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The effects of dietary nickel exposure on growth and reproduction of *Daphnia magna*

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ABSTRACT

Although there is growing evidence that dietborne metals can be toxic to various aquatic species, there is still insufficient knowledge to integrate this information in environmental risk assessment procedures. In this study, we investigated the effects of a 21-day exposure of *Daphnia magna* to a control diet (i.e. the green alga *Pseudokirchneriella subcapitata* containing <4.0 μg Ni/g dry wt) and five diets with elevated Ni concentrations (i.e. the same alga contaminated with Ni burdens between 33.7 and 837 μg Ni/g dry wt). A significant accumulation of dietborne Ni in *D. magna*, i.e. between 49.6 and 72.5 μg Ni/g dry wt, was observed when they were fed with diets containing between 85.6 and 837 μg Ni/g dry wt. This was paralleled by a significant reduction of reproduction (by 33.1%), measured as the total number of juvenile offspring per female and growth (by 9.1%), measured as the carapax length of 21-day-old females. Life-history analysis showed that the time to first brood of Ni exposed organisms was between 7.8 and 8.2 days, and occurred 0.7–1.1 days earlier than for the control organisms (time to first brood = 8.9 days). The number of offspring in the first brood was significantly reduced (by 21–33% compared to the control) in all dietary treatments. Longer exposure (≥8.9 days, i.e. from the second brood onwards) led to a reduction of brood size only when given diets containing 85.6 and 837 μg Ni/g dry wt. The results suggest that a variety of mechanisms may be involved in the effects of dietary Ni exposure, including altered resource allocation or targeted reproductive inhibition. While Ni exposure clearly altered the quality of the diet (measured as essential ω3 polyunsaturated fatty acid content and C:P ratio), we found no conclusive evidence that these diet quality shifts could have affected growth or total reproductive output. More research is required to fully understand the mechanisms of Ni toxicity associated with the dietary exposure route.

1. Introduction

Risk assessments of metals in freshwater ecosystems usually assume that toxicity is caused by waterborne metal only (Meyer et al., 2005). In this context the biotic ligand model (BLM) is a useful construct to predict changes in bioavailability and toxicity of waterborne metal as a function of the prevalent physico-chemical water characteristics (e.g., Di Toro et al., 2001; Paquin et al., 2002; De Schamphelaere and Janssen, 2002; Niyogi and Wood, 2004; Heijerick et al., 2005; Deleebeeck et al., 2008). Recently, however, evidence has been growing that dietary metal exposure can also cause adverse effects in aquatic organisms. Although initial research has largely focused on fish species (Meyer et al., 2005), interest in the effects on freshwater and marine invertebrates is growing. Hook and Fisher (2001a) demonstrated reproductive toxicity of dietary Ag to the freshwater cladocerans *Simocephalus* sp. and *Ceriodaphnia dubia*, fed with the chlorophyte *Chlorella vulgaris*. They also reported a decreased reproduction in the marine copepods *Acartia tonsa* and *Acartia hudsonica* after exposure to the diatom *Thallasiosira pseudonana* contaminated with Hg, Cd, Zn, Ag and Mn (Hook and Fisher, 2001b).

According to these authors, the reduced production of eggs was attributed to a disturbance of vitellogenesis, as evidenced by a decreased accumulation of yolk proteins in the ovary. The same mechanism was hypothesized to be involved in reproductive toxicity of dietary Zn to *Daphnia magna*, as only reproductive inhibition and no effects on growth (expressed as dry wt) or time to first brood were observed (De Schamphelaere et al., 2004). A link between reproductive toxicity and effects on molting-related processes has also been recently suggested (De Schamphelaere et al., 2008). According to Kooijman (2000), both observations suggest that dietary Zn directly hampers the conversion of energy reserves and resources into the reproductive output. Recent dietary studies with cladocerans feeding on Cu and Cd-contaminated green algae have reported inhibition of feeding rate, food assimilation, growth and/or reproduction (De Schamphelaere et al., 2007; Wang et al., 2007; Sofyan et al., 2007a, b). Moreover, when taking Cd body burdens as a reference, Geffard et al. (2008) demonstrated

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additive effects of waterborne and dietary Cd exposure to *D. magna* reproduction at low Cd concentrations (i.e. up to 25 μg/L), whereas the importance of dietary Cd on the daphnid contamination level decreases at higher Cd concentrations.

To our knowledge, only one study has been conducted on dietary Ni toxicity. Bielmyer et al. (2006) demonstrated increased mortality and reduced reproduction in the marine copepod *A. tonsa* feeding on Ni-enriched *T. pseudonana*. Similar research with freshwater crustaceans is lacking, but we hypothesized that, given the large body of evidence for other metals, dietary Ni exposure would also invoke toxicity in *D. magna*.

