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Pb-Diet

An investigation of the potential toxicity of dietary Pb to *Ceriodaphnia dubia*

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1. Introduction

There is increasing evidence that dietary metal exposure is readily assimilated by and can cause toxicity to aquatic invertebrates (see Meyer et al., 2005 for a review). In our laboratory we have recently demonstrated reproductive inhibition in *Daphnia magna* that were fed with live green algae (*Pseudokirchneriella subcapitata*) which were contaminated with Zn (De Schamphelaere et al., 2004), Cu (De Schamphelaere et al., 2007) and Ni (Evens et al., 2009). Dietary metal exposure can influence the reproduction of *Daphnia magna* directly (Cu) or indirectly (Zn). Dietary Cu toxicity may be caused by a direct effect on several toxicity mechanisms, such as increased metabolic cost, reduced energy acquisition (potentially via inhibition of digestive enzyme activity), targeted inhibition of reproduction (potentially via inhibition of vitellogenesis), and/or direct inhibition of molting (De Schamphelaere et al., 2007). .The "dietary" effects of Zn on reproduction of *Daphnia magna* are, in contrast, more likely an indirect effect of the quality of the algal food, which is influenced by Zn exposure (Evens et al. 2012a). The effect of dietary Ni toxicity is likely induced by a combination of both direct and indirect effects (Evens et al. 2012b).

The occurrence of dietary toxicity has also implications for the chronic biotic ligand model (BLM), which predicts chronic 'waterborne' metal toxicity as a function of the physicochemical characteristics of the water, thereby assuming that only the waterborne exposure route is important. This assumption may not be valid since in chronic ecotoxicity tests with *D. magna*, live algae are always administered as a food source for the daphnids, and those algae can take up metals before being ingested, thus creating a dietary exposure pathway to the daphnids. Hence, the derived BLM parameters (stability constants), which are assumed to describe competitive waterborne toxicity reduction by e.g. increased waterborne Ca^{2+}, H^{+} (decreased pH), in fact may, in part, contain a dietary toxicity component. Thus, there is an urgent need for a clearer understanding of the effects of dietary metal exposure and the interaction with the waterborne exposure route.

Previous research showed that dietary Pb toxicity had no effect on fish growth and survival when fed with diets containing up to 1000 µg Pb/g dry matter (Alves et al. 2006, Erickson et al. 2010). To our knowledge, there is only one study which investigated the effects of dietary Pb exposure to a freshwater invertebrate, i.e. *Hyalella azteca* (Besser et al., 2005). In contradiction with the fish data, Pb contaminated diets significantly affected – according to these authors - survival, growth and reproduction of *Hyalella azeteca.* However, a few issues make the results of this study ambiguous; e.g. 'control diet' was not specified, the chosen reproductive endpoint (juveniles/adult instead of juveniles/female) was not the most appropriate, and the way of statistically analyzing the data was not

the most adequate. Therefore, there is still insufficient knowledge to integrate dietary Pb toxicity in risk assessment processes. In this report, we present a study on dietary Pb toxicity to *Ceriodaphnia dubia* fed with a live diet of *Pseudokirchneriella subcapitata.*

2. Materials &Methods

2.1 Collection of natural water

All toxicity tests were conducted in modified natural water. The natural water was collected from L'Ourthe Orientale in Brisy, Belgium. This water has previously been used successfully for ecotoxicity testing in our lab for the development of the chronic Pb-BLM for *C. dubia* (Nys et al. 2012) and is known to be unpolluted and has a low hardness and low DOC concentration. The natural water was filtered through 0.45 µm and stored at 4°C in total darkness in 10L acid washed polyethylene barrels until use. 0.75 mM $CaCl_2$ was added to the Brisy water (hereafter called the modified Brisy medium), to assure adequate minimal Ca concentrations needed for *Ceriodaphnia dubia* growth and reproduction.

2.2 Experimental design

The diet experiment consisted of three different exposure settings: a waterborne exposure, a dietborne exposure and a combined diet- and waterborne exposure (Figure 1). The green algae *Pseudokirchneriella subcapitata* was exposed to 6 Pb concentrations and a control for 64h, after which the algae cells were harvested and fed to *C. dubia* in the diet toxicity test. The toxicity tests for all exposure routes were run simultaneously.

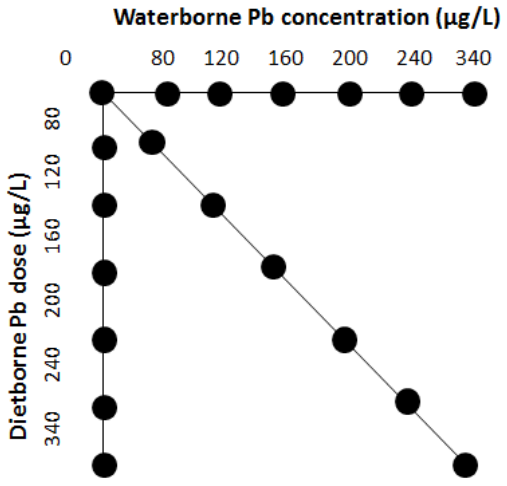

Figure 1 Experimental design of the Pb diet experiment.

2.3 Algae exposure

P. subcapitata starter cultures were obtained from the Culture Collection of Algae and Protozoa (CCAP 278/4, Argyll, Scotland). The algal pre-exposure culture was grown in carbon-filtered aerated tap water (Gent, Belgium) supplemented with the modified Provasoli's ES enrichment (Bold and Wynne, 1978) at 1/2 strength, and also with 1.4 mg/L $FeSO_4$, 15 mg/L NaH_2PO_4·, 150 mg/L $NaNO_3$ and 2.35 mg/L $MnCl_2$. This culture was grown for 5 days, i.e. until the end of the exponential growth phase, and subsequently harvested to initiate actual Pb exposures. Pb exposure of algae was performed in 1.5L glass-jars containing 1L of exposure medium. For the algae exposure 1 mM 3-N-morpholino-propane-sulfonic acid (MOPS) was added as pH buffer to the modified Brisy medium, to keep pH stable during the exposure. When pH was not buffered, precipitation was clearly visible in the algae exposure (Figure A7, see appendix). The added concentration of MOPS was chosen after a preliminary test, in which pH in the Brisy medium with 10^6 cells/ml was monitored during 64h (see appendix C). The algae were exposed to a Pb concentration range that was known to cause chronic waterborne toxicity to *C. dubia*: i.e. 80, 120, 160, 200, 240 and 340 µg Pb/L (Nys et al. 2012). The same concentration range was applied in the waterborne Pb exposure of *C. dubia*.. All chemicals were purchased from VWR International (Leuven, Belgium). Each Pb treatment of the algae exposures was performed with three replicates (i.e. 3 glass-jars). The control had 10 replicates, to ensure a sufficient amount of control algae for the subsequent *C. dubia* exposures.

P. subcapitata inoculation density was selected at 10^6 cells/mL after preliminary testing (see Appendix B). The algae exposure was performed under the same conditions as the *C. dubia* exposure: i.e. 25°C and a light cycle of 16h light and 8h dark, starting with 5h light at the beginning of the exposure. The cell density in all treatments was determined daily with a Coulter particle counter (Beckman-Analis, Namur, Belgium). pH was measured twice per day and adjusted with dilute NaOH or HCl when needed. After 64 h of exposure, the algae were harvested by centrifugation, and collected in 15 mL of the supernatant for storage in the dark at 4°C. Algae were stored 2 days before the start of the *C. dubia* exposure. Maximum storage duration was 9 days (end of *C. dubia* exposure).

2.4 Algal characteristics

Dry weight of the exposed algae was determined at the beginning of the *C. dubia* experiment. Dry weight was determined by filtering 10^8 cells over a washed and dried 0.45 µM filter (type Supor 450, PALL Life Sciences, Port Washington, NY, USA). Filters were dried for 24h and measured to the nearest 0.01 mg.
Pb burdens of the stored algae were determined at the beginning (1 day after centrifugation) and end (9 days after centrifugation) of the *C. dubia* exposure. For Pb burden analyses, 10^8 cells of the diet stock

suspensions were centrifuged in triplicate in 2 ml polyethylene Eppendorf vials at 13,000×*g* for 15 minutes in a Mikro 200 R centrifuge (Hettich Zentrifugen; Tuttlingen, Germany) and the supernatant was removed. The remaining pellet was resuspended in 1.2 ml of a 1 mmol/L solution of KCl to avoid cell wall disruption by a prolonged exposure to Na_2EDTA alone. After resuspension 0.1 mL of a 65 mM Na_2EDTA solution was added. Samples were homogeneously mixed and they were then left to react for 1 min (Slaveykova & Wilkinson, 2002) and then centrifuged for the second time exactly as described above. Subsequently, the supernatant was removed and stored. External Pb (adsorbed to the algae) was defined as the Pb quantity that was desorbed from the algal surface into the supernatant by this EDTA wash. The samples with EDTA solutions were acidified by adding 1% HNO_3 (v/v) The internal Pb burdens of the algae were determined by hot acid digestion (60 min, 110°C) of the remaining algae in 400 µL of ultrapure 14 mol/L HNO_3 (Normatom quality, obtained from VWR Prolabo, Leuven, Belgium) in the Eppendorf vials. The digests were diluted 10-fold with deionized water before analysis.

