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Interaction of cadmium toxicity in embryos and larvae of zebrafish (*Danio rerio*) with calcium and humic substances

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Abstract

The influence of humic substances (HS) and calcium (Ca) on cadmium (Cd) toxicity was determined using zebrafish (*Danio rerio*). Embryo and larvae of the zebrafish were exposed to various Cd concentrations (1.8; 2.8; 4.2; 6.2; 9.3 mg/l Cd) for 144 h. Combinations of low (0.2 mmol/l) and high (2 mmol/l) Ca, + HS (5 mg/l C) or − HS were used during Cd exposure. The toxicity of Cd was affected by (1) exposure concentration; (2) exposure time; (3) presence of HS; and (4) the Ca concentration. The results show that Ca and HS protect against Cd toxicity in zebrafish embryos. The best protection was in the high Ca − HS group, followed by high Ca + HS group and low Ca + HS group. The survival in the low Ca − HS group was the worse. Survival in the high Ca − HS group and the high Ca + HS group was similar with the exception of the highest Cd concentration (9.3 mg/l) where the survival of the high Ca + HS group was less than in the high Ca − HS group. The exposure system was modelled using a chemical equilibrium program (MINEQL +) to determine if the likely mechanism causing the anomalous result in the highest Cd concentration. The equilibrium model cannot explain these results, which suggests that this effect has a kinetic basis, such as time needed for Cd to displace Ca already bound by HS.

Keywords: Cadmium; Calcium; Humic substances; Toxicity; Embryo

1. Introduction

Cadmium (Cd) is widely distributed in the aquatic environment and damages aquatic animals (Gill et al., 1991) and interferes with the ionic and water balance in fish (Hwang et al., 1995). The toxicity of cadmium is well known to

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be affected by several water chemistry parameters, particularly calcium (Ca) and humic substances (Playle et al., 1993a,b; Playle, 1998; Richards et al., 1999). The toxicity of metals in aqueous solutions is also altered by the water pH and the presence of ligands, such as hydroxides, carbonates, chlorides, humic substances (HS) and others etc. (Janes and Playle, 1995; Sprague, 1995; Fent, 1998; Van Ginneken et al., 1999; Wood et al., 1999). HS are rich in organic ligands which form complexes with free metal ions and hydroxides, and the stability of the complexes generally follow the Irving–Williams series. These interactions have been described as chelation, complex binding, ion exchange, surface adsorption, coagulation and peptidization reactions (Petersen et al., 1987). The amount of metals complexed to HS depends on pH, ionic strength, and the concentration of metals and HS. The concentration of biologically available metals in natural water is highly affected by the amount and composition of humic material and dissolved inorganic species (Petersen et al., 1987).

Metal complexation with humic substances is generally thought to occur via the carboxylic and phenolic hydroxyl groups (Linnik and Nabivanets, 1984). Several studies revealed the beneficial effect of humus HS on the survival of fish. Gjessing (1981) found HS to decrease the survival of fish and algae in metals impacted water. During investigations made by Hollis et al. (1996), the addition of 5–20 mg C/l DOC reduced the toxicity of Cd to small rainbow trout (*Oncorhynchus mykiss*). Playle et al. (1993a,b) found a reduction in Cd binding to fish gills in the presence of dissolved organic matter (DOM). However, in studies with *Daphnia* sp., HS was found to increase the toxicity of Cd (Winner, 1984). Possibly, metals complexed with the HS may be released by ion exchange and the toxicity, therefore, increases which may be viewed as a negative biological effect of the HS. Similarly, Paarlberg (1984) found the tendency for mortality in *Gammarus* sp. rise with increasing HS, at identical pH and Ca concentrations and in the absence of metals, possibly because high levels of HS may bind Ca until it becomes limiting. Waters which are rich in HS and low in Ca will have most of the existing metal ions complexed with the HS (Petersen et al., 1987). Therefore, the interaction between HS and metals is more complex and other factors may also influence this system. Ca seems to have a key position in the metal-HS − Ca system. Meinelt et al. (1995) found an increase of zinc (Zn) toxicity in the presence of HS in water with elevated water hardness. In contrast, in water with a low Ca content Zn toxicity decreased when HS was added. Embryos and larvae of zebrafish were used for toxicity tests because of their convenience for laboratory studies (Westerfield, 1995).

The purpose of this study was to test the hypothesis that increased Ca content, which interacts with HS, increases the sensitivity of the developing embryos and larvae of zebrafish (*Danio rerio*) to Cd.

