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Reduction of growth and haemolymph Ca levels in the freshwater snail *Lymnaea stagnalis* chronically exposed to cobalt

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Abstract

The ecological risk assessment and the development of water-quality criteria for Co are currently still hampered by insufficient knowledge about the toxicity of Co to freshwater organisms. A relevant group of organisms, for which no toxicity data with Co are available, is the class of the herbivorous pulmonate freshwater snails, which fulfil a pivotal role in the consumption and decomposition of aquatic plants and epihyton. We measured the growth rate of the pond snail *Lymnaea stagnalis* chronically exposed for 28 days to a series of Co concentrations. The no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) for growth rate were 26 and 79 μg Co/L, respectively. Growth rate of snails exposed to 79 μg Co/L and higher concentrations was more impaired in the final 2 weeks of exposure than in the first 2 weeks of exposure. The reduced growth rate at 79 μg Co/L was accompanied by a reduced concentration of Ca in the haemolymph at the end of the exposure. Possible mechanisms of toxicity of Co to snail growth were suggested to be an impairment of Ca uptake and homeostasis and/or feeding inhibition. Although additional research is needed to investigate the relative importance of these mechanisms, as well as the interrelatedness between them, the toxicity data currently presented can assist in risk assessment and water-quality criteria development.

Keywords: Metal toxicity; Risk assessment; Water-quality criteria; Calcium homeostasis; Cobalt

1. Introduction

Cobalt (Co) is a naturally occurring essential element that is mainly found in the ores cobaltite, erythrite, and smaltite (Barceloux, 1999). Co is commercially refined from these ores and used in a variety of applications including metal alloys, pigments in textile manufacturing, drying agents in paints, and nutritional supplements (Diamond et al., 1992). Uncontaminated waters generally contain no more than a few micrograms of Co per liter (Marr et al., 1998). However, Co can occur at elevated concentrations as a result of, for instance, ore and coal mining, and discharges of certain textile dyes (Diamond et al., 1992). Yet, risk assessment or water-quality criteria setting for Co are currently still hampered by insufficient knowledge about the aquatic toxicity of Co.

Published chronic toxicity data that are potentially useful for this purpose are only available for a few cladocerans (*Daphnia magna*, *Ceriodaphnia dubia*) and fish (*Pimephales promelas*, *Brachydanio rerio*), with no observed effect concentrations (NOEC) ranging from 2.8 μg/L to more than 3800 μg/L (http://cfpub.epa.gov/ecotox; Diamond et al., 1992; Dave and Xiu, 1991). Hence, there is a clear need for chronic toxicity data for Co to other types of organisms that are of ecological relevance for the freshwater environment. One such relevant type of organisms is the class of pulmonate freshwater snails, such as the herbivorous pond snail *Lymnaea stagnalis*. These organisms fulfil a pivotal role in the consumption and decomposition of aquatic plants and epiphyton (Barnes, 1987).

Recently, it has become apparent that the growth of snails, due to their high-Ca requirements for shell formation,

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might be sensitive to metal exposure, especially when the metal interferes with Ca homeostasis (Grosell and Brix, 2004; Grosell et al., 2006). It has been shown that the pulmonate snails *L. stagnalis* and *Lymnaea pallustris* are among the most sensitive organisms for Pb (Grosell et al., 2006; Borgmann et al., 1978). Co has been shown to interfere with Ca uptake in freshwater fish, although a link with Co toxicity on endpoints at higher levels of biological organization, such as mortality or growth, has not yet been established (Richards and Playle, 1998; Comhaire et al., 1998). Here, we investigated the sensitivity of *L. stagnalis* growth to Co and also whether Co interferes with Ca homeostasis. To this end, we conducted a chronic toxicity bioassay with *L. stagnalis* in which we monitored growth rate during 28 days and determined Ca concentrations in the haemolymph at the end of the exposure period.

