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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 µg Co/L 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 two weeks of exposure than in the first two 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

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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.

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 (Mar 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 to more than 3,800 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, 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.

Materials and methods

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 12h light - 12h dark. Two-hundred three-week old snails were acclimated for 10 days in 50L 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 ± standard deviation).

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

77 was 1 mmol/L of $CaCl_2$, 0.4 mmol/L of $MgCl_2$ (hardness = 140 mg $CaCO_3$/L) 2.4
78 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.
79 pH ranged between 7.6 and 7.9 during testing (Table 1). Using this dilution water the
80 following treatments were prepared: a control (no added Co) and 3.2, 10, 32, 100, 320,
81 and 1000 µg Co/L (nominal concentration). Cobalt was added from a stock solution of
82 10 mg Co/L that was prepared by dissolving $CoCl_2$ in deionized water. Polyethylene
83 test containers were filled with 200 mL of experimental medium. Snails were randomly
84 assigned to the control or Co treatments. All snails were housed individually (one snail
85 per test container) and were tested simultaneously. Each treatment was tested on eight
86 animals. Tests were conducted at 20°C under a light-cycle of 12h light – 12h dark. At
87 test initiation and with every renewal, each snail was provided with an ad libitum food
88 ration of 55 mg lettuce during the first two weeks of the exposure and subsequently with
89 65 mg lettuce (approximately 2 cm^2 of leaf surface). The entire 200 mL of test medium
90 was replaced with fresh test medium twice a week (on the 4^{th}, 7^{th}, 11^{th}, 14^{th}, 18^{th}, 21^{st},
91 and 25^{th} day of exposure). Determinations of dissolved Co, pH, dissolved organic
92 carbon (DOC) and inorganic carbon (IC) were performed on freshly prepared test
93 solutions, on test solutions just before each renewal, and at the end of the experiment.
94 Snails were weighed to the nearest 0.1 mg at test initiation, after 14 days and after 28
95 days of exposure. Previous studies have shown that snail weight is very tightly
96 correlated to size (shell height or length) according to a third power function ($r=0.99$, N
97 $=47$, $P<0.0001$; Loose en Koene, in press; see also Koene et al., 2007). At the end of
98 the exposure period, haemolymph was extracted from the snails exposed to nominal
99 concentrations of 100 µg Co/L and lower. Extracted haemolymph quantities varied
100 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.

Chemical analyses of test solutions

All analyses, except pH, were performed on filtered samples (0.45 µm, Gelman Sciences, Ann Arbor, MI, USA). Dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC) were measured with a total organic carbon analyzer (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 (0.14N HNO_3, NORMATOM quality, VWR International, Belgium). The detection limit was 1 µg/L. Two certified reference samples, TMDA-62 and TM-25.2 (National Water Research Institute, Burlington, ON, Canada) with Co concentrations (mean ± 95% confidence interval) of 99.7 ± 7.8 µg/L and 12 ± 2.24 µg/L, respectively, were analyzed 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.

Data treatment

125 Using wet body weights at test initiation and after 14 days and 28 days of exposure,
126 specific growth rates (*r)* were calculated for the first 14 days and the final 14 days of
127 exposure with following equation:
128 $r_{1-2} = \{\ln(W_2/W_1)\}/(t_2-t_1)$ (Eq. 1)
129 where r_{1-2} = specific growth rate (d^{-1}) between t_1 and t_2; W_1, W_2 = weight of snails at t_1
130 and t_2, respectively; t_1 and t_2 = time from test initiation (days). Growth rate and
131 haemolymph Ca data in the Co treatments were statistically compared to those in the
132 control with the Jonckheere-Terpstra step-down trend test using the statistical software
133 package SPSS 15.0 (SPS Inc., Chicago, IL).
134
135 **Results**
136
137 Table 1 gives an overview of the chemistry measured during the toxicity tests.
138 Measured dissolved Co concentrations were 15% to 21% lower than the nominal
139 concentrations. The pH was between 7.6 and 7.9 and DOC concentrations were between
140 1.4 and 2.5 mg/L. There was a significant trend (Sign-test, $p<0.05$, n=9) of lower DOC
141 concentrations at higher Co concentrations (79 to 820 µg/L) than at lower Co
142 concentrations (2.6 to 26 µg/L).
143
144 No mortality was observed throughout the 28d-exposure period in any of the
145 concentrations investigated. Growth rate in first two weeks as well as in the final two
146 weeks of the exposure was significantly impaired at concentrations of 79 µg Co/L and
147 higher ($p<0.05$) (Figure 1), resulting in a no observed effect concentration (NOEC) of
148 26 µg Co/L. At concentrations of 79 µg Co/L and higher growth rate was clearly more

