



Chronic toxicity of Pb to *Chironomus riparius* in five natural waters

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1. Introduction

Chronic toxicity data of Pb with insect species are currently only available for *Chironomus tentans* (Diptera) larvae tested in standard medium (Grosell et al., 2006). In order to use chronic Pb Biotic Ligand Models (BLMs) for fish or daphnids (De Schamphelaere, 2010; Nys et al., 2012) for bioavailability normalization of the entire species sensitivity distribution, it is needed to demonstrate that these BLMs can be extrapolated across species. Insects are one group of species for which this should preferably be shown (Schlekat et al., 2010). It is common to test this using chronic metal toxicity tests performed in natural waters with a broad range of water chemistry covering the natural variation of pH, Ca and DOC (Schlekat et al., 2010; De Schamphelaere and Janssen, 2010). Until now, this type of work for Pb has already been performed with a snail, a rotifer and an aquatic plant (Esbaugh et al., 2012; Antunes and Holtze, 2010). However, similar Pb data for an insect were still lacking.

The aim of the present study was therefore to generate Pb chronic toxicity data with *Chironomus riparius*. The midge larvae were exposed for 14 days to a range of Pb concentrations in five natural waters with different water chemistry including differences in pH, Ca and DOC, the three most important factors influencing Pb bioavailability (De Schamphelaere, 2010).

2. Materials and methods

The natural waters were collected from 5 different locations in Bihain and Brisys (Belgium), Ankeveen (The Netherlands), Regge and Vyon (France). These sites have been used in similar BLM extrapolation studies with other metals (e.g., De Schamphelaere and Janssen, 2010). Water samples were filtered (0.2 µm) *in situ* and stored in the laboratory until use. The study was organized into three phases. In *phase 1*, the equilibration of Pb spiked waters and the solubility limits of Pb were investigated. Phase 1 was performed in September-October 2011 (i.e. within 1 month after sample collection). In *phase 2*, range-finding tests were conducted (November-December 2011). The final chronic toxicity tests were conducted in *phase 3* (March-April 2012).

2.1 Toxicity tests

Test organism. *Chironomus riparius* (Diptera) used in this study were obtained from the stock culture maintained at our laboratory. The initial starting cultures originated from ECT Oekotoxikologie GmbH (Germany) and AquaSence (the Netherlands). The organisms have been kept under standard culturing conditions for more than 3 years. Culture conditions are described in OECD (2004). *C. riparius* is held at 20±1°C with a photo-period of 16 hours light and 8 hours dark. The bottom of the culturing aquaria (5-L volume) is covered by a layer of quartz sand and a paper substrate (prepared by cutting unbleached brown papers). Culture water (EPA moderate hard water) (US EPA, 2000) was renewed once a week (75% renewal). The midges were fed with a fish flake food (TetraMin®) at approximately 100 mg per tank per day. For test initiation, the first instar larvae (i.e. 48 hours post hatching) were used.

The exposure system. The tests were conducted in 500-mL glass jars containing 300 mL of the test waters and no substrate. Each of the jars contained 10 organisms. The range-finding tests included a control and one Pb concentration (i.e. a concentration expected to be close to the solubility limit of each of the waters). In the final chronic tests, a control and 5 Pb concentrations with 3 replicates for each of the treatments were set up. The tests were conducted as a semi-static test in which water was renewed (\pm 75% renewal) 3 times during the exposure (i.e. at day 5, day 8 and day 11).

Test medium preparation. For each of the Pb concentrations, the water was thoroughly mixed with the desired amount of PbCl₂ stock solutions. The ratio of PbCl₂ stock solution and test medium was \leq 1:50. Waters spiked with Pb were equilibrated for 4 days under the same conditions as those used during the chronic exposure prior to introduction of the test organisms.

Test procedure. An overview of the test conditions is presented in Table 1.

Table 1. Test conditions of the 14-day tests with *Chironomus riparius*.

	Range-finding test	Dose response/chronic test
Duration of the test	14 days	14 days
Test vessel	500-mL glass jar	500-mL glass jar
Number of concentrations	1	5
Number of replicates per concentration	3	3
Number of organisms per concentration	10	10
Volume of water	300 mL	300 mL
Feeding	TetraMin® <u>Day 0-7:</u> 0.25 mg/organism/day <u>Day 8-14:</u> 0.5 mg/organism/day	TetraMin® <u>Day 0-7:</u> 0.25 mg/organism/day <u>Day 8-14:</u> 0.5 mg/organism/day
Renewal	3 times	3 times
Light-Dark cycle	16 hours – 8 hours	16 hours – 8 hours
Temperature	20 \pm 1°C	20 \pm 1°C
Dissolved oxygen concentrations	> 3 mg/L	> 3 mg/L

2.2 Physical and chemical analysis

All glass and plastic ware were acid washed (HCl 10% v/v) before used. The following parameters: pH, dissolved organic carbon (DOC) measured as non purgeable total organic carbon (NPOC), inorganic carbon (IC), Ca and total and filtered Pb were monitored at test initiation and before renewal. The filtered Pb was defined as the fraction passing through 0.45- μ m membrane syringe filters. Pb and Ca in the water were measured by flame atomic absorption spectroscopy (AAS) or a graphite furnace AAS. Dissolved organic carbon and

inorganic carbon were determined by the Total Organic Carbon analyzer (TOC-5000A) with Autosampler (ASI-5000A).

2.3 Test endpoints

The test endpoints used were survival and growth of the larvae after 14 days of exposure. Test jars were monitored daily to visually assess possible behavioral changes of the organisms.

