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Effects of heavy metals in river waters in Japan on immobility and mortality of *Daphnia magna* and *Oryzias latipes* larvae

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ABSTRACT: Samples of river waters containing high concentrations of zinc and other heavy metals but low concentrations of other anthropogenic contaminants were collected to investigate the relationship between toxicity of heavy metals and naturally present organic matters or hardness, as well as the effects of heavy metals on aquatic organisms. Acute toxicity tests were conducted for the water samples using *Daphnia magna* and medaka *Oryzias latipes*. Almost all the *D. magna* died in river waters containing high concentrations of zinc, but *O. latipes* in the same waters were hardly affected. Since the test organisms were not only exposed to zinc but also other heavy metals in the river waters, we examined the toxicity using toxic units composed of zinc, copper, lead, and cadmium. The results of a bioassay with the river waters showed that the mortality of *D. magna* did not depend solely on the total number of toxic units of heavy metals. The organic matters and the hardness of the river waters could decrease the acute toxicity of zinc and other heavy metals to *D. magna*.

KEY WORDS: ***Daphnia magna*, hardness, heavy metals, organic matter, rivers, zinc.**

INTRODUCTION

Numerous investigations have shown that several chemicals in river and sea waters immobilize,[1,2] kill,[3,4] inhibit the growth of,[5] or reduce reproduction of[6,7] aquatic organisms. The Ministry of the Environment of Japan has carried out risk assessment studies to identify those hazardous chemicals which will lead to future risk management in Japanese surface waters.[8] The risk is assessed by comparing the measured concentrations of toxic chemicals in the waters with the toxicities of the chemicals to aquatic organisms as determined in the laboratory. The results of these studies have shown that zinc and other chemicals pose a high risk to aquatic organisms. On the basis of these studies in 2003, the Ministry of the Environment of Japan published environmental quality standards for zinc in river and sea waters designed to protect aquatic organisms.[9]

Generally, the results of bioassays conducted in laboratories show only the toxicity of a certain chemical. Aquatic organisms in natural waters, however, are exposed to more than two chemicals simultaneously. Therefore, it is difficult to determine the risk that chemicals pose to aquatic organisms in natural waters on the basis of toxicity data of a single chemical measured in the laboratory. Moreover, the toxicity of a chemical to aquatic organisms depends on numerous factors, such as the dissolved organic matters, hardness, and the suspended particle load of river or lake waters.[4,10–13] To evaluate the toxicity of mixtures of chemicals to aquatic organisms, several studies have used the sum of their toxic units.[14–16] The number of toxic units equals the ratio of the measured concentration of a chemical to its effective concentration, i.e. the concentration that is toxic to aquatic organisms (e.g. concentration of a chemical causing death in 50% of the test organisms [LC_{50}], and concentration of a chemical causing immobilization in 50% of the organisms [EC_{50}]). However, there has been limited investigation on the effects of organic

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matters or hardness on the combined toxicity of heavy metals to aquatic organisms.

Zinc is a highly toxic chemical to aquatic organisms.[4,5] River and sea waters in Japan and in many other countries contain high concentrations of zinc and other heavy metals.[17–19] Moreover, high concentrations of zinc have been observed in tissues of amphipods and fish living in contaminated rivers or seas.[17,20,21] Therefore, it is important to ascertain the environmental toxicity of zinc to aquatic organisms with a bioassay using natural river water.

The aims of this study are to confirm concentrations of heavy metals in river waters, and their toxicity to *Daphnia magna* and *Oryzias latipes*. It was evaluated whether total organic carbon (TOC) and hardness in river waters would affect the toxicity of zinc and other heavy metals to aquatic organisms.

