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

Ryo Honda, Chihiro Tachi, Keisuke Yasuda, Tatsuki Hirata, Mana Noguchi, Hiroe Hara-Yamamura, Ryoko Yamamoto-Ikemoto and Toru Watanabe

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 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 x 10^16 CFU of E. coli was discharged from 6.1 x 10^6 m^3 of CSO, while 1.3 x 10^12 CFU of E. coli from 2.1 x 10^7 m^3 of effluent from the wastewater treatment plant (WWTP). This E. coli discharge was equivalent to 7.9 x 10^9 CFU/m^3 from CSO, and 6.2 x 10^4 CFU/m^3 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.

npj Clean Water (2020) 3:15; https://doi.org/10.1038/s41545-020-0059-5

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 concern. To prevent prevalence of ARB through environmental pathways, it is important to control the release of ARB from anthropogenic activity into the environment. Municipal wastewater is a major source of ARB in urban water environment in both developed and developing countries. 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.

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. According to USEPA, 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. However, there are a limited number of studies on ARB discharge from CSO. Garner et al. 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 loads 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 the 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...
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 process. 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 $10^3$–$10^9$ CFU/mL in sewage, and $10^1$–$10^3$ CFU/mL in secondary treatment effluents. E. coli concentrations in this study were $3.2 \times 10^3$–$1.0 \times 10^5$ CFU/mL in combined sewage, and $2.8 \times 10^1$–$1.7 \times 10^4$ CFU/mL in secondary treatment effluents, which were within the typical range of those reported in other WWTPs. 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, 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 CSO. 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

| 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. |
|---|
| **Table 1.** E. coli concentrations reported in combined sewage and secondary treatment effluent. |
| 2014 | July | Aug | Sep | Oct | Nov | Dec | 2015 | Jan | Feb | Mar | Apr | May |
| Sewage | STE | Sewage | STE | Sewage | STE | Sewage | STE | Sewage | STE | Sewage | STE | Sewage |
| E. coli (CFU/mL) | 7.1 × 10^3 | 6.9 × 10^4 | 3.4 × 10^4 | 6.4 × 10^4 | 3.2 × 10^5 | 1.4 × 10^9 | 8.2 × 10^4 | 2.8 × 10^5 | 8.6 × 10^4 | 1.7 × 10^5 | 2.5 × 10^5 | 1.0 × 10^6 |
| % AMR | 63% | 65% | 63% | 58% | 48% | 55% | 65% | 50% | 59% | 53% | 50% | 49% |
| % MAR | 37% | 35% | 37% | 42% | 45% | 45% | 35% | 47% | 41% | 47% | 49% | 51% |
| No. of isolates | 24 | 23 | 23 | 24 | 25 | 15 | 25 | 20 | 24 | 25 | 20 | 27 |

The values in brackets are standard deviations.
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 m$^3$/h in December and 9214 m$^3$/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
E. coli

Antibiotic resistant E. coli

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 $7.9 \times 10^9$ CFU/m$^3$ from CSO, and $6.2 \times 10^4$ CFU/m$^3$ 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 USEPA,[20] 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 WWTP.[25] 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.[12,24,29–32] 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 counter-measures 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 Lisbon\textsuperscript{19}, 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\textsuperscript{30,41}. 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 pipes\textsuperscript{32}. Sediments in sewer pipelines possibly contain ARB\textsuperscript{37,38}, 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 \textit{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\textsuperscript{11,44}. Moreover, some reported that chlorination possibly increases the abundance of ARB because antibiotic resistance is induced via stress on its cells\textsuperscript{11,45,46}. In some developing countries, chlorination is effective to reduce 3-log of ARB in secondary treatment effluent\textsuperscript{11,45,46}. This study also showed that ARB contained in the WWTP effluent is also an important measure to reduce ARB discharge from WWTP effluent in developing countries.

\section*{METHODS}

\subsection*{Combined sewer catchment}

The target combined sewer catchment was located in Kanazawa, Japan, and covered 29,900 of population and 4.04 km\textsuperscript{2} 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 m\textsuperscript{3}/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 m\textsuperscript{3}/h in wet-weather conditions, and 1200 m\textsuperscript{3}/h in dry-weather conditions. The combined sewage received at the WWTP was treated by a conventional activated sludge process, and chlorinated before discharge.

\subsection*{Quantification and isolation of \textit{Escherichia coli}}

\textit{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 (A045H407W, Advantec Toyo, Tokyo, Japan). Filters were then placed on Chromocult Acinetobacter Agar ES (Merck KGaA, Darmstadt, Germany), and incubated at 37°C for 24 h. After counting all colonies, 20–25 colonies of \textit{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.

\subsection*{Antibiotic-susceptibility test}

Susceptibility of each \textit{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 \textit{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-S18\textsuperscript{40}. 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

\begin{equation}
A_{i,j} = \frac{R_{i,j}}{N}
\end{equation}

where $A_{i,j}$: abundance of isolates resistant to antibiotic $i$, $R_{i,j}$: 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.\textsuperscript{41}. 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 \textit{E. coli} concentrations, abundance of antibiotic resistance, and multiple antibiotic resistance, respectively.

