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

3

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12

1 **ABSTRACT**

2

3 Sediment characteristics are well known to interfere with toxicity, mainly through

4 differences in terms of bioavailability. Here, with chironomids exposed to zinc in an artificial

5 and a field sediment, we investigated the differences of zinc accumulation and of effects on

6 the life cycle, at individual and population level. We used biology and energy-based modeling

7 to analyze the data at all the levels of biological organization. This permits a reliable

8 estimation of thresholds values. Bioavailability accounted for most of the differences between

9 the results for both sediments (a factor of 11 for differences from 20 to 100 depending on the

10 parameter which is considered). Taking into account bioavailability and background

11 variability, the differences relative to thresholds could be accounted for. However, it appeared

12 that, once the threshold was passed, effects were much more pronounced for organisms

13 exposed to artificial sediment compared to field sediment. We concluded that some sediment

14 characteristics can enhance toxicity, whatever the compound bioavailability, even if the latter

15 was the major source of differences in our study.

16

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

18

1 INTRODUCTION

2
3 Confounding factors, like accumulation kinetics or bioavailability influence the
4 interpretation of the results of bioassays, especially when these results are expressed in terms
5 of exposure concentration (Driscoll and Landrum, 1997 ; Hwang et al., 2001). In a previous
6 paper (Péry et al., in press), we used a mechanistic effects model to link effects and internal
7 concentration to avoid these confounding factors. We analysed copper-spiked sediment
8 growth tests with the midge *Chironomus riparius* to derive effects thresholds. No effect
9 concentration expressed in term of exposure concentration differed substantially between
10 sediments. In contrast, no effect concentration expressed in terms of body residues did not
11 depend on the sediment characteristics.

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

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

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

3 Effects of zinc-spiked sediments (a field one and an artificial one with substantially
4 different physicochemical characteristics) are studied on the life cycle of *C. riparius*. This
5 species is commonly used to assess the toxicity of field or spiked sediments, mainly because it
6 is common in the field at temperate latitudes and valuable for toxicity tests (Callaghan et al.,
7 2002). Its life cycle comprises aquatic stages (egg, four larval instars within the sediment,
8 pupa) and an aerial adult stage. Five tests are performed at individual level for both
9 sediments: a growth test for second to fourth instars larvae in ad libitum conditions, a growth
10 test for fourth instars larvae in food-limited conditions, a kinetics test for fourth instars larvae,
11 a reproduction test. These tests are analysed with biology-based models we developed (Péry
12 et al., 2003b ; Ducrot et al., 2004). The results are then used to derive effects at the population
13 level. To achieve this, we use our model which is based on our biology-based models and
14 allows the study of the effects of toxicants on the carrying capacity (Péry et al., 2004).

15

16 **MATERIALS AND METHODS**

17

18 *Sediment spiking*

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

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

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

8 9 *Growth and survival tests*

10 The test beakers were prepared three days before the beginning of each test. They
11 were filled with 0.1 litre sediment and 0.4 litre water (half from demineralised water and half
12 from an uncontaminated spring near our laboratory; the pH is 8.1 and the conductivity 400
13 $\mu\text{S}/\text{cm}$). They were set in a water bath at 21°C with a 16:8h light:dark photoperiod and water
14 was aerated. Using the results of previous tests, we chose exposure concentrations of 0, 700,
15 1400, 2100, 2800 and 3500 mg/kg for the field sediment, and 0, 20, 40, 80, 160 mg/kg for the
16 artificial sediment. Tests organisms came from our laboratory culture. At day 0 of the test, 10
17 organisms were introduced randomly into each glass-beakers (9 per concentration). Three
18 experiments were performed with respectively second (two days after hatching), third (four
19 days after hatching) and fourth instars (six days after hatching) larvae. Instar was checked by
20 measuring head capsule width. Midges were fed *ad libitum* each day with 0.6 mg Tetramin®
21 fish food (Tetrawerke, Melle, Germany) per individual as advised in (Péry et al., 2003a).
22 Conductivity, temperature, pH, dissolved oxygen, nitrate and ammonium concentrations were
23 measured daily. Growth effects were assessed by measuring length daily during 3 days. Each
24 day, we chose randomly three beakers per concentration for measurements, which
25 corresponded to about 30 length measurements per data point. Length was measured using a

