Do differences between metal body residues reflect the differences between effects for Chironomus riparius exposed to different sediments?

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Do differences between metal body residues reflect the differences between effects for *Chironomus riparius* exposed to different sediments ?

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ABSTRACT

Sediment characteristics are well known to interfere with toxicity, mainly through differences in terms of bioavailability. Here, with chironomids exposed to zinc in an artificial and a field sediment, we investigated the differences of zinc accumulation and of effects on the life cycle, at individual and population level. We used biology and energy-based modeling to analyze the data at all the levels of biological organization. This permits a reliable estimation of thresholds values. Bioavailability accounted for most of the differences between the results for both sediments (a factor of 11 for differences from 20 to 100 depending on the parameter which is considered). Taking into account bioavailability and background variability, the differences relative to thresholds could be accounted for. However, it appeared that, once the threshold was passed, effects were much more pronounced for organisms exposed to artificial sediment compared to field sediment. We concluded that some sediment characteristics can enhance toxicity, whatever the compound bioavailability, even if the latter was the major source of differences in our study.

Key words : *Chironomus riparius*, modeling, zinc, body residues, population, risk assessment.

INTRODUCTION

Confounding factors, like accumulation kinetics or bioavailability influence the interpretation of the results of bioassays, especially when these results are expressed in terms of exposure concentration (Driscoll and Landrum, 1997 ; Hwang et al., 2001). In a previous paper (Péry et al., in press), we used a mechanistic effects model to link effects and internal concentration to avoid these confounding factors. We analysed copper-spiked sediment growth tests with the midge *Chironomus riparius* to derive effects thresholds. No effect concentration expressed in term of exposure concentration differed substantially between sediments. In contrast, no effect concentration expressed in terms of body residues did not depend on the sediment characteristics.

However, sediment physicochemical characteristics, like particle size distribution or organic matter content, can also greatly influence survival, growth and reproduction of the midges and interfere with feeding levels apart from their influence on body residues. Ristola et al. (1999) showed that organic content was a confounding factor but not the only one. In their experiment with clean sediments, larvae grew best in sandy sediments with low organic carbon content (0.5 %). We also showed that, even in *ad libitum* feeding conditions, clean sediment characteristics had an effect on growth and reproduction (Péry et al., 2003a). There was a clear link between particle size distribution and growth (up to half a day of delay), and between lipid contents and reproduction (up to 25 % difference in fecundity).

Here, we plan to investigate the confounding influence of sediment characteristics on zinc toxicity at individual and population level. This change of scale permits to integrate in one parameter (here carrying capacity) all the effects observed at individual level. Comparisons are performed with effects parameters expressed in terms of body residues to assess any sediment confounding influence other than bioavailability. We should

consequently derive information about the necessity and the way to account for sediment characteristics in a risk assessment perspective.

Effects of zinc-spiked sediments (a field one and an artificial one with substantially different physicochemical characteristics) are studied on the life cycle of *C. riparius*. This species is commonly used to assess the toxicity of field or spiked sediments, mainly because it is common in the field at temperate latitudes and valuable for toxicity tests (Callaghan et al., 2002). Its life cycle comprises aquatic stages (egg, four larval instars within the sediment, pupa) and an aerial adult stage. Five tests are performed at individual level for both sediments: a growth test for second to fourth instars larvae in ad libitum conditions, a growth test for fourth instars larvae in food-limited conditions, a kinetics test for fourth instars larvae, a reproduction test. These tests are analysed with biology-based models we developed (Péry et al., 2003b ; Ducrot et al., 2004). The results are then used to derive effects at the population level. To achieve this, we use our model which is based on our biology-based models and allows the study of the effects of toxicants on the carrying capacity (Péry et al., 2004).

MATERIALS AND METHODS

Sediment spiking

We used two different sediments. The artificial sediment was silica sand with the following particle size distribution: 90 % between 50 and 200 µm, 10 % under 50 µm. 30 kg of sediments were maintained with 25 litres of water and a small amount of food (0.5 g Tetramin® fish food) for three weeks before spiking to allow bacterial development. The uncontaminated field sediment (Port-Galland) was taken in a tributary of the river Ain.

To spike zinc into sediment, we placed 1.98 kg wet sediment into 2 litre jars together with zinc (zinc sulfate heptahydrate) dissolved in 0.8 litres of water. Jars were then rolled during 6 hours, kept at test temperature and swirled manually each day for 10 days.

To test the efficiency of the spiking, zinc concentration of the compounds was measured in the spiked sediment and in the overlying water during the experiment at the last day. The measurements were performed by the Laboratoire Santé Environnement Hygiène, Lyon.

