Fate and seasonal change of *Escherichia coli* resistant to different antibiotic classes at each stage of conventional activated sludge process

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ABSTRACT

This study investigated the impact of each treatment stage of the activated sludge process on the fate of antibiotic resistant bacteria (ARB) in wastewater treatment plants (WWTPs). Wastewater and sludge samples were collected monthly at each stage of a commercial-scale WWTP. After 20–25 strains of indicator *Escherichia coli* were isolated from each sample on Chromocult Coliform Agar, antibiotic resistance of the isolates to amoxicillin (AMX), ciprofloxacin (CIP), norfloxacin (NFX), kanamycin (KM), sulfamethoxazole/trimethoprim (ST) and tetracycline (TC) were tested with the Kirby–Bauer disk diffusion method. As a result, activated sludge in the aeration tank and return sludge had higher abundance of antibiotic resistant *E. coli* than influent wastewater and secondary treatment effluent. AMX resistant *E. coli* was enriched in return sludge at the secondary clarifier. Higher temperature was also likely to cause an increase of AMX resistant *E. coli* in sludge. The antibiotic resistance profile of *E. coli* in secondary treatment effluent was more dependent on activated sludge than influent wastewater. These results suggested that activated sludge in WWTP possibly serves as a reservoir of ARB, and that behavior of ARB in WWTP differs by antibiotic classes.

Key words | activated sludge, amoxicillin (AMX), antimicrobial resistance (AMR), wastewater treatment

HIGHLIGHTS

- One-year monitoring of a full-scale WWTP was conducted to investigate fate of ARB in each treatment stage.
- Activated sludge and return sludge had higher abundance of AMR than wastewater and treated effluent.
- The increase of AMX resistance in activated sludge was because AMX resistant *E. coli* was enriched in sludge at secondary clarifier.
- Higher temperature was also likely to cause increase of AMX resistant *E. coli* in sludge.

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INTRODUCTION

The increased emergence of antibiotic resistant bacteria (ARB) is now a great concern for future human health. Recently, One Health approach, which is a collaborative effort of multiple disciplines to attain optimal health for people, animals and the environment, is recognized to be necessary to prevent possible burdens caused by antibiotic resistance on a global scale (Rousham *et al.* 2018). To prevent the prevalence of ARB through environmental pathways, it is important to control the release of ARB from anthropogenic activity into the environment (Pruden *et al.* 2013). Municipal wastewater is a major source of ARB in the urban water environment in both developed and developing countries (Koczura *et al.* 2012; Kotlarska *et al.* 2015; Rosas *et al.* 2015; Honda *et al.* 2016; Kumar *et al.* 2019). Wastewater treatment plants (WWTPs) are important focal points to control the spread of ARB from municipal wastewater. However, ARB in wastewater still remains in high abundance in secondary treatment effluents of WWTPs and are the major origin of ARB in water environments receiving the treated effluent (Ferreira da Silva *et al.* 2007; Łuczkiewicz *et al.* 2010; Koczura *et al.* 2012; Reinthaler *et al.* 2003; Kotlarska *et al.* 2015; Rosas *et al.* 2015; Zhang *et al.* 2015). One major reason is because activated sludge often has an equivalent or higher level of antibiotic resistance than wastewater (Łuczkiewicz *et al.* 2010; Rizzo *et al.* 2013; Reinthaler *et al.* 2003). These findings suggest that a WWTP possibly serves not only as the barrier of ARB to prevent its spread from wastewater into the water environment, but also as the reservoir to maintain ARB in its system. Activated sludge is known to form a microbial community which is distinct from that in wastewater (Ye & Zhang 2013; Shchegolkova *et al.* 2016). This is because the process configuration of the activated sludge process gives selective pressure to enrich microbes in sludge flocs with good settleability. This process configuration also possibly works to maintain ARB in activated sludge. A number of studies reported a higher abundance of ARB in treated effluent than influent wastewater. However, a limited number of studies investigated the change of ARB in each step of wastewater treatment. Łuczkiewicz *et al.* (2010) and Reinthaler *et al.* (2003) investigated the change of ARB abundance in influent wastewater to activated sludge, and activated sludge to treatment effluent. However, none of the past studies investigated the change of ARB abundance in primary clarifier effluent. Therefore, the change of ARB abundance from influent wastewater to primary clarifier effluent, and primary clarifier effluent to activated sludge is still veiled. Hence, it is important to know which stages of treatment affect the increase or decrease of ARB in wastewater treatment.

The objective of this study was to reveal the impact of each treatment stage in a conventional activated sludge process on the fate of ARB from wastewater. Change of *E. coli* concentrations and its abundance of antibiotic resistance to six antibiotics was monitored in a full-scale WWTP throughout a year. The seasonal change of ARB abundance was discussed.

METHODS

Sampling

Samples were collected at a full-scale WWTP which mainly received municipal wastewater from a populated area in the Hokuriku region in Japan. Fifty-mL of water samples of influent wastewater (INF), primary treatment effluent (PTE), activated sludge (AS), return sludge (EXS), and secondary treatment effluent (STE) were collected in dry weather monthly for 12 months from July 2014 to June 2015.

Isolation of indicator *E. coli* and antibiotic susceptibility test

Indicator *E. coli* was chosen as the target bacteria to trace the fate of ARB originated from fecal sources because it is commonly used as the representative fecal bacteria in many studies on ARB in water and wastewater (Koczura *et al.* 2012; Kotlarska *et al.* 2015; Łuczkiewicz *et al.* 2010; Reinthaler *et al.* 2003; Rosas *et al.* 2015). Isolation of *E. coli* and an antibiotic susceptibility test was conducted as described in Honda *et al.* (2020). After a sample was diluted with physiological saline water in an appropriate series, 1 mL of each diluted sample was filtered using 0.45 μm

cellulose-acetate membrane filters (A045H047 W, Advantec Toyo, Tokyo, Japan). Filters were then placed on Chromocult® Coliform Agar ES (Merck KGaA, Darmstadt, Germany), and incubated at 37 °C for 24 h. After counting all colonies, 20–25 colonies of *E. coli* were randomly picked up from each sample and put into PERLCORE Trypto-Soy Broth (Eiken Chemicals, Tokyo, Japan), and incubated at 37 °C overnight. Glycerol was added to a final concentration of 15–20% and the cultures were frozen and stored at −80 °C. The procedures for colony counting were conducted within 24 hours after sampling.

