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Original Article

Pharmaceutical concentrations and antimicrobial activity using hypersusceptible *Escherichia coli* lacking TolC component of multidrug efflux system in the Ayase River in Japan

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ABSTRACT — The pharmaceutical concentrations and antimicrobial activity in the Ayase River were investigated. The water sources of the Ayase River are agricultural wastewater and effluent from septic tanks during the irrigation season and effluent from a sewage treatment plant and septic tanks during the non-irrigation season. Acetaminophen, bezafibrate, carbamazepine, clarithromycin, and diphenhydramine levels were in the range of 1.9–495 ng/L. During the irrigation season, pharmaceutical concentrations were higher downstream than upstream due to the inflow of agricultural wastewater in upstream areas. However, during the non-irrigation season, since most of the water sources were effluent from a sewage treatment plant and septic tanks, pharmaceutical concentrations were extremely high from upstream to downstream. The pharmaceutical concentrations during the non-irrigation season were at the same levels as those downstream of the Arakawa River, which was polluted by the effluent from Japan's largest sewage treatment plant. This extremely high pharmaceutical concentration in the non-irrigated season was found only in the Ayase River but not in the nearby Nakagawa and Furutone rivers, which are both urban water sources. The maximum concentration of clarithromycin in the Ayase River was higher than the predicted no-effect concentration for green algae, indicating that the water from the Ayase River may affect aquatic organisms. Antimicrobial activity analysis in the Ayase River using hypersusceptible *Escherichia coli* lacking the TolC component of the multidrug efflux system revealed that the river water had antimicrobial activity, suggesting potential risks, such as bactericidal action and development of antibiotic resistance in river water.

Key words: Ayase River, Pharmaceutical, Antimicrobial activity, Hypersusceptible *Escherichia coli*, Multidrug efflux system, TolC

INTRODUCTION

The Ayase River flows through Saitama Prefecture and the Tokyo Metropolitan area. The name of Ayase River is derived from "Ayashi-no river". "Ayashi" is an old word meaning "strange". The flow of the Ayase River through the Saitama lowlands changes every time it rained heavily (Hirato and Murakami, 2014). Until the 15th century,

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the Ayase River was a distributary of the Arakawa River; however, in the 16th century, the Ayase River was separated from the Arakawa River for flood control. (Matsuura, 2018). Therefore, the water source of the Ayase River changed from rain in the mountains to wastewater in urban areas.

Agricultural canals were then constructed from the Arakawa and Tone rivers to the Saitama lowlands (Minegishi *et al*., 2000). Agricultural water is distributed to each rice field like arteries gradually diverge and become capillaries. On the other hand, agricultural and domestic wastewater from narrow drainage waterways finally flows into the Ayase and Nakagawa rivers like capillaries merge into veins.

Ayase River is a relatively small first-class river of Japan (i.e., the river maintained by the government in the River Act), with a channel length of 48 km and a basin area of 176 km^2. However, the basin population of Ayase River is over 1.15 million (Edogawa River Office, 2022a). Thus, the population density of the basin is extremely high compared with that of other first-class rivers, and the water quality is deteriorating. According to a water pollution survey (Ministry of Land, Infrastructure, Transport and Tourism, 2022), biochemical oxygen demand (BOD) of the Ayase River has been the worst for 15 consecutive years since 1980, and the Ayase River was said to be the dirtiest river among the first-class rivers in Japan (Edogawa River Office, 2022b).

Several studies have indicated that Ayase River water is contaminated with dioxins from herbicides used in rice fields and from incinerators (Minomo *et al*., 2011, 2018), total organic halogens from factories (Inaba *et al*., 1995, 1998), and surfactants from domestic and industrial wastewater (Yamazaki *et al*., 2000, 2001). In recent years, analytical technology has become more sophisticated, and nanogram-level pharmaceuticals in aquatic environments can be detected using high-performance liquid chromatography (HPLC)/tandem mass spectrometry (MS/MS) (Daughton and Ternes, 1999). Because pharmaceuticals have biological activity, there are concerns about their adverse effects on humans and wildlife (Oetken *et al*., 2005; Berninger *et al*., 2011).

