



# **ECO-LYME**

## **“Evaluating and improving the ecological relevance of *Lymnaea stagnalis* chronic metal toxicity estimates”**

### **Final report**

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## List of abbreviations

<b>ANOVA</b>	Analysis Of Variance
<b>BLM</b>	Biotic Ligand Model
<b>DOC</b>	Dissolved Organic Carbon
<b>EC<sub>x</sub></b>	Effect concentration resulting in a x% effect
<b>EQS</b>	Environmental Quality Standard
<b>EU</b>	European Union
<b>HC<sub>5</sub></b>	5 % Hazardous concentration
<b>ICP-MS</b>	Inductively coupled plasma mass spectrometry
<b>JFE</b>	Juvenile Feeding Experiment
<b>LC<sub>50</sub></b>	Lethal concentration resulting in 50% mortality
<b>LOEC</b>	Lowest Observed Effect Concentration
<b>NOEC</b>	No Observed Effect Concentration
<b>PNEC</b>	Predicted No Effect Concentration
<b>RAR</b>	Risk Assessment Report
<b>SD</b>	Standard Deviation
<b>SE</b>	Standard Error
<b>SEM</b>	Standard Error of the Mean
<b>SSD</b>	Species Sensitivity Distribution

## 1. Introduction

*Lymnaea stagnalis* appears to be a very widespread organism in Europe and frequently inhabits systems impacted by urban and agricultural activities (Loy et al., 2001), which suggests that they might be less sensitive to chemicals, including metals. However, based on laboratory ecotoxicity data, *Lymnaea stagnalis* is amongst the most sensitive organisms to Cu, Pb and Ni (Grosell et al., 2006; De Schampelaere et al., 2008; Schlekat et al., 2010 and Brix et al., 2011). It appears to be the number 1 in sensitivity rank for Ni, with a few exceptions for sensitive algae or higher plants at high pH levels. In this regard, *Lymnaea stagnalis* chronic toxicity data could be an important determining factor for the Species Sensitivity Distribution (SSD) based 5% hazardous concentration (HC<sub>5</sub>) estimates (and thus also for predicted no effect concentrations (PNEC) and Environmental Quality Standard (EQS) derivations), in that they “drive” the HC<sub>5</sub> to lower values.

Most chronic toxicity studies with freshwater snails are 14-day to 28-day juvenile growth studies in which lettuce is used as a food source. This may be a sub-optimal food source for juvenile snails. Moreover it has been demonstrated that juvenile growth limitation may be common in many published ecotoxicity datasets as a consequence of this incompletely developed feeding apparatus. Mechanistic modeling suggests that toxicant sensitivity may be greatly overestimated compared to well-fed juvenile *L. stagnalis* (Zimmer et al. 2012). It has also been demonstrated in our lab (Nys, 2011) and by Zimmer et al. (2012) that fish flakes provide a protein-rich diet that ensures a ‘normal’ unlimited, Von Bertalanfy growth pattern over the entire life-cycle, thus including the juvenile stage where the feeding apparatus is underdeveloped.

Within the overall aim to investigate the ecological relevance of the apparent hypersensitivity of *Lymnaea stagnalis* to copper as found by Brix et al. (2011), the effect of food source (lettuce vs. fish flakes) on copper toxicity was investigated for 28 days.

## 2. Materials & Methods

### 2.1. Test conditions and experimental design

An artificial fresh water was used for testing of its chemical composition can be found in Table 1. The medium was produced in one batch, and stored in 10 L polyethylene vessels at  $4 \pm 0.5$  °C in the dark. Every vessel of medium was continuously aerated for at least 72 hours at 20°C before use in order to obtain equilibrium conditions and in order to reach test temperature. Each replicate consisted of one juvenile snail in one 250 mL polyethylene test container filled with 100 ml test medium with static renewal (De Schampelaere 2008). Medium was renewed every other day. The aimed oxygen concentration was  $> 6\text{mg L}^{-1}$  in new and old medium. The aimed conductivity was  $600 \pm 200 \mu\text{S cm}^{-1}$  in new and old medium and the target pH for testing was  $8.0 \pm 0.5$ .

**Table 1.** Chemical composition of the medium used for testing.

Substance	Concentration
CaCl <sub>2</sub>	1 mmol L <sup>-1</sup>
MgCl <sub>2</sub>	0.4 mmol L <sup>-1</sup>
NaHCO <sub>3</sub>	3 mmol L <sup>-1</sup>
K <sub>2</sub> SO <sub>4</sub>	0.15 mmol L <sup>-1</sup>
DOC <sup>1,2</sup>	5 mg L <sup>-1</sup>

<sup>1</sup>Dissolved Organic Carbon

<sup>2</sup> Aldrich Humic Acid (AHA) was used as the DOC source

Ten nominal copper concentrations (in  $\mu\text{g L}^{-1}$ ) were tested (0, 2, 4, 8, 16, 32, 64, 128, 256, 512). Copper originated from a stock solution of CuCl<sub>2</sub> dissolved in deionized water. 7 replicates were used for each treatment (combination of copper concentration and food source). In total 140 organisms were tested. All replicates were randomized prior to the test and were divided randomly over multiple plates. This fully randomized set up was maintained during the entire duration of the test.

In order to quantify a standardized ad libitum food requirement for lettuce and Tetramin a pre-test was performed. Lettuce treated snails received  $0.025 \text{ mg} \pm 0.0005$  wet weight lettuce from day 1 till day 14 and  $0.050 \pm 0.0005$  mg wet weight lettuce every 2 days from day 15 till day 28. The lettuce was perforated from fresh organic farmed salad leaves in circular pellets with a diameter of 1 cm. Fish flakes treated snails received  $0.002 \pm 0.0005$  mg dry weight of Tetramin from day 1 till day 14 and  $0.004 \pm 0.0005$  mg dry weight Tetramin from day 15 till day 28. Tetramin flakes were cut into squares of  $0.25 \text{ cm}^2$ . A light cycle of 12 hours of dark and 12 hours of light was maintained. The test was performed at  $20^\circ\text{C} \pm 1^\circ\text{C}$

## 2.2. Test organisms

Adult snails were obtained from an in-house culture. This culture originates from an in-house culture from INRA (Rennes, France) a French public research institute for agriculture. Our in house culture was maintained in flow through carbon-filtered Ghent city tap water (pH=8.0 ± 0.5, Conductivity = 480 ± 30 µs cm<sup>-1</sup>, CaCO<sub>3</sub> 280 ± 20 mg L<sup>-1</sup>, NO<sub>2</sub><sup>-</sup> <0.1 mg L<sup>-1</sup>, NO<sub>3</sub><sup>-</sup> 10 ± 4 mg L<sup>-1</sup> and NH<sub>4</sub><sup>+</sup> <0.2 mg L<sup>-1</sup> at 20 °C ± 1 °C. The culture was fed organic farmed lettuce. Hatchlings of similar age (0-24 hours) were used in the test. To ensure synchronized hatching egg masses deposited within a time frame of 24 hours were transferred from the main culture to large static-renewal petri dishes (150 x20mm) at 20°C in the decarbonized tap water under a 12D-12L light cycle. When juveniles started to hatch, already hatched individuals were removed from the petri dish. The remaining egg masses were left to hatch for 24 hours. These juveniles of 0-24 hours old and with a mean size and as standard deviation of 1445 µm ± 93 µm (n=10) were used for testing.

## 2.3. Physico-chemical analyses

Copper, DOC, Inorganic carbon, Cl<sup>-</sup>, NH<sub>4</sub><sup>+</sup>, SO<sub>2</sub><sup>-</sup> and cation samples were taken on day 0, 14 and 28. Copper, DOC and IC samples (filtered and unfiltered) were taken on day 0, 2, 10, 18 and 25. Copper samples were immediately acidified to 1% with HNO<sub>3</sub> (Normaton quality, VWR international, Belgium) after taking them. Filtered samples were filtered with a 0.45 µm filter (Gelman Sciences, Ann Arbor, MI, USA). Copper samples with nominal concentrations 2, 4, 8, 16 and 32 (diluted 2x) µg L<sup>-1</sup> were analyzed with a graphite furnace atom absorption spectroscope (GFS Furnace auto sampler, Thermo Fisher Scientific Inc. Waltham, MA, USA). Copper samples with nominal concentrations 64, 128, 265 and 512 µg L<sup>-1</sup> were measured by flame atomic absorbance spectroscopy (SPECTRAA800, Varian, Mulgrave). DOC and IC samples were analyzed with a total organic carbon analyzer (TOC-5000 Shimadzu, Duisburg, Germany). Cations samples (Na<sup>+</sup>, Mg<sup>2+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Fe, Zn, Cd and Pb) were analyzed with an ICP-MS (Agilent 7700x, Agilent technologies, Santa Clara, USA). Chloride and sulphate samples were taken at the start and at the end of the test, and were measured with spectrophotometry (Aquamate, Thermo Electron Corporation; Chloride: Merck, Spectroquant 1.14897.001; Sulphate: Merck, Spectroquant 1.14548.001). pH of both new and old medium was measured at each renewal with a pH glass electrode (P407, Consort).

#### *2.4. Measured endpoints*

Endpoints measured were: survival (0<sup>th</sup>, 6<sup>th</sup>, 17<sup>th</sup>, 25<sup>th</sup> and 28<sup>th</sup> day), shell length (0<sup>th</sup>, 6<sup>th</sup>, 17<sup>th</sup>, 25<sup>th</sup> and 28<sup>th</sup> day) and dry blotted weight (28<sup>th</sup> day). Shell length was measured on a stereo microscope (Olympus SZX10, Olympus, Tokyo, Japan) with the Olympus cellSens Dimension software. Using a thin soft paint brush individuals were placed with the opening of the concha up. Subsequently the concha was measured from top to bottom) using the cellSens Dimension software. During measurements with the stereo microscope it was also possible to determine the survival of each organism with higher certainty than with the naked eye. Dry blotted weight was measured on the 28<sup>th</sup> day by carefully blotting the snail on paper tissue and weighing it on an analytical balance (Metler Toledo AX105 Delta Range, Columbus, Switzerland).