Growth and survival tests

The test beakers were prepared three days before the beginning of each test. They were filled with 0.1 litre sediment and 0.4 litre water (half from demineralised water and half from an uncontaminated spring near our laboratory; the pH is 8.1 and the conductivity 400 µS/cm). They were set in a water bath at 21°C with a 16:8h light:dark photoperiod and water was aerated. Using the results of previous tests, we chose exposure concentrations of 0, 700, 1400, 2100, 2800 and 3500 mg/kg for the field sediment, and 0, 20, 40, 80, 160 mg/kg for the artificial sediment. Tests organisms came from our laboratory culture. At day 0 of the test, 10 organisms were introduced randomly into each glass-beakers (9 per concentration). Three experiments were performed with respectively second (two days after hatching), third (four days after hatching) and fourth instars (six days after hatching) larvae. Instar was checked by measuring head capsule width. Midges were fed *ad libitum* each day with 0.6 mg Tetramin® fish food (Tetrawerke, Melle, Germany) per individual as advised in (Péry et al., 2003a). Conductivity, temperature, pH, dissolved oxygen, nitrate and ammonium concentrations were measured daily. Growth effects were assessed by measuring length daily during 3 days. Each day, we chose randomly three beakers per concentration for measurements, which corresponded to about 30 length measurements per data point. Length was measured using a

binocular microscope fitted with a calibrated eye-piece micrometer. Prior to measurements, organisms were killed using a solution of 20 % formaldehyde and 80% water. They were rinsed less than 10 seconds to avoid distortion of the shape.

An additional fourth instar growth test comparing *ad libitum* and food-limited conditions (0.1 mg food per larva per day) was performed with the artificial sediment to provide insights concerning the physiological mode of action of zinc (decrease of feeding rate or increase of growth costs) as presented previously (Péry et al., 2003b). We used the same concentrations as in the growth tests with 5 replicates per concentration and feeding level. Individuals were exposed at the beginning of fourth instar (6 days after hatching) during 4 days in *ad libitum* conditions and 5 days in food-limited conditions. In these conditions, if the physiological mode of action is decrease of feeding, a 60 % effect in *ad libitum* conditions would result in no effect in food-limited conditions. Indeed, as we showed in (Péry et al., 2003a), in food limited conditions, the time needed to incorporate food is not relevant to describe growth. What is relevant is the amount of food available. Consequently, if organisms exposed to zinc in food limited conditions can incorporate all the food available in 24 hours as the control organisms do, no difference relative to growth would be found between control and exposed organisms.

Reproduction tests

The assays were initiated with four days old larvae (end of the second larval instar). At day 0 of the test, 10 organisms were randomly introduced into each glass-beaker. There were 6 replicates per zinc concentration. We used exposure concentrations of 0, 350, 700, 1400, 2100 and 2800 for the field sediment, and 0, 20, 40, 80, 160 mg/kg for the artificial sediment. Test beakers were set in the same conditions as previously. Feeding level was 0.6 mg per larva per day. The beakers were covered with a net trap to prevent adults from escaping. From

the very start of emergences, imagoes were daily withdrawn from the test beakers by aspiration and counted. Females emerging from each replicate corresponding to a defined concentration were gathered into a bottle (Pyrex, one litre) containing a few quantity of water in order to receive the clutches and were immediately stored at 23°C after addition of two males per female, taken in the laboratory culture. Indeed, as emergence occurs earlier for males, not enough males were available from the test beakers to fertilize the emerging females. Clutches obtained in each bottle were daily removed. The number of eggs per egg-mass was then counted. Only one egg-mass can be laid down per female for *C. riparius*. Thus, the mean number of eggs per mass corresponds to a measurement of fecundity. Females of *C. riparius* remains receptive for reproduction during their whole life (Downe, 1973). Thus, the test ended when all females were dead (day 27 of the test in our case).

Kinetics tests

For each sediment, we followed zinc kinetics in fourth instar larvae for nominal concentrations 0, 700, 1400 and 2100 for the field sediment and 0, 20, 40 and 80 mg/kg for the artificial sediment. Only fourth instars larvae have sufficient dry weight to allow precise chemical measurements. Organisms were exposed to the sediments as described previously (10 organisms per beaker). Three groups of 10 organisms at day 0 and three beakers per concentration at day 1, 2 and 3, were randomly taken, then the larvae were let for ten minutes at room temperature in a 100 ml solution of 3×10^{-3} M EDTA to remove zinc from the surface of the organisms as we performed with copper in previous experiments (Péry et al., 2005). Afterwards, organisms were freezed at -80°C, freezed-dried and weighted. Before metal analysis, a hot acid mineralization was realised by add of acid nitric (1 ml by 0.5 g wet weight). After 12h 80°C, sample was adjust at a know volume (1 ml) with deionised water and analysed by flame atomic absorption spectrophotometer (AAS). Internal calibration was

equally applied with lobster hepatopancreas tissue (TORT-2, NRC-CNRC) as reference material (our value 176 ± 2 mg/kg; certified value 180 ± 6 mg/kg).

