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Mechanisms of chronic waterborne Zn toxicity in *Daphnia magna*

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Abstract

In order to gain better insights in the integrated response of *Daphnia magna* following chronic zinc exposure, several physiological parameters were measured in a time-dependent manner. *D. magna* juveniles were exposed for 21 days to dissolved Zn concentrations up to 340 μg/L. Next to standard endpoints such as mortality, growth and reproduction the following sub-lethal endpoints were measured: filtration and ingestion rate, respiration rate, energy reserves, internal Zn and total Ca concentrations in the organisms. Organisms exposed to 80 μg/L generally performed better than the Zn deprived control organisms. The former were used to elucidate the effects of higher Zn concentrations on the endpoints mentioned above. After 1 week, only 7% of the organisms exposed to 340 μg/L survived. Body Zn contents of these organisms were 281 ± 76 μg g dry weight and a 37% decrease of the Ca contents was observed. This suggests a competitive effect of Zn on Ca uptake. Filtration rate (−51%), individual weight (−58%) and energy reserves (−35%) also exhibited a decreasing trend as a function of increasing Zn exposure concentrations. During the second and third exposure week an overall repair process was observed. In the surviving organisms mortality and reproduction were only slightly affected. This can be explained by (over)compensation reactions at lower levels of biological organisation: Ca contents (+24%) and filtration rate (+90%) increased as a function of the exposure concentration while respiration rate decreased (−29%) resulting in energy reserves remaining constant as a function of Zn exposure. It is hypothesized that a disturbed Ca balance is probably the first cause for zinc toxicity effects in *D. magna*.

Keywords: Zinc sensitivity; Calcium; Filtration; Respiration; Energy

1. Introduction

Although Zn is an essential metal for all living organisms, toxicity effects will occur during exposure to elevated concentrations (Eisler, 1993). The sensitivity of different aquatic organisms toward Zn has been studied extensively using standard endpoints such as survival, growth and reproduction. However, an individual's performance is determined by the integrated phenotypic response of all its systems working in concert. In order to gain insights on the impact of chemical exposure on organisms, the simultaneous measurement of several biological variables is required (Hebel et al., 1997).

In metal-exposed *Daphnia magna*, several molecular and physiological traits have been measured including filtration, ingestion and assimilation rates (Bodar et al., 1988; Allen et al., 1995; Knops et al., 2001); respiration rate and enzymatic activity (Knops et al., 2001; Khangarot and Rathore, 2003; De Coen and Janssen, 1997a; De Coen et al., 2001); biochemical composition including protein, sugar, lipid, DNA and RNA contents (Bodar et al., 1988; Barber et al., 1994; De Coen and Janssen, 1997b, 2003); metallothioneins (Guan and Wang, 2004); ion turnover rates (Pane et al., 2003; Bianchini and Wood, 2002); and metal accumulation (Muyssen and Janssen, 2002; Yu and Wang, 2002). Compared to Cd and Cu, studies with Zn are relatively scarce and the underlying causes of chronic Zn toxicity to *D. magna* are not yet understood.

In most studies physiological and molecular effects were measured following acute exposure to the metal. Moreover, in many reports only a few physiological endpoints have been measured simultaneously, and often survival and reproduction are not reported, rendering an integrated effect analysis impossible. Pooling of results from several studies is very difficult due to the use of (i) different metal concentrations and/or culture media and hence metal bioavailability and (ii) genetically different daphnid clones differing in physiology and metal sensitivity.

Based on evidence with freshwater fish, the primary acute effect of an increase in waterborne Zn^{2+} is believed to be an impaired branchial Ca^{2+} influx that, in turn, leads to hypocalcaemia (Spry and Wood, 1985). During chronic exposure to sublethal concentrations of metals, fish often acclimate and nor-

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mal rates of ion transport are restored (McDonald and Wood, 1993; Hogstrand et al., 1995). This often involves a damage repair process in the gills (e.g. regeneration of Ca^{2+} barriers and faster protein-turnover) that results in an increased energetic cost at this site. Like other metabolic expenditure related to acclimation, this renders less energy available for other processes such as feeding, growth and reproduction (Calow, 1991; Wilson et al., 1994; Hogstrand et al., 1995). For daphnids, the effects of Zn toxicity on Ca homeostasis and the consequences for the organisms' physiology have never been demonstrated.