#### *2.5. Data treatment*

Data matrix construction and growth rate calculation was done with Microsoft Excel (Microsoft, Redmond, WA, USA). Matrices for lettuce and Tetramin treatments were exported into a .txt file and imported in R (R Foundation for Statistical Computing, Vienna, Austria) for further analysis. Dose response curves were generated and EC<sub>x</sub> values were calculated in R using the drc-package. Model selection was based on the highest likelihood. Other models with a slightly lower likelihood were also fitted to the data in order to check for a possible better fit. However the models with highest likelihood always gave the best fit. Growth rates ( $\mu$ ) in day<sup>-1</sup> for all individuals were calculated as the slope of LN(length) in function of time. A log-logistic function with 5 parameters (equation 1) was fitted to the growth rates of the Tetramin treatment. The lower limit parameter (c) was fixed to 0 since the growth rate can't be negative. For the lettuce treatment, a Weibull function (equation 2.) with 3 parameters was fitted to the growth rate data. A log-logistic function with 4 parameters (equation 3) was fitted to the survival data of the lettuce treatment, and a Weibull function with 4 parameters (equation 4) was fitted to the survival data of the Tetramin treatment. Survival data followed a binomial distribution (0 = dead, 1= alive) thus 50% Lethality Concentration (LC<sub>50</sub>) values with their 95% confidence intervals were calculated using the Trimmed Spearman-Kärber method since the requirements for the Probit method and the untrimmed Spearman-Kärber Method were not met (OECD No. 54, 2006 and Hamilton et al., 1977). LC<sub>50</sub> values were thus derived from the true binomially distributed data. LC<sub>10</sub> and LC<sub>20</sub> values cannot be derived via the Trimmed Spearman-Kärber method. Differences between Effect Concentrations and LC<sub>50</sub> values were tested with a Wheeler ratio test (Wheeler et al., 2006). No Observed Effect Concentrations (NOECs) and Lowest Observed Effect Concentrations (LOECs) for growth and dry blotted weight endpoints were obtained by Analysis Of Variance (ANOVA) when the normality and the homogeneity of variance assumptions

were fulfilled. When these assumptions were not fulfilled a non-parametric Pairwise Wilcoxon Rank Sum Test was used. For ANOVA's pairwise comparisons between concentrations were obtained by using Tukey's Honestly Significant Difference test. In order to predict length, dry blotted weight, growth rate and survival a generalized linear model was constructed via linear regression using backwards selection using quadratic parameters and all interactions to start with. For the survival dataset the Jonckheere-Terpstra test was used to determine the NOEC and the LOEC values.

## 2.6. Data comparison

In order to compare our toxicity values to other toxicity values in the available literature toxicity values were extracted with xyExtract software (Wilton P. Silva, 2011) and normalized for copper bioavailability with the specific chronic daphnia magna copper BLM (De Schamphelaere et al. 2004; De Schamphelaere et al. 2006; Van Sprang et al. 2008) to the Rhine reference water (The Netherlands) with following physico-chemistry: pH= 7.8, DOC = 2.8 mg L<sup>-1</sup>, Na = 36.80 mg L<sup>-1</sup>, Mg = 10.90 mg L<sup>-1</sup>, K = 5.7 mg L<sup>-1</sup>, Ca = 68.90 mg L<sup>-1</sup>, Cl = 81.50 mg L<sup>-1</sup>, SO<sub>4</sub>= 51.90 mg L<sup>-1</sup> and CO<sub>3</sub> = 147.83 mg L<sup>-1</sup>.

Three-parameter Weibull function

$$y = k \cdot \exp(-\exp(\beta(\ln(x) - f))) \quad \text{eq. 1.}$$

Three-parameter log-logistic function

$$y = \frac{k}{1 + \exp(\beta(\ln(x) - \ln(f)))} \quad \text{eq. 2.}$$

Four-parameter Weibull function

$$y = c + (k - c) \cdot \exp(-\exp(\beta(\ln(x) - f))) \quad \text{eq. 3.}$$

Four-parameter log-logistic function

$$y = c + \frac{k - c}{1 + \exp(\beta(\ln(x) - \ln(f)))} \quad \text{eq. 4.}$$

Where:

- $k$  = value of the maximal response (control)
- $y$  = predicted response (length, growth rate, survival or dry blotted weight)
- $x$  = dissolved cu-concentration ( $\mu\text{g L}^{-1}$ )
- $\beta$  =slope parameter
- $c$  = lower limit
- $d$  = upper limit
- $f$  = the inflection point

### 3. Results

#### 3.1. Test conditions

Temperature was  $20 \pm 1$  °C ( $n=10$ ) and conductivity was  $488 \pm 43$   $\mu\text{s cm}^{-1}$  ( $n=10$ ) in both new and old medium. Mean DOC content between the Lettuce and Tetramin treatment differed slightly. A Wilcoxon test was performed, proving this difference significant. This difference can be explained by the fact that dry Tetramin flakes partly dissolve in the test medium, therefore adding more DOC to the medium. Nevertheless, the results of our test can simply be compared by normalizing the  $EC_x$  values (see table 9). Further physico- chemistry can be found in table 2, below.

**Table 2.** Physico-chemistry of the medium during the test for both treatments.

Food Source	pH (mg/L)	DOC <sup>1</sup> (mg/L)	Ca (mg/L)	Mg (mg/L)	Na (mg/L)	K (mg/L)	SO4 (mg/L)	Cl (mg/L)	DIC <sup>2</sup> (mg/L)
Lettuce	$8.0 \pm 0.2$	$5.8 \pm 0.9$	31.96	4.27	74.02	11.91	NR	NR	40.25
Tetramin	$7.9 \pm 0.1$	$6.5 \pm 1.3$	31.96	4.27	74.02	11.91	NR	NR	40.25

<sup>1</sup> DOC= Dissolved Organic Carbon (Aldrich Humic Acid was used as the DOC source)

<sup>2</sup> DIC= Dissolved Inorganic Carbon

### 3.2. Length and Dry blotted weight

A comparison of the controls of both food types are shown for length and dry blotted weight in Figure 1 and Figure 2. Length vs. time was plotted for all replicates grouped per treatment (see supportive information Figure 15 (lettuce) and Figure 16 (Tetramin)).

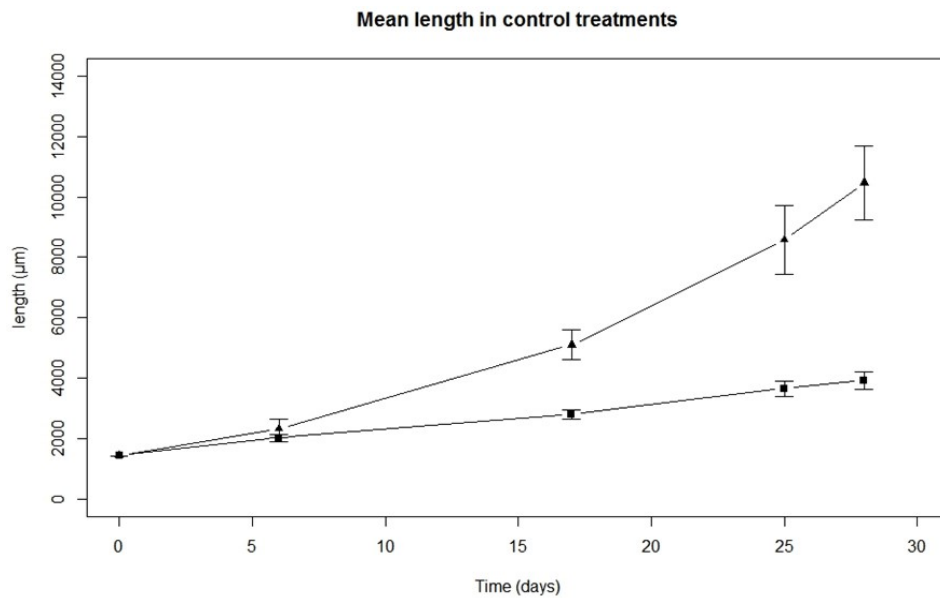


Fig. 1. Mean length in time with standard deviation flags of the control treatments of the lettuce treatment (black squares) and the Tetramin treatment (black triangles).

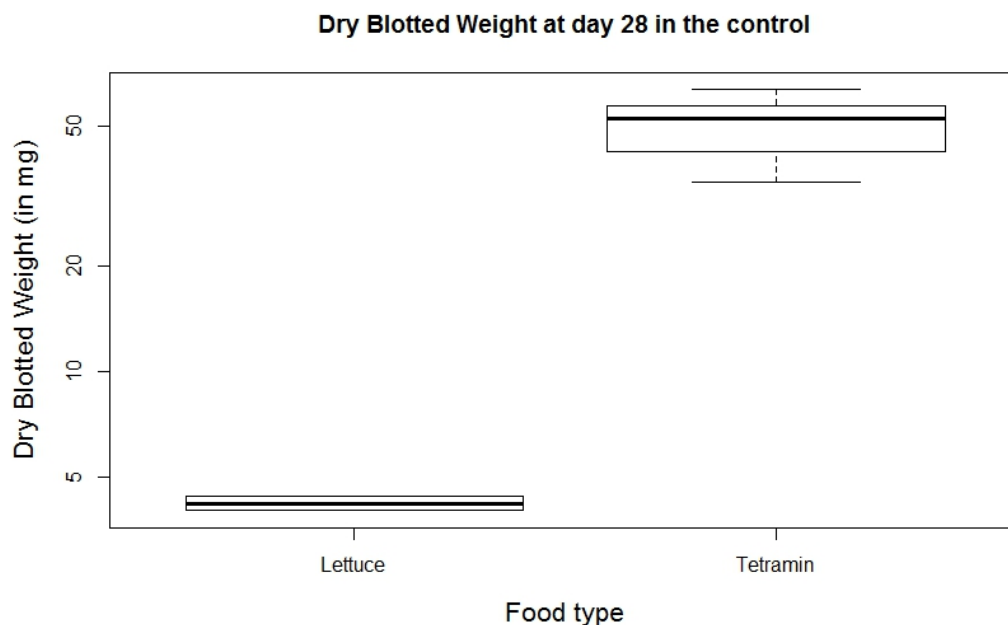


Fig. 2. Box and whisker plot of dry blotted weight at day 28 in control conditions, with median (thick black line), 5% quartile (lower box line), 95% quartile (upper box line), maximum and minimum response (whiskers).