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

4 An additional fourth instar growth test comparing *ad libitum* and food-limited
5 conditions (0.1 mg food per larva per day) was performed with the artificial sediment to
6 provide insights concerning the physiological mode of action of zinc (decrease of feeding rate
7 or increase of growth costs) as presented previously (Péry et al., 2003b). We used the same
8 concentrations as in the growth tests with 5 replicates per concentration and feeding level.
9 Individuals were exposed at the beginning of fourth instar (6 days after hatching) during 4
10 days in *ad libitum* conditions and 5 days in food-limited conditions. In these conditions, if the
11 physiological mode of action is decrease of feeding, a 60 % effect in *ad libitum* conditions
12 would result in no effect in food-limited conditions. Indeed, as we showed in (Péry et al.,
13 2003a), in food limited conditions, the time needed to incorporate food is not relevant to
14 describe growth. What is relevant is the amount of food available. Consequently, if organisms
15 exposed to zinc in food limited conditions can incorporate all the food available in 24 hours as
16 the control organisms do, no difference relative to growth would be found between control
17 and exposed organisms.

18

19 *Reproduction tests*

20 The assays were initiated with four days old larvae (end of the second larval instar). At
21 day 0 of the test, 10 organisms were randomly introduced into each glass-beaker. There were
22 6 replicates per zinc concentration. We used exposure concentrations of 0, 350, 700, 1400,
23 2100 and 2800 for the field sediment, and 0, 20, 40, 80, 160 mg/kg for the artificial sediment.
24 Test beakers were set in the same conditions as previously. Feeding level was 0.6 mg per
25 larva per day. The beakers were covered with a net trap to prevent adults from escaping. From

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

12

13 *Kinetics tests*

14 For each sediment, we followed zinc kinetics in fourth instar larvae for nominal
15 concentrations 0, 700, 1400 and 2100 for the field sediment and 0, 20, 40 and 80 mg/kg for
16 the artificial sediment. Only fourth instars larvae have sufficient dry weight to allow precise
17 chemical measurements. Organisms were exposed to the sediments as described previously
18 (10 organisms per beaker). Three groups of 10 organisms at day 0 and three beakers per
19 concentration at day 1, 2 and 3, were randomly taken, then the larvae were let for ten minutes
20 at room temperature in a 100 ml solution of 3×10^{-3} M EDTA to remove zinc from the surface
21 of the organisms as we performed with copper in previous experiments (Péry et al., 2005).
22 Afterwards, organisms were freezed at -80°C, freezed-dried and weighted. Before metal
23 analysis, a hot acid mineralization was realised by add of acid nitric (1 ml by 0.5 g wet
24 weight). After 12h 80°C, sample was adjust at a know volume (1 ml) with deionised water
25 and analysed by flame atomic absorption spectrophotometer (AAS). Internal calibration was

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

3

4 *Data analysis*

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

6

$$7 \quad \frac{dci}{dt}(t) = ke \times (ce(t) - ci(t)), \quad (1)$$

8

9 with ke the elimination rate, $ce(t)$ (either in mg/l or in mg/kg) the exposure concentration and
10 $ci(t)$ (in the same units) the concentration in the tissue scaled by the Bioconcentration Factor.

11 The scaled concentration $ci(t)$ is thus proportional to the concentration in the tissue, but has
12 the dimension of an external concentration. The initial internal concentration is estimated
13 through measurements of organisms at the beginning of the tests.

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

16

$$17 \quad dl^3 / dt \propto l^2 \Leftrightarrow dl / dt = a, \quad (2)$$

18

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

21 Two possible modes of action are distinguished: decrease of feeding rate or increase of
22 growth energetic costs. In case of an increase of the growth energy costs, these costs are
23 multiplied by a factor $1 + b \times (ci(t) - NEC)$, as soon as ci exceeded the NEC (Péry et al.,
24 2003), where NEC is the No Effect Concentration and b accounts for the level of toxicity once
25 the internal concentration is exceeded. Equation (2) becomes then :

1

$$2 \quad dl / dt = a / (1 + b \times (ci(t) - NEC)) , \quad (3)$$

3

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

5

$$6 \quad dl / dt = a \times (1 - b \times (ci(t) - NEC)) . \quad (4)$$

7

8 To analyze reproduction data, two possible modes of action are distinguished:

9 decrease of feeding rate or increase of reproduction energetic costs. In the case of feeding