MATERIALS AND METHODS

Sampling

Water samples were collected from five river systems in Japan (Fig. 1). Samples were collected from known contaminated and uncontaminated areas of each river system. Four river systems, the Namari (A), Kaishu (B), Miyata (C) and Watarase (D), have mineral ore deposits or smelting plants in their catchment basins. Up- and downstream of sampling site 1 of the Namari River, there are mineral ore deposits and a lead smelting plant, respectively. The deposits include sphalerite, galena, wurtzite, and pyrite.[21] In the catchment basin of the Kaishu River, there are mineral ore deposits that include pyrite, chalcopyrite, and sphalerite.[22] Upstream of sampling site 2 of the Miyata River, there is a smelting slag waste dump. On the west bank of the Watarase River, mineral ore deposits extend. The deposits include chalcopyrite, bornite, pyrite, sphalerite, galena, arsenopyrite, and tin stone.[23,24] The Itadori River (E), in contrast, is uncontaminated. These rivers have little inflow from households or factories. Surface water samples were collected in polyethylene bottles and stored at –20°C until bioassay was performed.

Chemical analysis

Concentrations of heavy metals, TOC, and hardness were measured according to the Japanese Industrial Standard methods.[25] The organic contents of the water samples were analyzed for differences between unfiltered and filtered (0.45-μm filters) samples in a preliminary experiment. A statistically significant difference between the organic contents in unfiltered waters and filtered waters

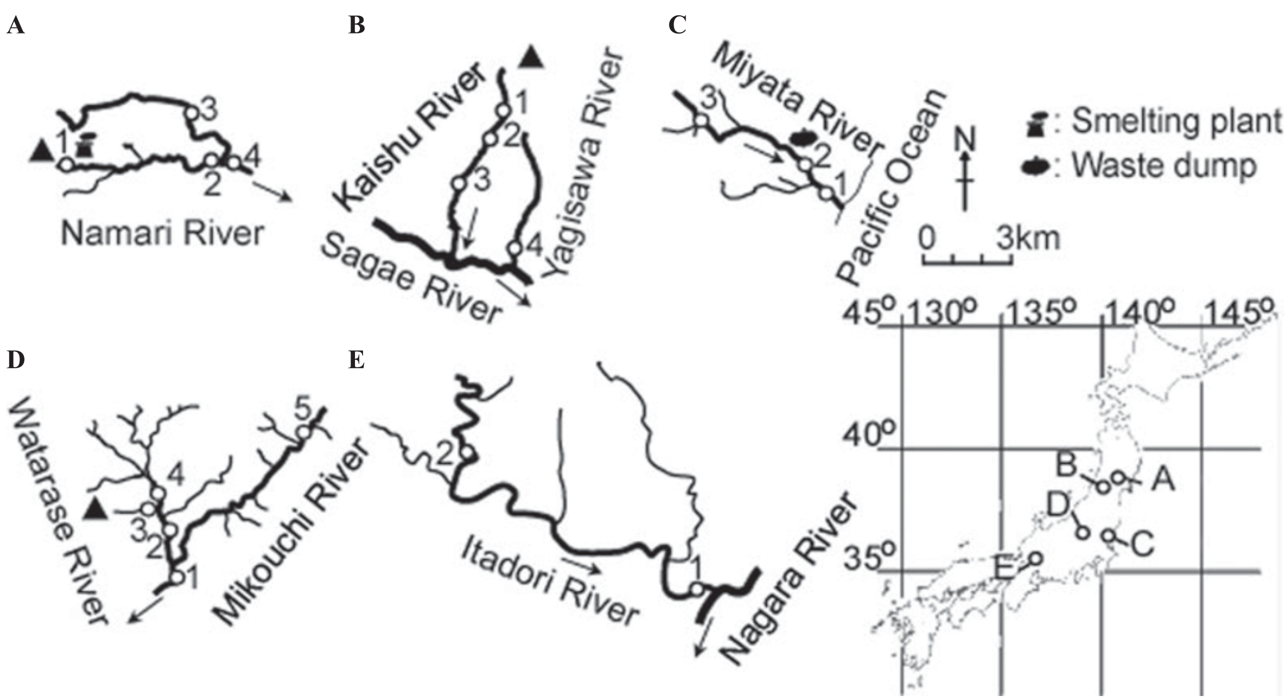

Fig. 1 Sampling sites in six river systems in Japan. Namari River (A), Kaishu River (B), Miyata River (C), Watarase River (D), and Itadori River (E), showing sampling sites (○) and ore deposits (▲).