The present study aimed at revealing the physiological processes that are affected by chronic Zn exposure in order to explain observed mortality and reproduction effects. To that end, we exposed *D. magna* to various Zn concentrations for 21 days and measured at regular intervals Zn and Ca body burdens, respiration rate, filtration and ingestion rates, energy reserves, growth, mortality and reproduction. A detailed knowledge of species-specific toxicity mechanisms of a metal will contribute to understanding the mechanisms underlying the recently developed Zn biotic ligand models (BLM for a review see Niyogi and Wood, 2004; Heijerick et al., 2005; Santore et al., 2002).

2. Materials and methods

2.1. Culturing of D. magna

D. magna Straus (clone K6) used in all our experiments was originally collected from a pond in Kiel (Antwerp, Belgium) and has been successfully cultured for over 15 years in biologically filtered and aerated Ghent (Belgium) tap water (pH 7.6, hardness 180–200 mg/L as $CaCO_3$, 2–3 mg/L DOC, 5–7 μg/L dissolved zinc, measured in aquaria). Experimental animals were cultured under semi-static conditions in polystyrene aquaria (one aquarium per test concentration). Tests were started with 300 juveniles in 3 L of medium and the culture volume was adjusted as a function of survival or removal of organisms for specific tests (see below) to reach a density of one organism per 10 mL until day 7 and of one organism per 20 mL afterwards. Tests were performed in a synthetic freshwater containing 2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.75 mM $NaHCO_3$ and 0.078 mM KCl and 4 mg/L dissolved organic carbon (DOC). Dissolved organic matter (DOM) was obtained from a natural source (Ankeveen, The Netherlands) in November 2001, concentrated by reverse osmosis and stored at −20 °C until use. A detailed description on the sampling site and procedure can be found in De Schamphelaere and Janssen (2004), although it has to be noted that the DOC used for the present study was sampled at another period the same year. Inevitably, the use of natural DOC from reverse osmosis isolation resulted in the addition of other elements to the test medium. Concentrations due to these additions were low but were nevertheless taken into account for speciation calculations (see further): 0.19 μM Mg, 1.3 μM Ca, 6.2 μM K, 46.6 μM SO_4, 46.8 μM Cl, 315 μM Na; 15 pM Cd, 51 pM Pb, 6.5 nM Cu, 8.1 nM Mn, 9.8 nM Zn, 29.3 nM Ni, 88.4 nM Fe and 175.4 nM Al. The pH of the test medium was 7.6 ± 0.2. Zn was added as $ZnCl_2$ and test concentrations were 3 (control background concentration), 80, 115, 170, 250 and 340 μg Zn/L, corresponding to free ion activities of 8.8 nM of Zn^{2+} activity in the control and 0.41, 0.63, 1.01, 1.59 and 2.27 μM Zn^{2+} activity in the Zn exposure concentrations (calculated using WHAM 6, Tipping, 1998, according to scenario 2 assumptions in Cheng et al., 2005). Animals were fed daily with the green algae *Pseudokirchneriella subcapitata*: 5×10^5, 7.5×10^5 and 1×10^6 cells/mL during each of the 3 subsequent weeks of exposure. The internal Zn concentration in the algal cells ranged from 1.5 to 3×10^{-9} μg/cell, which was demonstrated not to result in toxicity to *D. magna* (De Schamphelaere et al., 2004). Daily, the quantity of algae remaining in the aquaria was measured (Z1 Coulter Particle Counter, Beckman Coulter, Analis) and algal cells were added to reach the food levels described above. The temperature during culturing and testing was 20 ± 1 °C, with a light:dark cycle of 12 h:12 h. The medium was gently aerated and renewed two or three times a week. Tests were initiated using neonates (<24 h) originating from third to eighth brood females. Reagent-grade chemicals were used in all experiments.

2.2. Survival and reproduction

Three times a week the test medium was renewed and survival and reproduction (number of juveniles) was recorded.

2.3. Zn and Ca measurements in tissues and medium

A number of test organisms (15–60 organisms, depending on the size) was removed from the aquaria, rinsed with deionized water and placed in a 5 mM ethylenediaminetetraacetic acid solution (EDTA) for 20 min in order to remove adsorbed metal from the organisms (and thus retaining the internal concentration). After rinsing with deionized water, the organisms were oven-dried (50 °C) until no change of the dry mass was observed. Daphnids were weighed (Mettler H35, Germany) and digested in polypropylene tubes by adding 1 mL of 70% HNO_3 and subsequent heating in a microwave (in cycles of 4 min at 90, 160, 320 and 500 W). Cooled samples were diluted to 3 mL using bi-deionized water and stored until analysis. Zn and Ca concentrations were measured as described below.