Therefore, in the present study, we investigated dietary Ni toxicity by exposing the freshwater cladoceran *D. magna* for 21 days to a control and 5 different dietary Ni exposure levels, using mortality, body length (growth), and various reproductive traits as toxicological endpoints. The bioaccumulation of Ni was also measured. Since it is well known that exposure of algae to metals can cause changes in food quality (Meyer et al., 2005; De Schamphelaere et al., 2007; McLarnon-Riches et al., 1998), we also measured – in all diets used – two food quality parameters that are known to be of importance for *Daphnia* performance, i.e. the phosphorous content (measured as C:P ratio) and the concentration of ω3 poly unsaturated fatty acids in the food (PUFA) (Park et al., 2002; Weers et al., 1997; Becker and Boersma, 2005; Brett et al., 2006). It was anticipated that this would enable us to discriminate between direct effects of dietary metals and indirect effects due to altered food quality. The issue of food quality has so far only been addressed in very few dietary metal toxicity studies with aquatic invertebrates (e.g., De Schamphelaere et al., 2007).

2. Material and methods

2.1. Experimental design

The green alga *Pseudokirchneriella subcapitata* was exposed to a control and 5 Ni concentrations for 64 h. The differently treated algae were harvested, and their Ni contents and nutritional quality were measured. They were then used as food in a 21-day life-table experiment with *D. magna*, during which survival and reproduction were monitored daily. *Daphnia* growth was measured as length at the end of the experiment and at the same time, their Ni body burden was measured.

2.2. Algae exposure

An algal starter culture was maintained as described in De Schamphelaere et al. (2004), and harvested after 5 days (i.e. end of the exponential growth phase). The actual Ni exposures were performed in six conical polyethylene bags containing 12 L of a growth medium to which the modified Provasoli's ES enrichment at half strength (Provasoli et al., 1957), and 1.4 mg/L $FeSO_4{\cdot}7H_2O$, 15 mg/L $NaH_2PO_4{\cdot}2H_2O$, 150 mg/L $NaNO_3$ and 2.35 mg/L $MnCl_2{\cdot}4H_2O$ were added. The six treatments consisted of a control (no added Ni) and nominal concentrations of 225, 449, 898, 1800 and 3590 μg Ni/L. These concentrations were equal to the EC50/4, EC50/2, EC50, EC50 × 2, and EC50 × 4, respectively, where EC50 is the 72-h median effective concentration for an effect on growth rate as predicted for this particular medium with the algal-BLM developed by Deleebeeck et al. (2009). Furthermore, 3-N morpholino propane sulfonic acid (MOPS, Sigma–Aldrich, Steinheim, Germany) was added at a concentration of 3.6 mmol/L as a pH buffer to maintain pH around 8.0. *P. subcapitata* was inoculated in concentrations of 10^6 cells/ml, and exposed under conditions described in De Schamphelaere et al. (2004). The growth rate of algal biomass was determined according to OECD test guideline 201 (OECD, 2006): cell concentrations were daily determined with a Coulter particle counter (Beckman-Analis, Namur, Belgium), and average specific cell growth rates (μ) were subsequently quantified following the equation:

$$\mu = \frac{\ln(N_t) - \ln(N_0)}{t},$$

where N_0 and N_t denote the cell concentrations (mg dry wt/L) at the start and after t days of exposure, respectively. Initial and final algal biomass (as dry wt) were determined by filtering 10^8 cells through a dried (24 h at 60 °C) and pre-weighed 0.45 μm filter (PALL, Supor 800 Life Science filters, Ann Arbor, MI, USA). After drying the filters + algae for 24 h at 60 °C and weighing, the dry wt of the algal cells was calculated as the difference between dry wt after and before filtration of the algae (De Schamphelaere et al., 2004).

After 64 h of exposure, the algae were harvested by centrifugation, washed with 50 ml of modified M4 medium (see Section 2.4), and centrifuged again. After removing the supernatant, new M4 medium was added to resuspend the algae. The resulting concentrate was stored in dark at 4 °C throughout the daphnid testing period. These algal suspensions were diluted to biomass concentrations of 1.25, 2.5 and 3.75 mg dry wt/ml with modified M4 medium at the beginning of weeks 1–3 of the *Daphnia* assay, respectively. Individual daphnids were fed daily with 200 μL of these suspensions, so that each daphnid received 250, 500 and 750 μg dry wt biomass per day as the daily food ration during weeks 1–3 of the experiment, respectively (see also Section 2.4).