was not observed. Thus, TOC concentration is effectively equivalent to dissolved organic carbon (DOC) for these rivers. The concentration of zinc and copper was measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Flameless atomic absorption spectrophotometry was used for measurement of cadmium, lead, and hardness (as $CaCO_3$). TOC was determined by high-temperature combustion. Electrical conductivity (EC) was measured *in situ* by the four-alternating current (4-AC) electrode method.

Bioassay

Bioassays of the river water samples were conducted using *D. magna* and *O. latipes*. Tap water, supplied by the municipal waterworks of Tsukuba, Japan, was dechlorinated by a column of granular activated carbon and used as the control. Each bioassay was carried out for 48 h and toxic effects were observed at 24 h and at the end of the assay. Immobilization or death of the test organisms was confirmed under a stereomicroscope. If a test organism's heart did not pump for 15 s, we assumed that it was dead. We measured the pH and DOC of the water at the start and end of each test. The pH and DOC were measured by glass electrode and polarograph methods, respectively. In cases where the pH was outside the range 6.0–8.5 at the start of the test and some organisms were found dead or immobilized at the end of the test, an additional test was carried out after adjusting the pH to 6.0–8.5 with 0.1 N HCl or 0.1 N NaOH. If all organisms died, the test water was diluted with dechlorinated tap water and another bioassay was performed. The LC_{50} and EC_{50} values were calculated based on the probit method[26,27] using EcoTox-Statics v2.4 software (Yoshioka Y, pers. comm., 2004; http://www.intio.or.jp/jset/ecotox.htm).

Daphnia magna

An acute immobilization test for *D. magna* was carried out in accordance with test guideline 202 of the Organization for Economic Cooperation and Development (OECD).[27] Cultures of *Daphnia magna* have been maintained at the testing laboratory (National Institute for Environment Study [NIES], Tsukuba, Japan) since the mid-1980s. The origin of the colony was the United States of America Environmental Protection Authority (US-EPA). The parents of the daphnids used in the tests were acclimated at 20 ± 1°C under cool white fluorescent lights (<1000 lx) with an 8:16 h dark–light period for at least 28 days. They were maintained in dechlorinated tap water, and the water was renewed twice a week. The dechlorinated water was well aerated before using for cultivation. During acclimation, parent daphnids were fed daily with green algae *Scenedesmus* sp. Offspring that were born after the third larvae were used for the test. All parent daphnids produced more than 60 larvae within 21 days and did not produce any resting eggs. The *D. magna* larvae were used for bioassay within 24 h posthatch. Twenty daphnids, divided into four groups of five, were used for each test concentration and the control. Each group was placed in 20-mL glass vessels filled with 15 mL of test waters, i.e. river water, river water diluted with dechlorinated tap water, or dechlorinated tap water. The bioassay was conducted based on the static method under the same conditions of acclimation described above, and dechlorinated tap water was used for the control. During the test period, the test organisms were not fed and test solutions were not aerated to prevent the formation of metal oxides.

Oryzias latipes

An acute toxicity test for medaka *O. latipes* larvae was carried out according to OECD test guideline 203.[26] Fish were obtained from commercial fisheries and have been cultured at the NIES testing laboratory since mid-1980s. Parent fish were acclimated using a flow-through chamber at 24 ± 1°C under cool white fluorescent lights (<1000 lx) with an 8:16 h dark–light period for at least 28 days. They were cultured in dechlorinated tap water. During acclimation, parent fish were fed daily with brine shrimp *Artemia salina* and the culture water was aerated by pump. Larvae of *O. latipes* that had produced many eggs during acclimation were used for bioassay when they were less than 24 h old (<24 h posthatch). The bioassays were carried out using the static method under the same conditions as for acclimation in 200-mL glass beakers, each containing 100 mL of river water or dechlorinated tap water. During the test period, test organisms were not fed and test solutions were not aerated. Ten fish were placed in each vessel. The bioassays were performed in duplicate.

