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1 **Chronic toxicity of dietary copper to *Daphnia magna***

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Abstract

There is a growing concern that dietborne metal toxicity might be important in aquatic ecosystems. However, the science behind this matter is insufficiently developed to explicitly and accurately account for this in metals regulation or risk assessment. We investigated the effects of a chronic exposure of *Daphnia magna* to an elevated level of Cu (3,000 µg Cu/g dry wt) in their diet (the green alga *Pseudokirchneriella subcapitata*). Compared to daphnids fed with *P. subcapitata* containing a background of 10.6 µg Cu/g dry wt, daphnids fed for 21 days with this Cu-contaminated food accumulated a total copper body burden of 325 µg Cu/g dry wt, which is about 30-fold higher than the control body burden of 12.1 µg/g dry wt. The exposed daphnids experienced a 38% reduction of growth (measured as final dry body weight), a 50% reduction of reproduction (total number of juveniles produced per daphnid), and only produced three broods (vs. four broods by the control daphnids). Unlike most other studies, we were able to demonstrate that these effects were most likely not due to a reduced nutritional quality of the food, based on C:P ratios and fatty acid content and composition of the Cu-contaminated algae. Life-history analysis showed that time to first brood was not affected by dietary Cu, while the second and third broods were significantly delayed by 0.7 and 1.5 days, respectively. On the other hand, brood sizes of all three broods were significantly lower in Cu exposed daphnids, i.e. by 32% to 55%. The variety of effects observed suggest the possible, and perhaps simultaneous, involvement of several toxicity mechanisms such as increased metabolic cost, reduced energy acquisition (potentially via inhibition of digestive enzyme activity), targeted inhibition of reproduction (potentially via inhibition of vitellogenesis), and/or direct inhibition of molting. Further research is needed to differentiate between these postulated mechanisms of dietary Cu toxicity and to determine whether they act separately or in concert.

Keywords *Daphnia*; copper; dietborne metal exposure; metal toxicity

Introduction

There is growing evidence that dietborne metal toxicity might be important in aquatic ecosystems (Wikfors and Ukeles, 1982; Moreno-Garrido et al., 1999; Hook and Fisher, 2001a, 2001b, 2002; Clearwater et al., 2002; De Schamphelaere et al., 2004; Borgmann et al., 2005; Meyer et al., 2005). The current understanding of dietborne metal toxicity is, however, insufficiently developed and, as a consequence, the dietary exposure route is generally not considered explicitly in most existing regulations or risk assessments (Schlekat et al., 2001; Borgmann et al., 2005).

Recently, several studies have been published concerning the effects of dietary metal exposure on crustacean species. Hook and Fisher (2001a, 2002) reported the inhibition of reproduction in marine copepods fed with diatoms contaminated with Mn, Zn, Ag, Cd, or Hg and in cladocerans fed with green algae contaminated with Ag (Hook and Fisher, 2001b). The absence of more general stress responses (i.e., respiration and behavior) as well as reduced egg protein content, led these authors to hypothesize that dietary metals invoke an effect specifically targeting reproduction, potentially linked to the inhibition of processes related to vitellogenesis. Their hypothesis was supported by the fact that Cd had been shown to reduce vitellogenin production in blue crabs, *Callinectes sapidus* (Lee and Noone, 1995). More recently, De Schamphelaere et al. (2004) demonstrated a similar specific reproductive effect of dietary Zn (accumulated in green algae) on the cladoceran *Daphnia magna*, with no general stress response observed (no effects on survival, growth or feeding rate).

Earlier-reported effects of dietborne Cu exposure on crustaceans are less consistent. De Schamphelaere and Janssen (2004a) demonstrated a stimulation of *D. magna* growth and reproduction by dietary Cu (incorporated at internal concentrations between 680 and 1400 µg

Cu/g dry wt in the alga *Pseudokirchneriella subcapitata*) and hypothesized that this effect might be related to an enhanced digestive enzyme activity (supported by studies of Chen et al., 2002; and Kirchgessner et al., 1976). On the other hand, Chang and Sibley (1993) reported an inhibition of reproduction in *D. magna* fed with the Cu-contaminated green alga *Oocystis pusilla*. Unfortunately, the latter authors did not consider other organism traits such as growth or feeding rates. Therefore, it is unclear whether the reproductive inhibition was a targeted effect (like for dietary Zn exposure in *D. magna*, De Schamphelaere et al., 2004) or whether it was the result of a more general stress response (e.g., reduced feeding, increased metabolic costs). Although diet quality shifts due to pre-exposure of live algal food to Zn were not considered a likely explanation for inhibited reproduction in daphnids (De Schamphelaere et al., 2004), one cannot exclude *a priori* that diet quality shifts were important in the Chang and Sibley (1993) study with Cu.

Based on all this, the major aim of the present study was to investigate whether an elevated level of dietary Cu would specifically inhibit reproduction in daphnids (as for other metals), or whether a more general stress response would be invoked (e.g., effects on survival, growth and/or feeding rate). This was investigated by feeding *D. magna* in a 21-day chronic bioassay with the alga *P. subcapitata* which had been contaminated with a high internal Cu concentration. In parallel with the bioassay we also quantified diet quality in control and Cu-contaminated algal food as defined by the C:P ratio and the fatty acid composition. A level of Cu contamination of the food was selected that was higher than the one that previously resulted in stimulation of growth and reproduction in *D. magna* (De Schamphelaere and Janssen, 2004a) and that had been shown in a preliminary experiment to inhibit growth and reproduction in *D. magna* (unpublished data). The main focus of this study was not so much on assessing the effects of a realistically contaminated Cu-diet but rather on getting basic

information about possible physiological mechanisms of toxicity of dietborne Cu in a model crustacean.