2.3. Algal characteristics

Dry wt and Ni burdens of the stored algae were determined at the beginning of each of the 3 weeks of the *D. magna* experiment, whereas the molar C:P ratio and content of ω3 PUFAs were determined immediately after harvest. For Ni burden analyses, volumes of the diet stock suspensions containing 5 mg dry wt of algae were centrifuged in triplicate in 2 ml polyethylene vials at 13,000 × *g* for 15 min in a Mikro 200 R centrifuge (Hettich Zentrifugen; Tuttlingen, Germany). The remaining pellet was washed in a 5 mmol/L solution of Na_2EDTA for 20 min (Hassler et al., 2004) and then centrifuged for the second time as described above. External Ni was operationally defined as the metal quantity that was desorbed from the algal surface into the supernatant by this EDTA wash. Complete dissolution of metals in these EDTA solutions was guaranteed by adding HNO_3 to a concentration of 0.14 mol/L. The internal Ni burdens were determined by hot acid digestion (60 min, 110 °C) of the remaining algae in 400 μL of ultrapure 14 mol/L HNO_3 (Normatom quality, obtained from VWR Prolabo, Leuven, Belgium) in polypropylene vials (Laborimpex, Brussels, Belgium). The digests were diluted 10-fold with deionized water before analysis. This method allowed a recovery of 98% of Ni from a certified reference plankton sample (National Institute of Standards and Technology, SRM 2977). All Ni analyses were performed using graphite furnace atomic absorption spectrophotometry (SpectrAA 800 with Zeeman background correction, Varian, Mulgrave, Australia).

The organic carbon to dry wt ratio was determined by isolating an aliquot of known dry wt from the algal stock and analyzing its organic carbon content using a total organic carbon analyzer (TOC-5000; Shimadzu, Duisburg, Germany) (De Schamphelaere and Janssen, 2004). This ratio was 0.40 ± 0.04 g C/g dry wt (mean ± standard deviation, n = 18) and was not significantly different across the different Ni exposures. For total P burden analyses, the same digestion method in 14N HNO_3 as described for metal analyses was applied, except that the algae were washed with a 10 mmol/L solution of KCl prior to acid digestion. This ensures that P remaining in the intercellular medium of the algae was removed. After digestion, the samples were diluted 10-fold with deionized water and the pH was adjusted with NaOH to within the

interval pH 2.5–10, and the total P content of the sample was determined according to Clesceri et al. (1998) with the Spectroquant phosphate cell test (Merck). In brief, 5 ml of each solution was subsequently digested in a 5/1 (volumetric) mixture of concentrated HNO_3 (14 mol/L)/H_2SO_4 (12 mol/L) in order to hydrolyze (30 min, 120 °C) the organically bound P to orthophosphate. The liberated orthophosphate in the digest was converted into phosphomolybdic acid via reaction with ammonium molybdate and potassium antimonyl tartrate. The phosphomolybdic acid was reduced to the intensely colored molybdenum blue by the addition of ascorbic acid, which was colorimetrically quantified at 710 nm (Aquamate spectrophotometer; Thermo Electron Corporation; Cambridge, UK). Molar C:P ratios were then calculated from the carbon and phosphorus contents.

Fatty acid (FA) composition was determined as described in De Schamphelaere et al. (2007). In brief, fatty acid methyl esters (FAME) were prepared via a modified procedure of Lepage and Roy (1984), which implies a direct acid catalysed transesterification on 10–150 mg dry wt of sample. Eicosadienoic acid (20:2 ($n-6$)) was added as an internal standard prior to the reaction. FAME were extracted with hexane and, after evaporation of the solvent, prepared for injection in the chromatograph by dissolution in isooctane (2 mg/ml). Finally, quantitative determination was done on a Chrompack CP9001 gas-chromatograph.

2.4. Daphnia magna exposure

The 21-day exposure of *D. magna* to dietary Ni was performed according to OECD guideline No. 211 for testing of chemicals (OECD, 1998). The test organisms originated from a healthy stock culture of parent daphnids (clone K6), which has been successfully cultured for over 15 years in biologically filtered and aerated city tap water (Ghent, Belgium) (pH 7.6, hardness 180–200 mg/L as $CaCO_3$, 2–3 mg/L DOC) (Muyssen et al., 2006). The dissolved Ni concentration in this medium at the start of the experiment was 3.1 μg/L. The exposures were performed in M4 medium (Elendt and Bias, 1990), with two modifications. First, total Zn concentration was increased to 26 μg/L to ensure a bioavailable Zn concentration leading to optimal daphnid performance (cf. Muyssen et al., 2006). Second, the strong metal chelator EDTA in the medium was omitted and replaced with 4 mg/L of natural dissolved organic carbon (DOC). DOC was collected by reverse osmosis from an uncontaminated creek (Ruisseau de St-Martin, Bihain, Belgium), as described in De Schamphelaere et al. (2003). Inevitably, the use of natural DOC collected with reverse osmosis resulted in the addition of other trace elements to the exposure medium. Concentrations due to these additions were low (1.2 μg Cu/L, 6.2 μg Zn/L, 2.1 μg Mn/L, 660.9 μg Fe/L, 0.02 μg Cd/L, 1.1 μg Ni/L, 96.2 μg Al/L and 0.5 μg Pb/L), but were still taken into account for speciation calculations and predictions of waterborne Ni toxicity to *D. magna* with the BLM (see Section 2.5). The pH of the medium at the start of the test was 8.0.

