Ecological impact of the antibiotic ciprofloxacin on microbial community of aerobic activated sludge

Accepted to

Environmental Geochemistry and Health December, 2018

Dogun Kim[a], Luong N. Nguyen[b] and Seungdae Oh[a*]

[a] Department of Civil Engineering, Kyung Hee University, 1732 Deogyeong-daero, Giheung-gu, Yongin-si, Gyeonggi-do, 17104, Republic of Korea.

[b] Center for Technology in Water and Wastewater, School of Civil and Environmental Engineering, University of Technology Sydney, Sydney, NSW 2007, Australia

Corresponding author

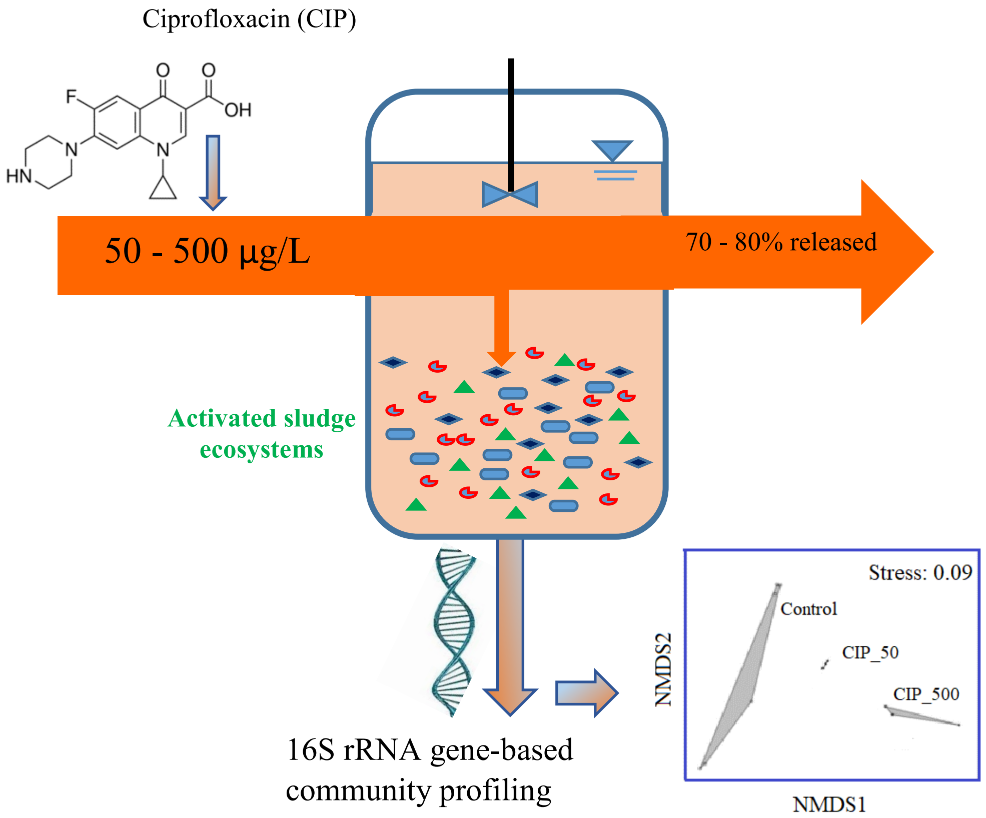
[*] Seungdae Oh

Email: soh@khu.ac.kr ; Tel: +82 (031) 201-3664

Highlight

- Activated sludge (AS) function was not affected by CIP at 50-500 μg/L
- AS microbial community evenness was decreased at CIP exposure of 500 μg/L
- Overall AS microbial community structure was changed at CIP exposure of 500 μg/L
- Ciprofloxacin (CIP) was poorly removed by AS

Graphical abstract



Abstract

This study investigated the effects and fate of the antibiotic ciprofloxacin (CIP) at environmentally relevant levels (50–500 µg/L) in activated sludge (AS) microbial communities under aerobic conditions. Exposure to 500 µg/L of CIP decreased species diversity by about 20% and significantly altered the phylogenetic structure of AS communities compared to those of control communities (no CIP exposure), while there were no significant changes upon exposure to 50 µg/L of CIP. Analysis of community composition revealed that exposure to 500 µg/L of CIP significantly reduced the relative abundance of *Rhodobacteraceae* and *Nakamurellaceae* by more than tenfold. These species frequently occur in AS communities across many full-scale wastewater treatment plants and are involved in key ecosystem functions (i.e., organic matter and nitrogen removal). Our analyses showed that 50–500 µg/L CIP was poorly removed in AS (about 20% removal), implying that the majority of CIP from AS processes may be released with either their effluents or waste sludge. We therefore strongly recommend further research on CIP residuals and/or post-treatment processes (e.g., anaerobic digestion) for waste streams that may cause ecological risks in receiving water bodies.

Key words: Ciprofloxacin (CIP); Activated sludge ecosystems; microbial community structure

1. Introduction

Recently, antibiotic residues and antibiotic resistant bacteria as well as genes have been considered new classes of water contaminants. Antibiotics have been widely used to prevent or treat microbial infections in human and veterinary application. The chemicals are indispensable in our modern society. However, their metabolism is poor and in some cases above 70% of administrative dose is released. Due to the biologically active properties, antibiotic residues can affect aquatic ecology and proliferate microbes that are persistent to antibiotics (Halling-Sørensen et al., 2000; Martínez, 2008). As an example, ciprofloxacin (CIP) was introduced in 1987 and become widely used for human and animal antibacterial therapies due to its most effective and safe medicines. The widespread use of CIP leads to its occurrence at relatively high concentration than others in its group. (Nguyen et al., 2018). As expected based on previous work (Larsson et al., 2007; Nguyen et al., 2017; Tran et al., 2018), Secondary effluent, hospital wastewater and pharmaceutical wastewater have been found with CIP at concentration of 10 – 200 μg/L and 6.5 – 31 mg/L. These concentrations exceed the predicted no-effect concentrations for several aquatic organisms (Robinson et al., 2005a).