The Ayase River has been cleaned by many measures, such as public sewage system construction, sludge removal, and transmission of relatively clean water from the Arakawa River (Ministry of Land, Infrastructure, Transport and Tourism, 2011). Citizen activities such as garbage cleaning and water pollution surveys have also been conducted. Therefore, clarifying and disclosing the current state of river pollution is an important environmental conservation activity. This study aimed to assess the current situation of pharmaceutical pollution in the Ayase River by analyzing pharmaceutical concentrations in river water and its impact on the ecosystem through a bioassay of antimicrobial activity using hypersusceptible *Escherichia coli* lacking the TolC component of multidrug efflux system.

MATERIALS AND METHODS

Reagent

Acetaminophen, bezafibrate, carbamazepine, diphenhydramine hydrochloride (hereafter, diphenhydramine), and sulpiride were purchased from FUJIFILM Wako Pure Chemical Corporation (Tokyo, Japan), and clarithromycin was from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other reagents were from FUJIFILM.

Sampling of river water

Figure 1 shows the sampling sites. Water samples from the Ayase River were collected at Hommuraseki Bridge (Site A, upstream site; 35°59'49"N, 139°38'19"E), Nawate Bridge (Site B, midstream site; 35°53'14"N, 139°44'29"E), and Shin-kahei Bridge (Site C, downstream site; 35°46'40"N, 139°49'28"E), on March 24, April 18, June 1, July 13, August 24, and October 26, 2017. Water samples from the Arakawa River were collected at Onari Bridge (Site D, midstream site; 36°02'59"N, 139°29'56"E), Jisui Bridge (Site E, midstream site; 35°53'32"N, 139°33'45"E), and Toda Bridge (Site F, downstream site; 35°47'60"N, 139°41'04"E) on July 21 and October 4, 2017, and January 16, 2018. Water samples from Nakagawa River and Ootoshi-Furutone River (hereafter, Furutone River) were collected at Nakagawa-Jindo Bridge (Site G, 35°58'53"N, 139°46'54"E) and Ushijima-Jindo Bridge (Site H, 35°97'98"N, 139°77'31"E), respectively, on January 29, February 23, March 15, April 19, May 19, June 23, July 21, August 22, September 22, October 24, November 27, and December 25, 2018. More than 1 L of surface water was collected at the center of the bridge using a HEYROHT water sampler (SIBATA Scientific Technology Ltd., Saitama, Japan).

River water pretreatment

River water was filtered through a filter paper (No. 6; Toyo Roshi Kaisha, Ltd., Tokyo, Japan), and 500 mL of filtrate was passed through a solid-phase extraction cartridge (Oasis HLB Plus, Waters Corporation, Milford, MA, USA) pre-conditioned with 10 mL of CH_3OH and 10 mL of H_2O at a flow rate of 10 mL/min. After drying the cartridge under air flow, the analytes were eluted with 10 mL of CH_3OH, and the solvent of the eluate was evap-

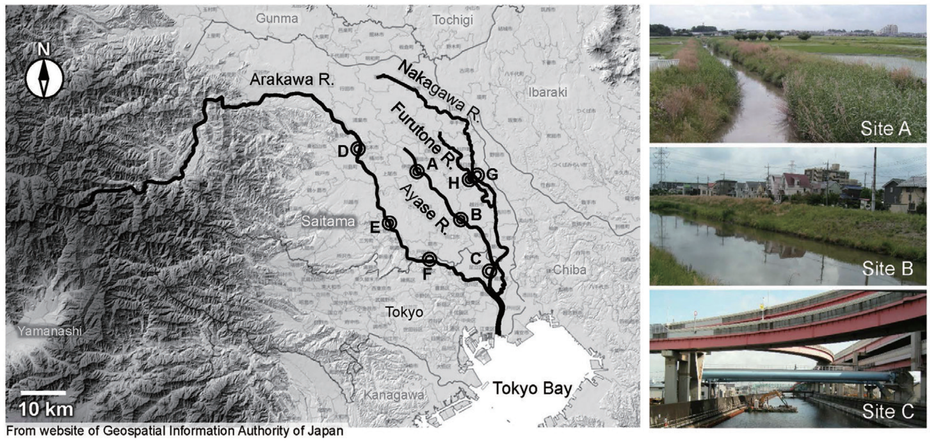

Fig. 1. Sampling locations.

orated to dryness. The residue was dissolved in 1 mL of CH_3OH and analyzed using HPLC/MS/MS. Six samples in these were analyzed using the bioassay for antimicrobial activity.