10 decrease, we assume that reduction of the feeding rate is proportional to $(c_e - NEC)$. We do

11 not use the kinetics module for we assume that when reaching the period of energetic

12 investment into reproduction (by the end of fourth instar), internal concentration has already

13 reached a plateau. The maximal amount of reproduction per female under *ad libitum* feeding

14 conditions for a given toxicity level is:

15

$$16 \quad N_{\max} = N_{\max 0} \times (1 - b(c_e - NEC)) , \quad (5)$$

17

18 where b accounts for the level of toxicity of the chemical for *C. riparius*. Similarly, in the

19 case of increase of the amount of energy required to produce eggs :

20

$$21 \quad N_{\max} = \frac{N_{\max 0}}{1 + b(c_e - NEC)} . \quad (6)$$

22

23 The estimation of parameters was done using a software, named Chimotox, previously

24 written in C++ and java to analyse data with *Chironomus riparius* (Péry et al., 2003b, Ducrot

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

3 4 *Effects on population*

5 The population modelling is detailed in (Péry et al., 2004). For a given feeding level
6 and a given exposure concentration, we can calculate the growth pattern of an individual, the
7 emergence of males and females and the mean number of eggs obtained for each female. The
8 emergence times for males and females are used to calculate mating probabilities. For a given
9 number of eggs at generation x , and a given feeding level Q , it is possible to predict the
10 number of eggs at generation $x+1$. The increase of number of eggs is the population growth
11 rate. Using a dichotomist method, our software calculate the minimum value feeding level Q_0
12 for which population growth rate is superior to 1. This corresponds to the equilibrium
13 situation for the population. For the value Q_0 , we then calculate the number of adults
14 produced per generation or per unity of time to account for the production of chironomids at
15 population equilibrium, which we consider as a carrying capacity (the maximum population
16 density of a given species in an area beyond which no significant increase can occur without
17 damage occurring to the resources upon which the population depends). We then plot on a
18 graph the carrying capacities obtained for the different exposure concentrations.

19 20 **RESULTS**

21 22 *Chemical analysis*

23 During all the experiments, temperature was constant (21 \pm 0.5°C) so as pH (between
24 8.1 and 8.4). Conductivity was between 350 and 450 μ S/cm, and the percentage of dissolved
25 oxygen was always above 90 %. Nitrate and ammonia level were always below 0.5 mg/l.

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

4 5 *Kinetics*

6 Control data were within the usual range for unexposed chironomids in our laboratory
7 (150 \pm 50 mg/kg), although organisms exposed to control artificial sediment contained more
8 zinc (196 \pm 15 mg/kg) than organisms exposed to control field sediment (120 \pm 40 mg/kg).
9 We found kinetics to be extremely rapid, for the maximum body residues was already reached
10 after one day of exposure in each experiment. We found bioconcentration factors of
11 respectively 6.62 and 0.594 for artificial and field sediments respectively (Figure 1). There is
12 thus a ratio of 11 between both bioconcentration factors accounting for substantial differences
13 in terms of bioavailability.

14 15 *Mode of action*

16 Length at the beginning of the experiment was 6.26 \pm 0.52 mm. For both sediment,
17 chironomids grew significantly during our experiments for all the concentrations (Student-t-
18 tests, $p < 0.01$). In *ad libitum* conditions, there was a significant difference between the results
19 obtained for control and 40 mg/kg (Student-t-tests, $p < 0.05$, 19 % of growth rate decrease) and
20 a very significant difference between control data and those obtained for 80 mg/kg ($p < 0.001$,
21 58 % of growth decrease). In food-limited conditions, there were respectively 8 % and 41 %
22 of growth decrease compared to control, and effects were significant ($p < 0.01$) for
23 concentration 80 mg/kg. We consequently reject decrease of feeding as a potential
24 physiological mode of action because, as written before, if this was the case, a 60 % effect in
25 *ad libitum* conditions would result in no effect in food-limited conditions

1

2 *Effects at individual level*

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

$$5 \quad p = \frac{x^{nH}}{LC50^{nH} + x^{nH}} \quad (6),$$

6 where p is the percentage of effects, x the concentration, $LC50$ the concentration leading to 50
7 % of effects, and nH the Hill number. This equation is a log-normal statistical model. $LC50$
8 values were 38.9 and 1588 mg/kg, nH values of 13.7 and 8.2 respectively for the artificial and
9 the field sediment. There was a ratio of more than 40 between $LC50$ values and Hill numbers,
10 indicating a stronger effects of zinc when spiked to artificial sediment, compared to field
11 sediments.

