Water Research 200 (2021) 117274

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Ecotoxicological risk assessment of metal cocktails based on maximum cumulative ratio during multi-generational exposures

Xuehua Sun[a], Li Zhang[a], Hongling Liu[a,*], Zunyao Wang[a], John P. Giesy[a,b,c]

[a] State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing, Jiangsu 210023, China
[b] Department of Veterinary Biomedical Sciences and Toxicology Center, University of Saskatchewan, Saskatoon, SK S7N 5B3, Canada
[c] Department of Environmental Science, Baylor University, Waco, Texas, USA

ARTICLE INFO

Article history:
Received 24 December 2020
Revised 13 May 2021
Accepted 17 May 2021
Available online 22 May 2021

Keywords:
crustacean
metal mixture
multi-generational effect
Leslie matrix
population dynamics
Tai Lake, China, Asia

ABSTRACT

Humans and wildlife are frequently exposed to complex mixtures of chemicals, with exposure rarely causing only one dominant effect. Consequently, there is an urgent need to develop strategies to assess exposures to multiple, hazardous chemicals and effects of such combinations. Here, the maximum cumulative ratio was used as part of a tiered approach to evaluate and prioritize risks of co-exposures to metals in 781 samples of surface water from Tai Lake, China. Multiple metals, including copper, lead, cadmium, nickel and zinc dominated the hazardous effects on aquatic organisms. Based on species sensitivity distributions developed from genus mean chronic values, crustaceans were the most susceptible to effects of metals. Results of a multi-generation experiment demonstrated adverse effects of mixtures of metals at environmentally relevant concentrations on growth and reproduction of the cladocerans, *Daphnia magna* and *Moina macrocopa*. Specifically, when exposed to metals body length and total number of offspring produced per adult female were less than the controls. Resistance of *D. magna* populations to mixtures of metals was significantly less, while, under similar conditions, *M. macrocopa* exhibited greater capacity to recover and the response to adverse effects occurred earlier. Demographic analysis models constructed using a Leslie matrix, used to predict population dynamics of the cladocerans, revealed that various effects of metal cocktails on individual-level endpoints was related to attenuation at the population level. By integrating all the observations, it was recommended that densities of populations of cladocerans in surface waters could be a useful parameter for indicating possible detrimental effects induced by toxic chemicals. Results of this study provide novel insights into risks posed by simultaneous exposure to multiple metals and reveal their potential adverse long-term effects on sensitive aquatic organisms.

1. Introduction

Multiple metals are generally present in surface waters, which can enter via multiple routes such as atmospheric deposition, industrial effluent discharge and surface runoff (Zeng et al., 2012). However, most regulatory approaches and assessments of the hazards of metals are based on results of toxicity tests of individual metals on individual species (Gama-Flores et al., 2007), which do not account for the possibility that simultaneous exposure to other metals may also contribute to the observed toxicity. Results of several studies have indicated that the combination of chemicals at concentrations less than the no observation effect concentrations could produce significant effects and that toxic potency can be strictly additive or supra-additive (Payne et al., 2001; Silva et al., 2002). For example, some mixtures of different metals, each individually causing $<10\%$ growth inhibition of *Hordeum vulgare*, yielded much greater inhibition (up to 66%) when dosed in combination (Nys et al. 2017). Thus, single metal exposures judged to be “safe” based on results of independent exposures, might pose significant risks in reality if there is co-exposure with other metals.

There was urgent need to develop strategies for assessing interactive effects of exposure to multiple hazardous metals. A key challenge in elucidating risks arises from the large number of potential combinations and concentrations of metals in the environment. To address the challenge, the maximum cumulative ratio (MCR) concept has been proposed (Price and Han, 2011). Tiered approaches that apply the MCR can identify combined exposures in cases where potential risks would be ignored and provide a

---

* Corresponding author.
E-mail address: hlliu@nju.edu.cn (H. Liu).

https://doi.org/10.1016/j.watres.2021.117274

quantitative measure of the magnitude of the toxicity that is underestimated by single-substance assessments. MCR methodology has been applied to crop protection products in surface waters in the U.S. (Vallotton and Price, 2016).

Tai Lake (Chinese: *Taihu*) is a shallow lake located in a densely populated, urbanized and industrialized area, south of the Yangtze Delta in China. Due to rapid industrialization and intensification of agriculture in the watershed surrounding Tai Lake, various contaminants such as nutrients and metals have been introduced into its surface water (Zhai et al., 2010). Urban effluents from metal processing, plating, and electronics industries are dominant sources of metals to Tai Lake (Liang et al., 2011). Long-term exposures to metals influence capacities of populations to cope with additional environmental stressors (Ríos-Arana et al., 2007). Therefore, the presence of multiple metal contaminants might pose threats to aquatic organisms in Tai Lake.

However, most published data are based on concentrations of chemicals that are greater than those detected in freshwater systems. Therefore, studies considering effects of metals on aquatic organisms at environmentally relevant concentrations are required (Dietrich et al., 2010a). In this study, MCR was used to identify whether single or multiple metals dominated the adverse effects on aquatic organisms in Tai Lake, and the adverse effects of multi-metal mixtures at environmentally realistic concentrations were studied.

The water flea is a common crustacean invertebrate in freshwater systems. This type of zooplankton grazes on algae and forms the base of the secondary producer food chain (Chen et al., 2014). Testing more than two successive generations provides more information regarding possible chronic, population level effects of metals (Dietrich et al., 2010b). A multi-generational study over three generations, using two model organisms, the cladocerans *Daphnia magna* and *Moina macrocopa* was conducted. The primary aim of the present study was to identify the “real culprit” causing adverse effects by multiple metals on organisms in an aquatic environment and investigate the effects of co-exposure to the most hazardous metals at environmental concentrations on growth, reproduction, and population dynamics in the two sensitive species. The secondary aim of the paper was to screen the most sensitive parameter of water flea as a candidate indicator indicating possible detrimental effects induced by multiple toxic chemicals. Therefore, results of this study allowed integration of effects of metal mixtures at environmentally relevant concentrations in risk-assessment frameworks.

2. Materials and Methods

2.1. Sample collection and analysis

Samples of water (500 mL) were collected twice in September 2019 (wet season) and January 2020 (dry season) from 52 sampling points in Tai Lake (Fig. S1 in the Supporting Information). Temperature, dissolved oxygen, turbidity, electrical conductivity, and pH of water samples were measured on site using YSI portable meters (6600V2-4, Ecosense, Ohio, USA). Then, water samples were filtered through 0.45-μm microporous membranes and packed in polyethylene bottles that were pre-washed according to national standard water quality reference (HJ 493-2009, Ministry of Ecology and Environment of the People’s Republic of China, 2009). After sampling, samples were preserved by adjusting pH to 1–2 by adding 5 ml concentrated nitric acid (HNO_3) to 500 ml water sample prior to quantification of copper (Cu), lead (Pb), cadmium (Cd), and zinc (Zn) concentrations, while those for quantification of hexavalent chromium (Cr(VI)) were preserved with NaOH (pH 8–9).

Concentrations of Cu, Pb, Cd, Zn, and Cr(VI) were determined by use of inductively coupled plasma–mass spectrometry (ICP–MS) following methods set by national standards (HJ 700-2014, Ministry of Ecology and Environment of the People’s Republic of China, 2014). Analyses were performed on blank and duplicate samples and a standard recovery test was used to determine the accuracy of the analysis. The relative deviation of the duplicate samples was $<$ 6.50% for all batch treatments and the recovery rates of each element were in the range 87–105%, which satisfied the quality requirements.

2.2. Chronic toxicity benchmarks and metal concentrations in Tai Lake

In addition to data obtained for 104 samples, concentrations of metals were obtained from the published literature by searching using the keywords “Tai Lake” and “metals.” Data were also provided by the Jiangsu Environmental Monitoring Center.

Aquatic benchmarks (BMs) are based on the most sensitive toxicity endpoint of the distribution for each aquatic taxa and support baseline risk assessments of individual metals (U.S. Environmental Protection Agency, 2019). The use of chronic BMs is optimal for long-term protection of aquatic organisms. Water chemistry characteristics such as pH, organic carbon content, hardness, as well as other edaphic factors and biological species, result in diverse BMs in various regions (Carlson, A., 1984). Therefore, chronic BMs of metals that have been established through quality screening and data standardization for the Tai Lake were selected first, followed by BMs for China and, finally, the values set by other agencies, such as the U.S. Environmental Protection Agency (U.S. EPA). If the values derived for Tai Lake and China differed more than five-fold, the geometric mean of the two BMs was used as the ultimate threshold (Table 1).

2.3. Methodology for risk assessment of metals

A tiered, MCR approach, based on dose additive models, was used to determine whether it was necessary to perform a detailed cumulative risk assessment for organisms that are simultaneously exposed to multiple metals Price and Han, 2011). Hazard quotients (HQ) for individual metals were calculated by dividing the metal concentration by the BM (Eq. 1). The hazard index (HI) was calculated as the sum of individual HQs (Eq. 2). The MCR for exposure to n pollutants was then calculated (Eqs. 3 and (4).

$$\mathrm{HQ}_i = \frac{C_i}{\mathrm{BM}_i}, \tag{1}$$

$$\mathrm{HI} = \sum \mathrm{HQ}_i, \tag{2}$$

$$\mathrm{MHQ} = \mathrm{MAX}(\mathrm{HQ}_i), \tag{3}$$

$$\mathrm{MCR}_i = \frac{\mathrm{HI}_i}{\mathrm{MHQ}_i}. \tag{4}$$

Where C_i (μg/L) is the measured environmental concentration of the i^{th} metal in surface water, BM_i denotes the chronic benchmark of the i^{th} metal, HQ_i is the hazard quotient of the i^{th} metal, HI is the sum of the individual values of HQ_i, and MHQ is the maximum of HQ_i. Based on results of MCR and HI for each sample, four groups of combined exposure were identified (Price et al., 2012). Each group indicates the potential hazard and proposes relevant management strategies:

- Group I: At least individual metals are of concern (MHQ $>$1).
- Group II: Risks posed by individual metals and multiple metals can be ignored (HI $<$ 1).

Table 1
Concentrations of metals and chronic toxicity benchmarks (BMs) in Tai Lake.

Metals	No. of samples	Concentration (μg/L) Maximum	Mean	Median	Minimum	Chronic BM (Reference)
Cr(VI)	348	75.50 (Chen et al., 2011)	3.10	2.00	0.005	5.44 (Liao et al., 2014) 14.22 (Wu et al., 2012)
Ni	63	224.00	14.31	3.00	0.550	3.08 (Tenzin et al., 2020)
Hg	58	0.14 (Wang et al., 2016)	0.03	0.03	0.005	0.47 (Zhang et al., 2012) 2.34 (Zhao et al., 2015)
Pb	538	28.00	3.54	2.51	0.009	63.80 (Zhao et al., 2015) 4.36 (Sun et al., 2020)
Cd	270	2.00	0.24	0.05	0.001	1.95 (Zhao et al., 2015) 0.24 (Wu et al., 2012) 0.23 (Yan et al., 2009) 0.12 (Wu et al., 2011)
Zn	296	240.00	20.07	11.13	0.160 (Yan et al., 2011)	52.75 (Zhao et al., 2015)
Cu	633	77.60 (Yan et al., 2011)	5.20	4.00	0.500	3.70 (Shi et al., 2014)
Se	180	4.00	0.71	0.60	0.200	5.00 (U.S. EPA, 2009)
Fe	46	1589.00	623.50	469.50	51.000	1000.00 (U.S. EPA, 2009)
Mn	22	88.00	48.18	43.50	26.000	50.00 (U.S. EPA, 2009)
As	530	15.60	2.36	1.90	0.010	56.55 (Zhang et al., 2015)

- Group III: Risks posed by individual metals could be ignored; however, the combined effect is a concern (MHQ $\leq$ 1 and HI $\geq$ 1). This group was divided into two subgroups:

Group IIIA: Toxicity of the combined exposure was dominated by one metal (MCR $<$ 2).

Group IIIB: Toxicity of combined exposure was accounted for by several metals (MCR $\geq$ 2).

HQs were calculated for each metal in mixtures based on the MCR during Tier I. In Tier II, sums of samples with an HQ $>$ 1.0 were calculated for each metal to identify the possible combination of metals that posed the greatest risk to organisms. Subsequently, species that were more sensitive to metals were identified based on the species sensitivity distributions (SSDs), which were developed following our previously reported method (Sun et al., 2020).