At each renewal Zn concentrations in the test media (total and dissolved) were measured. These samples as well as digested daphnid samples and Ca in the digested daphnid samples were analysed using flame (SpectrAA100-Zeeman; Varian, Mulgrave, Victoria, Australia) or a graphite furnace (SpectrAA300-Zeeman; Varian) atomic absorption spectrophotometer (AAS). Ten millilitres of water samples were acidified (pH < 1) with 0.14N HNO_3. Calibration standards and a reagent blank were analysed with every 10 samples. Two certified reference samples, TMDA-62 and TM-25.2 (National Water Research Institute, Burlington, Ont., Canada) with certified Zn concentrations (mean ± 95% confidence interval) of 110 ± 15.5 and 24 ± 4.6 μg L^{-1}, respectively, were analysed at the beginning and end of each series of Zn measurements. Measured values were always within 10% of the certified value. All results presented are dissolved concentrations. Measured Zn concentrations in the test media did not differ more than 10% from the

nominal reported concentrations. In the controls, an average Zn concentration of 3 μg Zn/L was measured.

2.4. Filtration and ingestion rate

Daphnids were removed from the aquaria and four replicates of four organisms were set up in polyethylene cups containing 40 mL of medium identical to that in the test aquaria (water characteristics, Zn concentration and food concentration). One additional replicate without organisms was used for calculation of the correction factor (A). At the beginning and after an 8-h period the algal concentration (*P. subcapitata*) was measured and the filtration rate (F) and the ingestion rate (I) were calculated following Gauld (1951):

$$F = \frac{V}{n}\frac{\ln C_0 - \ln C_t}{t} - A$$

$$A = \frac{\ln C_0 - \ln C'_t}{t}$$

$$I = F\sqrt{C_0 C_t}$$

where C_0 and C_t are the initial and final food concentrations (cells/mL), t the time of exposure, n the number of organisms per vessel, V the test volume and C'_t is the final cell concentration in the control cup (without daphnids added). The 8-h feeding period resulted in a sufficient difference between C_0 and C_t (10–30%) and minimized the settlement of algae at the bottom of the test vessels. Filtration and ingestion rates were divided by the organism's dry weight (obtained from another sample, see Section 2.3).

2.5. Respiration rate

Oxygen consumption was measured by transferring a number of organisms (see further) from the different Zn test concentrations to a glass syringe containing 5 mL of medium identical to that in the test aquaria. On days 2, 7, 14 and 21 two replicates of six, three, two and two organisms, respectively, were used. The syringes were left in a horizontal position in a 20 °C warm water bath. During a 2-h period every 30 min 0.5 mL of the medium was injected in an oxygen electrode (Oxygen Meter Model 781, Strathkelvin Instruments, Scotland) and the oxygen concentration was recorded. The respiration rate was calculated as the slope of the remaining O_2 in the syringe as a function of time, divided by the organism's dry weight (obtained from another sample, see Section 2.3).

2.6. Energy reserves

Energy reserves were measured as the sum of protein, carbohydrate and lipid content in the organisms. For each energy fraction and depending on the organisms' age (2, 7, 14 or 21 days) 30, 10, 5 and 5 organisms were used, respectively. Daphnids were collected, shock-frozen in liquid nitrogen and stored at −80 °C until analysis. The different energy contents (mg/mg dry weight of tissue) were measured spectrophotometrically and converted to mJ equivalents as described by De Coen and Janssen (1997b, 2003). Each sample was divided into three replicates during the experimental procedure.

2.7. Statistical analysis

Effects of the various Zn acclimation concentrations on different endpoints were compared using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test (Statistica software, Statsoft, Tulsa, OK, USA). Homogeneity of variance and normality were tested using Bartlett and Shapiro-Wilkinson W-test, respectively. Statements of significance are based on accepting $p < 0.05$.