\subsection*{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 (8750 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:

\begin{equation}
Q_{w(t)} = \sum_{i=1}^{2} q_{w(t),i}
\end{equation}

\begin{equation}
Q_{o(t)} = \sum_{i=1}^{3} q_{o(t),i}
\end{equation}

\begin{equation}
Q_{ot} = Q_{w(t)} + Q_{o(t)}
\end{equation}

where $Q_{w(t)}$: hourly flow rate of sewage sent to WWTP at time $t$, $Q_{o(t),i}$: 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 $t$, $Q_{ot}$: 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:

\begin{equation}
Q_{d(h)} = \sum_{t=1}^{24} Q_{w(t)} = 0 \land t \in h
\end{equation}

where $h$: hour of a day (0–23), $Q_{d(h)}$: hourly flow rate of total combined sewage at the same hour $h$ in dry weather, $N_{d(h)}$: the number of data points
where \( Q_{\text{day}(t)} \) is shown in Supplementary Fig. S1. The dilution factor of wastewater \( f_d(t) \) at time \( t \) was calculated as

\[
f_d(t) = \frac{Q_{\text{Nat}(t)}}{Q_{\text{day}(t)}}
\]

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 \( E.\ coli \) concentration in influent wastewater in the corresponding month \( m \). The exact value of the ARB discharge from 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:

\[
X_{\text{CSO}(t)} = C_{\text{WWT}(m)} \cdot Q_{\text{CSO}(t)}
\]

where \( X_{\text{CSO}} \): quantity of \( E.\ coli \) discharged from CSO at time \( t \), \( C_{\text{WWT}} \): \( E.\ coli \) concentration in influent wastewater in the corresponding month \( m \). Abundance of ARB in CSO was assumed to be equal to the influent wastewater.

This article is protected by copyright. All rights reserved.
34. Pärnänen, K. M. M. et al. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. Sci. Adv. 5, eaau9124 (2019).
35. Hendriksen, R. S. et al. Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. Nat. Commun. 10, 1124 (2019).
36. Exall, K. & Marsalek, J. A coagulant survey for chemically enhanced primary treatment of synthetic CSOs. Water Air. Soil Pollut. 224, 1414 (2013).
37. Li, J. G., Horneck, H., Averill, D., McCorquodale, J. A. & Biswas, N. High-rate retention treatment basins for CSO control in Windsor, Ontario. Water Qual. Res. J. Can. 39, 449–456 (2004).
38. McCorquodale, J. A., Griborio, A., Li, J., Horneck, H. & Biswas, N. Modeling a retention treatment basin for CSO. J. Environ. Eng. 133, 263–271 (2007).
39. David, L. M. & Matos, J. S. Combined sewer overflow emissions to bathing waters in Portugal. How to reduce in densely urbanized areas? Water Sci. Technol. 52, 183–190 (2005).
40. Jacopin, C. & Bertrand-Krajewski, J. L. Characterisation and settling of solids in an open, grassed, stormwater sewer network detention basin. Water Sci. Technol. 39, 135–144 (1999).
41. U.S. Environmental Protection Agency. Combined Sewer Overflow Technology Fact Sheet. EPA 832-F-99-042 (1999).
42. Anne-Sophie, M. H. et al. Temporal analysis of E. coli, TSS and wastewater micropollutant loads from combined sewer overflows: implications for management. Environ. Sci. Process. Impacts 17, 965–974 (2015).
43. Pijuan, M. et al. Sewers as potential reservoirs of antibiotic resistance. Sci. Total Environ. 605–606, 1047–1054 (2017).
44. Huang, J. J. et al. Inactivation and reactivation of antibiotic-resistant bacteria by chlorination in secondary effluents of a municipal wastewater treatment plant. Water Res. 45, 2775–2781 (2011).
45. Dodd, M. Potential impacts of disinfection processes on elimination and deactivation of antibiotic resistance genes during wastewater and wastewater treatment. J. Environ. Monit. 14, 1754–1771 (2012).
46. Murray, G. E., Tobin, R. S., Jenkins, B., Kushner, D. J. & Al, M. E. T. Effect of chlorination on antibiotic resistance profiles of sewage-related bacteria. Microbiology 48, 73–77 (1984).
47. Amaya, E. et al. Antibiotic resistance patterns of Escherichia coli isolates from different aquatic environmental sources in León, Nicaragua. Clin. Microbiol. Infect. 18, E347–E354 (2012).
48. Nordmann, P., Poirot, L., Toleman, M. A. & Walsh, T. R. Does broad-spectrum-lactam resistance due to NDM-1 herald the end of the antibiotic era for treatment of infections caused by Gram-negative bacteria? J. Antimicro. Chemother. 66, 689–692 (2011).
49. Novo, A. & Manaia, C. M. Factors influencing antibiotic resistance burden in municipal wastewater treatment plants. Appl. Microbiol. Biotechnol. 87, 1157–1166 (2010).
50. CLSI. Performance Standards for Antimicrobial Susceptibility Testing, 28th edn. CLSI supplement M100. (Clinical and Laboratory Standards Institute, 2008).
51. Magiorakos, A. P. et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 18, 268–281 (2012).

ACKNOWLEDGEMENTS
This research was supported by JST MIRAI Program (Grant No. JPMJMI18DC), Japan Society for the Promotion of Science (JSPS) through Grant-in-Aid for Scientific Research (B) [KAKENH Grant Nos. 19H02272, 18KK0114, 26289180, and 26281037], Nippon Life Insurance Foundation, and Hiramoto-gumi inc.

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.J.-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
Supplementary information is available for this paper at https://doi.org/10.1038/s41545-020-0059-5.
Correspondence and requests for materials should be addressed to R.H.
Reprints and permission information is available at http://www.nature.com/reprints
Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit https://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020