Antibiotic residues like CIP are indispensably collected into the wastewater treatment plants (WWTPs). Thus, WWTPs are considered as the hot spot for the development of antibiotic resistant bacteria and genes as well as source of antibiotics in the environment. To prevent the release, WWTPs should remove antibiotics effectively. Activated sludge is a main means for biological degradation of antibiotics. Biodegradation of CIP in activate sludge could potential lead to complete metabolisms or to by-products that has less toxicity. Since a substantial amount of CPC is released into WWTPs due to its extensive use in domestic and industrial applications, it is critical to address (1) whether CIP is removed effectively from the wastewater and (2) how CIP is removed from the AS.

Antibiotic residues can inhibit the microbial activity of activated sludge and thus influence the bioreactor performance on organic and nutrient removal. For instance, erythromycin caused adverse effects on the structure and chemistry of activated sludge when its presence at 10 mg/L (Louvet et al., 2010; Pala-Ozkok & Orhon, 2013). Recently, Wang et al. (2015) observed an inhibition on COD and nitrogen removal in an anoxic-aerobic reactor under the presence of antibiotic oxytetracycline of 10 mg/L. Kang et al. (2018) observed a change in the microbial community of aerobic granular and suspended activated sludge in the presence of sulfamethoxazole at 2 μg/L. Collectively, it appears that the toxicity of compound on the activated sludge function and microbial community depends on compound concentrations.

These results also highlight that it is more relevant to study at environmentally relevant concentration to generate more realistic findings.

Recently, the inception of next-generation sequencing technologies has paved the way for in-depth investigation of the microbial communities from different environmental matrixes including samples from WWTPs (Nguyen et al., 2019). This culture-independent techniques bypass the need to isolate and culture of microbes and allow the detection of new previous unknown microorganisms. Thus, NGS can be useful to evaluate any changes in the microbial communities under antibiotics treatment.

Therefore, this study aim to investigate the impact of CIP at environmentally relevant concentrations on the microbial community of activated sludge. Laboratory bioreactors were inoculated with AS and fed with CIP-containing substrates. Under the experiment period of two months, the biological functions of aerobic activated sludge were measured. Bacterial community dynamics were assessed using 16S rRNA gene sequencing and analysis.

2. Materials and Methods

2.1 Materials

Analytical grade (> 98% purity) of ciprofloxacin hydrochloride monohydrate was purchased from Sigma-Aldrich (Singapore). A stock solution containing 1 g/ L was prepared in Milli-Q water and stored at 4 °C prior to use within one month.

A synthetic feed (1 L) contained $C_6H_{12}O_6$ (1.83 g), NH_4Cl (30 mg), KH_2PO_4 (340 mg), K_2HPO_4 (600 mg), $MgSO_4.7H_2O$ (270 mg), $FeSO_4$ (10 mg) and 10 mL of 100 x trace element solution (ZnSO4.7H2O 0.35 mg, $MnSO_4.H_2O$ 0.21 mg, H_3BO_4 2.1 mg, $CoCl_2.2H_2O$ 1.4 mg, $CuCl_2.2H_20$ 0.07 mg, $NiSO_4.6H_2O$ 0.1 mg, $Na_2MoO_4.2H_2O$ 0.21 mg per liter) was used. The ratio of COD, total nitrogen and total phosphorous (COD: TN: TP) in the synthetic feed was 80: 5: 1 (Nguyen & Oh, 2019).

AS taken from an aeration tank of a municipal WWTP (Jurong, Singapore) was used an inoculum source. The AD was acclimated with synthetic feed and laboratory conditions for 1 month when COD removal and MLVSS concentration was stable. The adapted inoculum was then used for CIP exposure experiment.

2.2 CIP exposure in AS bioreactor

A set of nine identical reactors was developed from the AS inoculum (Section 2.1). Three of these reactors were control. Another six reactors were exposed to 50 and 500 μg/L of CIP for two months. The exposure concentrations were environmentally relevant levels in wastewater (Nguyen et al., 2018).

The reactors (0.6 L working volume) were operated in a fed-batch mode. One-third of a mixed liquor suspension was withdrew and replaced with a freshly-prepared synthetic feed and CIP every 3.5 days. The solid retention time, temperature and dissolved oxygen concentration were maintained at 10.5 days, 22 ± 1 °C and 4.8 ± 0.8 mg/L, respectively.

2.3 Analytical methods

2.3.1 AS bioreactor performance

The heterotrophic growth of AS microbial community was assessed through soluble chemical oxygen demand (sCOD) removal and mixed liquor volatile suspended solids (MLVSS). sCOD was measured using a HACH colorimetric method after filtering the samples through a 0.22-μm filter. MLVSS were measured following the APHA Standard Method 2540.