Determination of pharmaceutical concentrations using HPLC/MS/MS

An HPLC (1100 Series; Agilent Technologies, Inc., Santa Clara, CA, USA) connected to a triple quadrupole mass spectrometer (API-4000; AB Sciex LLC, Framingham, MA, USA) was used to determine the concentrations of acetaminophen, bezafibrate, carbamazepine, diphenhydramine, and sulpiride in river water. The samples were introduced into an Inertsil ODS-3 analytical column (2 mm i.d., 150 mm length, 5 µm particle size; GL Sciences Inc., Tokyo, Japan), and the analytes were eluted using a binary linear gradient of 0.5% CH_3COOH aqueous solution and CH_3CN. The percentage of CH_3CN was 20% for 0–10 min, ramped up at 8%/min for 10–20 min, and held at 100% for 20–30 min. The analytes of interest were ionized using an electrospray ionization interface and detected using the multiple reaction monitoring mode. The detected *m/z* of precursor ion and product ion were 152.1/110.1 for acetaminophen, 359.6/273.9 for bezafibrate, 237.1/194.1 for carbamazepine, 748.0/158.2 for clarithromycin, 255.7/166.9 for diphenhydramine, and 341.5/112.2 for sulpiride.

Disc diffusion assay

The antimicrobial activity of each sample from the river water (June 1, July 13, and August 24, 2017 for the Ayase River; July 21 and October 4, 2017 and January 16, 2018 for the Arakawa River) was determined by the disc diffusion assay according to the Clinical and Laboratory Standards Institute guidelines (Wayne, 2015). Briefly, the *Escherichia coli* K-12 strain NKE128 (*tolC*–deleted mutant of *E. coli* MG1655) (Nishino *et al*., 2008) was used. A total of 200 µL of 10^3 dilutions of overnight cultures were spread onto tryptic soy agar plates using a sterile spreader. The samples were absorbed using a paper disc (diameter: 6 mm, Toyo Roshi Kaisha). The discs were then placed on top of the inoculated plate and incubated overnight at 37°C. The zone of inhibition (mm) formed around each disc was measured to compare the antimicrobial activity of the samples.

RESULTS AND DISCUSSION

Pharmaceutical concentrations in Ayase River water

To assess the level of pharmaceutical pollution in the Ayase River, we collected water from three sampling sites in the Ayase River from March 24 to October 26, 2017, and analyzed the concentrations of six pharmaceuticals using HPLC/MS/MS. The six pharmaceuticals—acetaminophen, diphenhydramine, clarithromycin, carbamazepine, sulpiride, and bezafibrate—were selected based on previous reports highlighting their high detection frequency and concentration in aquatic environments (Azuma *et al*., 2016; Okawa *et al*., 2016; Seino *et al*., 2004a; Suzuki *et al*., 2010, 2021). All analytical results are listed in Table 1. The maximum, 75th percentile, median, 25th percentile, and minimum values for each pharmaceutical are shown in Fig. 2. Acetaminophen (median, 78 ng/L) was the most abundant, followed by sulpiride (55 ng/L), clarithromycin (53 ng/L), bezafibrate (28 ng/L), carbamazepine (15 ng/L), and diphenhydramine (7 ng/L). The

Table 1. Pharmaceutical concentrations in water from the Ayase River.

Date	Acetami-nophen	Sulpiride	Clarithro-mycin	Bezafibrate	Carbama-zepine	Diphen-hydramie	Sum of six pharmaceuticals
Site A (upstream)							
March 24, 2017	253	215	149	99	60	27	802
April 18, 2017	495	105	58	29	23	18	729
June 1, 2017	39	25	30	7	7	10	118
July 13, 2017	9	27	8	5	7	4	60
August 24, 2017	25	17	7	6	6	3	65
October 26, 2017	16	8	7	3	2	4	40
Site B (midstream)							
March 24, 2017	344	123	87	108	38	4	704
April 18, 2017	434	94	103	70	23	7	731
June 1, 2017	44	40	24	11	12	4	134
July 13, 2017	43	34	16	13	7	6	119
August 24, 2017	109	28	11	13	12	5	178
October 26, 2017	23	6	6	4	3	2	43
Site C (downstream)							
March 24, 2017	474	114	289	210	42	7	1136
April 18, 2017	209	125	221	87	28	97	766
June 1, 2017	110	70	49	42	18	9	297
July 13, 2017	33	110	79	35	31	52	340
August 24, 2017	87	79	56	27	18	32	299
October 26, 2017	70	19	64	33	9	17	212

Unit, ng/L.

