Water Supply

IWA PUBLISHING

Water Supply Vol 22 No 4, 3603 doi: 10.2166/ws.2022.003

Effect-based water quality assessment of rivers receiving discharges from legacy mines by using acute and chronic bioassays with two cladoceran species

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ABSTRACT

Information about the ecotoxicological impacts of surface waters that receive discharges from legacy mines is valuable to infer the ecological impacts on natural environment for managing mine discharges. In this study, we investigated behavioural and reproductive responses of two cladoceran species *Ceriodaphnia dubia* and *Daphnia magna* to water samples collected from metal-contaminated and reference rivers near legacy mines in Japan. The toxicity identification evaluation (TIE) of water samples that caused *D. magna* immobility was conducted to evaluate the key metals causing acute toxicity. The results of our water quality assessment performed using two cladoceran species demonstrated modest to significant adverse effects on their behaviour and reproduction, suggesting the potential for ecotoxicological impacts on natural populations and communities at several contaminated sites that received mine drainage. The results of TIE of water samples that caused *D. magna* immobility indicated likely contributions of Zn and Cu. These results imply that effect-based water quality assessments such as ours can provide direct and unique evidence of the ecotoxicological impacts of metals in river waters, which will be useful for better understanding and predicting the ecological effects of these metals in the natural environment.

Key words: abandoned mine, bioavailability, ecotoxicity, heavy metals, water flea, water pollution

HIGHLIGHTS

- Results of effect-based water quality assessment by using two cladoceran species suggest the potential ecotoxicological impacts at contaminated rivers receiving mine drainages in Japan.
- Toxicity identification evaluation of water samples suggested a major contribution of Zn and Cu.
- Effect-based water quality assessments can provide direct and unique evidence of the ecotoxicological impacts of metals.

INTRODUCTION

Worldwide, the water quality in aquatic environments such as rivers that receive mine discharges is observed to be impaired (Fields 2003). In such aquatic environments, elevated metal concentrations are observed because of the inflow of mine discharges (Gemici 2008; Byrne *et al.* 2013). Thus, for the management of mine discharges, information about the ecotoxicological impacts of metals on the aquatic environments is valuable to infer the ecological impacts on the natural environment.

The ecotoxicological impact of metals is often evaluated by measuring the metal concentrations in the environment and through biological surveys (Namba *et al.* 2020). However, measuring concentrations of a limited number of metals has several limitations in estimating the ecotoxicological impacts driven by water quality. For example, the metals or chemical compounds that are not measured or quantified may play an important role in determining the overall toxicity. In addition, the metal toxicity and bioavailability can be affected by several water quality parameters such as pH, hardness and dissolved organic matter, as well as by interactions among metals (Murano *et al.* 2007; Meyer *et al.* 2015). Biological surveys accompanied by collection of aquatic organisms such as fish and macroinvertebrates can help to directly assess the ecological consequences of exposure in real environments (Namba *et al.* 2020). However, biological surveys require a careful study

design, including selecting appropriate reference sites (Takeshita *et al.* 2020) and professional and time-consuming work in the field or/and laboratory.

Given these limitations and challenges, effect-based water quality assessment using bioassays present can play a complementary and important role in assessing the ecotoxicological impacts of metal contamination in aquatic environments. In this type of assessments, biological responses to a water sample are induced by the combined effects of all chemical mixtures in the water sample, and therefore, these responses reflect the whole toxicity of the water sample (van Dam & Chapman 2001). Effect-based water quality assessment has been used to identify the ecotoxicological impacts and risks of chemicals in effluents and surface waters (de Paiva Magalhães *et al.* 2014; De Baat *et al.* 2019). In Canada, the Metal Mining Environmental Effects Monitoring Program adopts effect-based water quality assessments to examine the adequacy of metal mining effluent regulations (Environment Canada 2012). The effect-based approach can be applied to identify the key chemicals that cause ecotoxicological impacts. In this regard, the U.S. Environmental Protection Agency (USEPA) developed the toxicity identification evaluation (TIE) method to identify key chemicals among thousands of potentially toxic compounds (USEPA 1991, 1993). The TIE approach was applied extensively to identify the major toxicity-inducing compounds in effluents, including industrial wastewaters (Maltby *et al.* 2000; Fang *et al.* 2012). The TIE can be useful for identifying the key metals in the natural waters into which the effluent from an abandoned mine is discharged.

Japan has more than 5000 legacy mines (JOGMEC, http://www.jogmec.go.jp/english/mp_control/mp_control_metal_10_000005.html) and approximately 100 mine drainages were treated with support from the Ministry of Economy, Trade and Industry for ensuring compliance with the effluent standards (Iwasaki *et al.* 2021). Elevated concentrations of metals such as Zn were also observed in river waters that received mine discharges of several legacy mines in Japan (e.g. Namba *et al.* 2021). Although several biological surveys were performed to investigate the ecological impacts in such metal-contaminated rivers (Iwasaki *et al.* 2011, 2020; Namba *et al.* 2021), the application of effect-based water quality assessments to the metal-contaminated rivers in Japan is surprisingly limited (Murano *et al.* 2007). Therefore, it is important to examine the applicability of such assessment based on bioassays and TIE method in Japan to develop an integrated approach with biological surveys and water quality measurements. Such an integrated approach will help us better understand and evaluate the ecotoxicological impacts in metal-contaminated rivers.

In this study, two cladoceran species, *Ceriodaphnia dubia* and *Daphnia magna* were used. These are standard test species used for the hazard assessment of chemicals and effluents and are sensitive to trace metals. We investigated the biological responses of these species to the water samples collected from metal-contaminated and reference rivers near legacy mines in Japan. By comparing the toxicity of the water samples from metal-contaminated rivers with that of the water samples from the reference rivers and culture media as controls, we assessed the ecotoxicity in the metal-contaminated rivers. In addition, the TIE results of the water samples that caused acute toxicity to *D. manga* were obtained to evaluate the key metals causing the acute toxicity.