The MCR approach can be used to investigate the magnitudes of toxicity underestimation by assessments of hazard based on individual metals (Price and Han, 2011). The proportion of the toxicity that is overlooked for each sample can be estimated (Eq. 5).

$$\text{Missed toxicity} = 1 - \frac{1}{\text{MCR}_i} \tag{5}$$

2.4. Multi-generation experiment

Based on results of the MCR, mixtures of Cu, Pb, nickel (Ni), Cd and Zn were selected to be tested for multi-generational effects on the most susceptible organisms, cladocerans. Stock solutions of $PbCl_2$, $CuCl_2•2H_2O$, $ZnCl_2$, $CdCl_2•2.5H_2O$, and $NiCl_2•2.5H_2O$ (all purity >99.00%, Sigma-Aldrich, St. Louis, MO, USA) were prepared as sources of Pb, Cu, Zn, Cd, and Ni, respectively. Two cladocerans, *D. magna* and *M. macrocopa*, were selected as test species for the multi-generation experiment. Based on the distribution of environmental concentrations of each metal (Table 1), organisms were exposed to one of the three concentrations: least, medium, and greatest (Table 2). In addition, tap water was aerated for >3 days and was used as a negative control to which responses could be referenced. Basic physical and chemical parameters of experimental water samples were as follows: pH: 7.60 ± 0.14, conductivity: 300 ± 9.80 μS/cm, dissolved oxygen: 8.15 ± 0.23 mg/L, alkalinity: 81.25 ± 2.17 mg/L ($CaCO_3$), and hardness: 155.50 ± 4.95 mg/L ($CaCO_3$). Experiments were conducted according to the Organization for Economic Co-operation and Development (OECD) guideline 211 (OECD Guidelines for the Testing of Chemicals, 2012). Further details regarding exposures to mixtures of metals are provided in the Supporting Information.

Table 2
Concentrations of metals to which *Daphnia magna* and *Moina macrocopa* were exposed.

Concentration (μg/L)	Ni	Pb	Cd	Zn	Cu
Least	0.51	0.06	0.51	0.27	0.54
Medium	0.65	0.23	0.51	0.27	0.81
Greatest	5.14	1.93	3.08	1.33	5.14

2.5. Leslie matrix and demographic analysis

The Leslie matrix is a matriarchal-based model developed by Leslie (1945) and Lewis (1977). It can project population growth over time based on the fecundity and survival rates of individual life stage classes. In this study, stage-specific survivorship and fecundity data were obtained from the multi-generation experiment. In a Leslie matrix analysis, the life history information is organized in a projection matrix $\boldsymbol{A}$ (Eq. 6).

$$\boldsymbol{A} = \begin{bmatrix} F_1 & F_2 & F_3 & F_4 & F_5 & F_6 \\ S_1 & 0 & 0 & 0 & 0 & 0 \\ 0 & S_2 & 0 & 0 & 0 & 0 \\ 0 & 0 & S_3 & 0 & 0 & 0 \\ 0 & 0 & 0 & S_4 & 0 & 0 \\ 0 & 0 & 0 & 0 & S_5 & 0 \end{bmatrix} \tag{6}$$

where F_i represents the total numbers of offspring per adult female produced at stage i and S_i represents the survival rate from stage i-1 to stage i.

The number of females at each stage at time t was calculated (Eq. 7).

$$N_t = [n_{1,t},\ n_{2,t},\ n_{3,t},\ n_{4,t},\ n_{5,t},\ n_{6,t}]^T \tag{7}$$

where n_{ij} is the number of organisms of stage i at the start of day j.

The numbers for the following days were determined (Eq. 8).

$$N_{t+1} = AN_t \tag{8}$$

The intrinsic rate of population growth (r) is an integrative indicator that is important for measuring the instantaneous rate of population development. It is the natural logarithm of the largest eigenvalue (λ) of matrix $\boldsymbol{A}$ (Eq. 9).

$$r = \ln(\lambda) \tag{9}$$

r can also be calculated using the survivorship and reproduction data (Eq. 10):

$$r = \frac{\ln R_0 \times R_0}{\sum x \times l_x \times m_x} \tag{10}$$

where R_0 is the net reproduce rate, l_x is the survive rate of individuals reaching age x, and m_x is the average number of live offspring produced per female of age x during the time interval x to $x+1$.

The Leslie matrix distinguishes various life stages and the same r value was obtained through equations 9 and 10 when setting one day as the life stage of test organisms. Monte Carlo-type stochastic simulations based on survival rate normal distribution were used to represent the 95% confidence interval (CI) based on 1000 iterations. However, the results of Monte Carlo-type stochastic simulations were hardly different from values predicted using the Leslie Matrix thus could not be effectively displayed.

2.6. Statistical analysis

Basic descriptive statistical analyses were performed using Microsoft Excel (version 2016; Microsoft, Redmond, USA) and data were processed using SPSS software (version 22.0; SPSS Inc., USA). Statistical drawing was performed using GraphPad Prism (GraphPad Prism Development Core Team, http://www.graphpad.com/scientific-software/prism/). Monte Carlo-type stochastic simulations were employed using PopTools (http://www.poptools.org) for a probabilistic estimation of population prediction based on the variability of the input parameters. Differences in growth and reproduction parameters were determined using one-way analysis of variance (ANOVA) assuming normal distributions based on the Shapiro–Wilk test, and homogeneity of variances was assessed by use of Levene's test. Then, significant differences were tested using Tukey's honestly significant difference (least significance difference, LSD) method, which was applied for multiple comparisons among means. For data that were not normally distributed, logarithmic transformation was applied and data were, then, rechecked for normality. Levels of significance were set to $p < 0.05$, $p < 0.01$, and $p < 0.001$.

3. Results and discussion

3.1. Concentrations of metals in Tai Lake surface water

Dissolved oxygen concentrations of water fluctuated within the ranges 3.48–13.89 and 8.91–11.08 mg/L during the wet and dry seasons, respectively. Similarly, the pH also varied substantially, ranging from 6.93 to 8.52 at 52 sampling sites, and was generally high in the wet season. For electrical conductivity, similar variations were observed among different seasons, ranging from 103.30 to 592.00 μS/cm.

Concentrations of the five metals in Tai Lake varied between seasons and among locations (Tables S1 and S2). Concentrations of Cu in the wet and dry seasons were 0.68–11 and 1.52–5.4 μg/L, respectively. Concentrations of Zn at most locations were < 30 μg/L, except for a few points where the concentration was >50 μg/L. Concentrations of Cd were similar during wet and dry seasons, with mean concentrations of 0.044 and 0.049 μg/L, respectively. During the wet season, ~33% of concentrations of Pb were less than the limit of detection. Concentrations of Cr(VI) were 0.71–10.5 and 1.53–3.73 μg/L during the wet and dry seasons, respectively. Concentrations of Cu, Zn, and Cr(VI) in the wet season were generally greater than those during the dry season. The opposite trend was observed for distributions of concentrations of Pb. Emissions from mechanical manufacturing industries were possibly the main source of metals in Tai Lake basin during the wet season, while metals accumulated in environmental media were the main source during the dry season (Yao et al. 2014). Differences in sources of metals might result in different distributions of the five metals in Tai Lake.

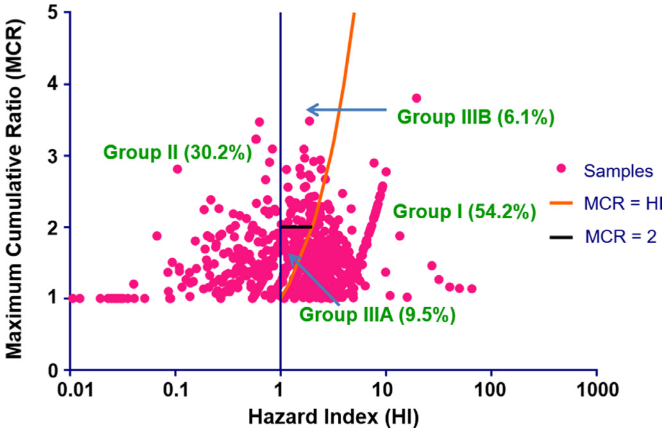

Fig. 1. Distributions of mixtures (red dots) in four groups based on the hazard index (HI) and maximum cumulative ratio (MCR) values.

When combined with recent values, published in the literature, 11 metals were detected in 781 samples from Tai Lake. The order of median concentrations in Tai Lake was as follows: Iron (Fe) > manganese (Mn) > Zn > Cu > Ni > Pb > Cr(VI) > arsenic (As) > selenium (Se) > Cd > mercury (Hg) (Table 1, Fig. S2A). Concentrations of the 11 metals were generally skewed and were best described by a log-normal distribution (Fig. S2B–L). In Tai Lake, concentrations of Fe and Mn, which are generally regarded as macroelements, were greater than concentrations of other metals, followed by those of Zn and Cu, which are classified as trace elements. However, concentrations of Se, which is also an essential trace element, in surface water were relatively low. Median concentrations of Ni (3.00 μg/L), which is not included in the Environmental Quality Standards for Surface Water (Ministry of Environment Protection of the People's Republic of China, 2002), were greater than concentrations of most of the studied metals. Metals considered to be priority pollutants in China, including Cr(VI), Pb, As, Cd, and Hg, were present at relatively small concentrations. For example, Cd and Hg concentrations were 10- to 100-fold less than those of other metals.

3.2. Evaluation of composite HQ of metals

Based on the calculated MCR and HI values, all samples could be categorized into one of four groups (Fig. 1). The percentages of samples in Groups I and II were 54.2% and 30.2%, respectively, while the percentage of samples in Group III was 15.6%. These results indicated that 69.8% of samples exceeded the level of concern based on the potential for the observed metal concentrations to cause adverse effects. Group III could be further divided into samples that are hazardous based on composite metals (Group IIIB; 6.1%) or a single metal (Group IIIA; 9.5%). In general, both the HI and n or MCR and n were significantly, positively correlated (Fig. S3). This suggests that the need for cumulative hazard assessment increases with the number of metals considered for aquatic environments. In addition, MCR values demonstrated that when the chemical-by-chemical approach (MHQ) was applied to assess the risks of metal mixtures, on average, 46.6% of toxicities of samples were missed by overlooking the toxicity attributed to co-exposure

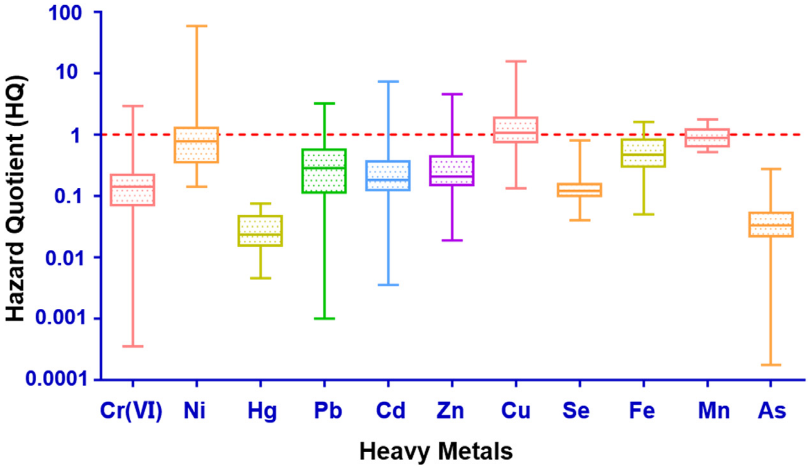

Fig. 2. Hazard quotients (HQs) of metals based on 781 water samples of Tai Lake.

during risk assessments. The results underscore that it is important to study combined effects of metals.

