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***Daphnia magna* to assess hot-spring drainage in the Shibukuro and Tama Rivers (Akita, Japan)**

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Abstract

We investigated the lethal toxicity of Shibukuro and Tama river water near the inflow of Tamagawa hot-spring water in Akita Prefecture, Japan. We first measured metal concentrations in both rivers. We detected iron, arsenic, and aluminum; the concentrations of each tended to decrease from upstream to downstream. We next examined the influence of river water on zebrafish *Danio rerio* and water flea *Daphnia magna*. We observed lethal effects in both species, with *Daphnia magna* more sensitive to toxicity than *Danio rerio*. For both species, the toxic effects of river water decreased with increasing distance downstream from the inflow of hot-spring water. Our results show that the metals discharged from Tamagawa hot spring have a negative effect on aquatic organisms.

Key words: bioassay • ecotoxicity • hot spring • metal • zebrafish

Declarations

Data Availability

The authors confirm that all data underlying the findings are fully available without restriction.

Animal Research (Ethics)

The fish which was used in the present study were handled according to guidelines of Akita Prefectural University and Kobe University.

Consent to Participate (Ethics)

This research did not involve human subjects, so clinical trial registration is not applicable.

Consent to Publish (Ethics)

The authors certify that this manuscript is our original unpublished work, has not been published elsewhere, and is not under consideration by another journal. All authors have approved the manuscript and agree with its submission.

Plant Reproducibility

The authors confirmed reproducibility.

Author Contribution

All authors listed on the current study contributed to the experimental design or data analysis. (Kyo Suwa; Sampling and Bioassay: Chiho Takahashi; chemical analysis: Yoshifumi Horie; All experiment except chemical analysis).

Conflict of Interest

The authors declare that they have no conflict of interest.

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1. Introduction

Lake Tazawa is the deepest lake in Japan (maximum water depth, 423.4 m) and was historically inhabited by various fish species such as *Oncorhynchus kawamurae*, *Salvelinus* spp, and *Anguilla japonica*, and by zooplankton such as rotifers and cladocerans (Nakabo 2011). However, in 1940, a waterway was created to introduce water from the nearby Tama River in order to use the lake water for power generation. Tamagawa hot-spring water, which is used for medical treatment, flows into Tama River via the Shibukuro River, which contains hypochlorous acid and has a pH of 3.5 or higher (Tamagawa hot spring water source: pH 1.1 to 1.2) (https://www.pref.akita.lg.jp/pages/archive/39675). Therefore, the water in Lake Tazawa before the influx of Tama River water had a pH close to neutral (6.7), but became acidified to a pH of about 4.2 around 1970 (https://www.pref.akita.lg.jp/pages/archive/39675). Many aquatic organisms disappeared from Lake Tazawa because of the rapid deterioration of water quality (Nakabo 2011). Hot-spring waters often contain naturally high concentrations of various metals. Therefore, if hot-spring drainage flows into a river, it may have serious adverse effects on the aquatic ecosystem. However, there have been few previous studies on the effects of hot-spring drainage on living organisms, and there are many details requiring clarification.

In the present study, we used the zebrafish (*Danio rerio*) and the water flea (*Daphnia magna*) to assess the effects of hot-spring drainage flow into a river. To date, these species have been used in bioassays assessing the negative effects of various rivers, for example the Tietê River, São Paulo, Brazil (Rodgher et al. 2005); the Tiber River basin in the Lazio Region, Italy (Cristiano et al. 2020); and the Pearl River Delta region, China (Fang et al. 2012). Fish and zooplankton have a food chain relationship, and together they can help to clarify the impact of environmental pollution on an ecosystem. The zebrafish is a small fish native to India with a body length of approximately 5 cm. It is a model fish for vertebrates and is regularly used for risk assessment of various environmental pollutants. For example, Severo et al. (2020) reported the negative ecological risks of pesticide contamination in a Brazilian river using zebrafish embryos, Li et al. (2016) reported the toxicity of sediments from the Yangtze River estuary using zebrafish embryos, and Rocha et al. (2011) assessed particle-bound pollutants in the Tietê River basin using zebrafish embryos.