METHODS

Study sites

Water sampling was performed at 24 sites located on four river basins in Hokkaido, Kinki, Chubu and Tohoku regions in the spring seasons of 2018–2020 (Figure 1). In this paper, the four river basins, Tokushibatsu, Ichi, Kiso and Waga River basins, are labelled as T, I, K and W, respectively. In Tokushibetu and Ichi River basins, we selected contaminated sites in a river that received mine discharge (site names including S) and reference sites (site names starting with R) in a nearby uncontaminated river within the same basin. S1a and S1b sites were sites upstream and downstream of the inflow of the mine discharge from the corresponding legacy mine, respectively. For these two River basins, multiple reference sites were selected to examine whether the water samples collected from a nearby river that was unaffected by mine drainage exhibited any toxicity. Sampling sites were carefully chosen so that the physicochemical conditions other than those related to metal contamination were similar between the reference and contaminated sites. The study sites with the same numbers had similar elevation levels, for example, T-S4 and T-R4. In the Kiso River basin, because we could not establish any reference sites in the rivers close to the contaminated site (K-S1), we established three reference sites with similar elevation (970–1,270 m) and catchment areas (6–14 km^2) in different tributaries within the basin. In the Waga River basin, we established three pairs of reference and contaminated sites near upstream and downstream of the inflow of tributaries containing mine discharges

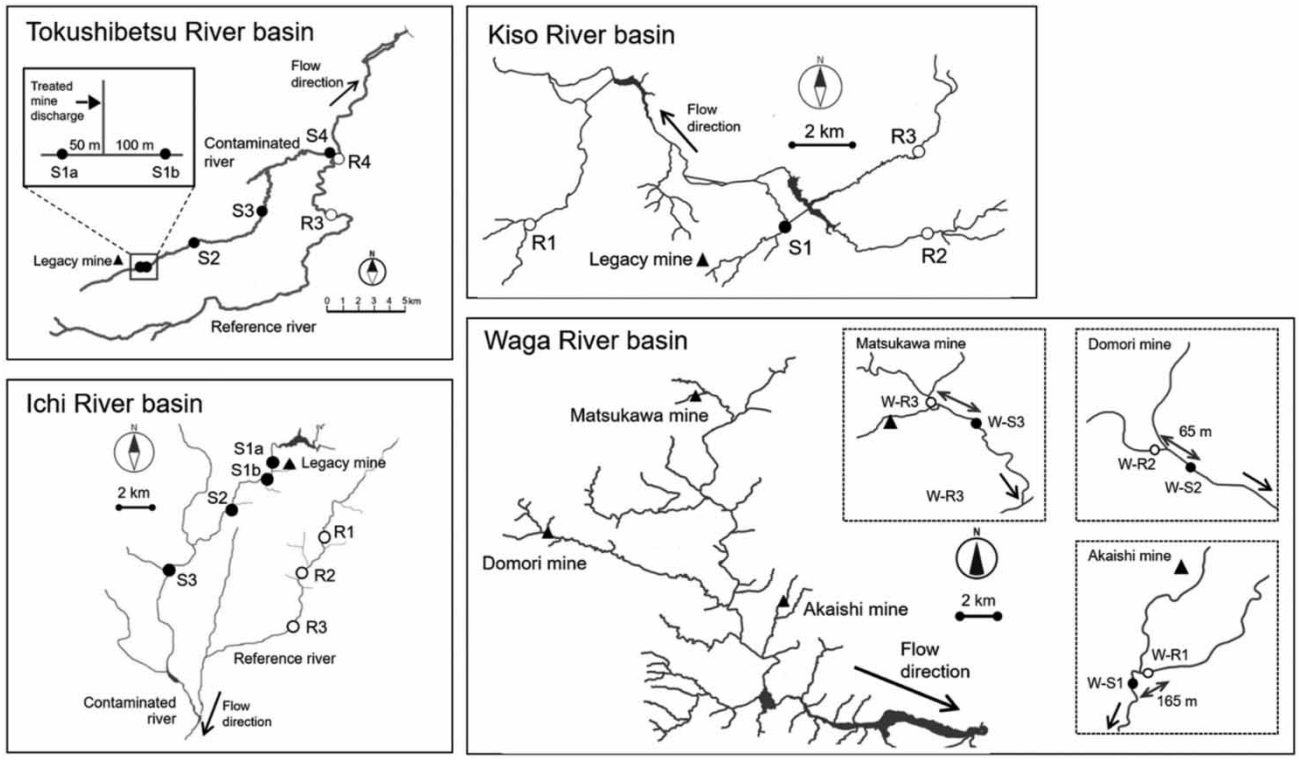

Figure 1 | Maps showing the study sites, including the reference sites in Tokushibetsu, Ichi, Kiso and Waga River basins. Open and filled circles indicate reference and contaminated sites, respectively. In the Waga River basin, three pairs of reference and contaminated sites near upstream and downstream of the inflow of tributaries containing mine discharges from the Akaishi, Domori and Matsukawa mines were established. The appropriate locations of these mines are shown using the solid triangles in this map.

from Akaishi (sites W-R1 and W-S1), Domori (W-R2 and W-S2) and Matsukawa (W-R3 and W-S3) mines in three different rivers. The distances between the inflow of mine discharge and the downstream contaminated sites for these legacy mines were approximately 165, 65 and 560 m, respectively.

Water sampling and water chemistry measurements

At each site, 10 L of river water for bioassays were collected in a 10-L polyethylene tank, transported to our laboratory and then filtered through a 60-μm nylon filter (DIN100, Rigosha, Tokyo, Japan). The filtered waters were stored in the dark at 4 °C until the toxicity tests were performed.

The water chemistry of a river water sample from each site was analysed. The results of water chemistry analyses of river water samples from the Hokkaido area are reported in Namba *et al.* (2021). Hence, the procedures for analysing the water samples from sites of the Kinki, Chubu and Tohoku regions are described here. Water temperature, dissolved oxygen (DO), pH and electrical conductivity were measured at each site by using multi parameter portable metres (Multi 3630IDS, Xylem Analytic Germany, Weilheim, Germany). Three 50-mL water samples were obtained for measuring the concentrations of four metals (Cd, Cu, Pb and Zn) and two 100-mL water samples were obtained for measuring the dissolved organic carbon (DOC) and major ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^- and SO_4^{2-}) at each site; these samples were obtained by filtering the water through a 0.45-μm hydrophilic polytetrafluoroethylene filter. The water samples for analysing the metals were acidified with 1% nitric acid. The water samples were transported under refrigeration. The metal concentrations were measured by inductively coupled plasma–sector field mass spectrometry (ELEMENT XR, Thermo Fisher Scientific Inc., Bremen, Germany). The DOC was measured using a total organic carbon analyser (TOC-L CPH, Shimazu Corp., Kyoto, Japan). The positive ions Mg^{2+}, Ca^{2+}, Na^+ and K^+ and the negative ions Cl^- and SO_4^{2-} were analysed by atomic absorption spectroscopy (Hitachi Z-2000, Hitachi High Technologies Corp., Tokyo, Japan) and ion chromatography (DIONEX ICS-1100, Thermo Fisher Scientific, Tokyo Japan), respectively.

Test species

Two cladoceran species, namely, *C. dubia* and *D. magna*, were used to conduct bioassays of the river water samples. Strains of *C. dubia* and *D. magna* were obtained from the National Institute for Environmental Studies in Japan.