Distributions of HQs of metals indicated that the maximum HQ values of individual metals in 781 samples collected from Tai Lake were > 1.0, except for Hg, Se, and As (Fig. 2). Based on the number of samples in which the single metal HQ exceeded 1.0 in the Tier I assessment, the order of the top five metals was Cu, Cd, Pb, Ni, and Zn, followed by Fe and Mn (Fig. S4). Greater hazards posed by Cu and Zn were consistent with previous results (Fu et al., 2016), which predicted that approximately 99.9% and 50.7% of aquatic organisms would be adversely affected by Cu and Zn, respectively, in Tai Lake surface water. Pb and Cd have been classified as priority pollutants in ambient waters in China and in the U.S. (EPA), and reportedly pose risks to organisms in Tai Lake (Jiang et al., 2012; Lei et al., 2016). In addition, Ni poses the greatest hazard to humans via ingestion due to relatively great concentrations in Tai Lake (Liang et al., 2011). Furthermore, Zn and Cu have potential effects on human health when they co-occur with other metals (Fu et al., 2013). Little attention has previously been paid to concentrations of Fe and Mn in water or the hazards they pose. According to the results of present study, Fe may have adverse effects on organisms. Because the chronic BM for Mn is not based on toxic potencies, but on other characteristics, Mn exposure may not pose a risk for aquatic organisms (U.S. EPA, 1986). Thus, in Tai Lake, mixtures of Cu, Pb, Cd, Ni, and Zn were considered as the combination with the greatest potential to cause adverse effects and should be given the greatest priority.

Based on the chronic SSD curves of the five metals, crustaceans, especially cladocerans such as daphnids, were the most susceptible to the effects of metals (Fig. S5). Previous analyses of SSD curves, based on acute metal exposures, indicated that invertebrates were more sensitive than vertebrates (Xin et al., 2015), which was consistent with the results of the chronic SSD curves in this study. Therefore, *D. magna* and *M. macrocopa* were selected as test species to explore the multi-generational effects of Cu, Pb, Ni, Cd, and Zn mixtures at environmentally relevant concentrations, which could provide insights into the adverse effects of prevailing metal exposure states on sensitive species in Tai Lake.

3.3. Multi-generational effects on growth

Metals influenced rates of survival of *D. magna* and *M. macrocopa*. Mortality followed a concentration–response pattern and was directly proportional to the concentrations of metals in mixtures (Table S3). Results of the exposure groups indicated that resistance of *D. magna* to metals weakened, whereby offspring became more sensitive. However, survival rates of later generations of *M. macrocopa* in the remaining treatments increased, and were significantly different from the trend observed for *D. magna*. Potential tolerance and recovery trends of *M. macrocopa* were previously demonstrated in a few life history variables (Gama-Flores et al., 2017).

Results of a previous study indicated that feeding behavior of *D. magna* was affected by exposure to small concentrations of multiple metals (Ni, Zn, Cu, and Cd), although the concentrations were 27–63 times less than concentrations that are lethal (Lari et al., 2017). In the present study, growth of *D. magna* was affected by mixtures of five metals. Compared with other endpoints, body length showed a stronger dose–response relationship over three generations (Fig. 3A, Table S4). Body lengths of adult female (F_0) *D. magna* were 9.85%–38.58% less than those of the control group, which indicated that exposure to the greatest concentration had the most significant effect. At the greatest concentration, the number of molts in the F_1 generation of *D. magna* was significantly less ($p < 0.001$) than that in the F_0 generation (Fig. S6B) and were inversely proportional to the concentrations of metal mixtures (Fig. 3B). This result was consistent with results of a previous study, which reported that growth of *D. magna* was inversely proportional to concentrations of binary mixtures of metals (Brun et al., 2019).

Significant multi-generational effects on growth of *M. macrocopa* were observed, compared with the control. Tolerance of *M. macrocopa* became greater in successive generations (Fig. 3C–D, Table S5). When exposed to the greatest concentration of mixture of metals, body lengths of females belonging to F_0 and F_1 generations were significantly less ($p < 0.001$). The opposite effect was observed in the F_2 generation exposed to the same concentration ($p < 0.05$). In addition, during exposure to the medium concentration, the inhibition of body length observed in the F_2 generation decreased when compared to the body lengths in the F_0 generation ($p < 0.01$; Fig. S6a). In the F_0 generation, a stronger dose–response relationship was observed for the number of molts, which was significantly inversely proportional to the exposure concentration (Fig. 3D). However, the number of molts of F_1 and F_2 generations were similar to that of the control. The results above are consistent with the results of a previous study (Gama-Flores et al., 2007) which showed that survival rate and reproduction of *M. macrocopa* were inversely proportion to the exposure duration and the $CdCl_2$ concentrations to which they were exposed. All results demonstrated that the toxicity of mixture of metals was caused by the combined effect of intensity (concentration) and duration (time) of exposure.

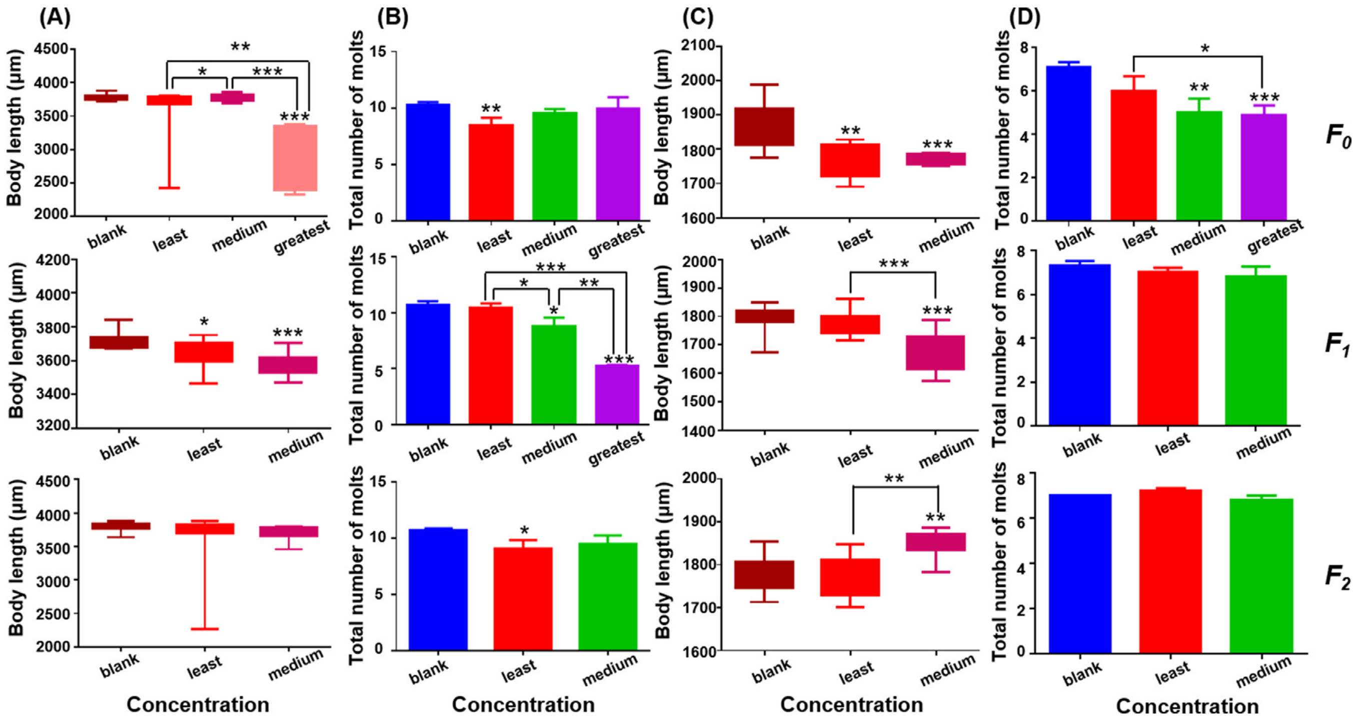

Fig. 3. Comparison of body length and total number of molts among concentrations. (A) Body length of *Daphnia magna*, (B) total number of molts of *D. magna*, (C) body length of *Moina macrocopa*, and (D) total number of molts of *M. macrocopa*. Each row represents a generation, with a total of three generations observed for each species. Each graph shows the respective effect based on treatment with different metal mixture concentrations (blank, least, medium, or greatest). Error bars represent the standard deviation of 10 independent measurements (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared with the control group).

3.4. Multi-generational effects on reproduction

Results of previous studies have demonstrated that several measurement endpoints of offspring were affected when adult female *D. magna* were exposed to a single metal (Guan and Wang, 2006). Similar effects were observed for *M. macrocopa* (Gama-Flores et al., 2017), thereby indicating that not only survival rates but also reproductive rates were affected when individuals were continuously exposed to Cd. In this study, effects of metal mixtures on reproduction appeared to be greater than that on the growth, especially in terms of the significant decrease in the number of offspring. Total numbers of offspring per adult female *D. magna* produced within 21 days exhibited a strong dose–response relationship and was inversely proportional to concentrations in all treatments (Fig. 4, Table S6). As a result of the exposure to the maximum concentration, total numbers of offspring were reduced by 58.3%, 82.6%, and 23.8% in F_0, F_1, and F_2 generations, respectively, compared with the controls. When exposed to the greatest concentration, the endpoint was reduced by 56.8% in the F_1 generation compared with the F_0 generation (Fig. S6C). Age at first spawning of *D. magna* gradually increased in successive generations under the same exposure (Fig. S6D). Generation times in the F_0 and F_1 generations also decreased significantly following exposure to the greatest concentration ($p < 0.001$). These results are consistent with previously reported results (Biesinger et al., 1986).

Statistically significant changes were also observed for *M. macrocopa* exposed to multiple metals compared with the controls (Fig. 4, Table S7). The number of offspring produced by *M. macrocopa* exposed to multiple metals was lower than that by the controls. This endpoint significantly differed between each generation, especially for the medium-exposure group, wherein it increased gradually with successive generations (Fig. S6c). At the medium concentration, a significant delay in age of first reproduction was observed for the F_0 generation ($p < 0.05$). A significant decline in the age of first reproduction for F_2 *M. macrocopa* was also observed in individuals exposed to the least concentration compared with the controls ($p < 0.01$). Hazardous effects induced by metal mixtures were observed on generation times of *M. macrocopa*. The generation times of *M. macrocopa* in F_0 and F_2 generations exposed to the medium and greatest concentrations of metal mixtures were significantly less than those of the control ($p < 0.05$).

In this study, the finite rate of increase (λ), which is used to evaluate the development of a population over a longer time period (An et al., 2009), exhibited similar trends as the intrinsic rate of population growth for both species. A difference in the intrinsic rate of population growth of *D. magna* was primarily observed in the F_1 generation. This indicator was significantly weak in the least- and medium-exposure groups compared with the control group ($p < 0.01$; Fig. 4, Table S6). The same reduction occurred in F_0 *M. macrocopa* ($p < 0.001$; Fig. 4), but the intrinsic rate of population growth significantly increased as a function of generation in *M. macrocopa* in the medium-exposure group, i.e., by 8.8% in the F_2 generation compared with the F_0 generation (Fig. S6f, Table S7). All reproduction endpoints showed that *M. macrocopa* exhibited greater capacity to recover but the response to adverse effects was earlier than *D. magna* under the same conditions. Environmental concentrations of the five metals were mostly higher than the concentrations used in the multi-generation experiment and it is important to note that there are more than five metals in Tai Lake. Thus, it is speculated that effects of multiple metals on these two cladocerans may be more critical in the actual environment and other organisms would also be adversely affected.