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

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

20

21 *Effects at population level*

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

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

4

5 **DISCUSSION**

6

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

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

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

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

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

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

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

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

7

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12

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- 14
- 15

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

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

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

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

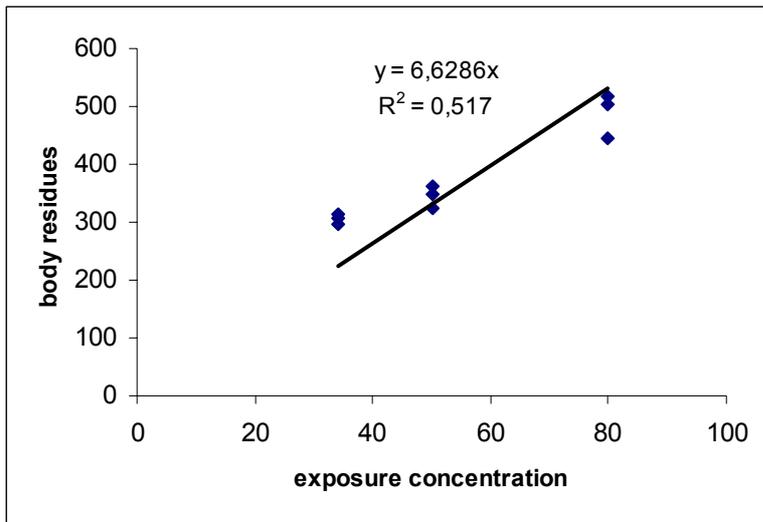
10 Figure 5. Carrying capacity as a function of zinc concentration inside the organisms (Plain
11 line: field spiked-sediment, dotted line : artificial spiked sediment).

12

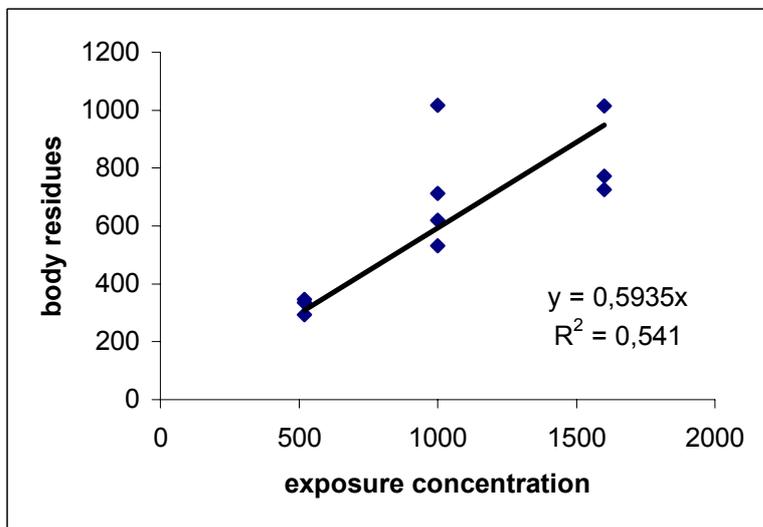
1 Table 1. Toxicity parameters from the growth data analysis as a function of sediment type and
 2 larval instar.

Instar	Sediment	<i>NEC</i> value and confidence interval	<i>b</i> 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