### 3.3. Predicting endpoints

In order to predict toxicity endpoints generalized linear models were constructed via linear regression, using backward selection from a full model using all interactions and quadratic effects. For length, dry blotted weight, growth rate and survival this resulted in respectively model 1, 2, 3 and 4. AIC criteria are respectively given in table 8,9,10 and 11. Predicted vs. observed curves are respectively shown in Figure 11, 12, 13, 14.

### 3.4. Dose-response curves and effect concentrations

All reported effect concentrations are based on dissolved copper and reported in Tables 3-5. Fitted concentration response curves with growth rate as an endpoint are shown in Figures 3 & 4. Fitted concentration response curves with dry blotted weight as endpoint are shown in Figures 5 & 6. Fitted concentration response curves with survival as endpoint are shown in Figure 7 & 8.

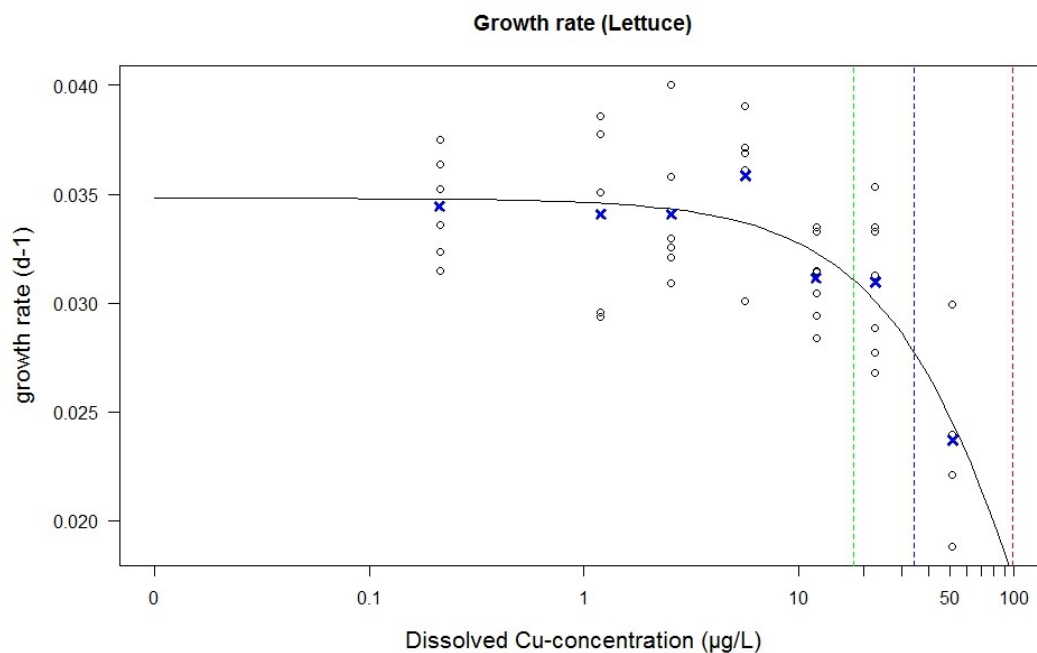


Fig 3. Concentration response data and fitted concentration response curves (Three-parameter Weibull function) for the Lettuce treatment, with mean growth rate per copper concentration (blue crosses), replicates (black circles), EC<sub>10</sub> (green dashed line), EC<sub>20</sub> (blue dashed line) and EC<sub>50</sub> (red dashed line).

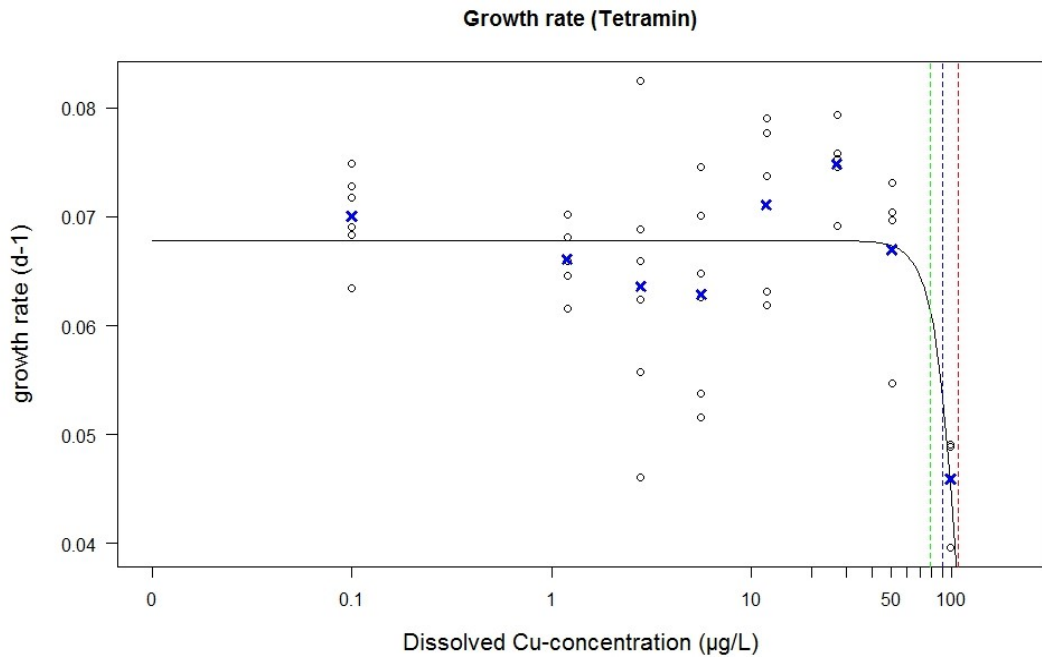


Fig 4. Concentration response data and fitted concentration response curves (Three parameter Weibull) for the Tetramin treatment, with mean growth rate per copper concentration (blue crosses), replicates (black circles), EC<sub>10</sub> (green dashed line), EC<sub>20</sub> (blue dashed line) and EC<sub>50</sub> (red dashed line).

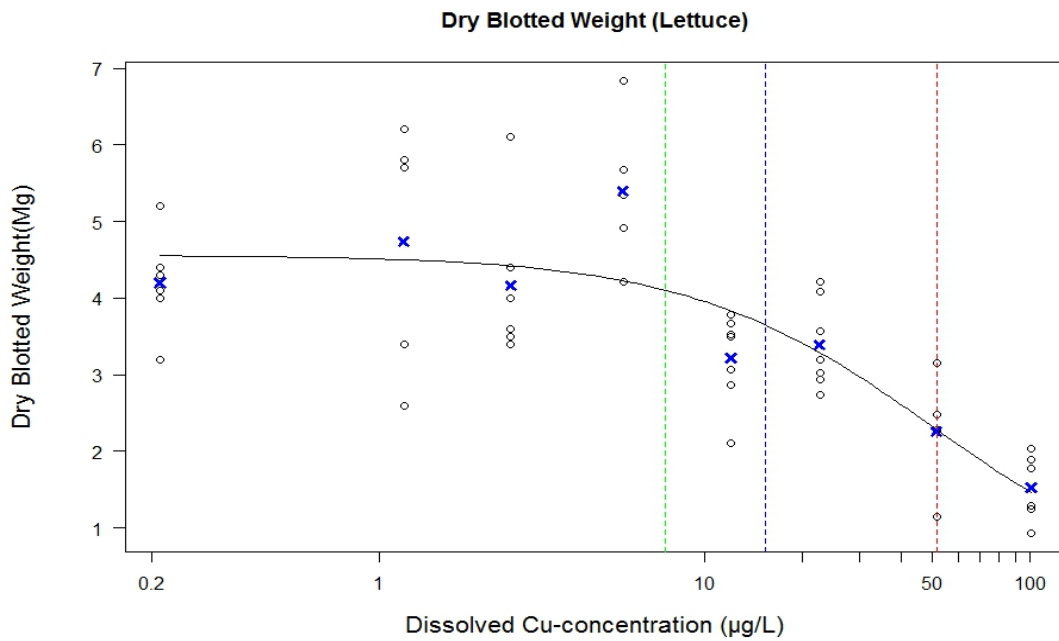


Fig 5. Concentration response data and fitted concentration response curves (Three parameter Log-logistic) for the Lettuce treatment, with mean dry blotted weight per copper concentration (blue crosses), replicates (black circles), EC<sub>10</sub> (green dashed line), EC<sub>20</sub> (blue dashed line) and EC<sub>50</sub> (red dashed line).

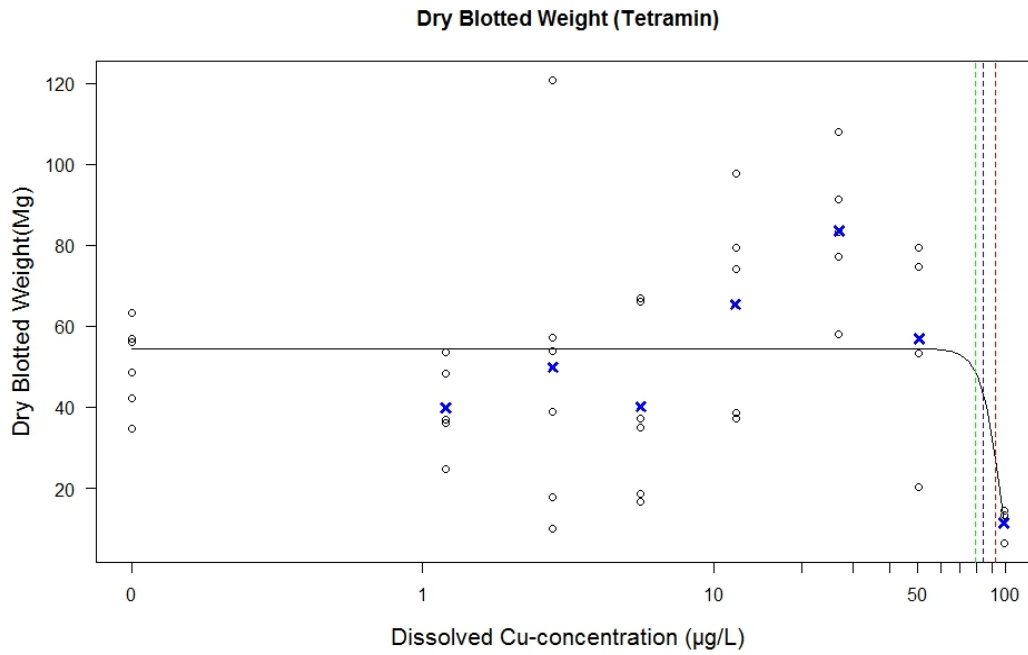


Fig 6. Concentration response data and fitted concentration response curves (Three parameter Weibull) for the Lettuce treatment, with mean dry blotted weight per copper concentration (blue crosses), replicates (black circles), EC<sub>10</sub> (green dashed line), EC<sub>20</sub> (blue dashed line) and EC<sub>50</sub> (red dashed line).