Daphnia magna is widely used worldwide as a standard test species for ecotoxicity because it is easy to culture in the laboratory, produces multiple generations, and is highly sensitive to chemicals (Evens et al. 2011). *Daphnia magna* is also regularly used for risk assessment of various environmental pollutants. For example, Zhang et al. (2020) studied the toxicity of heavily polluted river sediments on *Daphnia magna*. Giraudo et al. (2019) also reported that a major release of urban untreated wastewater into the St. Lawrence River (Quebec, Canada) altered the growth, reproduction, and redox status in experimentally exposed *Daphnia magna*. Thus zebrafish and *Daphnia magna* are well established as test organisms for revealing the effects of various environmental pollutants on fish and *Daphnia,* although it is still unclear if they are appropriate for the risk assessment of hot-spring drainage .

In the present study, therefore, we first measured the concentrations of metals in the collected river water to clarify the state of metal pollution. Next, we used these two species to perform bioassays of river water collected from the Shibukuro and Tama Rivers near the inflow of Tamagawa hot-spring water, to evaluate any effects on aquatic organisms.

2. Materials and Methods

2.1. Study area and sampling

The Shibukuro and Tama Rivers that were targeted in this study are located in the northeastern part of Akita Prefecture, Japan, to the west and south of Hachimantai Akita-Yakeyama volcano. The Shibukuro River has a channel length of 11 km and a basin area of 41 km^2 and joins the Tama River. The Tama River has a channel length of 103 km and a basin area of 1219 km^2 and joins the Omono River. The Tamagawa hot spring is located in the Tamagawa hot-spring explosion crater at the western foot of Akita-Yakeyama Volcano, which is located about 60 km northeast of Akita City. Most of the Tamagawa hot-spring water is neutralized at a neutralization treatment facility, but some flows directly into the upper section of the Shibukuro River. We selected three sampling sites in the Shibukuro and Tama Rivers. The survey points were selected to include those that are expected to show an effect on aquatic organisms and those that are not expected to show an effect (Fig. 1). Site A is located approximately 3.5 km downstream of the Tamagawa hot-spring facility, Site B is located approximately 3 km farther downstream, at the confluence of the Shibukuro and Tama Rivers, and Site C is located approximately 3 km farther downstream in the Tama River.

All water samples were collected from each site in August 2020. Surface water samples were collected from a depth of 0.3 m below the river surface. Twenty liters of water were taken from each site for chemical analysis for metals and for bioassays using *Danio rerio* and *Daphnia magna*. Water samples were transported and kept at 4 ℃ until chemical analysis or bioassay. Water quality parameters were measured by using a pH and EC meter (WQ-310; Horiba, Kyoto, Japan), a dissolved oxygen meter (OM-71; Horiba), and a thermometer (AD-5624; AND, Tokyo, Japan).

2.2. Bioassays using Danio rerio *and* Daphnia magna

We conducted a bioassay using *Danio rerio* following OECD TG 212 with minor modification (OECD 1998). First, the sampled river water, which was stored at 4 °C, was heated to 25 °C using a water bath. Next, a dilution series of six concentrations was prepared using dechlorinated water: control, 6.25%, 12.5%, 25%, 50%, and 100% test water. Fish eggs were obtained by natural mating and unfertilized or abnormally developed eggs were removed under a stereomicroscope. Fertilized eggs within four hours post fertilization were selected and exposed to each concentration for eight days post fertilization (five days post hatching). After exposure, 15 fertilized eggs were transferred to a 100-mL glass vessel (exposure volume, 60 mL) with four replicates for each concentration, for a total of 60 fertilized eggs per treatment. Eggs were observed at the same time each day under a stereomicroscope; abnormal eggs were noted, and dead eggs were removed immediately after observation. The water was changed every two days. The bioassay was conducted at 25 ± 2 °C and a photoperiod of 16 h light:8 h dark. After the test was completed, the survival rate was calculated.