149 inhibited during the final two weeks of the exposure than during the first two weeks of
150 exposure. Indeed at 79 µg Co/L, growth rate in the first two weeks was inhibited by
151 27% (compared to the control), while it was reduced by 88% during the final two
152 weeks. At concentrations of 270 and 860 µg Co/L, growth in the first two weeks was
153 reduced by 40% and 86%, respectively, while negative growth rate (net weight loss)
154 was observed during the final two weeks.
155
156 The Ca concentration in the haemolymph of *L. stagnalis* exposed for 28 days to 79 µg
157 Co/L, i.e. 2.49 mmoles Ca/L, was significantly lower than the Ca content in the control
158 snails, i.e. 2.49 mmoles Ca/L ($p<0.05$, Figure 2). Lower cobalt concentrations did not
159 significantly affect Ca concentrations in the haemolymph.
160
161 **Discussion**
162
163 The fact that measured dissolved Co concentrations were 15% to 21% lower than the
164 nominal concentrations may be due to adsorption to test container walls and particulate
165 matter in the test solutions originating from food addition and snail defecation. DOC
166 concentrations measured in solutions, i.e. between 1.4 and 2.5 mg/L (Table 1), were
167 between 1.1 and 2.2 mg/L higher than average DOC levels around 0.3 mg/L that are
168 typically recorder in fresh deionized water in our laboratory (De Schamphelaere and
169 Janssen, 2002). This increase is most likely due to biological activity of the snails in the
170 test containers (e.g., excretion of dissolved ligands resulting from food digestion). The
171 significant trend (Sign-test, $p<0.05$, $n=9$) of lower DOC concentrations at higher Co
172 concentrations (79 to 820 µg/L) than at lower Co concentrations (2.6 to 26 µg/L), could

173 suggest a lower biological activity (e.g., feeding activity) of the snails at higher Co
174 concentrations (see also further).
175
176 Growth rate of the snails was significantly reduced at concentrations of 79 µg Co/L and
177 higher, both during the first two weeks and during the final two weeks of the exposure,
178 resulting in a NOEC of 26 µg Co/L (Figure 1). It is interesting from a regulatory
179 perspective to compare this value with chronic NOEC's obtained with other freshwater
180 species. A literature and database search revealed chronic NOEC's lower than 50µg/L
181 for *Ceriodaphnia dubia* (reproduction), between 2.8 and 10 µg Co/L the cladoceran
182 *Daphnia magna* (reproduction), between 210 and >3,800 µg Co/L for the fish
183 *Pimephales promelas* (survival and reproduction), and between 60 and 3,800 µg Co/L
184 for the fish *Brachydanio rerio* (early life stage tests, embryo hatching and larval
185 survival) (http://cfpub.epa.gov/ecotox; Diamond et al., 1992; Dave and Xiu, 1991).
186 Although one might infer from this that the sensitivity of *L. stagnalis* growth to Co is
187 intermediate to that of cladocerans and fish, no definitive conclusions can be drawn
188 because different endpoints have been considered and because different test waters with
189 different chemistries have been used. For instance, water hardness - which is a
190 parameter known to affect aquatic Co toxicity (Diamond et al., 1992; Rathore et al.,
191 2003) – varied between 50 and 800 mg $CaCO_3$/L among all those studies, including the
192 present study. Further experimentation could help in determining relative species
193 sensitivities to Co and the effect of water chemistry parameters on Co toxicity to
194 different species.
195
196