2.5 *C. dubia* exposure

The chronic Pb toxicity tests were conducted following the USEPA protocol (USEPA, 2002). *C. dubia* juveniles originated from an in-house isoclonal lab culture which is being maintained at 25°C in carbon filtered Ghent city tap water to which selenium (1 µg Se/L) and vitamins (75 µg/L thiamine, 1 µg/L cyanocobalamine, 0.75 µg/L biotine) are added. Acclimation started with 140 juveniles (0-24h), which were cultured individually in polyethylene cups containing 20 mL of the modified Brisy medium. During the acclimation period daphnids were daily fed with clean (i.e. not exposed to Pb) *P. subcapitata* algae (2 10^5 cells/mL) which are cultured weekly in the lab and a YUT-mixture (Yeast-Urtica-Trout Chow mixture of 12 mg solids/L). Media were refreshed completely every other day. The diet toxicity test was initiated with juveniles (0-24h) of the 4^{th} brood, originating from acclimated parent animals which produced at least 8 juveniles in this brood. The offspring of one parent animal was evenly distributed among the Pb treatments within a test plate, as subscribed by the USEPA protocol (2002). For the waterborne and combined exposure route 6 Pb concentrations and a control were tested. After Pb spiking, media were equilibrated at 25°C for one day before use. The dietborne exposure route was tested in modified Brisy medium with no Pb spiked in the water. During the toxicity test daphnids were daily fed with a clean YUT-mixture (12 mg solids/L, not spiked with Pb) and 2 10^5 cells/mL of the stock suspensions of Pb-exposed algae. The daphnids in the waterborne exposure route treatments and those in the controls of the dietborne and combined exposure route treatments were fed with the control algae. The daphnids in the other treatments of the dietborne and combined exposure were fed with six dietary Pb concentrations (i.e., 6 algae stocks exposed to a different Pb concentration). Ecotoxicity tests were run

all simultaneously to exclude interference of potential temporal sensitivity variation of the daphnids with the further interpretation of the observations. The control and each Pb treatment of every exposure route received ten replicates. Tests were conducted in polyethylene cups consisting of 20 mL of test medium at 25°C under a light cycle of 16h light and 8h dark. Test media were renewed daily. Before renewal, fresh test media were adjusted to pH 7 by adding HCl or NaOH. Mortality and number of juveniles were scored daily. The toxicity tests were ended when 60% of the control animals had produced three broods (8 days).

2.6 Analytical chemistry

During the algae exposure samples for total and filtered (0.45 µM, Acrodisc, PALL Life Sciences, Port Washington, NY, USA) Pb, OC (organic carbon) and IC (inorganic carbon) measurement were taken at the start and end (64h) of the exposure. Samples for filtered Pb measurements were also taken at 16h and 40h after the start of the exposure. During the *C. dubia* exposure period samples of fresh and old (just after renewal) test media were collected regularly for analysis of total and filtered Pb, OC and IC. Total and filtered samples of fresh media were taken on day 0, day 3 and day 6. Filtered samples of old media were taken on day 1, day 4 and day 7. Samples for Pb measurement were acidified to 0.14 mol/L HNO_3 (Normatom quality, VWR Prolabo, Leuven, Belgium). Pb concentrations were measured using graphite furnace atomic absorption spectrophotometry (GFAAS Furnace Autosampler, Thermo Fisher Scientific Inc., Waltham, MA, USA). Ca concentrations were measured using a flame atomic absorption spectrophotometry (SpectrAA100, Varian, Mulgrave, Australia). DOC and DIC were measured with a Total Organic Carbon analyzer (TOC-5000, Shimadzu, Duisburg, Germany). pH of fresh and old media were measured daily with a pH glass electrode.

2.7 Concentration response analysis

Effect concentrations (EC10 and EC50) were calculated based on average filtered Pb concentrations. Total reproduction relative to the mean control reproduction (%) was used as endpoint. EC10, EC50s and corresponding 95% confidence intervals were determined with the drc-package in R 2.14.1 (R Development Core Team, Vienna, Austria) with a log-logistic concentration response model with three parameters:

$$y = \frac{100}{1+exp^{(b(\log(x)-\log(EC50)))}} \qquad \text{(Eq. 1)}$$

Where y= predicted reproduction (total number of offspring) relative to the average of the controls (%), b= a slope parameter, x= the mean filtered Pb concentration (µg/L), EC50= median effect concentration (µg/L).

3. Results and discussion

3.1 Algae exposure

Measured Pb concentrations gradually decreased during the algae exposure period (

Table 1 and Figure 2). After 40h of exposure filtered Pb concentrations were more or less stable. During the algae exposure filtered Pb concentration in the exposure solution was lowered 3- to 4-fold. At the end of the exposure period between 18 and 52 µg Pb/L was still in the filtered phase, which is on average 70% less than at the start of the exposure. It is assumed that the exposed algae were in equilibrium with these Pb concentrations.

Table 1 Measured Pb concentrations in solution during the algae exposure

Nominal conc (µg/L)	Start exposure (0h)		16h	40h	End of exposure (64h)
	Total Pb conc. (µg/L)	Filtered Pb conc. (µg/L)	Filtered Pb conc. (µg/L)	Filtered Pb conc. (µg/L)	Filtered Pb conc. (µg/L)
0	<DL[b]	<DL	<DL	<DL	<DL
80	62	48	27	16	18
120	101	71	37	26	22
160	138	120	49	34	32
200	205	147	66	46	39
240	235	193	80	56	46
340	281	215	92	64	52

[a] Concentration measured in the supernatant obtained after centrifugation of the algae
[b] <DL: measured concentration below detection limit (0.5 µg Pb/L)

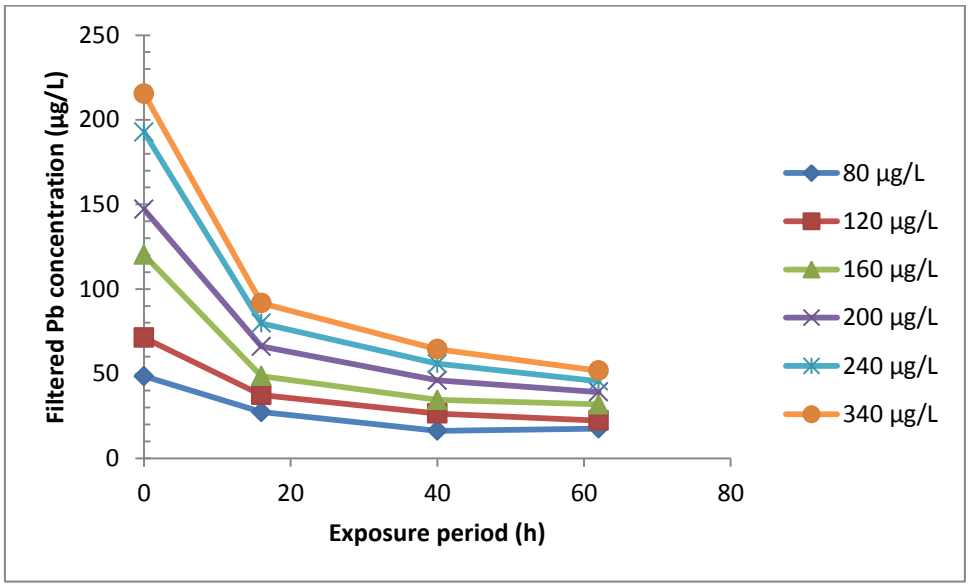

Figure 2 Filtered Pb concentrations for the different Pb treatments during the algae exposure.

Cell density increased slightly during the exposure period for all treatments (Figure 3). There were no significant differences in cell densities between Pb treatments after 16h and 40h of exposure (Kruskal-Wallis test: 16h: p=0.36, 40h: p=0.06). However, at the end of exposure (64h) cell densities in the control and 120 µg/L treatments were significantly higher than in the 340 µg/L treatment ($p<0.001$). pH was relatively stable during the exposure period and across Pb treatments (Figure 4). pH and cell density were relatively constant between the replicates of a Pb treatment (Table 2). Therefore, the replicates of every Pb treatment were pooled after centrifugation for the algal characteristics measurements and the feeding during the *C. dubia* exposure.

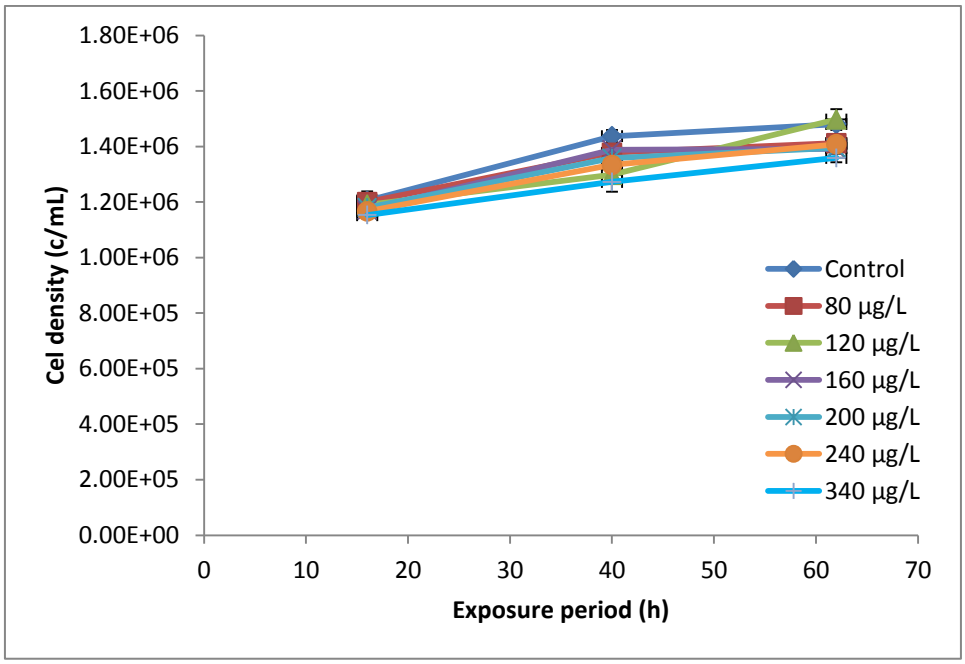

Figure 3 Cell density for the different treatments during the algae exposure. Error bars indicate standard errors.