Data analysis

The kinetics of chemical compounds are assumed to follow a one-compartment model:

$$\frac{dci}{dt}(t) = ke \times (ce(t) - ci(t)), \qquad (1)$$

with *ke* the elimination rate, *ce(t)* (either in mg/l or in mg/kg) the exposure concentration and *ci(t)* (in the same units) the concentration in the tissue scaled by the Bioconcentration Factor. The scaled concentration *ci(t)* is thus proportional to the concentration in the tissue, but has the dimension of an external concentration. The initial internal concentration is estimated through measurements of organisms at the beginning of the tests.

The analysis of growth data is detailed in Péry et al. (2003b). In *ad libitum* conditions, growth was linear for each instar, with a growth rate depending on the instar:

$$dl^3 / dt \propto l^2 \Leftrightarrow dl / dt = a, \qquad (2)$$

where *l* is the mean length of the organisms, *a* a constant accounting for feeding rate and depending on the instar and on the sex, and where *dt* represents a one day-period.

Two possible modes of action are distinguished: decrease of feeding rate or increase of growth energetic costs. In case of an increase of the growth energy costs, these costs are multiplied by a factor $1 + b \times (ci(t) - NEC)$, as soon as *ci* exceeded the *NEC* (Péry et al., 2003), where *NEC* is the No Effect Concentration and *b* accounts for the level of toxicity once the internal concentration is exceeded. Equation (2) becomes then :

$$dl/dt = a/(1 + b \times (ci(t) - NEC)) \quad , \qquad (3)$$

In case of decrease of feeding rate, equation (2) becomes :

$$dl/dt = a \times (1 - b \times (ci(t) - NEC)) \, . \qquad (4)$$

To analyze reproduction data, two possible modes of action are distinguished: decrease of feeding rate or increase of reproduction energetic costs. In the case of feeding decrease, we assume that reduction of the feeding rate is proportional to (c_e - *NEC*). We do not use the kinetics module for we assume that when reaching the period of energetic investment into reproduction (by the end of fourth instar), internal concentration has already reached a plateau. The maximal amount of reproduction per female under *ad libitum* feeding conditions for a given toxicity level is:

$$N_{max} = N_{max0} \times (1 - b(c_e - NEC)) \, , \qquad (5)$$

where *b* accounts for the level of toxicity of the chemical for *C. riparius*. Similarly, in the case of increase of the amount of energy required to produce eggs :

$$N_{max} = \frac{N_{max0}}{1 + b(c_e - NEC)} \quad . \qquad (6)$$

The estimation of parameters was done using a software, named Chimotox, previously written in C++ and java to analyse data with *Chironomus riparius* (Péry et al., 2003b, Ducrot

et al., 2004). The statistical analysis in the software is based on the Maximum Log-likelihood theory and provides a 95% confidence interval for estimates of all the parameters.

Effects on population

The population modelling is detailed in (Péry et al., 2004). For a given feeding level and a given exposure concentration, we can calculate the growth pattern of an individual, the emergence of males and females and the mean number of eggs obtained for each female. The emergence times for males and females are used to calculate mating probabilities. For a given number of eggs at generation *x*, and a given feeding level *Q*, it is possible to predict the number of eggs at generation *x+1*. The increase of number of eggs is the population growth rate. Using a dichotomist method, our software calculate the minimum value feeding level *Qo* for which population growth rate is superior to 1. This corresponds to the equilibrium situation for the population. For the value *Qo*, we then calculate the number of adults produced per generation or per unity of time to account for the production of chironomids at population equilibrium, which we consider as a carrying capacity (the maximum population density of a given species in an area beyond which no significant increase can occur without damage occurring to the resources upon which the population depends). We then plot on a graph the carrying capacities obtained for the different exposure concentrations.

RESULTS

Chemical analysis

During all the experiments, temperature was constant (21+/-0.5°C) so as pH (between 8.1 and 8.4). Conductivity was between 350 and 450 µS/cm, and the percentage of dissolved oxygen was always above 90 %. Nitrate and ammonia level were always below 0.5 mg/l.

Zinc spiking efficiency was 75 % and 95 % respectively in the experiment with natural and artificial sediment with a good linear relationship between nominal and actual concentrations. We used measured values for the data analysis.

Kinetics

Control data were within the usual range for unexposed chironomids in our laboratory (150+/-50 mg/kg), although organisms exposed to control artificial sediment contained more zinc (196 +/- 15 mg/kg) than organisms exposed to control field sediment (120 +/- 40 mg/kg). We found kinetics to be extremely rapid, for the maximum body residues was already reached after one day of exposure in each experiment. We found bioconcentration factors of respectively 6.62 and 0.594 for artificial and field sediments respectively (Figure 1). There is thus a ratio of 11 between both bioconcentration factors accounting for substantial differences in terms of bioavailability.

