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*Original Article*

# Evaluation of pharmaceutical concentrations and antimicrobial activity in river water from the Tone River system in Gunma Prefecture, Japan

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**ABSTRACT** — We investigated the characteristics of pharmaceutical concentrations and antimicrobial activities in river water from the Tone River system in Gunma Prefecture. The mean concentrations of diphenhydramine, clarithromycin, carbamazepine, and bezafibrate in the midstream of the Tone River were 8.6, 29, 3.8, and 8.1 ng/L, respectively. Their concentrations were nearly half of those in the midstream of the Ayase River, the main water source of which is wastewater. Seasonal variations in pharmaceutical concentrations were high in winter and low in late spring and autumn. This variation depended on the flow rate of the river water, which in turn depended on the rainfall in the upstream area. Except for bezafibrate, the pharmaceutical concentrations in river water did not change after 5 days of incubation at 30°C, indicating that biochemical degradation during the hot summer season was minimal. A comparison of the concentrations between the sampling locations revealed that the pharmaceutical load was proportional to basin population, and the annual fluxes of pharmaceuticals from Gunma Prefecture were estimated to be 98, 210, 28, and 53 kg/year, respectively. Disc diffusion assay of some samples of Tone River water extracts revealed inhibition zones owing to their antimicrobial activity. However, no relationship was observed between the diameter of the inhibition zone and clarithromycin concentration in the river water. These results suggest that the antimicrobial activities of the river samples were not dependent on clarithromycin. We are currently investigating the pollution and drug-resistant bacteria present in the Tone River in detail.

**Key words:** Tone River, Gunma Prefecture, Pharmaceutical, Antimicrobial activity, Hypersusceptible *Escherichia coli*, Multidrug efflux system

## INTRODUCTION

Gunma Prefecture, located in the northern part of the Kanto Region which includes the Tokyo metropolitan area, is the source of the Tone River. The Tone River has the second-longest channel length (322 km) and largest catchment area (16,840 km$^2$) in Japan. The river basin population is 13 million, accounting for approximately one-tenth of Japan's population (Ministry of Land, Infrastructure, Transport and Tourism, 2023a). Gunma Prefecture is rich in natural water from the Tone River, and 99% of its land is covered by the Tone River

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system (Gunma Prefectural Government, 2024a). However, the penetration rate of the public sewage system in Gunma Prefecture is the lowest (54.6%) among the Kanto regions (Japan Sewage Work Association, 2020), and water quality accidents occur frequently (Gunma Prefectural Government, 2024b). The Tone River water system supplies 3,650 km$^3$ of tap water to 27.5 million people in Tokyo and five prefectures (Ministry of Land, Infrastructure, Transport and Tourism, 2006), and there is a need to improve its water quality.

Recently, pharmaceuticals originating from human waste have been detected in river water (Daughton and Ternes, 1999). Consequently, there is growing concern regarding the effects of residual pharmaceuticals in river water on humans (Bruce *et al*., 2010) and wildlife (Oetken *et al*., 2005; Berninger *et al*., 2011). Moreover, bacteria may develop drug resistance owing to the presence of antibacterial substances in river water. Although the occurrence and fate of pharmaceuticals in the Tone River have been reported (Nakada *et al*., 2007), information on the relationship between pharmaceutical concentration and the antimicrobial activity of the Tone River water in Gunma Prefecture is lacking.

In this study, we investigated the pharmaceutical concentrations and antimicrobial activities of river water collected from six locations in the Tone River system flowing through Gunma Prefecture. The four pharmaceuticals—diphenhydramine, clarithromycin, carbamazepine, 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*., 2004; Suzuki *et al*., 2010, 2021). Subsequently, we analyzed the factors influencing seasonal and site variations in pharmaceutical concentrations. The biochemical degradation of pharmaceuticals was also investigated in incubation experiments, and the antimicrobial activity of river water was assayed using a disc diffusion assay to clarify the possibility of drug resistance development.

## MATERIALS AND METHODS

### Chemicals

Bezafibrate, carbamazepine, and diphenhydramine were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), and clarithromycin was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Additionally, acetonitrile, acetic acid, ammonium acetate, and pure water were used as mobile phases for high-performance liquid chromatography-tandem mass spectrometry (LC/MS/MS) and were obtained from FUJIFILM.