2. Material and methods

2.1. Test fish

Embryos and larvae of zebrafish were often used in toxicity. The biology and reproduction of zebrafish (short generation interval, short spawning interval, transparent eggs) make this species suitable as a test fish for toxicological research (Ehms, 1995). Adults reach 4–6 cm in length and sexual characteristics usually begin to develop at 4–5 months. Zebrafish are oviparous (Hisaoka and Battle, 1958; Eaton and Farley, 1974). Spawning is induced by light, and begins at daybreak under natural conditions. Eggs are 1.0–1.2 mm in diameter and have a transparent chorion; embryonic development is completed in 96 h at 26°C water temperature. For our experiments, we used zebrafish eggs which were obtained from stock bred for 8 years at the Institute for Freshwater Ecology and Inland Fisheries in Berlin, Germany. The original stocks were obtained in 1992 from the West Aquarium (Bad Lauterberg, Germany). The zebrafish we used as spawners had a length of 3.5–4.5 cm and were fed three times daily ad libitum with TETRAMIN (TETRA WERKE, Melle, Germany) and *Artemia* sp. nauplii.

2.2. Spawning aquarium section

Spawning zebrafish were kept at 26°C in modified glass aquaria each with a volume of 80 l (Meinelt, 1996). Five females and ten males zebrafish were placed in the egg sampling aquarium. Plastic plants were used as the spawning substrate. The bottom of each aquarium was made of a 4-mm metal grid, through which the spawned non-sticky eggs passed and are thus protected from the predatory parents. Below the metal grate, a funnel shaped base leads into a tube, through which the eggs were collected. The eggs were taken from the tube while opening a valve. Following sampling, the eggs were cleaned with 26°C of the particular used test medium.

2.3. Fish toxicity test method

The toxicity tests were performed as a embryo-larval-test over a 144 h period. Twenty eggs were placed in each test chamber (glass crystallizing dishes). The tests were replicated three times consecutively. Zebrafish eggs reaching the four- to eight-cell-stage were exposed to different concentrations of Cd ($CdCl_2 \times H_2O$) (1.8; 2.8; 4.2; 6.2; 9.3 mg/l Cd, nominal concentrations, based on Cd) for 144 h. Four kinds of reconstituted waters were used as test media produced by mixing constant amounts of salts ($CaCl_2 \cdot 2H_2O$; $MgSO_4 \cdot 7H_2O$; $NaHCO_3$; KCl) into deionized water. Thus, the test solutions differed only in the content of Cd, dissolved organic carbon and/or the amount of Ca and chloride (Table 1). The test waters were, (1) high Ca (2 mmol/l) − HS; (2) high Ca + HS (5 mg/l C); (3) low Ca (0.2 mmol/l) − HS and (4) low Ca + HS. The control waters were the same kinds of reconstituted waters without Cd additions. Prior to use the test waters were aerated and the oxygen content as well as the pH and temperature were measured. Details are given in Table 1. Every 24 h the test solution was replaced, and the mortality of embryos and larvae was recorded. Dead individuals were removed daily in each test chamber. All individuals were observed up to 144 h and at that time all embryos and larvae were counted.

The DOM was obtained by physical isolation methods. Water from Luther Marsh, Ontario, Canada (N 43°53′38″, W 80°24′08″) was centrifuged (Westphalia, $10\,000 \times g$), filtered (glass-fiber filter, 1.0 μm pore-size) and then filtered through a Pellicon 0.2 μm tangential flow filtration unit (Millipore, Bedford, MA, USA). Potential metal concentrations from the DOM were reduced by passage of the filtrate through a cation-exchange resin (H^+ form, AG50W-X8, BioRad). Concentrations of DOM were measured by high temperature combustion (Beckman 915B total carbon analyser) after sample acidification with 30% phosphoric acid to remove inorganic carbonates.