Survival: organisms were considered dead if no movement or reaction was observed to a mechanical stimulus. Organisms which were not found back in the test jars after 14 days were considered dead

Growth: whole body dry mass after 14 days of exposure was used. Prior to the drying process, the organisms were placed in clean EPA medium for 24 hours to clear their gut content. The organisms in each replicate were transferred to a labeled weighing pan (one per replicate) and dried at 60°C for 72 hours. After cooling in a desiccators, the organisms were weighed to the nearest 0.01 mg. Dead organisms were not weighed.

2.4 Data analysis

Prior to the analysis of variance (ANOVA), data were checked for normality of distribution and homogeneity of variance, using Kolmogorov-Smirnov and Bartlett's tests, respectively. Survival data were normalised by the arcsine square root transformation. Post-hoc comparisons between a Pb treatment and the control treatment were performed with a Dunnett test at a significance level of $p < 0.05$. The EC_{50} values and associated 95% confidence limits were calculated using the log-logistic model.

$$y = \frac{k}{1 + \left(\frac{x}{EC_{50}}\right)^\beta}$$

Where y = the predicted value of the endpoint, x = dissolved Pb concentration (mean of all filtered Pb concentrations in fresh medium and in old medium) ($\mu\text{g/L}$), k = the value of the endpoint at $x = 0$. For visualization of the dose response data and fitted dose response curves, data and fits were normalized relative to the fitted value of k (i.e. they were expressed as % of the fitted control value of the endpoint). All statistical analysis was performed with Statistica 6.0 software.

3. Results

The main characteristics of the water samples are presented in Table 2. The data show that the 5 waters collected represent a broad range of chemical compositions, especially in terms of pH (7.0 to 8.2), Ca concentrations (3.5 mg/L to 45 mg/L) and the DOC concentrations (3 mg/L to 15 mg/L).

Table 2. Main physical-chemical characteristics of the collected waters (at the time of the final chronic tests in *phase 3*).

Site (location, country)	Sample name	Water type	pH	DOC (mg/L)	Conductivity (μ S/cm)	Ca (mg/L)	Mg (mg/L)	PO ₄ ³⁻ (mg/L)	Total P (mg/L)	Pb Non-filtered (μ g/L)	Pb Filtered (0.45 μ m) (μ g/L)
St. Martin R (Bihain, Belgium)	Bihain	Stream	7.0	16.1	60	8.5	1.4	<0.05	<0.05	1.198	0.866
Ourthe Orientale (Brisy Belgium)	Brisy	Stream	7.6	3.5	148	3.4	3.4	<0.05	<0.05	< 0.5	< 0.5
Ankeveensche (The Netherlands)	Ankeveen	Ditch	8.1	9.3	795	42.8	9.9	<0.05	<0.05	< 0.5	< 0.5
Beneden Regge (Ommen, The Netherlands)	Regge	River	8.2	17	597	55	5.7	0.07	0.1	< 0.5	< 0.5
Le Vuyon (Trélon, France)	Voyon	Stream	7.6	8.9	146	17.8	3.6	<0.05	<0.05	0.972	0.565

3.1 Equilibration/solubility tests (*phase 1*)

Waters spiked with Pb were equilibrated under the same conditions as those used during the chronic exposure. The equilibration process was enhanced by means of mixing through constant gentle aeration. Figure 1 presents the amount of dissolved Pb measured 4 days and 7 days after spiking. In three waters, i.e. *Brisy*, *Bihain* and *Voyon*, after 4 days of equilibration there was >50% dissolved Pb noted at all concentrations tested. In *Ankeveen* water, >90% dissolved Pb was detected at $\leq 3200 \mu\text{g/L}$. At $10000 \mu\text{g/L}$, dissolved Pb represented only 47% of total Pb. For *Regge* water, 58% dissolved Pb was detected at $\leq 1000 \mu\text{g/L}$. It was slightly higher (79%) at $3200 \mu\text{g/L}$ and was 58% at $10000 \mu\text{g/L}$. The dissolved Pb concentrations at day 7 remained within 1.3-fold from those at day 4 in all waters. Based on the results of the equilibration, a 4-day equilibration was applied in the range-finding tests (*phase 2*) and the final chronic tests (*phase 2*). The results also show that for *Ankeveen* water, the solubility limit is situated at $\geq 1000 \mu\text{g/L}$ (nominal). For *Bihain*, *Brisy*, *Regge* and *Voyon* waters, although no clear solubility limits could be detected, the onset of Pb precipitation was observed at $\geq 3200 \mu\text{g/L}$ (nominal).

3.2 Range-finding tests (*phase 2*)

The range-finding toxicity tests were conducted simultaneously with all 5 waters. For each of the waters, a control and one Pb concentration were tested. The Pb concentrations were selected based on (i) the results of the equilibration (*phase 1*, section 3.1), (ii) Pb concentrations which were close to solubility limit of each waters (Figure 1); and (iii) the sensitivity of *C. tentans* or *C. riparius* to Pb reported in earlier studies (e.g., Grosell et al., 2006; B  chard et al., 2008). The results are shown in Table 3.

After 4 days of equilibration, 68% and 88% dissolved Pb was measured at $1000 \mu\text{g/L}$ in *Brisy* and *Bihain* waters, respectively. For *Ankeveen* and *Voyon* waters, there was >90% dissolved Pb noted at $3200 \mu\text{g/L}$. These fractions of dissolved Pb are in agreement with the data of the Equilibration/Solubility limits tests (*phase 1*). For *Regge* water ($10000 \mu\text{g/L}$), the dissolved Pb was 80% of the total Pb concentration and was slightly higher than the value measured during the equilibration (58%). The differences in dissolved Pb between fresh medium and old medium were 1.1-fold (*Bihain*, $1000 \mu\text{g/L}$), 1.8-fold (*Brisy*, $1000 \mu\text{g/L}$), 1.6-fold (*Ankeveen*, $3200 \mu\text{g/L}$), 2.3-fold (*Regge*, $10000 \mu\text{g/L}$) and 1.4-fold (*Voyon*, $3200 \mu\text{g/L}$).