The stock cultures of *C. dubia* were maintained in an 8:2 mixture (total hardness: 10–30 mg $CaCO_3$/L) of the slightly modified USEPA very soft reconstituted water and slightly modified USEPA moderately hard reconstituted water. The slightly modified USEPA very soft reconstituted water was obtained by adding 2 μg/L of vitamin B_{12} and 4.8 μg/L of sodium selenite (USEPA 2002; Environment Canada 2007) into USEPA very soft reconstituted water (USEPA 2002). The slightly modified USEPA moderately hard reconstituted water was obtained by adding 2-μg/L vitamin B_{12} and 4.8-μg/L sodium selenite (USEPA 2002; Environment Canada 2007) into the USEPA moderately hard reconstituted water (USEPA 2002). The cultures of immature *C. dubia* individuals were prepared in glass beakers containing 200 mL of the culture medium and 50 immature individuals. The adult *C. dubia* cultures were prepared in 500-mL glass beakers containing 400 mL of the culture medium and 30 adults. The culture medium was changed three times a week. The cultures were daily fed *Chlorella vulgaris* (Recenttec K. K., Tokyo, Japan), a green alga, at a concentration of 1×10^5 cells/mL, and yeast, cerophyll and trout chow (YCT, Recenttec K. K., Tokyo, Japan) at the appropriate concentration. The cultures were maintained at 25 °C $\pm$1 °C under a 16-h light and 8-h dark photoperiod.

The cultures of the immature *D. magna* individuals were prepared in glass beakers containing 200 mL of culture medium and 40 immature individuals. The adult *D. magna* cultures were prepared in glass beakers containing 1 L of the culture medium and 35 adults. The culture medium was changed three times a week. The cultures were fed *C. vulgaris* daily at a concentration of 5×10^5 cells/mL. The cultures were maintained at 21 °C$\pm$1 °C under a 16-h light and 8-h dark photoperiod (light intensity up to 15 μE/m^2/s).

The stock cultures used for the acute immobilisation toxicity tests were maintained in a 9:1 ratio of the slightly modified USEPA moderately hard reconstituted water and dechlorinated tap water (total hardness: 60–90 mg $CaCO_3$/L). As the water hardness of the river water samples was much lower than that of the routine stock cultures, it could affect reproduction of *D. manga*, and the stock cultures used for chronic toxicity tests were acclimated to low-hardness water for at least two generations. For the acclimation to low-hardness, stock cultures of *D. manga* were maintained in an 8:2 mixture of slightly modified USEPA very soft reconstituted water and dechlorinated tap water (total hardness: 10–30 mg $CaCO_3$/L).

Bioassays

All the bioassays were conducted under identical conditions of water temperature, photoperiod and light intensity for maintaining the stock cultures. The DO (measured using HI 2040-01, Hanna Instruments Japan Corp., Chiba, Japan), pH and water temperature (measured using AS800, AS ONE Corp., Osaka, Japan) in the test solutions were measured at the test solution renewal of the reproduction tests and at the beginning and end of the acute tests.

Reproduction test of *C. dubia*

The reproduction test using *C. dubia* was conducted according to the test methods described by USEPA (USEPA 2002) and Environment Canada (Environment Canada 2007). Before the test, individual cultures were performed as single cultures to provide test organisms. One neonate was taken from the stock cultures and placed in each of a series of 50-mL glass cups containing 15 mL of a 2:8 mixture of Volvic (Kirin Beverage Company Ltd, Tokyo, Japan) and the slightly modified USEPA very soft reconstituted water. Green alga *C. vulgaris* with 1×10^8 cells/mL (50 μL) and YCT (50 μL) were added daily into each glass cup. The individual organisms were transferred to new culture water once every two days. Neonates produced from the third or fourth broods were used for the tests.

Neonates (<24 h old) were individually transferred to a 50-mL glass cup containing 15 mL of exposure solution. Each treatment consisted of 10 replicates of a particular non-diluted river water sample or the medium used for single culture as the control. The survival of the test individual and number of offspring in each glass cup were observed for up to eight days. One replicate from the same parent in each of test solutions for Tokushibetsu River basin was excluded from data analysis because it presented lower reproductive output than the other replicates (i.e. nine replicates were analysed for the test with river waters collected from Tokushibetsu River basin). Tests were conducted under identical conditions of food supply as those adopted for maintaining single cultures.

Reproduction test of *D. magna*

The reproduction test using *D. magna* was conducted as a reference to the test method by the OECD test guideline 211 (OECD 2012). One neonate (<24 h old) from the second or later brood was taken from a stock culture and placed into a 100-mL polypropylene beaker with 40 mL of the exposure solution. Each treatment was carried out in 10 replicates for a particular non-diluted river water sample or the medium used for stock culture as the control. Further, 60 μL of green alga *C. vulgaris* containing 5×10^8 cells/mL was added daily into each beaker. The exposure solution was newly prepared and renewed once every two or three days. The number of offspring and mortality were checked daily in each replicate of each treatment.

Acute immobilisation test of *D. magna*

The acute immobilisation test using *D. magna* was conducted according to the test method prescribed by the OECD test guideline 202 (OECD 2004). Five neonates (<24 h old) from the second or later brood were taken from a stock culture and placed into a 100-mL polypropylene beaker with 50 mL of the exposure solution. Each treatment was performed in four replicates for a particular non-diluted river water solution or the medium used for stock culture as the control. The number of immobile individuals was counted in each beaker every 24 h for 48 h.

TIE was conducted for the river water samples, in which acute immobility was observed with reference to USEPA (USEPA 1991, 1993). First, the ethylenediamine tetraacetic acid (EDTA) addition tests of the river water samples were conducted to check whether the metal toxicants in the samples caused immobilisation. Second, based on results of the acute toxicity tests of four metals (Cd, Cu, Pb and Zn) in the low-hardness test medium and based on the concentrations of the four metals in the river water samples, the contributions of the metals to *D. magna* immobility in the water samples were evaluated.

Test solutions were prepared by adding EDTA disodium salt dihydrate (CAS No. 6381-92-6) (reagent grade, MP Bio Japan K.K., Tokyo, Japan) to the river water samples at concentrations of 0, 3 and 8 mg/L according to USEPA (1992). Each treatment for a river water was carried out in two replicates; for each replicated, 10 neonates (<24 h old) from the third or later brood were taken in a 100-mL polypropylene beaker with 50 mL of each test solution. The number of immobile individuals was counted in each beaker for 48 h.

Acute toxicity tests of Cd, Cu, Pb and Zn were conducted in a low-hardness medium. Stock solutions of the four metals were prepared by individually dissolving metal compounds, cadmium sulphate-water (3/8) (CAS No. 7790-84-3), lead (II) nitrate (CAS No. 10099-74-8), copper (II) sulphate pentahydrate (CAS No.7758-99-8) and zinc sulphate heptahydrate (CAS No. 7746-20-0) (reagent grade, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) in MilliQ water. Each test solution was obtained by adding a stock solution with an 8:2 mixture of the slightly modified USEPA very soft reconstituted and the slightly modified USEPA moderately hard reconstituted waters. Each toxicity test included a control and no less than five spiked metal concentrations. The prepared test solutions were allowed to equilibrate for at least 12 h. The number of immobile individuals was counted in each beaker for 48 h. The tested metal concentrations in the test solutions sampled at the beginning and end of each toxicity test were measured by the same analytical methods for measuring the water chemistry of river water samples. The geometric means of the measured metal concentrations at the beginning and end of the tests were calculated to estimate the toxicity. Before the start of the toxicity tests, the concentrations of the DOC and major ions (Mg^{2+}, Ca^{2+}, Na^{+}, K^{+}, Cl^{-} and SO_4^{2-}) in the control solution were measured three times.