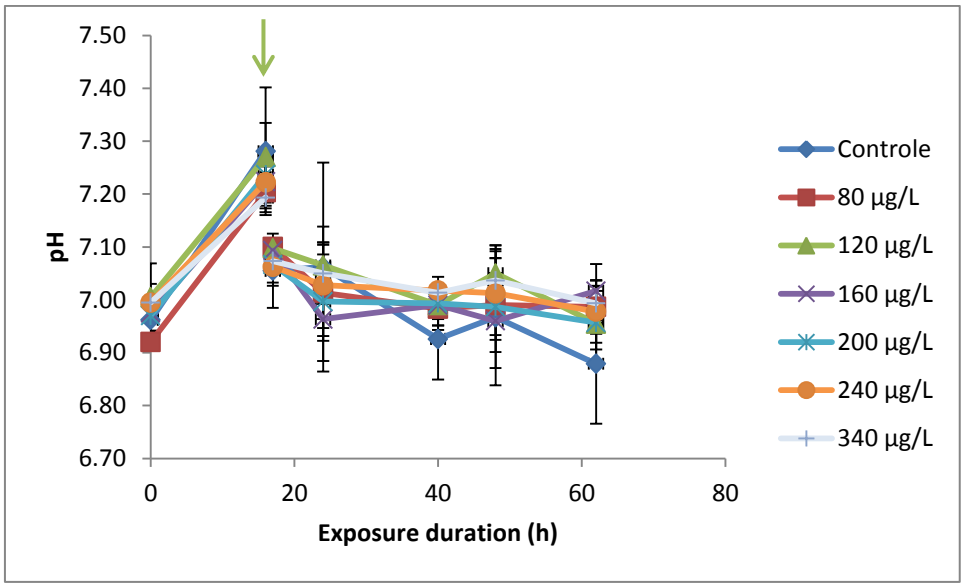

Figure 4 pH for the different Pb concentrations during the algae exposure. Error bars indicate standard deviations. The arrow indicates a pH adjustment after 16h of exposure.

Table 2 pH and cell densities during the algae exposure. Mean values for all Pb treatments are reported ± standard deviation.

Nom. Conc.	pH							Cell density (10^6 cells/mL)		
	0h	16h	16.02h[a]	24h	40h	48h	64h	16h	40h	64h
Controle	7.0±0.0	7.3±0.1	7.1±0.1	7.1±0.2	6.9±0.1	7.0±0.1	6.9±0.1	1.20±0.07	1.44±0.07	1.48±0.04
80 µg/L	6.9±0.0	7.2±0.0	7.1±0.0	7.0±0.1	7.0±0.0	7.0±0.1	7.0±0.1	1.20±0.07	1.38±0.08	1.41±0.01
120 µg/L	7.0±0.1	7.3±0.1	7.1±0.0	7.1±0.1	7.0±0.0	7.1±0.1	7.0±0.1	1.19±0.05	1.30±0.06	1.50±0.07
160 µg/L	7.0±0.0	7.2±0.1	7.1±0.0	7.0±0.0	7.0±0.0	7.0±0.0	7.0±0.0	1.17±0.01	1.39±0.01	1.39±0.01
200 µg/L	7.0±0.0	7.2±0.0	7.1±0.0	7.0±0.1	7.0±0.1	7.0±0.1	7.0±0.0	1.18±0.03	1.36±0.07	1.39±0.02
240 µg/L	7.0±0.0	7.2±0.0	7.1±0.0	7.0±0.1	7.0±0.0	7.0±0.1	7.0±0.1	1.17±0.04	1.33±0.04	1.41±0.05
340 µg/L	7.0±0.0	7.2±0.0	7.1±0.0	7.1±0.0	7.0±0.0	7.0±0.0	7.0±0.0	1.15±0.01	1.27±0.06	1.36±0.03

[a] pH measurement 1 minute after pH adjustment at 16h of exposure.

3.2 Algal characteristics

The results of the internal and external Pb burdens of the algal cells are listed in Table 3. The measured Pb concentrations in the EDTA and digested samples are listed in supportive information (Table S1). External and internal Pb concentrations increased with increasing exposure concentrations (Figure 5). However, for the external Pb concentrations a saturation effect was observed at higher Pb concentrations, which was also reported by Slaveykova & Wilkinson (2002). The external Pb concentrations decreased slightly at the end of the *C. dubia* exposure period in comparison with the measurements at the beginning of the exposure period. The internal Pb concentrations remained relatively constant while the algae were stored in the fridge, except for the algae at the highest Pb exposure concentration, for which the internal Pb concentration decreased with about 25%.

Table 3 Dry weight, calculated Pb conc at the surface of the algal cells (Pb_{extern}) and Pb conc in the algal cells(Pb_{intern}) at the start and end of the *C. dubia* exposure period.

Nominal Pb conc. in algae exposure	Start of C. dubia exposure (Day -1)			End of C. dubia exposure (Day 8)	
	Dry weight (mg/10^8cells)	Pb_{extern} (µg/g dry weight)	Pb_{intern} (µg/g dry weight)	Pb_{extern} (µg/g dry weight)	Pb_{intern} (µg/g dry weight)
Control	1.62±0.19	7±1	<DL	9±1	<DL
80 µg/L	1.80±0.07	891±103	206±22	576±160	333±200
120 µg/L	1.94±0.12	1095±94	330±99	863±80	348±33
160 µg/L	2.01±0.14	1361±317	697±264	1161±309	645±145
200 µg/L	1.82±0.09	1382±194	1162	964±204	1385±345
240 µg/L	2.43±0.02	1204±36	1421±113	1159±35	1812±309
340 µg/L	1.90±0.09	1638±600	3669±1154	1439±202	2739±334

<DL: detection limit for Pb_{intern} is 12 µg/g dry weight, for Pb_{extern} DL is 0.3 µg/g dry weight

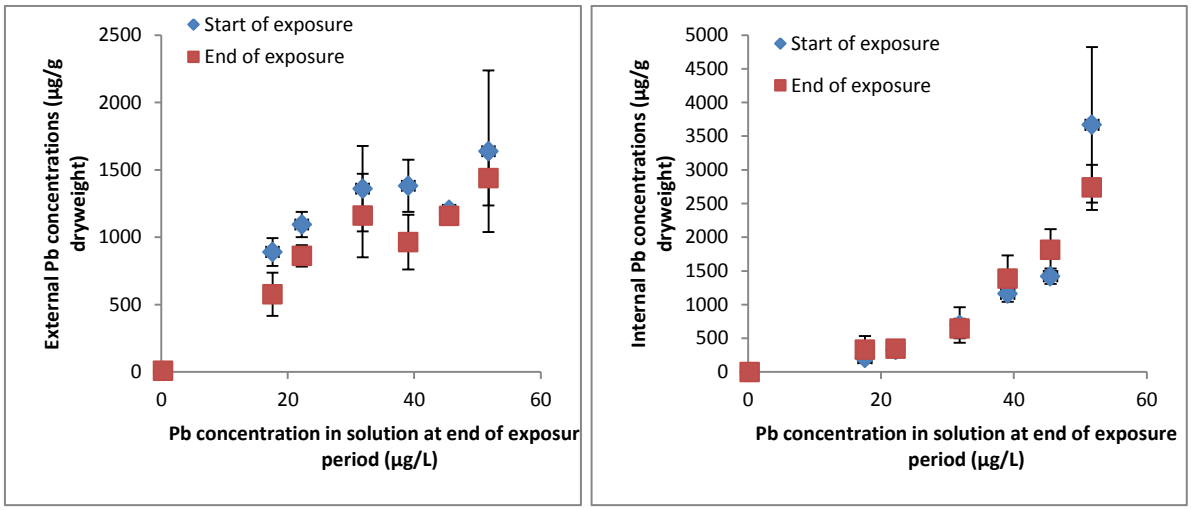

Figure 5 External and internal Pb concentrations of the algae cells. Error bars indicate standard deviation.

