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COMBINED EFFECTS OF DISSOLVED ORGANIC MATERIAL AND WATER HARDNESS ON TOXICITY OF CADMIUM TO *DAPHNIA MAGNA*

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Abstract—The interaction between dissolved organic material (DOM) and water hardness and their effects on the acute toxicity of cadmium (Cd) to *Daphnia magna* was studied. At an original hardness (0.1 mmol Ca + Mg per liter) of humic lake water (DOC 19.6 mg/L), Cd was significantly less toxic in the humic than in the reference (DOC $<$ 0.2 mg/L) water. Furthermore, after dilution down to 10% (dissolved organic carbon [DOC] 2.0 mg/L), the humic water still decreased the lethality significantly. The results suggest that the reduced toxicity of Cd in the lake water is due to complexation with DOC. An increase in water hardness (from 0.1 to 0.5 or 2.5 mmol Ca + Mg per liter) decreased the measured binding coefficient of Cd to DOM. In addition, the acute toxicity of Cd decreased, and the difference between the reference and humic water disappeared. As a conclusion, DOM in the soft lake water had a protective effect against Cd toxicity. In hard water, obviously, the added hardness cations, especially Ca^{2+}, effectively competed with Cd^{2+} for available binding sites in DOM. Simultaneously, Ca^{2+} ions interfered also with the uptake of Cd^{2+} either by competing in transport through cell membranes or by reducing membrane permeability.

Keywords—Dissolved organic material Water hardness Cadmium Toxicity Binding

INTRODUCTION

High concentration of dissolved organic material (DOM) is characteristic of boreal freshwaters and affects the chemistry and ecology of aquatic environments. Both low pH and the brownish color of surface waters are mostly contributed by humic substances, the major constituents of DOM. Humic substances are a heterogenous mixture of organic acids with high molecular weight and polyelectrolyte nature as caused by a wide variety of functional groups [1]. However, the physicochemical properties of humic substances are related to acidic functional groups that are predominantly carboxylic and secondarily phenolic hydroxylic groups. Because the majority of DOM contain these oxyfunctional groups, they have an important role in the metal complexation ability of DOM. In the case of soft cations, such as cadmium (Cd), soft ligands, such as nitrogen (N) and sulfur (S) containing functional groups of DOM may play a role [2,3].

The environmental fate of metals in natural waters is greatly dependent of their speciation, and the total metal concentration is hardly sufficient to explain their behavior in aquatic systems. The chemical form of metal ions is governed by physicochemical factors (e.g., salinity, pH, hardness, and DOM) that prevail in the local environment [4]. The presence of natural complexing agents such as DOM will directly influence the transport and bioavailability of metal [5]. In general, due to complexation, the free and effective concentration of metal ions is reduced in water, and thus also their bioavailability and toxicity to biota is lowered [6]. However, varying and even controversial effects of dissolved humic material on Cd bioavailability and toxicity are reported in different studies. In many cases, the toxicity or bioavailability of Cd is increased in the presence of DOM [7–9]. Some studies show that DOM may reduce the bioavailability of Cd [10–12].

In addition to DOM, low hardness of water is typical of Finnish freshwaters. Exluding the lakes in Lappland, the median total organic carbon (TOC) in Finnish lakes is 14 mg/L. The median color, pH, and total Ca + Mg hardness are 120 mg platinum per liter, 6.1, and 0.2 mmol/L, respectively [13]. Because water quality parameters such as DOM and hardness are important factors modifying the environmental fate and effects of heavy metals in boreal environment, they were considered relevant topics for this study. The objectives of this study were (1) to examine the effect of water hardness on Cd binding to DOM and (2) to study the effects of DOM and water hardness on the acute toxicity of Cd in natural humic water to *Daphnia magna* to see how these two environmental parameters may change the observed toxicity.