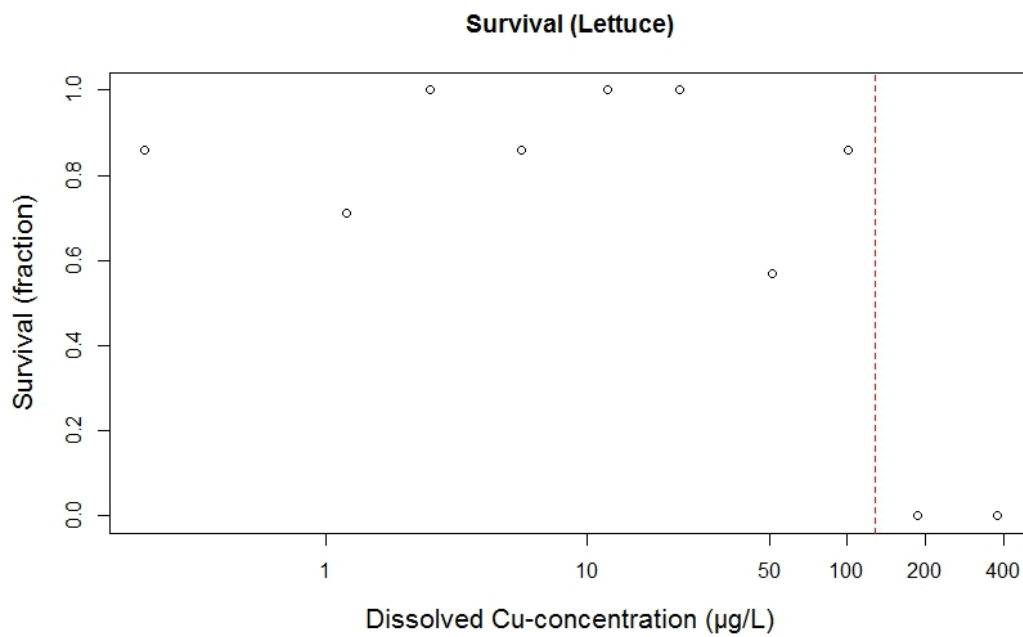


Fig 7. Concentration response data and fitted concentration response curves (Four parameter log-logistic) for the Lettuce treatment, fraction of survival within a the copper concentration (black circles), and LC50 (red dashed line).

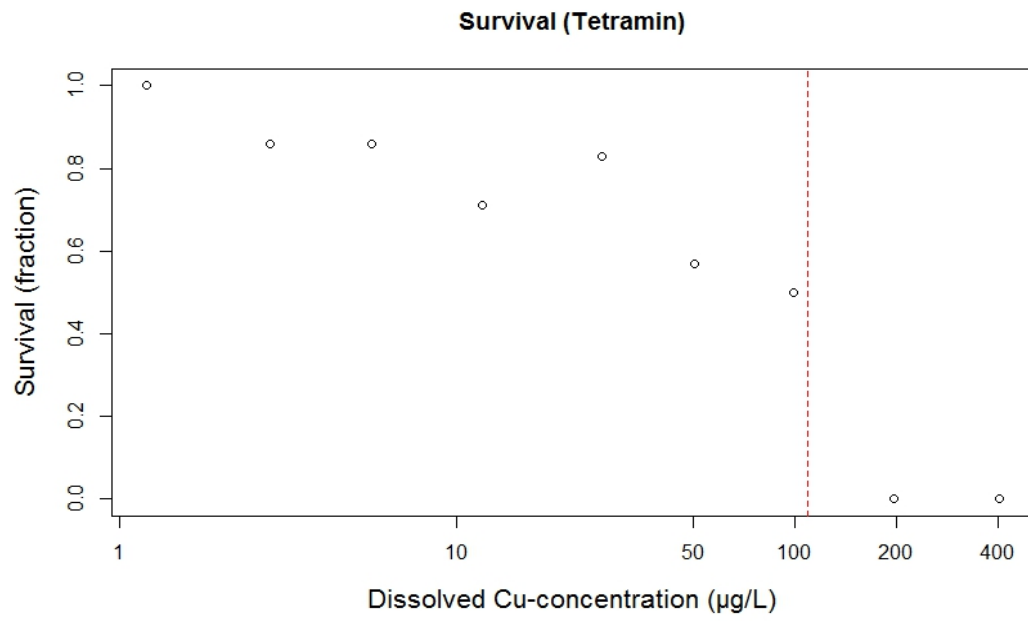


Fig 8. Concentration response data and fitted concentration response curves (Four parameter Weibull) for the Tetramin treatment, percentage of survival within a the copper concentration (black circles), LC<sub>50</sub> (red dashed line).

**Table 3.** Effect concentrations<sup>a</sup> of copper ( $\mu\text{g L}^{-1}$  as dissolved) obtained for the toxicity assays with *Lymnaea stagnalis* (numbers between parentheses are 95% confidence limits for EC<sub>x</sub> values), toxicity endpoint: **Growth rate**.

Food	28-d EC <sub>10</sub>	28-d EC <sub>20</sub>	28-d EC <sub>50</sub>	NOEC <sup>b</sup>	LOEC <sup>c</sup>
Lettuce	17 (6-28)	34 (20-48)	98 (77-119)	22	51
Tetramin	79 (32-136)	90 (77-103)	110 (-1397-1616) <sup>d</sup>	50	99

**Table 4.** Effect concentrations<sup>a</sup> of copper ( $\mu\text{g L}^{-1}$  as dissolved) obtained for the toxicity assays with *Lymnaea stagnalis* (numbers between parentheses are 95% confidence limits for EC<sub>x</sub> values, toxicity endpoint: **Dry blotted weight**).

Food	28-d EC <sub>10</sub>	28-d EC <sub>20</sub>	28-d EC <sub>50</sub>	NOEC <sup>b</sup>	LOEC <sup>c</sup>
Lettuce	7 (-1 -16)	15 (3-27)	51 (27-76)	22	51
Tetramin	78 (-138-296)	84 (-83-251)	92 (15-170)	50	99

**Table 5.** Effect concentrations of copper ( $\mu\text{g L}^{-1}$  as dissolved) obtained for the toxicity assays with *Lymnaea stagnalis*, toxicity endpoint: **Survival**.

Food	28-d LC <sub>50</sub> <sup>e</sup>	NOEC <sup>b</sup>	LOEC <sup>c</sup>
Lettuce	128 (113-145)	100	185
Tetramin	109 (73-163)	99	197

<sup>a</sup>EC<sub>10</sub>, EC<sub>20</sub> and EC<sub>50</sub> = the 10%, 20% and 50% effect concentration, respectively

<sup>b</sup>NOEC = No Observed Effect Concentration

<sup>c</sup>LOEC = Lowest Observed Effect Concentration

<sup>d</sup>Provides a rough estimate, but 95% confidence intervals are wide due to steepness of the curve, and thus insufficient partial effects

<sup>e</sup>LC<sub>50</sub>'s were calculated using the Trimmed Spearman-Kärber method (Hamilton et al. 1977)

To compare whether EC<sub>x</sub> values are statistically different from each other and to determine the most sensitive endpoint a Wheeler ratio test (Wheeler et al., 2006) was used, this test compares EC<sub>x</sub> values by subtracting the natural logarithm of the EC<sub>x</sub> values. If the 95% confidence interval of the ratio does not contain 1, the EC<sub>x</sub> values are statistically different from each other. Estimates with their 95% confidence intervals are given in Table 6.

**Table 6.** A comparison of EC<sub>x</sub> values performed through Wheeler ratio test. Estimates and their 95% confidence intervals are given for each comparison.

<b>Growth rate</b>	<b>28-d EC<sub>10</sub></b>	<b>28-d EC<sub>20</sub></b>	<b>28-d EC<sub>50</sub></b>
Tetramin vs. Lettuce	0.22 (0.10-0.45)*	0.38 (0.26-0.55)*	0.89 (0.06-13.18)

<b>Dry blotted weight</b>	<b>28-d EC<sub>10</sub></b>	<b>28-d EC<sub>20</sub></b>	<b>28-d EC<sub>50</sub></b>
Tetramin vs. Lettuce	0.09 (0.02-0.43)*	0.19 (0.05-0.71)*	0.55 (0.27-1.15)

<b>Survival</b>	<b>28-d LC<sub>10</sub></b>	<b>28-d LC<sub>20</sub></b>	<b>28-d LC<sub>50</sub></b>
Tetramin vs. Lettuce	0.31 (0.03-3.32)	0.42 (0.05-3.66)	1.17 (0.77-1.79)

<b>Growth rate vs Dry blotted weight</b>	<b>28-d EC<sub>10</sub></b>	<b>28-d EC<sub>20</sub></b>	<b>28-d EC<sub>50</sub></b>
Lettuce	2.43 (0.92-6.38)	2.27 (1.15-4.48)*	1.92 (1.23-2.99)*
Tetramin	1.01 (0.24-4.27)	0.96 (0.07-12.40)	1.20 (0.08-18.82)

<b>Survival vs. Growth rate</b>	<b>28-d E(L)C<sub>50</sub></b>
Lettuce	0.77 (0.61-0.96)*
Tetramin	1.01 (0.07-15.28)

\*Statistically significant

## 4. Discussion

### 4.1. The effect of food source on length, dry blotted weight growth rate

In our study we found a mean shell length in the Tetramin fed control after 28 days of  $1.04 \pm 0.12$  cm and a mean shell length in the lettuce fed control after 28 days of  $0.34 \pm 0.03$  cm. Meaning individuals fed with Tetramin in control conditions have on average a > 60% larger shell length after 28 days. The mean shell length after 28 days in Tetramin fed controls derived from our data corresponds with the mean shell length after 28 days in the Juvenile feeding experiment (JFE) with medium level amount of fish flakes shown in Figure 2 of Zimmer et al. (2012). The fact that our data corresponds with the medium level of fish flakes in Zimmer et al. (2012) and not clearly with the maximum level fish flake treatment might be due to the fact that the amount of Tetramin flakes given in our experiment did not entirely reflect ad libitum conditions. The difference in mean length between the Tetramin treatment and the lettuce treatment and thus the significant effect of food type on shell length can be explained by the fact that the protein content of Tetramin fish flakes is 47% (given by the manufacturer). Protein content of lettuce varies only between 20% (McKeehen et al. 1996) and 40% (Selck et al. 2006). The high protein content and softness of the fish flakes appear to make Tetramin fish flakes a better food for the juvenile snails than lettuce. Zimmer et al. (2012) postulated these findings for the TetraPhyll fish flakes. Food limitation might exaggerate the response to toxic stress and the interpretation of the results of juvenile growth tests may thus be biased when lettuce is used as the sole food source.