The control survivals were similar at $\geq 90\%$ except for the *Voyon* water (66.7%). The body mass of the control midges after 14 days, however, was variable among the waters and can be ranked as follows: *Bihain* > *Voyon* > *Regge* > *Brisy* > *Ankeveen*.

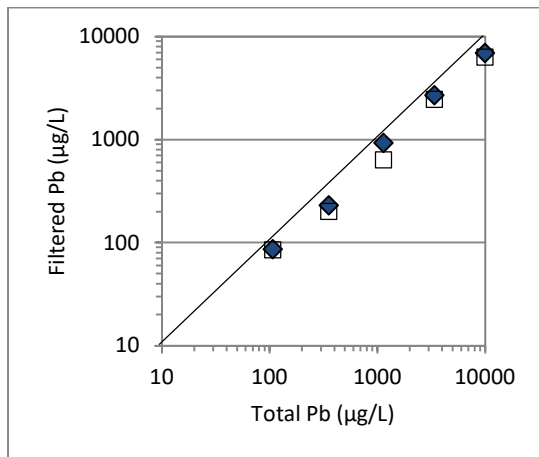
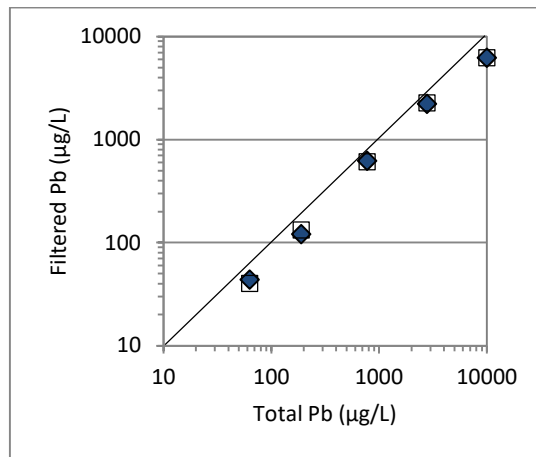
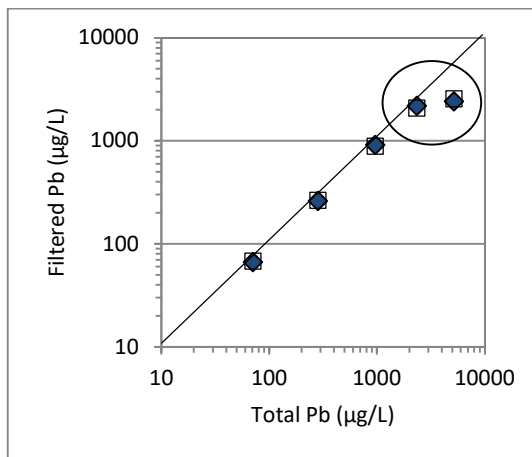
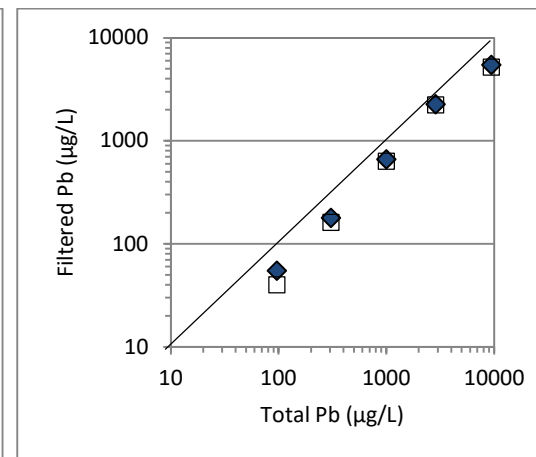
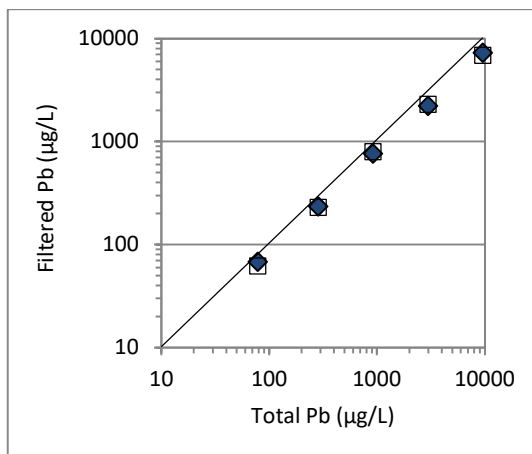
Bihain**Brisy****Ankeveen****Regge****Voyon**

Figure 1. Concentrations of filtered Pb at day 4 (◆) and day 7 (□) after spiking (phase 1).

No adverse effect on survival was noted at 1245 µg/L (measured, filtered) in *Bihain* water, whereas the total number of living midges was significantly reduced to 30% at 1180 µg/L in *Brisy* water. For *Ankeveen* and *Voyon* waters, no survival was noted at 3391 µg/L and 3291

µg/L, respectively. At 9717 µg/L in *Regge* water no living midges were found either after 14 days. The growth of *C. riparius* was not affected at 1245 µg Pb/L in *Bihain* water. For *Brisy* water, exposure to 1180 µg/L reduced the growth by 51%.

Table 3. Result of the range finding tests (*phase 2*). Data are expressed as mean ± sd (n≥2).