2.4. Statistics

Toxic effects of Cd in different test waters were determined by analysing the life times of the embryos and larvae in each of the five Cd concentrations and in the controls. The Log–Rank test (Rosner, 1995) was used to detect significant differences between the test groups in each investigation. The Log–Rank test provides a powerful and robust non-parametric procedure for testing for differences in mortality pattern in homogeneous populations. To improve the statistical power, the observations of the three investigations were com-

Table 1
Calcium and dissolved organic carbon content of the test waters[a]

Parameter	High Ca − HS	High Ca + HS	Low Ca − HS	Low Ca + HS
Calcium (mmol/l)	2	2	0.2	0.2
HS (dissolved organic carbon, mg/l C)	0	5	0	5

[a] Water conditions in all test waters were magnesium 0.5 mmol/l, potassium 0.077 mmol/l, sodium 0.77 mmol/l, temperature 26 ± 0.2°C, dissolved oxygen 7.0 ± 0.5 mg/l, pH 7.47–7.97.

bined in an additional analysis. The life times of individuals from the four test waters were in pairs tested against each other. For each population the survival function was estimated by the Kaplan–Meier-estimator (Kaplan and Meier, 1958). The Kaplan–Meier plots are used to visualize the differences in the survival rates. The cumulative survival function $S(x)$ (sometimes called survival function) is the complement of the cumulative distribution function $F(x)$, e.g. $S(x) = 1 - F(x)$, and gives the percentage of living individuals at time x. The Kaplan–Meier-estimator is the appropriate estimator of the cumulative survival function in the case of censored data. The statistical analyses were done using SPSS (1999).

3. Results

Fig. 1 shows in detail the survival of the developing embryos and larvae in the four test solutions for each Cd concentration tested. The low Ca − HS treatment had the lowest survival rates, and high Ca − HS always had the highest survival rates. The low Ca + HS treatment increased survival to 6.2 mg/l Cd Fig. 1E, but did not protect in the 9.3 mg/l Cd exposure (Fig. 1F). The high Ca + HS treatment protected fully against Cd up to 6.2 mg/l Cd Fig. 1B–E, but the high Ca + HS treatment only showed intermediate survival in the highest Cd treatment of 9.3 mg/l Cd Fig. 1F.

Since there were no significant differences between the three replications in each test water, the data of the analysis were pooled for further analysis. The in pairs comparisons of the survival of the developing embryos and larvae in the test solutions revealed highly significant differences ($P < 0.0001$). In one case (low Ca + HS vs. low Ca − HS) difference was found to be significant ($P = 0.023$).

3.1. Cd-embryo interaction model

Generally, our results show that Ca and HS protect against Cd toxicity in zebrafish embryos, and that Ca and HS together protect better than HS alone and as well as Ca alone (e.g. 100% protection). However, in the highest Cd concentration used, embryo survival was low in the high Ca + HS treatment than in the high Ca − HS treatment (Fig. 1F). We modelled our exposure system using a chemical equilibrium program (MINEQL$^+$, version 3.0) to determine if this seemingly anomalous result is reasonable chemically or if some other explanation is necessary (Schecher and McAvoy, 1992).

The Cd-embryo interaction model is illustrated in Fig. 2. In the low Ca − HS situation, most of the Cd-embryo binding sites are filled by Cd, resulting in high toxicity (Fig. 2A). In the low Ca + HS treatment, some of the Cd in solution is complexed by HS, so that less Cd binds to the embryo, resulting in lower Cd toxicity (Fig. 2B). In the high Ca − HS test water, the additional Ca in solution competes for the Cd binding sites on the embryo, reducing the binding of Cd on the embryo (Fig. 2C).

There are two possibilities for the high Ca + HS condition, both of which are supported by our results presented in Fig. 1. The lower survival in the presence of Ca and HS than in the presence of Ca alone is given in Fig. 2D, in which the HS binds so much Ca that not enough Ca is available to compete with Cd at the egg/embryo surface, resulting in more Cd binding to the egg/embryo than in the presence of Ca alone e.g. (Fig. 2C). The case of additive protective effects of Ca and HS is illustrated in Fig. 2E, in which some Ca is bound by HS and displaces some Cd from HS, but overall the additional Ca in solution competes with Cd for binding sites on the embryo and therefore reduces Cd toxicity. The modelling exercise was designed to determine which case, (Fig. 2D or 2E), is most likely given the separate protective effects of HS complexation and Ca competition.

We began a model simulation using the Ca and Cd values from a published Cd-gill model (Playle et al., 1993a,b; Playle, 1998). In that model, the conditional equilibrium stability constant (K) for Cd binding to the gills of the fathead minnow (*Pimephales promelas*) is $\log KC_{\text{d-gill}} = 8.6$. Ca binds to those sites about 4000 times more weakly than Cd does ($\log K_{\text{Ca-gill}} = 5.0$). The conditional equilibrium stability constant for Cd to DOM (roughly equivalent to HS) is $\log K_{\text{Cd-DOM}} = 7.4$