The bioassay using *Daphnia magna* followed OECD TG 202 with minor modification (OECD 2004). First, the sampled river water, which was stored at 4 °C, was heated to 25 °C using a water bath. Next, a dilution series of six concentrations was prepared using dechlorinated water: control, 6.25%, 12.5%, 25%, 50%, and 100% test water. We selected less than 24 hours *Daphnia* from the bred parent *Daphnia* and exposed them to each concentration for two days. After exposure, five larvae were distributed to a 100-mL glass vessel (exposure volume, 60 mL) with four replicates for each concentration, for a total of 20 larvae per treatment. Larvae were observed at the same time each day under a stereomicroscope; abnormal larvae were noted, and dead larvae were removed immediately after observation. The water was changed every two days. The bioassay was conducted at 20 ± 2 °C and a photoperiod of 16 h light:8 h dark. After the test was completed, the survival rate was calculated.

In all experiments, the *Danio rerio* and *Daphnia magna* were handled in a humane manner in accordance with the guidelines of Akita Prefectural University, Japan.

2.3. Chemical analysis for Fe, As, and Al

Sato et al. (2005) reported that the main metals flowing out of Tamagawa hot spring were iron (Fe), arsenic (As), and aluminum (Al). We therefore conducted chemical analyses for these three substances.

Fe concentrations were determined by using an *atomic absorption spectrophotometer (AA280FS, Agilent Technologies Japan, Ltd, Tokyo, Japan).* Each water sample (200 mL) was placed in a fluororesin beaker to which high-purity nitric acid (5 mL) was added. The beaker was heated for 1 h at 180 °C on a hot plate. After the sample cooled to room temperature, nitric acid (2 mL) was added. A portion of the sample was transferred to a test tube and used for flame atomic absorption spectroscopy (FAAS) analysis. Analytical conditions were as follows: flame type, air-acetylene; air mass flow rate, 13.50 L/min; acetylene flow rate, 2.00 L/min; measurement mode, spectral analysis; analytical wavelength, 248.3 nm. The blank value was 0.0350 mg/L.

As concentrations were determined by using an *inductively coupled plasma–mass spectrometer (ICP-MS; Agilent 7700x, Agilent Technologies Japan, Ltd).* Each water sample (25 mL) was placed in a fluororesin beaker to which nitric acid (0.25 mL) was added. The beaker was heated for 1 h at 150 °C on a hot plate. After the sample cooled to room temperature, a portion of the sample was transferred to a test tube and used for analysis. Analytical conditions were as follows: sampling depth, 10 mm; measurement mode, spectral analysis; plasma gas flow rate, 15 L/min; carrier gas flow rate, 0.35 L/min; high matrix introduction (HMI) gas flow rate, 0.60 L/min; collision cell gas (He) flow rate, 10 mL/min; nebulizer pump rotation speed, 0.1 rps; spray chamber temperature, 2 °C. The internal standard method (with yttrium [Y] as the internal standard) was used in ICP-MS analysis. The blank value was 0.000050 mg/L.

Al concentrations were determined by using an inductivity coupled plasma optical emission spectrometer *(ICP-OES; iCAP6300Duo, Thermo Fisher Scientific, Tokyo, Japan).* Each water sample (25 mL) was placed in a fluororesin beaker to which nitric acid (1 mL) was added. The beaker was heated for 1 h at 150 °C on a hot plate. After the sample cooled to room temperature, a portion of the sample was transferred to a test tube and used for analysis. Analytical conditions were as follows: ICP mode, multi-channel; plasma gas flow rate, 12 L/min; nebulizer gas flow rate, 0.50 L/min; auxiliary gas flow rate, 0.5 L/min; pump speed, 50 rpm. The internal standard method (with yttrium [Y] as the internal standard) was used in ICP-*OES* analysis. The blank value was 0.0063 mg/L.

2.4. Statistical analysis

Statistical analyses were conducted as reported previously (Horie et al. 2017). We used custom R code and the package *Rcmdr* (Fox and Bouchet-Valat 2018) to test for homogeneity of variance using Bartlett's test (significance level, 5%). If the null hypothesis (i.e., the data are homoscedastic) was not rejected, we tested for differences among treatments using Dunnett's test; otherwise, we used Steel's test.