Model calculation of metal speciation

Because the free-ion activity of the metals in water is responsible for the waterborne toxicity of the metal (de Paiva Magalhães *et al.* 2015), the speciation of Cd, Cu, Pb and Zn in the river waters causing *D. manga* immobility and the test solutions for *D. magna* acute toxicity tests of the metals were calculated using WHAM VI software (Tipping 1998). We assumed that the humic substances in the river waters and test solutions were almost only fulvic acid and accounted for 40% of the dissolved organic matter, which comprises 50% carbon (Dwane & Tipping 1998). Accordingly, for each solution, the concentration of the active fulvic acid was 0.8 times the DOC concentration. The input variables were the nominal water temperature, measured pH and measured concentrations (M) of the major ions (Ca, Mg, Na, K, Cl^{-} and SO_4^{2-}) and dissolved metals (Cd, Cu, Pb and Zn).

Evaluation of contributions of four metals to the acute toxicity of river waters to *D. magna*

To examine the contribution of each metal to the acute toxicity of a river water sample, the toxic unit (TU) values of the metal in the sample were calculated by using the following equation:

$$TU_{metal} = Concentration_{metal}/EC_x$$

where TU_{metal}, $Concentration_{metal}$ and EC_x indicate the acute toxic units of the metal, metal concentration in the river water and percentage effect concentration of the metal, respectively. In the present study, three TU values of each metal were calculated by dividing the estimated free-ion activity of the metal in the river water sample by EC_{10}, EC_{50} and EC_{90} values of the metal free-ion activity. A TU value greater than 1 for a metal in a river water sample, which was calculated based on EC_x, implies that the free-ion activity of the metal in the sample was higher than the x% effect ion activity.

To test whether the toxicity of water samples causing *D. manga* mobility can be explained by the combined toxicity of the trace metals contributing to *D. manga* immobility, we estimated the *D. manga* immobility induced by the metals in the water samples by using the zinc toxic equivalent quantity (Zn-TEQ); this method was previously adopted for studying Japanese waters (Matsuzaki 2011). The Zn-TEQ (unit: mM) is the sum of the concentration of Zn and concentrations of other coexisting trace metals converted to Zn equivalents by adopting a concentration addition approach that assumes these metals have the same mode of action:

$$Zn - TEQ = \left(\sum \frac{C_j}{RV_j}\right) \times RV_{Zn} + C_{Zn}$$

where j represents a metal other than Zn (e.g. Cu); C_j and C_{Zn} represent the concentrations of free-ion activities of metal j and Zn in a water sample, respectively; RV_j and RV_{Zn} indicate the reference toxicity values of ion activities of a metal j and Zn, respectively. In the present study, we used EC_{50} values [mM] of the free-ion activities for the metals considered in the *D. manga* immobility tests as the reference toxicity values. Then, we calculated the Zn-TEQ values. We used the Zn-TEQ values and log–logistic model estimated based on Zn free-ion activity to predict the immobility in the water samples. The predicted frequency of the immobilised test individuals, which was evaluated by multiplying the predicted immobility by 20, was compared with the observed frequency in the water samples.

Statistics

The effect of the exposure to a river water sample on the biological response was investigated by comparing the response to exposure of the river water sample with the response of a control solution. If the reproduction of test individuals in water samples from a contaminated site and the corresponding site did not differ from that in the control, a statistically significant difference between the contaminant and reference sites can indicate the adverse effect of the river water from the contaminated site on reproduction. Therefore, we compared the reproduction outputs of the individuals exposed to river waters from a contaminated site with those of the individuals exposed to a corresponding reference site.

Data analyses were conducted in R version 3.6.3 (R Core Team 2020). For all the statistical tests, the significance level was set to 0.05. Homogeneity of variance in data on reproduction was checked by the Levene test (*leveneTest* function of the *car* package). Reproduction data found to be homogeneous were analysed using the multiple comparison with the single-step method for adjusting the *P*-values with the *glht* function of the *multcomp* package to conduct pairwise comparisons of the reproduction outputs in a control solution and in water samples from a contaminated site and the corresponding reference site. The data that did not meet the assumption of homogeneity of variance were analysed by comparing all pairs by Welch's *t*-test with the Holm's correction (*pairwise.t.test* function of the *stats* package). For the Tokushibetsu basin, because the water samples from reference sites corresponding to the three contaminated sites T-S1a, T-S1b and T-S2 were not sampled, the results of the toxicity tests of the river water samples from the contaminated sites were compared with those of a control solution and those of water samples from downstream reference sites T-R3 and T-R4. In addition, the reproduction outputs in the water samples from sites S1a and S1b in each of Tokushibetsu and Ichi River basins were compared simultaneously by multiple comparison tests to investigate effects of the discharge of treated and non-treated mine drainage on the reproduction of *D. magna* and *C. dubia*.

The comparison of the frequency of immobilised individuals in a river water sample with that in a control solution was performed using Fisher's exact test (*fisher.test* function of the *stats* package). The 48-h EC_{10}, EC_{50} and EC_{90} values of the

four metals for *D. magna* immobility and the corresponding 95% confidence intervals were analysed using the *drc* package. Data were fitted to two-parameter log–logistic (LL.2) models with binomial-type error distributions using the *drm* function. The predicted immobility rate was modelled as

$$\text{Immobility rate} = \frac{1}{1 + \exp\left(b \times (\log(x) - \log(e))\right)}$$

where x is the free-ion activity of a metal and *b* (slope) and *e* (median effective concentration) are model parameters that need to be estimated. The goodness-of-fit test based on Pearson's statistic was conducted using the *modelFit* function to test whether the log–logistic model fitted the available data of each toxicity test. The significance of the two model parameters was examined by *t*-tests. Further, to examine the Zn-TEQ-based predictions, the predicted and observed frequencies of immobilised individuals in the river water samples, in which *D. manga* immobility was detected, were compared using Fisher's exact test.

RESULTS AND DISCUSSION

Water chemistry of the river water samples

Across all the study sites, the ranges of pH, DOC and water hardness were 7.0–8.0, 0.2–1.1 and 11–29 mg $CaCO_3$/L, respectively. The observed ranges were relatively narrow, and in the individual river basins, the pH and DOC and major ion concentrations at the contaminated sites were similar to the values from the corresponding reference sites. Summaries of the field and water chemistry measurements other than for Cd, Cu, Pb and Zn for the river water samples collected from the study sites are provided in Tables S1 and S2.

