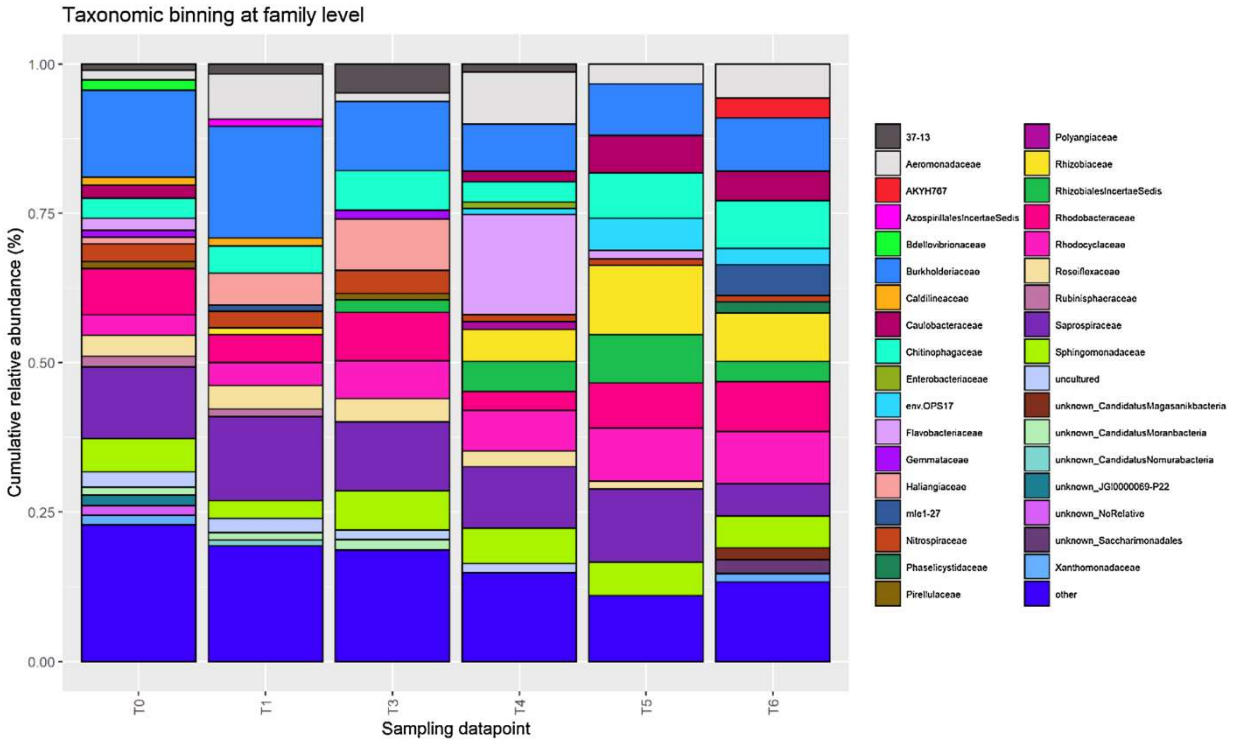

Fig. 5. Relative abundance of taxonomic groups at Family level in test reactor. Sampling timepoints as in scheme in Table 2 (T0 – day before first spiking of test system, T1 – the last day of spiking of test system with 0.0001 mg/L ciprofloxacin, T2 – a day after temperature change in bioreactors, T3 – the last day of spiking of test system with 0.001 mg/L ciprofloxacin, T4 – the last day of spiking of test system with 0.01 mg/L ciprofloxacin, T5 – the last day of the first week of test system spiking with 0.1 mg/L ciprofloxacin; control system shut down, T6 – the last day of spiking of test system with 0.1 mg/L ciprofloxacin).

for *Nitrosomonas* spp. and *Nitrospira* spp.

Following the drop in temperature in both reactors in week 14 (samples taken at T2)), no statistically significant changes in community structure between the two bioreactors were observed, indicating that the bioreactors responded alike to the change in temperature. Thereafter, a slight decrease in the relative abundance of the filamentous bacterial genus *Kouleothrix* was observed (Table S22 and S23, Supplementary Material). A further decrease in its relative abundance was noted in the test bioreactor after spiking with 0.1 mg/L ciprofloxacin. In contrast, in the control bioreactor, at the same sampling point (T5), the abundance of this genus increased leading to filamentous bulking, which hindered operation of this system for the last two weeks of the experiment resulting in the above-mentioned loss of nitrifying bacteria. The excessive filamentous growth was also observed in microscopic pictures of sludge (Supplementary Material, Table S6). The richness of the OTUs in samples from both bioreactors (Fig. 6, Table S25) was comparable over the whole experimental period (average 300 for control and 294 for test system). For the test system, a decrease in OTUs richness was observed at T6, the sampling time point when the test bioreactor was spiked with the highest antibiotic concentration. However, this difference showed not to be statistically significant.