MATERIAL AND METHODS

Experimental waters and chemicals

Water sample from Lake Louhilampi (Eastern Finland), considered a representative source of DOM, was collected just beneath the water surface. Some water quality parameters were measured immediately: pH 5.2, color 180 mg Pt per liter, conductivity 2.4 mS/m, and dissolved organic carbon (DOC) 19.6 mg/L. Total Ca + Mg hardness (Ca 2.055 mg/L and Mg 0.756 mg/L corresponding to 0.1 mmol Ca + Mg per liter or 8.3 mg/L $CaCO_3$ hardness with a Ca/Mg ratio of 5:3) was determined with atomic-absorption spectroscopy (AAS, Hitachi Z-2000 Polarized Zeeman AAS, Hitachi, Tokoyo, Japan).

Filtered (Whatman GF/C glassfiber filters, 1.6 μm, Whatman, Clifton, NJ, USA) water was stored in darkness (+5°C) until the start of experiments. Artificial humic-free reference water was made up of Milli-Q® grade water (DOC $<$ 0.2 mg/L) (Milli-Q, Bedford, MA, USA) by adding the following reagent-grade salts: $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, KCl 1.1 mg/

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L, and $NaHCO_3$ 13.0 mg/L (modified from Finnish Standards [14]). In experiments, the water hardness was modified by adding appropriate amounts of Ca and Mg salts (Ca/Mg ratio always 5:3). Because sublethal stress resulting from naturally low pH of humic water might have affect on the survival of daphnids, the pH was adjusted to 6.5 with additions of 0.1 mmol/L NaOH or HCl before experiments. Then the waters were filtered (0.2 μm, Nuclepore). The stock solution of cadmium was prepared from its chloride salt ($CdCl_2 \cdot H_2O$; chemical purity 99%; Sigma Chemical, St. Louis, MO, USA), and its final concentration was checked by AAS. Test concentrations were freshly prepared by adding stock solution to experimental waters. In the toxicity tests, the experimental concentrations of Ca and Cd were nominal and were not checked by AAS.

Determination of metal binding to dissolved organic material

The organic carbon normalized partition coefficients (K_{oc}) between Cd and DOM were determined by equilibrum dialysis [15,16]. First, the time dependence of binding was studied using contact times of 1, 2, 4, 7, 10, and 14 d, and Cd concentration was 300 μg/L. Further, the partition coefficients were determined at three different water hardnesses (0.1, 0.5, and 2.5 mmol Ca + Mg per liter) using five nominal Cd concentrations (40, 100, 300, 600, and 850 μg/L). Seven milliliters of lake water (DOC 19.6 mg/L) was added to a dialysis bag (Spectra/Por 6, mol wt cutoff 1,000 D) and placed into a glass jar containing 90 ml aqueous solution of Cd. To inhibit microbial activity, sodium azide (0.002%) was added. The jar was sealed with a Teflon®-lined cap and slowly shaken in the dark at 20°C for 4 d. Three or four replicates for each water hardness and Cd concentration were made, and a parallel experiment with DOM-free reference water was performed to ensure that equilibrum was achieved. The metal concentrations (both Cd and Ca) inside and outside of the dialysis bag were analyzed by AAS. The outside concentration (C_o) is considered to be freely dissolved metal, and the difference between the inside and outside concentration (C_p) is the metal bound to organic matter inside the bag. The K_{oc} (liters per kilogram) was calculated as

$$K_{oc} = C_p/(C_o \times \text{DOC}) \qquad (1)$$

where DOC is the concentration of dissolved organic carbon (kilograms per liter).

Another adsorption coefficient was determined by Freundlich isotherm [17]:

$$C_p = K \times C_o^n \qquad (2)$$

where C_p is the concentration of organically bound Cd (milligrams per kilogram of C), and C_o is the freely dissolved concentration (milligrams per liter) at the equilibrum. The empirical Freundlich constants K (adsorption coefficient, liters per kilogram) and n were determined by simple linear regression of the linear form of the Freundlich equation (log C_p vs log C_o). The slope is then n, and a value of $n = 1$ corresponds to a linear isotherm.