Table 1 lists the dissolved concentrations of Cd, Cu, Pb and Zn in the water samples from the Tokushibetsu, Ichi, Kiso and Waga River basins. In the Tokushibetsu and Ichi River basins, although the metal contamination levels varied depending on the site locations (e.g. sites further from the inflow of mine discharge were less contaminated), the dissolved metal concentrations at the contaminated sites were generally higher than those at the reference sites. For example, the dissolved concentrations of Zn at the contaminated sites in these two rivers ranged from 4.8 to 194.4 μg/L but those at the reference sites were below 2.5 μg/L. Similarly, in the Kiso River basin, the dissolved concentrations of all the four metals at a contaminated site were markedly higher than those at the three reference sites. In the Waga River basin, the dissolved Cu concentrations at the three contaminated sites were relatively higher than those at the three reference sites as well as those at the contaminated sites in the other basins.

Toxicity tests of the river water samples

Bioassays of the river waters collected from the four river basins near legacy mines and from the culture media as control were conducted using *D. magna* and *C. dubia* to examine the effects of metal contaminants in river waters on the biological responses of the two species. Results of controls in all toxicity tests of the river waters met the criteria of toxicity tests for validating performance in controls (USEPA 2002; OECD 2004, 2012). Figures 2 and 3 show as reproduction responses the total number of surviving offspring produced by *C. dubia* (Figure 2(a)–2(d)) and *D. manga* (Figure 3(a)–3(d)) test individuals exposed to each river water sample. Summaries of the total number of living offspring per replicate, pH, DO and water temperature in test solutions are listed in Tables S3–S10. All the *C. dubia* test individuals exposed to the river water sample from site K-S1 died before producing offspring (Figure 2(c)). The total number of living offspring was significantly lower in river water samples from I-S1a, I-S1b, I-S2, I-S3, K-S1 and W-S1 than in the control and corresponding reference sites (a 27–100% reduction) (Figure 2(b) and 2(d)). The results of *C. dubia* reproduction tests indicated a range of adverse biological effects of river water samples from these contaminated sites.

The total numbers of offspring produced by the *D. magna* test individuals in water samples mostly collected from the reference sites (i.e. I-R1, I-R2, K-R1, W-R1, W-R2, W-R3 and W-S2) were significantly higher than the total numbers in the control. All the *D. magna* test individuals exposed to the river water samples from sites I-S1b, I-S2, K-S1 and W-S1 died before producing offspring (Figure 3(b)–3(d)). The total number of living offspring was significantly lower in the river water samples from sites I-S1a, I-S1b, I-S2, I-S3, K-S1 and W-S1 than in the control and water samples from the corresponding reference sites (21–100% reduction) (Figure 3(b)–3(d)). The test individuals exposed to river water samples from sites K-R2 and W-S3 produced significantly less offspring than those exposed to the control (31% reduction) and to the river water sample from the

Table 1 | Dissolved concentrations of four metals in river waters from study sites in the Tokushibetsu (T), Ichi (I), Kiso (K) and Waga (W) River basins

River basin	Sites	Cd (μg/L)	Cu (μg/L)	Pb (μg/L)	Zn (μg/L)
Tokushibetsu[a]	T-R3	<0.005	0.1	0.04	0.1
	T-R4	<0.005	0.1	0.03	0.3
	T-S1a	0.13	1.0	0.69	24.0
	T-S1b	0.16	1.1	0.71	27.5
	T-S2	0.17	0.8	0.25	25.9
	T-S3	0.07	0.5	0.23	11.5
	T-S4	<0.005	0.3	0.05	4.8
Ichi	I-R1	0.008	0.20	<0.025	<2.5
	I-R2	<0.005	0.19	<0.025	<2.5
	I-R3	<0.005	0.18	<0.025	<2.5
	I-S1a	0.168	0.42	0.551	43.9
	I-S1b	1.277	2.6	0.673	194.4
	I-S2	0.878	2.6	0.294	143.2
	I-S3	0.180	1.1	<0.025	33.9
	LOQ[b]	0.005	0.025	0.025	2.5
Kiso	K-R1	<0.001	<0.1	<0.001	<2.5
	K-R2	<0.001	<0.1	<0.001	<2.5
	K-R3	<0.001	<0.1	<0.001	<2.5
	K-S1	0.894	2.4	0.168	170.5
	LOQ[b]	0.001	0.1	0.001	2.5
Waga	W-R1	<0.001	0.30	<0.025	0.9
	W-S1	1.606	34.5	0.067	69.0
	W-R2	0.039	0.8	0.086	4.9
	W-S2	0.073	6.1	0.142	9.9
	W-R3	0.007	0.9	<0.025	1.7
	W-S3	0.024	7.7	<0.025	12.5
	LOQ[b]	0.001	0.1	0.025	0.5

[a]Concentrations of four metals in river waters from study sites in the Tokushibetsu basin were referred from Namba *et al.* (2021).
[b]Limit of quantification.

corresponding reference site (27% reduction) (Figure 3(c) and 3(d)). The results of the *D. manga* reproduction tests indicated a range of adverse biological effects of the river water samples from the seven contaminated sites (i.e. I-S1a, I-S1b, I-S2, I-S3, K-S1, W-S1 and W-S3) and a reference site (K-R2).

Almost all the test individuals of *D. manga* exposed to the river water samples of sites I-S1b, I-S2, K-S1 and W-S1 exhibited immobility ($P<0.05$; Table S11), indicating the significant acute toxicity to *D. manga* by the river waters from these contaminated sites. Table S11 presents a summary of the pH, DO and water temperature in the test solutions.

These results clearly demonstrate the adverse effects of river waters from most of the contaminated sites in the Ichi, Kiso and Waga River basins on the biological responses of the two species, indicating the potential for ecotoxicological impacts on the natural populations and communities at these sites (Hickey & Clements 1998). In the Tokushibetsu River basin, no adverse effect was observed on the reproduction of the two species at any study site. The results of the biological surveys performed by collecting fish and macroinvertebrates in the Tokushibetsu River at the same time as the sampling of the river waters in this study showed the reduction of a few macroinvertebrate metrics such as the abundance of metal-susceptible heptageniid mayflies at sites T-S1a and T-S1b (Namba *et al.* 2021). However, Namba *et al.* (2021) did not detect any clear impacts on the abundances and conditions of four fish species collected at these sites. They suggested that the metal contamination levels at sites T-S1a and T-S1b may have been close to the threshold where some adverse effects on susceptible macroinvertebrates would be detected. Although further testing is required, these results based on the biological surveys together with the results of the toxicity tests performed in this study suggests that the sensitivity of reproduction bioassays of *D. manga* and *C. dubia* to metal contamination could be lower than that of the responses of metal-susceptible macroinvertebrates in the field.