3

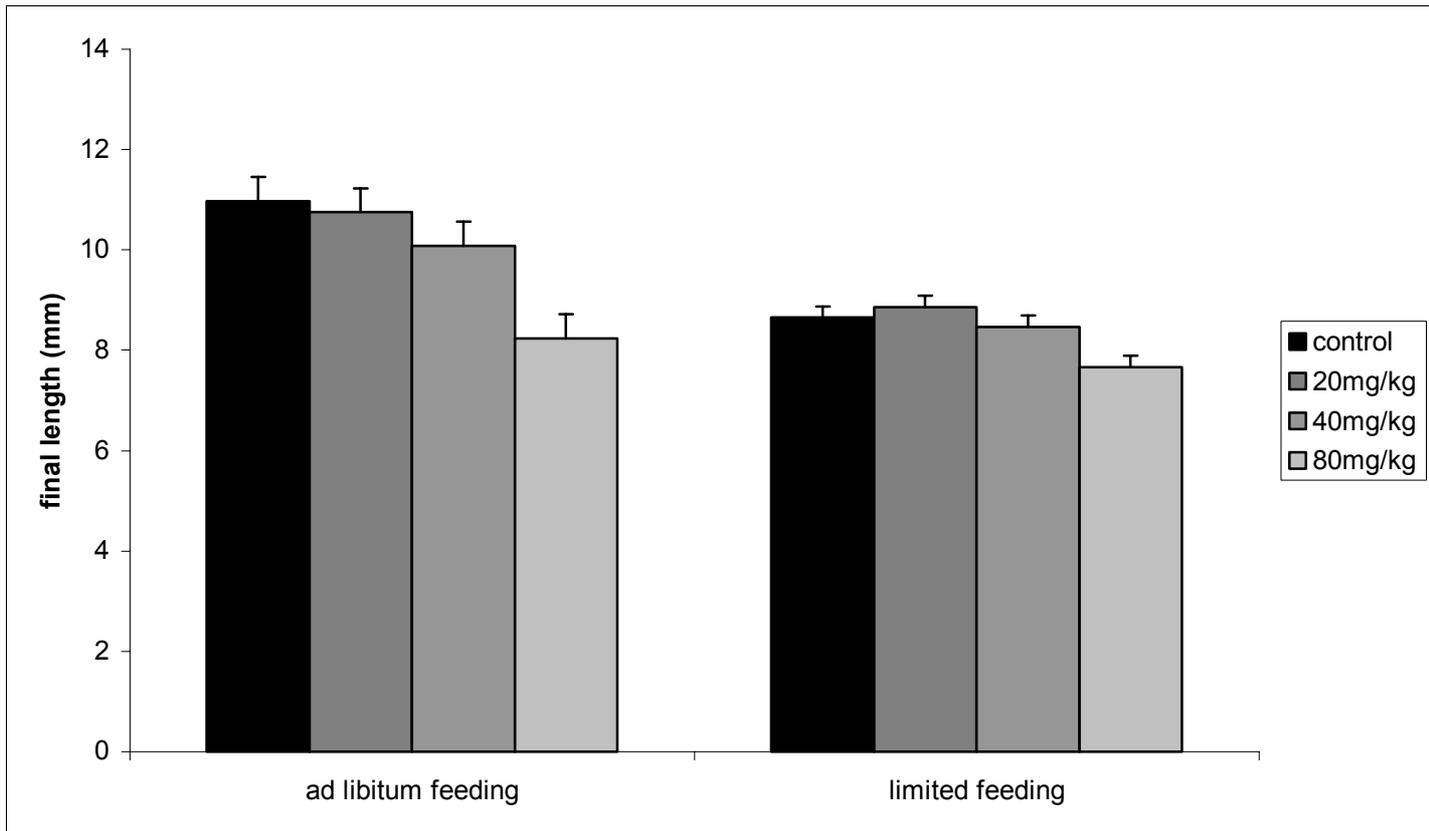


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2 Figure 1.