Mode of action

Length at the beginning of the experiment was 6.26+/-0.52 mm. For both sediment, chironomids grew significantly during our experiments for all the concentrations (Student-t-tests, $p<0.01$). In *ad libitum* conditions, there was a significant difference between the results obtained for control and 40 mg/kg (Student-t-tests, $p<0.05$, 19 % of growth rate decrease) and a very significant difference between control data and those obtained for 80 mg/kg ($p<0.001$, 58 % of growth decrease). In food-limited conditions, there were respectively 8 % and 41 % of growth decrease compared to control, and effects were significant ($p<0.01$) for concentration 80 mg/kg. We consequently reject decrease of feeding as a potential physiological mode of action because, as written before, if this was the case, a 60 % effect in *ad libitum* conditions would result in no effect in food-limited conditions

Effects at individual level

There was no effect on survival for any larval instar. On the contrary, emergence success was affected (Figure 3). We fitted the data with the Hill equation (Garric et al., 1990):

$$p = \frac{x^{nH}}{LC50^{nH} + x^{nH}} \quad (6),$$

where p is the percentage of effects, x the concentration, *LC50* the concentration leading to 50 % of effects, and *nH* the Hill number. This equation is a log-normal statistical model. *LC50* values were 38.9 and 1588 mg/kg, *nH* values of 13.7 and 8.2 respectively for the artificial and the field sediment. There was a ratio of more than 40 between *LC50* values and Hill numbers, indicating a stronger effects of zinc when spiked to artificial sediment, compared to field sediments.

Table 1 presents the data analysis from the growth tests (*NEC* and *b* values and 95% confidence intervals). For a given sediment, effects were quite similar between instars, second instars larvae being just slightly more sensitive to zinc. In contrast, *NEC* values between sediments are in a ratio between 19 and 25, and *b* values in a ratio from 48 to 100.

As for reproduction (Figure 4), we found *NEC* values of 24.7 and 0 mg/kg with 95 % confidence interval of [0 ; 33] and [0 ; 300], *b* values of 0.0253 and 0.00053 respectively for artificial and field sediment. In each case, 0 mg/kg was not among the rejected values for threshold of effects. We obtained a ratio of 47.7 between *b* values.

Effects at population level

The translation at population level is given by Figure 5. For a given body residue, effects appear to be more pronounced for organisms exposed to artificial sediments compared to field sediments. Moreover, the decrease of the carrying capacity is more rapid in artificial sediments. Population extinction occurs at respective concentrations of 350 and 1050 mg/kg

for artificial and field sediments. Beginning of carrying capacity decrease is around respective values of 150 and 350 mg/kg. There is thus a ratio from 2 to 3 between the effects at population level expected for both sediments.

DISCUSSION

In the present study, we were able to model effects of zinc at individual and population level for the species *C. riparius* at individual and population level. This modeling was greatly helped by the assessment of zinc physiological mode of action. As copper (Péry et al., 2004), zinc has a direct effect on growth. Part of the assimilated energy is consequently wasted due to zinc exposure.

We found very substantial differences between the results for the artificial and the field sediment. *NEC* values vary with a factor of about 20, whereas the values accounting for the level of effects (*b* values or *LC50*) vary in factor from 40 to 100. Differences relative to accumulation explain a large part of the difference (a factor of 11). In the same way, Burton et al. (2005) showed that accounting for acid volatile sulphide (AVS) and organic matter as traps for metals could considerably increase the relevance of zinc toxicity assessment for sediments. If now we take body residues into account, the differences between *NEC* values and between *b* values are respectively around 2 and from 4 to 10. In our study this resulted in differences at population level from 2 to 3.

The internal metal concentration taken into account in this study correspond to the total metal body burden. So, the accumulation trace metal in an aquatic invertebrate can therefore be interpreted in terms of two categories – firstly metal in metabolically available form, and secondly metal that has been detoxified and is no longer available to play any role in metabolism essential or deleterious (Rainbow, 2002). Metal detoxification can take place

by way of binding to inducible metal-binding proteins (i.e. Metallothioneins) or through the precipitation of metal into insoluble concretions (Brown, 1982; Roesijadi, 1992). Several authors had proposedcompartmentalization procedure for determining the subcellular partitioning (Mouneyrac et al, 1998; Wallace et al, 2003) in which the metabolically available form or metal sensitive fraction (metals associated with organelles and enzymes) correspond principally to metal concentration in the cytosol fraction except metal bind to metallothionein protein pool. In this case, the difference recorded in this study concerning the NEC estimation could be reduced with more relevant measurements of the body residues, by accounting for cytosolic part of the compound.