Non-metric multidimensional scaling (NMDS) plot generated based on Bray-Curtis distances suggested that four samples may be clustered together (control reactor at T4 and test reactor at T4, T5 and T6), and differ from the other datapoints, which seemed to form one distinct group (Figure S9, Supplementary Material). To further investigate this observation, dendrogram was constructed (Figure S10, Supplementary Material). It suggested that samples from the test reactor taken at T5 and T6 show high similarity to each other as well as samples from control and test reactor taken at T4. These samples were positioned on a clade separating them from the main cluster of the other samples. Furthermore, samples from control and test reactors taken at the same sampling points were positioned at the same clade showing high similarity between reactors (e.g. at T0, T1, T2, T3, and T4). To evaluate the statistical significance of the observed possible dissimilarities between data groups, PERMANOVA test was run. The results of this test showed no statistically significant differences between control and test reactors ($p = 0.145$). PCoA was constructed to visualize the positioning of centroids and dispersion of data (Figure S11, supplementary Material). The centroids were positioned in close proximity and dispersion of data seemed similar between the reactors, confirming the results of PERMANOVA test.

4. Discussion

Our work shows that CAS systems can be highly resilient to that incremental increases of large range of sub-inhibitory ciprofloxacin concentrations. A 151 day-long experiment with well-controlled laboratory CAS systems, including high frequent measurements of carbon and nitrogen transformations, PCR and plate-based counts along with high throughput sequencing community analyses uncovered a hitherto unknown resilience of sludge-based treatment to such sub-inhibitory antibiotics concentrations.

Four-concentrations of ciprofloxacin on the activated sludge core microbiome and resistome were investigated in controlled conditions where the contribution of pharmaceuticals and personal care products in the inflow was bypassed together with the continuous input of bacteria that normally significantly contribute to the overall resistome of a WWTP. Previous studies had investigated the effects of antibiotics (including ciprofloxacin) on activated sludge microbiomes in experimental setups comparable to the one described herein, without the continuous presence of external bacteria in the inflow [14,30,31]. These studies reported changes in ARGs and ARB distribution in their systems, as well as changes in the COD and N removal performances, however these effects were observed at extremely high dosages of spiked-in antibiotics (in the order of magnitude of several mg/L) which are substantially higher than the average concentrations of antibiotics typically measured in WWTP influents (~0.0001 mg/L). Our aim was to mimic and investigate the effect of antibiotic selection pressure in WWTPs within a range that encompasses realistic sub-inhibitory concentrations previously reported for ciprofloxacin [18] up to a concentration one order of magnitude below the clinical MIC for *Enterobacteriaceae* (according to [24]), here used as an upper reference point.

In our work, the relative abundances of most abundant phyla were stable in both bioreactors over the whole experimental period except for Proteobacteria at 0.1 mg/L ciprofloxacin spiking in the test bioreactor, similarly to what previously observed [32,33]. This indicates that members from this predominant lineage largely constituted the favoured species and proliferated in the test system at 0.1 mg/L ciprofloxacin spiking, such as: *Brevundimonas* spp., *Rhodobacter* spp., and *Zooglea* spp. According to Han & Andrade, [34] and Ryan & Pembroke, [35], *Brevundimonas* spp. may harbour intrinsic resistance to fluoroquinolones, which would explain its relative proliferation at elevated ciprofloxacin doses. *Rhodobacter* spp., a group of photosynthetic bacteria that can be capable of denitrifying, was previously reported to be resistant to tetracycline in activated sludge [36,37] and might be either intrinsically resistant to fluoroquinolones or rely on multidrug resistance mechanisms [38,39]. Meng et al., [33] observed increased relative abundance of members of some denitrifying bacteria lineages under elevated fluoroquinolones selection pressure (0.9 and 9 mg/L) suggesting that these may be tolerant and well-adapted to them. *Zooglea* spp., grows in activated sludge flocs and is known as one of the most important bacteria producing extracellular polymeric substances (EPS) in activated sludge [30]. In the presence of erythromycin, it was reported to become more abundant and to form protective biofilms [40], which may be a case also in our study, under 0.1 mg/L ciprofloxacin pressure.