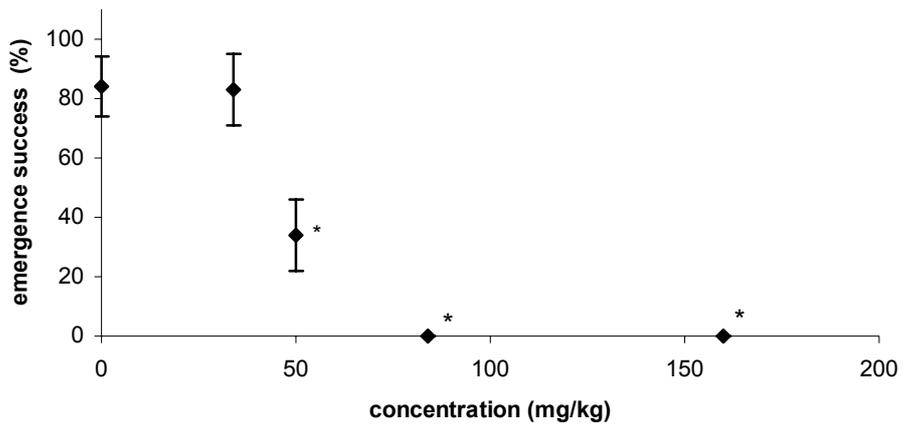
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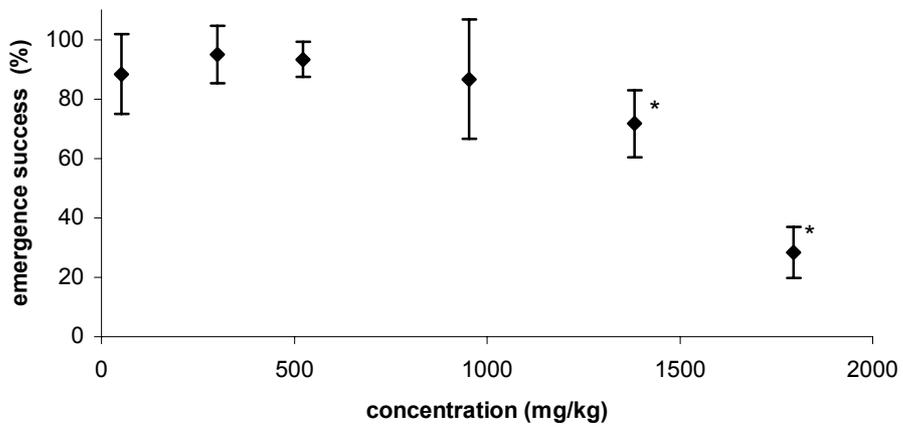
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2 Figure 2.

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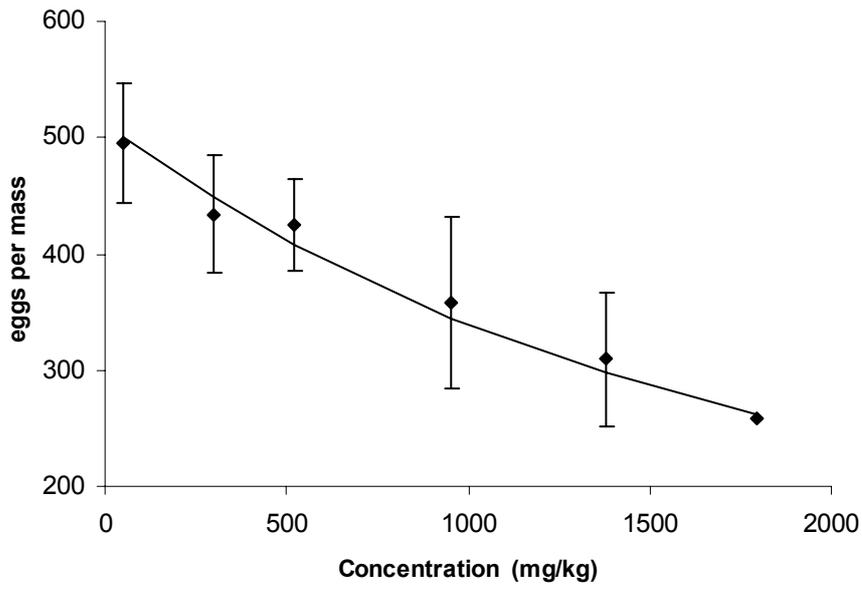