2. Materials and methods

2.1. Organisms

L. stagnalis (Linnaeus, 1758) originated from the breeding facility at the Vrije Universiteit in Amsterdam. These animals where reared and maintained in tap water (2 μg Cu/L, pH 8.4, hardness 150 mg $CaCO_3$/L) at 20 °C under a light-cycle of 12 h light–12 h dark. Two-hundred 3-week-old snails were acclimated for 10 days in 50 L of the test medium and to the conditions of the toxicity test (see further) prior to exposure to Co. Snails were 31 days old at test initiation and weighed 22.8±6.2 mg (mean wet weight±S.D.).

2.2. Toxicity experiments

Artificial freshwater used for testing was AFNOR test medium (Gomot, 1998) with hardness adjusted to the water hardness of the culture water and with additions of some essential elements to prevent deficiency in the control treatments. Final composition was 1 mmol/L of $CaCl_2$, 0.4 mmol/L of $MgCl_2$ (hardness = 140 mg $CaCO_3$/L) 2.4 mmol/L of $NaHCO_3$, and 0.15 mmol/L of K_2SO_4, 1 μg Cu/L, 3 μg Zn/L, and 1 μg Co/L. pH ranged between 7.6 and 7.9 during testing (Table 1). Using this dilution water the following treatments were prepared: a control (no added Co) and 3.2, 10, 32, 100, 320, and 1000 μg Co/L (nominal concentration). Cobalt was added from a stock solution of 10 mg Co/L that was prepared by dissolving $CoCl_2$ in deionized water. Polyethylene test containers were filled with 200 mL of experimental medium. Snails were randomly assigned to the control or Co treatments. All snails were housed individually (one snail per test container) and were tested simultaneously. Each treatment was tested on eight animals. Tests were conducted at 20 °C under a light-cycle of 12 h light–12 h dark. At test initiation and with every renewal, each snail was provided with an ad libitum food ration of 55 mg lettuce during the first 2 weeks of the exposure and subsequently with 65 mg lettuce (approximately 2 cm^2 of leaf surface). The entire 200 mL of test medium was replaced with fresh test medium twice a week (on the 4th, 7th, 11th, 14th, 18th, 21st, and 25th day of exposure). Determinations of dissolved Co, pH, dissolved organic carbon (DOC) and inorganic carbon (IC) were performed on freshly prepared test solutions, on test solutions just before each renewal, and at the end of the experiment. Snails were weighed to the nearest 0.1 mg at test initiation, after 14 and 28 days of exposure. Previous studies have shown that snail weight is very tightly correlated to size (shell height or length) according to a third power function ($r = 0.99$, $N = 47$, $p < 0.0001$; Loose and Koene, in press; see also Koene et al., 2007). At the end of the exposure period, haemolymph was extracted from the snails exposed to nominal concentrations of 100 μg Co/L and lower. Extracted haemolymph quantities varied between 7 and 48 μL (extracted volume was positively related to wet weight, $r = 0.84$). Snails exposed to higher Co concentrations did not grow sufficiently to obtain sufficient haemolymph for a reliable Ca analysis. Haemolymph was digested in 14 N HNO_3 (NORMATOM quality, VWR International, Belgium) and Ca was determined on the digested sample with flame atomic absorption spectrometry (SpectrAA100; Varian, Mulgrave, Australia) with a detection limit of 100 μg Ca/L. Measured Ca concentrations in the digested samples were well above this limit, i.e. all higher than 590 μg Ca/L.

Table 1
Chemical parameters measured during the ecotoxicity tests with *Lymnaea stagnalis*

Nominal Co (μg/L)	Measured dissolved Co (μg/L)	pH	DOC (mg/L)[a]
Control	<1	7.8±0.1	2.5±1.2 A
3.2	2.6±0.5	7.8±0.1	2.3±0.8 A
10	8.2±1.3	7.8±0.1	2.1±0.7 A
32	26±3	7.6±0.1	2.4±1.0 A
100	79±11	7.7±0.1	2.0±1.0 B
320	270±10	7.8±0.1	1.4±0.6 C
1000	860±20	7.9±0.2	1.4±0.9 C

[a]Data are presented as mean±standard deviation of all measured values (see materials and methods; $n = 9$). Dissolved organic carbon; reported DOC values are mean± standard deviation of values measured in test solutions immediately before the medium was renewed ($n = 7$). DOC concentrations followed by the same letter are not significantly different from each other (Sign test, $p < 0.05$).