CIP concentrations in the synthetic feed and effluent (i.e. supernatant withdraw from reactors) were measured for assessment of CIP removal. A high-performance liquid chromatography system (Shimadzu Asia Pacific Pte. Ltd) equipped with a Shim-Pack GIST Phenyl column (5 μm, 4.6 x 250 mm) and a UV–vis multiple wavelength detector was used. The mobile phase comprised of 60% acetonitrile and 40% Milli-Q water buffered with 25 mM NaH_2PO_4 at pH 2.5. The mobile phase was delivered at 1.8 mL/min through the column for 5 min. CIP was eluted and detected at 3.5 min and 280 nm, respectively. The limit of quantification for CIP using these conditions was approximately 10 μg/L. CIP removal in a fed-batch bioreactor was calculated using the following equation: removal (%) = $[(C_{inf} - C_{eff}) \times 100] \div C_{inf}$, where C_{inf} and C_{eff} denote the concentration of CIP in the reactor influent and effluent, respectively.

CIP concentration on sludge sample was measured following an ultrasonic solvent extraction method (Wijekoon et al., 2013). In brief, the sludge was freeze-dried for 24 hours and ground to a fine powder using a mortar and pestle. The powder was subsequently washed with 5 mL methanol in a 13-mL tube. The resulting slurry was mixed well with a vortex mixer and ultrasonicated at 30 °C for 10 min, after which the resultant suspension was spun by centrifugation at 2851 x g for 10 min. The supernatant was collected and the remaining solid mixture was subjected to another round of extraction. The supernatants from all extraction steps were combined, filtered by 0.22 μm, and subjected to HPLC analysis. Independent tests contained inactive (heat-killed biomass) and abiotic settings, showing an extraction efficiency of 74 ± 5%. The mass of CIP was calculated as: adsorption = T × C_{CIP} × E, where T (g), C_{CIP} (μg/g), and E (%) denote the total sludge mass in the reactor, the concentration of CIP extracted per one gram of sludge, and the extraction efficiency, respectively.

2.3.2 Microbial community analysis

Sludge samples were subjected to a DNA extraction protocol (PowerSoil ® DNA isolation kit, MOBIO, Carlsbad, CA, USA). The extracted DNA samples were tested for the concentration using Nanodrop 2300 and agarose gel electrophoresis. All obtained DNA concentrations were > 0.5 μg/μL with absorbance ratios (A_{260}/A_{280}) > 1.8.

The variable regions (V3-V4) on the 16S rRNA genes were amplified using the universal bacteria primers (341F-805R) at Macrogen Inc. (Seoul, Republic of Korea). The 16S rRNA gene amplicon products were sequenced using the MiSeq^{TM} platform at Macrogen Inc.

Paired-end (2 × 300 bp) 16S rRNA gene sequences were analyzed using the MiSeq SOP pipeline (Kozich et al., 2013). In brief, raw sequences were preprocessed with the following parameters, no ambiguous sequence, > 200 bp in length, and < 8 bp homopolymer, with other parameters at their default settings. The preprocessed sequences were chimera-checked using chimera.vsearch and then taxonomically classified with classify.seqs. Chimera sequences and those assigned to chloroplasts, mitochondria, archaea, eukaryotes, and unknown were excluded from further analyses. The remaining sequences were clustered into operational taxonomic units (OTUs) using a 97% nucleotide identity cutoff with the dist.seqs and cluster commands. The sequences were rarefied to the lowest number of sequences per sample to calculate alpha diversity indices across different datasets. The OTU level bacterial community composition data were used for beta diversity analysis. The 16S rRNA gene sequence datasets used in this study were deposited in GenBank under the following accession numbers: CIP_50_1 (SRS2340180), CIP_50_2 (SRS2340182), CIP_50_3 (SRS2340184), CIP_500_1 (SRS2340177), CIP_500_2 (SRS2340179), CIP_500_3 (SRS2340181), Control_0_1 (SRS2340183), Control_0_2 (SRS2340176), Control_0_3 (SRS2340220), Control_42_1 (SRS2340175), Control_42_2 (SRS2340198), and Control_42_3 (SRS2340197).

Statistical testing for differential community characteristics was conducted using the Mann-Whitney U test. Non-metric multidimensional scaling analysis and compositional similarity index were performed in PASS software with Bray-Curtis index.

3. Results and discussion

3.1 Impact of CIP on reactor function

CIP exposure did not affect the AS biological functions (i.e. organic matter removal and biomass yield). The sCOD removal rates in the CIP-exposed reactors were 88 ± 3.6% and 88 ± 2.0%, (n = 8) under CIP of 50 and 500 µg/L, respectively. The removal rates were marginally lower than the COD removal in the control reactor (91.6 ± 3.7%) with no statistical significance $P > 0.05$ Mann-Whitney U tests. Likewise, MLVSS concentrations were 0.79 ± 0.06, 0.79 ± 0.17, 0.74 ± 0.14 in the control, CIP_50 and CIP_500 reactors, respectively. Although no perturbation of CIP on AS was observed, the results showed noticeably lower MLVSS that that of conventional AS in this study. The reason could be due to the reactor operation without sludge returning in this study.

The impact of CIP on AS biological functions could depend on exposure concentrations and microbial consortium. At a magnitude dose of 2 mg/L CIP, Yi et al. (2017) observed a reduction of 12 and 15 % of phosphate and nitrogen removal in a sequencing batch reactors. Comparatively, the selected concentrations were low in this study. This presents a difficulty when discussion results of other studies as the level of CIP were different. Nevertheless, the results presented provide evidence that environmentally relevant concentrations of CIP did not have impact on AS biological function. Future investigations determining the effects of CIP on other metabolic activities of other important nutrients (e.g. nitrogen and phosphorous) in biological nitrogen removal and enhanced biological phosphorous removal process will be highly desirable.