Materials and methods

Experimental design

The green alga *P. subcapitata* was cultured in a control medium and medium containing an elevated Cu concentration for three days. The algae were harvested and their nutritional value (in terms of their C:P ratio and essential fatty acid content) and internal copper burden were determined. The algae were fed to *D. magna* in chronic bioassays where survival, feeding rate, growth and reproduction was recorded for 21 days.

Test media

Exposures of *P. subcapitata* and *D. magna* were performed in a reconstituted test medium containing natural dissolved organic carbon collected by reverse osmosis from the Ankeveensche Plassen, The Netherlands (a freshwater ditch belonging to a system of connect lakes; see De Schamphelaere et al., 2003 for a detailed description). The test media contained 10 mg dissolved organic carbon L^{-1}, 2.0 mM Ca, 0.50 mM Mg, 5.2 mM Na, 0.078 mM K, 0.50 mM SO_4, and 4.1 mM Cl. 3-N morpholino propane sulfonic acid (MOPS, Sigma-Aldrich, Steinheim, Germany) was added at a concentration of 3.6 mM as a pH buffer to maintain pH around 8. The alga test medium, but not the *Daphnia* test medium, contained additional macronutrients (NH_4, PO_4) and micronutrients (Fe, B, Mn, Zn, Co, and Mo) at the concentrations described in OECD test guideline 201 (OECD, 1984). The strong metal-chelator ethylene-diamine-tetra-acetate (EDTA) was omitted from the solution.

Algae exposure

Pseudokirchneriella subcapitata (CCAP 278/4) originated from the Culture Collection of Algae and Protozoa (currently hosted at the Scottish Association for Marine Science, Argyll, Scotland) and is continuously maintained in our laboratory according to procedures described elsewhere (De Schamphelaere et al., 2005). Log-phase algae were used to initiate the exposures. Algae were cultured for three days in a control test solution (no added Cu) and a solution with 500 µg/L added Cu (added as $CuCl_2$). Ten liters of test medium were prepared, spiked with copper and transferred into a polyethylene bag. The spiked media were equilibrated for 48 hours at 20°C and then each bag was inoculated with $5 \cdot 10^5$ cells ml^{-1}. Exposures were carried out under continuous illumination (240 µmol photons m^{-2} s^{-1}) and continuous aeration at 20 to 21°C. Initial and final biomass (dry weight) were determined in triplicate according to De Schamphelaere et al. (2004) and the biomass growth rate was determined according to OECD guideline 201 (1984). After 72 hours of exposure, the algae were harvested by centrifugation using a continuous-flow centrifuge with a volume of 300 mL at a flow rate of 2.5 mL s^{-1} and a g-force of 1,000 g (IEC Chemical centrifuge, International Equipment Company, USA). The supernatant was pipetted off and the resulting pellets were stored in darkness at 4°C in 300 mL of supernatant. Hence, the algae were stored in the same solution chemistry as the one they had been exposed in. This minimizes changes in internal copper burdens of the algae during the storage of the concentrated cell suspensions (De Schamphelaere and Janssen, 2004a).

Algal characteristics

Dry weight, internal copper content, molar C:P ratio, and fatty acid contents of the algae from the stored suspensions were determined at the start and at the end of the chronic *D. magna* bioassay. Dry weight determination and sample preparation for internal and external copper

burdens were carried out following procedures described in De Schamphelaere and Janssen (2004a). External copper is operationally defined as the copper that is desorbed from the algal surface by a 20 minute wash in a solution of a 5 mM ethylene-diamine-tetra-acetic acid solution. Internal copper is the copper remaining inside the cells after this treatment (De Schamphelaere and Janssen, 2004a). Copper analyses were performed with graphite furnace atomic absorption spectrophotometry (SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia). A reference plankton sample (BCR-414, Institute for Reference Materials and Measurements, Geel, Belgium) was analyzed using the same method. Measured copper levels were within 10% of the certified value. Carbon content was determined as explained in De Schamphelaere and Janssen (2004a). Total phosphorus content was determined by nitric acid – sulfuric acid digestion and colorimetric phosphate determination, according to APHA method 4500-P (Clesceri et al., 1998). Molar C:P ratios were calculated from the carbon and phosphorus contents.

Fatty acid composition was determined by gas chromatography. Fatty acid methyl esters (FAME) were prepared via a modified procedure of Lepage and Roy (1984). This method implies a direct acid catalysed transesterification without prior extraction of total fat on 10 to 150 mg dry weight of sample. An internal standard of eicosadienoic acid, 20:2(n-6), was added prior to the reaction. FAME were extracted with hexane and, after evaporation of the solvent, prepared for injection in the chromatograph by dissolution in iso-octane (2 mg/ml). Quantitative determination was done by a Chrompack CP9001 gas-chromatograph equipped with an autosampler and a temperature programmable on-column injector. Injections (0.2 µl) were performed on-column into a polar 50 m capillary column, BPX70 (SGE, Australia), with a diameter of 0.32 mm and a layer thickness of 0.25 µm connected to a 2.5 m methyl deactivated pre-column. The carrier gas was H_2, at a pressure of 100 kPa and the detection mode was flame ionization detection. The oven was programmed to rise from the initial

temperature of 85°C to 150°C at a rate of 30°C/min, from 150°C to 152°C at 0.1°C/min, from 152°C to 172°C at 0.65°C/min, from 172°C to 187°C at 25°C/min and remained at 187°C for 7 min. The injector was heated from 85°C to 190°C at 5°C/sec and remained at 190°C for 30 min. Identification was based on standard reference mixtures (Nu-Chek-Prep, Inc., U.S.A.) and calculations were performed by the software program Maestro (Chrompack).