2.3. Chemical analyses of test solutions

All analyses, except pH, were performed on filtered samples (0.45 μm; Gelman Sciences, Ann Arbor, MI, USA). DOC and dissolved inorganic carbon (DIC) were measured with a total organic carbon analyser (TOC-5000, Shimadzu, Duisburg, Germany). Dissolved Co concentrations were measured using a graphite furnace atomic absorption spectrophotometer (SpectrAA800 with Zeeman background correction; Varian, Mulgrave, Australia) after acidification of the samples (0.14 N HNO_3; NORMATOM quality, VWR Internatinal, Belgium). The detection limit was 1 μg/L. Two certified reference samples, TMDA-62 and TM-25.2 (National Water Research Institute, Burlington, Ont., Canada) with Co concentrations (mean±95% confidence interval) of 99.7±7.8 and 12±2.24 μg/L, respectively, were analysed at the beginning and end of each series of Co measurements. Measured values were always within the 95% confidence interval and did not deviate by more than 8% (higher reference) and 15% (lower reference) of the mean certified value.

2.4. Data treatment

Using wet body weights at test initiation and after 14 and 28 days of exposure, specific growth rates (r) were calculated for the first 14 days and the final 14 days of exposure with following equation:

$$r_{1-2} = \frac{\{\ln(W_2/W_1)\}}{(t_2 - t_1)}, \quad (1)$$

where r_{1-2} is the specific growth rate (day^{-1}) between t_1 and t_2; W_1 and W_2 are the weight of snails at t_1 and t_2, respectively; t_1 and t_2 are the time from test initiation (days). Growth rate and haemolymph Ca data in the Co treatments were statistically compared to those in the control with the Jonckheere–Terpstra step-down trend test using the statistical software package SPSS 15.0 (SPS Inc., Chicago, IL).

3. Results

Table 1 gives an overview of the chemistry measured during the toxicity tests. Measured dissolved Co concentrations were 15–21% lower than the nominal concentrations. The pH was between 7.6 and 7.9 and DOC concentrations were between 1.4 and 2.5 mg/L. There was a significant trend (Sign-test, $p<0.05$, $n=9$) of lower DOC concentrations at higher Co concentrations (79–820 μg/L) than at lower Co concentrations (2.6–26 μg/L).

No mortality was observed throughout the 28-day exposure period in any of the concentrations investigated. Growth rate in first 2 weeks as well as in the final 2 weeks of the exposure was significantly impaired at concentrations of 79 μg Co/L and higher ($p<0.05$) (Fig. 1), resulting in a NOEC of 26 μg Co/L. At concentrations of 79 μg Co/L and higher growth rate was clearly more inhibited during the final 2 weeks of the exposure than during the first 2 weeks of exposure. Indeed at 79 μg Co/L, growth rate in the first 2 weeks was inhibited by 27% (compared to the control), while it was reduced by 88% during the final 2 weeks. At concentrations of 270 and 860 μg Co/L, growth in the first 2 weeks was reduced by 40% and 86%, respectively, while negative growth rate (net weight loss) was observed during the final 2 weeks.