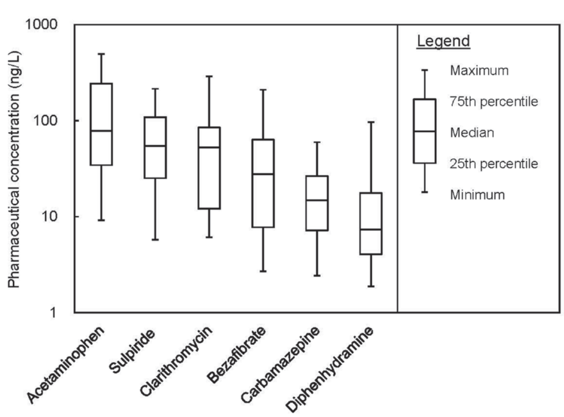

Fig. 2. Pharmaceutical concentrations in water from the Ayase River.

75th and 25th percentile values showed the same tendency as the medians. According to the National Database of Health Insurance Claims and Specific Health Checkups of Japan (NDB) Open Data (Ministry of Health, Labor and Welfare, 2018), the prescribed amount of pharmaceuticals in Japan during the fiscal year 2017 was 299,650 kg for acetaminophen, 13,362 kg for sulpiride, 75,624 kg for clarithromycin, 71,970 kg for bezafibrate, 40,884 kg for carbamazepine, and 205 kg for diphenhydramine. A strong correlation ($r = 0.74$) was observed between the prescribed amount of each pharmaceutical and its concentration in the river water, demonstrating that the pharmaceutical concentration in river water was strongly affected by the prescribed amounts. A correlation between pharmaceutical concentration and shipping or sales volume of pharmaceuticals has also been reported (Oosterhuis *et al*., 2012; Azuma *et al*., 2015). In contrast, the removal efficiency of pharmaceuticals by sewage treatment plants has been reported to vary greatly depending on the pharmaceutical (Suzuki *et al*., 2010). This might be one of the other factors affecting the pharmaceutical concentrations in the river water.

Table 2 lists correlation coefficients among pharmaceutical concentrations. The correlation coefficients among concentrations of acetaminophen, clarithromycin, carbamazepine, and bezafibrate were higher ($r = 0.64$–0.97) than that between concentrations of diphenhydramine and the other pharmaceuticals ($r = 0.06$–0.48). Since diphenhydramine is mainly used as an anti-itch ointment, its emission and degradability in the water environment may differ from those of other pharmaceuticals. On the other hand, the minimum correlation coefficients between the sum of the six pharmaceutical concentrations and each

Table 2. Correlation coefficients between individual pharmaceutical concentrations and the sum of six pharmaceutical concentrations.

	Acetaminophen	Sulpiride	Clarithromycin	Bezafibrate	Carbamazepine	Diphenhydramie
Sulpiride	0.64					
Clarithromycin	0.66	0.71				
Bezafibrate	0.73	0.69	0.92			
Carbamazepine	0.65	0.97	0.75	0.79		
Diphenhydramine	0.06	0.48	0.51	0.20	0.35	
Sum of six pharmaceuticals	0.91	0.84	0.89	0.90	0.85	0.34

pharmaceutical concentration ($r = 0.34$) were higher than that among each pharmaceutical ($r = 0.06$). Therefore, the sum of the six pharmaceutical concentrations was used for evaluation in the following discussion.