Sensitivity test

To confirm the sensitivity of the test organisms, a sensitivity test was carried out in duplicate. The method for the sensitivity test was the same as for the bioassay. *Daphnia magna* and *O. latipes* were exposed to zinc sulfate (CAS no. 7733-02-0, Wako Pure Chemical, Osaka, Japan), for 48 h. Zinc sulfate concentrations were 180, 320, 580, 1000, and 1800 μg/L in the sensitivity test of *D. magna* and 1800, 3200, 5800, 10 000, and 18 000 μg/L in the sensitivity test of *O. latipes*. The sensitivity tests were performed prior to the bioassays of the river water.

Toxic units

To determine the toxicity of the combined heavy metals, the expected toxicities of the heavy metals were expressed as toxic units (TU) and the toxic unit sum was calculated by equation 1:[15,16]

$$\sum TU = \sum_{i=1}^{a} \frac{[Me_i]}{\overline{MeLC_{50,i}}}, \tag{1}$$

where

$\sum TU$ = sum of toxic units

$[Me]$ = concentration of a heavy metal in river water (μg/L)

$\overline{MeLC_{50}}$ = mean toxicity value calculated using published 48-h LC_{50} values of each heavy metal (μg/L)

a = the number of different heavy metals included in the sum

The heavy metal concentrations in each river water sample were divided by the calculated mean toxicity of each heavy metal to *D. magna*. The mean toxicity was calculated using known 48-h LC_{50} values described in published papers.[4,5,7,30–33] If the concentration of a heavy metal was below the quantification limit (Zn 1, Cu 1, Pb 1, and Cd 0.25 μg/L), a value of half the quantification limit was used.[28]

RESULTS AND DISCUSSION

Heavy metal concentrations and other characteristics of the river waters and the results of the river water bioassays with *D. magna* and *O. latipes* are shown in Table 1. During the tests, the water temperature of the test solutions for *D. magna* and *O. latipes* were maintained at 20.1–20.8 and 23.4–24.9°C, respectively. No control organisms in the dechlorinated tap water died during the test period.

The waters of river systems with metal ore deposits in their catchments contained relatively high concentrations of zinc. These waters also contained relatively high concentrations of copper, lead, and cadmium. Organic matter concentrations were relatively low in these rivers. The TOC ranged <0.5–1.7 mg carbon/L (mg C/L) (Table 1). Samples from the lower reaches of the Miyata River, which flows through populated areas (sites 1 and 2, Fig. 1), contained higher levels of TOC than other river water samples. The waters of the Itadori River, for which the catchment is free from contaminant sources, contained only a small amount of heavy metals. Compared with typical rivers in Japan, the Namari River and Miyata River showed relatively high hardness values, 289 ± 140 (standard error [SE]) and 270 ± 80 mg/L (as $CaCO_3$), respectively, while the hardness of the other rivers was in the range 31 ± 4 mg/L (Table 1).

The 48-h LC_{50} values of zinc sulfate for *D. magna* and *O. latipes* were 280–400 and 6500–7700 μg Zn/L, respectively (Table 2). The LC_{50} values for *D. magna* were reported as 151 μg Zn/L[4] and 752 μg Zn/L.[5] There was no report on LC_{50} for *O. latipes*. The 96-h LC_{50}s of zinc sulfate for juvenile *Oncorhynchus mykiss* in soft and hard waters were reported to be 430 and 7210 μg Zn/L, respectively.[29]

The results of the acute toxicity tests for *D. magna* and *O. latipes* exposed to the river waters are shown in Table 1. *Daphnia magna* were immobilized or dead in 12 of the 18 river water samples, whereas *O. latipes* were dead in only 4 of the 18 river water samples (Table 1).