The susceptibility of each *E. coli* isolate to six antibiotics in five classes was tested by the Kirby–Bauer disk diffusion method: ciprofloxacin (CIP), norfloxacin (NFX) in the fluoroquinolone class, tetracycline (TC) in the tetracycline class, amoxicillin (AMX) in the β-lactam class, kanamycin (KM) in the aminoglycoside class and sulfamethoxazole/trimethoprim (ST) in the sulfonamide class. The isolated *E. coli* cultures were spread on Muller–Hinton agar (PERLCORE Sensitivity Test Agar, Eiken Chemicals, Tokyo, Japan), and antibiotic susceptibility test disks (KB Disk, Eiken Chemicals, Tokyo, Japan) were placed on the spread plates. Each antibiotic test disk contained 5 μg of CIP, 10 μg of NFX, 30 μg of TC, 25 μg of AMX, 30 μg of KM, or a combination of 23.75 μg of sulfamethoxazole and 1.25 μg of trimethoprim. After incubation at 37 °C for 18 h, the diameter of the inhibition zone around each antibiotic disk on the agar was measured. The resistance of the isolate to each antibiotic was determined from the criteria of zone diameter according to the protocol provided by the manufacturer, which followed CLSI standards M100-S18 (CLSI 2008). According to the test protocol, the isolates which had possible contamination were excluded from the result. In this study, 15 out of a total of 1490 isolates were excluded from the further analysis. Abundance of resistant isolates in each sample was calculated by:

$$A_{r,i} = \frac{R_{r,\ i}}{N} \tag{1}$$

where $A_{r,i}$ is the abundance of isolates resistant to antibiotic i; $R_{r,i}$ is the number of isolates determined as 'resistant' to antibiotic i; N is the effective number of isolates in the susceptibility test.

Statistical analysis

Correlation of resistance among the tested antibiotics was evaluated using the phi (φ) coefficient, which ranges from zero to unity, corresponding to the magnitude of association between two variables. The phi coefficient was calculated based on 2×2 contingency tables established for each combination of two antibiotics. A 2×2 contingency table is comprised of the number of isolates which were resistant to each combination of two antibiotics, resistant to either of the antibiotics and sensitive to each combination of two antibiotics. Principal component analysis (PCA) was performed on abundance of resistance to each antibiotic in each sample with statistical software R version 3.5.0.

RESULTS AND DISCUSSION

Abundance of antibiotic resistance *E. coli* throughout the process

Influent wastewater and secondary treatment effluent both contained approximately 40% of antibiotic resistant *E. coli* (Figure 1). However, the abundance of antibiotic resistant *E. coli* significantly increased to 50–60% in activated sludge and return sludge. This increase in abundance of antibiotic resistance in sludge was consistent with many of the past studies (Reinthaler *et al.* 2003; Ferreira da Silva *et al.* 2007; Łuczkiewicz *et al.* 2010; Zhang *et al.* 2015). In sludge samples, abundance of the single antibiotic resistance clearly increased, while abundance of multiple antibiotic resistance did not change significantly. Hence, the increase in abundance of antibiotic resistance in sludge was mainly caused by the increase of the single antibiotic resistance. Among the six tested antibiotics, resistance to AMX had higher abundance than the other antibiotics in all samples (Figure 2). Higher abundance of resistance to AMX in wastewater treatment was also reported at many WWTPs (Ferreira da Silva *et al.* 2007; Novo & Manaia 2010). In particular, activated sludge and return sludge had a significantly higher abundance of resistance to AMX in this WWTP. Hence, this increase of AMX resistance was suggested to contribute to the increase in abundance of antibiotic resistant *E. coli* in activated sludge and return sludge. No

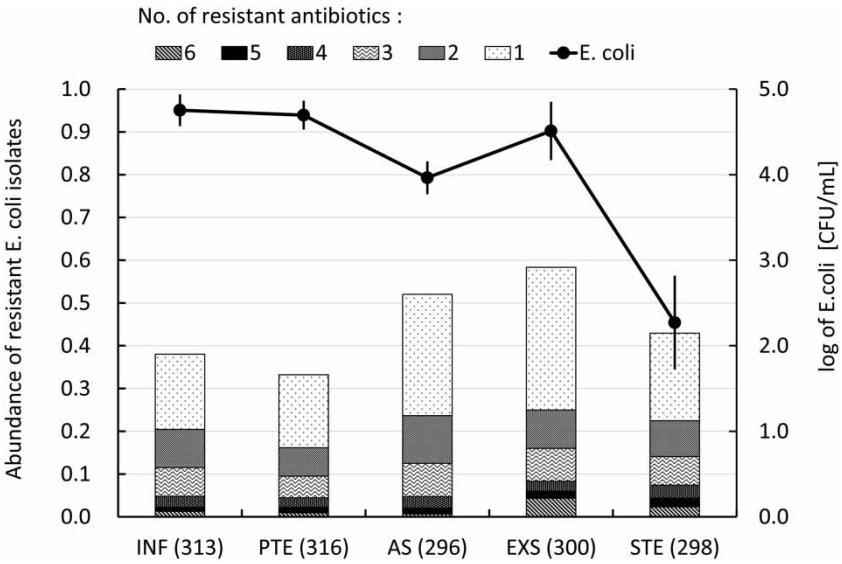

Figure 1 | Abundance of antibiotic resistant *E. coli* in influent wastewater (INF), primary treatment effluent (PTE), activated sludge (AS), return sludge (EXS), and secondary treatment effluent (STE). The values in brackets are the number of the tested isolates of indicator *E. coli*.