The Ca concentration in the haemolymph of *L. stagnalis* exposed for 28 days to 79 μg Co/L, i.e. 2.49 mmol Ca/L, was significantly lower than the Ca content in the control

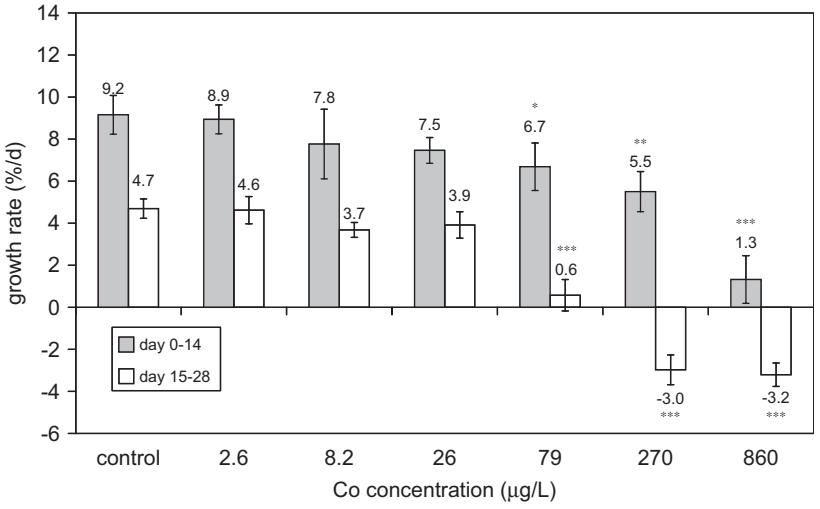

Fig. 1. Growth rate of *Lymnaea stagnalis* at different Co concentrations during the initial (day 0–14) and final 2 weeks of the exposure (day 15–28). Means ± standard error of the mean are shown. Values above the bars are the actual growth rates. Significant differences with the control, according to the Jonckheere–Terpstra test, are marked by $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$.

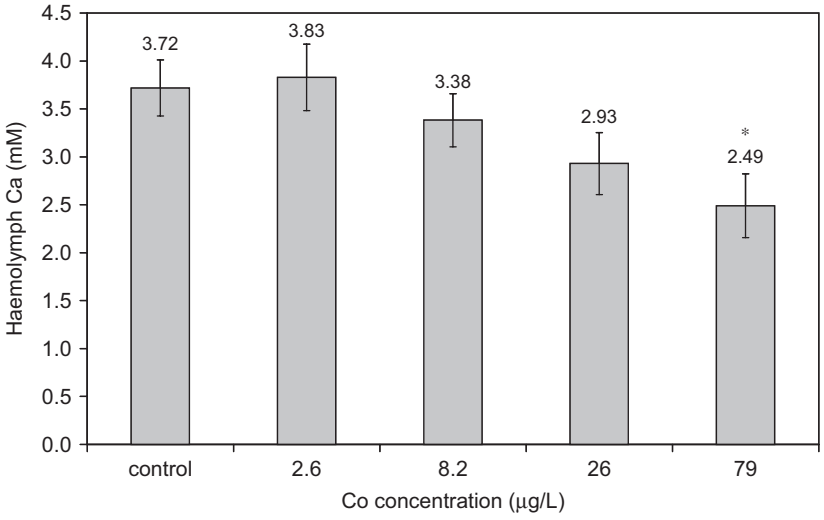

Fig. 2. Haemolymph Ca in *Lymnaea stagnalis* at different Co concentrations after 28 days of exposure. Means ± standard error of the mean are shown. Values above the bars are the actual haemolymph Ca concentrations. Significant differences with the control, according to the Jonckheere–Terpstra test, are marked by $^{*}p<0.05$.

snails, i.e. 2.49 mmol Ca/L ($p<0.05$; Fig. 2). Lower cobalt concentrations did not significantly affect Ca concentrations in the haemolymph.

4. Discussion

The fact that measured dissolved Co concentrations were 15–21% lower than the nominal concentrations may be due to adsorption to test container walls and particulate matter in the test solutions originating from food addition and snail defecation. DOC concentrations measured in solutions, i.e. between 1.4 and 2.5 mg/L (Table 1), were between 1.1 and 2.2 mg/L higher than average DOC levels around 0.3 mg/L that are typically recorded in fresh deionized water in our laboratory (De Schamphelaere and Janssen, 2002). This increase is most likely due to biological activity of the snails in the test containers (e.g., excretion of dissolved ligands resulting from food digestion). The significant trend (Sign-test, $p<0.05$, $n=9$) of lower DOC concentrations at higher Co concentrations (79–820 μg/L) than at lower Co concentrations (2.6–26 μg/L), could suggest a lower biological activity (e.g., feeding activity) of the snails at higher Co concentrations (see also further).