3. Results

3.1. Week 1

The results of the endpoints measured during the first week of Zn exposure are presented in Table 1. To facilitate comparison of endpoints, the observed trend as a function of the Zn concentration is indicated in the last column. Mortality gradually increased at the two highest concentrations (250 and 340 μg/L). At day 4, 82% and 53% survival was observed in these concentrations, respectively. At day 7, this was reduced to 40% and 7%, respectively. Zn significantly affected growth of the organisms: 7-day-old daphnids exposed to 340 μg/L were only half as large as the control organisms. These weight measurements also provide a first indication of sub-optimal physiology (Zn deficiency) in the control exposure: e.g. at day 2, control organisms had an average dry weight of 19.7 μg, while organisms exposed to 80 μg/L weighed 26.8 μg. Also for several of the other parameters, the daphnids exposed to 80 μg/L performed better. Therefore, potential trends between higher exposure concentrations and the 80 μg/L treatment were examined and indicated as such in Tables 1 and 2. Already at day 2, significant increases in the internal Zn concentrations as a function of increasing Zn exposure concentrations were observed. At day 7, the body concentrations ranged from 155 ± 27 μg Zn g^{-1} dry weight in the control to 281 ± 76 μg Zn g^{-1} dry weight in the (surviving) organisms exposed to 340 μg/L. Conversely, the total Ca concentration in the daphnids decreased with increasing Zn exposure (−37%). Filtration and respiration rates are expressed as a function of the organism's weight as these parameters are affected by the size of the daphnids. At day 2, the filtration and ingestion rate at concentrations ≥80 μg/L was significantly higher than in the control. At day 7, however a decrease (with 60%) from 3.41 ± 0.87 mL mg^{-1} h^{-1} in the control to 1.63 ± 0.02 mL mg^{-1} h^{-1} in 340 μg/L was noted. Corresponding ingestion rates at day 7 were $14.9 \pm 3.1 \times 10^5$ and $6.3 \pm 2.6 \times 10^5$ cells mg^{-1} h^{-1}, respectively. Respiration rates of organisms exposed to 80 μg/L were higher than these in the controls and decreased as a function of subsequent exposure concentration, except for 340 μg/L at day 7. When expressed as mJ $organism^{-1}$, the energy content of the daphnids correlated well to individual weight ($R^2 = 0.93$, $p < 0.001$). However, when expressed as mJ mg dry $weight^{-1}$ energy reserves at day 7 were

Table 1
Effects of Zn on *D. magna* during the first week of exposure

Endpoint	Day	Exposure concentration (μg Zn/L)						Trend
		Control	80	115	170	250	340	
Survival (%)	4	100	100	100	100	82	53	↓[a]
	7	95	98	95	93	40	7	↓[a]
Individual dry weight (μg)	2	19.7	26.8	24.6	27.1	25.3	23.9	–[a]
	7	99.8	107.2	100.8	85.1	73.3	44.8	↓[a]
Internal Zn body (μg Zn g^{-1} DW)	2	147 (24)^{AB}	137 (12)^{A}	181 (11)^{AC}	180 (13)^{AC}	221 (79)^{C}	200 (16)^{BC}	↑
	7	155 (27)^{A}	177 (13)^{AB}	191 (18)^{AB}	229 (30)^{BC}	255 (15)^{C}	281 (76)^{C}	↑
Total Ca body (mg Ca g^{-1} DW)	2	36.2 (9.2)^{A}	29.7 (4.4)^{A}	29.6 (3.6)^{A}	26.1 (1.4)^{A}	23.7 (1.1)^{B}	22.8 (0.3)^{B}	↓
	7	33.4 (4.5)^{A}	33.1 (3.0)^{A}	34.3 (4.1)^{A}	31.3 (4.2)^{A}	30.4 (4.2)^{A}	21.0 (3.1)^{B}	↓
Filtration rate (mL mg DW^{-1} h^{-1})	2	1.21 (0.34)^{A}	3.72 (1.21)^{B}	2.57 (0.66)^{AB}	3.16 (1.07)^{B}	3.31 (0.21)^{B}	3.11 (1.44)^{B}	–
	7	3.41 (0.88)^{B}	2.77 (1.42)^{B}	2.88 (0.34)^{B}	2.96 (0.80)^{B}	2.23 (0.75)^{AB}	1.35 (0.57)^{A}	↓
Ingestion rate ($\times 10^5$ cells mg DW^{-1} h^{-1})	2	6.96 (2.07)^{A}	17.18 (5.53)^{B}	11.92 (2.89)^{AB}	14.73 (4.74)^{B}	15.76 (7.88)^{B}	14.29 (6.58)^{B}	
	–							
	7	14.87 (3.10)^{B}	11.90 (5.66)^{B}	12.77 (1.10)^{B}	12.89 (2.85)^{B}	9.76 (3.22)^{AB}	6.31 (2.59)^{A}	↓
Respiration rate (μL O_2 mg DW^{-1} h^{-1})	2	12.40 (0.45)	14.75 (2.98)	8.90 (0.37)	10.87 (0.33)	10.40 (2.10)	9.45 (4.46)	↓[a]
	7	8.91 (1.60)	14.73 (1.98)	11.68 (0.35)	11.92 (1.04)	11.62 (0.49)	17.91 (2.37)	↑[a]
Energy reserves (mJ $organism^{-1}$)	2	315 (34)^{A}	364 (21)^{A}	516 (76)^{BC}	418 (104)^{AB}	572 (81)^{CD}	342 (38)^{A}	–
	7	1650 (265)^{B}	1818 (276)^{B}	1565 (188)^{B}	948 (207)^{A}	1187 (148)^{A}	–	↓