Chronic bioassays with daphnids

Chronic bioassays were performed according to OECD guideline No. 211 for testing of chemicals (OECD, 1998). Test organisms originated from a healthy *D. magna* clone which was cultured in the laboratory under standardized conditions in M4 medium (Elendt and Bias, 1991). At the start of each test, 10 juveniles (<24 hours old) were transferred individually to polyethylene cups containing 40 ml of test medium (i.e., 10 replicates of one organism per concentration). Every day the daphnids were fed with 60, 90 and 120 µg dry wt algae during the first, second, and third week of the exposure, respectively. This corresponds to 24, 36, and 48 µg C per day, respectively. These daily food rations are 4 to 8-fold lower than those recommended by the OECD test guideline and 5 times lower than those used in our earlier studies (De Schamphelaere and Janssen, 2004a, 2004b). Although it was anticipated that this would result in a total reproductive output lower than the 60 juveniles/daphnid as required by the test guideline, the lower food rations were needed to maintain the dissolved Cu levels in the *Daphnia* exposure vessels at a level low enough to avoid as much as possible toxic effects via the waterborne route (see also results section). This is because increasing the food rations increases the total amount of copper added to the test vessels and hence also increases the potential of the establishment of toxic copper concentrations in the water. The chosen food concentration was a trade-off between ensuring quantifiable reproduction and minimizing the built-up waterborne copper concentrations. Daphnids were transferred to fresh solutions on

day 2, 5, 7, 9, 12, 14, 16 and 19 of the exposure. Samples were taken from old test solutions to determine possible leaching of copper from the contaminated algal food. The number of algal cells remaining in the old test solutions was determined to calculate *per capita* algal ingestion rates of the daphnids, i.e. the amount of algae ingested per day and per individual daphnid (De Schamphelaere and Janssen, 2004a). Every day, parent mortality and the number of juveniles were noted in each replicate. At the end of the 21 day exposure period, mortality and the number juveniles was noted for the last time and parent daphnids were collected for dry weight and copper body burden determination as explained in De Schamphelaere and Janssen (2004a). Daphnids were fed for 8 hours with the control algae prior to copper body burden determination. This approach is recommended to ensure that ingested Cu is efficiently removed from the gut lumen (Gillis et al., 2005).

Chemical analyses

Copper concentrations in algal and daphnid exposure solutions were determined using a graphite furnace atomic absorption spectrophotometer (for Cu < 20 µg L^{-1}, SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia). Dissolved copper (filtration through 0.45 µm, Gelman Sciences, Ann Arbor, Michigan, U.S.) was measured daily in the algal exposure solutions and just prior to each test solution renewal in the *D. magna* tests.

Data treatment and statistics

All data are reported as mean ± standard deviation, unless noted otherwise. Non-parametric statistics, i.e. Mann-Whitney U, Spearman Rank Correlation and Wilcoxon Matched Pairs, were used in all instances and were carried out using Statistica 6.0 software (Statsoft, Tulsa, OK, U.S.A.)

Results

Results of the algae exposures and the analyses of algal composition are summarized in Table 1. *P. subcapitata* were exposed for 72 hours to measured concentrations of 1.9 µg Cu/L (control) and 494 µg Cu/L (Table 1). Compared to the control, the growth rate of the algae was reduced by 59% when exposed to 494 µg Cu/L (Table 1). Internal Cu concentrations in the algae during the *D. magna* bioassays were between 8.9 and 12.3 µg Cu/g dry wt (control) and between 2,720 and 3,290 µg Cu/g dry wt (494 µg Cu/L treatment). The molar C:P ratio was 12% lower in the Cu-treated (241) than in the control cells (274). Total fatty acids comprised 6.6% of the dry wt in control cells and 9.4% in Cu-treated cells. The most abundant fatty acids present in the control algae were palmitic (23% of all fatty acids on a weight basis), oleic (29%), linoleic (7.4%), linolenic (16%) and parinaric (or stearidonic) acid (4.4%), while the essential fatty acids eicosapentaenoic acid (EPA, 0.9%) and docosahexaenoic (DHA, <0.03%) were only present in traces or were undetectable, respectively. Copper-treated cells contained a similar concentration of EPA (4.5 nmol/g C) as the control cells (4.7 nmol/g C), but higher concentrations of palmitic (25% more than control) and oleic acid (170% more than control), and lower concentrations of linoleic (52% less) and linolenic acid (37% less). Total ω3-PUFA concentrations were 125.7 nmol/g C in control cells and 89.5 nmol/g C in Cu-treated cells, which is 29% less than in controls.

Results of the *Daphnia magna* tests are summarized in Table 2. The mean dissolved Cu concentration measured in old test solutions of the treatment fed with Cu contaminated algae was 36.5 µg/L (Table 2).

All daphnids in the control treatment and in the Cu-contaminated series survived the 21-day exposure. While 21-day old daphnids fed the control food had a body burden of 12.1 µg Cu/g

dry wt, daphnids fed Cu-contaminated algae had a significantly (MWU, $p<0.001$) higher Cu-burden at 325 µg Cu/g dry wt (Table 2). Daphnids fed Cu-contaminated algae exhibited a significant (MWU, $p<0.001$) 50% reduction of total reproduction and a significant (MWU, $p<0.01$) 38% reduction of growth over the 21-day period (Table 2). Although the time to first brood was not affected (MWU, $p>0.05$), the release of the second brood was significantly delayed by 0.7 days (MWU, $p<0.05$), and the release of the third brood by 1.5 days (MWU, $p<0.001$). Only 30% of the control daphnids were able to produce a 4^{th} brood while none of the daphnids exposed to Cu-contaminated food were able to do so within the 21-day exposure period. On the other hand, brood sizes of all three broods were significantly reduced by dietary Cu (MWU, $p<0.01$). The first, second, and third brood sizes in the dietary Cu treatment were decreased by 32%, 35% and 55%, respectively.