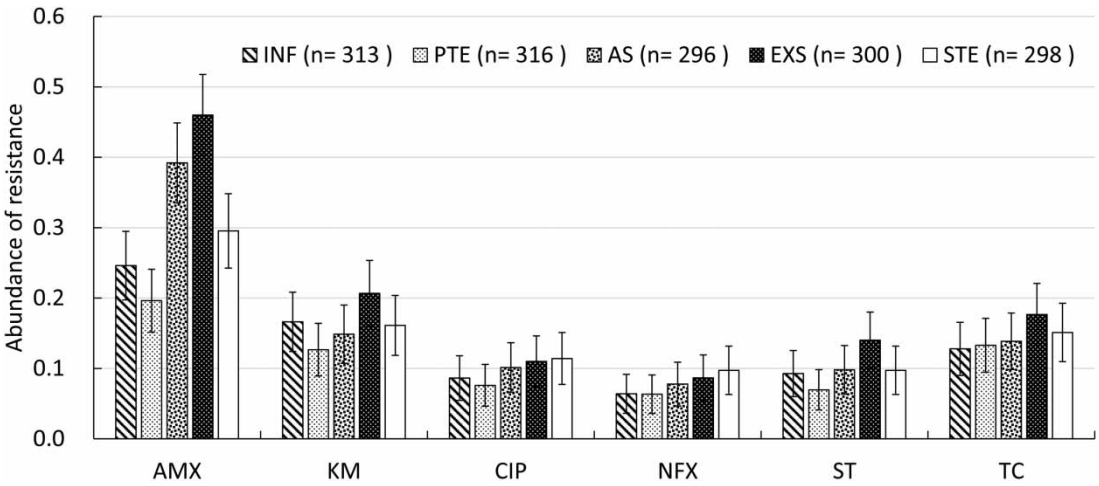

Figure 2 | Abundance of *E. coli* resistant to amoxicillin (AMX), kanamycin (KM), ciprofloxacin (CIP), norfloxacin (NFX), sulfamethoxazole/trimethoprim (ST) and tetracycline (TC) in influent wastewater (INF), primary treatment effluent (PTE), secondary treatment effluent (STE), activated sludge (AS) and return sludge (EXS).

significant correlation was found among the test antibiotics except between fluoroquinolone class antibiotics, CIP and NFX (Supplementary material, Table S1).

Change of antibiotic resistance *E. coli* at each treatment stage

A slight reduction was observed in ARB abundance, from 38 to 33%, by primary treatment, while the abundance of multiple antibiotic resistance was reduced from 20 to 16%, although it was not statistically significant ($p = 0.18$). The average abundance of resistance to AMX was also reduced from 23 to 19%, KM from 16 to 11%, and ST was reduced from 9.9 to 5.9%. The change in abundance of antibiotic resistant *E. coli* at each treatment stage on the same sampling day is compared in Figure 3. The abundance of resistance to AMX, KM, CIP, NFX and ST were often found with lower abundance in primary treatment effluent than influent wastewater (Figure 3; Supplementary material, Figure S1). Also, at the secondary clarifier, the abundance of