Seasonal variation

Since the seasonal variations in the sum of the pharmaceutical concentrations at the sampling sites of the upstream, midstream, and downstream regions showed a similar tendency (Table 1), we have used the concentrations in the midstream for discussion. Figure 3A illustrates the seasonal variation in the sum of the six pharmaceutical concentrations in Ayase River water. Pharmaceutical concentrations were high from March to April and low from May to October. Since high temperatures and strong solar irradiation were observed from May to September (Japan Meteorological Agency, 2017), it was considered that the pharmaceutical concentrations decreased due to microbial and solar decomposition in the summer season. However, previous research has shown that carbamazepine in sewage is hardly decomposed by bacteria (Nakada *et al*., 2007; Azuma *et al*., 2018) and that acetaminophen is hardly decomposed by sunlight (Kawabata *et al*., 2013), indicating that there are other reasons behind the seasonal variation in pharmaceutical concentrations. However, non-ionic surfactants and nonylphenols in Ayase River water during the non-irrigation season were reported to be higher than those during the irrigation season, and it was speculated that the reason for this was the decrease in agricultural wastewater discharge during the non-irrigated season (Yamazaki *et al*., 2000, 2001).

According to the database of the Water Information System (Ministry of Land, Infrastructure, Transport and Tourism, 2021), the discharge rates on March 24 and April 18, 2017 during the non-irrigation season were 1.8 and 2.0 m^3/sec, respectively. In contrast, those on June 1, July 13, August 24, and October 26, 2017 during the irrigation season were 4.1, 3.9, 4.8, and 10.5 m^3/sec, respectively (Fig. 3B). A strong correlation ($r = 0.96$) was observed between the pharmaceutical concentrations and the inverse of the river discharge rate at Site B, indicating that one of the factors contributing to this seasonal variation was the decrease in discharge during the non-irrigation season and the inflow of agricultural wastewater during the irrigation season. Furthermore, although the discharge from October 22 to 24 showed an annual maximum value of 20.0 to 40.3 m^3/sec, pharmaceutical concentrations on October 26 were extremely low. According to observations at the Koshigaya meteorological station, which is located 2 km east of Site B (Japan Meteorological Agency, 2017), there was 174 mm of rainfall from October 21 to 23, 2017, which was 14% of the annual rainfall (Fig. 3C). From the above results, it was clarified that the pharmaceutical concentrations in the Ayase River water were greatly affected by the amount of agricultural wastewater during the irrigation season, and their concentrations decreased because of dilution by heavy rain.

The relationship between the pharmaceutical source and load was also assessed. The coverage rate of public sewage systems in Saitama Prefecture was 80.8% in 2017 (Saitama Prefecture, 2022). Domestic wastewater from the Ayase River basin is discharged to the Nakagawa River after treatment by the public sewage system or discharged to the Ayase River after treatment using septic tanks. Moreover, treated water from the Moto-Arakawa River Water Recycling Center (sewage treatment plant) discharges to the most upstream regions of the Ayase River only during the non-irrigation season as a measure against the shortage of river water. The pharmaceutical load (μg/sec), calculated as the product of pharmaceutical concentrations in the river (ng/L) and discharge rate (m^3/sec), was high on March 24 (1,261 μg/sec) and April 18 (1,461 μg/sec) and low on June 23 (546 μg/sec), July 21 (462 μg/sec), and October 24 (451 μg/sec), 2017. The pharmaceutical sources during the non-irrigation season were both the sewage treatment plant and septic tanks, whereas septic tanks were the only source during the irrigation season. Therefore, the contribution of the sewage treatment plant to the pharmaceutical load during the non-irrigation season was estimated by comparing the

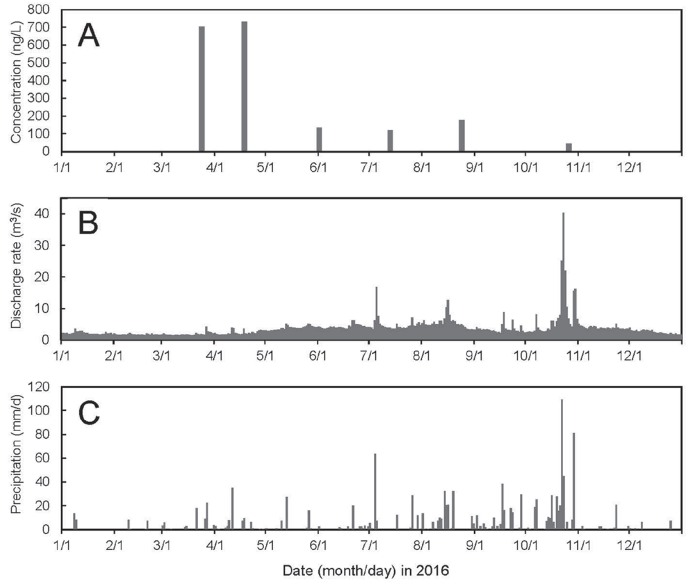

Fig. 3. Variations in total pharmaceutical concentration (A), discharge rate (B) and precipitation (C) at Site B of the Ayase River. Data of discharge rate and precipitation were from Ministry of Land, Infrastructure, Transport and Tourism, 2021 and Japan Meteorological Agency, 2017, respectively.