197 Detailed evaluation of the growth data revealed that, at concentrations of 79 µg Co/L
198 and higher growth rate was impaired to a clearly larger degree in the last two weeks of
199 exposure than in the first two weeks of the exposure (Figure 1). Growth at 79 µg Co/L
200 was nearly arrested during the final two weeks and negative growth (net weight loss)
201 occurred at 270 µg Co/L and higher (Figure 1). This observation, i.e. that the magnitude
202 of the inhibitory effects of Co on early growth rate in *L. stagnalis* increased with
203 increasing exposure duration, suggests that extrapolation of our results to longer
204 exposure durations (e.g., life cycle) should be performed cautiously. Indeed, adverse
205 effects of many chemicals on reproductive traits of many aquatic organisms, including
206 aquatic snails, occur at similar or lower concentrations than effects on growth.
207 For example, Cœurdassier et al. (2003), exposing *Lymnaea palustris* to Cd, found
208 similar median inhibitory concentrations (EC50) at for growth (58 µg/L) and
209 reproductive output (number of eggs or egg masses per individual) (60 µg/L), but
210 observed that embryos were unable to hatch at concentrations as low as 40 µg Cd/L.
211 Münzinger and Guarduci (1998), exposing *Biomphalaria glabrata* to Zn, observed a
212 reduction of not only growth rate at the lowest investigated concentration (500 µg/L),
213 but also a reduction of fecundity and embryonic hatching rate as well as a delayed
214 attainment of sexual maturity. Thus, there is at least some evidence that reproduction
215 and fertility of snails may be equally or even more sensitive to metal exposure than
216 growth. Hence, it would be instructive to perform additional studies in which the
217 reproductive output and fertility of *L. stagnalis* is determined as a function of the cobalt
218 concentration.
219

220 The reduced growth observed in the present study could potentially be explained by
221 impaired feeding activity of the snails. Indeed, although not quantified, we observed
222 that during this period (but not before), feeding of the snails exposed to concentrations
223 of 79 µg/L or higher was markedly inhibited (almost no lettuce was consumed). The
224 DOC concentration generated at these Co concentrations was significantly lower than
225 that in the lower Co treatments and in the control (Table 1). This also supports the idea
226 of a general impairment of biological activities, including feeding. Feeding inhibition is
227 a well-known response of aquatic organisms to chemicals exposure (e.g., Allen et al.,
228 1995). Crichton et al. (2004), for example reported feeding inhibition of *Lymnaea*
229 *peregra* following a 48-hour exposure to Cd. The mechanisms of feeding inhibition by
230 toxicants, however, are not always well understood. Allen et al. (1995) proposed that
231 contaminants adsorbed to or absorbed by the food could invoke inhibition of the
232 physical process of feeding (e.g., the scraping process preceding food ingestion by
233 snails), food avoidance (e.g., when organisms could "taste" the contaminant) or gut
234 poisoning. Next to this diet borne exposure route, physiological processes involved in
235 the feeding process may also be affected via the waterborne exposure route. Muyssen et
236 al. (2006) suggested that *Daphnia magna* exposed to Zn invoked a net loss of Ca from
237 the organism, possibly via the well-known inhibition of Ca uptake by Zn. This in turn
238 may have affected feeding rates, because Ca is needed for the muscle contraction
239 required for limb-movement-dependent feeding in these organisms (Muyssen et al.,
240 2006). If antagonism between Co and Ca in snails occurs, as it does in freshwater fish
241 (Comhaire et al., 1998), a loss of Ca from the snail following Co exposure may also
242 result in reduced muscle contraction and feeding activity. Obviously, additional
243 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 (Figure 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, De With (1978) 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.