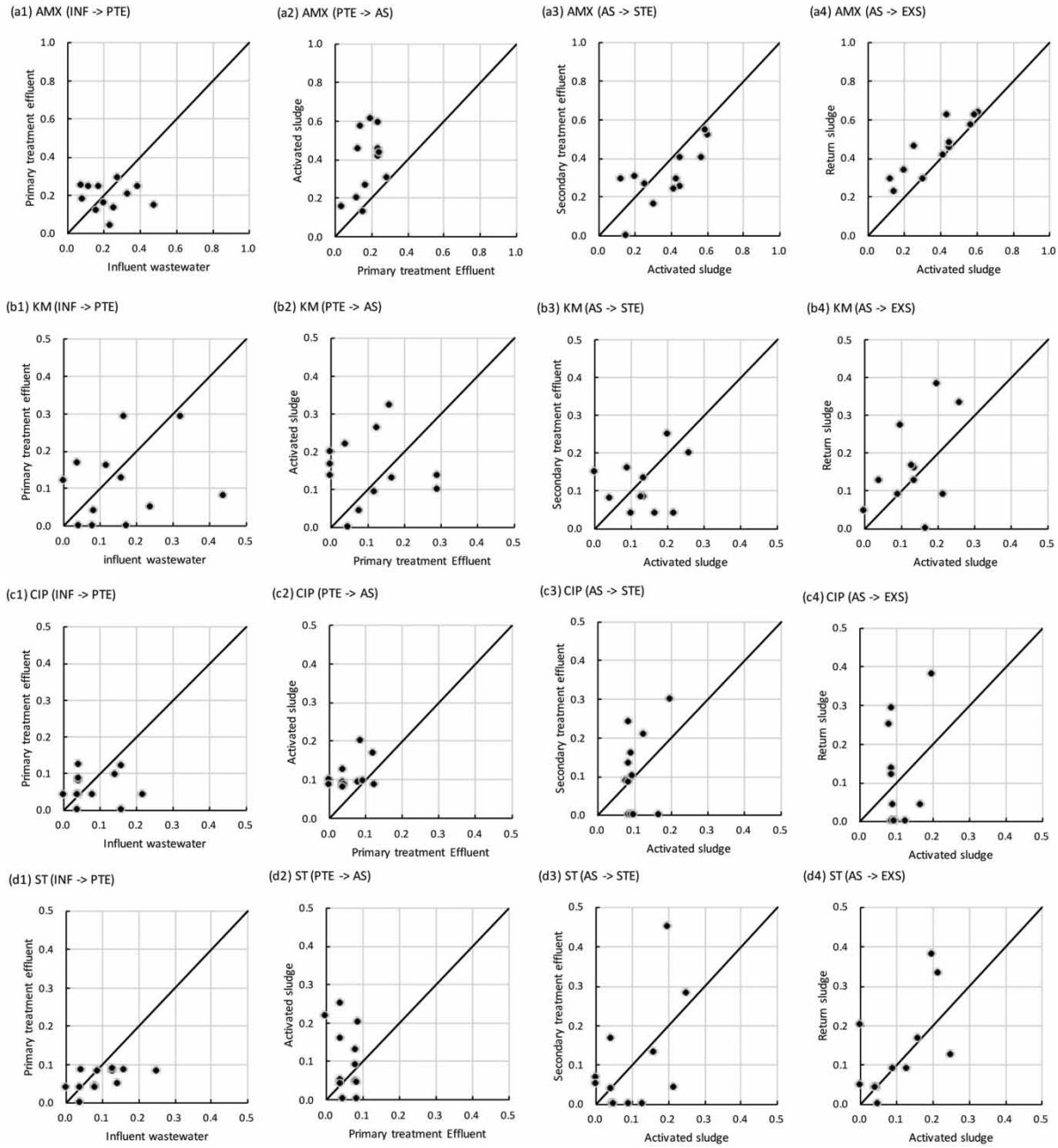

Figure 3 | Change in abundance of *E. coli* resistant to: (a) amoxicillin (AMX), (b) kanamycin (KM), (c) ciprofloxacin (CIP) and (d) sulfamethoxazole/trimethoprim (ST) at each treatment stages of (1) influent (INF) to primary treatment effluent (PTE), (2) primary treatment effluent (PTE) to activated sludge (AS), (3) activated sludge (AS) to secondary treatment effluent (STE), (4) activated sludge (AS) to return sludge (EXS).

resistance to AMX and KM frequently decreased in secondary treatment effluent and increased in return sludge (Figure 3(a4) and 3(b4)). These results suggest that some antibiotic resistant *E. coli* are possibly reduced in treatment effluent by sedimentation and enriched in return sludge. Especially, AMX resistant *E. coli* was remarkably enriched in return sludge (Figure 3(a4); Supplementary material, Figure S2). The abundance of resistance to AMX was significantly lower in secondary treatment effluent than return sludge ($p = 0.006$). This enrichment of AMX resistant

E. coli at secondary clarifier into return sludge probably caused a higher abundance of AMX resistance in activated sludge. One possible reason is the presence of selective pressure from the remaining AMX and other β-lactams. However, β-lactams were reportedly removed well in wastewater treatment (Matsuo *et al.* 2011; Yang *et al.* 2017) and not adsorbed in the sludge of primary sedimentation or final sedimentation (Matsuo *et al.* 2011). Hence, the effect of residue β-lactams on the enrichment of AMX resistance was probably limited. Another possible reason is because of hydrophobicity of the outer membrane of the AMX resistant strain. Godfrey *et al.* (1984) compared *Pseudomonas aeruginosa* strains with different lipopolysaccharides profiles of the outer membrane. According to Godfrey's study, relatively hydrophobic strains had higher resistance to hydrophilic antibiotics including β-lactams because of less permeability of the antibiotic to the cell membrane. Since AMX and β-lactams are hydrophilic antibiotics, *E. coli* strains with a hydrophobic outer membrane are relatively resistant to AMX. *E. coli* strains with a hydrophobic outer membrane tend to be adsorbed and partitioned to sludge flocs. Therefore, sludge samples of AS and EXS possibly had a higher abundance of AMX resistant *E. coli*.

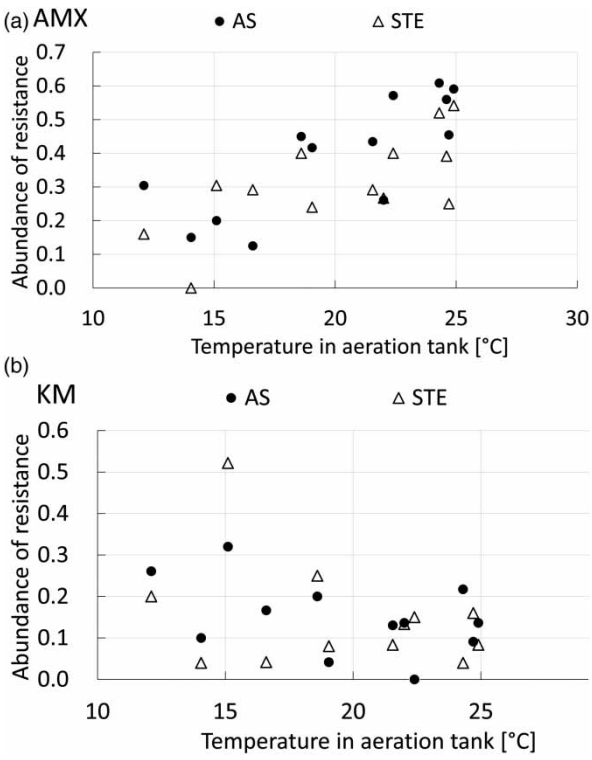

Figure 4 | Temperature dependency of resistance to: (a) amoxicillin (AMX) and (b) kanamycin (KM) in secondary treatment effluent (STE) and activated sludge (AS).

Seasonal change

Seasonal change was found in activated sludge and secondary treatment effluent (Figure 4). Under higher temperatures in summer, ARB to AMX in secondary treatment effluent was relatively higher than under lower temperatures in winter. A positive correlation was found between the abundance of AMX resistance and temperature in activated sludge ($R^2 = 0.76$) (Figure 4). This is possibly caused by the temperature dependency of β-lactamase activity and its production. The optimum temperature of β-lactamase is 25–45 °C and its activity decreases as the temperature drops (Behravan & Rangsaaz 2004; Hecky & Müller 2005). The production of β-lactamase was induced by the dose of the antibiotic under 17.5 °C or higher temperatures by *Pseudomonas fluorescens*, while no induction was observed at 8 °C (Orange 1994). Therefore, AMX resistance can be diminished under lower temperatures due to lower activity and/or production of β-lactamase. Consequently, *E. coli* strains with potential AMX resistance possibly become more tolerable to AMX and appear as resistant strains under higher temperatures.

