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Estimated discharge of antibiotic-resistant bacteria from combined sewer overflows of urban sewage system

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Yearly discharge of antibiotic-resistant bacteria (ARB) from combined sewer overflow (CSO) was estimated. The volume of CSO was estimated from operating data of the pumping station. In the target sewer catchment, 23% of the total of the volume of combined sewage was discharged untreated as CSO. Combined sewage contained 3-log larger *E. coli* than secondary treatment effluent although the abundance of antibiotic-resistant *E. coli* was not significantly different. In the target-combined sewer catchment, a yearly total of 4.8×10^{16} CFU of *E. coli* was discharged from 6.1×10^6 m³ of CSO, while 1.3×10^{12} CFU of *E. coli* from 2.1×10^7 m³ of effluent from the wastewater treatment plant (WWTP). This *E. coli* discharge was equivalent to 7.9×10^9 CFU/m³ from CSO, and 6.2×10^4 CFU/m³ from WWTP effluent. Consequently, a yearly total discharge of antibiotic-resistant *E. coli* from CSO was 3.7-log larger than the WWTP effluent. The small-flow CSO events, which had hourly flow rate smaller than five times of the average dry-weather flow, accounted for 43% of the total CSO volume, but 79% of the total discharge of antibiotic-resistant *E. coli* due to a small dilution factor with stormwater and frequent discharge. Reduction of small-flow CSO events would be important for effective reduction of ARB discharge from CSO.

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INTRODUCTION

Increased emergence of antibiotic-resistant bacteria (ARB) is now a great concern for future human health. Water and soil environments are now recognized as reservoirs and sources of ARB of clinical concern¹. To prevent prevalence of ARB through environmental pathways, it is important to control the release of ARB from anthropogenic activity into the environment². Municipal wastewater is a major source of ARB in urban water environment in both developed and developing countries^{3–8}. ARB in wastewater still remains at high abundance in secondary treatment effluents of WWTPs, and becomes the major source in water environment and drinking water resources^{4–6,9–14}.

Combined sewer overflow (CSO) is also a possible significant pathway of ARB discharge from municipal wastewater. In a combined sewer system, wastewater and stormwater are collected in the same pipe. When the sewage volume in a combined sewer system can exceed the capacity of WWTP due to heavy rainfall or snowmelt, the combined sewage, which contains both wastewater and stormwater, is designed to overflow and discharge directly to nearby water bodies without treatment. These overflows, called combined sewer overflow (CSO), are a significant source of fecal pollution in water bodies in many urban areas^{15–19}. According to USEPA²⁰, discharge of CSO was estimated to be 20% of the total annual discharge of combined sewage in the United States. Moreover, climate change is predicted to substantially increase the volume and frequency of CSO in some areas due to a change in the precipitation pattern^{21,22}. However, there are a limited number of studies on ARB discharge from CSO. Garner et al.²³ recently quantified antibiotic-resistant genes (ARG) at an urban stormwater outfall, and reported that a single CSO event could bring 1- to 3-log larger loadings of ARG than the dry-weather event. However, even though a single CSO event has a large discharge of ARB, the CSO

events occur only occasionally. Moreover, the volume of CSO in each event has a large fluctuation, depending on the quantity of stormwater received by the combined sewer system. Concentrations of ARB in CSO also change, depending on the quantity of stormwater, because ARB in wastewater is diluted with stormwater in the combined sewer pipes. On the other hand, the WWTP effluent is discharged continuously throughout a year, although it may contain less ARB than CSO. For effective reduction of ARB discharge, it is important to know which of the CSO and WWTP effluents has a larger impact on ARB discharge into the water environment and drinking water sources.

The objective of this study is to estimate ARB discharge from CSO and wastewater treatment effluents of an urban-combined sewer system, in order to investigate the quantitative impact of CSO on ARB discharge into water environment. A combined sewer catchment, where the entire quantity of CSO and combined sewage was monitored at pumping stations, was chosen as the target area in order to obtain an accurate volume of the entire combined sewage and CSO from operating data of the pumping stations. Antibiotic resistance of *E. coli*, as a representative of fecal-originated ARB, in the combined sewage and WWTP effluent, was monitored throughout a year. From these data, yearly discharge of antibiotic-resistant *E. coli* from CSO and WWTP effluents was estimated and compared with discussing their impact on ARB discharge from combined sewer systems to water environment.