It is also important to realize that usual variability between similar experiments with the midge *Chironomus riparius* has been within a factor of 3 in our laboratory. This statement corresponds in particular to our data from control charts with more than 40 tests (96-hours survival tests) with chironomids exposed to copper. In the same way, the differences in terms of body residues of control organisms in the present study is of a factor 1.6.

However, even if the differences between NEC values may be accounted for with kinetics and usual variability, once the threshold is passed, effects are much more pronounced for organisms exposed to artificial sediment compared to field sediment. Indeed, the differences observed for parameters *b* or *LC50* remain significant. For instance, emergence mortality was higher for artificial sediment. Moreover, for concentrations above 40 mg/kg, the emerging males were green and their body were twisted. Only a few emerging males were in such a state when exposed to zinc-spiked field sediment. Clearly, sediment characteristics (we still do not know which ones) can enhance toxicity, whatever the compound bioavailability, even if the latter is the major source of variability. Here the other sources led to differences of minimum 2 (the minimum ratio between differences between *b* and *NEC* values) to 9 (the maximum ratio between *b* values when accounting for the differences

relative to kinetics), and the differences between body residues leading to similar effects at population level were within a factor 3.

Our study confirms that accounting for body residues substantially reduces variability between toxicity tests and the level of safety factors necessary in risk assessment. However, this does not make the safety factors obsolete, because other sediment characteristics than the ability to capture compounds are probably involved in toxicity.

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Figure 1. Relationship between exposure concentration and body residues (in mg/kg) :upper figure: artificial sediment, lower figure: field sediment.

Figure 2. Relationship between length and zinc concentration for varying feeding diets in the physiological mode of action test.

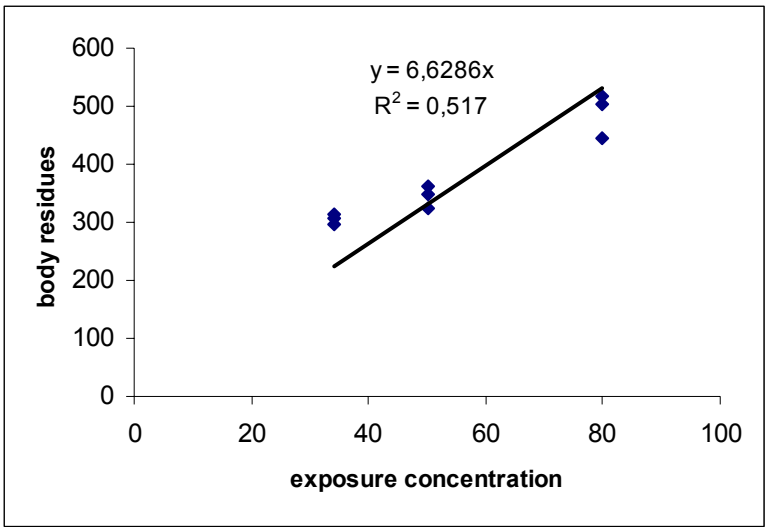
Figure 3. Emergence success as a function of concentration (upper figure: artificial sediment, lower figure: field sediment). The symbol * accounts for significant difference compared to control ($p<0.05$).

Figure 4. Fecundity (number of eggs per mass) as a function of concentration (upper figure: artificial sediment, lower figure: field sediment). The line accounts for model description.

Figure 5. Carrying capacity as a function of zinc concentration inside the organisms (Plain line: field spiked-sediment, dotted line : artificial spiked sediment).

Table 1. Toxicity parameters from the growth data analysis as a function of sediment type and larval instar.

Instar	Sediment	*NEC* value and confidence interval	*b* value
Second	Natural	695 [560 ; 785]	0.0020
Third	Natural	900 [550 ; 1100]	0.0012
Fourth	Natural	900 [800 ; 950]	0.0012
Second	Artificial	27.5 [14 ; 32]	0.097
Third	Artificial	45.8 [24 ; 39]	0.116
Fourth	Artificial	45.8 [24 ; 39]	0.116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body residues
exposure concentration
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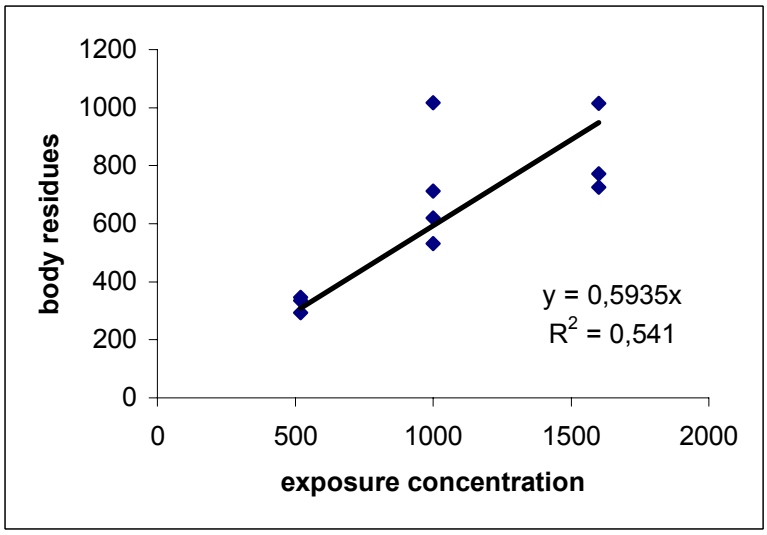
200
300
400
500
600
0
20
40
60
80
100