Meanwhile, the abundance of resistance to KM in secondary treatment effluent tends to become low at temperatures higher than 20 °C. KM is hardly biodegradable and is mainly removed by adsorption on sludge particles (Li & Zhang 2010). Since physical adsorption is an exothermic reaction, the adsorption capacity becomes lower at higher temperatures. Therefore, more KM is possibly adsorbed and present in sludge particles at lower temperatures. As a result, bacteria in sludge are exposed to higher selective pressures of KM, then ARB to KM increased in the sludge and secondary treatment effluent at lower temperatures. This enrichment of ARB to KM is likely to occur in sedimentation at the secondary clarifier rather than in the aeration tank because the abundance of resistance to KM in activated sludge did not have clear correlation with temperature (Figure 4). No significant seasonal trend was found in influent or resistance to other antibiotics (Supplementary material, Figures S4–S6).

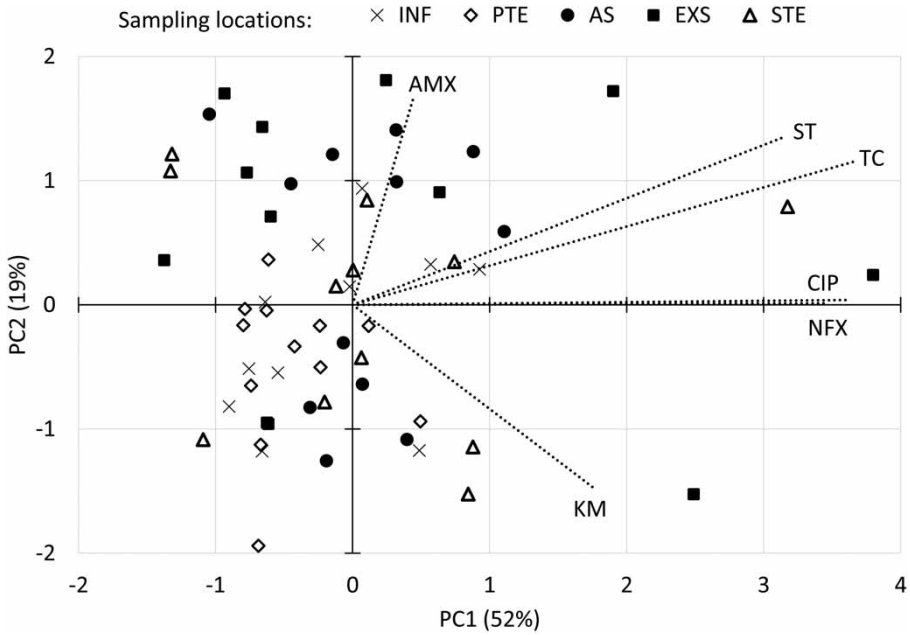

Figure 5 | Two-dimensional plots of principal component analysis on abundance of resistance to each antibiotic in influent wastewater (INF), primary treatment effluent (PTE), activated sludge (AS), return sludge (EXS), and secondary treatment effluent (STE).

Factors to determine the fate of antibiotic resistance in wastewater treatment

Principal component analysis (PCA) showed a different antibiotic resistance profile among influent wastewater, treatment effluents and sludge samples (Figure 5). In the PCA, the first principal component (PC1) indicated a high abundance of resistance to all antibiotics, especially to fluoroquinolones, ST and TC; the second principal component (PC2) indicated a high abundance of resistance to AMX and low abundance of KM. Most plots of influent wastewater and primary treatment effluent had relatively lower PC1 values, while secondary treatment effluent had a wide range of PC1 and PC2. These results showed a distinct difference of antibiotic resistance profile between influent wastewater and secondary treatment effluent.

The results of PCA indicated that the antibiotic resistance profile in influent wastewater and primary treatment effluent are similar. Meanwhile, the antibiotic resistance profile of secondary treatment effluent was not much affected by influent wastewater or primary treatment effluent. This implies that a significant change of antibiotic resistance profile seemed to occur in the aeration tank. Activated sludge had quite a different antibiotic resistance profile from primary treatment effluent but a closer profile to return sludge. This suggests that the antibiotic resistance profile in activated sludge was affected more by return sludge than primary treatment effluent. Mixed-liquor suspended solids (MLSS) concentrations of activated sludge and return sludge were 1,400 and 4,900 mg/L, respectively. Since the suspended solids concentration in raw wastewater was 30–150 mg/L, the amount of suspended solids in primary treatment effluent is much smaller than those in activated and return sludge. Therefore, ARB flora in activated sludge is more dependent on return sludge than primary treatment effluent. Moreover, the enrichment of ARB also possibly occurs in the aeration tank by induction of antibiotic resistance (Sulfikar *et al.* 2018) and horizontal gene transfer (Rizzo *et al.* 2013). Induction of antibiotic resistance, which occurs by the expression of resistance genes as responses to stresses on microbial cells, causes enhancement of antibiotic resistance. It possibly occurs in the aeration tank where sludge microbes are exposed to oxidation stress by aeration and virulence stress by micropollutants (Sulfikar *et al.* 2018). Horizontal gene transfer allows a microbial cell to acquire antibiotic resistance

genes from another cell by conjugation, transduction or transformation. The probability of horizontal gene transfer in the sludge microbial community was reported as 3–50 conjugation events per 1×10^5 recipient cells for 48 hours of cultivation (Li *et al.* 2018). In addition to enrichment in the secondary clarifier, these mechanisms could also contribute to an increase in abundance of antibiotic resistance in activated sludge, and also to the development of an antibiotic resistance profile in activated sludge which is distinct from influent wastewater. Meanwhile, the antibiotic resistance profile of secondary treatment is likely to be affected directly by activated sludge. Activated sludge had relatively higher PC2 values in summer and lower PC2 values in winter, probably because of the seasonal change in abundance of resistance to AMX and KM (Figure 4). Secondary treatment effluents also had low PC2 values when PC2 of activated sludge were low. This indicates that the resistance profile of secondary treatment is partly affected by activated sludge. However, antibiotic resistance in activated sludge was probably not the only factor to influence the resistance profile in secondary treatment effluent. The resistance profiles in secondary treatment effluent have larger variance than in activated sludge and did not always correlate with those in activated sludge. Therefore, the conditions in the secondary clarifier and chlorination also possibly influence the antibiotic resistance profile in secondary treatment effluent.