Animals and measurement of acute toxicity

Daphnia magna were obtained from a clone maintained at the University of Joensuu. Daphnids were cultured in artificial freshwater (pH between 6.5 and 7.1) according to Finnish standards [14] with day rhythm of 16 h light and 8 h dark at 20 ± 2°C. Daphnids were fed twice a week with green algae, *Monoraphidium contortum,* as the major food item.

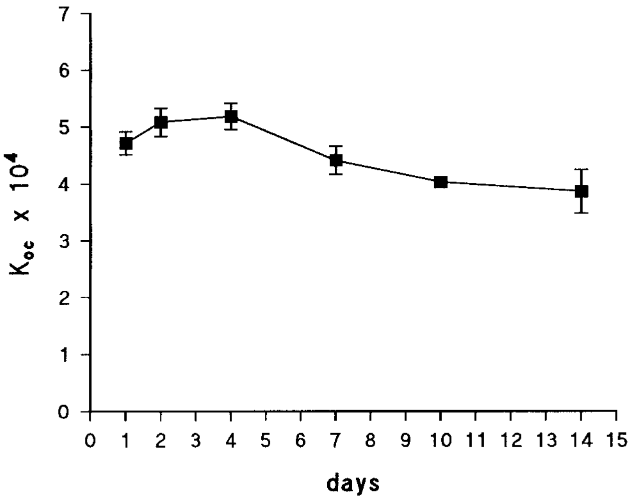

Fig. 1. The effect of dialysis time to the partition coefficients (K_{oc}) between cadmium and dissolved organic material in natural lake water (total hardness 0.1 mmol Ca + Mg per liter, dissolved organic carbon 19.6 mg/L).

Two series of experiments were initiated with neonatal (age <24 h) daphnids. In the first one, the effect of water hardness (0.1, 0.2, 0.5, 1.5, and 2.5 mmol Ca + Mg per liter) on the acute toxicity of Cd was examined in natural lake water. Parallel humic-free reference test was also conducted. The stock solution of Cd and experimental waters (10 ml in a 20-ml sealed glass vessel) were mixed just before the introduction of daphnids (five per each vessel). Three replicates were prepared for each test concentration. Then, test vials were transferred into the dark (20°C), and the mortality was observed after 24 and 48 h. No mortality was observed in natural lake water-only or reference water-only exposures. The experiments were performed at least twice with comparable results. In the second experiment, the effect of DOM on Cd toxicity was determined at constant water hardness. Lake water was diluted (1, 10, 25, 50, 75, and 90% resulting in nominal DOC content of 0.2, 2.0, 4.9, 9.8, 14.7, and 17.7 mg/L, respectively) with reference water at original hardness of 0.1 mmol Ca + Mg per liter. Undiluted lake water (DOC 19.6 mg/L) and a parallel humic-free reference water were also investigated.

Values of concentration causing 50% lethality (LC50) (48 h) with 95% confidence limits were calculated by the probit analysis of the SAS Institute [18]. The difference among LC50 values was considered significant if the 95% confidence limits did not overlap.

RESULTS

Binding of cadmium and calcium to dissolved organic material

Dissolved organic material in humic lake water exhibited a capacity to bind Cd, and the apparent equilibrium of binding was achieved after 2 d of dialysis (Fig. 1). The measured binding capacity was the same after 4 d exposure, but leakage of DOM from the bag was probably responsible for observed slight decrease in binding after 4 days (Fig. 1). Unfortunately, the leakage was not quantified by DOC measurement. To ensure the achievement of equilibrium and simultaneously to prevent any errors of too long dialysis time, the duration of dialysis was then fixed to 4 d for the following experiments.