load during the non-irrigation season with that during the irrigation season. The average load during the non-irrigation season was 1,362 µg/sec, and that during the irrigation season was 546 µg/sec. Assuming that the consumption of pharmaceuticals was constant throughout the year, the load from the sewage treatment plant in the non-irrigation season was calculated as the difference between the loads during the non-irrigation season and that during the irrigation season (816 µg/sec). This corresponded to 60% of the load during the non-irrigation season. This result indicated that the discharge of treated water from the sewage treatment plant into the Ayase River greatly contributed to the increase in pharmaceutical concentrations during the non-irrigation season.

Upstream to downstream variation

Rice fields spread around the upstream regions of the Ayase River, including Site A, and agricultural wastewater and effluent from septic tanks are the main water sources during the irrigation season. In the middle reaches of the Ayase River, the proportion of rice fields decreases, and the proportion of residential areas increases. Around Urawa-Misono, near Site B, residential area development can be seen with the expansion of the metropolitan area. Rice fields were not observed more than 4 km downstream from Site B. In the lower reaches, the Furu-Ayase, Den'u, and Kenaga rivers, which are polluted with factory and domestic wastewater, join the Ayase River, further polluting it. Site C is located approximately 3 km downstream from the confluence, and the polluted water remains there because of the tidal action in the zone.

Figure 4 (left) shows the variation in the sum of the six pharmaceutical concentrations in the Ayase River from upstream to downstream. During the irrigation season, the pharmaceutical concentrations generally increased as the Ayase River flowed down. A possible reason is that the pharmaceuticals from the upstream septic tank effluents were diluted by the large amount of wastewater from the rice fields, and as they flowed down, the proportion of effluent from the septic tank increased, resulting in higher pharmaceutical concentrations. On the other hand, during the non-irrigation season, no significant difference in pharmaceutical concentrations between upstream, midstream, and downstream regions was observed. This is because the amount of agricultural wastewater during this period is very small, and most of the river water in the Ayase River originates from the sewage treatment plant and septic tanks from upstream to downstream.

Comparison with the Arakawa River

The Arakawa River has a channel length of 173 km and a basin population of 9.3 million, which is the third largest population in Japanese rivers. Its water source is rainwater from Mt. Kobushigatake, at an altitude of 2,475 m.

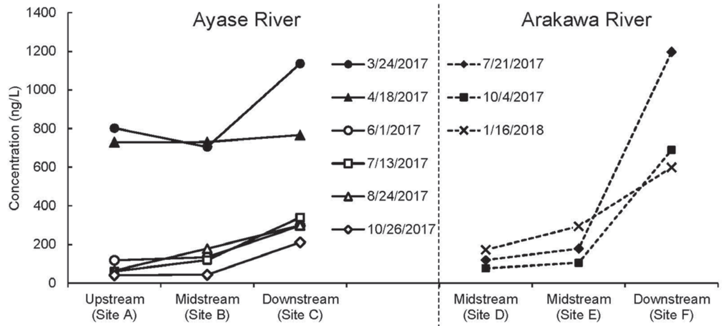

Fig. 4. Variations in pharmaceutical concentrations at the upstream, midstream, and downstream regions of the Ayase (left) and Arakawa (right) rivers.

The upstream flows through mountainous areas, such as Chichibu City, and the midstream flows south between large cities in the central part of Saitama Prefecture. The downstream is added to the discharged water from Japan's largest Arakawa Water Recycling Center (sewage treatment plant) and crosses the urban area of downtown Tokyo.