Daphnia magna and *O. latipes* were affected by various heavy metals present in the tested river waters (Table 1). Therefore, the combined toxicities of the heavy metals in the river water to the test organisms were evaluated based on ΣTU.[15,16] *Oryzias latipes* was little affected by the heavy metals contained in the river waters in this study. Therefore, the combined toxicities of heavy metals to *O. latipes* were not analyzed.

The LC_{50} values used to determine TU were obtained from published work: the reported 48-h LC_{50} values for *D. magna* exposed to zinc, copper, lead, and cadmium are 151–752,[4,5] this study 7–54,[4,7] 3610–4400,[7,30] and 20–118 μg/L.[7,31–33] We used mean toxicity values of zinc (340 ± 130 mg/L), copper (38 ± 16 mg/L), lead (4000 ± 400 mg/L), and cadmium (49 ± 10 mg/L) calculated from those values by assuming that all heavy metals were present as ions. TU were calculated by dividing the heavy metal concentration of each heavy metal in the water of each river by its calculated mean toxicity to *D. magna* (Fig. 2). In Figure 2, the mortality of *D. magna* and ΣTU are compared. Theoretically, when ΣTU is greater than one, half of the test organisms should be dead. In our results, the mortality of *D. magna* tended to be high when they were exposed to river waters with

Table 1 Chemical and physical properties of river waters and bioassay results for *Daphnia magna* and *Oryzias latipes*

River system	Site	Sampling date[†]	Heavy metal concentration (μg/L)				TOC (mg C/L)	Hardness as $CaCO_3$ (mg/L)	EC (μS/cm)	Cumulative number of immobilized or dead *D. magna*				pH of the *D. magna* test solution		DO of the *D. magna* test solution		Cumulative number of dead *O. latipes*		pH of the *O. latipes* test solution		DO of the *O. latipes* test solution	
										Immobility		Dead											
			Zn	Cu	Pb	Cd				24 h	48 h	24 h	48 h	0 h	48 h	0 h	48 h	24 h	48 h	0 h	48 h	0 h	48 h
Namari	1	29/5/2002	2600		21	12	<0.5	63	87	20	20	20	20	6.8	7.3	6.7	9.6	1	1	6.8	7.0–7.1	6.7	7.6–7.7
	2	29/5/2002	910	2	2	7.3	1	600	535	1	6	0	2	6.9	7.2	6.7	9.2	0	0	6.9	7.1	6.7	7.9
	3	29/5/2002	54	1	1	<0.25	1.4	43	63	0	1	0	0	7.4	7.6	7.3	9.3	0	0	7.4	7.0–7.1	7.3	7.9–8.0
	4	29/5/2002	400	1	1	4.3	1.2	450	447	0	0	0	0	7.1	7.3	7.3	9.3	0	0	7.1	7.5	7.3	8.0–8.3
Kaishu	1	28/5/2002	4900	90	38	23	0.5	64	71	20	20	20	20	7.0	7.2	8.0	8.5	2	19	7.0	7.2	7.6	7.7–7.8
	2	28/5/2002	1300	14	6	1.7	0.7	45	70	20	20	20	20	6.7	7.0	7.1	8.6	0	0	6.7	7.2	7.1	7.7–7.9
	3	28/5/2002	700	11	2	3.2	0.6	43	617	16	20	16	20	6.9	7.3	6.8	8.3	0	0	6.9	7.3–7.4	6.8	8.0
	4	28/5/2002	430	3	1	4.4	1.7	22	427	4	20	4	20	7.0	7.4	7.6	8.6	0	0	7.0	7.4	7.6	8.0
Miyata	1	16/10/2003	83	30	1	2.6	0.8	340	199	4	7	4	4	7.9	7.9	7.8	8.6	0	0	8.9	8.0	7.1	7.8–7.9
	2	16/10/2003	140	42	2	3.8	0.8	360	19	13	20	11	20	7.7	7.7	7.7	8.3	0	0	7.6	7.6–7.7	7.9	8.3
	3	16/10/2003	240	42	1	<0.25	1.2	110	13	0	6	0	3	7.8	7.9	8.3	8.5	0	5	8.0	7.8	6.4	7.4
Watarase	1	10/6/2003	5	3	<1	<0.25	0.6	23	103	0	0	0	0	9.0	7.5	7.4	7.9	0	0	8.3	7.6	8.1	7.6–7.7
	2	9/6/2003	13	8	1	0.4	<0.5	26	73	9	20	7	20	7.3	7.7	8.3	7.6	0	0	8.5	7.6	7.9	8.0
	3	9/6/2003	230	240	1	4.1	<0.5	49	137	20	20	20	20	7.8	7.8	8.0	8.5	2	9	7.0	7.7–7.6	8.0	7.8–8.0
	4	9/6/2003	7	3	<1	<0.25	<0.5	25	76	0	0	0	0	9.0	7.5	7.3	7.7	0	0	8.9	7.6	7.8	7.5–7.7
	5	10/6/2003	3	1	<1	<0.25	<0.5	13	42	0	0	0	0	8.4	7.5	7.4	7.7	0	0	8.1	7.7	7.6	7.8
Itadori	1	9/11/2003	1	<1	1	<0.25	<0.5	23	82	0	0	0	0	7.9	8.0	8.2	8.3	0	0	8.2	7.8	6.8	7.5–7.7
	2	9/11/2003	1	<1	1	<0.25	<0.5	23	75	0	0	0	0	7.6	7.6	8.3	8.3	0	0	8.4	7.7	7.0	7.5–7.6
Dechlorinated tap water			<1	<1	<1	<0.25		82	34	0	0	0	0	7.4–8.0	7.6–8.6	7.4–8.4	7.6–8.8	0	0	7.3–7.8	7.5–8.0	7.4–8.3	7.3–8.0