Table 1. The partition coefficients (K_{oc}) for binding of cadmium (Cd) to dissolved organic material in humic lake water at an original hardness of water (0.1 mmol Ca + Mg per liter) and at artificially elevated water hardness (0.5 and 2.5 mmol Ca$^+$Mg per liter)

Cd concentration		K_{oc} ± SD, water hardness (mmol/L)		
μg/L	μmol/L	0.1	0.5	2.5
40	0.36	$(8.1 \pm 0.9) \times 10^4$	$(1.8 \pm 0.4) \times 10^4$	$(8.2 \pm 2.1) \times 10^3$
100	0.89	$(8.6 \pm 0.8) \times 10^4$	$(1.6 \pm 0.2) \times 10^4$	$(6.4 \pm 1.2) \times 10^3$
300	2.67	$(5.2 \pm 0.2) \times 10^4$	$(1.4 \pm 0.3) \times 10^4$	$(4.8 \pm 0.7) \times 10^3$
600	5.34	$(4.2 \pm 0.4) \times 10^4$	$(1.3 \pm 0.3) \times 10^4$	$(4.7 \pm 0.3) \times 10^3$
850	7.56	$(4.1 \pm 0.3) \times 10^4$	$(1.1 \pm 0.2) \times 10^4$	$(5.0 \pm 0.8) \times 10^3$

An increase in the total Cd concentration causes a decrease in the Cd partition coefficient to DOC, which was observed at all three tested water hardnesses (Table 1). Also, in every Cd concentration tested, the binding of Cd to DOM in humic lake water decreased to about one tenth when water hardness was artificially elevated from the original to 2.5 mmol Ca + Mg per liter (Table 1). The partition coefficients for Ca binding to DOC in in the soft water (0.1 mmol Ca + Mg per liter) are listed in Table 2. There was no statistical difference between K_{oc} values of Ca in different Cd concentration tested. In the hard water, however, it was not possible to calculate the partition coefficient at all because the excess Ca led to equal concentrations of Ca both inside and outside the bag (raw data not shown).

A 10-fold decrease was also observed in the Freundlich adsorption constant (K) (Table 3), and the adsorption isotherms of Cd on DOC in three different hardnesses of water are shown in Fig. 2. Nonlinearity (slope $n < 1$) of the isotherms could be due to heterogenous binding sites with different affinities for Cd in humic molecules.

Toxicity of cadmium in humic water affected by water hardness

At an original (0.1 mmol Ca + Mg per liter) and slightly elevated (0.2 mmol Ca + Mg per liter) hardness, Cd was significantly less toxic in humic lake water (DOC 19.6 mg/L) than in the humic-free reference water (DOC <0.2 mg/L, Fig. 3). Because the concentration of the bioavailable species of cadmium (Cd^{2+}) in water was decreased due to the complexation to DOC (Table 1), the acute toxicity (48 h) was reduced in the humic water compared with the reference. In other words, complexation with DOM prevents Cd from producing a toxic effect in daphnids.

Althought an artificial increase in the total water hardness (from 0.1 to 0.5 or 2.5 mmol Ca + Mg per liter) in this study reduced the capacity of DOM in humic lake water to bind Cd (Table 1) and thus increased its effective and potentially toxic concentration in experimental water, the acute toxicity decreased, and no difference between humic and reference water was observed (Fig. 3). These results agree with previously measured uptake rates, which decreased by increased hardness and were equal in both experimental waters when hardness was elevated to 1.5 mmol Ca + Mg per liter [11].

The dilution of humic water with artificial freshwater with the same Ca–Mg hardness did not cause a linear change in the measured toxicity of Cd (Fig. 4). The LC50 value remain high until very low DOM concentrations. This result shows that in the soft waters even a low DOM amount may affect the behavior and effects of Cd.

DISCUSSION

Binding of cadmium to dissolved organic material

Cadmium tends to exist largely as the free divalent species in the absence of DOC and chloride in freshwater [19]. Because its chemical character as a soft metal ion, any complexation with inorganic ligands (such as hydroxide and carbonate anions) would not be preferred [20]. In most boreal natural waters, however, DOC is present at various levels and behaves as an important metal binding ligand. Cadmium has a moderate affinity for humic substances [21], but compared with some other metals such as copper, the complexation capacity of DOM for Cd is about an order of magnitude weaker [12,22,23]. The weaker binding of Cd to DOM can be explained by its chemical properties. Copper has an intermediate character between soft and hard metals, thus having more affinity to oxyfunctional groups than Cd [20].