3. Results and Discussion

3.1. Chemical analysis for Fe, As, and Al

Water quality parameters at the time of sampling were as follow (Table 1): Site A, pH 2.96, electrical conductivity (EC) 1292 µS/cm, dissolved oxygen (DO) 6.63 mg/L, and water temperature 20.6 °C; Site B, pH 3.30, EC 715 µS/cm, DO 4.57 mg/L, and water temperature 17.8 °C; Site C, pH 6.24, EC 103.9 µS/cm, DO 7.59 mg/L, and water temperature 19.1 °C.

Table 2 shows the measured concentrations of metals in each sampled river. The concentrations of the three metals at the three sites tended to decrease from the upstream to downstream (Fig. 2).

Sato et al. (2005) reported that the pH ranged between 2.9 and 5 between Sites A, B, and C, the Fe concentration ranged from not detectable to 9.8 mg/L, and the As concentration ranged from not detectable to 0.07 mg/L, with the concentrations decreasing from upstream to downstream. These results are almost in complete agreement with the results of the present study, indicating that the water quality of the Shibukuro and Tamag Rivers, near the inflow of Tamagawa hot-spring water, has not changed much between 2005 and 2020. In addition, our results revealed that the contaminant Al had the highest concentration in the Shibukuro and Tama Rivers, compared to Fe and As. Future studies should investigate the concentrations of other metal contaminants.

3.2. Bioassays using Danio rerio *and* Daphnia magna

The results of toxicity testing with zebrafish embryos and larvae are presented as the percent survival (Fig. 3). At Site A, all embryos died in the 100% treatment group, and the survival was significantly lower in the 12.5%, 25%, 50%, and 100% treatment groups than in the control. At Site B, 75% of the embryos died in the 100% treatment group, although there were no significant differences from the control in the 6.25%, 12.5%, 25%, and 50% treatment groups. On the other hand, there were no significant differences from the control group in survival rate in any of the treatment groups at Site C.

The results of toxicity testing with *Daphnia magna* are also presented as the percent survival (Fig. 4). At Site A, all larvae died in treatments of 12.5%, 25%, 50%, and 100%, and all treatment groups had significantly lower survival rates compared with the control group. At Site B, all larvae died in treatments of 25%, 50%, and 100%, and the survival rate was significantly lower in the 12.5%, 25%, 50%, and 100% treatment groups compared with the control group. At Site C, the survival rates of larvae in the 50% and 100% treatment groups were significantly lower than in the control group (35% and 85% mortality, respectively), although there were no significant differences in survival rates in the 6.25%, 12.5%, and 25% treatment groups compared with the control group (Fig. 4).

In the present study, the pH level of Sites A and B, where 100 and 75 % of the embryos died, were 2.96 and 3.30. Lethal effect of low pH level (acidic streams; pH of <5) was reported in previous study. Andrade et al (2016) revealed using zebrafish embryo that embryos exposed to pH below 3.5 showed 100% mortality, and the 96-h LC50 s value of 3.7 ± 0.03 pH units. In addition, Ghazy et al (2011) revealed that *Daphnia magna* exposed to 4.44 pH units showed 53% mortality, and the 48h-LC50s value of 4.37 pH units. These suggest that low pH level in the Shibukuro and Tama Rivers induced mortality in both zebrafish and *Daphnia magna*.

In the USEPA Water Quality Criteria, there are reported the acute toxicity of Al and As to aquatic animals. The 48-hr EC 50 for *D. magna* was ranged from 713.2 to 15,625 µg/L Al (Biesinger and Christensen.,1972; European Aluminum Association., 2009; Kimball., 1978; Shephard., 1983) and lethal effect for *D. rerio* was observed at 548 µg/L Al (Cardwell et al., 2018). The EC 50 for *D. magna* was ranged from 3,800 to 5,278 µg/L As (Anderson, 1946; Mount and Norberg., 1984; Lima et al., 1984) and the 96-hr LC 50 for *D. rerio* was 28.1 mg/L As (Tisler and Zagorc-Koncan., 2002). In the present study, As concentration in Site A was 90 µg/L As and was lower than the lethal concentration in both *D. magna* and *D. rerio*. On the other hand, Al concentration in Site A and B were 16.9 and 9.77 mg/L Al and were higher than the lethal concentration in both species. These suggest that Al contamination in the Shibukuro and Tama Rivers induced mortality in both zebrafish and *Daphnia magna*.