Per capita algal ingestion rates of Cu-exposed daphnids were significantly lower than in the controls (Wilcoxon Matched Pairs test, $p<0.02$, $N=7$) (Figure 1). The inhibition increases with time (Spearman Rank Correlation, $r=-0.86$, $p=0.01$, $N=7$), e.g., between 85% and 95% inhibition of control ingestion rates between day 5 and 12 of the exposure and between 62% and 70% between day 12 and 21. The inhibition is mainly due to the fact that ingestion rates of the control daphnids increased significantly with time (Spearman Rank Correlation, $r=0.86$, $p=0.01$, $N=7$), while they did not increase in the Cu-treated daphnids (Spearman Rank Correlation, $r=0.11$, $p=0.82$, $N=7$) (Figure 1).

Discussion

Before interpreting the outcome of the *D. magna* bioassays in terms of dietary metal toxicity, it is important to discuss potential confounding factors. The first is the unintentional

occurrence of an average Cu concentration of 36.5 µg Cu/L in solution during the dietary exposure. This has in the first place relevance for the estimation of the true dietary copper dose to which the daphnids have been subjected. Indeed, one could easily argue that too much internal Cu leached from the algae to be sure that the daphnids have actually been exposed to dietary Cu. A mass balance calculation was therefore performed to determine the relative importance of the different sources of the waterborne Cu in the test vessels and also to estimate this true dietary dose. This calculation was based on known additions of Cu to the test vessels and measurements of dissolved Cu at every test medium renewal (every two or three days). An exemplary calculation is presented below for exposure period between 7 and 9 days. At day 7 a dissolved Cu concentration of 30.6 µg/L was measured, representing a mass of 1,224 ng of Cu in the test vessel (30.6 µg/L x 0.04 L of test volume). Four important sources of this amount of copper need to be considered: (1) background Cu (2.3 µg/L x 0.04L = 92 ng Cu), (2) dissolved Cu that was directly transferred with every feeding event from the dissolved phase of the concentrated algal food suspension to the test solution (437 µg/L in algal suspension x 2 feedings of 200 µL of algal suspension = 172 ng Cu), (3) Cu that is rapidly desorbed from external binding sites on the algal surfaces when transferred into the daphnids' test vessels (2 feedings of 90 µg dry wt x 3554 µg Cu/g dry wt external Cu = 640 ng Cu), and (4) Cu that is leached from the internal compartments of the algae. The latter source was quantified by subtracting the three other sources from the total amount present, i.e. 320 ng Cu (1224-92-640-172). Now, between day 7 and day 9, a total amount of 540 ng of internal Cu was added (2 feedings of 90 µg dry wt x 3000 µg Cu/g dry wt). Hence, during this exposure period 31% of the Cu was retained, while 59% was leached. The same mass balance calculation was repeated for each exposure period in-between two test media renewals. This resulted in an average retention of internal Cu in the algae of 38% and an average leaching into the water of 62%. This means that internal Cu burdens of the contaminated algae fed to

the daphnids were between 3,000 µg/g dry wt (immediately after each feeding event) and 1160 µg/g dry wt (38% of 3,000). Multiplication of this internal Cu range with daily food ingestion rates (Figure 1), resulted in ingestion rates of Cu between 39 and 143 ng Cu per daphnid per day over the entire exposure period. This is 50 to 360-fold higher than the control daphnids, which ingested only between 0.4 and 0.8 ng Cu per daphnid per day. As a result, daphnids exposed to dietary Cu had body burdens of 325 µg Cu/g dry wt, which is 30-fold higher than the control body burden of 12.1 µg Cu/g dry wt. It is unlikely that this increased accumulation is the result of the waterborne concentration of 36.5 µg/L. Indeed, in a previous study, daphnids exposed for 21 days to a waterborne concentration of 35 µg Cu/L resulted in a body burden of only 31 µg Cu/g (De Schamphelaere and Janssen, 2004a). Moreover, the test medium used in the latter study was characterized by a higher copper bioavailability since DOC and hardness were similar while the pH of 6.8 was lower than the pH of 8 used in the present study (lower pH is higher bioavailability, De Schamphelaere and Janssen, 2004b). Hence, at 36.5 µg Cu/L and pH 8 - the conditions of the present dietary toxicity study - the body burden in the daphnids originating from waterborne Cu would be expected to be even lower than 31 µg Cu/g, which is 10-fold lower than the measured body burden. Thus daphnids exposed to dietary Cu in the present study accumulated at least 10-fold more Cu (325 µg Cu/g) than the amount that would be expected from waterborne contribution alone (<31 µg Cu/g). Hence, the above calculations and comparison clearly indicate that dietary Cu in the present study was ingested by the daphnids as well as assimilated into the tissues.

Although the presence of 36.5 µg dissolved Cu/L is clearly no indication of the absence of dietary Cu exposure, it is still important to discuss its potential contribution to the toxic effect observed. The experiment was specifically designed to minimize the bioavailability of copper by performing the test in a test solution with 10 mg DOC/L and a pH of 8. In waterborne-only