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), 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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296 **References**

297 Allen, Y.; Calow, P.; Baird, D.J. A mechanistic model of contaminant-induced feeding
298 inhibition in *Daphnia magna*. Environ. Toxicol. Chem. 14: 1625-1630; 1995.
299
300 Barceloux, D.G. Cobalt. J. Toxicol. Clin. Toxicol. 37:201-216; 1999.
301
302 Barnes, R.D. 1987. Invertebrate Zoology. Fifth Edition. Saunders College Publishing,
303 Philadelphia, PA, USA; 1987.
304
305 Borgmann, U.; Kramar, O.; Loveridge, C. Rates of mortality, growth, and biomass
306 production of *Lymnaea palustris* during chronic exposure to lead. J. Fish. Res. Board
307 Can. 35: 1109-1115; 1978.
308
309 Coeurdassier, De vaufleury, and P.-M. Badot, 2003. Bioconcentration of cadmium and
310 toxic effects on life-history traits of pond snails (*Lymnaea palustris* and *Lymnaea*
311 *stagnalis*) in laboratory bioassays. Arch. Environ. Contam. Toxicol. 45: 102-109; 2003
312
313 Comhaire, S.; Blust, R.; Vanginneken, L.; Verbost, P.M.; Vanderborght, O.L.J.
314 Branchial cobalt uptake in the carp, *Cyprinus carpio*: effect of calcium channel blockers
315 and calcium injection. Fish Physiol. Biochem. 18: 1-13; 1998.
316
317 Crichton, C.A.; Conrad, A.U.; Baird, D.J. Assessing stream grazer response to stress: A
318 post-exposure feeding bioassay using the freshwater snail *Lymnaea peregra* (Muller).
319 Bull. Environ. Contam. Toxicol. 72: 564-570; 2004.

Dave, G., Xiu, R. Toxicity of mercury, copper, nickel, lead and cobalt to embryos and larvae of zebra fish, *Brachydanio rerio*. Arch. Environ. Contam. Toxicol. 21: 126-134; 1991.

De Schamphelaere, K.A.C.; Janssen C.R. A biotic ligand model predicting acute copper toxicity for *Daphnia magna*: the effects of calcium, magnesium, sodium, potassium and pH. Environ. Sci. Technol. 36: 48-54; 2002.

DeWith, N.D.; Sminia, T. The effects of the nutritional state and the external calcium-concentration on the ionic composition of the haemolymph and on the calcium cells in the pulmonate freshwater snail *Lymnaea stagnalis*. Proceedings of the Koninklijke Nederlandse Akademie van Wetenschappen Series C-Biological and Medical Sciences 83: 217-227; 1980.

Diamond, J.M.; Winchester, E.L.; Mackler, D.G.; Rasnake, W.J.; Fanelli, J.K.; Gruber, D. Toxicity of cobalt to freshwater indicator species as a function of water hardness. Aquat. Toxicol. 22:163-180; 1992.

Gomot, A. Toxic effects of cadmium on reproduction, development, and hatching in the freshwater snail Lymnaea stagnalis for water quality monitoring. Ecotoxicol. Environ. Safety 41: 288-297; 1998.