Except for these genera, no statistically significant dissimilarities in the numbers and relative abundances of OTU were observed between control and test bioreactors at sampling data points based on Kruskal

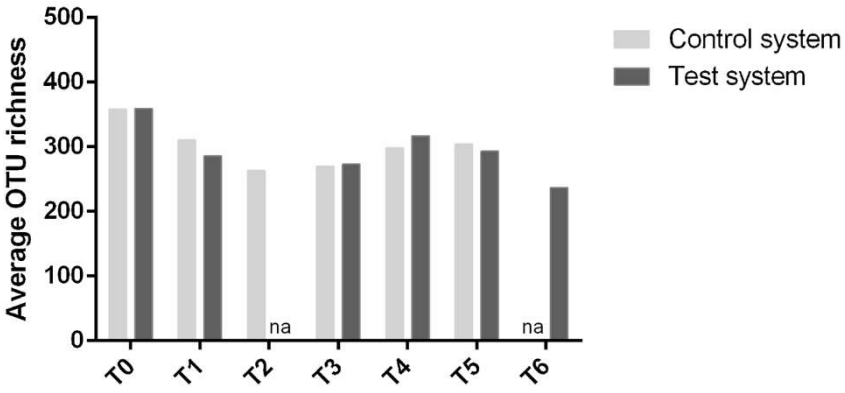

Fig. 6. OTU richness in the test and the control bioreactors at different sampling points. (T0 – day before first spiking of the test system, T1 – the last day of spiking of test system with 0.0001 mg/L ciprofloxacin, T2 – a day after temperature change in bioreactors, T3 – the last day of spiking of test system with 0.001 mg/L ciprofloxacin, T4 – the last day of spiking of test system with 0.01 mg/L ciprofloxacin, T5 – the last day of the first week of test system spiking with 0.1 mg/L ciprofloxacin; control system shut down, T6 – the last day of spiking of test system with 0.1 mg/L ciprofloxacin).

Wallis Rank Sum Test and Mann Whitney Test, PERMANOVA and PCoA. The NMDS and dendrogram clustering showed that OTU numbers and abundances for control and test reactor at the same timepoint clustered together, revealing high similarity between these two reactors. The diversity and abundance of bacterial taxa at phylum level was typical for activated sludge samples [32,15,41]. Richness of the bacterial community in the control reactor was comparable to the test bioreactor at the sampling datapoints over the whole experiment (no statistically significant differences with respect to the preceding sampling times were reported). which may explain the functional stability of both reactors. From a functional perspective, the two systems were indeed comparable throughout the whole experiment. Yet, filamentous bacterial bulking impeded the operation of the control reactor during the last two weeks of the experiment due to uncontrolled loss of nitrifying biomass via the effluent resulting from sludge separation problems in the secondary clarifier. This event was not observed in the test bioreactor, possibly due to the spiking with 0.1 mg/L ciprofloxacin that inhibited growth of filamentous bacteria (mostly *Kouleothrix* spp.).

Several studies suggested that antibiotics in WWTP influents may have an effect on activated sludge performance in general, and specifically on nitrification [42–46,21,33]. [47] observed that ciprofloxacin in concentrations of 0.2 and 2 mg/L affected the abundances of polyphosphate accumulating organisms, glycogen accumulating organisms and denitrifying bacteria, which were consistent with the decreased nutrient removal performance measured in these reactors. [48] noted decreased phosphorus removal in SBR spiked with 0.5 mg/L norfloxacin. These concentrations are order of magnitude higher than environmentally relevant concentrations typically found in wastewater [18]. In a study investigating effects of ciprofloxacin on nitritation, [43] reported that the presence of 0.1 mg/L ciprofloxacin resulted in temporary changes in bioreactor performance (inhibition of the ammonium oxidation process and reduced microbial biomass), and also observed a temporary change in the abundance of ammonia-oxidizing bacteria (AOB, i.e. *Nitrosomonas* spp.). In contrast, in our study, no statistically significant differences in N removal were observed between the control and test bioreactor, or between the antibiotic spiking intervals within the test bioreactor (even for 0.1 mg/L ciprofloxacin). Likewise, no significant differences were observed for the relative abundance of AOB (*Nitrosomonas* spp.) as well as nitrite oxidizing bacteria, (NOB, *Nitrospira* spp.) comparing both bioreactors at the sampling datapoints. Tolerance of *Nitrospira* spp. to fluoroquinolones was previously reported by [33] in a study surveying the effects of ciprofloxacin on membrane bioreactor systems.