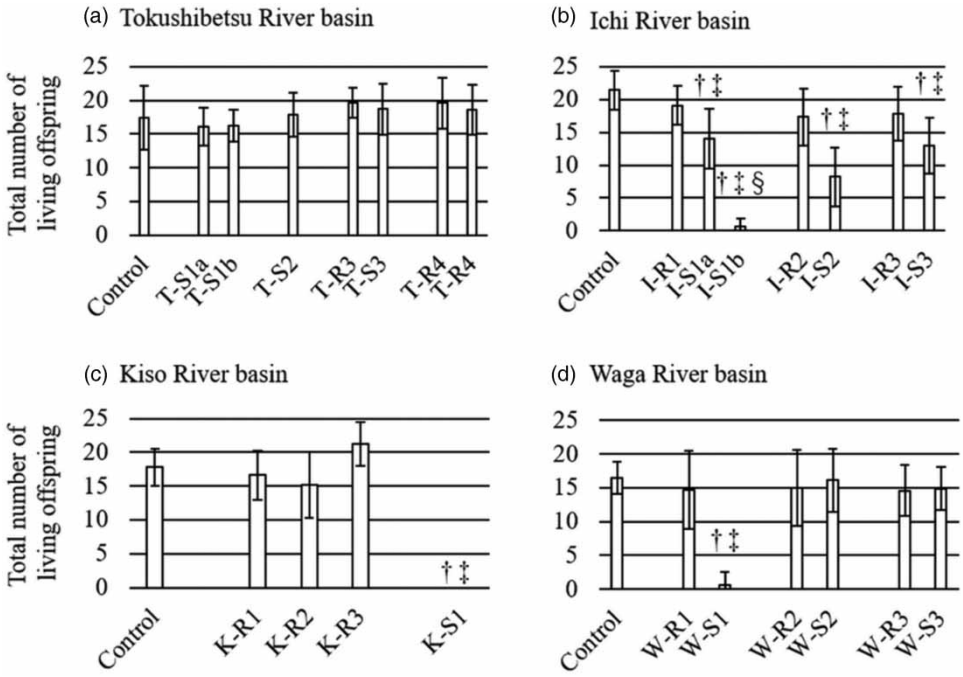

Figure 2 | Mean total numbers of the living offspring produced by *C. dubia* test individuals exposed to the control solution and river water samples from sites in the (a) Tokushibetsu, (b) Ichi, (c) Kiso and (d) Waga River basins. Error bars indicate standard deviations. † indicates that total number of produced offspring was significantly lower in a water sample from a site than in a control solution ($P < 0.05$). ‡ indicates that the total number of produced offspring was significantly lower in a sample from a contaminated site than that from the corresponding reference site ($P < 0.05$). § indicates that total number of produced offspring was significantly lower in a water sample from site S1b than in a water sample from site S1a in the (b) Ichi River basin ($P < 0.05$).

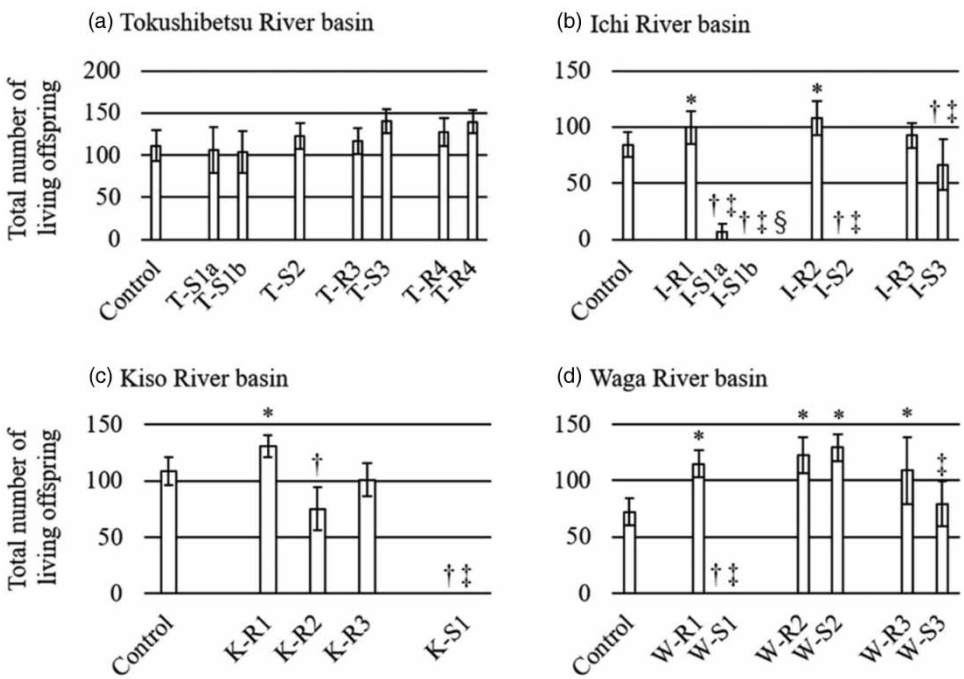

Figure 3 | Mean total numbers of living offspring produced by *D. magna* test individuals exposed to the control solution and river water samples from sites in the (a) Tokushibetsu, (b) Ichi, (c) Kiso and (d) Waga River basins. Error bars indicate standard deviations. * and † indicate that total number of produced offspring was significantly higher and lower in a water sample from a site than in a control solution, respectively ($P < 0.05$). ‡ indicates that indicates that total number of produced offspring was significantly lower in a sample from a contaminated site than that from the corresponding reference site ($P < 0.05$). § indicates that total number of produced offspring was significantly lower in a water sample from site S1b than in a water sample from site S1a in the (b) Ichi River basin ($P < 0.05$).

In addition, Iwasaki *et al.* (2012) investigated the ecological impacts of metal contamination with focus on Zn on benthic macroinvertebrate communities at the same study sites, excluding I-S1a in the Ichi River basin. Although their field study was conducted in 2007, the dissolved concentrations of the trace metals such as Cu and Zn were generally comparable at I-S1b and I-S2 (in their study, named as R1 and R2), and there were significant decreases in the abundance and richness of macroinvertebrates (such as mayfly abundance and richness) at these sites. Interestingly, we observed the immobility of (almost) all the individuals of *D. magna* in the acute toxicity of water samples collected from these sites. Considering that both the previous study on macroinvertebrates and our toxicity tests detected significant effects, the results of these two different approaches can be regarded as consistent. Furthermore, our results demonstrated the adverse effects of the water sample collected from site I-S3 on the reproduction of two species, suggesting the potential for the ecotoxicological impacts on natural communities at the site. Although an accurate comparison is difficult, Iwasaki *et al.* (2012) detected the reductions of a few heptageniid mayfly species at the same site (i.e. I-S3). These encouraging results emphasise the importance of further quantifying the relationships of the results of cladoceran toxicity tests with water samples to the ecological impacts observed in the field; this can enhance the value of the bioassays in predicting the field effects.

The total numbers of living offspring produced by *C. dubia* and *D. magna* test individuals were significantly lower in the water sample from site I-S1b than in the water sample from site I-S1a (Figures 2(b) and 3(b)). The observed differences in the *D. magna* mobility and in the reproduction of *D. magna* and *C. dubia* between sites I-S1a and I-S1b suggest that the increased adverse effects are likely associated with the inflow of mine drainage between these two sites. Biological surveys will be valuable to examine adverse effects on natural populations and communities at the sites where potential ecotoxicological impacts of metals are a concern.