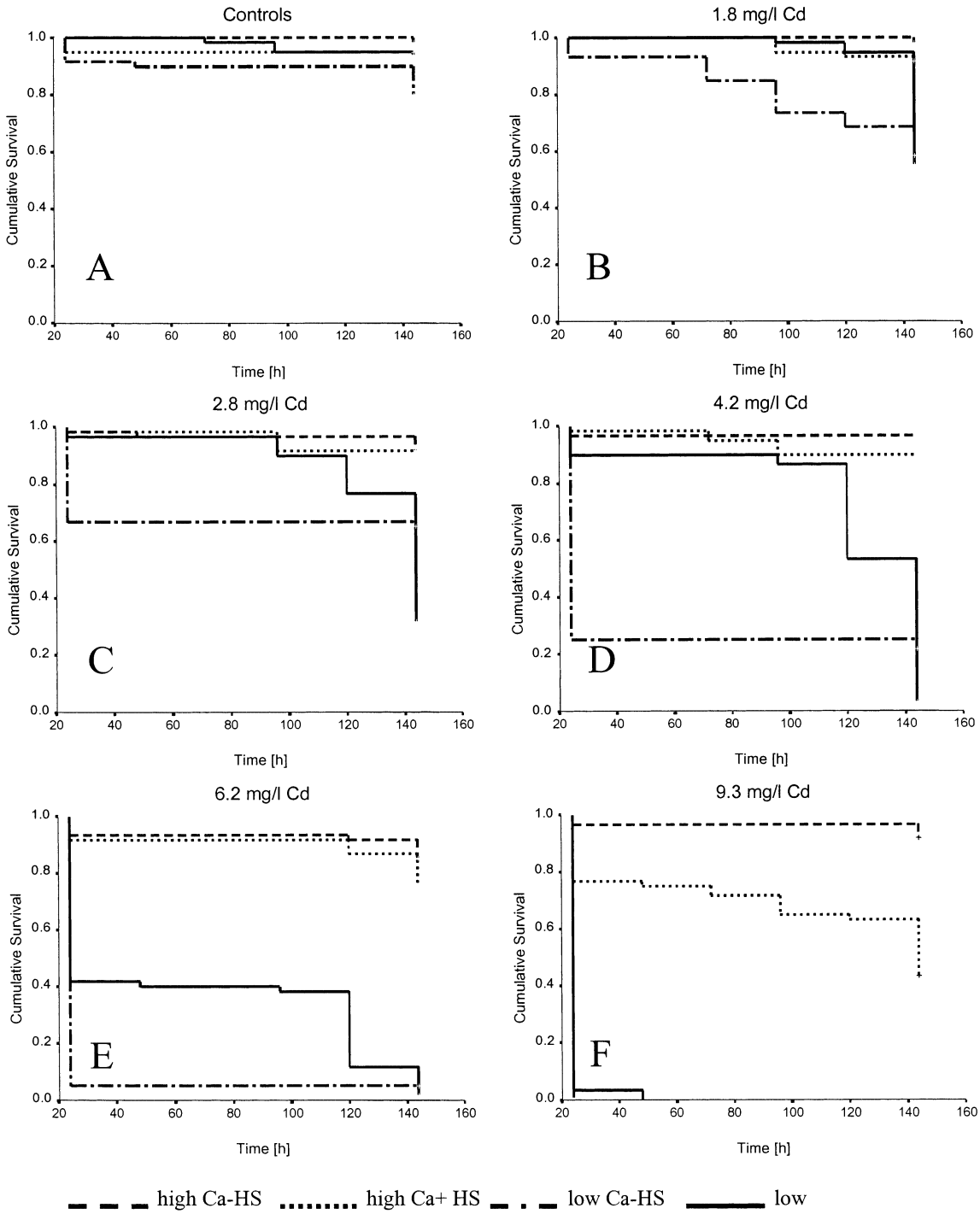

Fig. 1. Time dependent survival of five concentrations of Cd and controls in four test waters (Kaplan–Meier plots).