3.5. Multi-generational effects on population development

To visually show the long-term effects of exposures to environmentally relevant concentrations of metal mixtures on population development, demographic analysis models were constructed using a Leslie matrix based on multi-generational toxicity data of *D.*

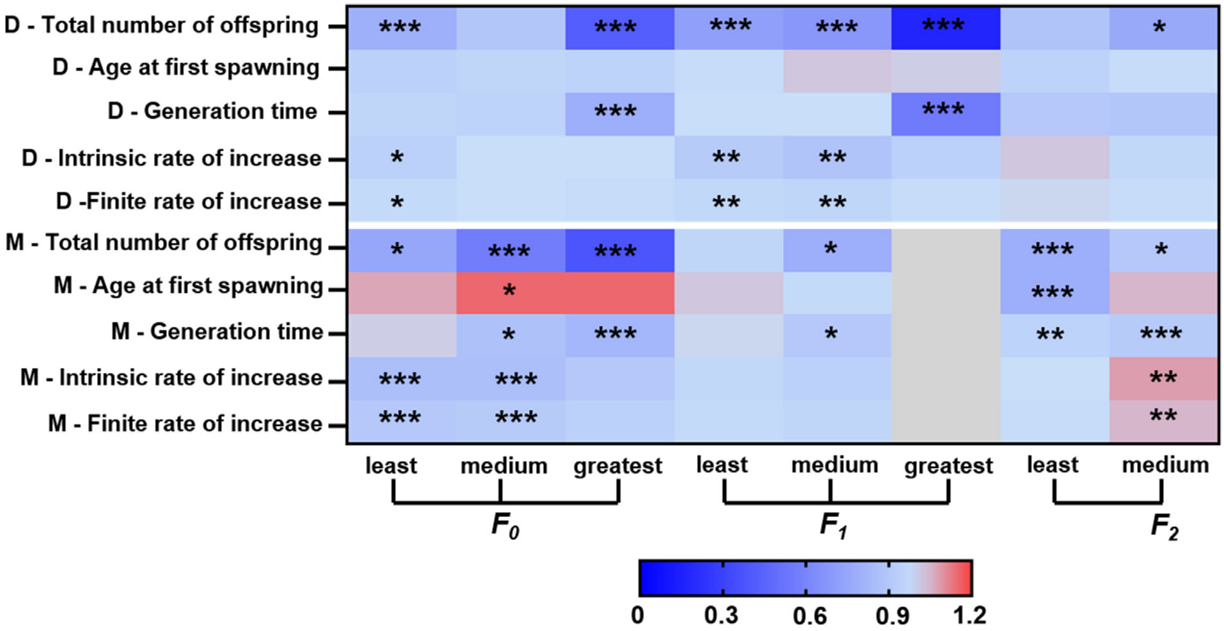

Fig. 4. Multi-generational effects induced by different concentrations of mixture of metals on reproduction of *Daphnia magna* and *Moina macrocopa*. Results are expressed as fold difference relative to the value of the negative control. Each value represents the mean ± SD of 10 independent experiments. No results are shown in the gray area owing to no neonates surviving for examination in subsequent experiments (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

magna and *M. macrocopa*. The initial population size was set as 10 for simulation of population dynamics. Analysis predicted only the number of *D. magna* after three years and *M. macrocopa* after two years due to their rapid reproduction rate. The contribution to sensitivity of the dominant eigenvalue (i.e., the infinite rate of increase) was predominantly related to the survival rate, whereas fecundity did not contribute significantly. Population growth showed that different effects of metal mixtures on individual-level endpoints could lead to attenuation at the population level over the next two or three years (Fig. 5, Fig. S7). The logarithmic value of the number of *D. magna* would be significantly lower than that of the control groups (by 2.40%–25.76%) in three years after exposure to multiple metals, and the adverse effects would strengthen in successive generations. A stage-structured population analysis, based on mortality and life-history traits revealed deteriorating groups had aging populations within the cumulative 1, 2 and 3 years. Proportions of adult-aged population (14−21 days) in F_2 *D. magna* each treatment over 3 years were as follows: medium (0.85%) > least (0.75%) > blank (0.32%). However, no maturity populations with stable reproduction occurred in F_1 *D. magna* exposed to the greatest concentration. Multi-generational exposure to greater concentrations of mixtures of metals were likely to cause greater effects on populations of *D. magna* and even cause extinction. Similarly, development of *M. macrocopa* would also be affected by metal cocktails and the influence on population size seemed to be more dramatic than for populations of *D. magna*, when only one generation was compared; however, the impact would be weakened in successive generations. Specifically, the logarithmic value of the population size of F_0 *M. macrocopa* was predicted to decline by 17.04%–25.45% over two years by exposure to mixture of metals compared with controls. In contrast, an increase compared with that in the blank group was predicted in F_2 population exposed to the medium concentration.

A number of scientific reports indicate that the densities of cladocerans in water bodies are declining (Barbiero et al., 2019), and *Daphnia* is no longer the dominant species in water bodies while *Moina* is gradually predominant, which is consistent with results of the multi-generational experiment in this study. The reason for the difference between the two species could be due to the shorter life cycle of *M. macrocopa* compared to that of *D. magna*, and the former can produce more offspring within relatively short periods, which may result in offspring sharing the toxic effects of metals entering the body of the adult females.

Due to the sensitivities to various substances and a number of parameters that can be measured simultaneously, cladocerans are considered as effective tools for toxicity assessment and monitoring of water quality (Zein et al., 2014). The present study shows that adverse effects of metal mixtures on water flea cannot be ignored despite the harmful effect of individual metals on organisms is negligible in most scenarios. Multi-generational effects showed that continuous exposure to low concentrations of multiple metals could lead to large population losses for dominant aquatic species *M. macrocopa*. Stage-based structure analysis for *D. magna* indicated the proportion of the adult-aged population changed dramatically under continuous exposure to greater concentrations of multiple metals within several years. *M. macrocopa* appeared to be more responsive to chemical stressors compared with *D. magna*, which suggested the potential use of *Moina* as important biomarker in water quality assessment. Reproduction endpoints, especially the total number of offspring, appeared to be more sensitive to exposure of cocktails, so the densities of cladocerans in water bodies could be an effective parameter indicating possible detrimental effects induced by toxic chemicals (Dodson et al., 1995). However, the metal monitoring results to which we used in our analysis most likely underestimate actual exposures because it is difficult to capture transient metal peak concentrations. Sexual reproduction in water flea and the associated production of resting eggs allows them to survive unfavorable environmental conditions (Issa et al., 2021), which might result in overestimation of ecotoxicological risks posed by metal cocktails in surface waters. Speciation of the metals in Tai Lake is much more complicated than that represented in this experiment, and toxicities can be affected by chemical properties of water bodies, such as hardness (Carlson, A., 1984). Further research should be performed to investigate the effects of different water chemistry characteristics on bioavailability and toxicity of metal mixture, as well as the mechanisms underlying the adverse effects induced by metal mixtures on organisms. To link laboratory data to situation

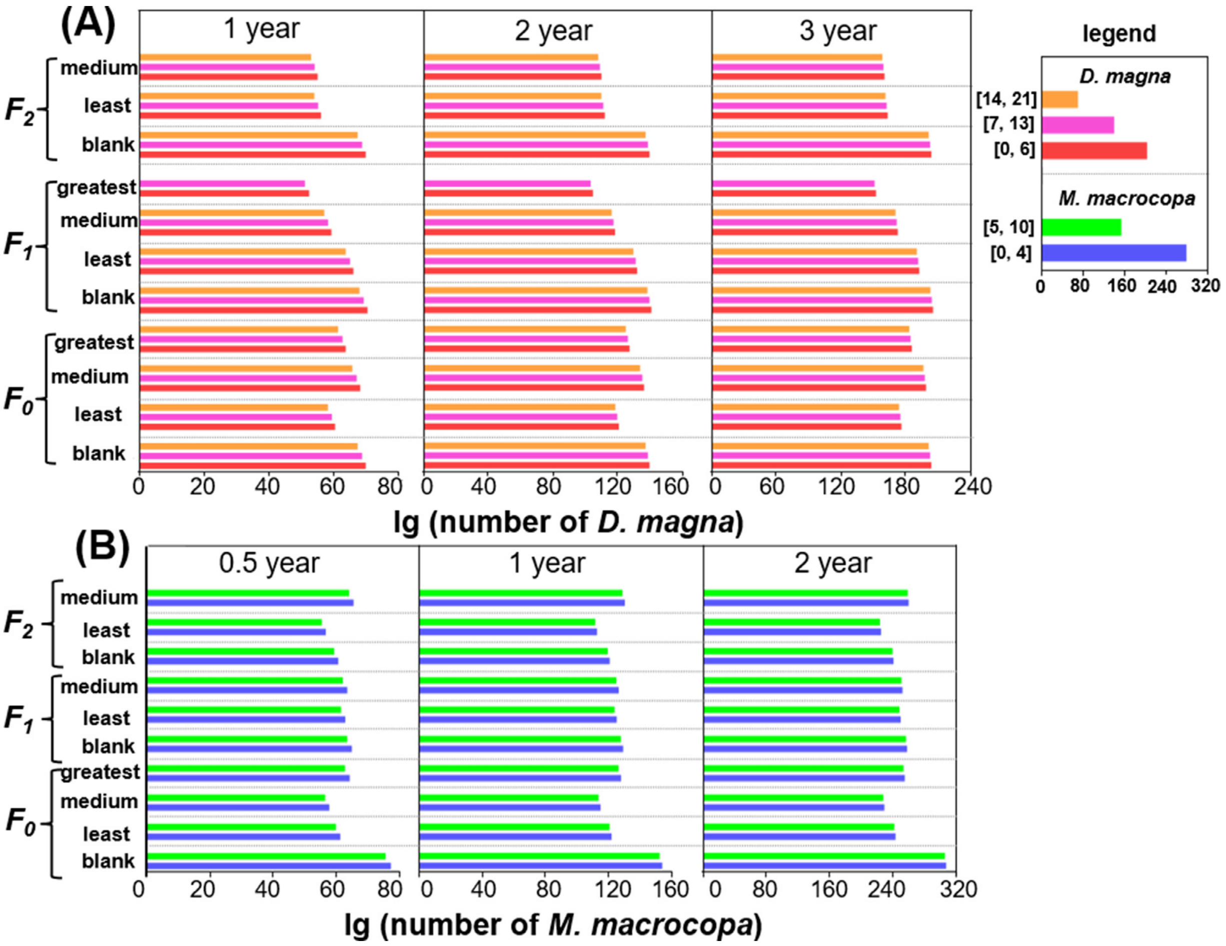

Fig. 5. Population projection of *Daphnia magna* (A) and *Moina macrocopa* (B) based on stage structure. The structure analysis intervals of *D. magna* are for three life stages: 0-6 days, 7-13 days and 14-21days within 1, 2 and 3 years while structure projection of *M. macrocopa* within 0.5, 1 and 2 years are based on two stages: 0-4 days and 5-10 days.

in the field, relationships between resting egg production and end points of interest (e.g., survivorship rate, fecundity) can be incorporated into a population model. Besides, whole-life-stage data on population structure and development based on density-dependent growth are needed due complexities of natural environments. Finally, more multi-generational studies are recommended since potential effects may be overlooked in single-generation experiments.

4. Conclusions

This study investigated distributions of multiple metals in Tai Lake, integrated individual-level measurements from multi-generational experiments and a simple demographic model to predict the long-term effects of metal cocktails at environmental concentrations on sensitive populations. Our results showed that co-exposure to mixtures of Cu, Pb, Cd, Ni, and Zn at environmental concentrations caused a series of adverse multi-generational effects on growth, reproduction, and population dynamics of sensitive species, *D. magna* and *M. macrocopa*, indicating that adverse effects caused by environmentally relevant concentrations of metal cocktails in Tai Lake should not be ignored. These findings highlight the immense value of population density of cladocerans as effective indicator for toxicity assessment and monitoring of water quality. The framework presented in the present study could address the challenge of assessing the hazards of metal mixtures in aquatic environments and the associated large datasets, and facilitate the consideration of the potential toxicities of metal mixtures based on environmentally relevant concentrations in risk assessment activities. This integrated approach can be used to develop research questions and management strategies for assessing ecological risk of hazardous chemicals.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was financially supported by the Major National Science and Technology Project of China [grant numbers 2018ZX07208001, 2017ZX07301002]; the National Natural Science Foundation of China [grant number 21677073]; and the National Key Research and Development Program of China [grant number 2018YFC1801505]. Portions of this research were supported by “Next generation solutions to ensure healthy water resources for future generations” funded by the Global Water Futures program, Canada First Research Excellence Fund [grant number #419205]. Prof. Giesy was supported by the “High Level Foreign Experts” program [grant number #GDT20143200016] funded by the State Administration of Foreign Experts Affairs, the P.R. China to Nanjing University, and the Einstein Professor Program of the Chinese Academy of Sciences. Prof. Giesy was also supported by the Canada Research Chair program and a Distinguished Visiting Professorship in the Department of Environmental Sciences at Baylor University, Waco, Texas, USA.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2021.117274.

References

An, W., Hu, J., Giesy, J.P., Yang, M., 2009. Extinction risk of exploited wild roach (*Rutilus rutilus*) populations due to chemical feminization. Environ. Sci. Technol. 43 (20), 7895–7901.

Barbiero, R.P., Rudstam, L.G., Watkins, J.M., Lesht, B.M., 2019. A cross-lake comparison of crustacean zooplankton communities in the Laurentian Great Lakes, 1997-2016. J. Great Lakes Res. 45 (3), 672–690.

Biesinger, K.E., Christensen, G.M., Fiandt, J.T., 1986. Effects of metal salt mixtures on *Daphnia magna* reproduction. Ecotoxicol. Environ. Saf. 11 (1), 9–14.