The clear decrease in binding of Cd to DOM was noticed with increasing Cd concentration or increasing water hardness (Table 1). This decrease might be related to either limited number of binding sites on DOM or to the heterogeneity of binding sites. The decrease in binding with increasing water hardness results from competition between Cd^{2+} and added water hardness cations—Ca^{2+} and Mg^{2+}—for binding sites in DOM. Because hard cations prefer oxygen (hard ligand) as

Table 2. The partition coefficients (K_{oc}) for binding of calcium to humic lake water at an original hardness of water (0.1 mmol Ca$^+$Mg per liter)

Cd concentration		
μg/L	μmol/L	K_{oc} ± Sd
40	0.36	$(1.5 \pm 0.1) \times 10^4$
100	0.89	$(1.5 \pm 0.3) \times 10^4$
300	2.67	$(1.1 \pm 0.1) \times 10^4$
600	5.34	$(1.3 \pm 0.1) \times 10^4$
850	7.56	$(1.3 \pm 0.4) \times 10^4$

Table 3. The Freundlich parameters (n and log K) and adsorption coefficients K (liters per kilogram) for cadmium binding on humic substances in lake water with three levels of water hardness (0.1, 0.5 and 2.5 mmol/L)[a]

Water hardness (mmol/L)	n	log K	K	r^2
0.1	0.75	4.6	3.7×10^4	0.99
0.5	0.85	4.0	1.1×10^4	1.00
2.5	0.81	3.6	0.4×10^4	0.99

[a] Coefficient for determination (r^2) for log-log regression is also presented.

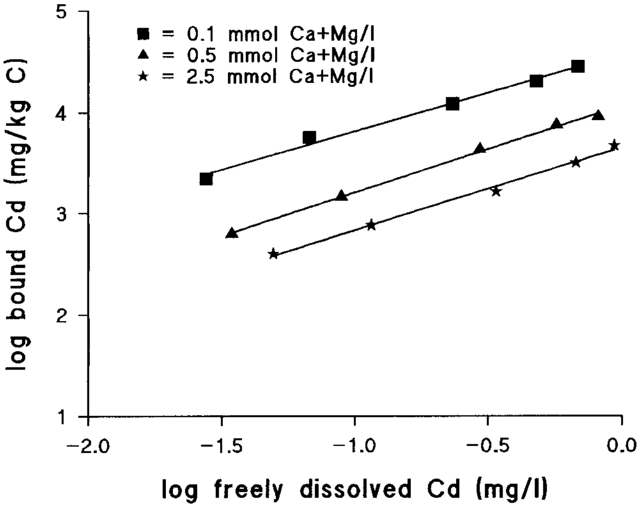

Fig. 2. Adsorption isotherms for cadmium on dissolved organic material in water from lake Louhilampi (dissolved organic carbon = 19.6 mg/L) at an original water hardness (0.1 mmol Ca + Mg per liter) and at artificially elevated hardness of water (0.5 and 2.5 mmol Ca + Mg per liter).