Our results show that water in the Shibukuro and Tama Rivers, where water enters from the Tamagawa hot spring, had negative toxic effects on *Danio rerio* and *Daphnia magna*. The lowest observed effect concentrations (LOECs) for survival at each sampling site are shown in Table 3. For both *Danio rerio* and *Daphnia magna*, the LOEC for survival increases from upstream to downstream, indicating that the toxic effect is lower with increasing distance downstream. In addition, the LOEC for survival is higher for *Danio rerio* than for *Daphnia magna*.

Martins et al. (2007) reviewed the differences in sensitivity to toxicity between zebrafish and *Daphnia magna* using various types of chemicals including metals, pesticides, organic chemicals, and solvents. They found that, in acute toxicity tests, *Daphnia magna* responded to a greater variety of chemicals with a higher sensitivity than zebrafish. Wittlerová et al. (2020) also reported that *Daphnia magna* was more sensitive than zebrafish in toxicity tests using hospital wastewater. Our results also show that *Daphnia magna* was more sensitive to toxicity than *Danio rerio*. In addition, there are reports of the trophic transfer of Zn (Liu et al. 2002) and TiO_2 nanoparticles (Zhu et al. 2010) from *Daphnia magna* to *Danio rerio*. This indicates that, in order to protect river ecosystems from environmental pollution, future studies should perform bioassays that consider the food chain. To our knowledge, ours is the first report to reveal the negative influence of metals discharged from Tamagawa hot spring on aquatic organisms, and to detect Fe, As, and Al in the rivers receiving the discharge.

4. Conclusions

We first measured the concentrations of iron, arsenic, and aluminum in the Shibukuro and Tama Rivers. The concentration of each metal tended to decrease in a downstream direction from the point of inflow. We then used bioassays and observed lethal effects from river water in both the zebrafish *Danio rerio* and the water flea *Daphnia magna*. *Daphnia magna* was more sensitive to toxicity than *Danio rerio*. To our knowledge, this is the first study to use bioassays with *Danio rerio* and *Daphnia magna* to clarify the lethal effects of hot-spring drainage in the Shibukuro and Tama Rivers.

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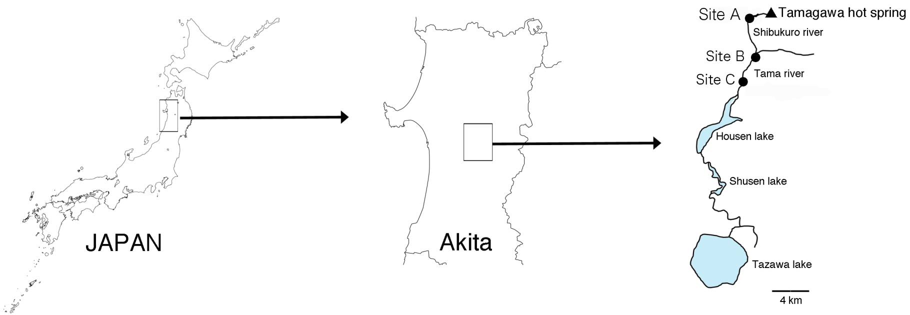
Figures

Fig. 1 Sampling locations (Sites A–C) on the Shibukuro and Tama Rivers, Akita Prefecture, Japan

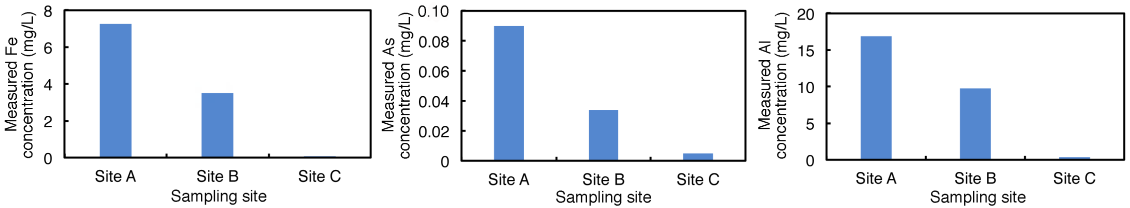
Fig. 2 Fe, As, and Al concentrations in river water from upstream (Site A) to downstream (Site C)