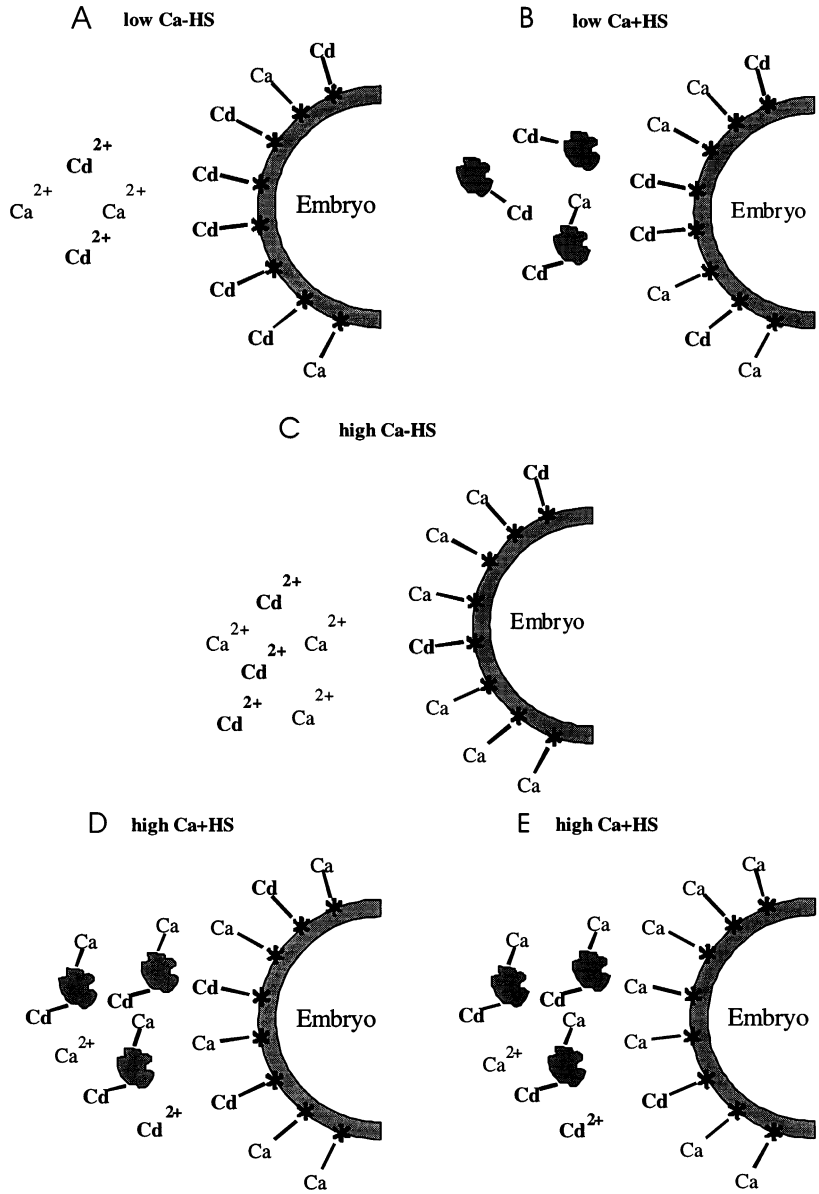

Fig. 2. Binding of Cd and Ca ions to humic substances and/or embryo (rationale of the binding site model).

(Playle et al., 1993a,b; Playle, 1998). There was no binding constant determined for Ca to DOM, but the value must be < 7.4 according to the Irving–Williams order (e.g. Ma et al., 1999), and a range of Ca-DOM binding constants of $\log K_{\text{Ca-DOM}} = 4–6$, depending on water pH, were found by Römkens et al. (1996).

Starting from these assumptions we mimicked the general results presented in Figs. 1A to 1E which are conceptually presented in Figs. 2A to 2C. Through trial and error during initial modelling we chose the general conditions of 20 μM Cd, 0.2 or 2.0 mM Ca, 0 or 15 μM HS, 100 nmol/l binding sites on the embryo, with the

formation of otavite (precipitated $CdCO_3$) removed from the calculations. For HS, 15 μM is 3 μmol binding sites per mg C, enough sites to bind a reasonable amount of the 20 μM Cd; 3 μmol binding sites per mg C was reported for copper (Cu) by Ma et al. (1999), although it is thirty times higher than the number of sites used in Playle et al. (1993a) or in Richards et al. (1999). We chose 100 nmol/l binding sites on the embryo, representing at most 0.5% of the 20 μM total Cd in the calculations, to simulate a great excess of water volume compared with binding sites on the embryo. Otavite was removed from consideration in all calculations, because if its formation was included only about 0.05 μM of the 20 μM Cd added theoretically remained in solution, which would clearly not translate into the Cd toxicity we observed. In addition, no precipitation was observed during the exposures in even our highest Cd exposures.