3.2 Effect of CIP on microbial community

3.2.1 Microbial community diversity

CIP addition did not influence AS microbial richness but decreased evenness (Table 1). The community indexes used was species richness and evenness. The species richness (Observed species) were 272 ± 30, 260 ± 28 and 262 ± 71 ($n=3$) in the control, CIP_50 and CIP_500 communities, respectively ($P > 0.05$, Whitney U test). On the other hand, statistical significance in species evenness was observed between communities in the CIP_500 and control reactors (3.2 ± 0.29 *vs* 2.6 ± 0.3). The communities in control and CIP_50 reactor, however, showed no difference in species evenness (3.2 ± 0.29 *vs* 3.2 ± 0.1). The decrease in species evenness suggest increase/decrease of different taxa abundance in the AS community under CIP addition.

[TABLE 1]

Table 1: Alpha-diversity indices of the control and CIP-exposed communities

	Chao	Shannon
Control [a]	273 ± 30	3.20 ± 0.29
CIP_50	260 ± 28	3.20 ± 0.08
CIP_500	262 ± 71	2.65 ± 0.3

[a] A total of six samples taken at day 0 (n = 3) and day 42 (n = 3) were combined into the control group for analysis.

3.2.2 Microbial community structure

AS microbial community structure changed significantly under exposure of CIP at 500 µg/L (Fig. 1). Non-metric multidimensional scaling (NMDS) analysis based on the Euclidean distance metric (for bacterial community composition at the OTU level) indicated a level of change in community structure (Fig. 1a). Within each group of reactors, the Euclidean distance dissimilarity index was 0.34 ± 0.16, 0.14 ± 0.02 and 0.23 ± 0.05 in control, CIP_50 and CIP_500, respectively. A low distance dissimilarity index suggests the community structure among the three replicate communities was similar. The pairwise distances between communities in control *vs* CIP_50 and control *vs* CIP_500 were higher than the distances within communities; 0.37 ± 0.05 and 0.52 ± 0.07, respectively. Permutational multivariate analysis of variance (PERMANOVA) test revealed that these pairwise distances were significant difference (Bonferroni-corrected $P > 0.05$) between communities of the control and CIP_500 reactors (Fig. 1b).

The structure and diversity of microbial communities are governed by stochastic processes, such as diversification, dispersal, and ecological drift, and deterministic processes, such as selection (Zhou & Ning, 2017). Therefore, the lower Euclidean distance dissimilarity in the presence of CIP_50 and CIP_500 suggests a decreased stochasticity of microbial communities due to a clear deterministic stress induced by CIP. It has also been reported that recalcitrant and toxic pollutants, such as heavy metals and antimicrobial agents, in biological treatment plants limited the microbial diversity (Balcom et al., 2016). Other factors considered detrimental are, influent characteristic, pH and dissolved oxygen (Nascimento et al., 2018; Zou et al., 2018).

[FIGURE 1]

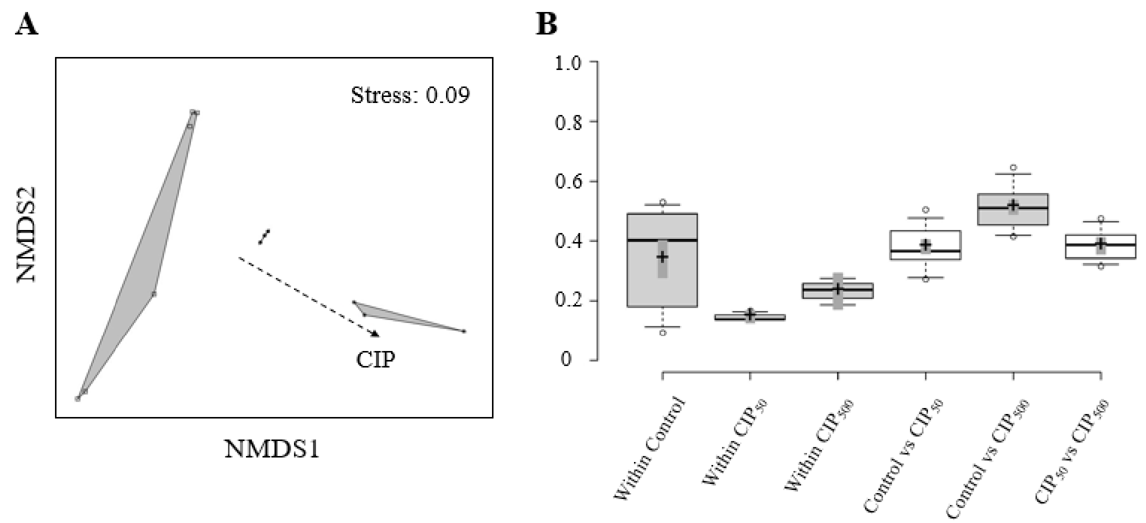


Figure 1: Shifts in community phylogenetic structure under CIP addition in activated sludge. NMDS analysis of community structure using the Euclidean distance metric (A). Open circles, solid circles and solid square represent the Control, CIP_50 and CIP_500, respectively. Euclidean distance within and between groups (B). The whiskers of the box represent the minimum and maximum values. The bottom and top of the box are the first and third quartiles, respectively, and the line inside the box denotes the median.