DO, dissolved oxygen; EC, electrical conductivity; TOC, total organic carbon.
[†]Day/month/year

Table 2 LC_{50} values of zinc sulfate for *Daphnia magna* and *Oryzias latipes*

Chemical	*D. magna* 48-h LC_{50} (μg Zn/L) (95% CL)	*O. latipes* 48-h LC_{50} (μg Zn/L) (95% CL)
$ZnSO_4$	400 (270–560)	6 500 (5400–7800)
	280 (240–300)	7 700 (6300–11 000)

CL, confidence limits.

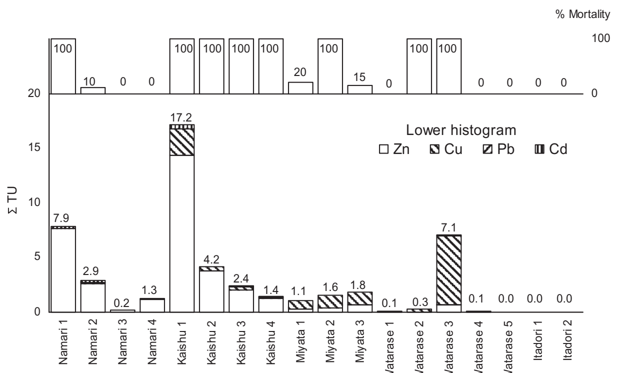

Fig. 2 Relationships between ΣTU and toxicity to *Daphnia magna*. The upper bar graph shows percent mortality and the lower histogram shows ΣTU for heavy metals.

high TU values for zinc (e.g. Namari River 1 and Kaishu River 1–4). Also, when ΣTU was greater than one, mortality was high, even if the TU of zinc was less than one (e.g. Watarase River 3). However, in some cases, the bioassay results did not show the expected relationship between the mortality and ΣTU. Specifically, Namari River 2 and 4 and Miyata River 1 and 3 waters had ΣTU higher than one, but mortality was less than 50%. Watarase River 2 had low ΣTU, but the mortality was 100%. It is suggested that Watarase River 2 contained other heavy metals, which we did not measure.