Brun, N.R., Fields, P.D., Horsfield, S., Mirbahai, L., Ebert, D., Colbourne, J.K., Fent, K., 2019. Mixtures of aluminum and indium induce more than additive phenotypic and toxicogenomic responses in *Daphnia magna*. Environ. Sci. Technol. 53 (3), 1639–1649.

Carlson, A., 1984. Guidelines for Deriving Numerical Aquatic Site-Specific Water Quality Criteria by Modifying National Criteria. Environmental Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency.

Chen, L., Zhou, B., Xu, B., Wu, F., Fu, Z., 2011. Cadmium and chromium concentrations and their ecological risks in the water body of Taihu Lake, East China. Chin. J. Ecol. 30 (10), 2290–2296.

Chen, Y., Huang, J., Xing, L., Liu, H., Giesy, J.P., Yu, H., Zhang, X., 2014. Effects of multigenerational exposures of *D. magna* to environmentally relevant concentrations of pentachlorophenol. Environ. Sci. Pollut. Res Int. 21 (1), 234–243.

Dietrich, S., Dammel, S., Ploessl, F., Bracher, F., Laforsch, C., 2010a. Effects of a pharmaceutical mixture at environmentally relevant concentrations on the amphipod *Gammarus fossarum*. Mar. Freshwater Res. 61 (2), 196–203.

Dietrich, S., Ploessl, F., Bracher, F., Laforsch, C., 2010b. Single and combined toxicity of pharmaceuticals at environmentally relevant concentrations in *Daphnia magna*—A multigenerational study. Chemosphere 79 (1), 60–66.

Dodson, S.I., Hanazato, T., Gorski, P.R., 1995. Behavioral-responses of daphnia-pulex exposed to carbaryl and chaoborus kairomone. Environ. Toxicol. Chem. 14 (1), 43–50.

Fu, J., Hu, X., Tao, X., Yu, H., Zhang, X., 2013. Risk and toxicity assessments of heavy metals in sediments and fishes from the Yangtze River and Taihu Lake. China. Chemosphere 93 (9), 1887–1895.

Fu, Z., Wu, F., Chen, L., Xu, B., Feng, C., Bai, Y., Liao, H., Sun, S., Giesy, J.P., Guo, W., 2016. Copper and zinc, but not other priority toxic metals, pose risks to native aquatic species in a large urban lake in Eastern China. Environ. Pollut. 219, 1069–1076.

Gama-Flores, J.L., Huidobro-Salas, M.E., Sarma, S.S.S., Nandini, S., 2017. Four transgenerational demographic performance of *Moina macrocopa* exposed to chronic levels of cadmium. Dose-Response 15 (3), 1559325817723732.

Gama-Flores, J.L., Sarma, S.S.S., Nandini, S., 2007. Exposure time-dependent cadmium toxicity to *Moina macrocopa* (Cladocera): A life table demographic study. Aquat. Ecol. 41 (4), 639–648.

Guan, R., Wang, W.X., 2006. Multigenerational cadmium acclimation and biokinetics in *Daphnia magna*. Environ. Pollut. 141 (2), 343–352.

Issa, S., Simonsen, A., Jaspers, V.L.B., Einum, S., 2021. Population dynamics and resting egg production in *Daphnia*: Interactive effects of mercury, population density and temperature. Sci. Total Environ. 755.

Jiang, X., Wang, W., Wang, S., Zhang, B., Hu, J., 2012. Initial identification of heavy metals contamination in Taihu Lake, a eutrophic lake in China. J. Environ. Sci. (China) 24 (9), 1539–1548.

Lari, E., Gauthier, P., Mohaddes, E., Pyle, G.G., 2017. Interactive toxicity of Ni, Zn, Cu, and Cd on *Daphnia magna* at lethal and sub-lethal concentrations. J. Hazard. Mater. 334, 21–28.

Lei, P., Zhang, H., Shan, B., Zhang, B., 2016. Distribution, diffusive fluxes, and toxicity of heavy metals and PAHs in pore water profiles from the northern bays of Taihu Lake. Environ. Sci. Pollut. Res Int. 23 (21), 22072–22083.

Leslie, P.H., 1945. On the use of matrices in certain population mathematics. Biometrika 33 (3), 183–212.

Lewis, E.G., 1977. On the generation and growth of a population. Biomathematics 221–225.

Liang, F., Yang, S., Sun, C., 2011. Primary health risk analysis of metals in surface water of Taihu Lake. China. Bull. Environ. Contam. Toxicol. 87 (4), 404–408.

Liao, J., Liang, F., Yang, S., He, H., Sun, C., Gao, S., Cui, Y., 2014. Derivation of freshwater quality criteria for hexavalent chromium for protection of aquatic organisms in China. Asian J. Ecotoxicol. 9 (2), 306–318.

Ministry of Ecology and Environment of the People's Republic of China, 2009. Water quality sampling—Technical regulation of the preservation and handling of samples. Available online: http://www.mee.gov.cn/ywgz/fgbz/bz/bzwb/jcffbz/200910/t20091010_162157.shtml. (Accessed 16 Aug 2019).

Ministry of Ecology and Environment of the People's Republic of China, 2014. Water quality—Determination of 65 elements Inductively coupled plasma-mass spectrometry. Available online: http://www.mee.gov.cn/ywgz/fgbz/bz/bzwb/jcffbz/201405/t20140523_275576.shtml. (Accessed 16 Aug 2019).

Ministry of Environment protection of the People's Republic of China, 2002. Environmental Quality Standards for Surface Water (GB 3838–2002). Available online: http://www.mee.gov.cn/ywgz/fgbz/bz/bzwb/shjbh/shjzlbz/200206/t20020601_66497.shtml. (Accessed 16 Aug 2019).

Nys, C., Versieren, L., Cordery, K.I., Blust, R., Smolders, E., De Schamphelaere, K.A.C., 2017. Systematic evaluation of chronic metal-mixture toxicity to three species and implications for risk assessment. Environ. Sci. Technol. 51 (8), 4615–4623.

OECD guidelines for the testing of chemicals, 2012. Test No. 211 *Daphnia magna* Reproduction Test. Available online: https://www.oecd-ilibrary.org/environment/test-no-211-daphnia-magna-reproduction-test_9789264185203-en. (Accessed 16 Oct 2018).

Payne, J., Scholze, M., Kortenkamp, A., 2001. Mixtures of four organochlorines enhance human breast cancer cell proliferation. Environ. Health Perspect. 109 (4), 391–397.

Price, P., Han, X., Junghans, M., Kunz, P., Watts, C., Leverett, D., 2012. An application of a decision tree for assessing effects from exposures to multiple substances to the assessment of human and ecological effects from combined exposures to chemicals observed in surface waters and wastewater effluents. Environ. Sci. Eur. 24, 34.

Price, P.S., Han, X., 2011. Maximum cumulative ratio (MCR) as a tool for assessing the value of performing a cumulative risk assessment. Int. J. Environ. Res. Public Health 8 (6), 2212–2225.

Ríos-Arana, J.V., Walsh, E.J., Ortiz, M., 2007. Interaction effects of multi-metal solutions (As, Cr, Cu, Ni, Pb and Zn) on life history traits in the rotifer *Plationus patulus*. J. Environ. Sci. Health A Toxic Hazard. Subst. Environ. Eng. 42 (10), 1473–1481.

Shi, R., Yang, C., Su, R., Jin, J., Chen, Y., Liu, H., Giesy, J.P., Yu, H., 2014. Weighted species sensitivity distribution method to derive site-specific quality criteria for copper in Tai Lake. China. Environ. Sci. Pollut. Res Int. 21 (22), 12968–12978.

Silva, E., Rajapakse, N., Kortenkamp, A., 2002. Something from "nothing"—Eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environ. Sci. Technol. 36 (8), 1751–1756.

Sun, X., Sun, C., Liu, H., 2020. Weighted species sensitivity distribution method to derive site-specific quality criteria of lead for protection of aquatic life in Tai Lake. Environ. Chem. 39 (6), 1578–1589.

Tenzin, Q., Wang, C., Wang, M., Shi, H., Wang, Y., 2020. The water quality criteria of nickel and the influence affected by water hardness to protect freshwater aquatic organisms in China. J. Northwest. Univ. Nat. Sci. Ed. 50 (1), 75–83.

U.S. Environmental Protection Agency, 1986. Quality criteria for water. Available online: https://nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=00001MGA.txt. (Accessed 26 May 2019).

U.S. Environmental Protection Agency, 2009. National recommended water quality criteria. Available online: https://www.epa.gov/wqc/national-recommended-water-quality-criteria-aquatic-life-criteria-table. (Accessed 26 May 2019).

U.S. Environmental Protection Agency, 2019. Available online: https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/aquatic-life-benchmarks-and-ecological-risk. Office of Pesticide Programs' Aquatic Life Benchmarks. (Accessed 26 Feb 2019).

Vallotton, N., Price, P.S., 2016. Use of the maximum cumulative ratio as an approach for prioritizing aquatic coexposure to plant protection products: A case study of a large surface water monitoring database. Environ. Sci. Technol. 50 (10), 5286–5293.

Wang, W., Fan, X., Huang, C., Zheng, H., Chen, Z., Fan, B., Xu, C., 2016. Monitoring and comparison analysis of heavy metals in the five great lakes in Jiangsu Province. J. Lake Sci. 28 (3), 494–501.

Wu, F., Feng, C., Zhang, R., Li, Y., Du, D., 2012. Derivation of water quality criteria for representative water-body pollutants in China. Sci. China Earth Sci. 55 (6), 900–906.

Wu, F., Meng, W., Cao, Y., Li, H., Zhang, R., Feng, C., Yan, Z., 2011. Derivation of aquatic life water quality criteria for cadmium in freshwater in China. Res. Environ. Sci. 24 (2), 172–184.

Xin, Z., Wenchao, Z., Zhenguang, Y., Yiguo, H., Zhengtao, L., Xianliang, Y., Xiaonan, W., Tingting, L., Liming, Z., 2015. Species sensitivity analysis of heavy metals to freshwater organisms. Ecotoxicology 24 (7–8), 1621–1631.

Yan, S., Yu, H., Li, H., Zhang, L., Wang, X., Xu, J., 2011. Spatial patterns of dissolved heavy metal in surrounding rivers of Lake Taihu during dry seasons. Environ. Sci. Technol. 34 (11), 6–10 63.

Yan, Z., Wei, M., Zhengtao, L.I.U., Ruozhen, Y.U., Yahui, Z., 2009. Biological criteria for freshwater Cd in China. Acta Sci. Circum. 29 (11), 2393–2406.

Yao, H., Qian, X., Gao, H.L., Wang, Y.L., Xia, B.S., 2014. Seasonal and spatial variations of heavy metals in two typical Chinese rivers: Concentrations, environmental risks, and possible sources. Int. J. Environ. Res. Public Health 11 (11), 11860–11878.

Zein, M.A., McElmurry, S.P., Kashian, D.R., Savolainen, P.T., Pitts, D.K., 2014. Optical bioassay for measuring sublethal toxicity of insecticides in *Daphnia pulex*. Environ. Toxicol. Chem. 33 (1), 144–151.

Zeng, J., Yang, L., Chen, X., Chuai, X., Wu, Q.L., 2012. Spatial distribution and seasonal variation of heavy metals in water and sediments of Taihu Lake. Pol. J. Environ. Stud. 21 (5), 1489–1496.

Zhai, S., Hu, W., Zhu, Z., 2010. Ecological impacts of water transfers on Lake Taihu from the Yangtze River. China. Ecol. Eng. 36 (4), 406–420.

Zhang, J., Yan, Z., Gao, F., Wu, J., Pei, S., Zhou, J., Liu, Z., 2015. Development of aquatic life criteria for arsenic species and its application in Liao River. Acta Sci. Circum. 35 (4), 1164–1173.

Zhang, R., Wu, F., Li, H., Feng, C., Guo, G., 2012. Deriving aquatic water quality criteria for inorganic mercury in China by species sensitivity distributions. Acta Sci. Circum. 32 (2), 440–449.

Zhao, Q., Hou, J., Wang, C., Wang, P., Miao, L., Lv, B., Gu, Q., 2015. Deriving aquatic water quality criteria for heavy metals in Taihu Lake by probabilistic species Sensitivity distribution. Asian J. Ecotoxicol. 10 (6), 121–128.