343 Grosell, M.; Brix, K.V. Why are freshwater pulmonate snails so sensitive to chronic
344 metal exposure? SETAC Globe 5: 43-45; 2004.
345
346 Grosell, M.; Gerdes, R.M.; Brix, K.V. Chronic toxicity of lead to three freshwater
347 invertebrates – *Brachionus calyciflorus*, *Chironomus tentans*, and *Lymnaea stagnalis*.
348 Environ. Toxicol. Chem. 25: 97-104; 2006.
349
350 Koene, J.M.; Montagne-Wajer, K.; Ter Maat, A. Aspects of body size and mate choice
351 in the simultaneously hermaphroditic pond snail *Lymnaea stagnalis*. Animal Biol. 57:
352 247-259; 2007
353
354 Loose, M.J.; Koene, J.M. Sperm transfer is affected by mating history in the
355 simultaneously hermaphroditic snail *Lymnaea stagnalis*. Invertebrate Biology, in press.
356
357 Marr, J.C.A.; Hansen, J.A.; Meyer, J.S.; Cacela, D.; Podrabsky, T.; Lipton, J.; Bergman,
358 H.L. Toxicity of cobalt and copper to rainbow trout: application of a mechanistic model
359 for predicting survival. Aquat. Toxicol. 43: 225-238; 1998.
360
361 Münzinger, A.; Guarducci, M.-L. 1988. The effect of low zinc concentrations on some
362 demographic parameters of *Biomphalaria glabrata* (Say), mollusca: gastropoda. Aquat.
363 Toxicol. 12: 51-61; 1988
364
365 Muyssen, B.T.A.; De Schamphelaere, K.A.C.; Janssen, C.R. Mechanisms of chronic
366 waterborne Zn toxicity in *Daphnia magna*. Aquat. Toxicol. 77: 393-401; 2006.

OECD. Draft Guidance Document for on the Statistical Analysis of Ecotoxicity Data. Environmental Health and Safety Publications; Series on Testing and Assessment; Environment Directorate; Organisation for Economic Co-operation and Development, Paris; 2005.

Rathore, R.S.; Khangarot, B.S. Effects of water hardness and metal concentration on a freshwater *Tubifex tubifex* Muller. Water Air and Soil Pollution 142: 341-356; 2003.

Richards, J.G.; Playle, R.C. Cobalt binding to gills of rainbow trout (*Oncorhynchus mykiss*): an equilibrium model. Comp. Biochem. Physiol. C Comp. Pharmacol. 119: 185-197; 1998.

Rogers, J.T.; Richards, J.G.; Wood, C.M. Ionoregulatory disruption as the acute toxic mechanism for lead in the rainbow trout (*Oncorhynchus mykiss*). Aquat Toxicol 64: 215-234; 2003.

Van Der Borght, O.; Van Puymbroeck, S. Calcium metabolism in a freshwater mollusc: quantitative importance of water and food as supply for calcium during growth. Nature 210: 791-793; 1966.

Figure legends

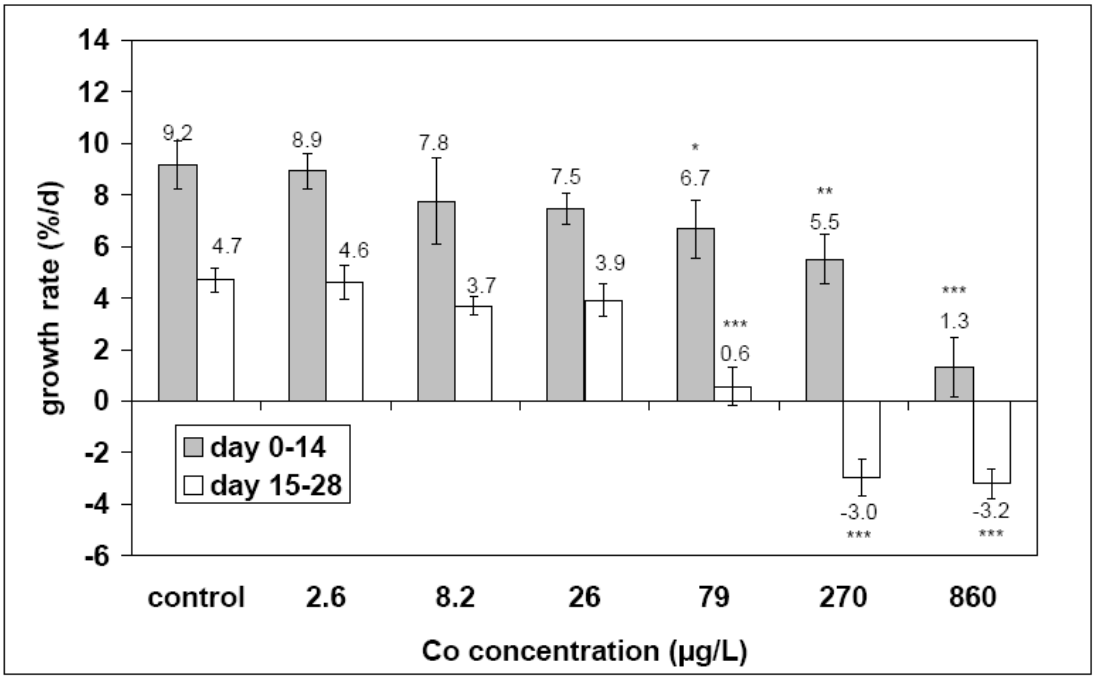
Figure 1 Growth rate of *Lymnaea stagnalis* at different Co concentrations during the initial (day 0-14) and final two weeks of the exposure (day 15-28). Means ± standard error of the mean are shown. Values above the bars are the actual growth rates. Significant differences with the control, according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