While [43] observed alterations of functional properties in the CAS bioreactors induced by application of a single high concentration (0.1 mg/L) dose of ciprofloxacin, the lack of changes in functional properties as well as AOB and NOB abundance observed in our study may originate from the duration of antibiotic-spiking. Although no direct support for this proposition was found in the literature, the stepwise increasing doses of ciprofloxacin might have resulted in AOB and NOB becoming insensitive to elevated concentrations of ciprofloxacin. A similar observation for erythromycin was made by Du et al. [42], who reported that stepwise increasing doses of antibiotic (1–50 mg/L) did not result in inhibition of nitrification in sludge, even if bioreactors were treated with concentration an order of magnitudes higher (400 mg/L), which in turn inhibited nitrogen removal when directly introduced. These results suggest that acclimation of sludge microbiota by introducing antibiotic concentrations several orders of magnitude below the MIC for longer times may induce higher tolerance of nitrifying bacteria towards such antibiotic concentrations. Functional stability could also be contextualized with functional redundancy [49], where ciprofloxacin-resistant AOB and NOB replaced sensitive strains following the imposed stress, leading to unnoticed changes in the overall performance of the bioreactor. This might have happened for example through point mutations in chromosomal DNA gyrase genes, a common quinolone resistant mechanism [50].

The lack of effects of spiking with sub-inhibitory antibiotic concentrations on functionality and bacterial community composition in the test bioreactor may also be related to the possible adsorption of the antibiotic compounds to sludge organic matter and EPS. Ciprofloxacin was previously reported to sorb to EPS, proteins, and humic acids naturally produced by sludge bacteria growing in biofilms, which can proliferate in response to antibiotic exposure [31,51]. This implies that even though local antibiotic concentrations might be higher in the microenvironment of a sludge floc, the substance is not biologically available, and that the EPS-adsorbed ciprofloxacin fraction is removed from the system every SRT. Sorption of ciprofloxacin onto EPS would also reduce the concentration in the aqueous phase, which in turn lowers its effect on planktonic bacteria. Secreted EPS may provide a mechanism that can actively protect bacterial cells from unfavourable conditions, as indicated by Kong et al. [32], who demonstrated that the concentration of proteins and polysaccharides constituting sludge biofilm increased with elevated ofloxacin concentrations in wastewater. Thus, EPS seems an important factor in determining an actual antibiotic selection pressure on bacteria in sludge. Nevertheless, while this may be true in the case of fluoroquinolones, this mechanism might not be true for other antibiotics, and future studies should investigate the effects of other chemically distinct antibiotics and antimicrobial compounds on WWTP microbiomes contextually with the EPS.

From the perspective of ARGs and ARB, we specifically focused on the impact of residual ciprofloxacin concentrations on resistant bacterial levels and on the relative abundance of *qnrS*. This gene is a model for plasmid mediated quinolone resistance (PMQR) [50], and therefore a proxy for HGT (horizontal gene transfer) of fluoroquinolone resistance. While the *qnr* genes do not typically confer resistance to high ciprofloxacin concentrations (i.e. $>$ 1 mg/L) they confer resistance to sub-inhibitory (MIC) fluoroquinolone concentrations (up to 0.1−0.5 mg/L) [8], and unlike other mechanisms of resistance (e.g. multi drug efflux pumps) they are fluoroquinolones specific. Additionally, previous studies have shown that bacteria that harbour *qnr* genes have significantly higher probabilities of acquiring mutations that confer MIC levels of resistance [52]. In our study, no significant effects of antibiotic spiking on ARB and *qnrS* abundance were observed. *Qnr*-harbouring plasmids, as well as other mechanisms conferring resistance to fluoroquinolones, are often present in WWTPs [53,54]. It is possible that the sludge microbiome being already adapted to the WWTP conditions, was not affected by the tested conditions, and that the whole duration of the experiment was not enough to allow for observable loss or development of ciprofloxacin-resistant determinants (among ARGs and ARB).