Supporting Information

Ecotoxicological risk assessment of metal cocktails based on maximum cumulative ratio during multi-generational exposures

Xuehua Sun[a], *Li Zhang*[a], *Hongling Liu*[a*], *Zunyao Wang*[a], *John P. Giesy*[a,b,c]

[a] State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing, Jiangsu 210023, China

[b] Department of Veterinary Biomedical Sciences and Toxicology Center, University of Saskatchewan, Saskatoon, SK S7N 5B3, Canada

[c] Department of Environmental Science, Baylor University, Waco, Texas, USA

*Corresponding author; E-mail: hlliu@nju.edu.cn

Keywords: crustacean; metal mixture; multi-generational effect; Leslie matrix; population dynamics; Tai Lake, China, Asia.

Materials and methods

S.1. Test organisms

Test organisms *Daphnia magna* and *Moina macrocopa* used in this study were all derived from a stock colony that was initiated from few individuals and have been cultured in the laboratory for several years at 22 ± 1 °C using a 16/8 h light/dark cycle. Culture medium is changed every two days and clones are fed with a suspension of the green alga *Scenedesmus obliquus* two times per day. Initially, 24 h tests were conducted with K_2CrO_3 (0.15、0.3、0.6、1.2、2.4、4.8 mg/L) as a positive control toxicant according to International Organization for Standardization (ISO) 6341-2012 to determine sensitivities and responsiveness of *Daphnia magna* and *Moina macrocopa* (ISO, 2012). Results of positive control showed test organisms met the requirements of reproduction test.

S.2. Metal mixtures exposure assay

This study used semi-static tests. Ten newly hatched neonates (F_0; <24 h old) of the third brood of age-synchronized adult females were randomly transferred into 50 mL polytetrafluoroethylene beakers containing 20 mL of the test solution; every beaker contained one neonate so the experimental unit was “beaker”. Neonates were fed at a specific time and the test solutions were changed daily during the exposure. Growth parameters, including shelling and swimming conditions, were measured twice per day and shells were promptly removed with disposable plastic pipettes. Once F_1 neonates were observed, F_0 individuals were removed from vessels and the numbers of newly hatched neonates of each adult female were recorded. For *D. magna*, on the 11th day of F_0 generation, ten neonates per beaker were selected as the F_1 generation to carry out the experiment. For *M. macrocopa*, neonates were randomly collected on the sixth day of the F_0 generation. The experiment was terminated when the neonates (F_2) of generation F_1 of *D. magna* and *M. macrocopa* were 21 and 10 days old, respectively. For F_0, F_1, and F_2 (21 and 10 days old)-generation females, body length from the apex of the helmet to the base of the tail spine was measured using a multipurpose zoom microscope (Nikon AZ 100). In addition, ages at first brood, numbers of molts, and numbers of offspring per adult female within 21 or 10 days were recorded. Female F_1 *D. magna* and *M. macrocopa* exposed to the greatest concentrations of mixtures of metals all died on approximately day 10 and 4 respectively, which left no sufficient neonates to continue the F_2 experiment.

Table S1. Concentrations of metals in the wet season at each site (unit: μg/L)[a].

Sites	Cu	Zn	Cd	Pb	Cr(VI)	Sites	Cu	Zn	Cd	Pb	Cr(VI)
S1	5.74	14.5	0.068	0.348	6.30	**S27**	3.62	26.8	0.038	0.635	5.26
S2	5.63	15.4	0.047	0.234	6.46	**S28**	4.07	15.1	0.041	-	5.37
S3	5.79	12.4	0.041	0.158	6.88	**S29**	4.06	9.31	0.033	-	4.45
S4	5.87	11.9	0.042	0.130	6.87	**S30**	3.92	11.8	0.035	-	4.81
S5	7.72	14.2	0.051	0.277	7.87	**S31**	3.99	7.09	0.037	-	5.10
S6	7.12	14.9	0.041	0.140	7.70	**S32**	3.93	9.61	0.035	0.009	5.16
S7	6.51	30.2	0.043	0.221	7.26	**S33**	3.49	23.6	0.028	-	4.79
S8	3.44	12.9	0.030	0.276	6.85	**S34**	2.91	10.1	0.024	-	4.37
S9	3.62	13.1	0.029	2.53	6.81	**S35**	2.21	28.4	0.049	0.286	0.71
S10	5.86	19.2	0.055	0.410	7.47	**S36**	2.50	12.0	0.018	-	4.38
S11	7.14	10.3	0.035	0.277	9.81	**S37**	2.64	41.1	0.021	-	4.33
S12	11.0	6.58	0.029	0.096	8.26	**Caoqiao River**	4.71	16.3	0.032	0.103	6.04
S13	7.17	11.7	0.055	1.85	7.27	**Chendong Port**	4.57	6.75	0.031	-	5.09
S14	7.79	15.8	0.047	0.260	8.75	**Dagang River**	0.68	3.10	0.003	-	1.65
S15	10.3	10.2	0.031	-	9.01	**Dapu Port**	4.10	4.61	0.032	-	5.55
S16	7.03	13.9	0.169	0.250	8.23	**Guandu Port**	4.17	4.75	0.023	0.323	4.88
S17	6.55	18.1	0.054	0.267	6.84	**Hongxiang Port**	5.16	5.92	0.031	0.031	5.24
S18	3.28	19.6	0.037	0.284	7.63	**Liangxi River**	2.76	8.98	0.033	-	4.08
S19	4.03	23.5	0.051	-	7.03	**Shedu Port**	8.17	8.24	0.044	0.251	5.30
S20	6.53	217	0.111	0.875	7.80	**Taige Canal**	10.15	15.2	0.034	-	7.83
S21	5.99	51.1	0.053	0.445	6.08	**Wangyu River**	3.82	14.5	0.04	0.067	4.98
S22	4.89	8.40	0.048	0.010	4.97	**Wuxi Port**	2.01	19.7	0.061	0.515	0.93
S23	6.59	23.4	0.084	2.86	6.25	**Wujin Port**	4.11	6.51	0.026	-	7.66
S24	4.90	6.36	0.026	0.201	5.20	**Xiaoxi Port**	3.01	7.73	0.023	0.454	4.24
S25	4.47	19.8	0.041	0.268	5.50	**Yincun Port**	6.75	18.9	0.057	0.158	6.09
S26	3.21	11.7	0.121	0.143	4.90	**Zhihu Port**	6.16	8.28	0.035	-	10.5

---

[a] Note: "-" is below the detection of limit.

Table S2. Concentrations of metals in the dry season at each site (unit: μg/L)[a].

Sites	Cu	Zn	Cd	Pb	Cr(VI)	Sites	Cu	Zn	Cd	Pb	Cr(VI)
S1	3.72	7.42	0.039	0.222	1.95	**S27**	4.03	9.91	0.047	0.962	2.26
S2	3.69	8.86	0.044	0.543	2.65	**S28**	3.89	8.01	0.060	0.688	2.37
S3	2.25	5.95	0.048	0.244	2.27	**S29**	4.14	10.88	0.052	0.947	2.52
S4	3.27	10.09	0.053	0.338	2.10	**S30**	3.91	14.06	0.063	0.724	2.56
S5	2.98	7.90	0.047	0.604	2.95	**S31**	3.72	6.39	0.054	1.058	2.44
S6	3.85	14.53	0.055	0.904	2.51	**S32**	2.65	7.34	0.067	3.476	2.45
S7	5.40	89.14	0.053	0.237	2.51	**S33**	3.67	8.37	0.058	1.057	2.35
S8	2.71	5.42	0.037	0.206	2.45	**S34**	3.78	9.52	0.077	1.165	2.48
S9	2.57	8.42	0.032	0.098	2.23	**S35**	2.15	5.02	0.037	0.292	2.27
S10	3.01	6.58	0.032	0.106	2.77	**S36**	4.08	6.60	0.039	0.319	2.14
S11	3.30	7.19	0.038	0.173	2.34	**S37**	3.26	7.42	0.044	0.666	3.73
S12	3.05	4.92	0.041	0.147	2.31	**Caoqiao River**	3.35	11.13	0.043	0.315	2.56
S13	3.56	4.65	0.044	0.150	2.40	**Chendong Port**	3.88	6.51	0.053	0.924	3.10
S14	4.78	7.72	0.046	0.126	3.65	**Dagang River**	1.52	8.53	0.038	0.799	1.53
S15	5.29	9.33	0.050	0.431	3.62	**Dapu Port**	3.69	6.29	0.056	0.297	2.87
S16	5.16	8.53	0.051	0.591	3.50	**Guandu Port**	3.74	6.01	0.049	0.201	2.82
S17	4.22	12.56	0.056	0.612	2.25	**Hongxiang Port**	4.18	7.86	0.051	0.592	3.09
S18	3.67	7.99	0.054	0.231	2.97	**Liangxi River**	2.74	7.93	0.035	0.436	2.49
S19	3.65	10.87	0.062	4.874	2.36	**Shedu Port**	4.38	12.63	0.043	0.244	2.84
S20	2.84	3.34	0.038	-	1.75	**Taige Canal**	2.16	7.53	0.033	0.351	3.25
S21	3.18	7.94	0.055	0.450	1.86	**Wangyu River**	2.81	6.13	0.053	0.939	2.38
S22	3.37	9.34	0.044	1.895	2.20	**Wuxi Port**	3.05	6.56	0.089	0.558	3.13
S23	3.24	9.67	0.038	0.226	2.51	**Wujin Port**	4.30	9.28	0.050	0.671	3.05
S24	3.48	6.43	0.062	1.646	2.20	**Xiaoxi Port**	3.77	12.57	0.068	0.811	2.32
S25	3.95	9.86	0.048	1.085	2.44	**Yincun Port**	5.34	8.51	0.040	0.782	2.95
S26	2.11	5.91	0.034	0.780	2.35	**Zhihu Port**	3.67	8.46	0.041	0.275	2.96

---

[a] Note: "-" is below the detection of limit.

Table S3. Rates of survival of three generations of 21 d - *Daphnia magna*, 10 d - *Moina macrocopa* treated with different concentrations. Each value represents the mean ± SD of ten independent experiments.

<table>
<tr><th colspan="5">Survival rate of <i>D. magna</i> at 21 d (%)</th></tr>
<tr><th>Generation</th><th>Blank</th><th>Least concentration</th><th>Medium concentration</th><th>Greatest concentration</th></tr>
<tr><td>F_0</td><td>100.00±0.00</td><td>80.00±14.43</td><td>80.00±16.58</td><td>60.00±14.14</td></tr>
<tr><td>F_1</td><td>100.00±0.00</td><td>90.00±17.32</td><td>80.00±16.58</td><td>0.00</td></tr>
<tr><td>F_2</td><td>100.00±0.00</td><td>70.00±27.39</td><td>70.00±22.36</td><td>0.00</td></tr>
<tr><th colspan="5">Survival rate of <i>M. macrocopa</i> at 10 d (%)</th></tr>
<tr><td>F_0</td><td>100.00±0.00</td><td>90.00±22.36</td><td>60.00±22.36</td><td>0.00</td></tr>
<tr><td>F_1</td><td>100.00±0.00</td><td>100.00±0.00</td><td>90.00±22.36</td><td>0.00</td></tr>
<tr><td>F_2</td><td>100.00±0.00</td><td>100.00±0.00</td><td>80.00±27.39</td><td>0.00</td></tr>
</table>

Table S4. Comparisons among endpoints based on growth of *Daphnia magna* in different generations. Each value represents the mean ± SD of ten independent experiments (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared with the control group).

Body length (μm)				
Generation	Blank	Least concentration	Medium concentration	Greatest concentration
F_0	3780.24±46.83	3578.47±470.72	3790.67±53.66	2984.74±480.19***
F_1	3719.87±50.71	3647.12±85.82*	3585.87±71.76***	
F_2	3797.59±78.19	3579.62±579.48	3702.94±118.91	
Total number of molts				
F_0	10.30±0.67	8.50±2.01**	9.50±1.27	9.90±3.35
F_1	10.70±1.06	10.40±1.35	8.80±2.44*	5.20±0.42***
F_2	10.70±0.48	9.10±2.28*	9.50±2.32	

Table S5. Comparisons among endpoints based on growth of *Moina macrocopa* in different generations. Each value represents the mean ± SD of ten independent experiments (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared with the control group).