3.2.3 Dissecting phylogenetic community structure

At class level, CIP addition at 500 µg/L caused significant changes in the relative abundance of *Alphaproteobacteria*, *Gammaproteobacteria* and *Flavobacteria*. While *Alphaproteobacteria* was increased 17.7 ± 2.8 (Control) to 25.6 ± 3.4 (CIP_50) and 36.7 ± 5 (CIP_500), *Gammaproteobacteria* was significantly decreased from 32.9 ± 10.3 (Control) to 6.5 ± 4 (CIP_50) and 11.2 ± 5 (CIP_500) (Fig 2). *Alpha*- and *Gammaproteobacteria* are two major classes of the *proteobacteria* phylum and are dominant in AS system. These classes contain mainly aerobic heterotrophs contributing to the organic matters removal in AS. Although, the *Gammaproteobacteria* abundance was decreased, the supplemented by other class (i.e. *Alphaproteobacteria*) could maintained the COD removal obtained in this study. Noteworthy, the predominance of *Alphaproteobacteria* over *Gammaproteobacteria* indicated the persistent of *Alphaproteobacteria* to CIP disturbance. *Flavobacteria* was also enriched in the CIP_500 reactors, suggesting their proliferation under CIP exposure.

Further analysis of microbial community into family level revealed that *Caulobacteraceae*, *Aeromonadaceae*, *Flavobacteriaceae*, *Cytophagaceae*, and *Microbacteriaceae* were significantly increased in CIP_500. The genus of *Caulobacter*, a member of *Caulobacteraceae*, is a heterotrophic aerobes frequently found in aquatic environments attached to solid surfaces. It has a stalk to core on solid surfaces, which elongates under a poor nutrient condition to increase the mass flux of nutrients to the cell (Klein et al., 2013), which enables the *Caulobacter* survive under a hard condition. The *Caulobacteraceae* family (*Alphaproteobacteria*) and the *Myxococcales* (*Deltaproteobacteria*) order were identified. The presence of Caulobacter species in AS is intriguing because these organisms are typically found in water and are considered to be oligotrophic (i.e. adapted to conditions with low nutrient availability) (Corpe et al., 1996; Pang et al., 2006). *Aeromonadaceae* was reported to be dominant under stressed conditions. It was reported that *Aeromonas caviae*, *Aeromonas allosaccharophila*, *Aeromonas salmonicida* and *Aeromonas veronii* were found in aerobic biofilm in the presence of 0-50 mg/L streptomycin (Selvaraj et al., 2018). Also, *Aeromonadaceae* was the eighth most abundant families in the sludge from an electro-bioreactor (ElNaker et al., 2018). *Aeromonas* is considered as one of the core genera of BOD-removal because it is capable of predation on cell biomass generating chitin-degradation enzymes (Chong et al., 2012). Thus, the increase in *Aeromonadaceae* would be a result of the stress given by CIP and of the predation of the cells deactivated by CIP.

The increase in *Flavobacteriaceae* and *Cytophagaceae* (phylum *Bacteroidetes*) can be attributed to the ability of *Bacteroidetes* to degrade complex organic substances. *Bacteroidetes* decomposes dead cells and organic micropollutants into simple organic compounds, i.e., ethanol and lactate, which can be utilized by other species. It has also been reported that the relative abundance of *Flavobacteriaceae* was higher in the sludge from an electro-bioreactor than that from a control reactor (ElNaker et al., 2018). Thus, *Bacteroidetes* can be sustained and contribute to refresh microbial communities under hard environments (Zhang et al., 2014). Also, the increase of *Bacteroidetes* strongly suggests that CIP is a clear stress to the microbial community of activated sludge.

A significant increase of *Microbacteriaceae* would be attributed to the increase in several genera which are persistent to antibiotics. *Agromyces mediolanus*, *Microbacterium lacticum* and *Microbacterium maritypicum* were detected in the presence of 0.1-50 mg/L streptomycin within an aerobic biofilm, while they were not detected in the absence of streptomycin (Selvaraj et al., 2018).

The family *Nakamurellaceae* and the sole genus identified so far, i.e., *Nakamurella,* was significantly decreased in CIP_500, indicating that 500 µg/L CIP could induce selective stress to *Nakamurella*. The decrease in *Nakamurella* in activated sludge was also recently observed in the presence of 5 µg/L non-steroidal anti-inflammatory pharmaceuticals, such as diclofenac, ibuprofen and naproxen (Jiang et al., 2017). It was also found that the anti-inflammatory pharmaceuticals could induce the oxidative stress of microorganisms in activated sludge and the damages in mitochondrial function, as indicated by the increase in superoxide dismutase activity and the decrease in succinate dehydrogenase activity (Jiang et al., 2017). Meanwhile, it was also reported that *Nakamurella, Thiomonas* and unclassified reads were increased in the presence of trivalent cations, i.e. Fe^{3+} and Al^{3+}.

The relative abundance of *Rhodobacteraceae* decreased dramatically as CIP concentration was increased. A significant decrease in *Paracoccus yeei* at >1 mg/L streptomycin was also reported by Selvaraj et al. (2018). *Rhodobacteraceae* plays an important role in organic substances degradation (Hong et al., 2008), denitrification and phosphorus accumulation (Sun, 2015). Therefore, the decrease in *Rhodobacteraceae* would result in a poor COD removal, phosphorus removal, and denitrification at high CIP concentrations. Figure 3 showed that the decrease in the genera Paracoccus, Albidovulum and Amaricoccus contributed to the decrease in *Rhodobacteraceae*.