1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body residues
exposure concentration
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200
400
600
800
1000
1200
0
500
1000
1500
2000

2 Figure 1.

3

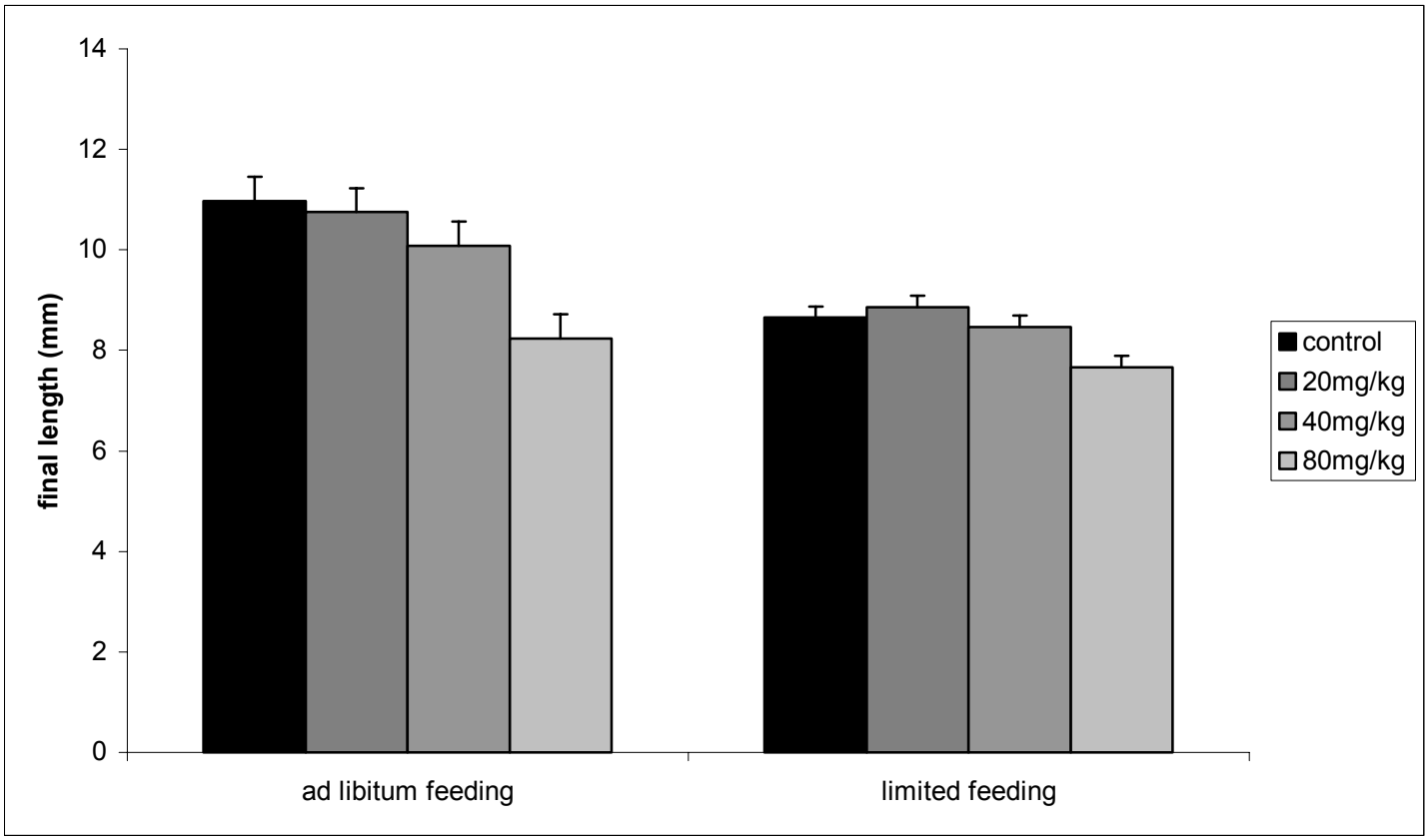
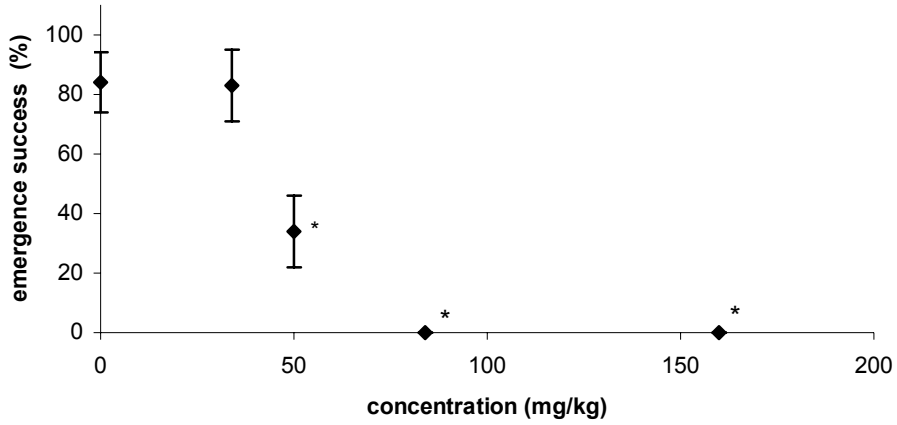