For the final model we used the binding constants given in Table 2, with Cd binding to the embryo about 40 times more strongly than Ca ($\log K_{\text{Cd-embryo}} = 8.6$, $\log K_{\text{Ca-embryo}} = 7.0$), and with Cd binding to HS about 250 times more strongly than Ca ($\log K_{\text{Cd-HS}} = 7.4$, $\log K_{\text{Ca-HS}} = 5.0$). These values yielded results which overall correspond to those of Figs. 1A to 1E and Figs. 2A to 2C. As long as Cd is modelled to bind more strongly than Ca to the embryo and to HS (i.e. according to the Irving–Williams order), qualitatively similar results to those in Table 2 will be obtained.

Simulation of the low Ca − HS treatment yielded 75% of total Cd as Cd^{2+} in solution, with 75% of the embryo sites filled by Cd (Table 2), representing our most toxic Cd conditions. Simulation of the low Ca + HS treatment yielded just 26% Cd^{2+} in solution, because 65% of the Cd was bound by HS (Table 2). The amount of Cd bound by the embryo decreased to 51% of the embryo sites filled by Cd, representing the moderate protective effect of HS (Fig. 1, 2B).

In the high Ca − HS simulation, Cd^{2+} comprised 63% of the total Cd in solution (with more $CdCl^+$ formed because of the extra Cl added with the Ca), but there was little Cd binding to the embryos, with just 20% of the embryo sites filled with Cd (Table 2). In theory, Ca occupied the remaining binding sites of the embryo. This illustrates the protective effect of Ca against Cd toxicity in zebrafish embryos (Fig. 1, 2C).

The high Ca + HS simulation yielded 39% Cd^{2+} in solution (higher than in the low Ca + HS condition) but there was less Cd binding to the embryos, with just 14% of the sites filled by Cd (Table 2). Although the additional Ca in solution theoretically displaced some Cd from HS, so that only 50% of the HS sites were filled by Cd compared with 86% filled by Cd in the low Ca + HS condition, the additional waterborne Ca was able to compete with Cd for binding to the embryo.

Table 2
Calculated percent speciation of 20 μM Cd in water, or percent of total HS and embryo binding sites filled by Cd or by Ca (in brackets), for the Cd treatments[a]

Complex	Percent speciation				
	log *K*	High Ca − HS	High Ca + HS	Low Ca − HS	Low Ca + HS
Cd^{2+}	0	63	39	75	26
$CdCl^+$	2.0	29	18	16	6
$CdCO_3$ (aq)	5.4	6	3	6	2
$CdHCO_3^+$	12.4	1	1	2	1
Cd − HS	7.4	–	37 (50)	–	65 (86)
Ca − HS	5.0	–	− (50)	–	− (13)
Cd-embryo	8.6	<1 (20)	<1 (14)	<1 (75)	<1 (51)
Ca-embryo	7.0	− (80)	− (86)	− (25)	− (49)

[a] Model assumptions, 20 μM Cd, 0 or 15 μM HS sites, 0.2 or 2.0 mM Ca, 2.25 or 6.85 mM Cl, 100 nM embryo sites, 0.5 mM Mg, 0.08 mM K, 0.77 mM Na, pH 7.6, 25°C, system open to the atmosphere, otavite precipitation not considered.

This behaviour is illustrated in Fig. 2E, in which Ca and HS together protect better than either agent alone. That is, the addition of the separate protective effects of Ca and of HS does not produce a situation where Ca and HS together protect less well than Ca alone, which is the result illustrated in Fig. 2D.

Further modelling demonstrated that it is impossible to simulate a protective effect of HS (e.g. Fig. 2B) and still yield the situation illustrated in Fig. 2D. For example, the introduction of additional sites on HS that bind Ca, but do not bind Cd, will produce the situation in which enough Ca is complexed by HS that there is little Ca available to compete with Cd at the embryo surface, so that binding of Cd to the embryo increases in the presence of HS (e.g. Fig. 2D). However, this scenario also simulates greater binding of Cd to the embryo in the low Ca + HS treatment compared with the low Ca − HS treatment, which does not fit the data in Figs. 1A to 1E. Similarly, simulating a condition which violates the lrving–Williams order so that Ca binds to HS more strongly than does Cd (e.g. $\log K_{\text{Ca-HS}} > \log K_{\text{Cd-HS}}$) produces a situation in which more Cd binds to the embryo in the presence of HS than in its absence, but this result applies to both the low and high Ca treatments.