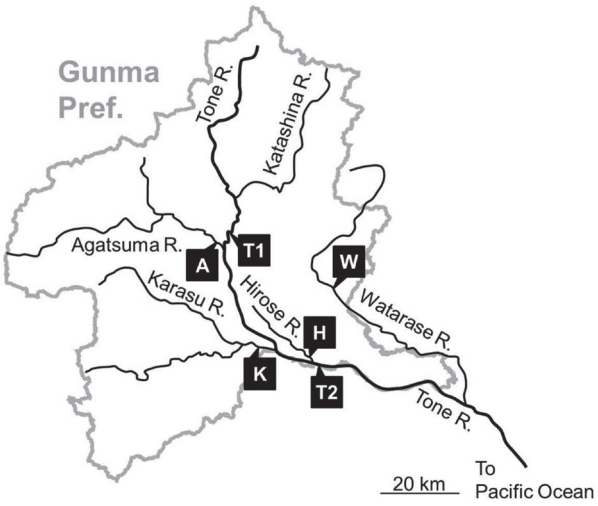


**Fig. 1.** Sampling locations in Gunma Prefecture.

### Sampling of river water

Figure 1 shows the sampling locations in Gunma Prefecture. To investigate the seasonal variation in the pharmaceutical concentrations, river water was collected at the midstream of the Tone River (Jobu-Ohashi Bridge, 36°24'91"N, 139°27'21"E, Site T2) and midstream of the Watarase River (Kin'o Bridge, 36°40'00"N, 139°32'92"E, Site W), monthly (January 28, February 25, March 13, April 17, May 13, June 22, July 13, August 22, September 8, October 20, November 25, and December 16, 2019). Moreover, to investigate differences among the pharmaceutical concentrations at the sites of Tone River and its main tributaries, river water was collected at four additional sites on March 26–27 and September 8, 2019. The sites were upstream of the Tone River (Miyata Bridge, 36°52'61"N, 139°02'08"E, Site T1), downstream of the Agatsuma River (Agatsuma-Shimbashi Bridge, 36°50'16"N, 139°01'24"E, Site A), downstream of the Karasu River (Iwakura Bridge 36°28'23"N, 139°11'77"E, Site K) and downstream of the Hirose River (Nakajima Bridge, 36°26'20"N, 139°25'20"E, Site H).

### Determination of pharmaceutical ingredients

The four pharmaceuticals in river water were extracted using a Waters Oasis HLB Plus cartridge (Milford, MA, USA). The water samples (filtered, 250 mL) were passed through a cartridge, and the analytes were eluted from the cartridge using 10 mL of methanol. The methanol in the

extract solution was evaporated to dryness, and the residue was dissolved in 1 mL of ethanol. This solution was then subjected to LC/MS/MS.

SCIEX QTRAP 6500 (Framingham, MA, USA) was used to identify the four pharmaceuticals. The mobile phase comprised a mixture of acetonitrile and water containing 0.5% acetic acid and 10 mM ammonium acetate. The percentage of acetonitrile was 20% for 0–3 min, increased to 100% for 3–13 min, and maintained at 100% for 13–20 min. The mobile phase was delivered at the rate of 0.2 mL/min through GL Sciences (Tokyo, Japan) Inertsil ODS-3 (2 mm i.d. ×150 mm, particle size 5 μm). The ion source and MS parameters were optimized for each analyte.

### Biochemical degradability of pharmaceuticals in river water

Approximately 3 L of river water was collected from Site T2 on January 27, 2020. The sampled water was dispensed into four bottles of 1 L in 500 mL aliquots. Two bottles were stored in an incubator at a temperature of 30°C, and the other two bottles were in a refrigerator at 5°C. The pharmaceuticals in the water samples before and after 5 days incubation were analyzed using the above method.