exposure using the same test water, we have previously determined a 21-day no observed effect concentration (NOEC) for *D. magna* reproduction of 120 µg Cu/L and a 50% inhibitive concentration for reproduction (EC50) of 167 µg Cu/L (De Schamphelaere and Janssen, 2004b). In our present exposure to dietary Cu, we observed 50% inhibition of reproduction at an average dissolved Cu concentration of 36.5 µg Cu/L (Table 2), which is about 5 times lower than the waterborne EC50 and 3 times lower than the NOEC. Additionally, *D. magna* exposed to 35 µg waterborne Cu/L - under conditions of slightly higher bioavailability (pH 6.8 vs. pH 8.0, see also above) - and fed with *P. subcapitata* contaminated with much less Cu, i.e. 330 µg Cu/g dry wt, exhibited a slightly stimulated reproduction, rather than an inhibition (De Schamphelaere and Janssen, 2004a). Obviously, it could be argued that a fair comparison between these earlier studies and the present study is not possible, since the 5-fold lower food concentration used in the exposures of the present study might have increased the sensitivity of the daphnids to waterborne copper. However, a recent study with *D. magna* at our laboratory (Gevaert, 2004) - in a similar test water except lower DOC (pH 7.8, $CaCl_2$ 1.44 mM, $MgSO_4$ 0.36 mM, $NaHCO_3$ 0.75 mM, KCl 0.075 mM, DOC 4 mg/L = 2.5-fold lower than in the present study) - yielded the same 21d-NOEC for reproduction of 55 µg dissolved Cu/L at both normal (same as in De Schamphelaere and Janssen, 2004a, 2004b) and 5-fold reduced food concentration (same as in present study) and not-significantly-different 21d-EC50's of 84 µg/L and 76 µg/L at normal and reduced food concentration, respectively. This clearly shows that a five-fold reduction of food concentration does not affect chronic toxicity of waterborne Cu to *D. magna*. Also, despite the 2.5-fold lower DOC concentration used by Gevaert (2004) (=2.5-fold higher bioavailability), the waterborne concentration of 76 µg Cu/L that causes 50% reduction of reproduction at the low food concentration (same as used in the present study), is still two-fold higher than the waterborne concentration of 36.5 µg waterborne Cu/L in the present exposure to dietary copper that also reduced reproduction by

50%. The combination of all these arguments makes it likely that the adverse effects on *D. magna*, observed in the present study, were mainly due to the ingestion and/or assimilation of Cu-contaminated food and not to the dissolved Cu concentration present in the water.

A second potential confounding factor is that metal-contaminated food may have had a reduced nutritional quality (Meyer et al., 2005; Clearwater et al., 2002). Thus, the observed effects might be an indirect result of food quality rather than a direct toxicological impact of ingested metal (Clearwater et al., 2002; Meyer et al., 2005). According to several authors, the key factors governing nutritional food quality to *D. magna* and other cladocerans are the C:P ratio and the essential fatty acid content and composition of the food (Sundbom and Vrede, 1997; Park et al., 2002). Exposure to copper increased the oleic acid and palmitic acid content of algae and reduced linoleic and linolenic acid (Table 1). McLarnon Riches et al. (1998) found a similar trend for oleic acid and linoleic acid, but not for palmitic or linolenic acid. We did not observe any effects on stearidonic acid and eicosapentaenoic acid (EPA). EPA is selectively stored in eggs of *D. magna* and reproduction is therefore the major drain of EPA from the females (Becker and Boersma, 2005). Since EPA levels in both control and Cu-contaminated food were very similar, the observed differences in reproduction cannot be related to EPA requirements of *D. magna.* Linolenic acid, which can be converted by daphnids into EPA (von Elert, 2002) was clearly lower in Cu-contaminated algae than in control algae, but since the conversion rate is low (von Elert, 2002) it is unlikely that the difference in linolenic acid could explain the marked reduction in reproduction that we observed. Additionally, EPA and linolenic acid are both known to accelerate maturation in daphnids, as indicated by a reduced time to first brood in *Daphnia galeata* (Sundbom and Vrede, 1997). Although linolenic acid was clearly lower in Cu-contaminated algae than in control algae, this reduction was apparently not large enough to affect time to first brood in *D.*

magna in our study (Table 2). This also suggests that the linolenic acid content of the Cu-exposed algae was most likely non-limiting. Finally, Park et al. (2002) derived an empirical equation relating growth rate of *D. magna* to C:P ratio and total ω3-poly-unsaturated-fatty-acid (PUFA) content of the food. Based on this equation and the data reported in Table 1, we estimated growth rates of daphnids of 37% of body mass increase per day for both the control and the dietary Cu treatment. These predicted growth rates are the same because in both control and Cu-contaminated food treatments neither the C:P ratio nor the ω3-PUFA-content were limiting (according to a comparison with limiting levels cited by Park et al., 2002). Indeed, the C:P ratio of both food sources was below the limiting level of 300, indicating that enough phosphorous was available. Also, the ω3-PUFA-content of both food sources was higher than the limiting level of 20 nmol/g C (Table 1). Thus, the observed differences in fatty acids and ω3- PUFA between control and Cu-exposed algae were not important enough to affect growth rate of the daphnids. We can summarize that, taking into account current knowledge about diet quality for *D. magna*, it is not likely that the adverse effects observed in our study are due to Cu-induced changes in nutritional quality of the food.

D. magna fed with algae pre-exposed to 500 µg Cu/L and with a copper burden of 3,000 µg Cu/g dry wt (Table 1), clearly exhibited impaired growth and reproduction compared to daphnids fed with control algae with a copper burden of only 10.6 µg Cu/g dry wt (Table 2). Chang and Sibley (1993) also demonstrated impaired reproduction upon feeding *D. magna* with Cu-contaminated algae. However, these authors did not consider the above-mentioned confounding factors. This prohibits a straight-forward comparison with our data. Earlier, we reported a stimulation of daphnid growth and reproduction when *D. magna* were fed with the same algal species with lower internal copper burdens between 327 and 1,360 µg Cu/g dry wt (De Schamphelaere and Janssen, 2004a). Additional experimentation, in which daphnids are

exposed to a wide range of dietary Cu doses in a single experiment, is needed to investigate whether responses of daphnids to dietary Cu exhibit a hormesis-like pattern.

The toxic response to dietary copper in *D. magna* is not the same as the one observed following exposure to dietary Zn (De Schamphelaere et al., 2004). While dietary Zn exclusively inhibited reproduction (De Schamphelaere et al., 2004), dietary Cu reduced reproduction, growth and *per capita* algal ingestion rate (Table 2, Figure 1). This implies that a broad spectrum of mechanisms and processes may be involved in the overall stress response of *D. magna* to dietary Cu exposure.