Data are expressed as means with standard deviations between brackets. Mean values sharing the same superscript letter are not significantly different. When applicable, trends are based on significant differences (ANOVA followed by Duncan's multiple range test) between 340 and 80 μg Zn/L ($p < 0.05$).
(–) No value or no trend.
[a] Due to the absence or limited number of replicates the trend was based on a difference of minimally 20% between 340 and 80 μg Zn/L.

Table 2
Effects of Zn on *D. magna* during the second and third week of exposure

Endpoint	Day	Exposure concentration (μg Zn/L)				Trend
		Control	80	115	170	
Survival (%)	14	89	94	88	81	–[a]
	21	84	91	86	74	–[a]
Reproduction (juveniles/female)	14	5.2	5.3	4.9	4.6	–[a]
	21	26.3	25.6	24.4	21.9	–[a]
Individual dry weight (μg)	14	277	226	264	297	–[a]
	21	277	353	337	235	↓[a]
Internal Zn body (μg Zn g^{-1} DW)	14	106 (6)^{A}	105 (18)^{A}	137 (17)^{AB}	152 (23)^{B}	↑
	21	96 (15)^{A}	103 (5)^{AB}	131 (16)^{B}	173 (26)^{C}	↑
Total Ca body (mg Ca g^{-1} DW)	14	25.0 (5.7)^{A}	21.7 (1.3)^{A}	23.5 (3.0)^{A}	20.8 (3.4)^{A}	–
	21	33.9 (2.6)^{AB}	30.6 (3.0)^{A}	33.0 (1.3)^{AB}	37.8 (2.1)^{B}	↑
Filtration rate (mL mg DW^{-1} h^{-1})	14	1.18 (0.42)^{BC}	1.34 (0.41)^{C}	0.72 (0.19)^{AB}	0.44 (0.17)^{A}	↓
	21	0.58 (0.09)^{A}	0.62 (0.09)^{A}	0.71 (0.09)^{A}	1.18 (0.19)^{B}	↑
Ingestion rate ($\times 10^5$ cells mg DW^{-1} h^{-1})	14	7.32 (2.44)^{BC}	8.19 (2.25)^{C}	4.48 (1.10)^{AB}	2.88 (1.04)^{A}	↓
	21	5.17 (0.78)^{A}	2.37 (0.73)^{A}	6.11 (0.73)^{A}	10.22 (1.54)^{B}	↑
Respiration rate (μL O_2 mg DW^{-1} h^{-1})	14	7.61 (–)	11.06 (2.23)	8.97 (–)	5.44 (0.89)	↓[a]
	21	7.05 (0.78)	7.62 (0.08)	4.46 (0.16)	5.45 (–)	↓[a]
Energy reserves (mJ $organism^{-1}$)	14	5111 (460)^{AB}	6355 (348)^{C}	5923 (740)^{BC}	4058 (373)^{A}	↓
	21	3946 (905)	5508 (772)	4652 (771)	5900 (123)	–[a]

Data are expressed as means with standard deviations between brackets. Mean values sharing the same superscript letter are not significantly different. When applicable, trends are based on significant differences (ANOVA followed by Duncan's multiple range test) between 170 and 80 μg Zn/L.
(–) No trend.
[a] Due to the absence or limited number of replicates the trend was based on a difference of minimally 20% between 170 and 80 μg Zn/L.

significantly lower than at day 2 for Zn concentrations from 115 to 250 μg/L.