### Estimation of river water flow

The flow rate of river water at each sampling site was estimated using data from the Water Information System (Ministry of Land, Infrastructure, Transport and Tourism, 2023b) as follows: The flow rate at Site T1 was the monthly mean flow rate at the Iwamoto Observatory, which was located 10 km upstream of Site T1 because large tributaries do not join the Tone River from the Iwamoto Observatory to Site T1. The flow rate at Site A was the mean value of the flow rate at the Murakami Observatory (11 km upstream from Site A) and the difference between the flow rates at the Maebashi Observatory (14 km downstream from the junction of the Tone and Agatsuma rivers) and the Iwamoto Observatory (12 km upstream from the junction), because Site A is located almost at the center of the three observatories. The flow rate at Site K was the same as that at the Shimmachi Observatory because the Shimmachi Observatory is located within 1 km of Site K. The flow rate at Site H was 70% of the difference between the flow rate at the Futto Observatory (11 km downstream from the junction of the Tone and Hirose rivers) and that at the Yattajima Observatory (8 km upstream from the junction) because the Hirose River and two small tributaries joined these observatories. A value of 70% was determined based on the width of the Hirose River (60 m) and the two small rivers (each 14 m). The flow rate at Site T2 was the mean of that at the Yattajima Observatory (8 km upstream from Site T2) and the Futto Observatory (11 km downstream from Site T2) because Site T2 is located almost at the center of the two observatories. The flow rate at Site W was the mean of those at the Takatsudo Observatory (12 km upstream from Site W) and the Hazikabashi Observatory (12 km downstream from Site W) because Site W is located almost at the center of the two observatories.

### Estimation of basin population

The basin population at each sampling site was estimated using website data (Gunma Prefectural Government, 2020; Midori City Office, 2020) as follows. The basin population at Site T1 was the sum of the populations in Minakami Town, Numata City, Katashina Village, Kawaba Village, and Showa Village. The basin population at Site A was the sum of the populations in Nakanojo Town, Naganohara Town, Tsumagoi Village, Kusatsu Town, Takayama Village, and Higashi-Agatsuma Town. The basin population at Site K was the sum of the populations in Annaka City, Fujioka City, Tomioka City, Shimonita Town, Nanmoku Village, Kanra Town, Ueno Village, Kanna Town, and half of Takasaki City, which has two large rivers (Karasu and Tone). The basin population at Site H was the sum of the populations in Isesaki City and half of that in Maebashi City, which has two large rivers (Hirose and Tone). The basin population at Site T2 was the sum of the populations in Shibukawa City, Hanto Village, Yoshioka Town, Tamamura Town, and half of Takasaki City, as well as the basin populations at sites T1, A, K, and H. The basin population at Site W was the sum of the populations in the Omama and Azuma regions of Midori City.

### Disc diffusion assay

The antimicrobial activities of the river water extracts collected at Site T2 on February 25, April 17, June 22, August 22, October 20, and December 16, 2019, were assayed using a disc diffusion assay according to the guidelines of the Clinical and Laboratory Standards Institute (Wayne, 2015). 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 then absorbed on paper discs. The discs were placed on top of the inoculated plate and incubated overnight at 37°C. The zones of inhibition (mm) formed around each