According to the energy allocation theory, the inhibition of growth and reproduction may be explained in terms of reduced energy acquisition and/or increased energy consumption (e.g., Kooijman, 2000; Nogueira et al., 2004). Increased metabolic costs to withstand toxicant stress and inhibition of food assimilation can both result in a reduction of growth and reproduction. Increased metabolic activity could for example be required for restoration of bio-molecules that are damaged by redox-cycling induced by accumulated Cu (Mason and Jenkins, 1995) or for detoxification processes such as metallothionein production (Amiard et al., 2006) or copper storage in granules (Bryan and Gibbs, 1983). Bossuyt and coworkers also suggested that increased energy expenditure for detoxification of accumulated Cu caused reduction in energy reserves, growth and reproduction. They demonstrated that *D. magna* was able to detoxify copper up to body burdens of around 100 µg Cu/g dry wt without affecting energy reserves, growth or reproduction (Bossuyt et al., 2005), while body burdens between 150 and 350 µg Cu/g dry wt clearly reduced energy reserves, growth and reproduction (Bossuyt and Janssen, 2005a). The internal body burden of 325 µg Cu/g observed in the present study could thus also explain the reduction of growth and reproduction.

However, reduced energy reserves can also be the result of reduced energy acquisition (as opposed to increased energy consumption), it is also possible that reduced energy acquisition has contributed to the observed toxic effects in the present study. Toxicants can reduce energy acquisition by inhibiting food ingestion, food assimilation efficiency, or a combination of both (Allen et al., 1995; Kooijman, 2000). Exposure to dissolved copper has been demonstrated to result in feeding inhibition in *D. magna* (Flickinger et al., 1982; Ferrando et al., 1993), but it is not easy to discriminate between the relative importance of waterborne and dietary exposure routes in these studies. Our study revealed that *per capita* ingestion rates of *D. magna* exposed to dietary Cu were inhibited (Figure 1), meaning that an exposed individual ingested significantly less food than a non-exposed individual. However, it is known that *per capita* feeding rates of *Daphnia* are proportional to size (Evers and Kooijman, 1989; Porter et al., 1982; Kooijman, 2000) and not accounting for this factor can lead to incorrect interpretations about toxicity mechanisms.

While at the end of the exposure (between day 19 to day 21) *per capita* ingestion rates in the Cu-exposed daphnids were 38% lower than in control daphnids (Table 2), the Cu exposed daphnids also weighed 38% less than control daphnids (Table 2). Assuming a daphnid's *per capita* ingestion rate is proportional to its squared length, l^2 (Kooijman, 2000), and assuming *D. magna* dry weight to be a power function of length with an average exponent of 2.4 (Porter et al., 1982), we calculated that the size-corrected ingestion rates of Cu-exposed and control daphnids differed by only 7%. Thus, it is likely that the inhibition of *per capita* ingestion rates by dietary Cu does not reflect an inhibition of the physical process of ingestion (e.g., related to toxicant-induced reduction of appendage beat rate as suggested by Allen et al., 1995), but that this observation is merely a reflection of reduced growth. This view is supported by the observation that the relative inhibition of *per capita* ingestion rates increased with time (Figure 1), mainly because ingestion rates of the Cu-exposed daphnids did not increase

significantly between the 5th and 21st day of exposure. Such an increase would be expected in normally growing and developing daphnids, as demonstrated by the observations of the control daphnids (Figure 1).

Even if the physical process of ingestion itself is not affected, it is still possible that energy acquisition is reduced through a decrease of assimilation efficiency. This could be the result of inhibition of digestive enzyme activity in the gut. Indeed, following Allen et al. (1995) this is the likely cause of inhibition of energy acquisition in *D. magna* for a range of contaminants associated with algal cells, including the metal Cd. Chen et al. (2002) demonstrated that high Cu concentrations inhibited digestive enzyme activity in the digestive fluids of several benthic invertebrates. It is thus possible that a reduction of digestive enzyme activity is involved in the inhibition of growth and reproduction in *D. magna* exposed to elevated levels of dietary Cu. Supported by the fact that Chen et al. (2002) found a stimulation of digestive enzyme activity at low copper levels, we also considered this mechanism to be the most plausible for explaining enhanced growth and reproduction of *D. magna* at dietary copper levels lower than the one investigated in the present study (De Schamphelaere et al., 2004).

Although it is tempting to relate growth and reproductive responses of *D. magna* at different dietary copper concentrations to digestive processes and effects on assimilation efficiency, the absence of data on growth and energy consumption throughout the exposure period makes it impossible to rule out the explanation of increased metabolic costs to cope with copper stress. Furthermore, although both mechanisms can partially explain most observations, they do not necessarily explain all observations. This follows from the fact that time to first brood (about 12 days) was not significantly affected by dietary Cu, while the size of this brood was significantly reduced by 35% (Table 2). Different authors have indicated that increased metabolic cost or reduced food assimilation would both result in a slower maturation and,

hence, a delayed onset of reproduction in *D. magna* (Kooijman, 2000; Nogueira et al., 2004). Therefore, it is rather unlikely that energy acquisition and/or consumption were adversely affected by dietary Cu before the 12th day of exposure and it is rather likely that another toxicity mechanism is needed to explain the reduction of the first brood size.

Several studies have suggested that the mechanisms of reproductive toxicity of dietborne metals to marine copepods (Hg, Ag, Cd, Zn, Mn), blue crabs (Cd), freshwater cladocerans (Ag), and *D. magna* (Zn) may be related to the inhibition of processes involved in vitellogenesis (Hook and Fisher, 2001a, 2001b, 2002; Lee and Noone, 1995; De Schamphelaere et al., 2004). This mechanism may not only explain the reduction of the first brood size, but also of the sizes of the second and the third broods in Cu-exposed *D. magna*.