RESULTS

Antibiotic-resistant bacteria in combined sewage and secondary treatment effluents

The abundance of antibiotic-resistant *E. coli* in combined sewage and secondary treatment effluents had no significant change and

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seasonal trend (Table 1). The annual average of the abundance of *E. coli* resistant to at least one antibiotic slightly increased from 37% in combined sewage to 42% in secondary treatment effluents; however, there was no statistically significant difference ($p = 0.45$). Many studies reported that ARB abundance showed no significant change or a slight increase through the treatment^{9,10,12,13}. However, a change in the abundance of antibiotic resistance between combined sewage and secondary treatment effluents was highly fluctuated, and had no consistent trend in monthly data. The abundance of multiple antibiotic resistance in combined sewage and secondary treatment effluents was also highly fluctuated on a monthly basis, but not significantly different as the annual average ($p = 0.67$). The abundance values of *E. coli* with multiple antibiotic resistance were 26 and 23% in combined sewage and secondary treatment effluents as the annual average, respectively.

Although the abundance of antibiotic-resistant *E. coli* in combined sewage was comparable with secondary treatment effluents, combined sewage contained significantly higher concentration of *E. coli* than secondary treatment effluents ($p < 0.001$) (Table 1). *E. coli* concentrations reportedly ranged 10^4 – 10^5 CFU/mL in sewage^{12,24,25}, and 10^1 – 10^3 CFU/mL in secondary treatment effluents^{12,24,26}. *E. coli* concentrations in this study were 3.2×10^4 – 1.0×10^5 CFU/mL in combined sewage, and 2.8×10^1 – 1.7×10^3 CFU/mL in secondary treatment effluents, which were within the typical range of those reported in other WWTPs^{12,24,26}. At the target WWTP, secondary treatment effluent was discharged after chlorination. The log-removal value of *E. coli* by chlorination at the target WWTP was 3.8-log (Supplementary Fig. S2). Therefore, combined sewage was estimated to contain 6.3-log larger *E. coli* than WWTP effluent at the target WWTP. Meanwhile, *E. coli* concentrations in combined sewage did not have significant seasonal change throughout a year. Standard deviation of *E. coli* concentrations in combined sewage was 0.16-log. *E. coli* in secondary treatment effluent had a slightly larger seasonal fluctuation than combined sewage, with 0.54-log of standard deviation. The observed fluctuations in this study had no consistent seasonal trend against temperature or precipitation. It is probably because they were originated from daily fluctuation rather than seasonal conditions. In this study, *E. coli* sampling was conducted once a month. No data were taken for daily and hourly fluctuation of *E. coli* concentrations in combined sewage and secondary treatment effluent. According to the past studies^{12,24,26}, fluctuations of *E. coli* concentrations are reported to be small within 0.5-log of standard deviation in wastewater, 2-log of standard deviation in secondary treatment effluent. These were consistent with the observed fluctuations in this study. Therefore, *E. coli* concentrations in combined sewage and secondary treatment effluent in this study were within the typical range and had seasonal fluctuations reported at other WWTPs.

Quantity of combined sewer overflow

In the target sewer catchment, 23% of total volume of combined sewage was discharged untreated as CSO. Although there are a limited number of studies that estimated CSO volume, it was comparable to estimation in the United States by USEPA (2004), which reported that 20% of combined sewage was discharged as CSO²⁰. The volume of CSO and the number of CSO events fluctuated, depending on seasonal change in precipitation conditions. In the target catchment, the volume of CSO was large in August and December because of large precipitation (Fig. 1). Daily CSO volume was highly correlated with daily precipitation (Fig. 2). This indicates that the CSO volume is highly affected by precipitation in this combined sewer catchment. The frequency of CSO events and the flow rate of CSO in each event varies a lot, depending on not only precipitation but also rainfall intensity and duration. In the target catchment, the total volume of CSO in

Table 1. *E. coli* population and abundance of resistant isolates to at least one antibiotic (% AMR) and multiple antibiotics (% MAR) in combined sewage and secondary treatment effluent.