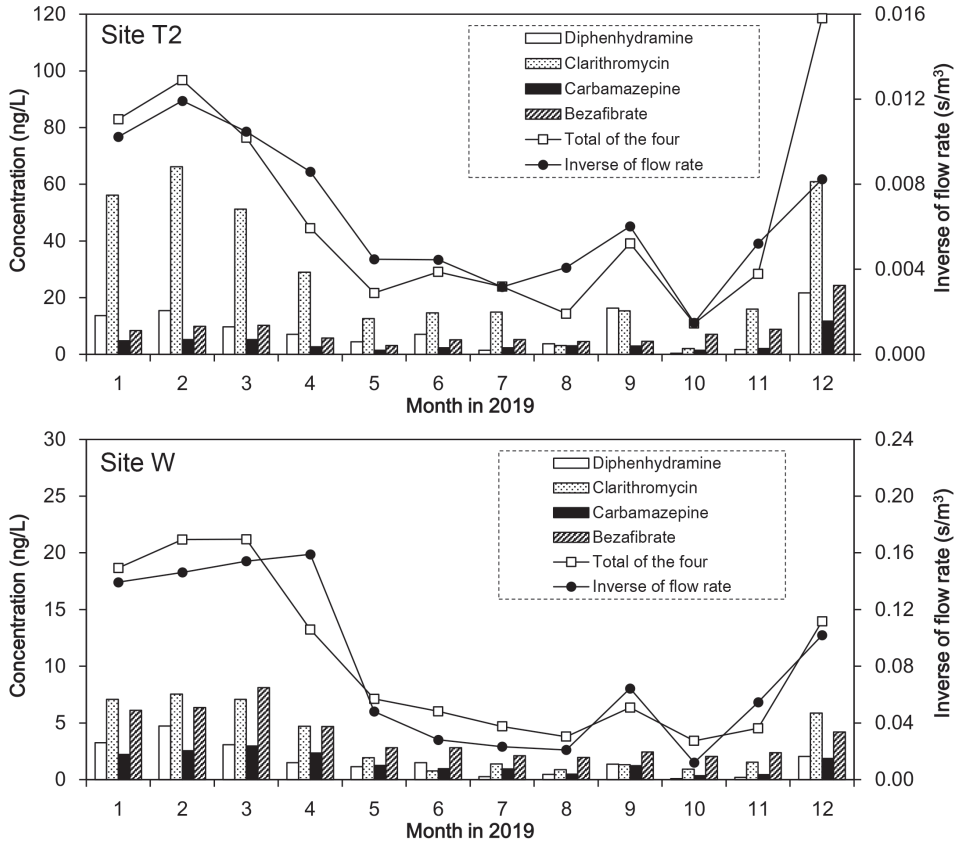


**Fig. 2.** Monthly variation in the pharmaceutical concentrations and inverse of the flow rate at Site T2 (top) and Site W (bottom).

disc were measured to compare the antimicrobial activities of the samples.

## RESULTS AND DISCUSSION

### Monthly variation in the pharmaceutical concentrations

The mainstream of the Tone River flows through the central area of Gunma Prefecture, and its largest tributary, the Watarase River, flows through the eastern area (Fig. 1). To clarify the characteristics of pharmaceutical pollution in the Tone and Watarase rivers, river water was collected every month in 2019 at Site T2 (midstream of the Tone River) and Site W (midstream of the Watarase River), and the concentrations of four pharmaceuticals were determined. Figure 2 (bar graph) shows the analytical results. The annual mean concentrations of diphenhydramine, clarithromycin, carbamazepine, and bezafibrate at Site T2 were 8.6, 29, 3.8, and 8.1 ng/L, respectively. The total concentration of the four pharmaceuticals was 49 ng/L. This was nearly half of the midstream area of the Ayase River (98 ng/L, Murahashi *et al*., 2022), which had the highest level of domestic wastewater pollution based on BOD in Japan from 1980 to 1994 (Edogawa River Office, 2022). Meanwhile, the annual concentrations at Site W were 1.6, 3.4, 1.5, and 3.8 ng/L, with a total concentration of 10 ng/L. This was approximately one order of magnitude lower than that in the midstream area of the Ayase River.

Seasonal variations in pharmaceutical concentrations were relatively high from January to March and in December and low from May to November at both sampling sites. The authors previously reported that the pharmaceutical concentrations in the Ayase River, whose main water sources are rice fields and domestic wastewater, were inversely proportional to the flow rate of the riv-

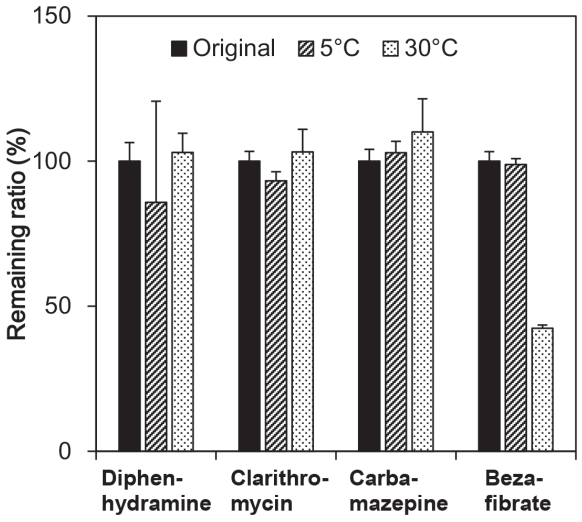


**Fig. 3.** Degradation of the pharmaceuticals in the river water after 5-d incubation at 5°C and 30°C. Concentration of the original river water was set at 100%. Boxes and bars represent the mean and SD ($n$=2), respectively.