A Langmuir isotherm was fitted to the external Pb concentrations data with Statistica 7.0 (StatSoft Inc, Tulsa, USA) (data from measurements before start of *C. dubia* exposure):

$$Pb_{ads} = Pb_{ads,max}\frac{K_L[Pb^{2+}]}{1+K_L[Pb^{2+}]} \qquad \text{(Eq. 2)}$$

Where Pb_{ads} is the amount of adsorbed Pb to the algal cell (mol/g DWT), $Pb_{ads,max}$ is the maximum amount of Pb adsorbed to algal cell (mol/g DWT), K_L is the Langmuir adsorption constant (L/mol) and Pb^{2+} is the Pb free ion activity (mol/L). The maximum concentration of Pb sorbed to the algal cells ($Pb_{ads,max}$) was determined to be 8.47 µmol/g DWT and log K_L was determined to be 9.37 (pH 7, fulvic acid concentration of 3.6 mg/L). The Langmuir adsorption constant calculated for this study is 4 orders of magnitude higher than the Langmuir adsorption constant of another green algae species, *Chlorella kesslerii,* reported by Slaveykova & Wilkinson, (2002) (log K_L of 5.04 L/mol, pH 6, no fulvic acid in exposure medium). This difference in adsorption constant can potentially be explained by both biotic and abiotic factors. First, the adsorption of metals can vary among species. Second, Pb adsorption increases with increasing pH (Slaveykova & Wilkinson, 2003) and in the presence of fulvic acid (Slaveykova et al., 2003).

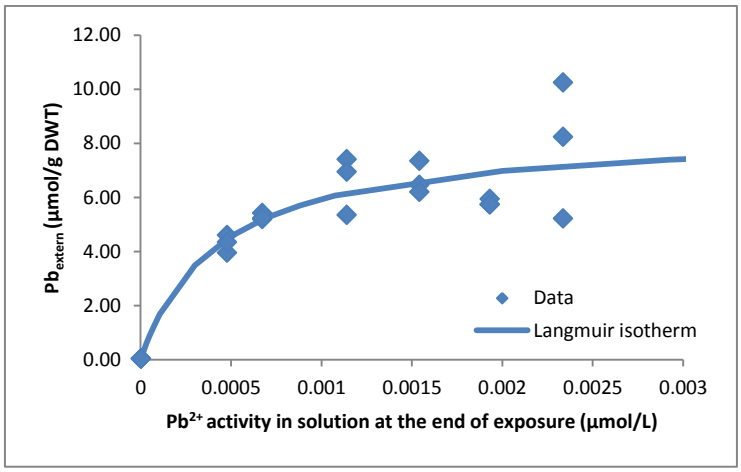

Figure 6 Langmuir isotherm fitted to the adsorption data

3.3 *C. dubia* dietary toxicity experiment

The measured waterborne Pb concentrations during the *C. dubia* exposure are listed in Table 4. More than 90% of the total Pb in the fresh medium was in the filtered phase. Pb concentrations in the control medium were always below the detection limit (DL=0.5 µg Pb/L). Pb concentrations of old medium in the dietborne exposure slightly increased when the daphndis were fed with algae that were exposed to higher Pb concentrations. However, the Pb concentrations in the medium of the dietborne exposure stayed well below the waterborne EC10 previously reported for the same modified Brisy medium, i.e. 69 µg filtered Pb/L (Nys et al. 2012). Filtered Pb concentrations in the old media of the waterborne and combined exposure were similar. The filtered Pb concentrations in the old media of these exposures were 40% lower than the filtered Pb concentration in the fresh media.

Mean measured Organic Carbon (OC) and Inorganic Carbon (IC) concentrations for all exposure routes are listed in Table 5. Total and dissolved OC concentrations were similar in the fresh medium. The Dissolved Organic Carbon (DOC) concentration was on average 2.8 mg/L in the fresh medium. DOC concentrations increased slightly in the old medium for all exposure routes. The Dissolved Inorganic Carbon (DIC) concentration was on average 3.2 mg/L in the fresh medium. DIC concentrations increased slightly in the old medium for all exposure routes. The pH of the fresh medium was on average 7.0 (

Table 6). pH remained relatively stable during the exposure, with pH increasing up to a maximum of 7.4.

Table 4 Measured Pb concentrations in the fresh and old exposure media during the *C. dubia* exposure. Concentrations of the fresh medium are measured before adding food (algae and YUT) to the medium of the waterborne, combined and dietborne (control concentrations) exposure.

Nominal conc. of *C. dubia* or algal exposure (µg/L)	Total Pb conc. of fresh medium (µg/L)	Filtered Pb conc. of fresh medium (µg/L)	Filtered Pb conc. of old medium of the dietborne exposure (µg/L)	Filtered Pb conc. of old medium of the combined exposure (µg/L)	Filtered Pb conc. of old medium of the waterborne exposure (µg/L)
Control	<DL	<DL	<DL	<DL	<DL
80	63±5	60±6	<DL	34±2.96	33±2.33
120	99±3	95±9	<DL	61±6.38	58±5.12
160	142±12	131±7	0.50±0.12	84±3.15	77±5.48
200	183±14	170±8	0.85±0.32	113±9.82	105±9.87
240	222±24	211±29	1.35±0.41	134±15.4	135±17.6
340	294±46	271±47	1.65±0.61	182±28	173±31

Table 5 Measured IC and OC concentrations in the fresh and old exposure media during the *C. dubia* exposure

	Total Fresh	Dissolved Fresh	Filtered Dietborne	Filtered Combined	Filtered Waterborne
IC (mg/L)	3.1±0.3	3.2±0.2	3.7±0.1	3.6±0.0	3.6±0.1
OC (mg/L)	2.6±0.2	2.8±0.2	3.2±0.1	3.4±0.1	3.3±0.2

Table 6 pH in the fresh and old exposure media during the *C. dubia* exposure

Nominal conc. of C. dubia or algal exposure (µg/L)	Mean pH of the fresh medium	Mean pH of the old medium of the dietborne exposure	Mean pH of the old medium of the combined exposure	Mean pH of the old medium of the waterborne exposure
control	7.0±0.1	7.2±0.4	7.1±0.2	7.2±0.2
80	7.0±0.1	7.4±0.4	7.1±0.1	7.1±0.1
120	7.0±0.1	7.4±0.4	7.0±0.1	7.0±0.1
160	7.0±0.1	7.3±0.3	7.0±0.1	7.0±0.1
200	6.9±0.1	7.2±0.2	7.0±0.1	7.0±0.1
240	7.0±0.1	7.2±0.2	6.9±0.0	6.9±0.1
340	6.9±0.1	7.1±0.1	6.9±0.0	6.9±0.1

The toxicity tests of all exposure routes met the validity criteria for *C. dubia testing* of the USEPA (2002) (Table 7). Mean control reproduction per parent animal was well above the required amount of 15 juveniles for all exposure routes. In all exposure routes more than 60% of the surviving parent animals in the control had 3 broods. The concentration response data and curves of all exposure routes are shown in Figure 7. The waterborne and the combined exposure route show similar toxicity responses. The effect concentrations were not significantly different between both exposure routes (ratio Wheeler test (Wheeler et al. (2002)), in all cases $p<0.001$). The dietary exposure route showed no significant differences in mean reproduction between treatments (F-test, $F=0.465$, $p=0.8$). No effect of dietborne

toxicity was observed even with the algae exposed to the highest Pb concentration (52 µg/L, Pb concentration with which the exposed algae was in equilibrium).

In conclusion, we did not observed dietary toxicity with algae that were in equilibrium with up to 52 µg filtered Pb/L, and that contained up to 3669 µg internal Pb/g dry weight. We did not observe differences in toxicity between the waterborne and combined exposure.

Table 7 Biological characteristics of the *C. dubia* exposure and EC10, EC20 and EC50. 95% confindence intervals on effect concentrations are mentioned between brackets.

Exposure route	Mean control reproduction (# of offspring/ parent animal; ±st. dev.)	Amount of parent animals in the control with at least 3 broods (%)	EC10 (µg/L)	EC20 (µg/L)	EC50 (µg/L)
Waterborne	23.9±6.8	90	68.9 (57.4-80.4)	77.4 (68.0-86.8)	94.4 (88.1-100.8)
Combined	19.2±8.7	80	74.7 (60.4-88.9)	82.2 (70.3-94.1)	96.8 (88.4-105.3)
Dietborne	20.0±7.8	80	>52	>52	>52

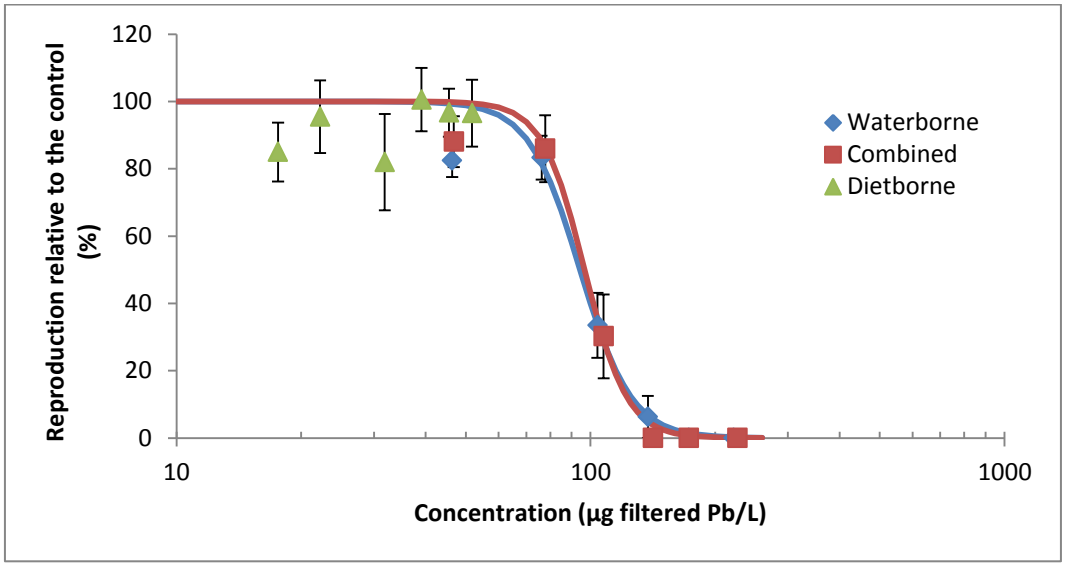

Figure 7 Concentration responses of the different exposure routes. Fitted curves are log-logistic dose response curves with 2 parameters. Error bars indicate standard errors. Reproduction of the waterborne and combined exposure are plotted in function of the average of measured filtered Pb concentrations in the fresh and old medium. Reproduction of the dietborne exposure is plotted in function of the filtered Pb concentration at the end of the algae exposure.