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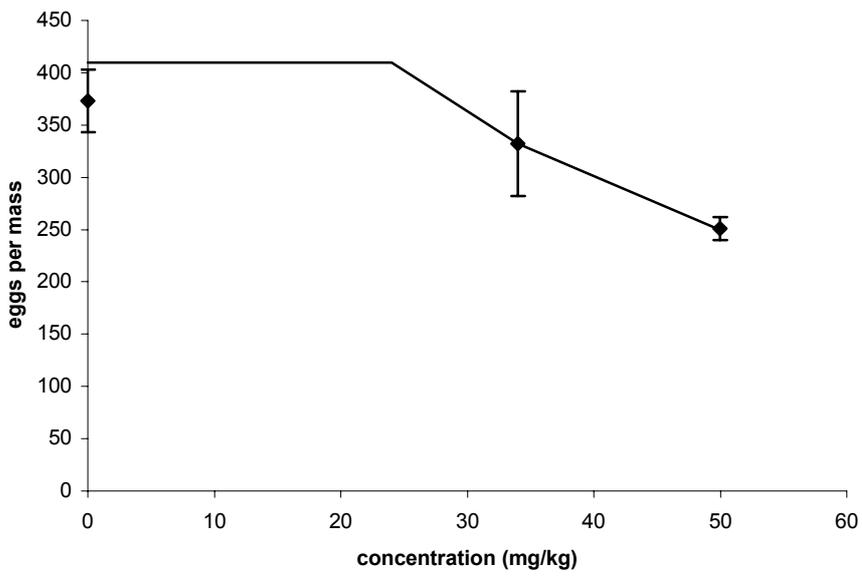
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4 Figure 3.

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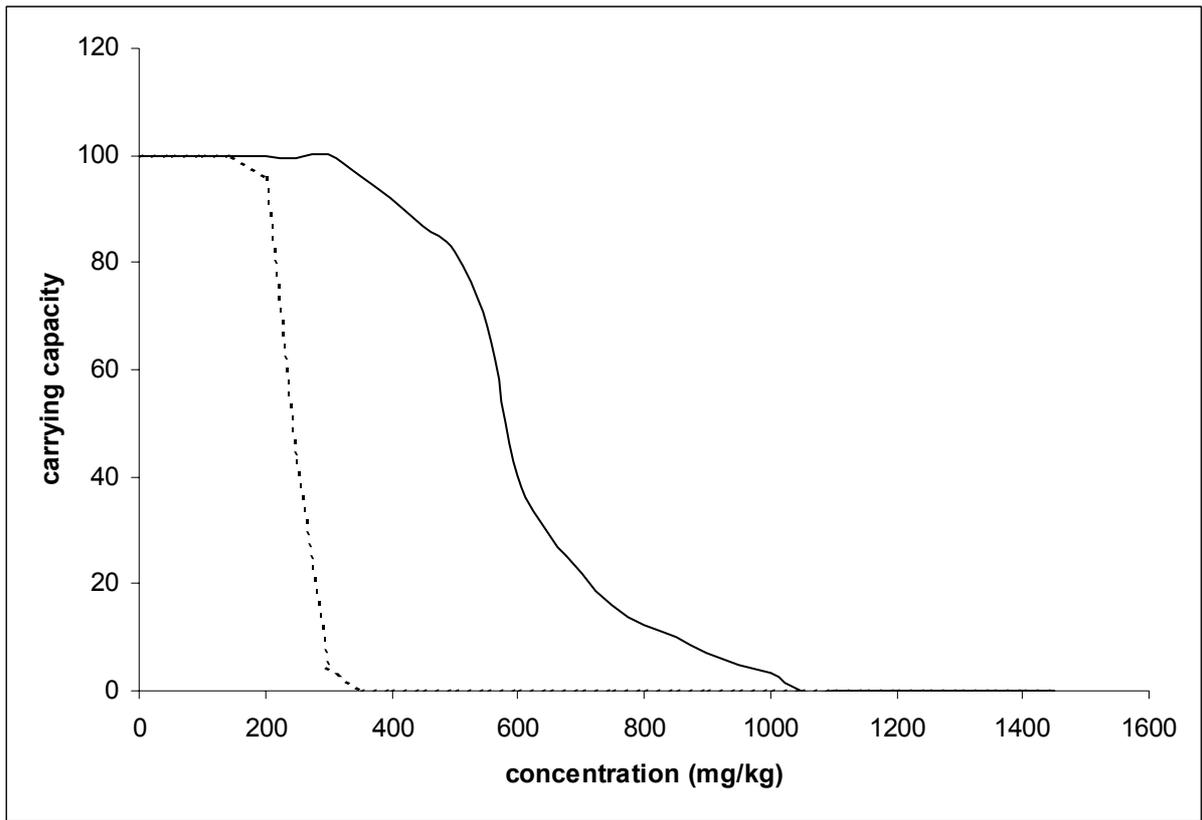
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3 Figure 4.

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2 Figure 5.