At the start of the experiment, 10 juveniles (<24 h old) were transferred individually to polyethylene cups containing 45 ml of test medium. The daphnids were fed daily with 250, 500 and 750 μg algal dry wt during weeks 1–3, respectively. Every other day, the medium was completely renewed and the pH of the 'old' medium was determined, while reproduction was recorded daily as the number of juveniles released from the brood pouch. Mortality was assessed daily, whereas carapax length – defined as the linear distance between top of the head and the base of the apical spine – was determined on the last day of the test. At the end of each week, old test medium was sampled and filtered (PALL Life Sciences Acrodisc, pore size 0.45 μm, pretreated with HNO_3) and the Ni concentration was determined to assess the quantity of Ni being eliminated from and/or desorbing from the algae into the *Daphnia* exposure medium. The surviving parent organisms at the end of the test were used for the determination of *Daphnia* dry wt and Ni bioaccumulation by hot acid digestion, as explained in De Schamphelaere and Janssen (2004). Daphnids were fed with control algae for 4 h prior to Ni body burden determination, which is recommended to ensure the ingested Ni is efficiently removed from the gut lumen (Gillis et al., 2005). All Ni analyses were performed with graphite furnace atomic absorption spectrophotometry (SpectrAA 800 with Zeeman background correction, Varian, Mulgrave, Australia).

2.5. Data treatment and statistics

Speciation calculations were performed using Visual Minteq (Windows Ver 2.53; free download from http://www.lwr.kth.se/english/OurSoftWare/Vminteq/index.htm) for the algal exposure media, and with the Windermere Humic Aqueous Model (WHAM)—model VI for the modified M4 medium of the daphnid experiment (Tipping, 1998). Calculations with the latter were performed using the optimal calibrated set of parameter values as described by Van Laer et al. (2006). Activities of Fe^{3+} and Al^{3+} were estimated to be controlled by their colloidal solid phases, i.e. $Fe(OH)_3$ and $Al(OH)_3$, respectively, and were thus estimated from pH and the solubility product of these hydroxides (Van Laer et al., 2006).

All observations related to algae or *Daphnia* are reported as mean ± standard deviation. Statistical comparisons between exposures and controls were carried out with the Mann–Whitney-U test (MWU; Siegel and Castellan, 1988), with the exception for the C:P ratio of algae, where comparisons among all treatments were performed using Duncan's multiple range test. All statistical comparisons, as well as correlation analysis were performed with Statistica 6.0 software (Statsoft, Tulsa, OK, USA).

3. Results

3.1. Algae exposure and properties

Measured Ni concentrations, algal Ni burdens, growth rates and food quality parameters are given in Table 1. Over the whole range of Ni exposures applied to the algae, internal ($p = 0.0002$, $r^2 = 0.98$) and external ($p = 0.0003$, $r^2 = 0.97$) Ni burdens of the algae increased linearly with increasing Ni concentration in the exposure medium, although a particularly strong increase in the Ni burdens could be noted between the exposures to 900 and 1700 μg Ni/L. The proportion of internal and external Ni burdens was relatively constant across all treatments, constituting 11–19% and 81–89% of total Ni, respectively.

The molar C:P ratio of the algae was significantly lower in all Ni exposures (C:P = 140–178) compared to the control (C:P = 230) (Table 1), although no significant linear trend ($r^2 = 0.19$, $p = 0.39$) as a function of increasing Ni exposure concentrations was detected.

The two most abundant ω3 PUFAs (together representing >98% of the total ω3 PUFA content in all diets) were α-linolenic acid (ALA, 66–86% of total ω3 PUFA content) and stearidonic acid (SDA, 14–34%) (Table 1). All other individual ω3 PUFA molecules were present in the diets at very low levels. The total ω3 PUFA contents did not show a significant linear correlation with the Ni exposure concentration ($p = 0.29$), but the data reveal an identifiable pattern of two distinct groups of treatments: (1) the algae exposed to the control, 231 and 463 μg Ni/L exhibit an average of 165 μmol/g C of ω3 PUFA and (2) the algae exposed to 900 μg/L and higher concentrations, had a lower average of 111 μmol/g C of ω3 PUFA (Table 1).

3.2. Daphnia exposure and effects

The results of the 21-day exposure are presented in Table 2. The pH of the exposure media never deviated by more than 0.5 pH units

Table 1
Data on algae exposure and properties of the different algal diets cultured at six different Ni exposure concentrations.[a].