14
12
10
8
6
4
2
0
final length (mm)
ad libitum feeding
limited feeding
control
20mg/kg
40mg/kg
80mg/kg

Figure 2.



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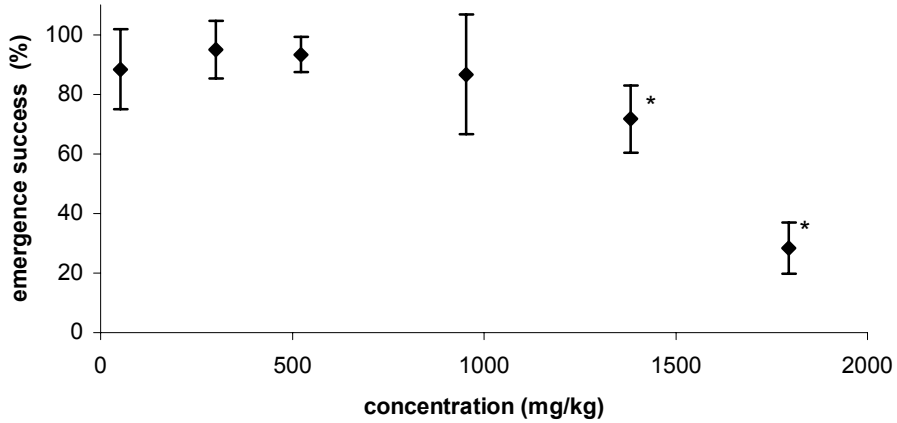

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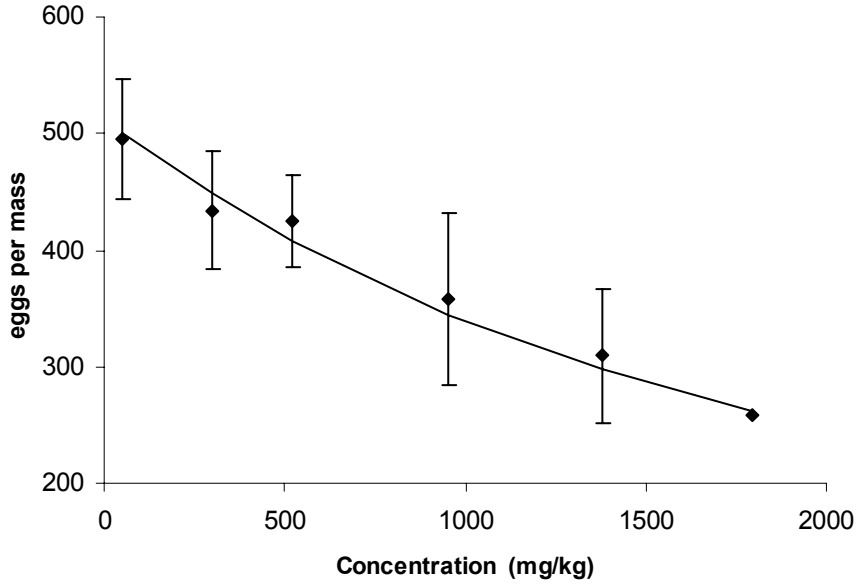
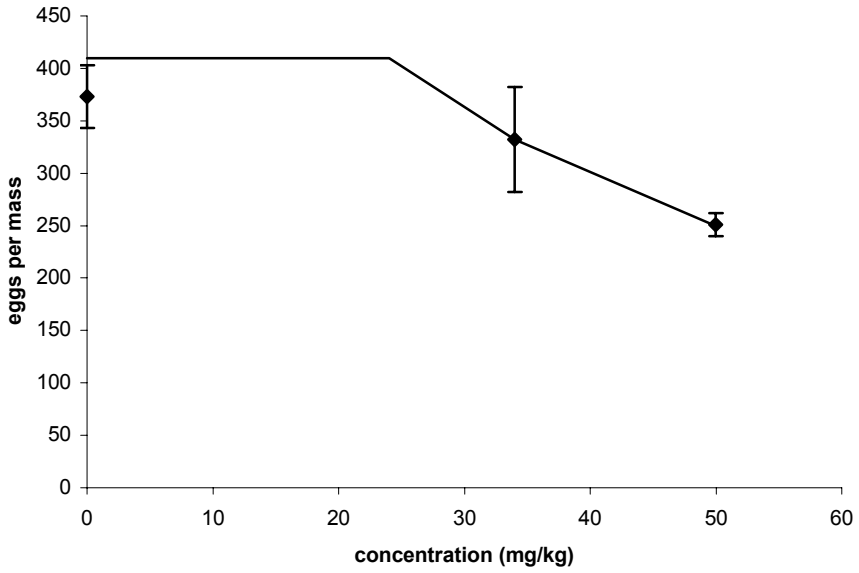
3