Interestingly, the third brood size of Cu-exposed daphnids is more reduced (i.e., by 55%) than the first and second broods (i.e., by 35%) (Table 2). This could be due to an increase of the targeted effect on vitellogenesis with time. The increase of reproductive toxicity with time was also observed following dietary Zn exposure of *D. magna* (De Schamphelaere et al., 2004). Another possible explanation is that adverse effects on energy acquisition and/or consumption, as discussed above, were initiated during instar A3, i.e. the instar between the release of the first and second brood and also the instar during which the third brood is provisioned, i.e. between day 12 and day 15 of the exposure (we use the instar nomenclature of Nogueira et al., 2004). The hypothesis of the initiation of effects on energy acquisition after day 12 may be supported by the increased instar duration of the instars A3 and A4 (Table 2). Indeed, Nogueira et al. (2004) demonstrated that food deprivation during an instar of *D. magna* resulted in an increased duration of this instar. Although this makes the energy acquisition hypothesis again very attractive, it is not definite because it is, to our knowledge,

currently unknown whether or not increased metabolic costs imposed by toxicant stress can also affect instar duration in *D. magna*. It can also not be ruled out that dietary copper affected instar duration directly by interfering with the ecdysteroid metabolism, which regulates the moult cycle, such as observed in *D. magna* exposed to Cd (Bodar et al., 1990). Since reduced energy acquisition and/or increased energy consumption are required to explain the reduced size of the Cu-exposed daphnids at the end of the experiment (Table 2), at least one of these mechanisms most likely also contributed at least partially to the aggravated effect on the third brood size. The current dataset does, however, not allow us to explain why energy acquisition and/or consumption are most probably only affected after 12 days of exposure to dietary copper, i.e. after instar A2, and not before this time.

Although our finding that dietary Cu can cause toxic effects on *D. magna* is important from a mechanistic point of view, it must be acknowledged that the Cu concentration to which the algal food was exposed, i.e. 494 µg/L, as well as the internal Cu concentration in the algal food that was needed to invoke toxicity in *D. magna*, i.e. 3,000 µg Cu/g dry wt, will not often be found in natural environments. Also, *D. magna* reproduction has been shown to be inhibited by 50% at a three-fold lower waterborne concentration, i.e. 167 µg Cu/L (see above). One may therefore suggest that, even under such extremely polluted conditions, copper toxicity will be primarily caused by waterborne exposure and less by the dietary component. Additionally, given the 59% reduced growth rate of the algae (Table 1) at internal copper burdens causing dietary toxicity to *D. magna*, it could be argued that algal food may disappear from natural systems at Cu concentrations lower than those that could lead to dietary toxicity. For all these reasons, one could argue that dietborne Cu toxicity to crustaceans is of limited concern in freshwater systems. However, it must be kept in mind that the above reasoning is only valid for the presently investigated simple system of a single

crustacean species (*D. magna*) grazing on a single species of algal food (*P. subcapitata*). It is for instance well-known that there are considerable inter-species differences in bioconcentration factors of copper in green algae (up to factor 10; Yan and Pan, 2002) as well as in he amount of internalized copper at which algae are still able to thrive in natural systems (more than two orders of magnitude; De Schamphelaere et al., 2005). Thus, the dietary Cu dose at a given waterborne concentration in a natural system may be highly dependent on the local algal species community as well as on the food preference of local crustaceans. Also, because there is considerable inter-species differences of waterborne copper toxicity among crustaceans (Brix et al., 2001; Bossuyt and Janssen, 2005b), it is possible that sensitivity to dietary copper also exhibits an important among-species variation in crustaceans. Finally, waterborne and dietary sensitivities do not necessarily correlate and, hence, the relative importance of dietary and waterborne Cu exposure routes to toxicity may also vary among species. Thus, although it is acknowledged that the current study does not necessarily represent a naturally realistic scenario, the fact alone that dietary Cu exerts toxicity on a model crustacean warrants further research into the inter-species variability of sensitivity to dietary copper, including research about the importance of the algal species used a food source.

Conclusions and research perspectives

D. magna fed for 21 days with algal food (*P. subcapitata*) contaminated with 3,000 µg Cu/g dry wt accumulated a total copper body burden of 325 µg Cu/g dry wt and clearly experienced inhibited growth and reproduction. We have demonstrated that these toxic effects were most likely not the result of elevated dissolved copper concentrations in the exposure solutions (waterborne exposure) or of a reduced nutritional quality of the algal food. Analysis of life-history and other biological traits such as algal ingestion rate, growth, time-to-first brood,

instar duration and brood size suggest the possible involvement of several toxicity mechanisms such as increased energy consumption, reduced energy acquisition (potentially via inhibition of digestive enzyme activity), targeted inhibition of reproduction (potentially via inhibition of vitellogenesis), or even direct effects on moulting. The data also seem to suggest that direct inhibition of reproduction occurs from the first brood, but that all other effects are most probably initiated after the release of the first brood (12^{th} day of exposure). A distinct explanation for this observation is lacking. Clearly more research is needed to differentiate between these postulated mechanisms of dietary Cu toxicity, to determine which mechanisms act separately or in concert on each biological trait, and to determine how these mechanisms vary during a chronic exposure or with the age of a daphnid.