3.2. Weeks 2 and 3

Results of the second and third week of exposure are presented in Table 2. Because at day 7 (i.e. before the start of reproduction) all organisms alive at 250 and 340 μg/L were used for measuring the physiological endpoints, no 14 and 21 days survival and reproduction data are available for these concentrations. At lower concentrations survival and reproduction was only moderately affected. The number of offspring per female ranged from 26.3 to 21.9 in the control and 170 μg Zn/L, respectively. At days 14 and 21 an overall decrease in internal Zn was observed, with a maximum body burden of 173 ± 26 μg Zn/g dry weight when exposed to 170 μg/L (versus 229 ± 30 μg Zn/g dry weight at day 7). No significant differences in Ca content between treatments were found at day 14, while at day 21 the Ca content of organisms exposed to 170 μg/L was higher than that of organisms exposed to lower concentrations. Filtration and ingestion rates were significantly lower than these noted at day 7. At day 14, a decreasing trend in filtration rate as a function of increasing Zn concentration was (as observed at day 7) was still present. At day 21, however, the opposite was found with a significantly increased filtration rate at 170 μg/L (1.2 ± 0.2 mL mg^{-1} h^{-1} compared to 0.8 ± 0.1 mL mg^{-1} h^{-1} in the control). The same conclusions could be made based on ingestion rates. Respiration rates decreased as organisms grew older and larger. The highest respiration rates (11.1 and 7.6 μL O_2 mg^{-1} h^{-1} for days 14 and 21, respectively) were again recorded at 80 μg/L and decreased at higher exposure concentrations. Total energy reserves were no longer correlated with the organism's weight ($R^2 = 0.21$, $p = 0.25$). Exposure to 80 μg/L resulted in significantly higher energy reserves than in the control organisms. At higher concentrations energy reserves clearly decreased on day 14, but slightly increased at 170 μg/L on day 21. Furthermore, it was observed that energy reserves on day 21 were generally lower than on day 14, except for 170 μg/L where a 45% increase was noted. This energy loss was highest in the control (23% difference).

4. Discussion

At the start of this section we would like to point to the fact that organisms exposed to 80 μg/L seemed to perform equally well as or better than organisms exposed to control conditions (without added Zn) in terms of filtration rate, energy reserves and individual weight. The Zn concentration of 80 μg/L corresponds to a free Zn^{2+} ion activity of 0.41 μM. This effect may be due to hormesis (i.e. the stimulatory effect of sub-lethal concentrations), but is more likely associated with the fact that organisms cultured without added Zn suffer from Zn deficiency, while organisms cultured at higher concentrations perform better as was previously demonstrated (Muyssen and Janssen, 2001; Muyssen et al., 2005). In that study with the same *D. magna* clone deficiency effects were observed at Zn activities ≤11 nM

Zn^{2+} (calculated from a total concentration of 200 μg/L) and the upper boundary of the optimal range was situated between 0.29 and 1.30 μM Zn^{2+} (calculated from total concentrations of 450 and 600 μg/L; Muyssen and Janssen, 2001). For this reason trends as a function of the Zn concentration were explored by comparing the highest test concentration to the 80 μg/L treatment.

4.1. Week 1

The acute effect of an increase in waterborne Zn on freshwater fish is believed to be a competitively inhibited branchial Ca influx that, in turn, leads to hypocalcaemia (Spry and Wood, 1985; Hogstrand et al., 1995). This competitive interaction between Ca and Zn at the gill surface can also explain the well-known protective effect of increased water hardness on fish exposed to Zn (Pagenkopf, 1983). The same effect of water hardness has also been demonstrated in *D. magna* (Winner and Gauss, 1986; Heijerick et al., 2003, 2005) thus indicating that Ca and Zn also in this species share similar uptake pathways, i.e. a Ca^{2+}-channel (Hogstrand et al., 1995). Our experiments confirmed these findings in another way: the total Ca content of the daphnids significantly decreased during the first week of exposure. Ca body burdens in daphnids exposed to 340 μg/L were up to 37% lower than in the controls. In fish, uni-directional Zn influx increased during the first week of exposure to waterborne Zn, an effect that may be largely explained by the changes in K_m for Ca (Hogstrand et al., 1995). These authors speculated that the initial response of the fish to elevated Zn is to compensate for a reduced availability of Ca by markedly increasing the affinity of a dual Ca/Zn transporter. The increase in internal Zn in *D. magna* as observed from day 2 to day 7 might be explained by similar mechanisms. The increase was highest (40%) at 340 μg/L.