The relationship between the mortality of *D. magna* and ΣTU indicates that the toxicity of heavy metals in river waters to *D. magna* did not depend solely on heavy metal concentrations. It is generally known that when organic chemicals form a chelate with heavy metal ions, the toxicity of the heavy metals to aquatic organisms is reduced. Hardness in water also reduces the toxicity of heavy metals to aquatic organisms. Paulaskis and Winner[11] studied the acute (72-h) toxicity of zinc to *D. magna* in water with different hardness values (50–200 mg/L as $CaCO_3$) and humic acid concentrations (0.00–1.50 mg/L), and found that increases in either the humic acid concentration or hardness resulted in proportional decreases in acute zinc toxicity. Oikari *et al.*[4] found that the toxic effects of zinc, copper, and lead on *D. magna* in natural waters decreased in the presence of humic acids. Pettinen *et al.*[12] studied the interaction between dissolved organic matters and water hardness and their effects on the acute toxicity of cadmium to *D. magna*. They found that dissolved organic matters in soft water had a protective effect against cadmium toxicity and that calcium ions interfered with the uptake of cadmium ions, either by competing with them for transport through membranes or by reducing membrane permeability. Because TOC is known to contain organic chemicals (e.g. humic acids) that form chelates with metals, we measured TOC in the river waters in this study. To examine whether the toxicity of heavy metals was affected by the organic matters contained in river waters, we compared TOC, which is an index of water pollution by organic matter, with ΣTU. Because all *D. magna* were dead in eight of the 18 bioassays (Fig. 2), we carried out additional bioassays for those river waters by gradually diluting the river water samples with dechlorinated tap water (Table 3 and Fig. 3). Two distinctive features can be found in Figure 3. First, in river water collected from the same site, as the degree of dilution increased or ΣTU decreased, mortality of *D. magna* decreased. Second, for similar ΣTU, regardless of sampling sites or rivers, mortality of *D. magna* decreased as TOC or hardness increased. We observe this feature especially in areas (i), (ii), and (iii) of Figure 3. In areas (i) and (ii), although the highest ΣTU was in waters from the Miyata River, mortality was lowest in those waters because of their high TOC and hardness. In areas (i) and (ii), both organic matter (TOC) and hardness can be considered important contributors to the decreased toxicity of heavy metals to living organisms including *D. magna*. In area (iii), the Kaishu River waters showed the lowest mortality of *D. magna* despite having the highest ΣTU. The hardness of each river water was almost the same and may not have contributed to the decrease in the toxicity of heavy metals to *D. magna* in area (iii). TOC of the Kaishu River water was higher than for other river waters in area (iii). This result indicates that in area (iii), organic matters in the river water decreases the toxicity of heavy metals to *D. magna*.

Table 3 Results of bioassays using *Daphnia magna* for river water diluted with dechlorinated tap water