er water, which mainly depended on the wastewater from the rice fields during the irrigation season (Murahashi *et al*., 2022). The monthly variations in the total of the four pharmaceutical concentrations and the inverse of the flow rate, estimated using data from the Water Information System website (Ministry of Land, Infrastructure, Transport and Tourism, 2023b), are also shown in Fig. 2 (line graph). At both sampling sites, variations in pharmaceutical concentrations were similar to those in the inverse of river flow, and high correlations ($r$=0.92 and 0.96 at sites T2 and W, respectively) were observed between the two. These results revealed that the pharmaceutical concentrations in the midstream waters of the Tone and Watarase rivers were inversely proportional to the water flow rate, as in the case of the Ayase River.

The main water source for the Tone and Watarase rivers is rain, which falls in mountainous areas further upstream. For the Tone River, a high correlation ($r$=0.85) was observed between the flow rate at Site T2 and the precipitation at the Numata Weather Observatory (Japan Meteorological Agency, 2020) located further upstream. In the case of the Watarase River, a high correlation ($r$=0.91) was also observed between the flow rate at Site W and the precipitation at the Kurohone Weather Observatory (Japan Meteorological Agency, 2020) located further upstream. These high correlations suggest that the flow rates of the Tone and Watarase rivers are highly dependent on precipitation.

## Biochemical degradation of the pharmaceuticals in river water

Some pharmaceuticals undergo biochemical decomposition in river waters (Azuma *et al*., 2018). Although the pharmaceutical concentrations in the Tone River showed seasonal variations depending on river flow in this study, pharmaceuticals possibly decomposed biochemically in river water during the hot summer season. Therefore, river water collected from the Tone River was incubated for 5 d at 5°C and 30°C, assuming temperatures of winter and summer, respectively. Subsequently, residual pharmaceuticals were determined.

Figure 3 shows the remaining ratio (%) of the pharmaceuticals after incubation compared to that before incubation. The remaining ratios of the three pharmaceuticals (diphenhydramine, clarithromycin, and carbamazepine) were almost 100% at both 5°C and 30°C, indicating that these three pharmaceuticals hardly decomposed within 5 d. However, bezafibrate decreased to 42% when incubated at 30°C for 5 d. The remaining ratio showed a daily reduction of 16%. The length of the river from Site T1, upstream, to Site T2, in the midstream of the Tone River, is approximately 40 km, and assuming the flow speed of the river water is 0.5 m/s, it takes only 1 d from Site T1 to Site T2. Therefore, this result suggests that the effect of pharmaceutical degradation was small, even in the summer season, and the main factor for the seasonal variation in pharmaceutical concentrations was the change in river flow rate.

## Comparison of the sampling locations for the pharmaceutical concentrations and their loads

The water source of the Tone River is in the northern area of Gunma Prefecture, and the flow rate of the river water gradually increases as it joins many tributaries. The large tributaries contain the Katashina, Agatsuma, Karasu, Hirose, and Watarase rivers, in that order, from upstream to downstream (Fig. 1). Both the Katashina and Agatsuma rivers flow from upstream to downstream through mountainous areas with low population densities. The Karasu River flows through the mountainous areas in the upstream region and Takasaki City, the most populous city in Gunma Prefecture, in the downstream region. The Hirose River is shorter than the others but flows through Maebashi City, the second most populous city. The Watarase River flows through mountainous areas in the upstream region and through mid-populous cities in the plains from midstream to downstream. In this study, to clarify the differences in pharmaceuti-