<table>
<tr><th colspan="5">Body length (μm)</th></tr>
<tr><th>Generation</th><th>Blank</th><th>Least concentration</th><th>Medium concentration</th><th>Greatest concentration</th></tr>
<tr><td>F_0</td><td>1871.21±69.08</td><td>1767.63±49.38**</td><td>1774.17±16.01***</td><td></td></tr>
<tr><td>F_1</td><td>1791.20±46.80</td><td>1773.44±44.81</td><td>1672.07±70.30***</td><td></td></tr>
<tr><td>F_2</td><td>1779.33±42.00</td><td>1776.40±49.24</td><td>1846.58±31.46**</td><td></td></tr>
<tr><th colspan="5">Total number of molts</th></tr>
<tr><td>F_0</td><td>7.10±0.74</td><td>6.00±2.16</td><td>5.00±2.05**</td><td>4.90±1.37***</td></tr>
<tr><td>F_1</td><td>7.30±0.67</td><td>7.00±0.67</td><td>6.80±1.48</td><td>7.00±0.00</td></tr>
<tr><td>F_2</td><td>7.20±0.00</td><td>7.20±0.42</td><td>6.80±0.63</td><td></td></tr>
</table>

Table S6. Comparisons among endpoints based on reproduction of *Daphnia magna* in different generations. Each value represents the mean ± SD of ten independent experiments (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared with the control group).

Generation	Blank	Least concentration	Medium concentration	Greatest concentration
Total number of offspring per adult female produced within 21 days				
F_0	90.60±6.55	71.25±9.88***	80.40±19.43	37.78±16.70***
F_1	94.10±5.90	67.80±15.72***	63.67±13.52***	16.33±6.98***
F_2	89.10±9.18	78.00±22.57	67.90±29.54*	
Age at first spawning (d)				
F_0	6.90±0.57	6.50±0.76	6.60±0.70	6.56±0.53
F_1	7.10±0.32	7.00±0.00	7.33±1.00	7.22±0.44
F_2	7.70±0.48	7.29±0.49	7.60±0.52	
Generation time (d)				
F_0	13.17±0.46	12.70±0.89	12.51±1.18	10.24±1.22***
F_1	13.20±0.43	13.26±1.29	13.22±1.48	7.25±0.43***
F_2	15.03±0.45	13.60±2.52	13.42±2.95	
Intrinsic rate of population growth (r; ind/day)				
F_0	0.34±0.01	0.32±0.02*	0.34±0.01	0.34±0.04
F_1	0.34±0.02	0.31±0.02**	0.30±0.03**	0.32±0.09
F_2	0.30±0.01	0.31±0.05	0.29±0.01	
Finite rate of increase (λ; ind/day)				
F_0	1.41±0.02	1.38±0.03*	1.41±0.02	1.40±0.06
F_1	1.41±0.02	1.37±0.03**	1.35±0.04**	1.39±0.12
F_2	1.35±0.02	1.36±0.07	1.33±0.03	

Table S7. Comparisons among endpoints based on reproduction of *Moina macrocopa* in different generations. Each value represents the mean ± SD of ten independent experiments (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared with the control group).

Generation	Blank	Least concentration	Medium concentration	Greatest concentration
Total number of offspring per adult female produced within 10 days				
F_0	121.40±20.50	90.89±31.71*	68.22±31.71***	45.60±25.05***
F_1	122.20±7.55	117.20±7.50	94.90±39.68*	
F_2	114.30±8.23	88.90±11.32***	103.10±14.48*	
Age at first spawning (d)				
F_0	3.10±0.32	3.33±0.50	3.56±0.53*	3.56±1.01
F_1	4.00±0.00	4.10±0.32	3.90±0.74	
F_2	4.00±0.00	3.10±0.32***	4.20±0.79	
Generation time (d)				
F_0	6.67±0.42	6.82±1.11	5.96±0.88*	5.50±0.74***
F_1	7.23±0.25	7.29±0.25	6.51±1.25*	
F_2	7.51±0.23	7.17±0.15**	6.83±0.51***	
Intrinsic rate of population growth (r; ind/day)				
F_0	0.72±0.03	0.62±0.05***	0.62±0.07***	0.65±0.10
F_1	0.67±0.02	0.65±0.02	0.63±0.10	
F_2	0.63±0.02	0.63±0.02	0.68±0.05**	
Finite rate of increase (λ; ind/day)				
F_0	2.05±0.06	1.85±0.10***	1.87±0.12***	1.93±0.19
F_1	1.95±0.04	1.92±0.04	1.88±0.17	
F_2	1.88±0.04	1.87±0.04	1.97±0.10**	

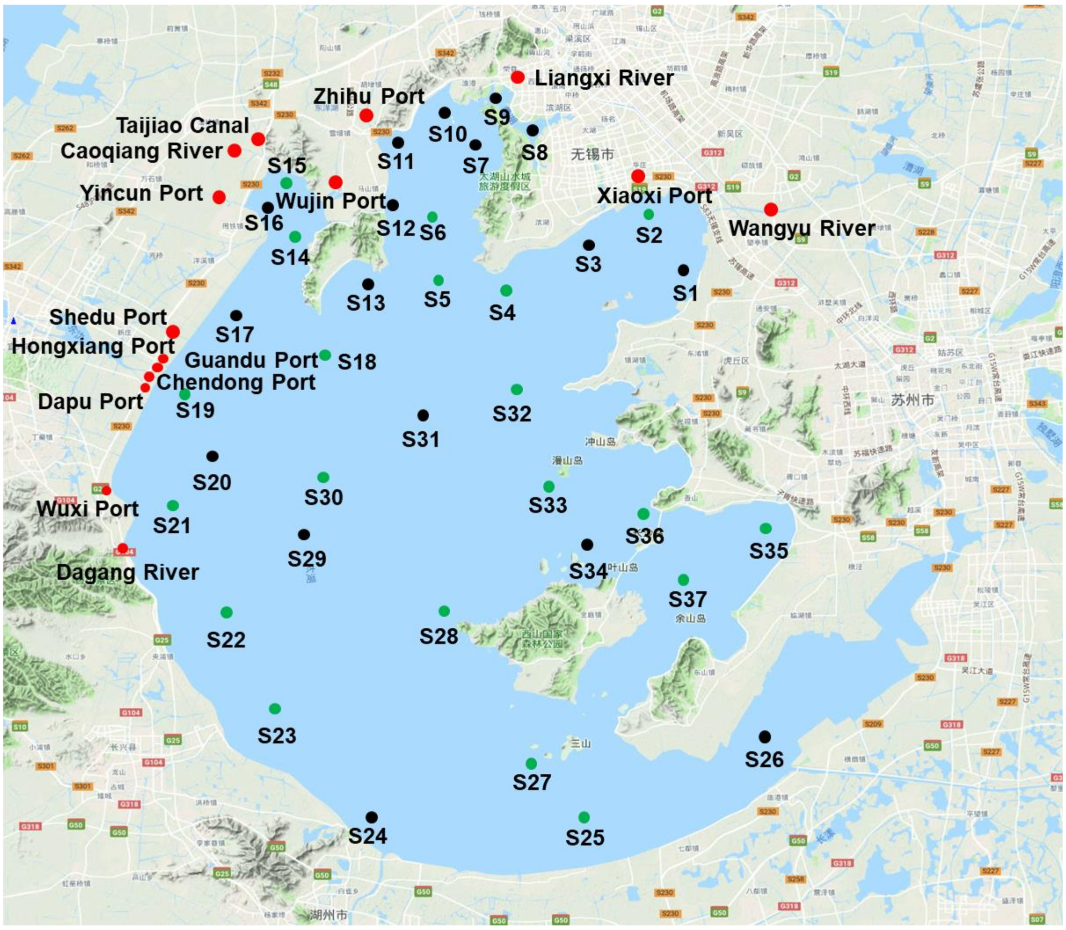

Fig. S1. Map of Tai Lake showing sampling locations.

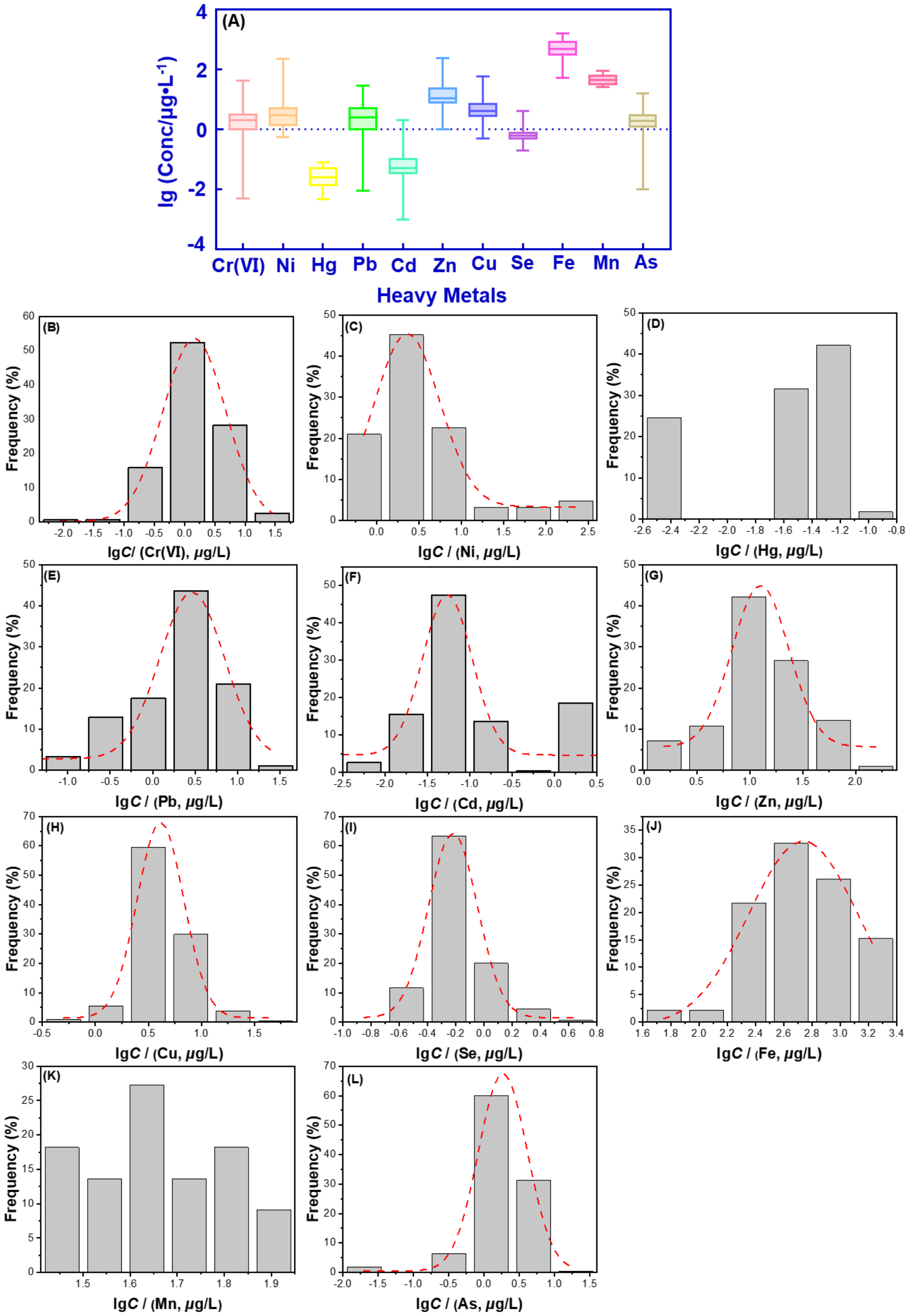

Fig. S2. Concentrations (A) and frequency distributions (B–L) of 11 metals (μg/L). (B)-(L) represent chromium (Cr(VI)), nickel (Ni), mercury (Hg), lead (Pb), cadmium (Cd), zinc (Zn), copper (Cu), selenium (Se), iron (Fe), manganese (Mn) and arsenic (As), respectively.