Fig. 3 Results of *Danio rerio* short-term toxicity tests using river water. Columns and error bars represent means ± SEM (n = 4 per exposure group). Asterisks indicate statistically significant differences compared with the control (Dunnett's test or Steel's test; $P < 0.05$)

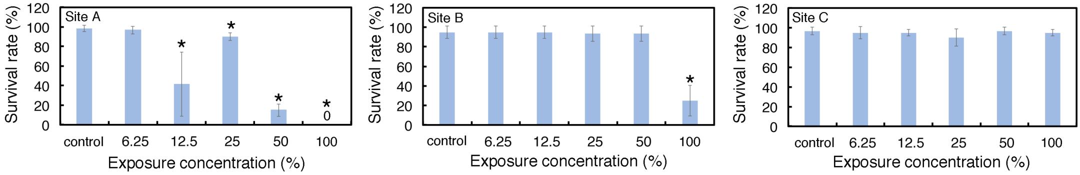
Fig. 4 Results of *Daphnia magna* acute toxicity tests using river water. Columns and error bars represent means ± SEM (n = 4 per exposure group). Asterisks indicate statistically significant differences compared with the control (Dunnett's test or Steel's test; $P < 0.05$)

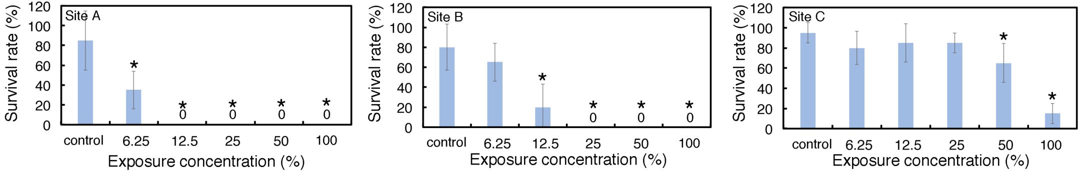


JAPAN
Akita
Site A
Tamagawa hot spring
Shibukuro river
Site B
Site C
Tama river
Housen lake
Shusen lake
Tazawa lake
4 km



Measured Fe concentration (mg/L)
8
6
4
2
0
Site A
Site B
Site C
Sampling site
Measured As concentration (mg/L)
0.10
0.08
0.06
0.04
0.02
0
Site A
Site B
Site C
Sampling site
Measured Al concentration (mg/L)
20
15
10
5
0
Site A
Site B
Site C
Sampling site


Site A
Site B
Site C
Survival rate (%)
0
20
40
60
80
100
120
*
0
control
6.25
12.5
25
50
100
Exposure concentration (%)



Site A
Site B
Site C
Survival rate (%)
Exposure concentration (%)
control
6.25
12.5
25
50
100
0
20
40
60
80
100
120

Table 1 Water quality in river water from Sites A–C.

Sampling site	Parameters			
	pH	Conductivity (μS/cm)	Oxygen dissolved (mg/L)	Temperature (°C)
Site A	2.96	1292	6.63	20.6
Site B	3.30	715	4.57	17.8
Site C	6.24	103.9	7.59	19.1

Table 2 Fe, As, and Al concentrations in river water from Sites A–C.

Sampling site	Measured concentration (mg/L)		
	Fe	As	Al
Site A	7.24	0.090	16.9
Site B	3.50	0.034	9.77
Site C	0.05	0.005	0.34

Table 3 Comparison of the LOEC for a lethal endpoint between *Danio rerio* and *Daphnia magna*. The concentration is given as the percentage of undiluted river water (100% = no dilution).

Sampling site	LOEC (%)	
	Danio rerio	*Daphnia magna*
Site A	12.5	<6.25
Site B	100	12.5
Site C	no effect	50