Waters	Pb (µg/L)			Survival (%)	Growth (mg/org)	
	Nominal	Measured				
		Fresh medium Total	Fresh medium Filtered			Old medium Filtered
Bihain	Control	<1	<0.5	<0.5	96.7±5.8	0.56±0.03
	1000	1245±260	850±128	807±140	96.7±5.8	0.54±0.17
Brisy	Control	<0.5	<0.5	<0.5	93.3±11.6	0.35±0.04
	1000	1180±51	1038±25	560±210	30±30*	0.18±0.02*
Ankeveen	Control	<0.5	<0.5	<0.5	90±0	0.3±0.04
	3200	3391±127	3201±141	2007±407	0	- ^a
Regge	Control	<0.5	<0.5	<0.5	93.3±10	0.42±0.007
	10000	9717±394	8059±1813	3540±103	0	- ^a
Voyon	Control	<0.5	<0.5	<0.5	66.7±11.6	0.48±0.06
	3200	3291±139	3179±7	2197±173	0	- ^a

*: significantly different from the control (Fisher exact-test, p<0.05 for survival; Dunnet test for growth)

a: no growth data because no surviving organism were available after 14 days of exposure

3.3 Final chronic toxicity tests (*phase 3*)

3.3.1 Water chemistry

Based on the results of the range-finding toxicity tests, three waters were selected for conducting the chronic tests: *Brisy*, *Ankeveen* and *Regge*. *Voyon* was not used because of sub-optimal control survival; *Bihain* was not selected due to anticipated potential issues with speciation modeling for this water, due to typically elevated Fe and Al concentrations. To fulfill the need of testing 5 waters, two modified waters were made. They were as follows: (1) *Brisy* water enriched with Ca (100 mg Ca/L; denoted as *Brisy high Ca*) and (2) *Ankeveen* water adjusted for pH (pH = 6.5; denoted as *Ankeveen low pH*). The modification of Ca and pH were done prior to the spiking of Pb. The concentrations of Pb for each of the waters were selected based on the results of the range-finding tests. All 5 chronic tests were performed simultaneously. At each renewal, dissolved Pb in *fresh medium* and *old medium* was measured. Table 4 represents the main characteristics of the tested waters. More detailed data are presented in Annex.

Table 4. Main physical-chemical characteristics of tested waters during the final chronic tests (*phase 3*)

		pH	pH	DOC (filtered)	DOC (filtered)	Ca (filtered)	Ca (filtered)	Pb (total)	Pb (filtered)	Pb (filtered)
		Fresh medium	Old medium	Fresh Medium ^a	Old Medium ^a	Fresh Medium ^a	Old Medium ^a	Fresh Medium ^a	Fresh Medium ^a	Old Medium ^a
Brisy	Control	7.6	7.5	4.1±0.63	7.1±1.52	9.7±0.12	10.7±0.14	<0.5	<0.5	<0.5
	100	7.6	7.1	3.8±0.73	6.7±1.25	11.5±4.23	10.5±0.72	67±5.4	46±22.3	15±4.3
	180	7.6	7.2	4.1±1.1	7.9±1.92	11.7±4.36	12.8±5.25	176±57.9	98±13.3	29±7.5
	320	7.6	7.0	4.2±0.88	7.8±1.94	10.9±2.73	13±5.29	359±149	250±0	138±35
	560	7.6	7.4	3.6±0.24	7.1±1.3	11.6±4.22	12.3±4.45	519±112	310±22.5	140±75.7
	1000	7.6	7.6	3.8±0.26	6.9±0.79	9.2±0.6	13±4.73	1312±159	1104±89.6	553±14.8
	Brisy high Ca	Control	7.5	7.5	4.1±0.8	7.0±1.05	107±2.65	111±5.87	<0.5	<0.5
180	7.5	7.1	4.6±0.88	7.2±0.85	100±3.79	108±2.29	169±78.4	111±14.4	25±6.8	
320	7.5	7.2	4.6±1.4	8.0±1.12	98±2	107±5.53	362±135	187±66.4	53±20.8	
560	7.5	7.1	4.6±1.3	8.2±0.65	98±25	110±6.41	620±120	466±24.8	221±19.6	
1000	7.5	7.4	4.6±1.3	8.5±1.51	115±18.7	100±7.57	1364±79.2	1221±51.8	705±39.8	
1800	7.5	7.5	4.5±0.79	8.1±0.47	112±14.2	104±11.35	1839±137	1618±137	514±148	
Ankeveen	Control	8.2	8.1	9.5±0.4	12.3±0.86	50.1±0.6	51.2±0.49	<0.5	<0.5	<0.5
	180	8.2	8.2	10±0.69	14±0.81	50.3±2.02	54.2±2.22	172±10.2	164±17.5	69±21.2
	320	8.2	8.0	8.8±0.26	12.2±1	48.1±1.63	49.1±1.5	425±11.9	388±19.7	195±34
	560	8.2	8.0	9.4±1.05	13.3±2.14	48.5±1.4	49.2±1.1	594±0	542±55.2	225±49.1
	1000	8.2	8.0	8.7±0.79	12.2±1.13	47.3±1.4	45±8.19	1270±203	1200±278	788±104
	1800	8.2	8.0	8.4±0.26	11.8±1.13	46.3±0.4	49.2±2.01	1689±102	1639±122	601±330
	Ankeveen low pH	Control	7.2	7.1	10.1±1.22	13.5±3.7	51.8±0.6	51.3±1.1	<0.5	<0.5
100	7.2	7.3	8.9±0.62	12.9±4	48.6±2.3	49.7±1.97	80±2.8	74±4.2	32±10.9	
180	7.2	7.4	8.9±0.68	11.7±2.72	48.7±1.96	50.6±4.36	168±5.8	152±23.5	44±19.8	
320	7.2	7.4	9.1±0.8	11.8±2.83	48.3±1.59	50.5±2.17	438±28.2	404±9.1	208±29.6	
560	7.2	7.4	8.9±0.95	11.3±2.6	47.7±2.34	50.8±4.11	638±37.2	573±49.9	292±52.1	
1000	7.2	7.3	8.6±0.65	11.8±2.71	49±2.23	50.8±1.19	1136±401	1092±379	448±155	
Regge	Control	7.9	7.8	17.2±2.9	21.6±1.32	53.7±1.43	74.9±0.92	<0.5	<0.5	<0.5
	320	7.9	7.2	14.1±0.58	13.3±1.08	16.1±0.55	17.8±7.53	318±18.5	298±2.7	138±12.2
	560	7.9	7.1	14.4±0.98	13.9±2.52	15.8±0.3	14.6±1.81	542±11.9	513±8.7	242±15.5
	1000	7.9	7.1	14.6±1.31	14.8±4.02	16.4±0.76	15.6±1.91	1033±20	963±52	576±44.8
	1800	7.9	7.1	13.6±0.32	13.4±0.6	18.6±3.64	13.7±1.44	1702±24.8	1473±122	543±125
	3200	7.8	7.5	14.5±0.35	15±1.06	18.1±2.27	23.2±6.82	2992±47	2856±114	960±157