Nominal exposure concentration (μg/L)	Control	225 μg/L	449 μg/L	898 μg/L	1800 μg/L	3590 μg/L
Measured Ni concentration (μg/L)	7.5	230.5	462.9	900.4	1699	3484
Biomass growth rate (d^{-1})	0.82	0.75	0.77	0.70	0.22	0.17
Internal Ni (μg/g dry wt)[a]	<4.0[b]	33.7 ± 3.6	68.5 ± 12.5	85.6 ± 6.7	391 ± 23	837 ± 33
External Ni (μg/g dry wt)[a]	5.5 ± 4.3	204 ± 50	499 ± 135	387 ± 99	1680 ± 584	4050 ± 1460
Molar C:P ratio[a,c,d]	230 ± 3[A]	169 ± 0[B,E]	178 ± 1[C]	155 ± 3[B]	140 ± 5[D]	166 ± 1[E]
ω3 polyunsaturated fatty acids (μmol/g C)[e]						
Total content	146	189	161	95.1	119	119
α linolenic acid 18:3 ($n-3$) (ALA)	95.5	151	130	76.7	102.6	102.8
Stearidonic acid 18:4 ($n-3$) (SDA)	49.9	37.9	31.5	17.2	16.7	16.6

[a] Data are presented as mean ± standard deviation ($n = 3$).
[b] Lower than detection limit.
[c] C:P ratios not sharing the same letter are significantly different from each other (Duncan, $p < 0.05$).
[d] Significance level $p = 0.05$.
[e] Mean of two replicates.

Table 2
Experimental conditions (pH, dissolved Ni) and results of the chronic *D. magna* exposure to 6 different dietary Ni levels.[a].

Ni concentration in exposures of algal diets	Control	225 μg/L	449 μg/L	898 μg/L	1800 μg/L	3590 μg/L
Internal Ni burden of algal diet	<4.0	33.7 ± 3.6	68.5 ± 12.5	85.6 ± 6.7	391 ± 23	837 ± 33
Average pH during exposure	7.82	7.68	7.65	7.76	7.55	7.50
Dissolved Ni week 1 (μg/L)	<DL[b]	4.2	7.3	8.7	26.3	111.3
Dissolved Ni week 2 (μg/L)	<DL	4.7	12.8	15.4	62.5	177.7
Dissolved Ni week 3 (μg/L)	<DL	5.1	16.8	15.9	105.5	132.8
D. magna Ni body burden at day 21 (μg/g)	<DL[c]	<DL	<DL	54.0	49.6	72.5
Survival (%)	90	100	100	100	100	90
Length at day 21 (mm)	3.75 ± 0.17	3.73 ± 0.18	3.64 ± 0.12	3.36 ± 0.11*	3.44 ± 0.19*	3.37 ± 0.12*
Total reproduction (# juveniles/daphnid)	99.8 ± 14.1	103.5 ± 27.3	100.6 ± 8.8	66.5 ± 9.5*	69.5 ± 14.3*	64.1 ± 10.3*
Time to first brood (days)	8.9 ± 0.3	8.1 ± 0.6*	7.8 ± 0.6*	8.2 ± 0.4*	8.1 ± 0.3*	8.2 ± 0.4*
First brood size (# juveniles)	13.8 ± 2.7	10.9 ± 2.6*	9.2 ± 2.7*	9.6 ± 2.6*	9.5 ± 4.4*	8.7 ± 1.6*
Second brood size (# juveniles)	18.7 ± 4.2	16.1 ± 5.2	17.4 ± 3.6	12.5 ± 4.9*	13.9 ± 3.4*	12.1 ± 3.2*
Third brood size (# juveniles)	25.8 ± 5.2	25.4 ± 3.5	22.2 ± 5.1	16.4 ± 3.9*	15.9 ± 5.6*	14.8 ± 1.9*
Fourth brood size (# juveniles)	21.6 ± 6.4	26.8 ± 6.1	25.9 ± 4.2	13.2 ± 3.6*	17.4 ± 4.9	14.9 ± 3.2*
Fifth brood size (# juveniles)	19.8 ± 7.7	24.2 ± 10.3	25.7 ± 4.2	14.8 ± 6.2*	16.0 ± 1.8*	12.3 ± 3.5*
Intrinsic rate of natural increase (r_m)	0.36 ± 0.01	0.37 ± 0.02	0.37 ± 0.02	0.34 ± 0.02	0.35 ± 0.03	0.34 ± 0.01

* Significant differences between the control exposure and elevated dietary Ni exposures (Mann–Whitney-U test; $p < 0.05$).
[a] Data on toxicological endpoints are presented as mean ± standard deviation ($n = 9$ for control and highest Ni exposure, $n = 10$ for other treatments).
[b] Below detection limit of 5 μg/L.
[c] Detection limit for *D. magna* Ni burdens = 4.0 μg Ni/g dry wt.

from the target of pH 8. While waterborne dissolved Ni in the control remained below the detection limit, higher levels of dietary exposure resulted in the introduction of increasing amounts of dissolved Ni in the test media, up to a maximum of 178 μg/L. *D. magna* Ni body burdens remained below the detection limit in the control and in the two lowest dietary exposure levels. Daphnids exposed to dietary Ni concentrations of 85.6 μg/g dry wt in the diet and higher exhibited increased body burdens between 49.6 and 72.5 μg/g dry wt.