As already indicated by Pane et al. (2003), we realize that it is difficult to demonstrate toxicant-induced effects on Ca-homeostasis in daphnids based on total Ca contents because the majority of their Ca body load is situated in the exoskeleton which is shed during each molt. Due to the rapid calcification process to form a new carapax and the associated Ca-uptake from solution there is no real steady-state rate of Ca-influx. In future experiments this issue will be further taken into account and investigated. *Daphnia* seems to be the genus with the highest specific content of Ca known among freshwater crustacean zooplankters, with typical values around a 2–5% Ca content (on dry weight basis) at ambient Ca concentrations above 5 mg Ca/L (Hessen and Rukke, 2000). This corresponds to the measurements of the present study: 21–38 mg Ca/g dry weight. Alstad et al. (1999) found that the specific Ca content of *D. magna* decreased from 4.2% to 1% of the body mass when changing the Ca content of the exposure water from 10 to 0.52 mg Ca/L in the water. Low Ca concentrations in *Daphnia* spp. affected both survival and reproduction. Saturation levels in *D. magna* are suggested to be situated at 5–6 mg/L (in the water) corresponding to Ca body contents ≥2% dry weight (Hamza et al., 1998; Hessen et al., 2000; Hessen and Rukke, 2000).

Ca plays an integral role in a wide variety of biological processes, including signal transduction, gene expression, cell proliferation, apoptosis and the coordination of muscle excitation–contraction coupling (Carafoli, 2002). Due to this complexity and the lack of reported experiments on this topic, it is difficult to link the decreased Ca contents in *D. magna* directly to one of the other endpoints measured. The reduced filtration rate observed at day 7 when exposed to 340 μg Zn/L might be the result of a disturbance in cell signaling and muscle contraction. Even in sponges, distinguished from other metazoa by the absence of specialized nerve or muscle cells, movement and tissue contraction (due to the contraction of actin and myosin bundles in the myocytes) is mediated by the presence of Ca (Lorenz et al., 1996). The hypothesized role of Ca in daphnid movement and filtration is the object of future experiments. Several authors report a reduction in filtration rate in *D. magna* and other cladoceran species following metal exposure (including Zn) through the water and/or food (Wong, 1992; Barata et al., 2002). However, in many of these studies no explanation for this reduction is provided. Ferrando and Andreu (1993) suggested an interference with neural transmission in *D. magna* exposed to Cu. Other reports link the toxic effect of Cd to gut poisoning (Griffiths, 1980; Allen et al., 1995; Barata et al., 2002). Independent of the cause of this reduced filtration rate, a decrease in food uptake will lead to a decrease in energy reserves and weight of the test organisms, as demonstrated by our results.

The effect of metals on daphnid respiration is rather complex as is also obvious from the contradictory results in literature. On the one hand, respiration is expected to increase as a result of an increased metabolic activity, e.g. an increased protein-turnover, needed to cope with toxicant stress (Calow, 1991). Such results were found by Barber et al. (1990) for *D. magna* following acute (48 h) Cd exposure. On the other hand, the majority of experiments did not demonstrate any trend (Barber et al., 1990; Knops et al., 2001), and even decreasing respiration rates as a function of the metal concentration were reported by Khangarot and Rathore (2003) and Pane et al. (2003). One direct reason for these latter observations can be that the metal causes structural damage to the gas exchange surface. Knops et al. (2001) suggest several possibilities for an unchanged metabolic cost and respiration rate under toxicant stress. One of these is that additional costs associated with repair processes are masked by other toxicant effects. This may result from reduced metabolic costs associated with locomotory activity and food acquisition. Filtration rate and respiration are linked as the movement of the filtration apparatus requires energy (and thus oxygen). Therefore, if filtration rate decreases due to metal exposure, respiration rate will also decrease to some extent. Moreover, the water current produced by the movements of the thoracic limbs is also thought to be important for oxygen extraction (Pirow et al., 1999). A second possibility is that stress-induced energy demands may have been too small, compared to overall metabolic costs, to be detected. For instance, costs for metallothionein synthesis are estimated to account for less than 5% of total metabolic costs in daphnids (Barber et al., 1990). It is difficult to extrapolate these findings to our results. At the more 'optimal' 80 μg/L exposure, respiration rates were higher than in the control. During the first week, at higher Zn concentrations no real trend was observed except for the remarkably higher oxygen consumption at day

7 when exposed to 340 μg/L. During the first week no correlation between filtration and respiration was found (Kendall's tau > 0.05).