A comparison between the Tetramin and the lettuce treatment of the mean dry blotted weight on day 28 in the control treatments ( $< 0.25 \mu\text{g L}^{-1}$ ) is shown in Figure 1. This Figure suggests a significant influence of food on dry blotted weight. The difference in dry blotted weight at day 28 between food types in control conditions is highly significant ( $p\text{-value} = 9 \cdot 10^{-7}$ ). Confirming our reasoning that Tetramin appears to be a better food source for juvenile *L. stagnalis* and that this endpoint might also show an exaggerated response to toxic stress when test individuals are food limited by an improper food source such as lettuce (Zimmer et al. 2012).

When comparing mean growth rates within copper concentrations between lettuce and Tetramin a significant difference in mean growth rate between lettuce and Tetramin was found ( $p\text{-value} = 4.052 \cdot 10^{-9}$ ). These findings confirm our reasoning based on the effects on dry blotted weight and length at day 28. Tetramin fish flakes appears to be a better food for the juvenile snails than lettuce, which may be due to its softness and its higher protein content in comparison to lettuce.

#### 4.2. The effect of food source and copper toxicity on growth rate, dry blotted weight and survival

Growth rate calculation was based on shell lengths on day 0, 6, 17, 25 and 28 and largely correlates with length after 28 days and dry blotted weight after 28 days (See supportive information). For growth rate, reliable  $EC_x$  values (see Table 3) could be derived via dose response curve analysis except for the  $EC_{50}$  of the Tetramin treatment. The calculated  $EC_{50}$  ( $110 \mu\text{g L}^{-1}$ ) for growth rate in Tetramin fed organisms is a reasonably good estimate when analyzing the fitted concentration response curve, however the 95% confidence intervals are exceptionally large due to the steep dose-response curve. Growth rate was calculated for every individual that survived for 28 days. No individuals survived at the test concentration of  $197 \mu\text{g L}^{-1}$ , which is 2-fold higher than the concentration ( $99 \mu\text{g L}^{-1}$ ) where organisms still survived. The  $EC_{50}$  value thus lies within  $99 \mu\text{g L}^{-1}$  and  $197 \mu\text{g L}^{-1}$  boundaries as it also was estimated by the dose response analysis between those boundaries. Thus this  $EC_{50}$  of  $110 \mu\text{g L}^{-1}$  is a good estimate. Comparing  $EC_{10}$ ,  $EC_{20}$  and  $EC_{50}$  values with their 95% confidence intervals via the Wheeler ratio test renders estimates showing significant differences between  $EC_{10}$  and  $EC_{20}$  values of the lettuce and Tetramin treatments (see Table 6). Thus the lettuce treatment is significantly more sensitive for copper at the growth rate endpoint, however only at the  $EC_{10}$  and  $EC_{20}$  level and not at the  $EC_{50}$  level. NOEC and LOEC values also differ between the lettuce and the Tetramin treatments. Both observed effect concentrations are 2-fold larger for the Tetramin treatment, confirming the findings derived from the  $EC_x$  values. This strongly indicates that lettuce fed snails are more sensitive for copper toxicity and thus that food type has an impact on copper toxicity for *L. stagnalis*.

Dry blotted weight was measured on day 28 and largely correlates with length after 28 days and growth rate (See supportive information). For dry blotted weight  $EC_x$  values (see table 4) could be derived via dose response curve analysis.  $EC_{10}$ ,  $EC_{20}$  and  $EC_{50}$  values with their 95% confidence intervals were compared via the Wheeler ratio test (see Table 6). This shows significant differences between the  $EC_{10}$  and  $EC_{20}$  values of the lettuce and Tetramin treatments. Thus the lettuce fed *L. stagnalis* individuals are also at this endpoint significantly more sensitive for copper, however not at  $EC_{50}$  level. NOEC and LOEC values also differ between the lettuce and Tetramin treatments. Both observed effect concentrations are over 2-fold larger for the Tetramin treatments, confirming the findings derived from the  $EC_x$  values. This strongly indicates that lettuce snails are more sensitive for copper toxicity and thus that food type has an impact on copper toxicity for *L. stagnalis*.

No significant differences were found in survival between both food types when comparing  $LC_{50}$ 's. This is in line with our results for dry blotted weight and growth rate which also do not differ at the  $EC_{50}$  level.

When comparing EC<sub>x</sub> values between growth rate and dry blotted weight within food treatment via the wheeler ratio test (see table 6), no significant difference is found between EC<sub>10</sub> values for growth rate & dry blotted weight for both food types. A significant difference was found for EC<sub>20</sub> and EC<sub>50</sub> values between growth rate & blotted weight for *L. stagnalis* individuals that received lettuce as food. No significant difference was found for EC<sub>20</sub> and EC<sub>50</sub> values between growth rate and dry blotted weight for *L. stagnalis* individuals that received Tetramin as food. Taking into account the EC<sub>x</sub> values, this implies that dry blotted weight is a more sensitive endpoint than growth rate.

#### 4.3. Determining the most sensitive endpoint

The EC<sub>x</sub> values and their 95% confidence intervals of different endpoints were compared with the Wheeler ratio test. We see that in Tetramin treatments no significant difference is found between the EC<sub>x</sub> values for different endpoints. However in lettuce treatments a significant higher sensitivity at the growth rate and dry blotted weight endpoint is found in comparison to survival, which is not the case for Tetramin. Lettuce fed *L. stagnalis* is thus more sensitive at size related endpoints. From the EC<sub>x</sub> comparisons in Table 6 we see that the most sensitive endpoint for lettuce is dry blotted weight. As mentioned before, no significant differences in sensitivity at different endpoints is observed for Tetramin.

#### 4.4. Comparison of our data to literature data

Another recent study on the toxic effect of copper on the freshwater snail, *L. stagnalis*, is Brix et al. (2011). In order to adequately compare our data to the data published in Brix et al. (2011) we have to compare the weight in the control, survival and weight endpoints, since these were tested in both our study and the study from Brix et al. (2011). A comparison of all normalized effect parameters for both endpoints can be found in table 7 and in figure 9.

For the control we see that for our Tetramin fed snails in control conditions (0.1 µg cu L<sup>-1</sup>) have a mean ± SD dry blotted weight of: 50.3 ± 10.6 mg (n=7) after 28 days, from figure 2 in Brix et al. (2011) we have extracted a mean ± SEM control weight of: 66.9 ± 9.90 mg after 30 days (1.2 µg cu L<sup>-1</sup>). Our control performed less well than the control in Brix. et al. (2011). However our individuals have grown 2 days less than those from Brix et al. (2011). Also as mentioned above the amount of food given in the Tetramin treatment, might not have reflected true ad libitum conditions as in Zimmer et al. (2010).

In the case of survival, our normalized NOEC values for both food types are considerably higher than the normalized NOEC in Brix et al. (2011) (see table 7). This indicates that the *L. stagnalis* individuals used in this study were considerably less sensitive to copper at the survival endpoint.

This argument is confirmed when the normalized EC<sub>x</sub> values are compared for the much more sensitive endpoint: weight. Our dry blotted weight endpoint was measured as described in Brix et al. (2011) and is thus highly comparable. In table 7 we can see that the *L. stagnalis* individuals are also more sensitive to copper in the study from Brix et al. (2011) as the normalized EC<sub>x</sub> values from our Tetramin fed individuals are up to 13 times higher than the normalized data from Brix et al. (2011).

**Table 7.** A comparison of the effect parameter values normalized physico-chemistry of the Rhine (The Netherlands) for different endpoints between Brix et al. (2011) and Berteloot et al. (2014).

Endpoint	Effect parameter	Brix et al. (2011) (in µg Cu L <sup>-1</sup> )	Berteloot et. al. (2014) Lettuce (in µg Cu L <sup>-1</sup> )	Berteloot et al. (2014) Tetramin (in µg Cu L <sup>-1</sup> )
Survival	NOEC	3.73	23.1	19.5
Weight	EC10	1.17	2.10	16.0
Weight	EC20	1.80	4.35	16.3
Weight	EC50	3.86	8.41	17.7

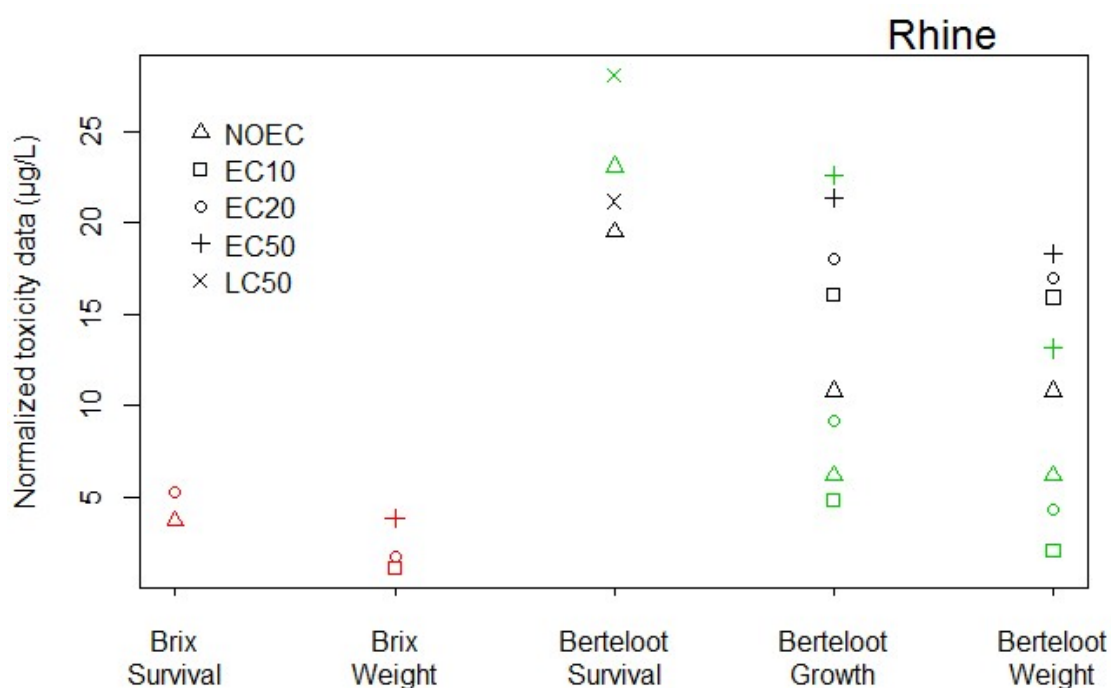


Fig 9. A comparison of the effect parameter values normalized physico-chemistry of the Rhine (The Netherlands) for different endpoints between Brix et al. and Berteloot et al. 2014. With Red = mix of lettuce carrots & sweet potato, Green= lettuce and Black = Tetramin fish flakes.