The effect-based water quality assessment based on the reproductive responses of both *D. magna* and *C. dubia* detected similar adverse effects for the water samples collected from the contaminated sites, except for a site W-S3. At site W-S3, there was a significant reduction in the reproduction response of *D. magna* compared with the corresponding reference site (W-R3), but not so for *C. dubia*. This difference may be attributed to a greater total number of offspring reproduced by *D. magna* in the water sample from a reference site W-R3 than in the control.

The exposure of the water sample from reference site K-R2 caused an adverse effect (~25% reduction) on the reproduction of *D. manga* but not for *C. dubia*. As the water chemical properties of the site were not different from those of the water samples from the other reference sites, the underlying mechanism that caused the adverse effect is unclear in this study. These results at least suggest the modest potential for identifying the ecotoxicological impacts of the water at the site. Collecting water samples at numerous points of time, e.g. during different seasons of the year can be valuable to reach a more compelling conclusion on the potential ecotoxicological impacts on the natural communities at this site.

Toxicity identification evaluations of river waters

Results of controls in EDTA addition tests of river waters and acute toxicity tests of Cd, Cu, Pb and Zn met the validity criterion of presence of no more than 10% of immobile individuals in controls (OECD 2004). Although almost all the test individuals of *D. manga* exhibited immobility in the river water samples collected from sites I-S1b, I-S2, K-S1 and W-S1, no acute toxicity (0% immobility) was detected in all tested water samples after the addition of EDTA (both 3 and 8 mg/L; see Table S12 for the summary of the test results). These results indicated that the cation metals probably contributed to the observed acute toxicity of the river water samples.

The log–logistic models were fitted to the acute toxicity test results of Cd, Cu, Pb and Zn to estimate the acute toxicity values of free-ion activities of the metals (Figure 4). The log–logistic models of the free-ion activities of Cd, Cu and Zn had similar slopes (the model parameter, *b*), while that for Pb free-ion activity had a steeper slope (Table 2). The results of the toxicity tests and summaries of water chemistry measurements of the test medium, pH, DO and water temperature in the test solutions are included in Tables S13–S18. Although, based on our results, the underlying reasons are uncertain, it should be noted that the measured concentrations of dissolved Cu in different test solutions decreased to less than half at the end of the test (Table S16).

The TU values of Cd, Cu, Pb and Zn in the river water samples that caused acute toxicity to *D. manga* were estimated by dividing the estimated free-ion activities of the metals in the river water samples (see Table S19) by the toxicity values of the metal free-ion activities (Table 3). These values are shown in Figure 5. The Cu and Zn free-ion activities in river water samples from sites I-S1b, I-S2 and K-S1 were higher than the EC_{10} value of Cu and EC_{50} value of Zn, respectively (Figure 5(a)–5(c)). These results indicated that Cu and Zn contributed to the acute toxicity of the water samples to *D. manga* and that the

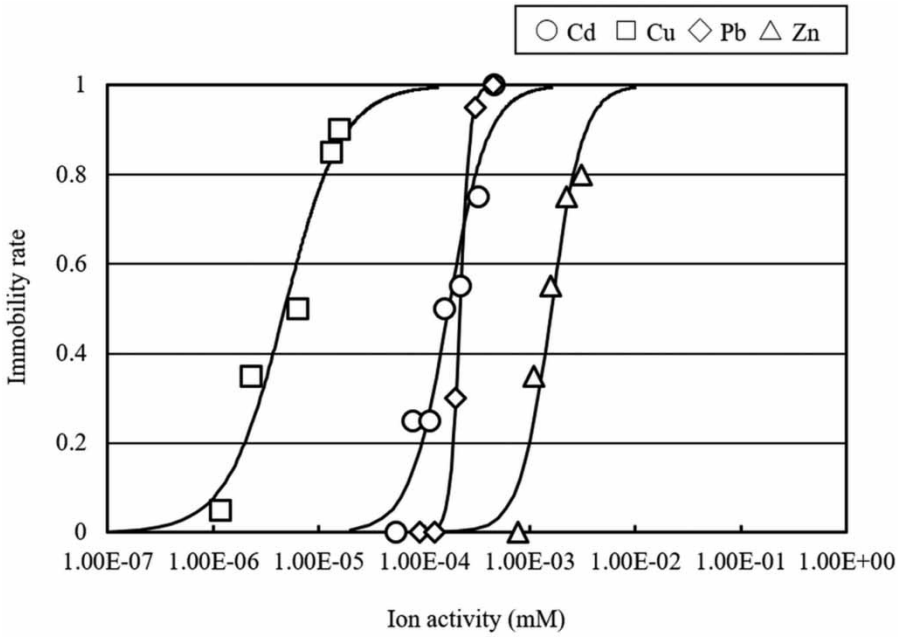

Figure 4 | *D. magna* immobility rates (48 h) against the free-ion activities [mM] of Cd, Cu, Pb and Zn. Circle, square, rhombus and triangle indicate the results for Cd, Cu, Pb and Zn, respectively. A solid line indicates a dose–response curve from a log–logistic model for immobility.

Table 2 | Parameter values in the log–logistic models for the immobility of *D. magna*

	Model parameters[a]		Model fit		
Target metal	***b* (95% C.I.)**	***e***	***df***	χ^2	***P*-value**
Cadmium	−2.36 (−3.11–−1.61)	1.70×10^{-4}	5	7.436	0.19
Copper	−1.59 (−2.16–−1.02)	4.75×10^{-6}	3	3.065	0.38
Lead	−9.31 (−14.01–−4.61)	2.18×10^{-4}	3	0.194	0.98
Zinc	−2.90 (−4.03–−1.76)	1.60×10^{-3}	3	4.782	0.19

[a]All parameter values were significantly different from zero (*t*-test, $P < 0.05$).

Table 3 | Toxicity values of Cu, Cd, Pb and Zn ion activities for the immobility of *D. magna*

	Toxicity values of ion activity (mM)		
Target metal	**EC_{10} (95% C.I.)**	**EC_{50} (95% C.I.)**	**EC_{90} (95% C.I.)**
Cadmium	6.70×10^{-5} (4.43×10^{-5}–8.97×10^{-5})	1.70×10^{-4} (1.40×10^{-4}–2.01×10^{-4})	4.32×10^{-4} (2.79×10^{-4}–5.85×10^{-4})
Copper	1.19×10^{-6} (4.47×10^{-7}–1.94×10^{-6})	4.75×10^{-6} (3.23×10^{-6}–6.27×10^{-6})	1.89×10^{-5} (8.40×10^{-6}–2.95×10^{-5})
Lead	1.72×10^{-4} (1.49×10^{-4}–1.96×10^{-4})	2.18×10^{-4} (1.97×10^{-4}–2.39×10^{-4})	2.76×10^{-4} (2.30×10^{-4}–3.23×10^{-4})
Zinc	7.51×10^{-4} (4.97×10^{-4}–1.00×10^{-3})	1.60×10^{-3} (1.34×10^{-3}–1.87×10^{-3})	3.42×10^{-3} (2.26×10^{-3}–4.58×10^{-3})

contribution of Zn was higher than that of Cu in the water samples. In contrast, the Zn and Cu free-ion activities in the river water sample from site W-S1 were higher than the EC_{10} value of Zn free-ion activity and EC_{90} value of Cu free-ion activity, respectively (Figure 5(d)), suggesting that Cu had a stronger influence than Zn on the acute toxicity to *D. magna*.