		2014 Jul	Aug	Sep	Oct	Nov	Dec	2015 Jan	Feb	Mar	Apr	May	Jun	Annual average
<i>E. coli</i> (CFU/mL)	Sewage	7.1×10^4	3.4×10^4	9.3×10^4	8.2×10^4	7.1×10^4	3.2×10^4	6.9×10^4	5.3×10^4	3.8×10^4	5.9×10^4	1.0×10^5	6.7×10^4	6.0×10^4 ($\pm 1.4 \times 10^0$)
	STE	1.4×10^3	6.5×10^1	1.7×10^3	8.6×10^1	8.1×10^1	2.8×10^1	6.4×10^1	1.6×10^2	2.5×10^2	2.8×10^2	2.1×10^2	7.9×10^1	2.0×10^2 ($\pm 3.5 \times 10^0$)
% AMR	Sewage	63%	17%	48%	25%	26%	40%	56%	28%	20%	52%	20%	52%	37% ($\pm 16\%$)
	STE	56%	32%	54%	27%	50%	44%	16%	74%	33%	40%	33%	45%	42% ($\pm 15\%$)
% MAR	Sewage	38%	17%	43%	4%	17%	48%	44%	24%	12%	28%	12%	19%	26% ($\pm 14\%$)
	STE	4%	20%	17%	13%	45%	24%	4%	43%	25%	28%	25%	25%	23% ($\pm 12\%$)
No. of isolates	Sewage	24	23	23	24	23	25	25	25	25	25	25	21	Total 288
	STE	25	25	24	15	20	25	25	23	24	25	24	20	275

STE secondary treatment effluent.
The values in brackets are standard deviations.

Fig. 1 Monthly precipitation (top), volume of combined sewer overflow (CSO) and effluent from wastewater treatment plant (WWTP) (bottom).

Fig. 2 Correlation between the volume of combined sewer overflow (CSO) and daily precipitation. The dashed line is the regression line.

August and December was comparable; however, the flow rate and frequency of CSO events had different trends. Median values of the flow rate in the CSO event were $3555 \text{ m}^3/\text{h}$ in December and $9214 \text{ m}^3/\text{h}$ in August. The total duration of CSO events was

313 h in December, and 93 h in August. Therefore, CSO events in December were mostly small-flow and long overflows, while those in August were large-flow and short overflows. This seasonal difference in CSO flow rate has a large impact on ARB discharge to the receiving water body. ARB in combined sewage is mainly originated from municipal wastewater, which was constantly discharged independent of weather conditions. Meanwhile, the flow rate of stormwater was highly fluctuated by precipitation. Combined sewage contained a higher concentration of ARB when it received a smaller quantity of stormwater, and vice versa. Therefore, in this catchment, CSOs in December probably had a larger impact on ARB discharge than other months.

Quantity of antibiotic-resistant bacteria discharged from CSO and WWTP effluents

Yearly quantity of antibiotic-resistant *E. coli* discharged from CSO and WWTP effluents was estimated from the abundance of antibiotic resistance, *E. coli* concentrations, and the flow rate of combined sewage and secondary treatment effluents. Yearly quantity of antibiotic-resistant *E. coli* discharged from CSO was much larger than the WWTP effluent (Fig. 3). Yearly quantity of *E. coli* with multiple antibiotic resistance also discharged more from CSO than WWTP effluents. The annual average of *E. coli* discharge

Fig. 3 Estimated discharge of antibiotic-resistant *E. coli* from combined sewer overflow (CSO) and effluents from wastewater treatment plant (WWTP). %AMR abundance of resistance to at least one antibiotic agent; %MAR abundance of resistance to multiple antibiotic classes.

Fig. 4 Cumulative proportion of discharge of antibiotic-resistant *E. coli* and CSO by hourly flow rate of CSO normalized by flow rate of combined sewage at the same hour in dry weather.

was equivalent to 7.9×10^9 CFU/m³ from CSO, and 6.2×10^4 CFU/m³ from WWTP effluents. Yearly volume of CSO corresponded only to 29% of secondary treatment effluent. However, yearly quantity of *E. coli* discharged from CSO was 4.6-log larger than that from WWTP effluent. The main reason was because CSO contained much higher concentrations of *E. coli* than the WWTP effluent, while the abundance of antibiotic-resistant *E. coli* was not significantly different between combined sewage and WWTP effluent. As discussed above, CSO events with smaller flow rates had more impact on discharge of antibiotic-resistant *E. coli*. Figure 4 shows the contribution of CSO to its volume and ARB discharge by hourly flow rate normalized by dry-weather flow of combined sewage. The figure indicates that 43% of total volume of CSO was discharged by small-flow CSO events with normalized hourly flow rates <5, which means flow rates smaller than 5 times of the dry-weather flow of combined sewage. Meanwhile, the quantity of antibiotic-resistant *E. coli*, which was discharged from the small-flow CSO events, accounted for 79% of the total quantity of antibiotic-resistant *E. coli* discharged from CSOs. CSO events with normalized hourly flow rates <2 accounted for only 16% of the total CSO volume, but 42% of the total discharge of antibiotic-resistant *E. coli*. These results clearly indicated that small-flow CSO events have a larger impact on ARB discharge than large-flow CSO events.