3.4 Adding the results of an earlier test

Here we also report the results of an earlier test we conducted with higher cell densities (1.5 10^7 cells/mL) employed during the algae exposures. The high cell densities exhausted the Pb concentrations in the exposure solutions almost completely, which led to low internal and external Pb concentration (see appendix A, Table A2). However, these results are useful to compare with the other data collected

for this project, and covers exposures to low internal algal Pb concentrations. The results of the dietborne exposure from the earlier test are in line with the data of the dietborne exposure from the previously described test in this report (3.2 and 3.3 of this report). Exposure to low dietary Pb concentrations did not have an influence on the reproduction of *C. dubia* (

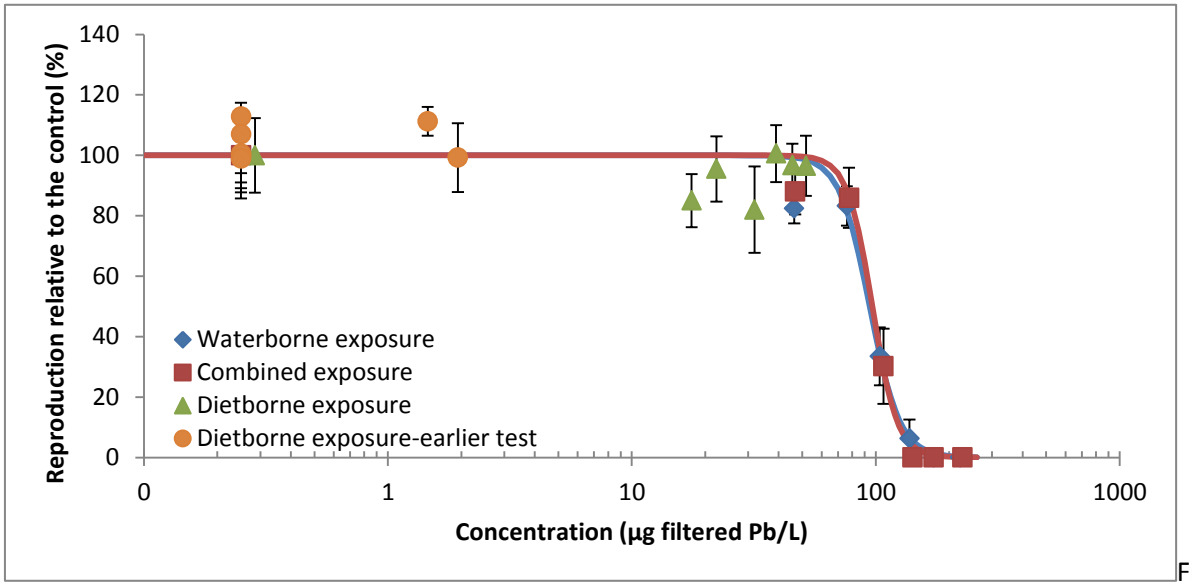

Figure 8). Further, there was no significant relation between the internal Pb concentration of the algae and the reproduction of *C. dubia* in the dietborne exposure of the both tests (p=0.44, Figure 9). This comparison adds to the conclusions previously mentioned in this report: there is no dietary toxicity observed for *C. dubia* fed with algae assumed to be in equilibrium with up to 52 µg filtered Pb/L or containing up to 3669 µg internal Pb/g dry wt.

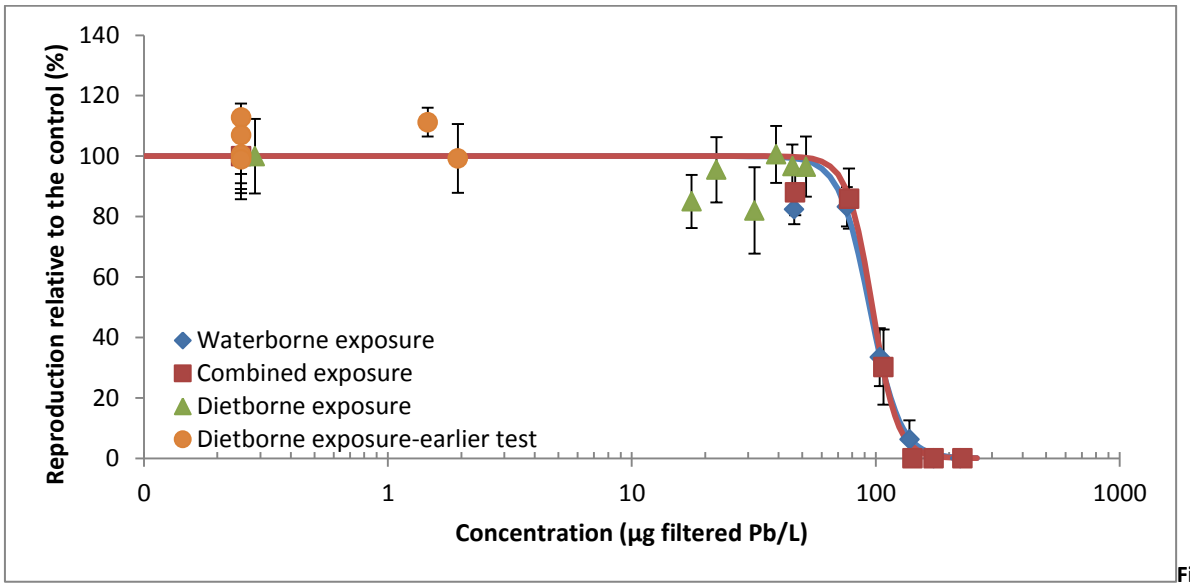

Figure 8 Concentration responses of the different exposure routes. Data from the dietborne exposure of the earlier test are added. Fitted curves are log-logistic dose response curves with 2 parameters. Error bars indicate standard errors. Reproduction of the waterborne and combined exposure are plotted in function of the average of measured filtered Pb concentrations in the fresh and old medium. Reproduction of the dietborne exposure is plotted in function of the filtered Pb concentration at the end of the algae exposure.

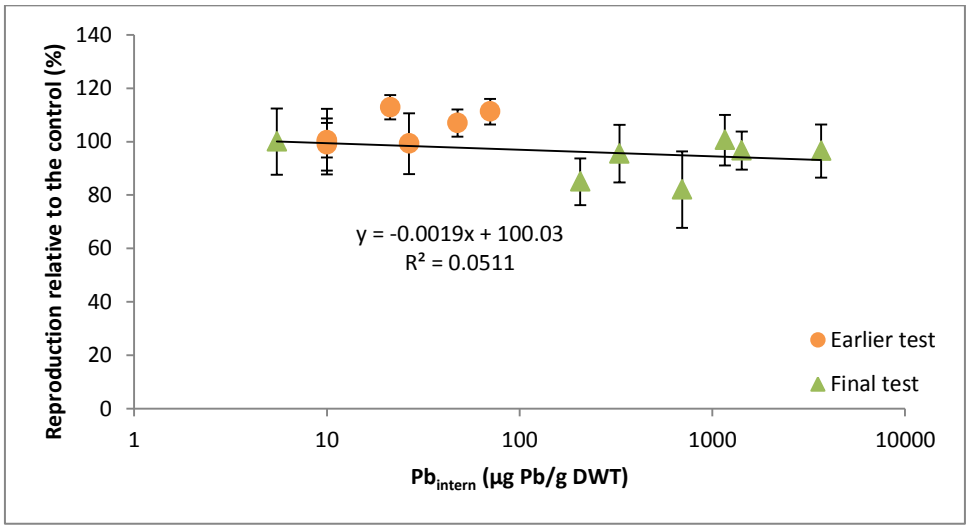

Figure 9 Internal Pb concentration in function of the reproduction relative to the control during the *C. dubia* exposure. Error bars indicate standard errors.

3.5 Preliminary characterization of potential dietary toxicity to *C. dubia* in the field

The data of the dietary toxicity test can be used to evaluate the possible effects associated with the exposure of *C. dubia* populations to realistic (dietary) Pb concentrations in the environment. In what will follow, a worst-case comparison is made in which we consider the fraction of suspended solids in monitored water bodies to be fully edible for *C. dubia*. This is of course a simplification of reality, as the suspended solids fraction in fresh water environment contains next to algae also other 'solids', like clay,

sand and silt. Pb concentrations associated with suspended solids for four German river basins (Donau, Elbe, Rhine and Wesser) measured in the period of 2005-2007 were obtained from the German Environmental Protection Agency, Umweltbundesamt (UBA):

http://gis.uba.de/website/web/atlantis/karten/76_464-Bericht.htm

Concentrations of suspended solids in these river basins were taken from Van Sprang et al. (2009). Of course, the concentration of suspended solids varies between rivers within a river basin, but for simplicity an average value was used in this preliminary assessment. For every river basin, the highest and lowest measured Pb concentrations in the suspended solids fraction (mg Pb/kg SS) were selected for the evaluation of potential dietary toxicity (Table 8).