Mortality was ≤10% in all treatments. The 21-day length and total reproduction were significantly affected ($p < 0.05$) in the daphnids fed with algal diets containing 85.6 μg Ni/g dry wt (= lowest observed effect concentration, LOEC) or more. The reduction of length and total reproduction is very similar in daphnids fed with diets containing 85.6 μg Ni/g dry wt or more. The six treatments can thus be divided into two groups of similar reproduction and growth, i.e. the group of daphnids fed with the control algae and those exposed to the two lowest dietary Ni levels on the one hand, and the group of daphnids exposed to the three highest dietary levels on the other. Reproduction and length in the latter group were on average 33.1% and 9.6% lower than in the control treatment, respectively.

When reproduction is examined at the level of individual broods, we observed a significant reduction of the first brood size (compared to the control) for daphnids exposed to all dietary Ni concentrations. From the second brood onwards, however, only daphnids exposed to concentrations ≥85.6 μg Ni/g dry wt exhibited reduced brood sizes. The time to first brood, indicative of the rate of maturation, was significantly reduced at all dietary Ni exposures compared to the control.

4. Discussion

4.1. Bioaccumulation and effects of dietary Ni in Daphnia magna

Measured internal Ni in the algae accounted for only 11–19% of the measured total Ni (internal + external Ni) associated with the algae. However, given the contact time of 20 min between the algae and the EDTA used to distinguish between internally and externally bound Ni (see Section 2.3), and assuming a first-order efflux rate constant of Ni from algae in the presence of EDTA equal to 2.4%/min (Worms et al., 2007), the true internal Ni burden may have been as much as 40% higher than the one reported in Table 1. As it is the true internal metal burden that is considered to be available for ingestion (De Schamphelaere et al., 2007), our EDTA washing method has probably led to an underestimation of the amount of dietary Ni that was available for ingestion by the daphnids.

Both efflux and desorption of Ni from the algal food contributed to increased concentrations of waterborne Ni in the *D. magna* exposure media during the 21-day exposure (Table 2). Thus, it cannot

be excluded *a priori* that the observed Ni accumulation and toxic effects in *D. magna* have been caused by this waterborne exposure route, instead of by feeding on the Ni contaminated diet. Two lines of evidence, however, do suggest that the contribution of dietborne Ni to both accumulation and toxicity has been more important than the contribution of waterborne Ni. First, while waterborne Ni concentrations were low and very similar in the dietary treatments with algae containing 68.5 and 85.6 μg Ni/g dry wt, significant Ni bioaccumulation (54 μg/g dry wt) and reduced growth (by 7.8%) and reproduction (by 34%) were only observed in the latter treatment. If waterborne Ni had been the cause of the observed bioaccumulation and toxicity, one would have expected similar bioaccumulation and effects in both treatments. Second, the biotic ligand model (BLM) for chronic waterborne Ni toxicity (Deleebeeck et al., 2008) predicted that a concentration of 160 μg Ni/L was needed for reducing total reproduction by 10%. However, only in the highest dietary Ni treatment (and only in the second week of exposure) has the waterborne Ni concentration exceeded 160 μg/L, albeit only slightly (i.e. 177 μg/L, Table 2). Since reproductive inhibition in the three highest dietary exposures (algae with internal Ni between 85.6 and 837 μg/g) was considerably higher than 10%, i.e. 30% to 36% (Table 2), dietborne Ni itself must have been the main contributor to the observed reproductive inhibition.

In support of this, it is noted that dietary Ni accumulation and toxicity showed a very similar pattern, resulting in a statistically significant correlation of accumulated Ni with growth ($r^2 = 0.92$, $p = 0.002$) and total reproduction ($r^2 = 0.97$, $p = 0.0003$). The absence of significant accumulation and effects at the two lowest exposure levels indicates that the internal Ni burden was tightly regulated without a significant expense at the organism level. Fed with the algae containing 85.6 μg Ni/g and higher (i.e. the three highest exposure levels), the *D. magna* regulation mechanism may have been overwhelmed and this resulted in an important bioaccumulation (49.6–72.5 μg Ni/g dry wt) accompanied with toxicity. Pane et al. (2004) observed that a higher body burden, i.e. ≥87.6 μg Ni/g dry wt, was needed to cause reproductive inhibition in *D. magna* following waterborne exposure, suggesting a difference in the toxicity of Ni taken up via different exposure routes.