DISCUSSION

Discharge of ARB from CSO occurs occasionally depending on precipitation conditions, while ARB in the WWTP effluent is

continuously discharged independent of weather conditions. This study showed, on yearly quantity basis, that CSO had a much larger impact on discharge of antibiotic-resistant *E. coli* from a combined sewer system than the WWTP effluent. The major reason was because CSO contained a much larger concentration of fecal bacteria than secondary treatment effluent. This study targeted a typical combined sewer catchment in Japan; however, the basic conditions in this catchment are applicable to many combined sewer catchments in other countries. *E. coli* concentrations in combined sewer and secondary treatment effluents observed in this study were in the typical range reported in many other WWTPs, as described above. The abundance of antibiotic resistance in *E. coli* was not significantly different between combined sewage and secondary treatment effluents ($p = 0.45$). Many of past studies in other countries also reported that the abundance of antibiotic-resistant *E. coli* did not significantly change. Therefore, the finding of this study on large impacts of CSO on ARB discharge are considered to be consistent in many combined sewer catchments in other countries.

Nevertheless, the volume of CSO is highly dependent on catchment conditions. CSO volume varies among cities and countries mainly because of the difference in precipitation patterns. There are a limited number of studies on catchment-scale CSO volume. In this study, the volume of CSO corresponded to 29% of secondary treatment effluent. This was in similar proportion with estimation by USEPA²⁰, in which 20% of combined sewage was discharged as CSO. As the least case in Sweden, the volume of CSO was only 0.4% of the total flow to the WWTP²². As event basis, CSO flow patterns are often affected by climate conditions. In tropical regions, where rainfall is often short and intense, CSO flows reach at a very high peak within half an hour, and end within an hour^{27,28}, while CSO flows in temperate regions are rather smaller and longer to last for several hours or sometimes for days^{16,17,23}. This suggests that CSOs in temperate regions could have more pollutant loads due to less dilution and longer duration. Compared with CSO quantity, *E. coli* concentrations in sewage have much smaller variations among locations. *E. coli* in sewage is reportedly 10^4 – 10^5 CFU/mL, both in temperate and tropical countries^{12,24,25,29–32}. This indicates that CSO contains 2- to 5-log larger *E. coli* than that in WWTP effluents^{12,24}. Therefore, even if the volume of CSO is much smaller than the WWTP effluent, CSO has a larger impact on *E. coli* discharge than the WWTP effluent in many combined sewer catchments. On the other hand, the abundance of antibiotic resistance in sewage is likely to be affected by economical and sanitation conditions in the country, rather than climate conditions. Urban sewage reflects gut microbiota and antibiotic resistome of the population^{33,34}. Pärnänen et al.³⁵ recently reported that antibiotic resistome in urban sewage had systematic differences between Europe/North America and Africa/Asia/South America, and that low abundance of antibiotic-resistance genes was associated with low Human Development Index (HDI) of the country. Consequently, among three possible factors to affect ARB discharge from CSO, CSO flow patterns are mainly affected by climate conditions; abundance of antibiotic resistance by economical development conditions; *E. coli* concentrations have less variation among locations.

The results of this study suggest that it is important to control ARB discharge from CSO for reduction of ARB discharge from urban wastewater into water environment. Various countermeasures have been proposed and introduced to reduce pollutant loading of CSO on receiving water bodies, such as retention basins for stormwater storage, chemically enhanced primary treatment, etc.^{36–38}. However, performance of these measures is limited when CSO has a large-flow rate. This study showed that small-flow CSO events contributed to a relatively larger portion of the total ARB discharge from CSO. It is because small-flow CSO contained a high concentration of *E. coli* due to less dilution with stormwater. Therefore, reduction of ARB from small-flow CSO events would be

important and efficient measures to reduce ARB discharge from a combined sewer catchment. In a case study in Lisbon³⁹, installation of stormwater storage was predicted to reduce up to 40% of CSO volume. In the United States and France, retention treatment basin, which is stormwater tank with settling ability, was proved to be effective to reduce pollutant loading and fecal coliform from CSO^{40,41}. These measures are also probably effective to reduce ARB discharge from CSO. In this study, the first flush from the sewer pipeline was not considered. The first flush possibly contains high loading of pollutants from wastewater and deposits in sewer pipes⁴². Sediments in sewer pipelines possibly contain ARB⁴³, and could be flushed at the beginning of a runoff event. In such cases, more ARB could be discharged at the beginning of CSO discharge. Retention basins are effective to reduce the impacts of the first flush. Installation of these measures is expected also for control of ARB release to water bodies.