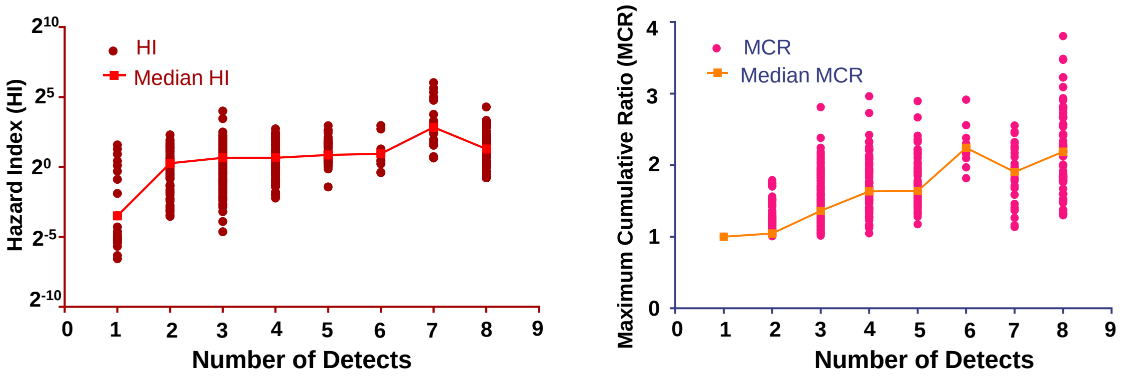

Fig. S3. Relationship (Kendall's tau-b value) between the hazard index (HI), maximum cumulative ratio (MCR) and *n* (left: HI-*n*; right: MCR-*n*). Proportions of samples with HI exceeding 1.0 was 69.8%. Based on all grouped samples, HI was significantly and positively correlated with *n*, for which Kendall's tau-b value was 0.306. Values of MCR ranged from 1.0 to 3.8. Positive correlation between MCR and *n* (0.608) was stronger than HI- *n*.

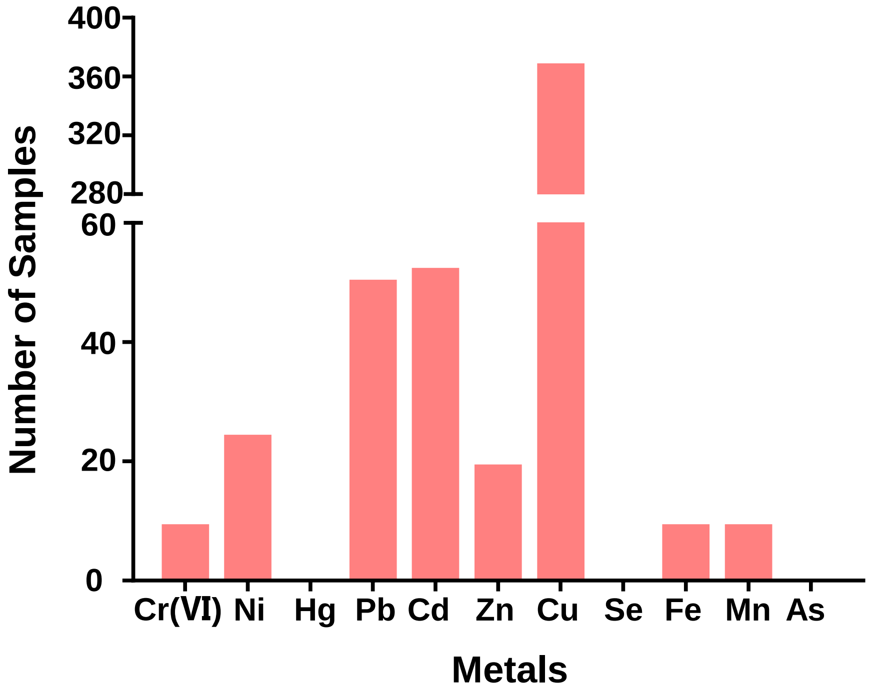

Fig. S4. Numbers of samples in which the hazard quotient (HQ) of a single metal exceed 1.0.

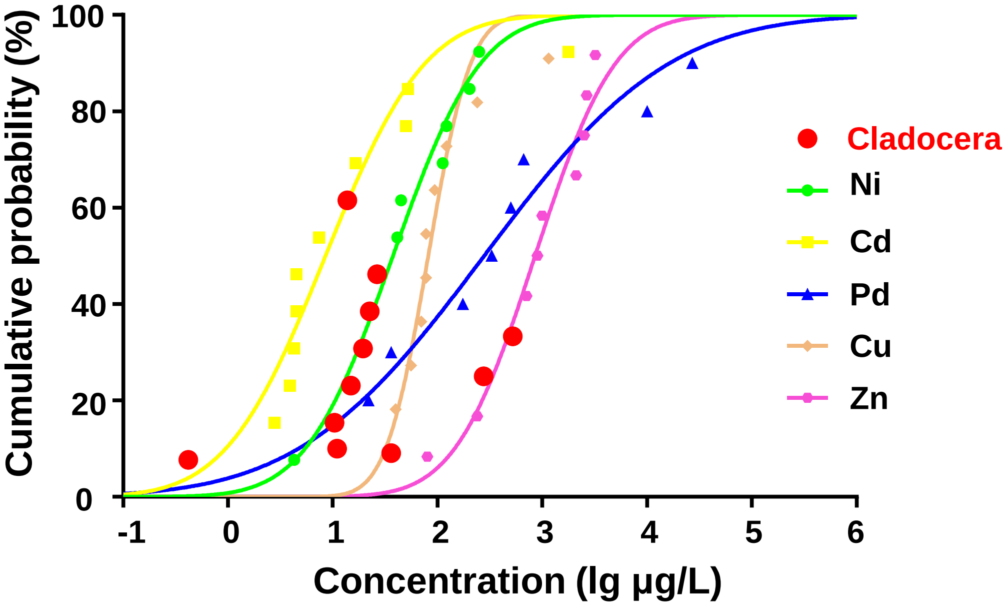

Fig. S5. Species sensitivity distributions for freshwater organisms based on chronic toxicity data for five metals.

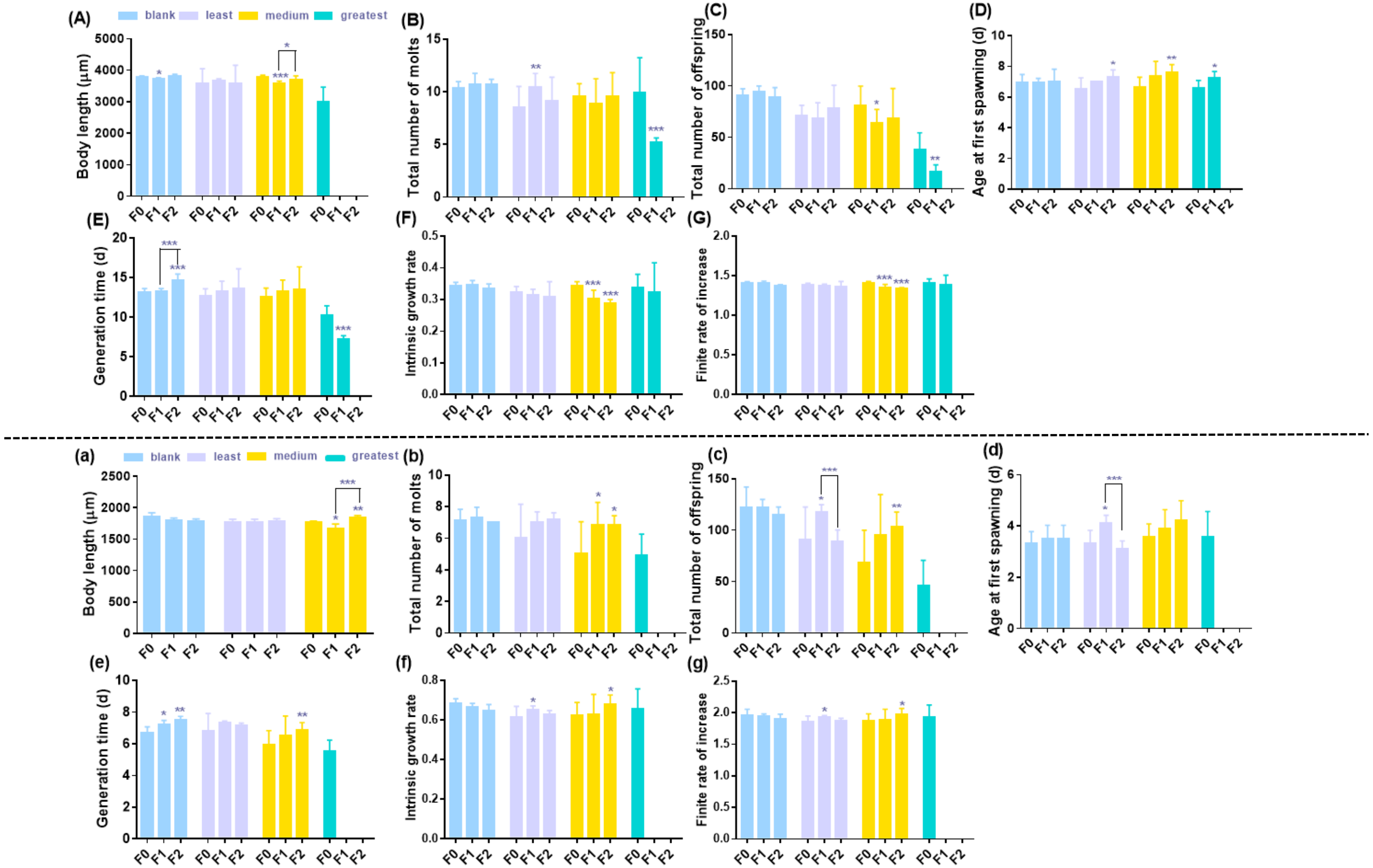

Fig. S6. Effects induced by different concentrations of metal mixtures on growth and reproduction of *Daphnia magna* (A–G) and *Moina macrocopa* (a–g) in different generations. The error bar represents the standard deviation of ten independent measurements (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared with the control group).

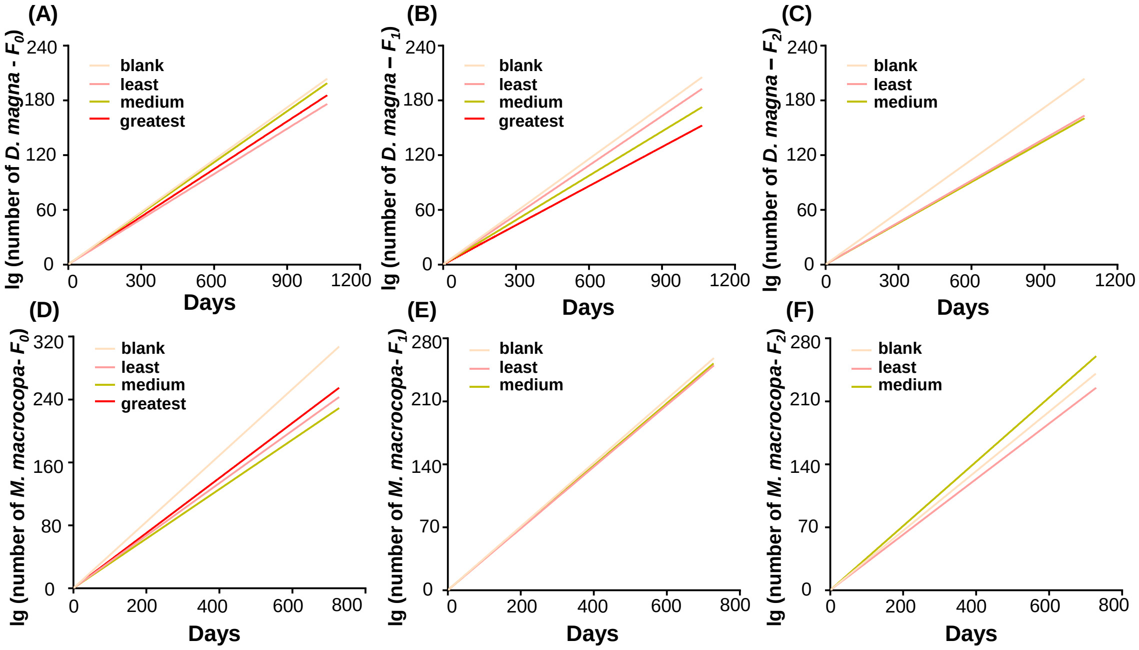

Fig. S7. Population projection of *Daphnia magna* and *Moina macrocopa*. (A–C) Logarithmic value of the number of *D. magna* in F_0, F_1 and F_2 population after three years, respectively, and (D–F) Logarithmic value of the number of *M. macrocopa* in F_0, F_1 and F_2 population after two years, respectively.

References

International Organization for Standardization, 2012. Water quality-Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) – Acute toxicity test. https://www.iso.org/standard/54614.html, Accessed date: 26 September 2019.