Growth rate of the snails was significantly reduced at concentrations of 79 μg Co/L and higher, both during the first 2 weeks and during the final 2 weeks of the exposure, resulting in a NOEC of 26 μg Co/L (Fig. 1). It is interesting from a regulatory perspective to compare this value with chronic NOEC's obtained with other freshwater species. A literature and database search revealed chronic NOEC's lower than 50 μg/L for *C. dubia* (reproduction), between 2.8 and 10 μg Co/L the cladoceran *D. magna* (reproduction), between 210 and >3800 μg Co/L for the fish *P. promelas* (survival and reproduction), and between 60 and 3800 μg Co/L for the fish *B. rerio* (early life stage tests, embryo hatching, and larval survival) (http://cfpub.epa.gov/ecotox; Diamond et al., 1992; Dave and Xiu, 1991). Although one might infer from this that the sensitivity of *L. stagnalis* growth to Co is intermediate to that of cladocerans and fish, no definitive conclusions can be drawn because different endpoints have been considered and because different test waters with different chemistries have been used. For instance, water hardness—which is a parameter known to affect aquatic Co toxicity (Diamond et al., 1992; Rathore and Khangarot, 2003)—varied between 50 and 800 mg $CaCO_3$/L among all those studies, including the present study. Further experimentation could help in determining relative species sensitivities to Co and the effect of water chemistry parameters on Co toxicity to different species.

Detailed evaluation of the growth data revealed that at concentrations of 79 μg Co/L and higher growth rate was impaired to a clearly larger degree in the last 2 weeks of exposure than in the first 2 weeks of the exposure (Fig. 1). Growth at 79 μg Co/L was nearly arrested during the final 2 weeks and negative growth (net weight loss) occurred at 270 μg Co/L and higher (Fig. 1). This observation, i.e. that the magnitude of the inhibitory effects of Co on early growth rate in *L. stagnalis* increased with increasing exposure duration, suggests that extrapolation of our results to longer exposure durations (e.g., life cycle) should be performed cautiously. Indeed, adverse effects of many chemicals on reproductive traits of many aquatic organisms, including aquatic snails, occur at similar or lower concentrations than effects on growth.

For example, Coeurdassier et al. (2003), exposing *Lymnaea palustris* to Cd, found similar median inhibitory concentrations (EC50) for growth (58 μg/L) and reproductive output (number of eggs or egg masses per individual) (60 μg/L), but observed that embryos were unable to hatch at concentrations as low as 40 μg Cd/L. Münzinger and Guarducci (1988), exposing *Biomphalaria glabrata* to Zn, observed a reduction of not only growth rate at the lowest investigated concentration (500 μg/L), but also a reduction of fecundity and embryonic hatching rate as well as a delayed attainment of sexual maturity. Thus, there is at least some evidence that reproduction and fertility of snails may be equally or even more sensitive to metal exposure than growth. Hence, it would be instructive to perform additional studies in which the reproductive output and fertility of *L. stagnalis* is determined as a function of the cobalt concentration.