^a n=3

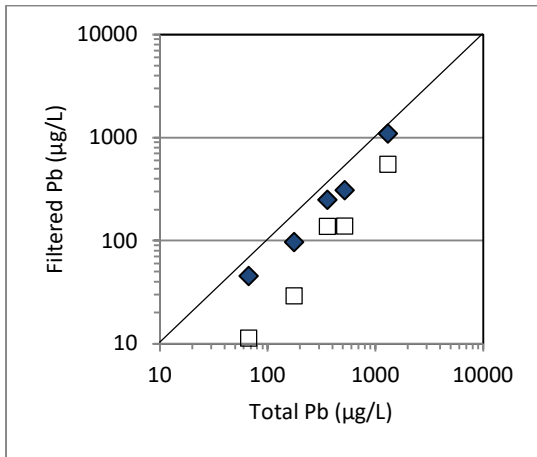
Figure 2 presents the concentrations of dissolved Pb during the chronic tests. The dissolved Pb concentrations in fresh medium were 55-84% (*Brisy*) and 87-97% (*Ankeveen*, and *Regge*) of the total Pb concentrations. The fractions of dissolved Pb are in agreement with the data of the equilibration and the range-finding phases. For *Brisy high Ca* water, the fractions of dissolved Pb were 52-89% of the total Pb concentrations. The amount of dissolved Pb in fresh medium was >90% in *Ankeveen low pH* water.

The results also show the decrease of dissolved Pb between fresh medium and old medium. This decrease was more pronounced in comparison with the difference of dissolved Pb between day 4 and day 7 observed in the equilibration (3.1; Figure 1). The decline of dissolved Pb in old medium was similar to that observed in the range-finding tests. For example, in the range-finding tests dissolved Pb reduced to 47% in old medium (*Brisy*, 1180 µg/L). In the chronic toxicity tests, 50% decline of dissolved Pb was noted (*Brisy*, 1312 µg/L). The presence of test organisms and food particles provided during the tests is a likely explanation for the decrease in dissolved Pb between fresh medium and old medium.

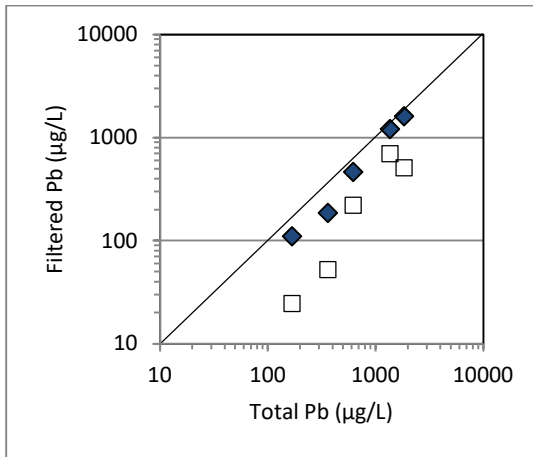
In *Brisy*, *Brisy high Ca*, *Ankeveen* and *Ankeveen low pH* waters, the pH values, DOC and Ca concentrations varied little between the controls and Pb spiked waters. There was no difference in Ca concentrations between fresh medium and old medium. The DOC concentrations in old medium were 2.5 to 4.5 mg/L higher than that of the fresh medium. This could be attributed to the addition of food and activity of the organisms during the tests.

In *Regge* control water, the DOC concentration in the old medium was also higher than that of the fresh medium (4.4 mg/L). Still in this control, an unexplained increase of Ca with 20 mg/L between fresh medium and old medium was noted. In Pb spiked *Regge* water, however, there was little difference in DOC and Ca concentrations between fresh medium and old medium. In comparison with the control, the DOC and Ca concentrations in all Pb spiked treatments in *Regge* water were considerably lower than in the controls. This reduction of DOC and Ca contents is difficult to explain with the current dataset, but it could be related to Pb-DOC-Ca co-precipitation. In any case, the fact that Ca is considerably different between control and Pb treatments, would seriously compromise the reliability of any derived NOEC or ECx values for *Regge* water and we therefore decided not to report them at all.