The genus *Paracoccus* was decrease significantly in the presence of CIP and was almost negligible at CIP_500, indicating the adverse effects of CIP on *Paracoccus* growth. There has been no study reporting the decrease in *Paracoccus* in the presence of an antibiotics. However, the inhibition of denitrification, which is the most important function of *Paracoccus* in activated sludge, was observed. The denitrification in an activated sludge was inhibited in the presence of 2-5 mg/L tetracycline via the release of extracellular polymeric substances (EPS), which unprotected the sludge from the toxic compounds (Chen et al., 2015). Ho et al. (2015) observed that the denitrification ratio of Yangtze Estuary sediments was decreased by approximately 40% when they were put into artificial seawater containing 5 µg/L sulfamethazine. At the same time, denitrification genes encoding nitrite reductase and nitrous oxide reductase were also decreased substantially. The denitrification rate and denitrification genes were decreased in the presence of the mixture of antibiotics, i.e., sulfamethazine, thiamphenicol, oxytetracycline, erythromycin and norfloxacin (Hou et al., 2017). However, some studies reported the increase of the relative abundance of *Paracoccus.* The relative abundance of *Paracoccus* was increased in the presence of the mixture of antibiotics was observed in a laboratory scale partial-nitrification biofilter filled with microbial carriers, fed with synthetic wastewater without a carbon source (Gonzalez-Martinez et al., 2018). Fifty (50) % of ammonium was partially oxidized to nitrate in the absence of antibiotics. However, ammonium was increased, nitrite was decreased to be negligible and the attached biomass was decreased significantly, when the mixture of antibiotics with different mechanisms, which are azithromycin (macrolide), norfloxacin (quinolone), trimethoprim and sulfamethoxazole (sulfonamide), at 2-9 mg/L each, was introduced. Afterwards, ammonium was decreased, nitrate was increased and the total nitrogen was decreased gradually, indicating the increase of denitrification activity, as the reactor was operated for 60 days in the presence of the antibiotics mixture. At the same time, the abundance of the genera of denitrification metabolism, i.e., *Paracoccus, Rhodobacter, Brevundimonas, Alicycliphilus, Acinetobacter, Acidovorax and Alcaligenes*, were increased with the dominance of *Paracoccus,* while *Nitrosomonas* was dominant in the absence of the antibiotics.

The genus Albidovulum was decreased to a negligible level in the communities of CIP50 and CIP500, indicating that Albidovulum is very sensitive to CIP. Albidovulum was hardly found in literature. Albidovulum was detected in the biofilm of the PVC carriers filled in a high-activity ammonia removal reactor (González-Martínez et al., 2013), but no more reported, so far.

The genus Amaricoccus was decreased as CIP concentration was increased. A significant decrease of Amaricoccus in activated sludge was also observed in the presence of non-steroidal anti-inflammatory pharmaceuticals (Jiang et al., 2017). However, Amaricoccus was reported to be resistant to sulfamethoxazole (SMX) when acetate was used as the major carbon source. Kor-Bicakci et al. (2016) reported that Amaricoccus was dominant in the presence of SMX and acetate (400 mg COD/L) as a sole carbon source, in addition, the relative abundance of Amaricoccus was increased as SMX concentration was increased from 0 (zero) to 200 mg/L. Also, the relative abundance of Amaricoccus, along with some heterotrophs of nonspecific oxidizing enzymes (oxygenases) such as Bryobacter, Pontibacter, Cryomorpha, and Dyadobacter, was increased in an SBR reactor in the presence of 100 μg/L malathion, when peptone and sodium acetate (87% and 13% of influent COD, respectively) were supplied as carbon sources. This suggests that Amaricoccus would biologically oxidize malathion. Given those, the decrease in Amaricoccus in this study would be attributed to the use of glucose as the carbon source, where the co-metabolism of toxic organic compounds was not encouraged. It was supported by Wang et al. (2016) showed that Amaricoccus was negligible when sulphamethoxazole, norfloxacin, prednisolone, naproxen and ibuprofen were introduced at 50 μg/L each, to a granular MBR reactor using glucose.