## 5. Conclusions

In this research we have found indications demonstrating that the food source plays an important role in size related endpoints such as dry blotted weight, length and growth rate. That lettuce fed *L. stagnalis* individuals are more sensitive for copper toxicity at size related endpoints than Tetramin fed individuals. However there appears to be no difference in survival between snails fed with Tetramin and snails fed with lettuce exposed to copper. We have also found that our *L. stagnalis* individuals are less sensitive to copper than other studies in literature such as Brix et al. (2011). Ultimately, there is a need for data on adaptive potential, food preference in nature and population growth in nature of *L. stagnalis* in order to determine and predict its sensitivity to copper more accurately.

## 6. References

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## 7. Supportive information

### 7.1. The von Bertalanffy model

In order to determine whether the snails used in our experiment followed a von Bertalanffy pattern (von Bertalanffy, 1934), we fitted the model presented as in Zimmer et al. 2012 (Model 5) to our data by adapting two parameters on which  $r_B$  is based: the food level (a factor for the quantity of food given) and the food quality factor.

$$\frac{dL}{dt} = r_B \cdot (L_\infty - L) \quad (\text{M.5})$$

With:

- $r_B$  = the von Bertalanffy growth rate constant ( $\text{days}^{-1}$ )
- $L_\infty$  = the maximum shell length at abundant food (cm)
- $L$  = shell length (cm)

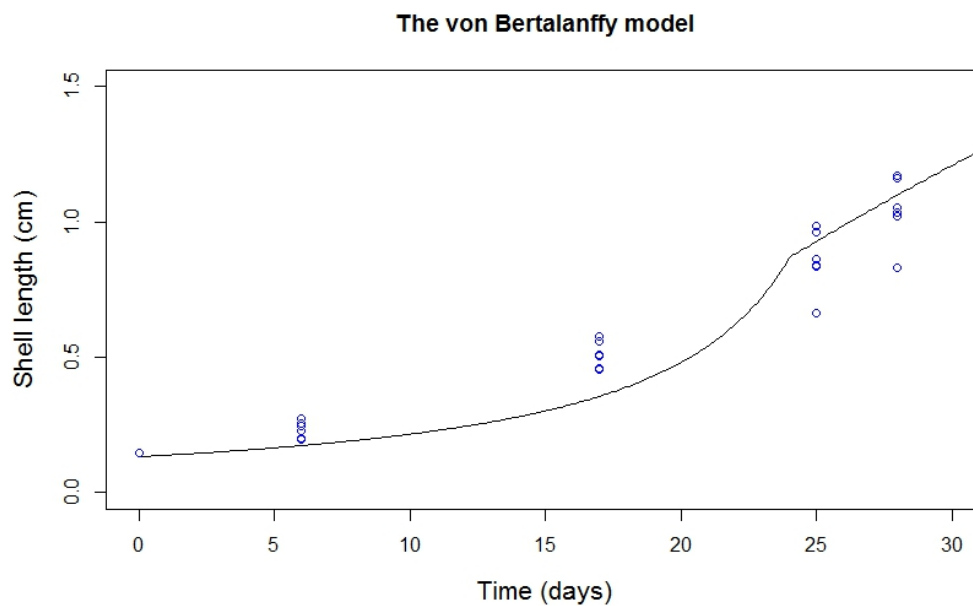


Fig. 10. A von Bertalanffy model simulation for 30 days (black line), fitted to our data of the Tetramin fed control treatment (blue circles).

## 7.2. Predicting endpoints

### Length (R<sup>2</sup> adjusted =0.94)

$$\begin{aligned} \text{LOG}(\text{LENGTH}(\mu\text{m})) = & 3.178 + 1.490 \cdot 10^{-2} \cdot \text{DAY} + 6.222 \cdot 10^{-4} \cdot \text{FOOD} - 1.025 \cdot 10^{-3} \cdot [\text{Cu}_{\text{diss}}] \\ & + 1.517 \cdot 10^{-2} \cdot \text{DAY} \cdot \text{FOOD} - 7.267 \cdot 10^{-5} \cdot \text{DAY} \cdot [\text{Cu}_{\text{diss}}] - 1.213 \cdot 10^{-5} \cdot [\text{Cu}_{\text{diss}}]^2 \end{aligned} \quad (\text{M.1})$$

**Table 8.** Summary of the terms in the model for growth (model 1) with the units, parameter estimates, standard error (SE), significance (P-value) and the explained variation (%).

Predictor	Unit	Parameter estimate	SE	P-value	Explained variation (%)
Day	(-)	$1.490 \cdot 10^{-2}$	$4.686 \cdot 10^{-4}$	$<2.2 \cdot 10^{-16}$	68.5
Food	(-)	$6.222 \cdot 10^{-4}$	$1.114 \cdot 10^{-2}$	$9.555 \cdot 10^{-1}$	1.98
[Cu <sub>diss</sub> ]	μg L <sup>-1</sup>	$-1.025 \cdot 10^{-3}$	$4.176 \cdot 10^{-4}$	$1.451 \cdot 10^{-2}$	0.20
Day · Food	(-)	$1.517 \cdot 10^{-2}$	$5.978 \cdot 10^{-4}$	$<2.2 \cdot 10^{-16}$	0.92
Day · [Cu <sub>diss</sub> ]	Day·μg L <sup>-1</sup>	$-7.267 \cdot 10^{-5}$	$9.756 \cdot 10^{-6}$	$5.35 \cdot 10^{-13}$	0.13
[Cu <sub>diss</sub> ] <sup>2</sup>	μg L <sup>-2</sup>	$-1.213 \cdot 10^{-5}$	$3.934 \cdot 10^{-6}$	$2.284 \cdot 10^{-3}$	0.01

This model can accurately predict the length of *L. stagnalis* individual with 100% of the predictions within a factor 2 of the observed values (fig. 11).

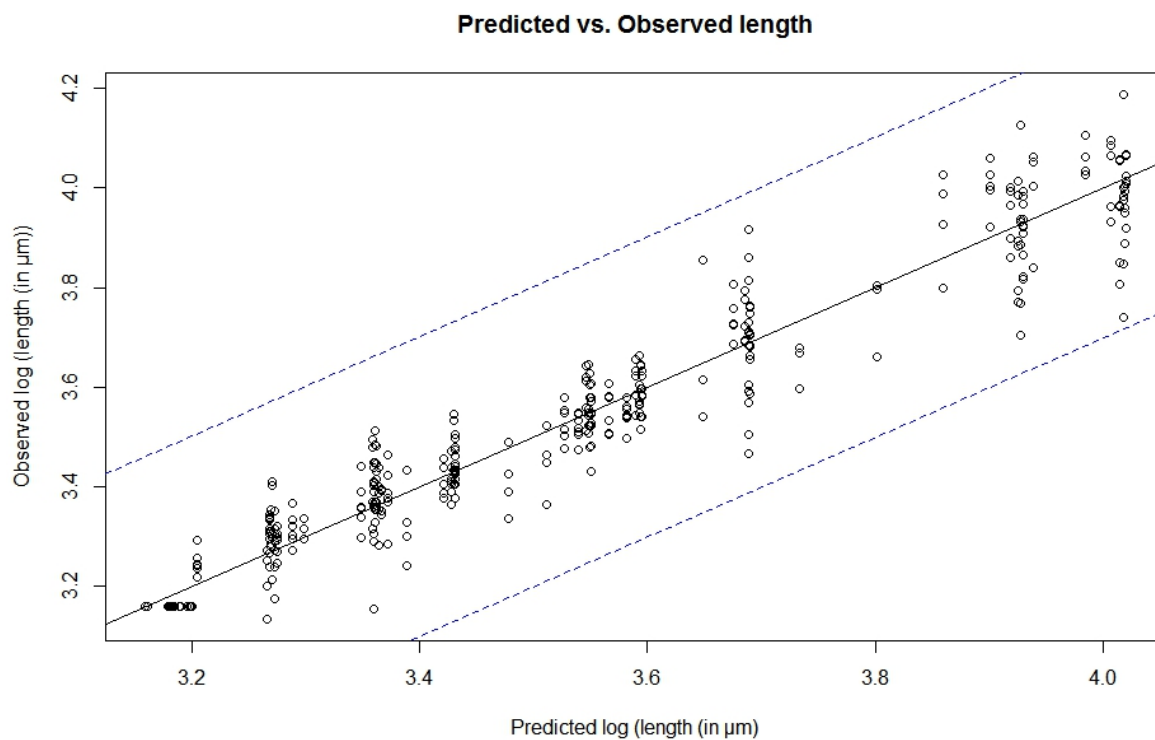


Fig. 11. Predicted values (black line) compared to the observed values (black circles) for model 1, with factor 2 differences (blue dashed lines).

Dry blotted weight ( $R^2$  adjusted =0.89)

$$\text{LOG(DRY BLOTTED WEIGHT(Mg))} = 5.982 \cdot 10^{-1} + 1.085 \cdot \text{FOOD} - 4.949 \cdot 10^{-5} \cdot [\text{Cu}_{\text{diss}}]^2 \quad (\text{M.2})$$

**Table 9.** Summary of the terms in the model for growth (model 2) with the units, parameter estimates, standard error (SE), significance (p-value) and the explained variation (%).