Our simulations suggest that the observed protective effect of HS alone is inconsistent with the lowest embryo survival in Ca + HS then with Ca alone (Fig. 1F), and that the condition illustrated in Figs. 2B and 2D are mutually exclusive. These simulations were run using an equilibrium chemistry program, perhaps the reason for the seemingly anomalous result of ours from Fig. 1F is a kinetic limitation in the Cd exposures. A kinetic effect is also implicated because the largest difference in embryo survival between the high Ca + HS treatment and the high Ca − HS treatment is in the first hours of the exposures (Fig. 1F).

4. Discussion

As in the investigations made with Zn (Meinelt et al., 1995), the interaction between metal-HS-Ca increases the toxicity of the metal Cd in presence of higher Ca ion concentrations and HS in the highest treatment groups. Therefore, our hypothesis, HS and higher Ca levels increase the toxicity of Cd to early developmental stages of zebrafish is true for high Cd conditions. Fig. 1 illustrates that in the Cd-exposed groups the addition of HS in the high Ca waters lead to a decreased survival of the zebrafish in 9.3 mg/l Cd. In the low Ca waters the addition of HS lead to an elevated or prolonged survival of the juveniles in all Cd concentrations tested. Low Ca − HS always showed the highest mortality. The results of our investigations prove that both Ca and HS reduce the toxicity of Cd (Table 2).

One sign of Cd poisoning is a loss in Ca because Cd and Ca are competitive ions. Both Cd and Ca have an ionic radius of ~ 1.2 Å. Verbost et al. (1989) suggested that both cations enter fish gills by the same pathway. The addition of 20 μg/l Cd suppressed the Ca influx in fish larvae by 32–45% (Chang et al., 1997). Exposure to Cd will lead to a drop in body Ca content resulting in for example, toxicity or in slowed growth. Reduced plasma Ca levels and resulting hypocalcemia after Cd exposure have been proposed as the fundamental mechanism of Cd toxicity (Giles, 1984). For developing fish embryos and larvae, Ca uptake may be reduced by the presence of Cd. Newly hatched tilapia larvae were less sensitive to Cd than 3 day old larvae (Hwang et al., 1995). These changes in sensitivity to Cd were suggested to be related to different ion uptake efficiencies during larval development (Hwang et al., 1995). Hwang et al. (1994, 1995) and Chang et al. (1997) suggest that a rapid increase in Ca influx after hatching is to obtain more Ca which is important for normal embryonic and larval development. In the presence of Cd, this change in Ca uptake efficiency associated with development leads to increased amounts of Cd accumulation as well as Ca. Once Cd is accumulated, the Ca uptake efficiency decreases leading to further deterioration of subsequent Ca-uptake. Therefore, the normal developmental pattern of increasing Ca uptake, which is important for developing larvae, is harmful when the fish is confronted with Cd. Our investigations support this assumption.

With respect to our results in the high Ca − HS and the low Ca − HS treatments, the protective effect of Ca ions is visible as hypothesised. This may be due to the competition between Cd and Ca ions and the interfered uptake of Ca by Cd. The '3 Cs', which conceptually clarify the effects of water chemistry on metal toxicity are Concentration, Competition and Complexation (Playle, 1998) that can be considered as a biogeochemical modelling of the metal toxicity influencing factors. Ca competes with metals for binding sites (Playle et al., 1993b). At fish gills Ca competition reduces Cd uptake (Wicklund and Runn, 1988). The Cd ions are available for the embryo and larvae and compete with the Ca ions as mentioned by Chang et al. (1997). The Ca ions alone, therefore, have that protective effect in the presence of Cd (high Ca vs. low Ca treatments).

The protective effect of the HS is visible in the low Ca + HS treatment (Fig. 1). Due to the binding affinity of the metals on HS follows the Irving–Williams series (Sigg and Stumm, 1991), Cd has a higher affinity to HS than Ca does. For this reason, the functional groups of the HS should be preferably loaded with the Cd ions and in a low Ca water, HS, therefore, acts as a protective factor. As Cd is complexed with HS and the concentration of freely available Cd is lowered by the HS, a protective effect is observed in the low Ca + HS versus low Ca − HS.

In the high Ca + HS treatment the survival of the embryos was reduced at the highest Cd concentration. Due to the high concentration of Ca ions, the functional groups of the HS are potentially filled with Ca. For this reason the Ca content in the solution could be reduced and therefore the protective effect of these Ca ions reduced. In addition, there is theoretically more uncomplexed Cd^{2+} in the water (Table 2). The binding site of Cd in high Ca + HS to the embryos and larvae is potentially elevated compared with high Ca − HS, and the toxicity increases as shown in the 9.3 mg/l Cd treatment. This case explains the increased toxicity of Cd in high Ca + HS (Fig. 2D). However, our modelling exercise (Section 3.1) suggests that this effect has a kinetic basis.