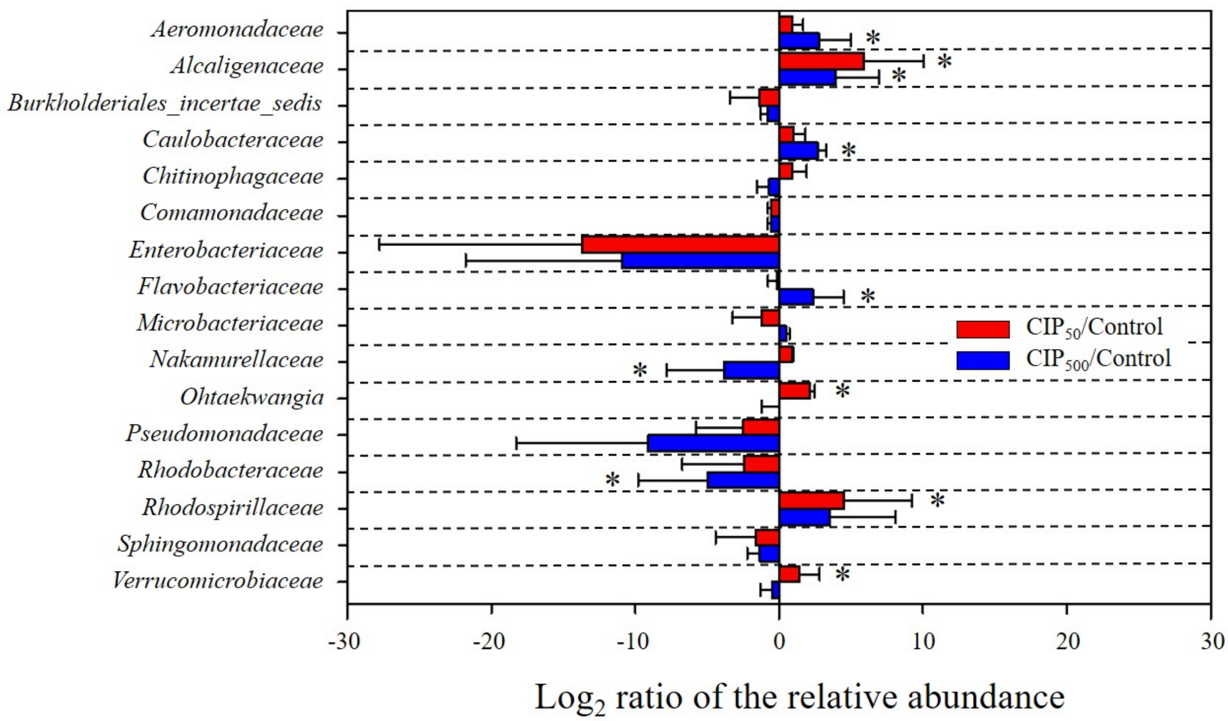


Fig 2. Log_2-transfomred relative abundance of CIP-exposed communities. The families of CIP-exposed communities compared to the control communities were classified with > 1% of the average relative abundance. The asterisk indicates statistically differential abundance ($P < 0.05$ by Mann-Whitney U test) between the CIP-exposed and control communities.