This study also showed that ARB contained in the WWTP effluent still has a certain impact on ARB discharge from a combined sewer catchment, even though antibiotic-resistant *E. coli* discharge was 3.7-log smaller than CSO. Secondary treatment effluent is usually chlorinated before it is discharged into natural water bodies. However, it still contains a certain level of ARB^{11,44}. Moreover, some reported that chlorination possibly increases the abundance of ARB because antibiotic resistance is induced via stress on its cells^{11,45,46}. In some developing countries, chlorination is often emitted at WWTPs due to economical reasons. Abundance of antibiotic resistance at wastewater treatment in developing countries is reported to be higher than that in developed countries^{3,13,47}. In such countries, therefore, ARB discharge from secondary treatment effluent would have a certain impact. As in the case of NDM-1, developing countries are recently concerned as the origin of ARB. ARB emerging in one country can spread to other countries and continents within a couple of years⁴⁸. Chlorination is effective to reduce 3-log of ARB in secondary treatment effluent^{44,49}. Disinfection of secondary treatment effluent is also an important measure to reduce ARB discharge from WWTP effluent in developing countries.

METHODS

Combined sewer catchment

The target -combined sewer catchment was located in Kanazawa, Japan, and covered 29,900 of population and 4.04 km² of area. Yearly average precipitation in this area was 2400 mm. The combined sewage in the target catchment mainly consisted of municipal wastewater and stormwater runoff. The quantity of combined sewage was 28,300 m³/day as daily average. All the combined sewage from the coverage area was received at the three pumping stations. At the pumping stations, the combined sewage within the treatment capacity was sent to the WWTP for treatment. When the combined sewage exceeded the treatment capacity of the WWTP in wet-weather conditions, the exceeded quantity of combined sewage was discharged untreated as CSO from the pumping stations to the neighboring river. The WWTP received 4000 m³/h in wet-weather conditions, and 1200 m³/h in dry-weather conditions. The combined sewage received at the WWTP was treated by a conventional activated sludge process, and chlorinated before discharge.

Quantification and isolation of *Escherichia coli*

E. coli was chosen as the target bacteria to represent ARB, which originated from the fecal sources. Fifty milliliters of water samples of influent wastewater (INF) and secondary treatment effluent (STE) before chlorination were collected monthly in the morning from July 2014 until June 2015 at the WWTP. 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 (A045H047W, 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 picked up from each sample 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 until colony counting were conducted within 24 h after sampling.

Antibiotic-susceptibility test

Susceptibility of each *E. coli* isolate to six antibiotics in four classes was tested by Kirby–Bauer disk diffusion method: ciprofloxacin (CIP), norfloxacin (NFX) in fluoroquinolone class, tetracycline (TC) in tetracycline class, amoxicillin (AMX) in β-lactam class, kanamycin (KM) in aminoglycoside class, and sulfamethoxazole/trimethoprim (ST) in 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 (Supplementary Table S1), which followed CLSI standard M100-S18⁵⁰. According to the test protocol, the isolates, which had possible contamination, were excluded from the result. Hence, the effective number of isolates was 15–25 per sample, as shown in Table 1. The abundance of resistant isolates in each sample was calculated by

$$A_{r,i} = \frac{R_{r,i}}{N} \quad (1)$$

where $A_{r,i}$: abundance of isolates resistant to antibiotic i , $R_{r,i}$: number of isolates determined as “resistance” to antibiotic i , N : the effective number of isolates in the susceptibility test.

The abundance of multiple antibiotic resistance (MAR) was calculated as abundance of isolates non-susceptible to three or more antibiotic categories, according to the definition of multidrug resistance by Magiorakos et al.⁵¹. Paired-sample t test was used for monthly data of sewage and secondary treatment effluent in order to test the statistical difference by treatment of *E. coli* concentrations, abundance of antibiotic resistance, and multiple antibiotic resistance, respectively.