The reduced growth observed in the present study could potentially be explained by impaired feeding activity of the snails. Indeed, although not quantified, we observed that during this period (but not before), feeding of the snails exposed to concentrations of 79 μg/L or higher was markedly inhibited (almost no lettuce was consumed). The DOC concentration generated at these Co concentrations was significantly lower than that in the lower Co treatments and in the control (Table 1). This also supports the idea of a general impairment of biological activities, including feeding. Feeding inhibition is a well-known response of aquatic organisms to chemicals exposure (e.g., Allen et al., 1995). Crichton et al. (2004), for example, reported feeding inhibition of *Lymnaea peregra* following a 48-h exposure to Cd. The mechanisms of feeding inhibition by toxicants, however, are not always well understood. Allen et al. (1995) proposed that contaminants adsorbed to or absorbed by the food could invoke inhibition of the physical process of feeding (e.g., the scraping process preceding food ingestion by snails), food avoidance (e.g., when organisms could "taste" the contaminant) or gut poisoning. Next to this diet borne exposure route, physiological processes involved in the feeding process may also be affected via the waterborne exposure route. Muyssen et al. (2006) suggested that *D. magna* exposed to Zn invoked a net loss of Ca from the organism, possibly via the well-known inhibition of Ca uptake by Zn. This in turn may have affected feeding rates, because Ca is needed for the muscle contraction required for limb-movement-dependent feeding in these organisms (Muyssen et al., 2006). If antagonism between Co and Ca in snails occurs, as it does in freshwater fish (Comhaire et al., 1998), a loss

of Ca from the snail following Co exposure may also result in reduced muscle contraction and feeding activity. Obviously, additional experimentation would be required to quantitatively relate feeding inhibition in snails to Co exposure (and possibly also to Ca loss), possibly using a similar methodology for measuring feeding inhibition as suggested by Crichton et al. (2004).

Next to the inhibition of growth, we also observed that the Ca concentration in the haemolymph of *L. stagnalis* exposed for 28 days to 79 μg Co/L was significantly lower than in the control snails (Fig. 2). Interestingly, the Co concentration at which Ca in the haemolymph was affected is the same as the one at which growth is significantly impaired.

The lower Ca in the haemolymph of snails exposed to Co may be explained by inhibition of Ca uptake by Co. Antagonism between Co and Ca has previously been proposed as an explanation as to why increased Ca (or water hardness) reduced Co uptake and toxicity in fish and invertebrates (Richards and Playle, 1998; Diamond et al., 1992; Comhaire et al., 1998). Grosell et al. (2006) suggested that the inhibition of Ca uptake by metals could potentially impair snail growth if Ca influx would become limiting for growth of the shell, which consists almost entirely of $CaCO_3$ (Van Der Borght and Van Puymbroeck, 1966). Grosell et al. (2006) also suggested that the very high-Ca requirements of freshwater snails could possibly explain why this species is amongst those that are most sensitive to Pb, another Ca antagonist (Rogers et al., 2003). If the reduced Ca haemolymph levels observed in the present study indeed reflect a reduced Ca influx, this mechanism possibly explains the toxicity of Co to snail growth. Alternatively, reduced Ca levels in the haemolymph may also impair growth by invoking reduced feeding, via a similar mechanism as proposed by Muyssen et al. (2006).

However, it is also possible that reduced Ca in the haemolymph is not the cause but rather the consequence of reduced feeding. Indeed, DeWith and Sminia (1980) reported Ca haemolymph concentrations between 4.2 and 5.1 mM in non-starved adult *L. stagnalis* during a 10-day growth period. When snails were starved a significant reduction in haemolymph Ca was observed to below 4 mM during the whole experimental period. The author suggested that the decrease was partly due to the fact that snails under normal conditions gain part of their Ca from the diet. Another possible explanation was that reduced metabolic activity due to reduced feeding resulted in decreased CO_2 production, increased pH and reduced Ca levels in haemolymph. Both mechanisms may be an alternative explanation for the lower Ca concentration observed in Co exposed snails.

5. Conclusion

When *L. stagnalis* was exposed for 28 days to a range of cobalt concentrations, it was observed that cobalt affected the growth rate, the Ca concentrations in the haemolymph and the feeding at and above dissolved concentrations of 79 μg Co/LOEC (lowest observed effect concentration), with a NOEC of 26 μg Co/L. Although several physiological explanations are possible for linking these observed effects, clearly more research is needed to further elucidate the mechanism of chronic toxicity of Co to freshwater snails.

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Human and animal welfare

All experiments in this study were conducted in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

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