Predictor	Unit	Parameter estimate	SE	p-value	Explained variation (%)
Food	(-)	1.085	$1.114 \cdot 10^{-2}$	$<2.2 \cdot 10^{-16}$	93.2
$[\text{Cu}_{\text{diss}}]^2$	$\mu\text{g L}^{-2}$	$-4.949 \cdot 10^{-5}$	$3.934 \cdot 10^{-6}$	$6.470 \cdot 10^{-10}$	6.80

This model can accurately predict the dry blotted weight of *L. stagnalis* individuals in the linear growth phase with 88% of the predictions within the factor 2 ranges (fig. 12).

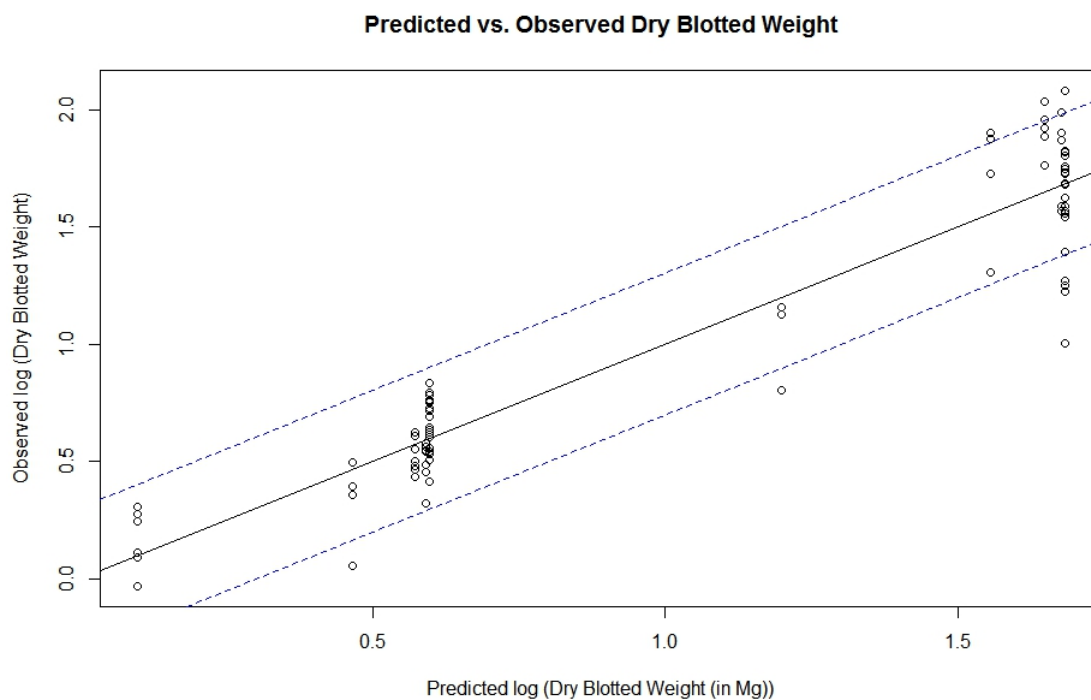


Fig. 12. Predicted values (black line) compared to the observed values (black circles) for model 2, with factor 2 differences (blue dashed lines).

Growth rate ( $R^2$  adjusted = 0.89)

$$\text{GROWTH RATE (DAYS}^{-1}\text{)} = 3.495 \cdot 10^{-2} + 3.495 \cdot 10^{-2} \cdot \text{FOOD} - 1.671 \cdot 10^{-4} [\text{Cu}_{\text{Diss}}] \quad (\text{M.3})$$

**Table 10.** Summary of the predictors in the model for (model 3) with the units, parameter estimates, standard error (SE), significance (p-value) and the explained variation (%)

Predictor	Unit	Parameter estimate	SE	p-value	Explained variation (%)
Food	(-)	$3.495 \cdot 10^{-2}$	$2.746 \cdot 10^{-2}$	$< 2.2 \cdot 10^{-16}$	92.5
$[\text{Cu}_{\text{diss}}]$	$\mu\text{g L}^{-1}$	$-1.671 \cdot 10^{-4}$	$2.226 \cdot 10^{-3}$	$3.798 \cdot 10^{-10}$	7.50

This model can accurately predict the growth rate of *L. stagnalis* individuals in the linear growth rate phase with 100% of the predictions well within the factor 2 ranges (fig. 13).

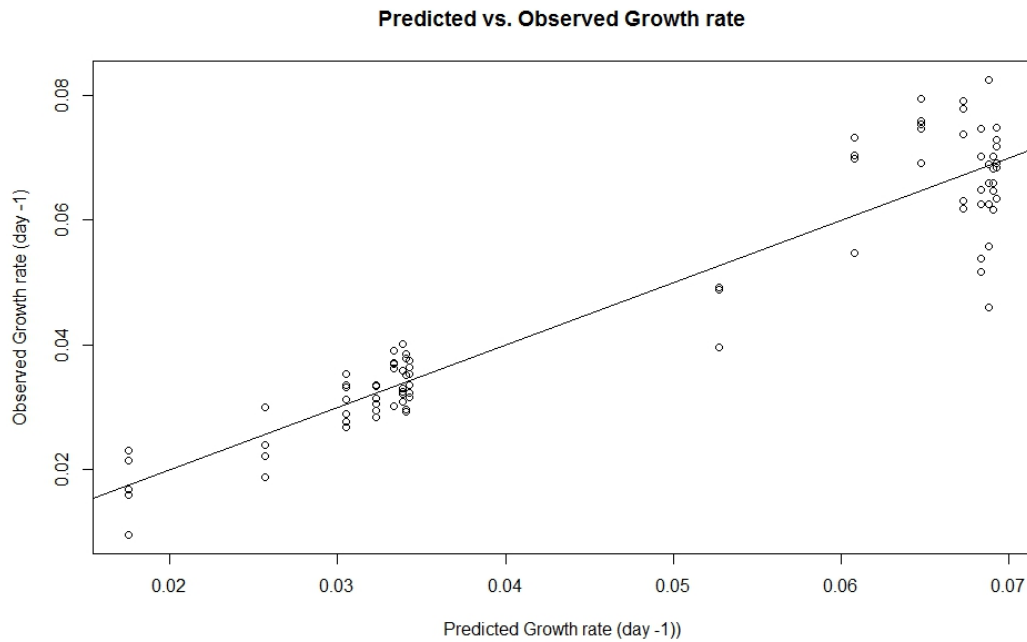


Fig. 13. Predicted values (black line) compared to the observed values (black circles) for model 3, with factor 2 differences outside of the plot.

Survival (Explained variation 73%)

$$\text{SURVIVAL} = 2.323 + 7.341 \cdot 10^{-1} \cdot \text{FOOD} - 1.119 \cdot 10^{-2} [\text{Cu}_{\text{diss}}] + 6.451 \cdot 10^{-4} \cdot \text{DAY} - 6.332 \cdot 10^{-4} \cdot \text{DAY} \cdot [\text{Cu}_{\text{diss}}] \quad (\text{M.4})$$

**Table 11.** Summary of the terms in the model for survival (model 4) with the units, parameter estimates, standard error (SE), significance (p-value) and the explained variation.

Predictor	Unit	parameter estimate	SE	p-value	Explained variation (%)
Day	(-)	$6.451 \cdot 10^{-4}$	$2.632 \cdot 10^{-2}$	$9.805 \cdot 10^{-1}$	1.44
Food	(-)	$7.341 \cdot 10^{-1}$	$3.212 \cdot 10^{-1}$	$2.510 \cdot 10^{-2}$	1.57
[Cu <sub>diss</sub> ]	µg L <sup>-1</sup>	$-1.119 \cdot 10^{-2}$	$4.651 \cdot 10^{-3}$	$1.850 \cdot 10^{-2}$	68.5
Day · [Cu <sub>diss</sub> ]	Day · µg L <sup>-1</sup>	$-6.332 \cdot 10^{-4}$	$2.685 \cdot 10^{-4}$	$2.100 \cdot 10^{-2}$	6.07

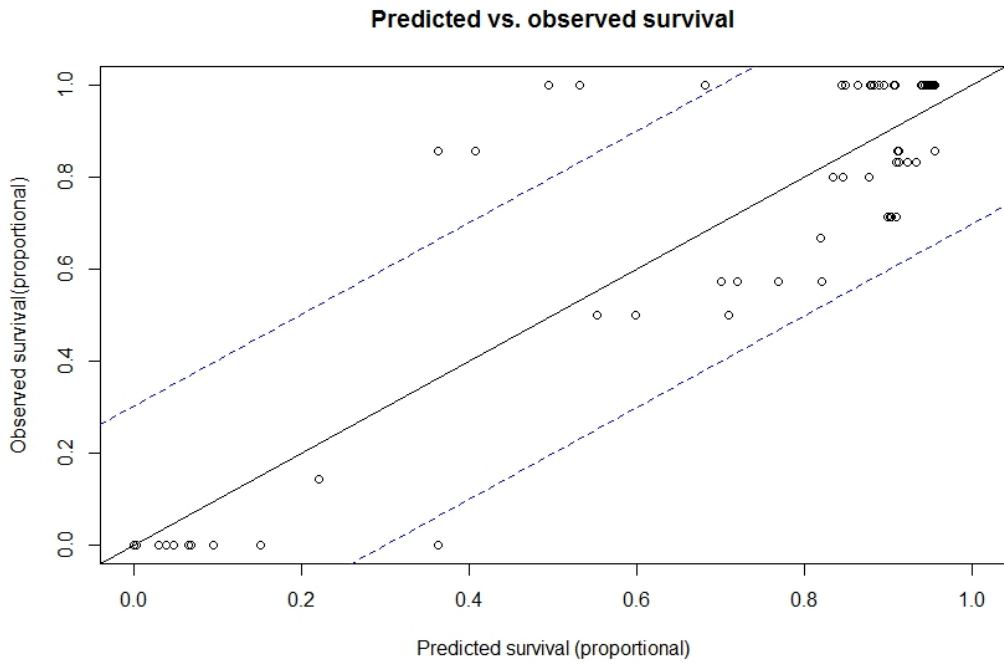


Fig. 14. Predicted values (black line) compared to the observed values (black circles) for model 4, with factor 2 differences (blue dashed lines).