Volume of CSO

In the target-combined sewer catchment, all combined sewage was collected into the three pumping stations. Pumping stations Nos. 1 and 2 had two outlets each: one for WWTP and the other for CSO discharge. Pumping station No. 3 was used only for CSO discharge. Hourly operation data of the three pumping stations from July 1, 2014 until June 30, 2015 (8759 time points per outlet) were used to calculate hourly flow rate of the total combined sewage, sewage sent to WWTP for treatment, and CSO according to the following equations:

$$Q_{w(t)} = \sum_{i=1}^2 q_{w(t),i} \quad (2)$$

$$Q_{o(t)} = \sum_{i=1}^3 q_{o(t),i} \quad (3)$$

$$Q_{in(t)} = Q_{w(t)} + Q_{o(t)} \quad (4)$$

where $Q_{w(t)}$: hourly flow rate of sewage sent to WWTP at time t , $q_{w(t),i}$: hourly flow rate of sewage sent to WWTP from pumping station i at time t , $Q_{o(t)}$: hourly flow rate of CSO at time t , $q_{o(t),i}$: hourly flow rate of CSO from pumping station i at time t , $Q_{in(t)}$: hourly flow rate of all combined sewage in the target catchment at time t .

Dilution factors of wastewater with stormwater were estimated as an hourly flow rate of combined sewage divided by the average hourly flow rate of combined sewage in dry weather at the same hour of the day. The average hourly flow rate of sewage in dry weather was calculated as the average of flow rate at each hour of a day when CSO discharge $Q_{o(t)} = 0$, according to the equations below:

$$Q_{dry(h)} = \frac{\sum \{Q_{in(t)} | Q_{o(t)} = 0 \wedge t \in h\}}{N_{d(h)}} \quad (5)$$

where h : hour of a day ($= 0 \sim 23$), $Q_{dry(h)}$: hourly flow rate of total combined sewage at the same hour h in dry weather, $N_{d(h)}$: the number of data points

where $Q_{o(t)} = 0$ at h . The average hourly flow rate of sewage in dry weather $Q_{dry(h)}$ is shown in Supplementary Fig. S1. The dilution factor of wastewater f_d at time t was calculated as

$$f_{d(t)} = \frac{Q_{in(t)}}{Q_{dry(h)}} \quad (6)$$

Estimation of *E. coli* and ARB discharge from CSO

Concentrations of *E. coli* in CSO were estimated by considering dilution of wastewater with stormwater. Dilution factors of wastewater with stormwater were calculated hourly by Eq. 6. The quantity of *E. coli* discharged from CSO was then estimated from the hourly dilution factor of CSO, monthly data of *E. coli* in influent wastewater, and quantity of CSO, according to the equation below:

$$X_{CSO(t)} = \frac{C_{WW(m)} \cdot Q_{o(t)}}{f_{d(t)}} \quad (7)$$

where $X_{CSO(t)}$: quantity of *E. coli* discharged from CSO at time t , $C_{WW(m)}$: *E. coli* concentration in influent wastewater in the corresponding month m . Abundance of ARB in CSO was assumed to be equal to the influent wastewater. Impacts of the first flush from sewer pipelines were not considered in this study.

The quantity of *E. coli* discharged via WWTP effluent was estimated from the monthly data of *E. coli* in secondary treatment effluent, quantity of WWTP effluent, and log-removal values of *E. coli* by chlorination, according to the equation below:

$$X_{DIS(t)} = C_{STE(m)} \cdot R_{Cl} \cdot Q_{w(t)} \quad (8)$$

where $X_{DIS(t)}$: quantity of *E. coli* discharged via WWTP effluent at time t , $C_{STE(t)}$: *E. coli* concentration in secondary treatment effluent in the corresponding month m , R_{Cl} : log-removal value rate of *E. coli* in the chlorination step. Log-removal rate of *E. coli* in chlorination at the target WWTP was 3.8-log (Supplementary Fig. S2).

DATA AVAILABILITY

The source data of the figures are available from Mendeley Data with the identifier <https://doi.org/10.17632/78ds4m9nrf.1>. Remaining data that support the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

R.H. contributed to plan and design of this research, analysis of flow data, data interpretation, and writing the paper. C.T., K.Y., T.H., and M.N. contributed to sampling and analysis of antibiotic-resistant *E. coli*. K.Y. also contributed to analysis of flow data. H.H.-Y. contributed to literature review and discussion. R.Y.-I. and T.W. contributed to plan and design of this research.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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