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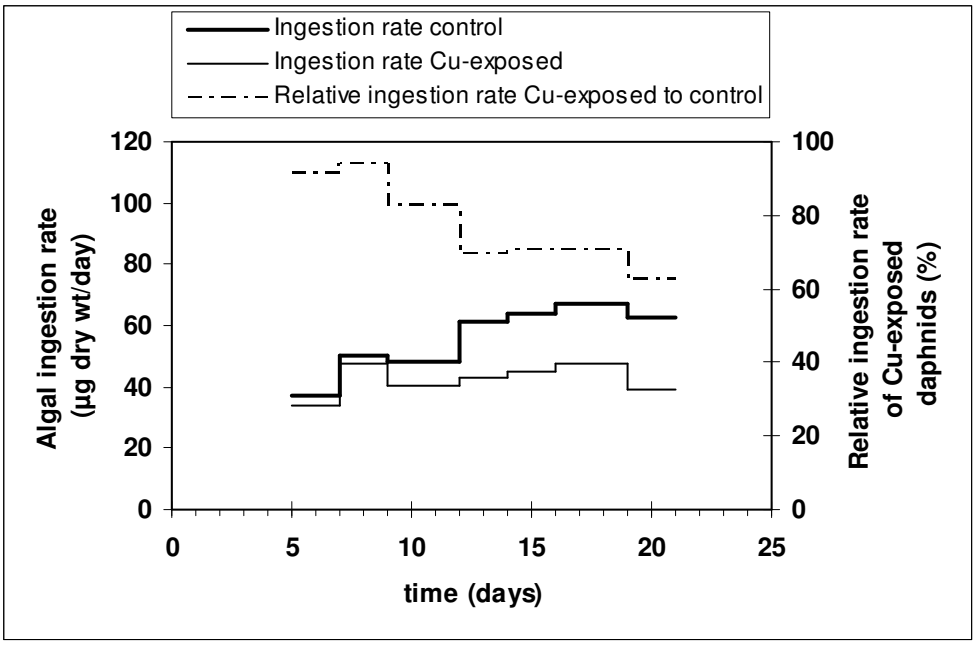

Figure 1 Algal ingestion rate (left axis) of *Daphnia magna* fed with control and Cu-contaminated algae and relative ingestion rates of Cu-exposed daphnids compared to control daphnids (right axis).

Table 1 Characteristics of algae cultured in control medium (no added Cu) and in medium containing 500 µg Cu/L.

	Control treatment		500 µg Cu/L treatment	
Disolved Cu during algal exposure (±stdev, n=4)	1.9±0.3 (2.3-1.7)[a]		494±42 (535-437)[a]	
Biomass growth rate (d^{-1})	0.881		0.364	
Internal Cu (µg/g dry wt) (±stdev, n=3) [b]				
Day 0	12.3±2.9		2,720±160	
Day 21	8.9±0.2		3,290±80	
Mean of day 0 and day 21	10.6		3,000	
External Cu (µg/g dry wt) (±stdev, n=3) [b]				
Day 0	<1.2		3,053±6	
Day 21	<1.2		4,055±10	
Mean of day 0 and day 21	<1.2		3,554	
Molar C:P ratio	274		241	
Fatty acids [c]	nmol/g C	mg/g dry wt	nmol/g C	mg/g dry wt
Palmitic acid, 16:0	145.5	14.9	182.5	18.7
Oleic acid, 18:1(n-9)	170.1	19.2	452.7	51.2
Linoleic acid, 18:2(n-6)	43.7	4.9	21.3	2.4
Linolenic acid*, 18:3(n-3)	94.9	10.6	59.7	6.7
Stearidonic acid*, 18:4(n-3)	26.1	2.9	25.3	2.8
EPA*,20:5(n-3) [d]	4.72	0.57	4.52	0.59
DHA*, 22:6(n-3) [e]	<0.15	< 0.02	<0.15	< 0.02
Total ω3-PUFA content [f]	125.7	14.0 [c]	89.5	10.0
Total fatty acid content		65.7		93.5

[a] Figures between parentheses are the initial and final concentration, respectively

[b] External copper is operationally defined as the copper that is desorbed from the algal surface by a 20 minute wash in a solution of a 5 mM ethylene-diamine-tetra-acetic acid solution. Internal copper is the copper remaining inside the cells after this treatment (De Schamphelaere and Janssen, 2004a; see also Materials and Methods).

[c] Fatty acid nomenclature reported after comma, concentrations given in two different units in columns 2-5 (nmol/g C and mg/g dry wt)

[d] EPA = eicosapentaenoic acid

[e] DHA = docosahexaenoic acid

[f] ω3-PUFA = omega-3 poly-unsaturated fatty acids; they are marked with a * next to their name in the first column

Table 2 Results of the chronic bioassays with *Daphnia magna* fed with control algae and algae pre-exposed to 500 µg Cu/L (nominal). Data are reported as mean ± standard deviation where applicable. Significant differences between daphnids fed Cu-contaminated and control food are indicated by * $p<0.05$, ** $p<0.01$, *** $p<0.001$; NS = not significant (Mann Whitney U test).

	Algae used as food source	
	Control algae	Algae exposed to 500 µg Cu/L
Dissolved Cu (µg/L) [a]	2.4±0.7	36.5±14.6
Survival (%)	100	100
Dry wt of daphnids at day 21 of exposure (µg)	252±32	156±32 **
Daphnid whole body burden of Cu at day 21 of exposure (µg Cu/g dry wt)	12.1±2.9	325±46 ***
Total reproduction in 21 days (juveniles/daphnid)	19.8±5.0	9.9±1.7 ***
Time to 1st brood (days)	12.1±1.1	12.2±0.8 NS
Time to 2nd brood (days)	15.2±1.0	15.9±0.6 *
Time to 3rd brood (days)	18.4±0.7	19.9±0.6 ***
1st brood size (juveniles/brood)	4.4±1.1	3.0±0.8 **
2nd brood size (juveniles/brood)	6.3±1.5	4.1±0.6 **
3rd brood size (juveniles/brood)	6.2±2.2	2.8±1.1 **
4th brood size (juveniles/brood)	8.3±2.2	No 4th brood

[a] Measured in old test solutions (n=9); concentration in fresh solution was 2.3 µg Cu/L in both treatments