Comparing the organism-level responses observed in our study with other studies of dietary metal effects, we found that the response of *D. magna* to dietary Ni exposure is fundamentally different from the ones observed following exposure of *D. magna* to dietary Zn (De Schamphelaere et al., 2004), freshwater cladocerans to Ag (Hook and Fisher, 2001a), and marine copepods to Hg, Cd, Zn, Ag, and Mn (Hook and Fisher, 2001b). In all these studies, a targeted effect on reproductive endpoints only (and not on growth or survival) was observed. However, the observed response to dietary Ni, i.e. concurrent inhibitory effects on both growth *and* reproduction, is in line with observations following exposure of *D. magna* to elevated dietary Cu (De Schamphelaere et al., 2007). The occurrence of concurrent effects on growth and reproduction implicates that an alteration of energy allocation is likely involved, be it reduced nutrient assimilation and/or increased metabolic costs (Kooijman, 2000; Nogueira et al., 2004). But, similarly as argued in our Cu study (De Schamphelaere et al., 2007), a contribution of a direct reproductive effect (cf. Zn, De Schamphelaere et al., 2004) cannot be excluded on the basis of our dataset.

Interestingly, while the effect of dietary Ni on brood sizes of the 2nd and further broods was fully in parallel with the observations on total reproduction and growth (Table 2) (i.e. significant inhibition at the three highest dietary levels but not at the two lowest), the observations made for time to first brood and the size of the first brood are clearly different. Dietary Ni brought about a *reduced* first brood size but, also, an *enhanced* maturation rate (reduced time to first brood). However, since both of these effects occurred to the same degree in *all five* dietary Ni treatments, despite the highly varying Ni contents in the diet exposure and levels of accumulated Ni inside the daphnids, it is difficult to relate this observation to the Ni exposure itself. An alternative explanation is that this observation is related to a nutritional quality factor of the algae (e.g., C:P ratio, see Section 4.2).

Since time to first brood and size of first brood are among the most important life-history traits determining population growth rate and since the effects of dietary Ni on both traits are in directions that have opposite effects on population growth rate, the net result is that the population growth rate was not significantly affected in any of the dietary Ni treatments, with r_m values between 0.34 and 0.37/day (Table 2). This suggests that adverse effects in true natural populations may be limited, although it should be stressed that the above calculation does not account for possible multigenerational effects. Pane et al. (2004) have shown a larger chronic impact of waterborne Ni on *D. magna* during the second generation of exposure compared with the first generation. Further research is required to test if this could also be the case for dietary Ni exposure.

4.2. Could differences in food quality have influenced Daphnia magna response?

We observed that the C:P ratio of the algae significantly decreased as a function of the Ni content (Table 1). Park et al. (2002) established a dietary P-limiting level at a molar C:P ratio of 300. C:P ratios above this threshold would impair *D. magna* performance. Since C:P ratios in the control and in all treatments are considerably below this threshold, i.e. between 140 and 230, the P-content of the algal food was most likely not limiting in any of the treatments. Furthermore, the P-content of all Ni contaminated diets was higher (C:P ratio lower) than in the control. Thus, altered C:P ratios do not explain the observed effects on growth and total reproduction. It is interesting, however, that the decreased C:P ratio (observed in *all* dietary treatments compared to the control) shows the same pattern as the above-mentioned effects on the first brood (i.e. increased maturation rate and reduced first brood size, which were also observed in *all* dietary treatments, see Section 4.1, Table 2). Further research is, however, needed to test if increased P-content can alter maturation rate (time to first brood) and/or first brood size in *D. magna*.

Regarding the FA content, eicosapentaenoic acid (EPA, 20:5 ($n-3$)) is considered as one of the most important determinants of food quality for daphnids (Stanley-Samuelson, 1994; Weers et al., 1997; von Elert, 2004; Becker and Boersma, 2005; Brett et al., 2006). In the absence of significant amounts of dietary EPA, as in the present study, the content of the ω3 PUFA precursors of these molecules, i.e. α-linolenic acid (ALA, 18:3 ($n-3$)) and stearidonic acid (SDA, 18:4 ($n-3$)) are indicative of food quality as they can be biochemically converted into EPA by daphnids after uptake via the diet (Weers et al., 1997). Exposure to Ni significantly affected the total ω3 PUFA content of the algal food in our study, as well as the content of the individual ω3 PUFA molecules. McLarnon-Riches et al. (1998) showed that Cu, Zn and Cd exposure affects FA composition in *P. subcapitata*. De Schamphelaere et al. (2007) reported a decreased ω3 PUFA content following Cu exposure in the same algae. To our knowledge, this is the first report showing that elevated Ni exposure causes a distinct reduction of the total ω3 PUFA content of a green alga species.