Control of ARB discharge from WWTPs

Secondary treatment effluent still contains similar or a higher abundance of antibiotic resistant *E. coli* than influent wastewater. Meanwhile, the total population of *E. coli* was reduced by almost 2-log from influent wastewater to secondary treatment effluent. Secondary treatment effluent is often chlorinated further before discharge to reduce the fecal bacteria population. Therefore, the total population of antibiotic resistant *E. coli* was substantially reduced in wastewater treatment. However, there are still surviving populations of ARB in treated effluent (Huang *et al.* 2011; Yuan *et al.* 2016). Moreover, it has been reported that chlorination possibly increases the abundance of ARB because antibiotic resistance is induced via stress on their cells (Murray *et al.* 1984; Huang *et al.* 2011; Dodd 2012). Among alternative disinfection techniques, ozonation is reported to be effective to reduce ARB and antibiotic resistance genes (ARGs) in secondary treatment effluent (Sharma *et al.* 2016). Ultraviolet disinfection is not very effective to reduce the abundance of ARB and ARGs although it is still effective in reducing the total number of fecal bacteria. In developing countries, chlorination is often emitted at WWTPs due to economic reasons. In such countries, secondary treatment effluent which contains high antibiotic resistance would be directly discharged into natural water. The abundance of antibiotic resistance in wastewater treatment in developing countries is reported to be higher than that in developed countries (Amaya *et al.* 2012; Zhang *et al.* 2015; Honda *et al.* 2016). As in the case of NDM-1, developing countries recently became as the origin of emerging ARB. ARB emerging in one country can spread to other countries and continents within a couple of years (Nordmann *et al.* 2011). Disinfection of treatment effluent in developing countries is an important measure, not only for the local environment but also to prevent the prevalence of ARB over the countries.

Excess sludge is also a potential source ARB discharge from a WWTP into the water and soil environment. The final release of ARB to the environment via excess sludge depends on subsequent sludge treatment. Incineration is the most secure way to prevent the release of ARB from the discharged sludge although it is not always economically feasible or efficient. Anaerobic digestion is one of the effective ways to reduce ARB release via sludge. It can reduce the abundance of ARB in sludge as well as the sludge volume. Anaerobic digestion at higher temperatures has greater removal of antibiotic resistant genes (Lapara & Diehl 2010; Ma *et al.* 2011). Therefore, thermophilic anaerobic digestion is preferable to mesophilic digestion for the reduction of ARB in sludge. Digested sludge, as well as excess sludge, is then landfilled after dewatering and drying. Solid waste landfill is another critical node of ARB release to the water and soil environment because landfill leachate contains a high abundance of ARB and multiple resistance (Threedeach *et al.* 2012; Zhang *et al.* 2018). Sanitary landfill and proper treatment of leachate is essential to prevent the release of ARB from solid waste landfill. However, antibiotic resistance genes were still found in high concentrations in downstream water and soil even though leachate was

treated (Threedeach *et al.* 2012). Further studies are expected to investigate the impact of solid waste landfill on the release of ARB into the water and soil environment and its reduction measures. Digestion sludge could also be a potential source of ARB because it is sometimes spread in agricultural fields as organic fertilizer. Excess sludge and digested sludge are often composted before being used as fertilizer. A considerable population of fecal bacteria in sludge are killed during the composting process due to high temperature (Déportes *et al.* 1998; Hassen *et al.* 2001; Wéry *et al.* 2008). However, ARB is likely to be more persistent in the composting process (Storteboom *et al.* 2007; Su *et al.* 2015), although Liao *et al.* (2018) reported that ARB was reduced in hyperthermophilic composting. Therefore, composted sludge could be a source of ARB in the soil environment. Moreover, in rural areas and in developing countries, digested sludge is sometimes directly spread on agricultural fields as liquid manure. Such a use of sludge as manure not only causes a substantial release of ARB in soil and groundwater but also may harm farmers' health through possible direct exposure to ARB. Direct use of sludge should be avoided and shifted to the use of composted sludge to prevent the release of ARB as well as to protect farmers' health. The release of ARB via sludge disposal should also be further assessed to understand the whole environmental pathways of ARB spread.

CONCLUSIONS

According to the one-year monitoring of a full-scale WWTP, activated sludge in the aeration tank and return sludge tended to have a higher abundance of antibiotic resistant *E. coli* than influent wastewater and secondary treatment effluent. The increase of antibiotic resistance in activated sludge was likely to be caused by an increase of resistance to AMX. This increase of AMX resistance in activated sludge was probably because AMX resistant *E. coli* was enriched into return sludge at the secondary clarifier. AMX resistant *E. coli* was also affected by temperature and increased in summer rather than winter. The antibiotic resistance profile of *E. coli* in secondary treatment effluent was more dependent on activated sludge than influent wastewater. These results suggested that activated sludge in WWTPs possibly serves as a reservoir of ARB, although the behavior of ARB in WWTP differs by antibiotic class.

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DATA AVAILABILITY STATEMENT

Data cannot be made publicly available; readers should contact the corresponding author for details.

REFERENCES

Amaya, E., Reyes, D., Paniagua, M., Calderón, S., Rashid, M.-U., Colque, P., Kühn, I., Möllby, R., Weintraub, A. & Nord, C. E. 2012 Antibiotic resistance patterns of *Escherichia coli* isolates from different aquatic environmental sources in León, Nicaragua. *Clin. Microbiol. Infect.* **18** (9), E347–E354.

Behravan, J. & Rangsaaz, Z. 2004 Detection and characterization of beta-lactam resistance in Bacillus Cereus PTCC 1015. *Sci. World J.* **4**, 622–627.

CLSI (Clinical and Laboratory Standards Institute) 2008 *Performance Standards for Antimicrobial Susceptibility Testing*, 18th edn. CLSI supplement M100. Clinical and Laboratory Standards Institute, Wayne, PA.