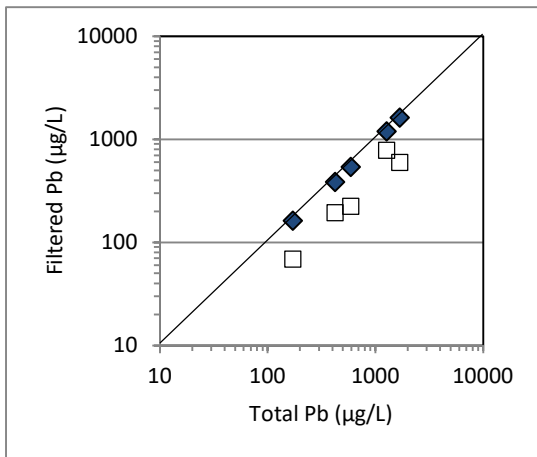
Brisy



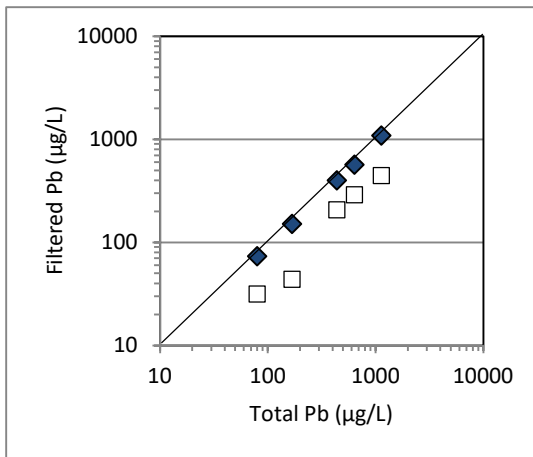
Brisy high Ca



Ankeveen



Ankeveen low pH



Regge

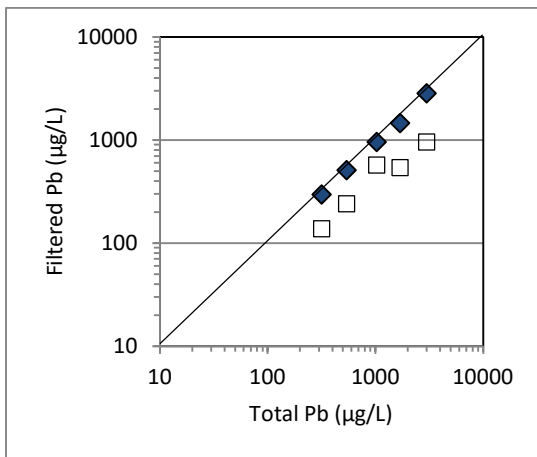


Figure 2. Mean dissolved Pb measured in fresh medium (■) and old medium (□) (n=3) (phase 3)

3.3.2 Toxicity

During the chronic tests, water temperatures were 18-19°C and dissolved oxygen was always >5 mg/L. The control survival in *Brisy*, *Brisy high Ca*, *Ankeveen low pH* and *Regge* waters ranged from 70% to 80%. For *Ankeveen*, survival was 63.3% in the control, but was >70% in some of the Pb treatments. These control survivals were slightly lower in comparison with those of the range-finding tests. The use of midge larvae from different sub-populations for range-finding tests (phase 2) and chronic tests (phase 3) could be a reason for this variability in midge survival.

Survival (as % relative to control survival) of *C. riparius* exposed to Pb in different waters is shown in Figure 3. For *Brisy* water survival at 829 µg/L was 45.8±14.4% (p<0.05). For *Brisy high Ca*, survival at 1066 µg/L was 8.7±7.5% (p<0.05). For *Ankeveen*, *Ankeveen low pH* and *Regge* waters no adverse effect on survival was noted in all Pb concentration tested (*Ankeveen*: ≤1120 µg/L; *Ankeveen low pH*: ≤770 µg/L; *Regge*: ≤1908 µg/L).

Figure 4 presents the growth of *C. riparius* in different waters. The control body mass of the midges was considerably lower than those in the ≤225 µg/L (*Brisy*) and in the ≤829 µg/L (*Brisy high Ca*). Further investigation is needed to explain for the low control growth in these waters (relative to the lower Pb treatments). The data on growth of *Brisy* and *Brisy high Ca*, were therefore not used for derivation of NOEC, LOEC or EC_x values. For *Ankeveen*, *Ankeveen low pH* a clear dose-response relationship between the growth and Pb concentration was noted. The body mass was significantly reduced at ≥770 µg/L in *Ankeveen* and *Ankeveen low pH*.

Based on those toxicity test results that are considered reliable, the NOEC, LOEC, EC₁₀, EC₂₀ and EC₅₀ values for survival and growth are presented in Table 5. Combining these values with the whole dose response data, it can be concluded that, on the basis of mean filtered Pb, there appears to be limited to no protective effect of increasing Ca (*Brisy* and *Brisy high Ca* survival data) and that the effect of pH (between 7.2 and 8.2) on chronic toxicity is also limited to non-existing (*Ankeveen low pH* vs. *Ankeveen* growth). Those data can be used (together with the raw concentration response data (Figure 3 and Figure 4) for BLM modeling and to check if the limited effects of Ca and pH are better explained by the fish BLM (De Schampelaere, 2010) or by the *Ceriodaphnia* BLM (Nys et al., 2012).