### 7.3. Correlation plots

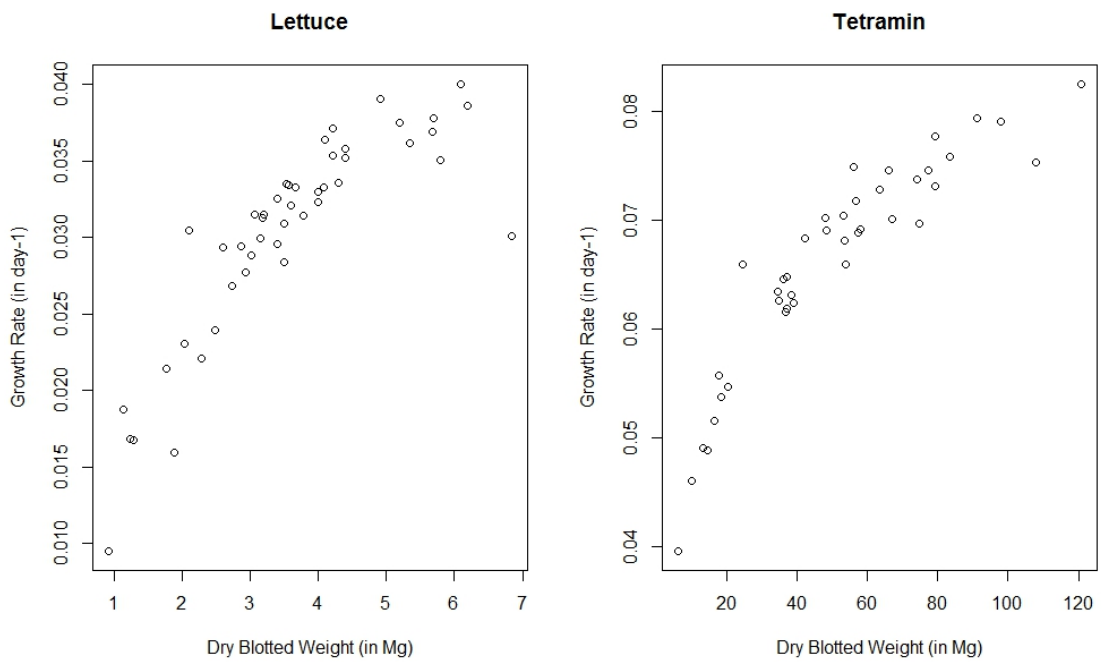


Fig 15. Correlation plots for dry blotted weight vs. growth rate, for both Tetramin and Lettuce fed individuals

#### 7.4. Length vs.time curves

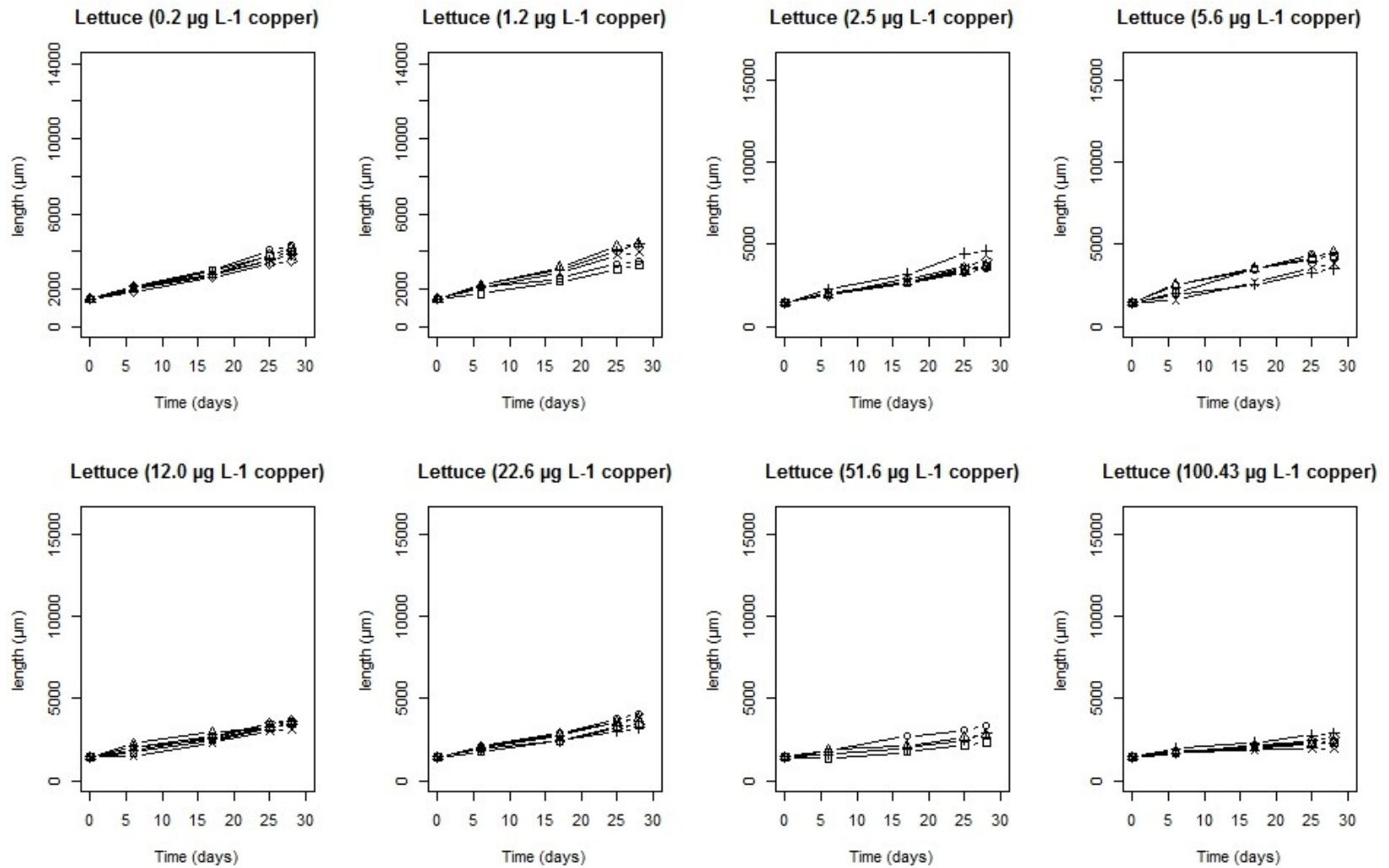


Fig. 16. Length in time curves of all survived Lettuce fed individuals within each tested concentration of copper.

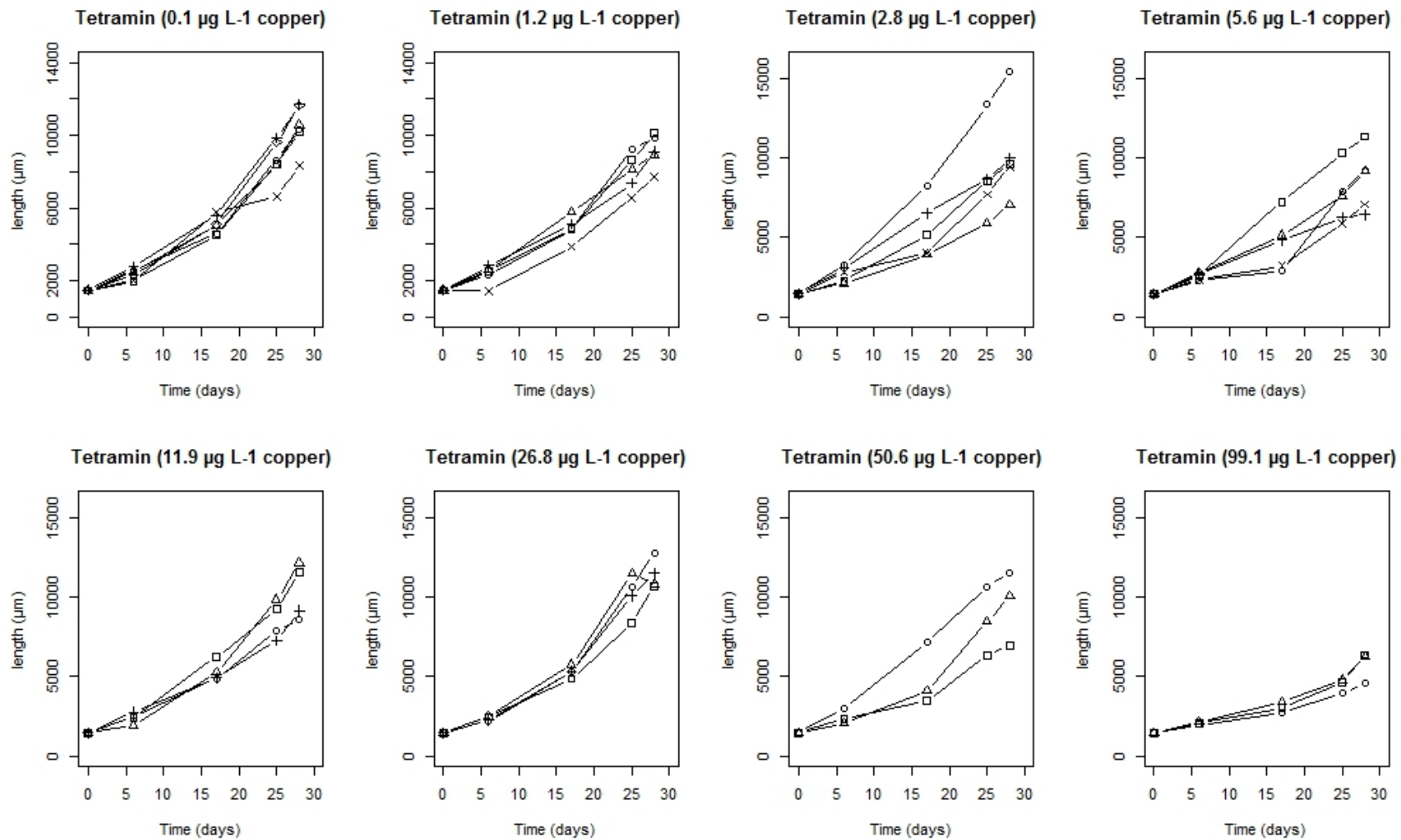


Fig. 17. Length in time curves of all survived Tetramin fed individuals within each tested concentration of copper