Déportes, I., Benoit-Guyod, J. L., Zmirou, D. & Bouvier, M. C. 1998 Microbial disinfection capacity of municipal solid waste (MSW) composting. *J. Appl. Microbiol.* **85** (2), 238–246.

Dodd, M. 2012 Potential impacts of disinfection processes on elimination and deactivation of antibiotic resistance genes during water and wastewater treatment. *J. Environ. Monit.* **14**, 1754–1771.

Ferreira da Silva, M., Vaz-Moreira, I., Gonzalez-Pajuelo, M., Nunes, O. C. & Manaia, C. M. 2007 Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. *FEMS Microbiol. Ecol.* **60** (1), 166–176.

Godfrey, A. J., Hatlelid, L. & Bryan, L. E. 1984 Correlation between lipopolysaccharide structure and permeability resistance in beta-lactam-resistant *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **26** (2), 181–186.

Hassen, A., Belguith, K., Jedidi, N., Cherif, A., Cherif, M. & Boudabous, A. 2001 Microbial characterization during composting of municipal solid waste. *Bioresour. Technol.* **80** (3), 217–225.

Hecky, J. & Müller, K. M. 2005 Structural perturbation and compensation by directed evolution at physiological temperature leads to thermostabilization of β-lactamase. *Biochemistry* **44**, 12640–12654. https://doi.org/10.1021/bi0501885.

Honda, R., Watanabe, T., Sawaittayotin, V., Masago, Y., Chulasak, R., Tanong, K., Tushara Chaminda, G., Wongsila, K., Sienglum, C., Sunthonwatthanaphong, V., Poonnotok, A., Chiemchaisri, W., Chiemchaisri, C., Furumai, H. & Yamamoto, K. 2016 Impacts of urbanization on the prevalence of antibiotic resistant *Escherichia coli* in the Chaophraya River and its tributaries. *Water Sci. Technol.* **73** (2), 362–374.

Honda, R., Tachi, C., Yasuda, K., Hirata, T., Noguchi, M., Hara-Yamamura, H., Yamamoto-Ikemoto, R. & Watanabe, T. 2020 Estimated discharge of antibiotic-resistant bacteria from combined sewer overflows of urban sewage system. *NPJ Clean Water* **3**, 1–7. https://doi.org/10.1038/s41545-020-0059-5.

Huang, J. J., Hu, H. Y., Tang, F., Li, Y., Lu, S. Q. & Lu, Y. 2011 Inactivation and reactivation of antibiotic-resistant bacteria by chlorination in secondary effluents of a municipal wastewater treatment plant. *Water Res.* **45** (9), 2775–2781.

Koczura, R., Mokracka, J., Jabłońska, L., Gozdecka, E., Kubek, M. & Kaznowski, A. 2012 Antimicrobial resistance of integron-harboring *Escherichia coli* isolates from clinical samples, wastewater treatment plant and river water. *Sci. Total Environ.* **414**, 680–685.

Kotlarska, E., Łuczkiewicz, A., Pisowacka, M. & Burzyński, A. 2015 Antibiotic resistance and prevalence of Class 1 and 2 integrons in *Escherichia coli* isolated from two wastewater treatment plants, and their receiving waters (Gulf of Gdansk, Baltic Sea, Poland). *Environ. Sci. Pollut. Res.* **22** (3), 2018–2030.

Kumar, M., Ram, B., Honda, R., Poopipattana, C., Canh, V. D., Chaminda, T. & Furumai, H. 2019 Concurrence of antibiotic resistant bacteria (ARB), viruses, pharmaceuticals and personal care products (PPCPs) in ambient waters of Guwahati, India: Urban vulnerability and resilience perspective. *Sci. Total Environ.* **693**, 133640. doi:10.1016/j.scitotenv.2019.13364.

Lapara, T. M. & Diehl, D. L. 2010 Effect of temperature on the fate of genes encoding TC resistance and the integrase of class 1 integrons within anaerobic and aerobic digesters treating municipal wastewater solids. *Environ. Sci. Technol.* **44** (23), 9128–9133.

Li, B. & Zhang, T. 2010 Biodegradation and adsorption of antibiotics in the activated sludge process. *Environ. Sci. Technol.* **44** (9), 3468–3473.

Li, L., Dechesne, A., He, Z., Madsen, J. S., Nesme, J., Sørensen, S. J. & Smets, B. F. 2018 Estimating the transfer range of plasmids encoding antimicrobial resistance in a wastewater treatment plant microbial community. *Environ. Sci. Technol. Lett.* **5** (5), 260–265. http://pubs.acs.org/doi/10.1021/acs.estlett.8b00105.

Liao, H., Lu, X., Rensing, C., Friman, V. P., Geisen, S., Chen, Z., Yu, Z., Wei, Z., Zhou, S. & Zhu, Y. 2018 Hyperthermophilic composting accelerates the removal of antibiotic resistance genes and mobile genetic elements in sewage sludge. *Environ. Sci. Technol.* **52** (1), 266–276.

Łuczkiewicz, A., Jankowska, K., Fudala-Książek, S. & Olańczuk-Neyman, K. 2010 Antimicrobial resistance of fecal indicators in municipal wastewater treatment plant. *Water Res.* **44** (17), 5089–5097.

Ma, Y., Wilson, C. A., Novak, J. T., Riffat, R., Aynur, S., Murthy, S. & Pruden, A. 2011 Effect of various sludge digestion conditions on sulfonamide, macrolide, and tetracycline resistance genes and Class I integrons. *Environ. Sci. Technol.* **45** (18), 7855–7861.

Matsuo, H., Sakamoto, H., Arizono, K. & Shinohara, R. 2011 Behavior of pharmaceuticals in waste water treatment plant in Japan. *Bull. Environ. Contam. Toxicol.* **87**, 31–35. https://doi.org/10.1007/s00128-011-0299-7.

Murray, G. E., Tobin, R. S., Junkins, B., Kushner, D. J. & Al, M. E. T. 1984 Effect of chlorination on antibiotic resistance profiles of sewage- related bacteria. *Microbiology* **48** (1), 73–77.