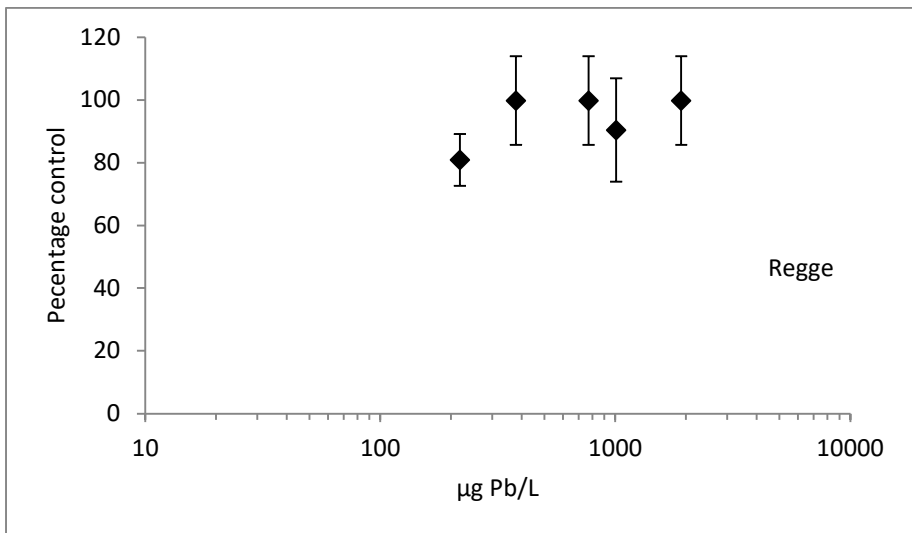
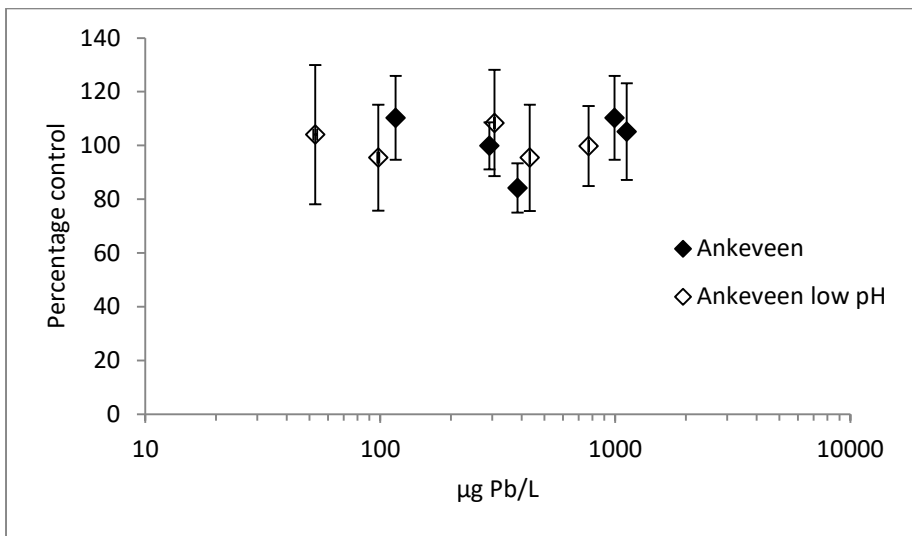
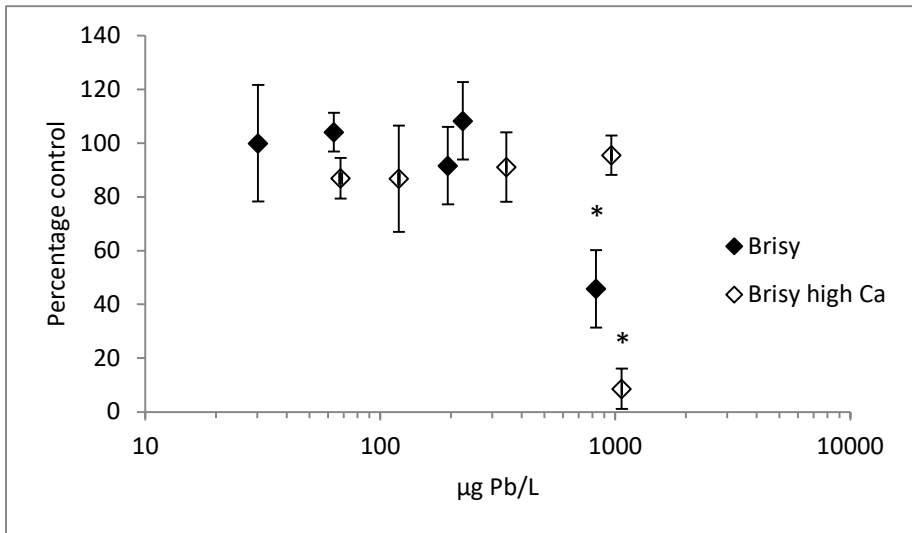


Figure 3. Survival of *Chironomus riparius* as a function of the mean filtered Pb in different natural waters. Data are expressed as percentage of the mean observed control survival (n=3). * indicates significantly different from the control (p<0.05).