These values were compared with the concentrations of Pb associated with the highest dietary exposure treatment (algae exposed to nominal concentration of 320 µg Pb/L and in equilibrium with 52 µg filtered Pb/L, which did not result in reproductive effects on *C. dubia*), under 4 different scenario's (Table 9): I) Pb concentration of suspended solids in exposure was based on the sum of internal and external Pb concentration of algae, but only the algae were considered as edible suspended solids, while YCT was not, II) Pb concentration of suspended solids in exposure was based on internal Pb concentrations of algae only (assuming that the external bound Pb will desorb very rapidly when the algae are brought in water with low Pb concentrations like in the non-spiked Brisy control medium), but only algae were considered as edible suspended solids, while YCT was not, III) Pb concentration of suspended solids in exposure was based on the sum of internal and external Pb concentration of algae, and both algae and (non-Pb-contaminated) YTC were considered as edible suspended solids, IV) Pb concentration of suspended solids in exposure was based only on internal Pb concentration of algae, and both algae and (non-Pb-contaminated) YTC were considered as edible suspended solids.

Table 8 Pb concentrations associated with the suspended solids for four river basins in Germany.

River basin	Concentration of suspended solids in water[a] (kg SS/L)	Minimum Pb loadings of the suspended solids[b] (mg Pb/kg SS)	Maximum Pb loadings of the suspended solids[b] (mg Pb/kg SS)	Minimum Pb conc related with suspended solids (µg/L)	Maximum Pb conc related with suspended solids (µg/L)
Donau	3.56E-05	10	51	0.36	1.82
Elbe	2.82E-05	11	386	0.30	10.88
Rhine	1.70E-05	13	460	0.21	7.82
Weser	1.75E-05	22	1500	0.39	26.25

[a] Taken from Van Sprang et al. 2009
[b] Taken from http://gis.uba.de/website/web/atlantis/karten/76_464-Bericht.htm

Table 9 Calculation of the dietary Pb concentration associated with the highest algae exposure under different scenario's.

Scenarios:	Pb loadings associated with suspended solids (mg Pb/kg DWT)	Dry weight associated with algae (kg DWT/L)	Dry weight associated with YCT (kg DWT/L)	Total dry weight in exposure (kg DWT/L)	Pb conc. related with suspended solids in exposure (µg/L)
I) Only algae (internal+external Pb conc)	5307	3.24E-06	0	3.24E-06	17.2
II) Only algae (Internal Pb conc)	3669	3.24E-06	0	3.24E-06	11.9
III) Corrected for YCT (external+internal Pb conc)	1129	3.24E-06	1.20E-05	1.52E-05	17.2
IV) Corrected for YCT (internal conc)	781	3.24E-06	1.20E-05	1.52E-05	11.9

An evaluation of possible dietary toxicity was made on the basis of a 'dietary toxicity ratio' (DTR). This dietary toxicity ratio evaluates dietary toxicity by dividing the measured environmental concentration associated with suspended solids ($MEC_{diet,}$ Table 8) with the no-observable effect concentration for dietary toxicity observed in this study (Table 9) (Eq. 3). However, it is important to keep in mind that the NOEC observed in this study was the concentration of the highest tested algae exposure and is an unbounded NOEC. This NOEC is therefore called $NOEC_{diet,unbounded}$. When the DTR is higher than 1, there can be a potential dietary toxicity of Pb in the environment.

$$Dietary\ toxicity\ ratio = \frac{MEC_{diet}}{NOEC_{diet,unbounded}} \qquad \text{Eq. 3}$$

A first preliminary characterization of potential dietary toxicity to *C. dubia* populations in the environment was made by comparing the Pb loading of the suspended solids (mg Pb/kg SS) in the river basins (MEC_{diet}) by the Pb loadings of the suspended solids in the algae exposure (mg Pb/kg DWT, Table 9, column 2) under the four different scenario's ($NOEC_{diet,unbounded}$). The dietary toxicity ratios for the four different scenarios are summarized in Table 10. The minimum Pb loading of the suspended solids (mg Pb/kg SS) in the river basins were always lower than Pb loadings of the suspended solids in the *C. dubia* exposure. Thus, for the minimum measured environmental Pb loading there is no potential dietary toxicity for all scenario's in the different river basins (DTR<1). For scenario I and II there is no potential dietary toxicity at the maximum measured Pb loadings for any of the river basins. However, for the maximum measured environmental Pb loadings in the Weser basin potential dietary toxicity cannot be excluded under scenario III and IV, DTR 1.3 and 1.9, respectively.

Another way to characterize potential dietary toxicity is by comparing the concentration of Pb associated with the suspended solids in the water column (µg/L). For this evaluation the MEC_{diet} is the minimum and maximum Pb concentration related with suspended solids for the four different river basins (µg/L),

calculated based on the Pb loadings of the suspended solids and the concentration of suspended solids in the river basin. While, the $NOEC_{diet,unbounded}$ in this case is the Pb concentration associated with the suspended solids (algae and/or YCT) in the *C. dubia* exposure solution, calculated based on the Pb loadings of the suspended solids and total dry weight concentration in the exposure under the different scenarios. This $NOEC_{diet,unbounded}$ is equal between scenario I and III between scenario II and IV, so only two comparisons are needed. For the Donau, Elbe and Rhine basins, there seems to be no potential dietary toxicity for both the minimal and maximal dietary toxicity under all scenario's. However, for the Weser basins, the potential for dietary toxicity cannot be excluded at the maximum measured Pb concentrations for all scenario's.

However, it remains uncertain if and how the *C. dubia* populations will be affected under the high Pb concentrations in the Weser basin, as the data was compared with an unbounded NOEC and we do not have information about the dietary toxicity of algae loaded with higher Pb concentrations. In addition, this uncertainty is reinforced considering the worst case assumption (all Pb associated with suspended solids is available to cause dietary toxicity) used in this comparison analysis.

Table 10 Dietary toxicity ratios for the Pb loading of suspended solids (mg Pb/kg SS) related to dietary toxicity of Pb to *C. dubia* in four different river basins in Germany under 4 scenario's

River basin	Scenario I		Scenario II		Scenario III		Scenario IV	
	Minimum Pb conc. associated with suspended solids	Maximum Pb conc. associated with suspended solids	Minimum Pb conc. associated with suspended solids	Maximum Pb conc. associated with suspended solids	Minimum Pb conc. associated with suspended solids	Maximum Pb conc. associated with suspended solids	Minimum Pb conc. associated with suspended solids	Maximum Pb conc. associated with suspended solids
Donau	0.00	0.01	0.00	0.01	0.01	0.05	0.01	0.07
Elbe	0.00	0.07	0.00	0.11	0.01	0.34	0.01	0.49
Rhine	0.00	0.09	0.00	0.13	0.01	0.41	0.02	0.59
Weser	0.00	0.28	0.01	0.41	0.02	1.33	0.03	1.92

Table 11 Dietary toxicity ratios for the Pb concentration associated with suspended solids in the water (µg/L) related to dietary toxicity of Pb to *C. dubia* in four different river basins in Germany under 4 scenario's.

River basin	Scenario I & III		Scenario II & IV	
	Minimum Pb conc. related to suspended solids	Maximum Pb conc. related to suspended solids	Minimum Pb conc. related to suspended solids	Maximum Pb conc. related to suspended solids
Donau	0.02	0.11	0.03	0.15
Elbe	0.02	0.63	0.03	0.91
Rhine	0.01	0.45	0.02	0.66
Weser	0.02	1.53	0.03	2.21

4. General conclusions

We investigated dietary toxicity of Pb to *Ceriodaphnia dubia* by comparing waterborne, dietary and combined exposures with a control. We did not observe dietary toxicity with algae that could be considered to be in equilibrium with 52 µg filtered Pb/L and that contained 3669 µg internal Pb/g dry weight. Also, the presence of a contaminated diet did not affect the waterborne effect concentrations of Pb either, i.e. there was no difference in waterborne Pb toxicity to *C. dubia* between the waterborne and combined exposure. A preliminary evaluation of potential dietary toxicity under field conditions in four German river basins showed that Pb concentrations measured in suspended solids (both expressed as mg Pb/kg dry wt or as µ/L of Pb associated with suspended solids) basins are lower than the highest dietary Pb concentration to which *C. dubia* was exposed and under which it did not experience reduced reproductive performance for three out of four river basins (Donau, Elbe and Rhine). The maximum measured dietary Pb concentration in the Weber basin was 1.3 to 2.2 times higher than the Pb concentration measured during the dietary toxicity test depending on the scenario. However, since comparisons were made with an unbounded dietary Pb NOEC, the potential for dietary toxicity to *C. dubia* in the Weser basin cannot be confirmed or excluded.