River system	Site	Dilution ratio	TOC (mg C/L)	Hardness as $CaCO_3$ (mg/L)	ΣTU	Cumulative number of dead or immobilized *D. magna*: Immobilized 24 h	Immobilized 48 h	Dead 24 h	Dead 48 h	pH of the *D. magna* test solution: 0 h	48 h	DO of the *D. magna* test solution: 0 h	48 h
Namari	1	1/3	0.25	76	2.6	0	16	0	16	7.0	7.3	7.2	9.2
Namari	1	2/3	0.25	69	5.3	15	20	15	20	6.9	7.0	7.5	9.4
Kaishu	1	1/81	0.25	82	0.21	0	0	0	0	7.8	7.7	8.0	9.0
Kaishu	1	1/27	0.26	81	0.64	0	8	0	6	7.8	7.6	7.8	8.9
Kaishu	1	1/9	0.28	80	1.9	20	20	20	20	7.6	7.6	7.8	8.3
Kaishu	2	1/3	0.4	70	1.4	5	13	5	13	7.0	7.3	6.7	8.5
Kaishu	2	2/3	0.55	57	2.8	9	15	9	15	7.0	7.1	6.8	8.2
Kaishu	2		0.7	45	4.2	20	20	20	20	6.7	7.0	7.1	8.6
Miyata	2	1/3	0.43	175	0.53	4	5	3	4	7.3	7.7	7.9	8.6
Miyata	2	2/3	0.62	267	1.1	1	6	0	3	7.4	7.7	8.0	8.3
Miyata	2		0.8	360	1.6	5	20	1	10	7.6	7.7	7.9	8.4
Watarase	2	1/3	0.25	63	0.09	0	0	0	0	8.0	8.3	8.3	7.8
Watarase	2	2/3	0.25	45	0.17	1	7	1	4	8.3	8.1	8.1	7.5
Watarase	2		0.25	26	0.26	9	18	7	16	7.3	8.3	8.3	7.6
Watarase	3	1/27	0.25	81	0.26	0	0	0	0	7.8	7.9	8.1	8.8
Watarase	3	1/9	0.25	78	0.79	0	6	0	5	7.7	7.8	7.8	8.7
Watarase	3	1/3	0.25	71	2.4	20	20	20	20	7.6	7.7	8.1	8.6
Dechlorinated tap water			<1	82		0	0	0	0	7.5–7.8	7.8–8.0	7.4–8.4	7.6–8.8

TOC and hardness of diluted test waters were estimated from measured TOC and hardness values of the river water sample and those of dechlorinated tap water.

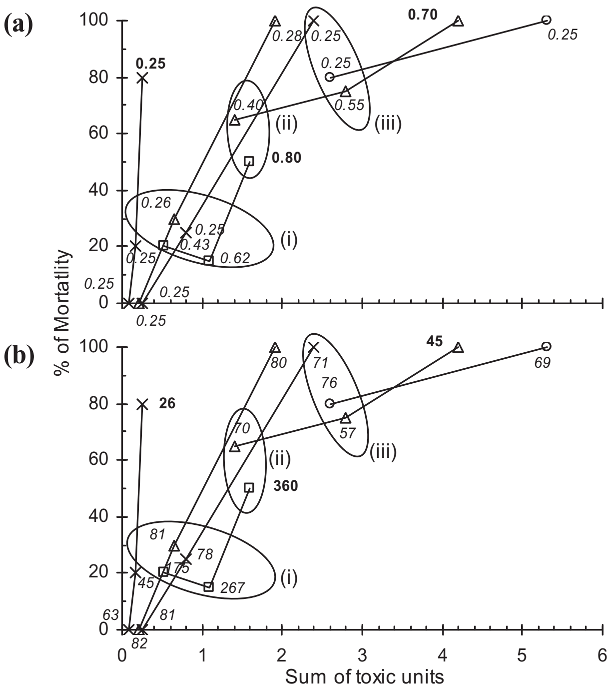

Fig. 3 Relationships between ΣTU and mortality of *Daphnia magna* for different levels of (a) TOC and (b) hardness for the Namari River (○), Kaishu River (△), Miyata River (□), and Watarase River (×). Italicized numbers show estimated TOC (mg C/L) or hardness (mg/L) of test solutions diluted with dechlorinated tap water (TOC < 0.5 mg C/L, hardness as $CaCO_3$ = 82 mg/L). Bold numbers show TOC (mg C/L) or hardness (mg/L) of undiluted test solutions. If a heavy metal concentration or TOC or hardness of a sample was below the quantification limit, a value of half the limit was used for the analysis. (i), (ii), and (iii) indicate groups of distinct trends.

It has been reported that the combined toxicity of heavy metals to aquatic organisms is synergistic–antagonistic,[34–36] and pH[37,38] is an important factor for toxicity. Further studies are required to define the combined toxicity of heavy metals in natural water.

CONCLUSION

River waters contained more than two heavy metals. The combined toxicity of heavy metals to *D. magna* did not depend only on the heavy metals. Organic matter content (TOC) and hardness of river waters reduced the toxicity of heavy metals to *D. magna*.

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