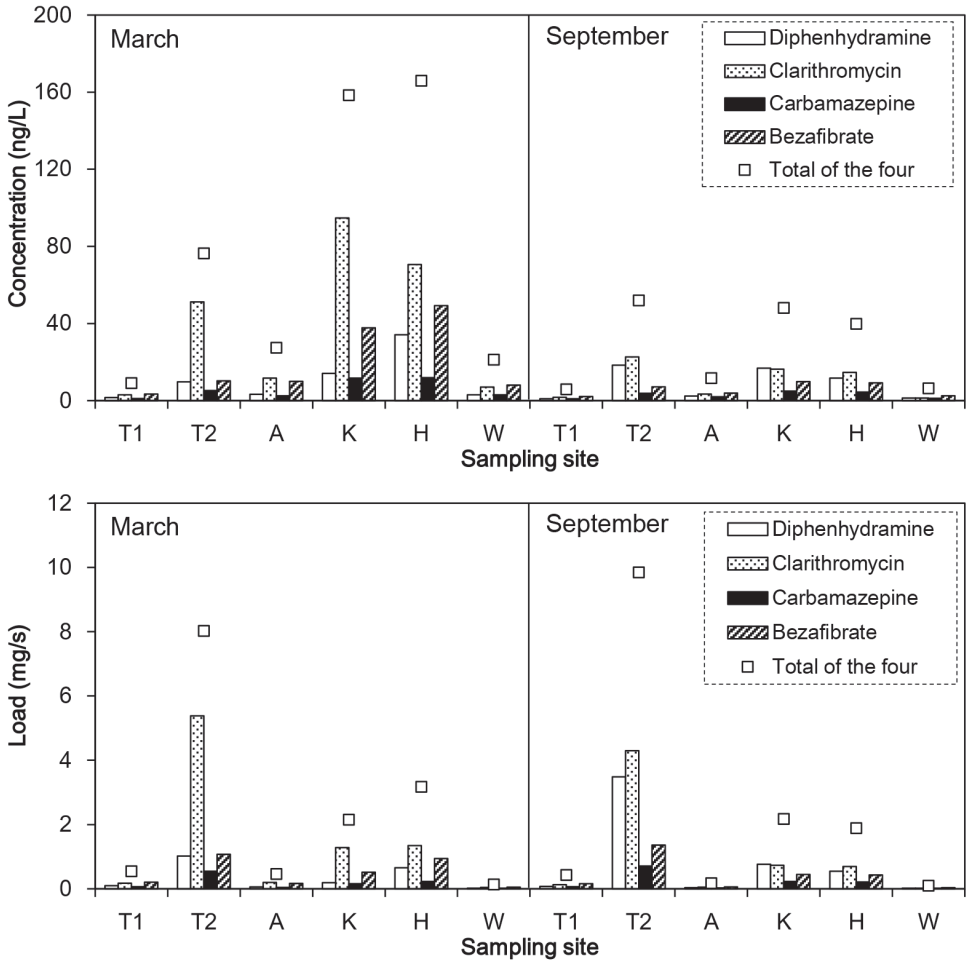


**Fig. 4.** Comparison of sampling locations for the pharmaceutical concentrations (top) and the pharmaceutical loads (bottom) in March (left) and September (right).

cal pollution between the Tone River (upstream and midstream) and its main tributaries, water was collected from six locations in March and September 2019. Subsequently, the pharmaceutical contents were determined.

Figure 4 (top) shows the four individual pharmaceutical concentrations (bar graph) and the total of the four (square symbols) at the six sampling locations. In March, the pharmaceutical concentrations at sites T2, K, and H, which flowed through large cities, were higher than those at sites T1, A, and W, which flowed through mountainous areas. In September, although pharmaceutical concentrations were lower than those in March, the high-low relationship of the pharmaceutical concentrations by sampling site was similar to that in March.

Pharmaceutical load (mg/s) was calculated using the formula $C \times F \times (10^3/10^6)$, where $C$ is the pharmaceutical concentration (ng/L), $F$ is the flow rate of river water ($m^3/s$), $10^3$ is litter in one cubic meter, $10^6$ is nanograms in one milligram. Figure 4 (bottom) shows the four individual pharmaceutical loads (bar graph) and the total of the four (square symbols) at the six sampling locations. Although the pharmaceutical concentrations differed between March and September, the pharmaceutical loads at each location were similar. Therefore, we discuss the use of mean loads on these two occasions. The mean load of the four pharmaceuticals at Site T1 upstream of the Tone River was 0.49 mg/s. As the Agatsuma, the Karasu, and the Hirose rivers joined the Tone River, their loads of 0.32, 2.2, and 1.8 mg/s were added to the load of the Tone River, successively. The total load was calculated to