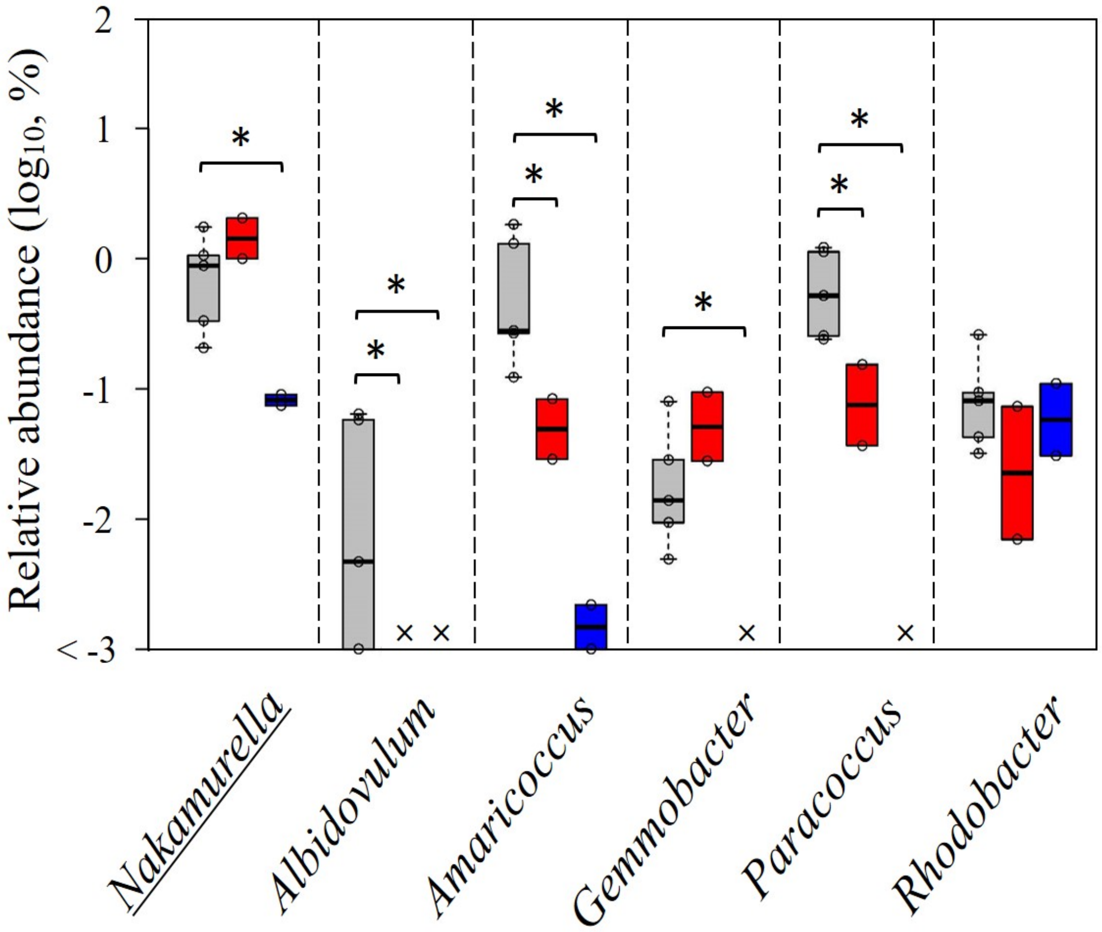


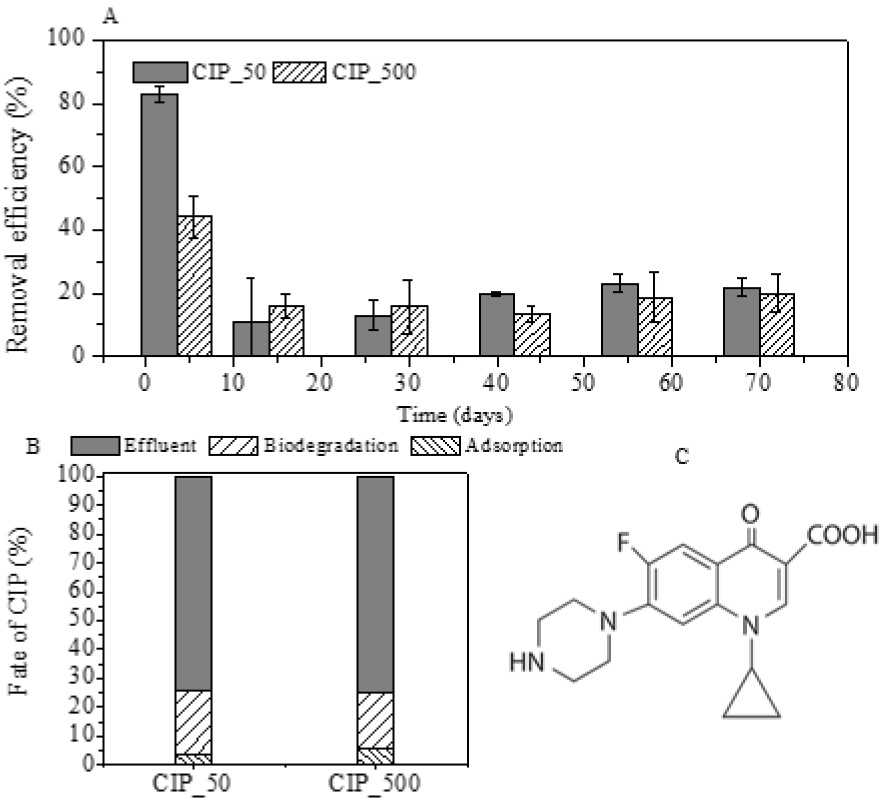
Figure 3. Boxplot of relative abundance of genera in reduced families. Base 10 logarithm values represent the relative abundance of genera among reduced families over CIP exposure. Grey color denotes the relative abundance of the control communities. Red color is the relative abundance of CIP_{50} communities. Blue color indicates the relative abundance of CIP_{500} communities. The small circles in the boxplot represents the data points. The asterisk represents statistically differential abundance ($P < 0.05$ by Mann-Whitney U test) between the CIP-exposed and control communities. The underlined genus belongs to the family of *Nakamurellaceae*. Other genera belong to the family of *Rhodobacteraceae*. The cross mark represents non-detected community.

3.2 Removal of CIP in bioreactor

Results presented here indicated that AS was not effective for CIP removal (Fig. 3). The removal efficiency of CIP by activated sludge were <20 % over the experimental period, with exception at the first feeding cycle (i.e. when CIP was first added into the system) (Fig. 3A). The persistence of CIP to biological treatment processes is likely due to its chemical properties. The centre code of CIP contains fluoride and carboxyl functional groups, which renders it less susceptible to oxidative catabolism (Tadkaew et al., 2011). In addition, it is difficult to attach onto sludge and be degraded by microorganism due to its low distribution coefficient between the aqueous and sludge phase (i.e. partition coefficient ([Octanol]/ [Water] = 0.28). Our results are in consistent with the previous studies in various biological treatment processes. Only 15% of CIP is reported to be removed by biological transformation (Li & Zhang, 2010) in activated sludge process. An MBR which was operated at mesophilic (38 °C) and high sludge concentration (15 g/L) could eliminate 52% of CIP after 12 h retention time (Dorival-García et al., 2013). More than 70% of the CIP influent was passed the full scale CAS into digested sludge (Lindberg et al., 2006). CIP is also persistent under anaerobic conditions. The anaerobic sulphate-reducing bacteria system could have biodegraded 28% of 5 mg/L CIP under solid retention time of 25 days (Jia et al., 2018).

[FIGURE 4]

The low removal of CIP could lead to the release of substantial account of CIP into the downstream environment since waste activated sludge is the main barrier. For example, at environmentally relevant concentration (50 – 500 µg/L), a range of 40 to 400 µg/L of CIP could remain in the effluents in this study. These range of concentration could cause potential health hazard to the downstream aquatic organisms since the effective concentration (EC50) of some organisms have been reported to be lower than 400 µg/L. The EC50 of the two common fresh water cyanobacteria (*Microcystis aeruginosa*) and duckweed (*Lemna minor*) was 17 µg/L and 203 µg/L, respectively (Robinson et al., 2005b). CIP inhibited the total carbon utilization of natural marine biofilms (Johansson et al., 2014). Another potential long-term impact is the development of antibiotic resistant bacteria. Overall, due to the persistence to biological treatment process and the potential health hazards to aquatic environment, more efficient technologies are needed for the treatment of CIP.



4. Conclusion

Results indicated that 500 µg/L CIP did not have impact on the heterotrophic function of AS (i.e. COD removal). However, CIP changed the AS microbial community species evenness and structure. Exposure of 500 µg/L CIP resulted in a species evenness decrease that was coincided with the decrease and increase in the abundance of two major groups *Gammaproteobacteria* and *Alphaproteobacteria*, respectively. Results also show that AS was not effective for the removal of CIP. Although the heterotrophic function was not affected, alternation of AS microbial community and ineffectiveness in CIP removal suggest the development of other treatment means for CIP-containing waste.

5. Acknowledgements

This work was supported by a grant from the National Research Foundation of Korea (NRF) funded by the Korean government (MSIP; Ministry of Science, ICT & Future Planning (No. 2017076367).

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