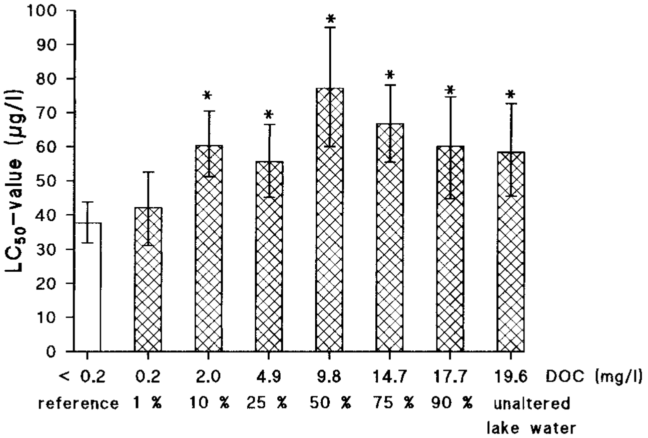

Fig. 4. The effects of dilution of natural humic water on the acute toxicity (concentration causing 50% lethality [LC50]; 48 h) of cadmium to neonatal *Daphnia magna* at a natural hardness of lake water (0.1 mmol/L). The dissolved organic carbon concentration is expressed both as percent to maximal and milligrams per liter. The error bar denotes 95% confidence limits of LC50 value, and the asterisk denotes a statistical difference.

donor atom [24] and the complex formation between Cd^{2+} and humic material is documented mainly to occure by carboxylic (−COOH) and phenolic hydroxylic (−OH) groups of humics [25], the competition exists. However, Ca^{2+} is more effective than Mg^{2+} in interfering organic complexation of Cd^{2+} [19] and might be explained by similarity of Cd^{2+} and Ca^{2+} ions (identical charge and almost equal ionic radius).

In spite of effective competition with Ca^{2+} for the binding sites in hard water, Cd^{2+} was still binding to DOM to a certain extent (Table 1). Because Cd^{2+} is chemically classified to be a soft cation, it has also a high affinity for soft ligands such as N and/or S containing functional groups of DOM [2,3], and competition with a hard cation such as Ca^{2+} might be less pronounced in these sites. Despite of minor Cd binding in other than oxyfunctional sites, the predominate Cd complexation sites in humic substances are still the carboxylic groups because of their prevalence [26]. Cleven and van Leeuwen [27] have observed that the binding of Cd to humic acid is quite similar to the Cd binding to homofunctional polycarboxylic acids.

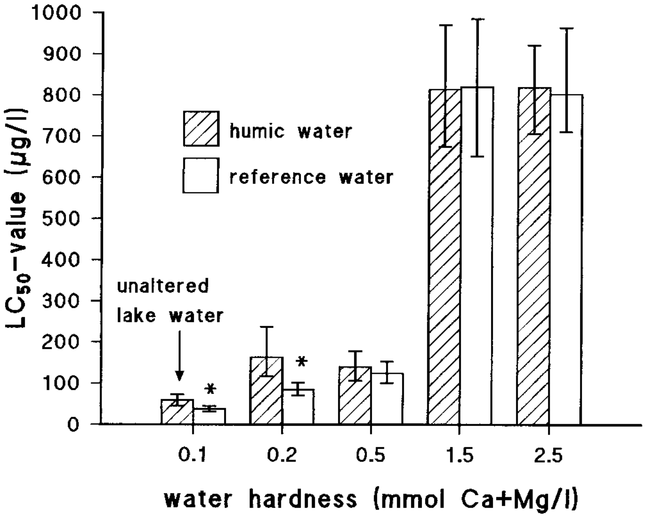

Fig. 3. Acute toxicity (concentration causing 50% lethality [LC50]; 48 h) of cadmium in humic and reference water to neonatal *Daphnia magna* as affected by water hardness. The error bar denotes 95% confidence limits of LC50 value, and the asterisk denotes a statistical difference between humic and reference water.

Toxicity of cadmium in humic water affected by water hardness

In our previous work [11], it was shown that the uptake rate of Cd was significantly faster in the reference than in the humic lake water at natural hardness level (0.1 mmol Ca + Mg per liter). This result supports our toxicity data presented in this paper. If the lethal body residue of Cd in *D. magna* is the same in both conditions, then the acutely toxic level of Cd in daphnids was reached at lower Cd concentration in reference than in humic water. In the study of Stackhouse and Benson [10], a 50-mg/L humic acid concentration in water significantly reduced the bioaccumulation of Cd to daphnids compared with the reference. In this study, even 10% dilution (DOC 2.0 mg/L) of the lake water was effective enough to significantly decrease the acute toxicity (Fig. 4) after 48 h, although Stackhouse and Benson [10] did not observed any influence of lower concentration of humic acid (0.5 or 5.0 mg/L) on the Cd bioaccumulation.