4 Figure 3.

5



1



2

3 Figure 4.

4

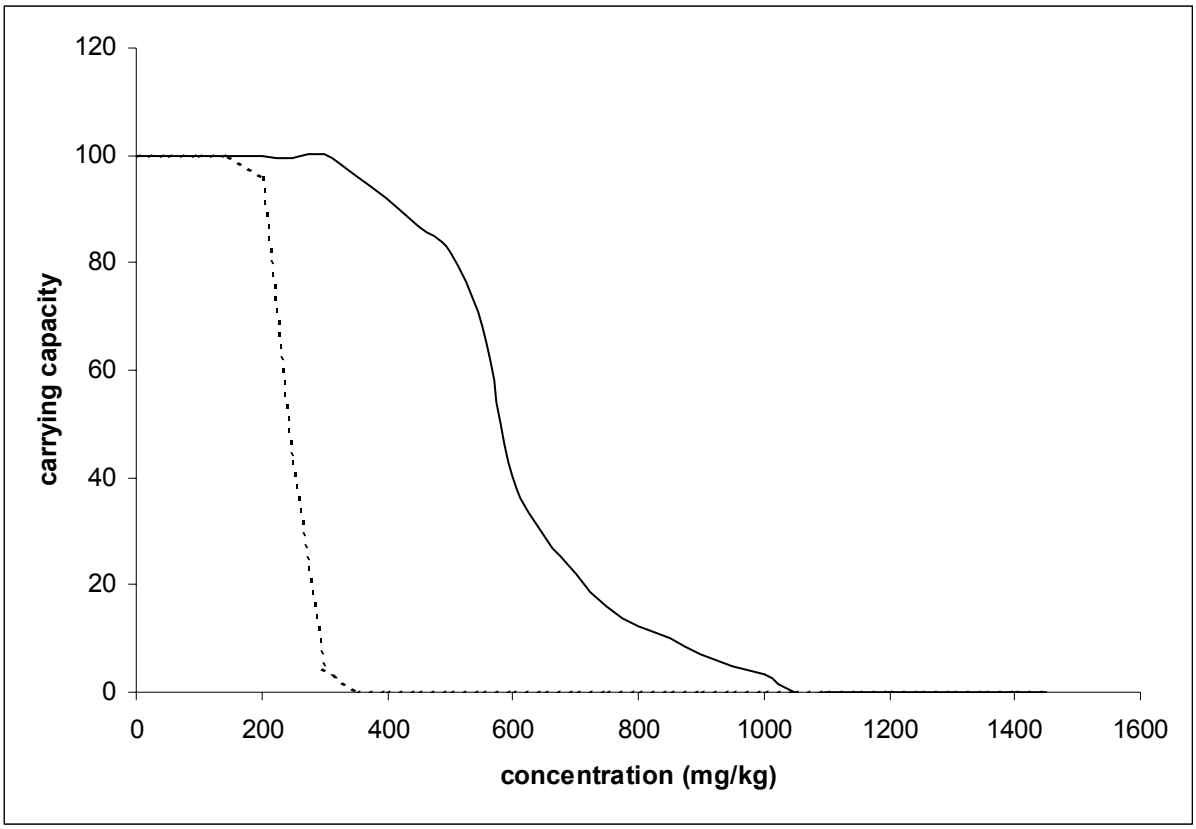

120
100
80
60
40
20
0
carrying capacity
0
200
400
600
800
1000
1200
1400
1600
concentration (mg/kg)

Figure 5.