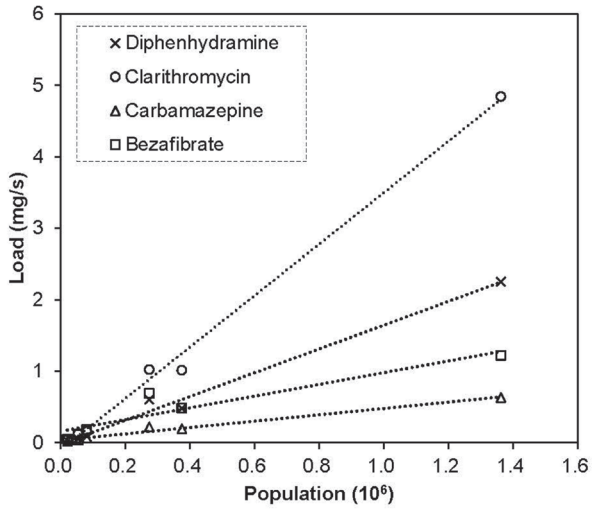


**Fig. 5.** Relationship between basin population and the pharmaceutical load.

be 4.8 mg/s. The difference (4.1 mg/s) from the load of Site T2 (8.9 mg/s) was estimated to be the load of treated water from the prefecture's largest Ken'o Sewage Treatment Plant discharged into the T1-T2 region.

Next, the relationship between pharmaceutical loads and basin populations was analyzed. Figure 5 shows the relationship between basin population and pharmaceutical loads at the six sampling sites. There was a linear proportional relationship between the two, with a high correlation coefficient of 0.905–0.998, indicating that pharmaceutical loads were highly dependent on the basin population. Therefore, it is possible to estimate the annual fluxes of pharmaceuticals discharged into the Tone River by all residents of Gunma Prefecture. The annual flux (kg/year) was calculated using the formula $L\times(P_G/P_B)\times(3.15\times10^7/10^6)$, where $L$ is the pharmaceutical load (mg/s), $P_G$ is the population in Gunma Prefecture (1.89 million people), $P_B$ is the basin population, $3.15\times10^7$ is seconds in one year, and $10^6$ is milligrams per kilogram. Using data from Site T2, which had the largest basin population, the annual fluxes of diphenhydramine, clarithromycin, carbamazepine, and bezafibrate were found to be 98, 210, 28, and 53 kg/year, respectively.

According to the NDB Open Data (Ministry of Health, Labour and Welfare, 2021), the weight of clarithromycin prescribed in Gunma Prefecture in the fiscal year 2019 was 127 kg at out-of-hospital pharmacies for outpatients, 272 kg at in-hospital pharmacies for outpatients, and 13 kg for inpatients, with a total of 412 kg. Meanwhile, the prescribed amounts of carbamazepine were 567, 163, and 38 kg for a total of 768 kg. According to the package inserts for Clarith® (clarithromycin) and Tegretol® (carbamazepine), the urinary excretion rates of pharmaceuticals without metabolism are approximately 50% and 2–3%, respectively (Taisho Pharmaceutical, 2023; Sun Pharma, 2023). Therefore, the two pharmaceuticals excreted in the urine of all Gunma Prefectural residents were estimated using the formula $W_P\times E_P/100$, where $W_P$ is the weight of the pharmaceutical prescribed in Gunma Prefecture, and $E_P$ is the urinary excretion rate of the pharmaceuticals. These values were 206 and 15– 23 kg/year for clarithromycin and carbamazepine, respectively. These values are similar to the annual fluxes (210 and 28 kg/year, respectively), indicating that clarithromycin and carbamazepine are stable during sewage treatment and in aquatic environments.

## Antimicrobial activity

As described above, the pharmaceutical concentrations in river water collected midstream of the Tone River were nearly half of those in river water from the Ayase River, which is relatively highly polluted in the Kanto region. Moreover, drug-resistant bacteria have been reported in the water of the Tone River (Hata *et al*., 2015). Therefore, there are concerns that bacteria could develop drug resistance due to the presence of antibacterial substances, such as antibiotics and synthetic antimicrobials, in river water. To investigate the characteristics of the antimicrobial activity of the Tone River water, we assayed the antimicrobial activity of river water extracts collected at Site T2 in February, April, June, August, October, and December.