Nordmann, P., Poirel, L., Toleman, M. A. & Walsh, T. R. 2011 Does broad-spectrum β-lactam resistance due to NDM-1 herald the end of the antibiotic era for treatment of infections caused by Gram-negative bacteria? *J. Antimicrob. Chemother.* **66** (4), 689–692.

Novo, A. & Manaia, C. M. 2010 Factors influencing antibiotic resistance burden in municipal wastewater treatment plants. *Appl. Microbiol. Biotechnol.* **87** (3), 1157–1166.

Orange, N. 1994 Growth temperature regulates the induction of β-lactamase in *Pseudomonas fluorescens* through modulation of the outer membrane permeation of a β-lactam-inducing antibiotic. *Microbiology* **140**, 3125–3130. https://doi.org/10.1099/13500872-140-11-3125.

Pruden, A., Joakim Larsson, D. G., Amézquita, A., Collignon, P., Brandt, K. K., Graham, D. W., Lazorchak, J. M., Suzuki, S., Silley, P., Snape, J. R., Topp, E., Zhang, T. & Zhu, Y. G. 2013 Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. *Environmental Health Perspectives* **121** (8), 878–885.

Reinthaler, F., Posch, J., Feierl, G., Wust, G., Haas, D., Ruckenbauer, G., Mascher, F. & Marth, E. 2003 Antibiotic resistance of *E. coli* in sewage and sludge. *Water Res.* **37** (8), 1685–1690.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C. & Fatta-Kassinos, D. 2013 Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. *Sci. Total Environ.* **447**, 345–360. https://doi.org/10.1016/j.scitotenv.2013.01.032.

Rosas, I., Salinas, E., Martínez, L., Cruz-Còrdova, A., Gonzàlez-Pedrajo, B., Espinosa, N. & Amábile-Cuevas, C. F. 2015

Characterization of *Escherichia coli* isolates from an urban lake receiving water from a wastewater treatment plant in Mexico City: fecal pollution and antibiotic resistance. *Curr. Microbiol.* **71** (4), 490–495.

Rousham, E. K., Unicomb, L. & Islam, M. A. 2018 Human, animal and environmental contributors to antibiotic resistance in low-resource settings: integrating behavioural, epidemiological and One Health approaches. *Proceedings of the Royal Society B: Biological Sciences* **285** (1876), 20180332.

Sharma, V. K., Johnson, N., Cizmas, L., McDonald, T. J. & Kim, H. 2016 A review of the influence of treatment strategies on antibiotic resistant bacteria and antibiotic resistance genes. *Chemosphere* **150**, 702–714.

Shchegolkova, N. M., Krasnov, G. S., Belova, A. A., Dmitriev, A. A., Kharitonov, S. L., Klimina, K. M. & Kudryavtseva, A. V. 2016 Microbial community structure of activated sludge in treatment plants with different wastewater compositions. *Front. Microbiol.* **7**, 1–15. https://doi.org/10.3389/fmicb.2016.00090.

Storteboom, H. N., Kim, S.-C., Doesken, K. C., Carlson, K. H., Davis, J. G. & Pruden, A. 2007 Response of antibiotics and resistance genes to high-intensity and low-intensity manure management. *J. Environ. Qual.* **36** (6), 1695–1703.

Su, J. Q., Wei, B., Ou-Yang, W. Y., Huang, F. Y., Zhao, Y., Xu, H. J. & Zhu, Y. G. 2015 Antibiotic resistome and its association with bacterial communities during sewage sludge composting. *Environ. Sci. Technol.* **49** (12), 7356–7363.

Sulfikar, H. R., Noguchi, M., Yamamoto-Ikemoto, R. & Watanabe, T. 2018 Effect of sedimentation and aeration on antibiotic resistance induction in the activated sludge process. *J. Water Environ. Technol.* **16** (2), 94–105.

Threedeach, S., Chiemchaisri, W., Watanabe, T., Chiemchaisri, C., Honda, R. & Yamamoto, K. 2012 Antibiotic resistance of *Escherichia coli* in leachates from municipal solid waste landfills: comparison between semi-aerobic and anaerobic operations. *Bioresour. Technol.* **113**, 253–258.

Wéry, N., Lhoutellier, C., Ducray, F., Delgenès, J. P. & Godon, J. J. 2008 Behaviour of pathogenic and indicator bacteria during urban wastewater treatment and sludge composting, as revealed by quantitative PCR. *Water Res.* **42** (1–2), 53–62.

Yang, Y., Ok, Y. S., Kim, K. H., Kwon, E. E. & Tsang, Y. F. 2017 Occurrences and removal of pharmaceuticals and personal care products (PPCPs) in drinking water and water/sewage treatment plants: a review. *Sci. Total Environ.* **596–597**, 303–320. https://doi.org/10.1016/j.scitotenv.2017.04.102.

Ye, L. & Zhang, T. 2013 Bacterial communities in different sections of a municipal wastewater treatment plant revealed by 16S rDNA 454 pyrosequencing. *Appl. Microbiol. Biotechnol.* **97** (6), 2681–2690. https://doi.org/10.1007/s00253-012-4082-4.

Yuan, Q. B., Guo, M. T., Wei, W. J. & Yang, J. 2016 Reductions of bacterial antibiotic resistance through five biological treatment processes treated municipal wastewater. *Environ. Sci. Pollut. Res.* **23** (19), 19495–19503.

Zhang, S., Han, B., Gu, J., Wang, C., Wang, P., Ma, Y., Cao, J. & He, Z. 2015 Fate of antibiotic resistant cultivable heterotrophic bacteria and antibiotic resistance genes in wastewater treatment processes. *Chemosphere* **135**, 138–145.

Zhang, J., Sui, Q., Tong, J., Zhong, H., Wang, Y., Chen, M. & Wei, Y. 2018 Soil types influence the fate of antibiotic-resistant bacteria and antibiotic resistance genes following the land application of sludge composts. *Environ. Int.* **118**, 34–43.

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