Figure 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 * for $p<0.05$

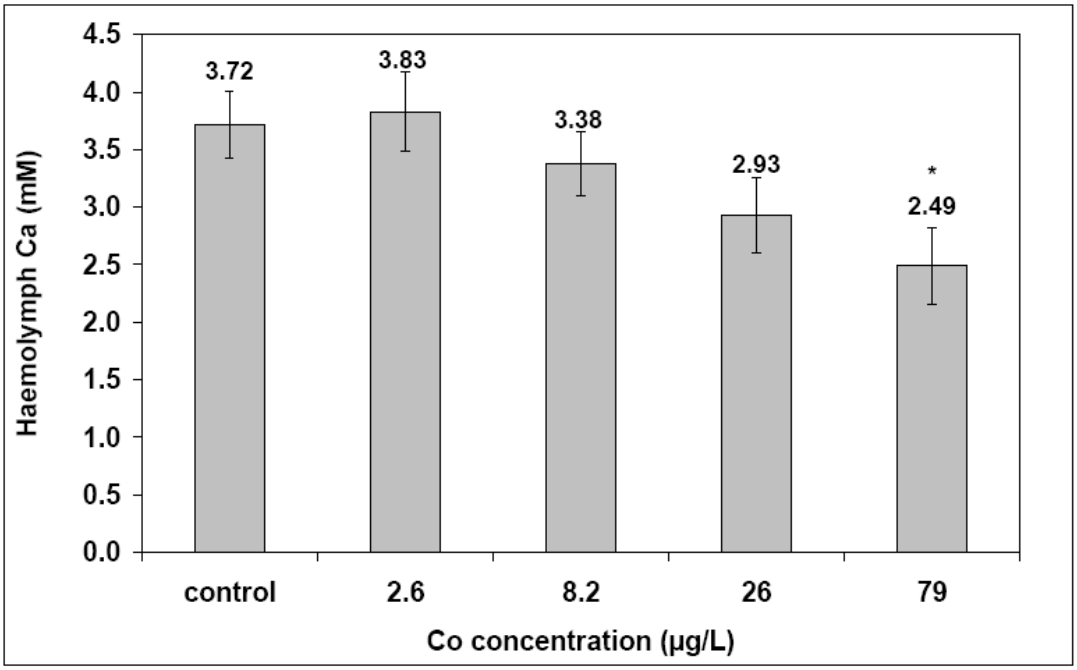
400 Table 1 Chemical parameters measured during the ecotoxicity tests with *Lymnaea*
401 *stagnalis*. Data are presented as mean ± standard deviation of all measured values (see
402 materials and methods; n=9).

Nominal Co (µg/L)	Measured dissolved Co (µg/L)	pH	DOC (mg/L) [1]
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]

403 [1] Dissolved organic carbon; reported DOC values are mean± standard deviation of
404 values measured in test solutions immediately before the medium was renewed (n=7).
405 DOC concentrations followed by the same letter are not significantly different from
406 each other (Sign test, $p<0.05$)



407
408 Figure 1



409
410 Figure 2