Supplementary information:

Table S1Results of Pb measurements in the samples of the algal digestion. External Pb was desorbed from the algal cells by 1.3 mL of a 5 mM Na_2EDTA solution. Algal cells were digested in 400 µL of 14N HNO_3 (Normaton quality) and diluted to 1% acid for analysis

Pb conc. in algae exposure (µg/L)	Start of C. dubia exposure (Day -1)			End of C. dubia exposure (Day 8)	
	Dry weight ($mg/10^8$cell)	Pb_{EDTA} (µg/L)	Pb_{digest} (µg/L)	Pb_{EDTA} (µg/L)	Pb_{digest} (µg/L)
Control	1.62	12±1	<DL	15±18	<DL
80	1.80	1656±125	93±6	1079±256	150±84
120	1.94	2196±50	160±38	1702±70	169±6
160	2.01	2784±459	349±109	2312±70	324±51
200	1.82	3270±294	529	1824±423	630±125
240	2.43	2993±60	863±60	2882±846	1101±177
340	1.90	3186±1021	1743±468	2756±261	1301±99

Appendix-Preliminary tests

In this appendix a series of preliminary tests executed in the framework of the Pb dietary project are discussed. Within this project we started with a dietary experiment that was similar to the experiment discussed in the report, but the algae exposure was inoculated with high cell densities. The results of this test are discussed in part A of the appendix. This first experiment (named 'earlier' experiment throughout the report) was followed by an experiment in which it was tested what the optimal cell density was to inoculate the algae exposures, and this is discussed in part B of the appendix. Finally, before executing the final experiment discussed in the main report, we also executed an experiment, which tested how pH in the algae exposure is influenced by different possible pH buffering treatments. The results of the pH buffering experiment are discussed in part C of the appendix.

A) Earlier dietary test

In what follows will the data be reported of a previous executed dietary test, which did not represent a full dietary toxicity rang, because the Pb concentrations in the algae exposure were completely exhausted, due to a combination of high algae inoculation densities and high pH in the unbuffered medium, which may have caused anorganic Pb precipitation, and internal Pb concentrations in the algae were low. However, the data is useful to compare with the other data collected for this project, and covers exposures to low internal Pb concentrations.

1. Materials and methods:

The experiment was performed as previously described in the materials and methods, except for these exceptions:

- There was no MOPS buffer added to the algal exposure medium
- Algae were inoculated at 2 10^7 cells/mL.
- Nominal Pb concentrations in the algal and the *C. dubia* exposures were 18, 32, 56, 100, 180 and 320 µg/L.
- Algae exposure was performed in erlemeyers-flasks filled with 150 mL of the modified Brisy medium. Erlenmeyers were incubated under continuous light in 25°C (120 µmol photons $m^{-2}s^{-1}$).
- Pb concentrations in the algae exposure was only measured at the start and at the end of the exposure.

2. Results

2.1 Algae exposure

Measured Pb concentrations for all Pb treatments in the algal exposure are shown in Figure A1.a and Table A1. Total Pb concentrations of the fresh medium were close to nominal values. Dissolved Pb concentrations in the fresh medium were between 30-50% lower than the total Pb concentrations. Dissolved Pb concentrations at the end of the exposure period were mostly lower than the detection limit (0.5 µg/L) and were at most 2 µg Pb/L. During the algae exposure the Pb supply was completely exhausted. It is therefore unlikely that the algae were in equilibrium with the Pb exposure concentrations.

Cell density did not increase during the exposure period (Figure A1.b). There were no significant differences between treatments in cell density at the end of the exposure period. pH raised dramatically during the algae exposure due to algal activity, from pH 7 to pH 10 (Figure A2). Speciation calculations predict that at the observed pH 10, precipitation of Pb hydroxides is likely to occur for the Pb treatments above 54 µg filtered Pb/L (nominal concentration of 100 µg/L).

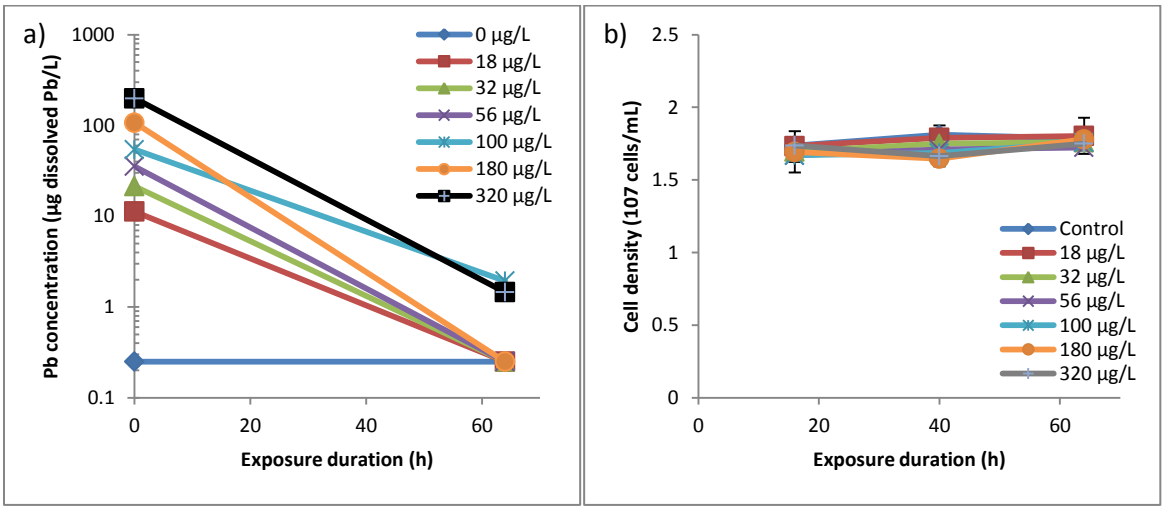

Figure A1 a) Filtered Pb concentrations in solution in the algae exposure, measured at the start and the end of the exposure period. b) Cell densities for the different treatments during algae exposure. Error bars indicate standard deviation.

Tabel A1 Measured Pb concentrations in solution at the start and end of the algal exposure period.

Nominal conc. (µg/L)	Start of exposure (0h)		End of exposure (64h)
	Tot Pb conc. (µg/L)	Diss Pb conc. (µg/L)	Diss Pb conc. (µg/L)
0	0.89	<DL	<DL
18	17	11	<DL
32	30	21	<DL
56	50	35	<DL
100	113	54	1.94
180	185	108	<DL
320	276	199	1.46

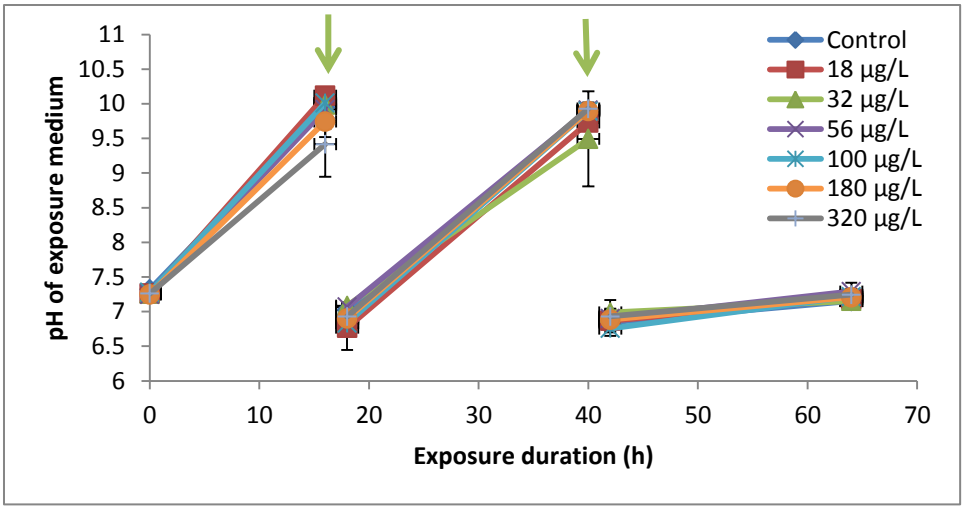

Figure A2: pH for the different Pb concentrations during the algae exposure. Error bars indicate standard deviations. The arrow indicates a pH adjustment after 16h and 40h of exposure.

2.2 Algal characteristics

Internal and external Pb concentrations for the different Pb treatments are listed in Table A2. Internal Pb concentrations were lower than the detection limit (DL=20 µg Pb/g DWT) for the control and the two lowest Pb treatments. Internal Pb concentrations increased with increasing Pb concentration in the fresh medium. However, internal Pb concentrations were low in comparison with the other experiment, due to the high exposure cell density and subsequent exhaustion of the Pb concentration in the exposure solutions. External Pb concentration also increased with increasing Pb exposure concentration, but for this experiment no saturation in external Pb was observed (Figure A3).