4.2. Weeks 2 and 3

During chronic exposure to sublethal concentrations of metals, fish often acclimate. The Ca influx is 'corrected' by restoration of functional transport sites and the system is tuned to limit the influx of Zn by a persistent reduction in the affinities for both ions (Hogstrand et al., 1995). In the present study, we also observed a restoration of the Ca body content at day 14 and even an increase in total Ca body content at day 21 in the highest remaining test concentration (170 μg Zn/L). At day 14 the Ca body contents were lower than at other time points, but this may be related to the moment in the molting cycle at which the organisms were sampled. Also, from day 7 to day 21, the Zn body burdens decreased in all Zn concentrations. However, as this decrease was not higher at elevated Zn concentrations (a decrease of 24% and 38% from day 7 to day 21, at 170 μg Zn/L and control, respectively), it is assumed that this was rather the result of the change in surface area:volume ratios and related uptake kinetics (Yu and Wang, 2002) rather than a transporter-mediated reduction in Zn uptake. As the Ca body content was restored, filtration rate and energy reserves followed the same trend, although with a certain time-lag as this was only observed at day 21 and not yet at day 14.

A possible energetic cost, as reflected by energy reserves of the organisms, associated with repair processes could not be determined. On the one hand one could state that the metabolic expenditure related to this acclimation renders less energy available for other processes including feeding, growth and reproduction (Calow, 1991; Wilson et al., 1994; Hogstrand et al., 1995). This can be confirmed by our data on filtration rate, energy reserves and growth at 14, but not day 21. On the other hand, if we assume that Ca content is linked directly to the filtration rate, then the possibly minor energetic costs for the restoration of the Ca content is masked by the larger gain in energy reserves due to a restoration of filtration rate. During the second and third week, recovery in most endpoints is accompanied by a decrease in respiration rate, thus suggesting that metabolic costs are also reduced. The final outcome of these combined effects at the organismal and population level can be deduced from the survival and reproduction data. From day 14 to day 21, some additional mortality was observed ranging from 2% to 7%. Overall, it may be concluded that repair mechanisms worked sufficiently for this endpoint. Total reproduction is affected by 17% on day 21, which might be mainly an effect of the reduced energy reserves at days 7 and 14. Based on the energy reserves measured at day 21, it can be hypothesized that reproduction would have recovered more if the test would have been continued beyond 21 days.

Bioconcentration factors (BCFs) were calculated as the ratio of the Zn body concentration and the Zn concentration in the water. When the $\log_{10}$(BCF) is plotted as a function of the $\log_{10}$(water concentration) an indication of the regulation mechanisms of the metal can be obtained (McGeer et al., 2003). If an inverse linear relationship is observed with a slope of approximately −1, the metal is actively regulated. If metals are accumulating – whether or not in detoxified form – the slope will approximate zero or higher. At day 2 of our experiment a slope of −0.93 was calculated; while at the other time points the slope was −0.89. Generally, based on these BCFs the role of storage/detoxification mechanisms, such as metallothioneins, was limited in the present study. In *D. magna* exposed to Cu and Zn, indications of storage/detoxification (based on BCFs) were only found after several generations of acclimation to concentrations above the optimal concentration range (Muyssen and Janssen, 2002; Bossuyt and Janssen, 2005). The induction of metallothionein-like proteins following Cd exposure was demonstrated by Bodar et al. (1990) and Stuhlbacher et al. (1992). More recently, Guan and Wang (2004) did quantify metallothionein concentrations in *D. magna* following Cd exposure. A maximal four-fold increase compared to the control was observed. Considering a factor of 10 for the wet weight–dry weight conversion and a binding capacity of 7 mol Cd mol^{-1} MT (Stillman, 1995), approximately 40 μg Cd/g dry weight can be detoxified by metallothioneins. Based on their data, the slope of the inverse linear relationship between $\log_{10}$(BCF) and $\log_{10}$(water concentration) was found to be <0.60, clearly indicating the difference in regulation between the essential metal Zn and the non-essential metal Cd.

5. Conclusions

From the conducted experiments we formulate the following hypothesis concerning the mechanisms of chronic Zn toxicity in *D. magna*. Zn inhibits Ca uptake, eventually resulting in reduced total Ca body contents of the organisms. When Ca reduction is severe enough, the organisms die as a result of hypocalcaemia. At sub-lethal reductions of Ca body contents, the movement and filtration rate is inhibited leading to a decreased food uptake. Consequently less energy is available for the organisms, which ultimately results in reduced growth, and reproduction. From 14 days of exposure and onwards, however, general repair processes were clearly observed: Ca body contents were restored to normal levels, as were feeding rates and available energy reserves.

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