Figure 4 (right) shows the pharmaceutical concentrations in river water collected from the midstream (Sites D and E) and downstream (Site F) of the Arakawa River. The pharmaceutical concentration increased as it flowed down; in particular, an extra-high concentration was observed downstream at Site F. This was attributed to the wastewater inflow from Japan's largest municipal sewage treatment plant. The pharmaceutical concentration in the Ayase River during the irrigation season was at the same level as that in the middle reaches of the Arakawa River. In contrast, the concentration during the non-irrigation season was comparable to the high concentration in the lower reaches of the Arakawa River.

Comparison with neighboring Nakagawa and Furutone rivers

To clarify whether the high pharmaceutical concentration from upstream to downstream during the non-irrigation season is peculiar to the Ayase River or common to rivers with urban water sources, river water was collected from the midstream of the Nakagawa and Furutone rivers near the Ayase River, and the pharmaceuticals were analyzed. The results are shown in Fig. 5 (A). Both rivers had higher pharmaceutical concentrations during the non-irrigation season and lower concentrations during the irrigation season. As shown in Fig. 5 (B), the pharmaceutical concentrations of the Nakagawa and Furutone rivers were inversely proportional to the river discharge rate, as in the case of the Ayase River.

However, the maximum pharmaceutical concentrations in the Nakagawa and Furutone rivers were 103 and 100 ng/L, respectively. These concentrations were approximately 1/8 of that in the Ayase River (704–802 ng/L). This is because agricultural canals are connected upstream of the Nakagawa and Furutone rivers, and pharmaceuticals in these rivers are diluted by canal water. This result clarified that the high pharmaceutical concentrations from the upstream to the midstream of the Ayase River during the non-irrigation season is peculiar to the Ayase River.

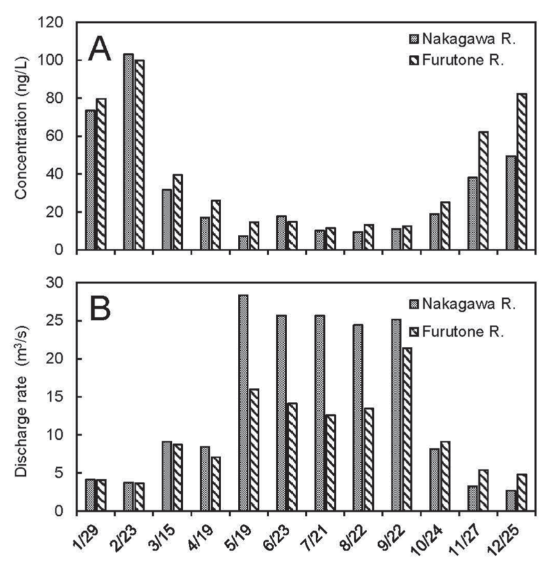

Fig. 5. Pharmaceutical concentration in water (A) and discharge rate (B) in the Nakagawa and Furutone rivers. Data of discharge rate were from Ministry of Land, Infrastructure, Transport and Tourism, 2021.

Effect on human health and ecosystem

In this study, it was clarified that the pharmaceutical concentrations in Ayase River water is high over the entire range from upstream to downstream during the non-irrigation season. Therefore, there are concerns regarding its impact on humans and ecosystems. As for human health, there is no need to worry because the water from Ayase River is not used as raw water for waterworks. Even if a human drinks one litter of river water, the intake of each pharmaceutical is at the sub-microgram level at most, which is less than 1/10,000 of the usual daily dose of tens to hundreds of milligrams. Therefore, adverse effects are unlikely to occur.

The effects of pharmaceuticals on wildlife have been evaluated by comparing the measured environmental concentrations (MEC) with predicted no-effect concentrations (PNEC) (Komori and Suzuki, 2009; Nishino *et al*., 2020). The Ministry of the Environment has set the PNEC for clarithromycin at 0.069 µg/L (Ministry of the Environment, 2002). This value is the EC_{50} value (6.9 µg/L) obtained from the growth inhibitory effect of green algae (*Pseudokirchneriella subcapitata*) divided by an assessment factor of 100. The maximum concentration of clarithromycin in Ayase River water analyzed in this study was 0.289 µg/L. Since this concentration exceeded the PNEC, Ayase River water has a potential risk of adverse effects on wildlife. It has been reported that pharmaceutical concentration in river water exceeded PNEC not only at Tamagawa and Sumida rivers in Tokyo, but also at urban rivers in Fukuoka City, Osaka City, Hyogo Prefecture, and Nagoya City (Nishino *et al*., 2020). These suggest that pharmaceutical concentration in urban river water generally exceed the PNEC, and it is important to monitor pharmaceutical concentrations in whole of country.