To test the hypothesis that it is binding to DOM that reduces the Cd toxicity, we can calculate the fraction of Cd that is freely dissolved (f_{free}) from the measured K_{oc} values (Table 1) or Freundlich adsorption coefficient (K) (Table 3) and the known DOC concentration:

$$f_{\text{free}} = 1/(1 + K \times \text{DOC}) \qquad (3)$$

where the DOC concentration is expressed as kilograms per liter and K is liters per kilogram. If the measured K_{oc} at the lowest Cd concentration and original water hardness is taken from Table 1 (the conditions of toxicity tests), the fraction of freely dissolved Cd can be calculated to be 39% of the total. Similarly, using the adsorption coefficient K of the Freundlich isotherm for the soft water results, the fraction of freely dissolved Cd is 58% of the total. The measured LC50 value of Cd in humic water at original hardness is 58.3 μg/L. If we assume that only the freely dissolved Cd is accumulating into the organisms and causing the effect, we can calculate that in these conditions the LC50 value of freely dissolved Cd is 22.7 μg/L or 33.8 μg/L when using the K_{oc} from dialysis or Freundlich K, respectively. The LC50 value for Cd in DOM-free reference water at the same hardness is 37.7 μg/L (95% confidence limits: 31–42 μg/L).

Several other studies have shown, however, that the presence of DOM in freshwater or seawater could also enhance the bioaccumulation, toxicity, or uptake rate of Cd by aquatic organisms [7–9]. No clear explanation on increased bioavailability of Cd in humic water is found, although some theories of facilitated uptake of Cd have been suggested. Based on our results ([8,11] and present study), it is important that, besides DOM, other water quality parameters, such as water hardness, should be taken into account in the experiments before concluding that the observations are a result of a certain parameter. In the case of adding isolated humic matter to experimental water [7,9] instead of using natural humic water as such, it might be possible that added humic substances form complexes with hardness or salinity metal ions. Enhanced toxicity or bioaccumulation of Cd could be the indirect effect of DOM due to reduced hardness or salinity because the uptake of Cd to aquatic invertebrates is observed to be faster in soft or low-salinity water than in hard or high-salinity water [11,28,29]. It is possible also in our experimental setup that the organisms in humic water still experience it as a softer environment than that characterized by the Ca–Mg hardness due to the fact that a proportion of total Ca^{2+} and Mg^{2+} is bound to DOM. That the calculated effective freely dissolved concentration of Cd was slightly lower that the measured LC50 in reference water might indicate this. Nevertheless, in the current and a previous study [11], DOM in soft lake water had a protective role against Cd toxicity. Because calcium is an essential element to biota, the specific uptake mechanisms for Ca^{2+} exists. The nonessential and toxic Cd has no own uptake pathway, and it appears that Cd^{2+} could be taken up via the existing mechanisms for Ca^{2+} due to their similar charge and size. It has been shown, for example, that Cd uptake by the gills of aquatic invertebrates and fish occurs via Ca channels [30–33]. If so, these divalent ions might compete for uptake by daphnids as well as binding to DOM. In this study, the concentration of Ca^{2+} in hard water was many orders of magnitude higher than that of Cd^{2+}, and it seems that Ca^{2+} was able to effectively compete with Cd^{2+} in both binding to DOC and in entering to the animal. In addition, increased hardness of experimental waters might have caused physiological changes in daphnids, such as stabilization of cell membranes, and thus affected permeability. Gundersen and Curtis [34] have observed that the permeability of fish gill was reduced when concentration of Ca^{2+} increased in aqueous environment, and this phenomena can be extended to cover also invertebrates, although the uptake route could be other than gills only. In hard water, obviously, Ca^{2+} had an antagonistic effect on Cd toxicity, and the effect of DOC on Cd bioavailability to daphnids became covered with the physicochemical or physiological effects of added Ca^{2+} ions in experimental system.

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