4.1. Modelling discussion

Our Cd-embryo modelling simulated the separate protective effects of Ca and HS against Cd binding to the embryo and, therefore, Cd toxicity. Ca competes for the potential Cd binding sites on the embryo and for the HS binding sites in the water column. When Ca and HS are combined, the model calculations produce case figure 2E, in which the protective effects of Ca and HS together are greater than they are separately. The model calculations did not predict case 2D, in which HS decreases the protective effect of Ca, this seemingly anomalous scenario was suggested by our results from the highest Cd exposure (Fig. 1F), in which HS seemed to reduce the protective effect of Ca against Cd toxicity. Most of the difference between embryo survival in the high Ca + HS and the high Ca − HS treatments occurred at the beginning of the Cd exposure, which suggests, along with the inability of equilibrium modelling to mimic this result, that a kinetic constraint may be the reason for this apparent anomaly.

A possible kinetic constraint is the initial binding of Ca to HS before the Cd was added to the system. If Ca is bound to HS, it may take time for the added Cd to displace Ca from HS, and, therefore, more time is available for Cd to bind directly to the embryo surface and exert toxic effects. This phenomenon was shown by Ma et al. (1999), in which Ca bound to humic acid slowed the rate of Cu complexation by the humic acid. This effect occurred at concentrations of ≥ 0.1 mM Ca, and was consistent with slow reaction kinetics of disjunctive reactions (Ma et al., 1999), where in our case, Ca must dissociate from HS before Cd will bind to the HS (e.g. Morel and Hering, 1993; Hudson, 1998). If this kinetic constraint existed in our highest Cd exposure, the anomalous result of lower protection in the high Ca + HS treatment compared with high Ca alone would not have occurred if Cd had been added to the HS solution before Ca was added, which would yield the theoretical equilibrium prediction given in Fig. 2E and in Table 2. This possibility merits further research.

The Cd-embryo model results presented in Table 2 yield some insight into the effects of water chemistry in modifying metal toxicity in aquatic organisms. A complexing agent like HS reduces the concentration of waterborne cationic metals such as Cd^{2+}, resulting in less Cd^{2+} available to bind to biological surfaces such as the zebrafish embryo. However, the reduction is not necessarily as much as might be expected by the calculated decrease in Cd^{2+} concentration, because the biological surface itself also strongly binds Cd^{2+} (e.g. Playle, 1998). That is, although Cd^{2+} decreased from 75 to 26% of total Cd, the amount of Cd bound to the embryo only decreased from 7% of the embryo sites filled by Cd to 51% Cd (Table 2). In the high Ca − HS conditions the opposite situation occurs, the percent Cd^{2+} decreased from 75 to 63% as $CdCl^{+}$ formation increased because of the greater chloride concentration in the water, but the number of embryo sites filled by Cd decreased disproportionately through Ca competition (from 75 to 20% filled by Cd, Table 2).

In the modelled high Ca + HS condition, the additional Ca displaces some Cd from HS, so that only 50% of the HS sites are filled by Cd compared with 65% filled by Cd in the low Ca + HS situation (Table 2). Therefore, there is a greater percentage of Cd^{2+} in solution than in the low Ca + HS treatment (39 vs. 26%). However, Ca also competes with Cd for binding sites on the embryo, so that ultimately less Cd will theoretically bind to the embryo (14% in the high Ca + HS treatment vs. 51% in the low Ca + HS treatment). This simulation reflects exactly recent Cd toxicity results for *Daphnia magna*, in which the complexation capacity of DOM for Cd was indeed decreased in the presence of Ca, but the additional Ca simultaneously reduced the uptake of Cd at the surface of the animals (Penttinen et al., 1998).

5. Conclusions

Both Ca and HS have a detoxification potential against metals. Because of the interaction of the metals, the HS and the binding site of the juvenile fish the influence of HS on the Cd toxicity is complex. That means that the toxicity of Cd to early developmental stages of zebrafish is strongly dependent on the concentration of Ca in the water, the competition of metal and Ca − HS and the test fish, as well as the physiological needs of the exposed organisms (Hwang et al., 1994, 1995; Chang et al., 1997). Finally, can state that both the Ca ion and the HS interact with Cd ions. In natural waters with low Ca concentrations, HS interact strongly with Cd ions. The Ca concentration is, therefore, the decisive factor in cadmium toxicity.

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