Antibacterial activity

Drug-resistant bacteria have been detected in rivers in Japan, raising concerns regarding their spread to humans (Seino *et al*., 2004b; Nishiyama *et al*., 2015). In this study, clarithromycin was detected in Ayase River water at concentrations that may affect the ecosystem. Since wild-type *Escherichia coli* excretes penetrated antibiotics via drug efflux system, antibacterial activity in river water can be detected with high sensitivity by using *Escherichia coli* lacking TolC component of multidrug efflux system. The antibacterial activity of extracts from the Ayase and Arakawa river waters were assayed using hypersusceptible *Escherichia coli* lacking the TolC component of the multidrug efflux system. The results are shown in Fig. 6, and the diameters of the inhibition zones exposed to the extract from the river water are listed in Table 3. Antibacterial activity was detected and found to be highly correlated (r = 0.95) with the concentration of clarithromycin in the river sample. Antibiotics such as clarithromycin and synthetic antibacterial drugs are considered to contribute to antibacterial activity. Trace levels of pharmaceuticals present in river water indicate potential risks,

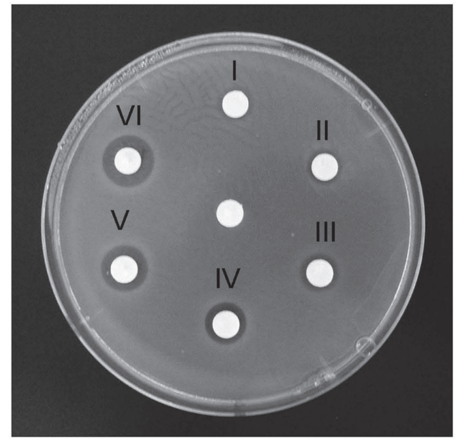

Fig. 6. Antibacterial activity of extracts from river water against *tolC*-deleted mutant of *E. coli* MG1655, as observed using the disc diffusion assay. Samples were extracted from Ayase River water in June 1 (I), July 13 (II), and August 24 (III), and Arakawa River in July 21 (IV), October 4 (V), and January 16 (VI). Methanol was used as the control (center).

Table 3. Diameter of inhibition zone exposed by the extract from river water.

River	Sampling date	Diameter of inhibition zone (mm)	Concentration of clarithromycin in river water (ng/L)
Ayase	June 1, 2017	6.5	34
Ayase	July 13, 2017	7.0	34
Ayase	August 24, 2017	8.0	25
Arakawa	July 21, 2017	9.0	91
Arakawa	October 4, 2017	11.0	109
Arakawa	January 16, 2018	12.0	132

such as bactericidal action and development of antibiotic resistance in river water. In addition, this assay system was useful for the comprehensive evaluation of antimicrobial agents present in aquatic environments.

It has been reported that Ayase River water has cytotoxicity and genotoxicity (Fujiwara *et al*., 2001; Nakajima *et al*., 2007). Our study revealed that it has the antibacterial activity. These results suggest that Ayase River water has a potential risk of adverse effects on ecosystem. We propose three measures to deal with this issue. The first is to introduce water from the Arakawa or Tone rivers, instead of treated water from the sewage treatment plant, to the upstream regions of the Ayase River during the non-irrigation season. The second is to increase the penetration rate of public sewage systems and reduce the number of septic tanks. The third is the introduction of ozone and powder-activated carbon treatment in sewage treatment. This treatment has been reported to remove carbamazepine more efficiently (Azuma *et al*., 2019; Westerhoff *et al*., 2005) than other methods, including chlorine and activated sludge treatment (Stackelberg *et al*., 2007; Kubota *et al*., 2008; Kim *et al*., 2010). Monitoring the changes in pollution levels is an important and essential activity under environmental conservation. The results of this study are valuable for assessing the impact of the river conservation activities and for developing new strategies for further conservation. We will continue to investigate the existence of pharmaceuticals in the water environment of the Tokyo metropolitan area and participate in environmental conservation activities through research activities.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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