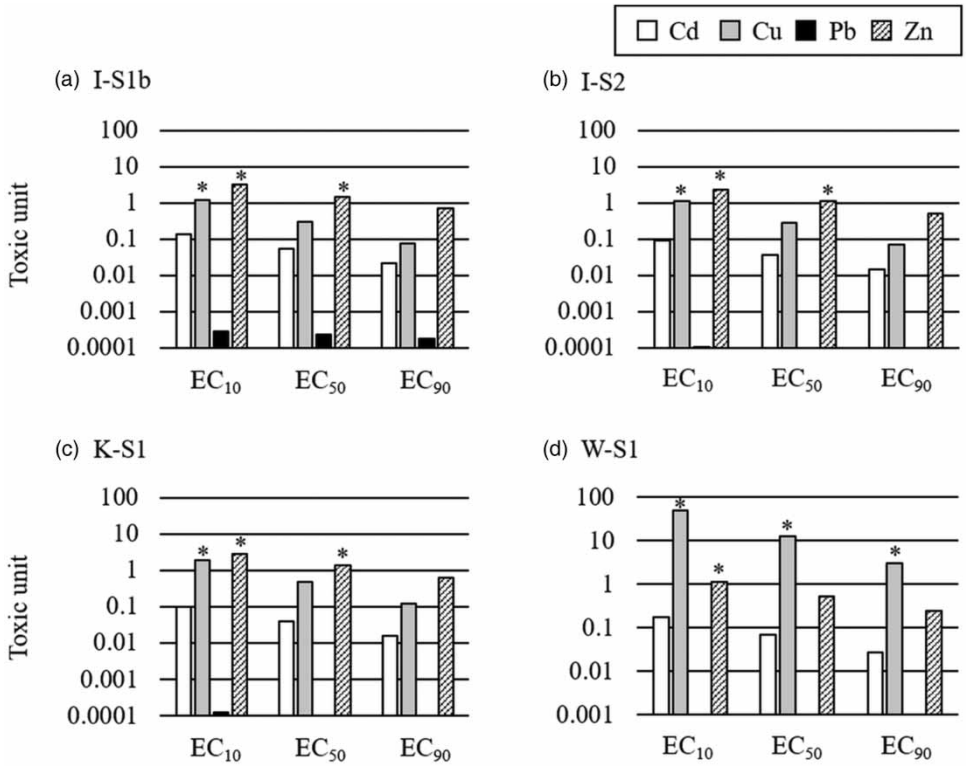

Figure 5 | Toxic units of Cd, Cu, Pb and Zn for the water samples from four sampling sites (a) I-S1b, (b) I-S2, (c) K-S1 and (d) W-S1. The toxic units of a metal for a water sample were obtained by dividing the free-ion activity of the metal [mM] in the sample by 10, 50 and 90% effect concentrations (EC_{10}, EC_{50} and EC_{90}, respectively) of free ion of the metal [mM]. * indicates that the toxic unit of a metal was greater than 1.

The Zn-TEQ values for water samples from sites I-S1b, I-S2, K-S1 and W-S1 were calculated by using the water concentrations and acute toxicity values of the free-ion activities of Cu and Zn, which dominantly contributed to *D. magna* immobility in the water samples (Figure 5). The Zn-TEQ and predicted immobility were 2.87×10^{-3} mM and 0.84, respectively, for site I-S1b; 2.22×10^{-3} mM and 0.72, respectively, for site I-S2; 2.95×10^{-3} mM and 0.85, respectively, for site K-S1 and 2.07×10^{-3} mM and 1.0, respectively, for site W-S1. The predicted frequency of the immobilised test individuals, which was calculated by multiplying the predicted immobility by 20, was not significantly different from the frequency of immobility (i.e. 20 individuals; 19 individuals for I-S2) observed in each of the four water samples (Fisher's exact test: $P = 0.23$ for I-S1b, $P = 0.09$ for I-S2, $P = 0.23$ for K-S1 and $P = 1.00$ for W-S1). These results suggest that the acute toxicity of the four water samples to *D. magna* was reasonably predicted by the concentration addition of the ion activities of Zn and Cu. The suitability of using the concentration addition of Cu and Zn free-ion activities was supported by the similar slopes of the concentration–response curves (Figure 4 and Table 2; see Kortenkamp *et al.* (2009) for more details). Previous studies also suggested that the toxicity of a Cu and Zn mixture was, on average, additive based on the speciated concentrations of Cu and Zn (Santore & Ryan 2015).

To estimate the mixture toxicity of Cu and Zn, we did not consider their competitions with major ions such as Ca and Mg at the binding sites of organisms. Such binding can reduce the bioavailability of trace metals. However, the water hardness of the river waters tested in this study (14–29 mg $CaCO_3$/L) may have been sufficiently low to neglect the influence of water hardness on the toxicity when estimating the mixture toxicity in the river waters. Although further testing is required, our results at least provide a case that in low-hardness river waters, the mixture toxicity of the trace metals such as Cu and Zn that have similar slopes of the concentration–response curves can be predicted by a simple concentration addition approach.

CONCLUSIONS

In this study, an effect-based water quality assessment by using two cladoceran species demonstrated modest to significant adverse effects on their mobility and reproduction, suggesting the potential for the ecotoxicological impacts on natural

populations and communities at the contaminated sites that received inflow of mine drainages in Japan. The TIE results for the water samples that caused *D. magna* immobility indicated major contributions of Zn and Cu, although the degrees of contributions varied with the sites. For the sites where the toxic effects on mobility or/and reproduction were observed, field biological surveys, in which macroinvertebrates are sampled (Namba *et al.* 2020) will be useful to examine the relationships between the observed effects on natural populations and communities and those based on bioassays. Although preliminary, we observed some consistency between the results of bioassays performed in this study and those of the field survey results available in the published literature. Establishing such relationships is valuable to predict the ecotoxicological impacts on natural populations and communities from laboratory bioassays with the field-collected water samples. We suggest that effect-based water quality assessments such as ours can provide direct and unique evidence of the ecotoxicological impacts of metals in river waters. Such evidence will be useful for better understanding and predicting the ecological effects in the natural environment.

ACKNOWLEDGEMENTS

The following grant information was disclosed by the authors: Environment Research and Technology Development Fund: JPMEERF20185R01. We thank M. Kamo and H. Namba for their assistance in the field surveys and A. Ueta and K. Uchino for their assistance in the laboratory experiments. This paper does not necessarily reflect the policies or views of any government agencies.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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