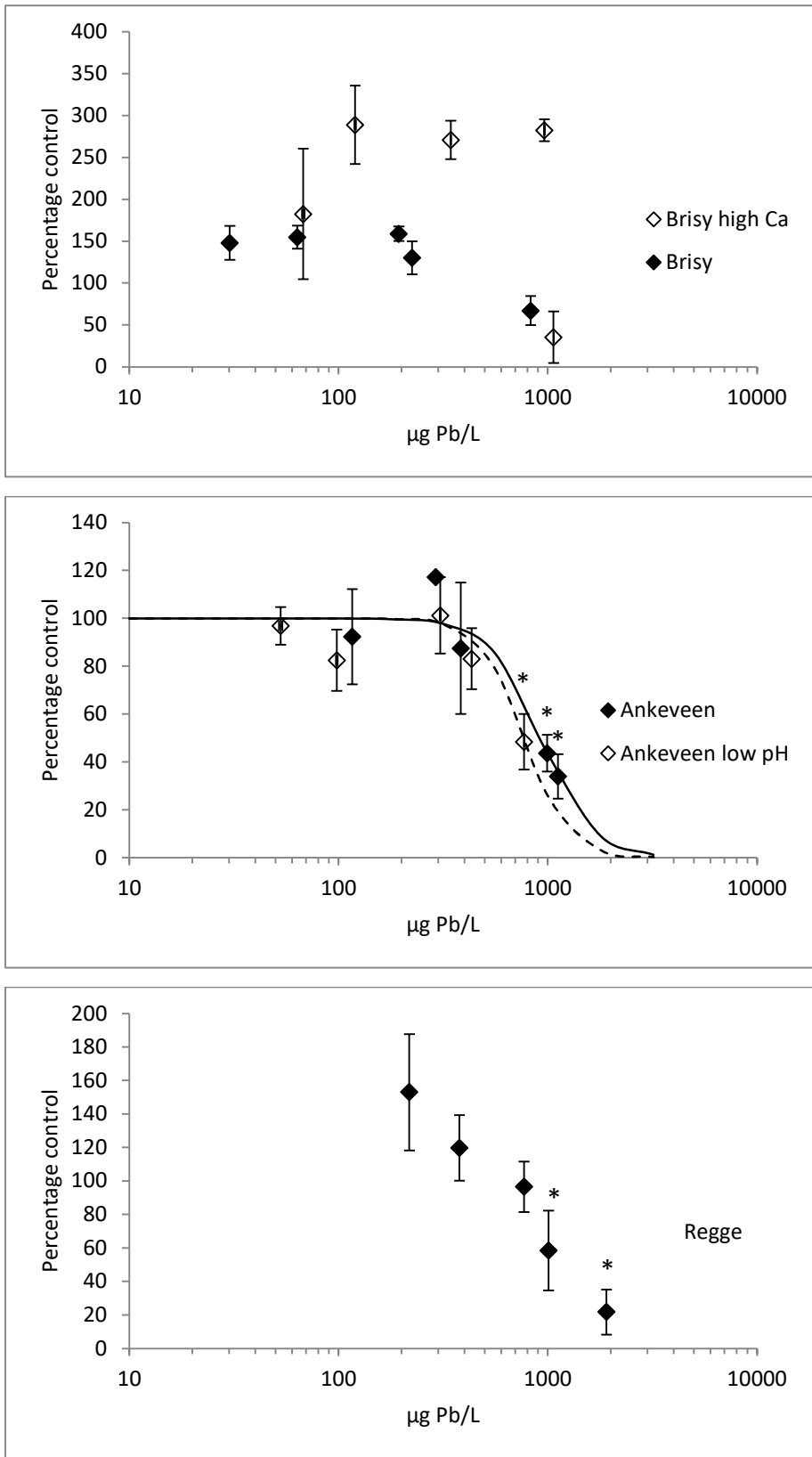


Figure 4. Growth of *Chironomus riparius* as a function of the mean filtered Pb in different natural waters. Data are expressed as a percentage of the control (n=3). Control body mass at day 14: *Brisys*: 0.55 ± 0.06 mg/org; *Brisys high Ca*: 0.31 ± 0.04 mg/org; *Ankeveen*: 1.03 ± 0.14 mg/org; *Ankeveen low pH*: 0.99 ± 0.18 mg/org; *Regge*: 0.78 ± 0.08 mg/org. *: significantly different from the control ($p < 0.05$).

Table 5. Summary of the effect concentration for dissolved Pb ($\mu\text{g/L}$) for 5 waters. These were calculated based on average of values measured in fresh medium and old medium.

Waters	14-day EC ₁₀ (95% CL)	14-day EC ₂₀ (95% CL)	14-day EC ₅₀ (95% CL)	14d-NOEC	14d-LOEC
Brisy					
<i>Survival</i>	-	-	~829 ^a	225	829
<i>Growth</i> ^c	Not calculated	Not calculated	Not calculated	Not calculated	Not calculated
Brisy high Ca					
<i>Survival</i>	-	-	963-1066 ^b	963	1066
<i>Growth</i> ^c	Not calculated	Not calculated	Not calculated	Not calculated	Not calculated
Ankeveen					
<i>Survival</i>	-	-	>1120	≥1120	>1120
<i>Growth</i>	495 (156-1564)	623 (274-1414)	925 (698-1227)	384	994
Ankeveen low pH					
<i>Survival</i>	-	-	>770	≥770	>770
<i>Growth</i>	461 (257-828)	551 (373-813)	775 (650-924)	433	770
Regge					
<i>Survival</i>	-	-	>1908	≥1908	>1908
<i>Growth</i> ^c	Not calculated	Not calculated	Not calculated	Not calculated	Not calculated

^a No dose response curve could be fitted because only one treatment with a partial response was available (EC₅₀ is estimated as the mean filtered concentration at the treatment where about 50% effect was observed (see Figure 3)

^b No reliable dose response curve could be fitted, but the true EC₅₀ should be between these two concentrations encompassing the 50% effect level (see Figure 3).

^c Not calculated due to non-reliable dose-response data (due to unexplained and considerably lower growth in controls than in lower Pb treatments).

4. Conclusions

We have performed chronic 14-day toxicity tests with Pb and the midge *C. riparius* in three natural waters and two modified natural waters. Due to unexplained changes in water chemistry in Pb spiked treatments of one water (*Regge*) and/or considerable (and not fully explainable) low control growth compared to low Pb treatments (*Regge, Brisy, Brisy high Ca*) not all dose response data for the growth endpoint can be considered reliable. Despite this, reliable data for further BLM modeling are nonetheless available for five waters (for survival) and for two waters (for growth). Overall, those data suggest limited to no effects of pH and Ca on chronic Pb toxicity, when expressed as filtered Pb concentration. Future modeling is needed to test whether or not these limited effects are correctly predicted by the fish or *C. dubia* BLM.

5. References

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