We also posit that the observed lack of changes in the surveyed antibiotic resistance determinants is ascribable to the community effect of these complex microbial populations. The MIC for a given antibiotic of a certain bacterium might indeed depend on the surrounding microenvironment and can significantly change when the same bacterium is tested alone or in a complex community, as described by [55]. It is therefore possible that the selective pressure of the antibiotic concentrations hereby described was lower than postulated, and that changes could not be appreciated over the relatively short time frame. Such observation is important as it provides experimental evidences that can support/complement previously proposed ciprofloxacin concentration thresholds predicted to assert a selective pressure on natural microbial communities [56].

Over the course of the experiment, considerable fluctuations in the relative abundance of ciprofloxacin resistant bacteria and *qnrS* gene copy number were observed in both bioreactors (Fig. 3). However, these were not attributed to the spiking of ciprofloxacin, as they were observed in both the test and the control bioreactor. Even at weeks 21 and 22 of the test system (spiked with 0.1 mg/L of ciprofloxacin) the highest relative abundance of sub-MIC resistant bacteria (on 0.1 mg/L ciprofloxacin plates) was no higher than that observed at a peak from week 10, when spiking concentration where 1000 times lower (Fig. 3, and Supplementary Fig. 5). However, changes in the percentage of ARB

from the two surveyed systems (in both sludge and effluents) followed similar fluctuation patterns like those observed for *qnrS*, suggesting that the numerical observations of the growth on media supplemented with ciprofloxacin (i.e the phenotype) is linked to the distribution of *qnrS* (i.e. the genotype). In support of this is (i) the scarce distribution of resistant bacteria plated on 1 and 4 mg/L of ciprofloxacin, a concentration for which *qnr* genes do not typically confer resistance to, and (ii) the distribution of *qnrS* among randomly selected sludge isolates plated on NA supplemented with/without cefotaxime, which shows that *qnrS* is profusely distributed in sludge bacteria resistant to 0.1 mg/L ciprofloxacin, at least among the cultivated fractions. Finally, it cannot be ruled out the contribution of DNA gyrase and topoisomerase point mutations already present in the activated sludge at the time of the inoculum, since the sludge originated from a real WWTP.

The displayed fluctuations of the tested ARB and *qnrS* in sludge and effluents, were interestingly synchronized over the investigated time between the test and the control bioreactors despite the two systems being independent from each other. We posit that this phenomenon was observable as a consequence of the relatively long SRT adopted, together with the multiple sampling times within each SRT timeframe. These fluctuations of the cultivable and the molecular targets reflected ecological fluctuations naturally occurring in the sludge biocenosis, that were appreciable simply because such bacteria were probably also hosts of the *qnrS* gene (and possibly other quinolone-resistance genes) within the investigated set up. These results highlight the embedded complexity and dynamics of bacterial communities in such systems, which despite steady physical, chemical, and operational conditions, are subjected to internal 'dynamic equilibria' with temporal expansion and reduction of certain species, probably driven by ecological parameters, such as predation and niche competition. The temporal synchrony of these fluctuations between the two systems reflects a phenomenon previously described also in membrane bioreactors, which sees dependent activated sludge systems evolving similarly upon the same operational parameters [57], and even at a regional scale in full-scale activated sludge bioreactors [58].

5. Conclusions

This work provides evidence that sub-inhibitory concentrations of ciprofloxacin typical of those present in urban WWTPs do not substantially alter the core CAS microbiome. Significant taxonomic changes were observed only at 0.1 mg/L ciprofloxacin, a concentration 1000 times higher than usually found in WWTPs. Based on these results it is presumed that in full-scale WWTPs ciprofloxacin is not expected to cause significant alteration of CAS bioreactor functionality or sludge bacterial community composition. In contrast, temporal fluctuations in CAS bioreactors facilitated by unknown mechanical or ecological parameters may have strong impact on ARB and ARGs abundance. Hence, care should be practiced when attempting to infer cause-effect relationships between CAS performance and the abundance of ARGs and ARB, and multiple sampling of CAS should be conducted to fully understand system performance (e.g. monthly samplings or within SRT samplings). Furthermore, future studies need to investigate the combinatorial effect of multiple antimicrobial compounds for a more realistic depiction of how antibiotic affect CAS performance and bacterial resistance.

Authors' contribution

K. Slipko and R.B.M. Marano conducted the experiments, analyzed the data, and wrote the manuscript (Equal contribution)

V. Merkus contributed to bioreactors' data

M. Wögerbauer, J. Krampe,and E. Jurkevitch supervised the work

E. Cytryn and N. Kreuzinger supervised the work, contributed to data analysis, provided resources

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 associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jece.2020.104783.

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