The pattern of algal ω3 PUFA reductions closely follows the pattern of effects on growth and total reproduction, in that they were all observed in the three highest dietary treatments. Despite a significant correlation between the ω3 PUFA content and growth ($R^2 = 0.75$ and $p = 0.03$) as well as reproduction ($R^2 = 0.80$ and $p = 0.02$), it is unlikely that the reduction of algal ω3 PUFA could have contributed to the observed reduction of growth and repro-

duction of *D. magna*. This is because the critical threshold for total ω3 PUFA limitation in *D. magna* has been reported as 30 μmol/g C in the diet (Park et al., 2002), whereas the ω3 PUFA content of all diets used in the present study (including those containing the highest Ni) was >95 μmol/g C. It thus seems more likely that the toxic effects observed have been caused by dietary Ni itself. Further research is, however, recommended to more definitively discriminate between effects of dietary Ni exposure and diet quality shifts, e.g., by using Ni enriched diets that do not differ in PUFA content from the control.

4.3. Implications for the BLM and for ecological risk assessment

Our finding that dietary Ni can cause toxic effects on *D. magna* is important from a mechanistic point of view, but the ecological relevance of this finding has to be explored before implications for ecological risk assessment are formulated. The LOEC for dietary exposure was 85.6 μg Ni/g dry wt in the algal food and exposure to 900 μg/L of Ni (dissolved) or 133 μg/L of bioavailable Ni concentration (i.e. Ni^{2+}) was needed to achieve this level. This is in the same order of magnitude as the reproductive EC50 (Ni^{2+}) for *D. magna* (115 μg/L) that we predicted (with the chronic BLM, Deleebeeck et al., 2008) for the test water used. This suggests that the chronic toxicity observed previously in standard 'waterborne' exposures may have been partly due to an indirect exposure via the diet because algae accumulated Ni from the exposure medium before they were ingested by the daphnids. This has obvious implications for the realism of the chronic BLM (Deleebeeck et al., 2008), which predicts chronic 'waterborne' nickel toxicity as a function of the physico-chemical characteristics of the water, thereby assuming that only the waterborne exposure route is of importance. Our present data show that this assumption may not be valid and that the derived model parameters (stability constants), which are assumed to describe competitive toxicity reduction by increased waterborne Ca^{2+}, Mg^{2+} and H^+ (decreased pH), in fact may partly contain a dietary toxicity component. The fact that decreasing H^+ (increasing pH) does affect chronic but not acute toxicity (Deleebeeck et al., 2008), lends some support to this hypothesis. Indeed, in acute toxicity experiments no food is added, so dietary exposure is not possible. In chronic exposures, algal food is added and it is well known that metal adsorption and internalization in algal cells increases with increasing pH (Crist et al., 1988; Worms and Wilkinson, 2007). Thus, increasing the pH of the chronic *Daphnia* test medium increases the potential exposure of *Daphnia* to dietary Ni and this may explain why toxicity occurs at lower waterborne Ni concentrations at higher pH levels, as observed by Deleebeeck et al. (2008). A clear distinction of the effect of both exposure routes as well as of interactions between those is necessary for the further refinement of the current chronic Ni BLM for *Daphnia*.

Another question is whether dietary toxicity could actually occur in the field. Clearly, the answer is yes, as the algal growth rate at the dietary LOEC was only reduced by 15%. It could thus be argued that algal food may reach Ni burdens that are toxic to *D. magna* at waterborne Ni concentrations under which they will still be present in contaminated natural systems. Furthermore, it must be kept in mind that our study only represents a system of a single crustacean species (*D. magna*) grazing on a single species of algal food (*P. subcapitata*). To extrapolate the results of this study reliably to the level of ecosystems, one should recognize the possibility of inter-species differences of algal bioconcentration factors of Ni and of dietary Ni sensitivity in crustaceans. Indeed, species that are more susceptible to dietary Ni than *D. magna* and which are feeding on algae with higher bioconcentration factors may experience dietary toxicity at lower free Ni^{2+} concentrations than those applied in the present study.

5. Conclusions

D. magna exposed during 21 days to dietary Ni concentrations between 85.6 and 837.3 μg/g dry wt of algal food accumulated a total Ni body burden ranging from 49.6 to 72.5 μg/g dry wt, and experienced significant inhibition of reproduction and growth by 33.1 and 9.6% compared to the control treatment, respectively. These concurrent effects on growth and reproduction suggest that an alteration of resource allocation is likely involved, although a contribution of direct effects on reproductive processes cannot be ruled out. While Ni exposure clearly altered the quality of the diet (measured as essential ω3 PUFA content and C:P ratio), we found no conclusive evidence that these diet quality shifts could have affected growth or total reproductive output. Overall, our data demonstrate that the dietary exposure route may be important to consider in risk assessment exercises, especially because toxic algal Ni-burdens already occurred at waterborne concentrations similar to those that cause waterborne toxicity in *D. magna*. This also indicates that a clearer understanding of the effects of both exposure routes as well as of interactions between them is necessary for further refinement of the chronic Ni BLM for *D. magna*.

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