Figure 6 shows a photograph of the Petri dish used in the diffusion assay. The addition of clarithromycin revealed the inhibition zones due to antimicrobial activities in a dose-dependent manner at a load of 1–16 µg onto paper discs (Fig. 6 A). A linear relationship ($r$ = 0.997) was observed between the logarithm of the clarithromycin concentration and the diameter of the inhibition zone. When the river water extracts were loaded onto the discs, inhibition zones were observed in samples collected in June (both low and high load) and February, October, and December (only high load), as shown in Fig. 6 B and C. Table 1 summarizes the diameter of the inhibition zone and the amount of clarithromycin in the loaded sample. The ascending order of clarithromycin concentration in river water was February>December>April>June, whereas that of the inhibition zone diameter was June>October>December> February. These results suggest that the antimicrobial activities of the river samples were not dependent on clarithromycin. Therefore, the June sample likely contained antibacterial substances other than clarithromycin; however, this remains unknown.

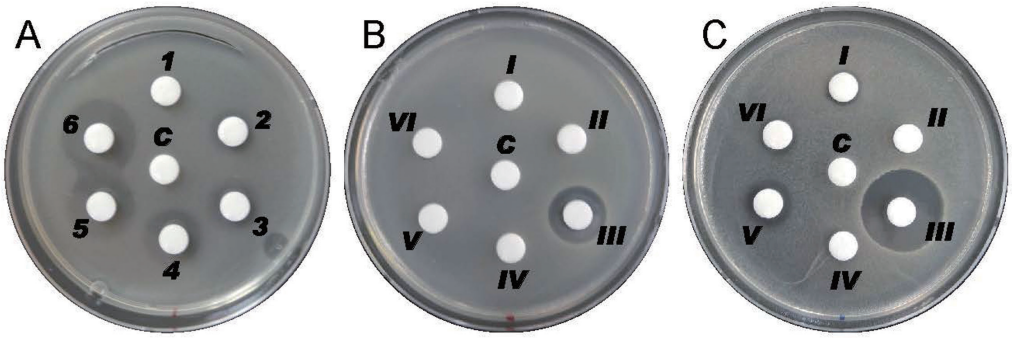


**Fig. 6.** Antimicrobial activities of authentic clarithromycin (A) and extracts from river water at low load (0.04 L river water equivalent, B) and high load (0.2 L equivalent, C) against tolC-deleted mutant of *E. coli* MG1655, as observed using the disc diffusion assay. A: Load was 0.5 µg (*1*), 1 µg (*2*), 2 µg (*3*), 4 µg (*4*), 8 µg (*5*), 16 µg (*6*). B, C: Sampling dates were February 25 (*I*), April 17 (*II*), June 22 (*III*), August 22 (*IV*), October 20 (*V*), and December 16 (*VI*), 2019. Methanol was used as the control (*C*).

**Table 1.** Diameter of inhibition zone by disc diffusion assay and the amount of clarithromycin contained in the loaded sample.

| Sampling date | Low load | | High load | |
|---|---|---|---|---|
| | Diameter(mm) | Clarithromycin(ng) | Diameter(mm) | Clarithromycin(ng) |
| February 25 | ND (<8) | 2.6 | 9 | 13 |
| April 17 | ND (<8) | 1.2 | ND (<8) | 5.8 |
| June 22 | 16.5 | 0.58 | 23.5 | 2.9 |
| August 22 | ND (<8) | 0.13 | ND (<8) | 0.63 |
| October 20 | ND (<8) | 0.08 | 12 | 0.41 |
| December 16 | ND (<8) | 2.4 | 10 | 12 |

Low load, 0.04 L river water equivalent; high load, 0.2 L river water equivalent.
The clarithromycin concentration (ng/L) was determined using LC/MS/MS and multiplied by the volume (L) of the river water equivalent. ND, not detected.

Pharmaceuticals discharged from pharmaceutical manufacturing factories have increased their concentrations in river waters (Cardoso *et al*., 2014). Moreover, surfactants and dyes exhibit antimicrobial activity (Nishino *et al*., 2004; Nishino *et al*., 2008). Therefore, the antibacterial substances in the extract collected in June may have originated from factories. Most studies have only evaluated pharmaceuticals derived from human waste in river water (Suzuki *et al*., 2021); however, antibacterial substances derived from factories must also be closely monitored. Currently, we are investigating the drug-resistant bacteria present in the Tone River water. As numerous wildlife species inhabit the Tone River and the water is used as raw water for drinking, we hope to clarify the pharmaceutical concentrations and drug-resistant bacteria in river water to purify the Tone River.

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

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