Table A2 Dry weight, calculated Pb conc at the surface of the algal cells (Pb_{extern}) and Pb conc in the algal cells(Pb_{intern})

Nominal Pb conc. in algae exposure (µg/L)	Cell dry weight (mg/10^8cell)	Pb_{extern} (µg Pb/g DWT)	Pb_{intern} (µg Pb/g DWT)
0	0.93±0.20	4±1	<DL
18	0.99±0.04	33±3	<DL
32	1.04±0.05	51±5	<DL
56	1.02±0.02	95±5	21±1
100	1.11±0.07	114±11	27±0
180	1.11±0.21	185±40	48±10
320	1.03±0.06	320±32	70±4

<DL: detection limit for Pb_{intern} is 20 µg/g DWT

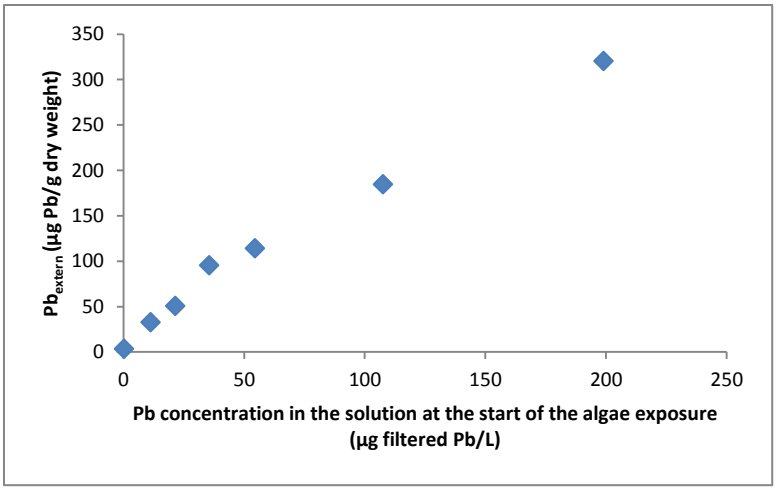

Figure A3 External Pb concentrations in function of the filtered Pb concentration in the exposure solution at the start of the exposure.

2.3 *C. dubia* diet toxicity experiment

The measured Pb concentrations in the *C. dubia* exposure are listed in Table A3. On average 70% of the total Pb in the fresh medium was in the filtered phase. During the exposure the fraction of Pb in the filtered phase decreased with 30% in both the waterborne and combined exposure. Filtered Pb concentrations in the control medium were always below the detection limit (DL=0.5 µg/L). In addition, Pb concentrations in the dietborne exposure were also below the detection limit.

Measured Organic Carbon (OC) and Inorganic Carbon (IC) concentrations are summarized in Table A4. IC and DOC concentrations were relatively stable during the exposure and were one average 4.7 and 3.7 mg/L, respectively. Mean pH of the fresh medium was 7.3 (Table A4). pH remained relatively stable during the exposure for all routes and measured 7.4 at most.

The toxicity tests of all exposure routes met the validity criteria for *C. dubia* testing of the USEPA (2002) (Table A5). Control reproduction was always higher than the required amount of 15 juveniles for all exposure routes. Control reproduction did not differ significantly between exposure routes (ANOVA: p=0.2, F=1.6). At least 80% of the control animals had 3 broods within the exposure period. Concentration responses for all exposure routes are shown in Figure A4. The corresponding effect concentrations are reported in Table A5. Effect concentrations did not significantly differ between the waterborne and combined exposure (ratio-wheeler test). The dietborne exposure did not decrease reproduction within the tested range (ANOVA: p=0.8, F=0.5). However, internal Pb concentrations in the algae used for the dietborne exposure were low (max 70 µg Pb/g DWT). Based on the results of this experiment, we can only conclude that there is no dietary toxicity up to an internal concentration of 70±4 µg Pb/g DWT.

Table A3 Measured Pb concentrations in the fresh and old exposure media during the *C. dubia* exposure. Mean measured values are reported ±st. dev.

Nominal conc. of *C. dubia* or algal exposure	Total Pb conc of fresh medium (µg/L)	Filtered Pb conc. of fresh medium µg/L	Filtered Pb conc. of old medium of the waterborne exposure (µg/L)	Filtered Pb conc. of old medium of the dietborne exposure (µg/L)	Filtered Pb conc of the old medium of the combined exposure (µg/L)
0	1±0	<DL	<DL	<DL	<DL
18	13±6	10±2	6±1	<DL	5±2
32	25±4	18±3	10±1	<DL	10±2
56	47±9	31±6	22±3	<DL	20±5
100	113±24	69±8	48±3	<DL	49±7
180	171±23	120±14	94±14	<DL	91±11
320	259±21	175±13	134±19	<DL	145±20

Table A4 Measured IC and OC concentrations and pH in the fresh and old exposure media during the *C. dubia* exposure

	Total fresh medium	Filtered fresh medium	Filtered Dietborne	Filtered Combined	Filtered Waterborne
IC (mg/L)	4.7±0.0	4.5±0.3	4.8±0.3	4.9±0.3	4.5±0.3
DOC (mg/L)	3.5±0.4	3.5±0.4	3.6±0.7	3.8±0.7	3.8±0.5
pH	7.3±0.0		7.4±0.0	7.3±0.1	7.3±0.0

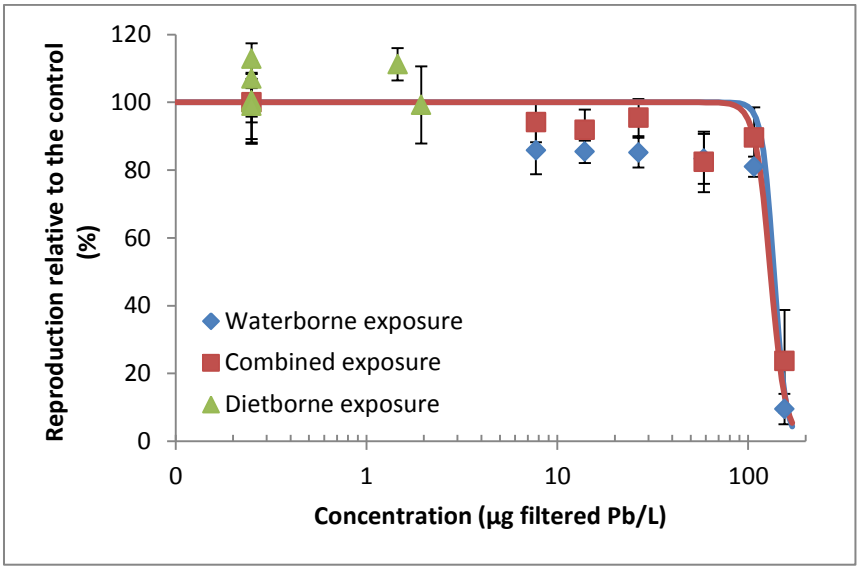

Figure A4 Concentration responses of the different exposure routes. Fitted curves are log-logistic dose response curves with 2 parameters.

Tabel A5 Biological characteristics of the *C. dubia* exposure and EC10 and EC50. 95% confidence intervals on effect concentrations are mentioned between brackets.

Exposure route	Mean control reproduction (# of offspring/ parent animal; ±st. dev.)	Amount of parent animals in the control with at least 3 broods (%)	EC10 (µg/L)	EC50 (µg/L)
Waterborne	24.0±3.2	100	109 (95-124)	132 (120-143)
Combined	22.2±8.4	90	118 (77-159)	142 (121-162)
Dietborne	18.7±7.2	80	>2	>2

B) Preliminary cell density test

A preliminary cell density test was conducted to determine the most ideal cell density to inoculate the algae exposure.

Materials & Methods

During the preliminary cell density test the algae were exposed to three different Pb concentrations (0, 150 and 300 µg/L) at three different cell densities (2E05, 1E06 and 2E06 cells/mL). Every combination (9 in total) received 3 replicates. The algae were exposed for 64h. pH was measured two times daily and adjusted with diluted HCl when needed. Cell densities were measured daily with a Coulter particle counter (Beckman-Analis, Namur, Belgium). Samples for DOC were taken daily and samples for filtered Pb analysis were taken three times daily. Chemical analysis were conducted as described in the main report. After 64h of exposure the Pb burdens of the exposed algae were determined as described in the main report.

Results

pH of the exposure medium during the preliminary cell density test is shown in Figure A5. pH increased up to 9.6 in the first 24 hours of the exposure and remained than more or less equal for the rest of the exposure period. The filtered Pb concentration of the exposure medium is plotted in Figure A6. The filtered Pb concentration in the exposure medium decreased steadily during the first 20h of the exposure and remained more or less constant during the rest of the exposure period. The decrease in filtered Pb concentration increased with higher cell densities, but was never lower than 60 µg/L. Thus, a substantial amount of the Pb was still in solution after 64h of exposure. Cell density increased up to 6-fold during Pb exposure for the 2E05 cells/mL initial cell density (Figure A7.a). An increase in cell density was also noticed for the control Pb treatment at the 1E06 cells/mL initial cell density (Figure A7.b), but less pronounced than for the 2E05 cells/mL treatment. The cell densities of

the other Pb (150 & 300 µg/L) and celldensity (2E06 cells/mL) treatments remained more or less equal (Figure A7.b and .c).

After 64h of exposure the algae cells were concentrated by centrifugation. However, the 2E05 cells/mL cell density did not settle after centrifugation and was therefore not further considered in the Pb burden analysis or as a possible inoculation cell density for the algae exposure in the diet toxicity test. Results of the Pb burden analysis are given in Table A6. Pb burdens were highest in the 1E06 cells/mL cell density treatment. Control algae showed low body burdens of Pb, likely due to some small concentrations of Pb (1.2 µg total Pb/L) in the Brisy testing water. However, the actual diet toxicity test was run with a different batch of Brisy water, sampled at a later date, which was not contaminated with Pb ((Pb measurements below detection limit, DL=0.5 µg/L), see Table 1).

Based on these results 1E06 cells/mL was chosen as the inoculation cell density of the algae exposure.

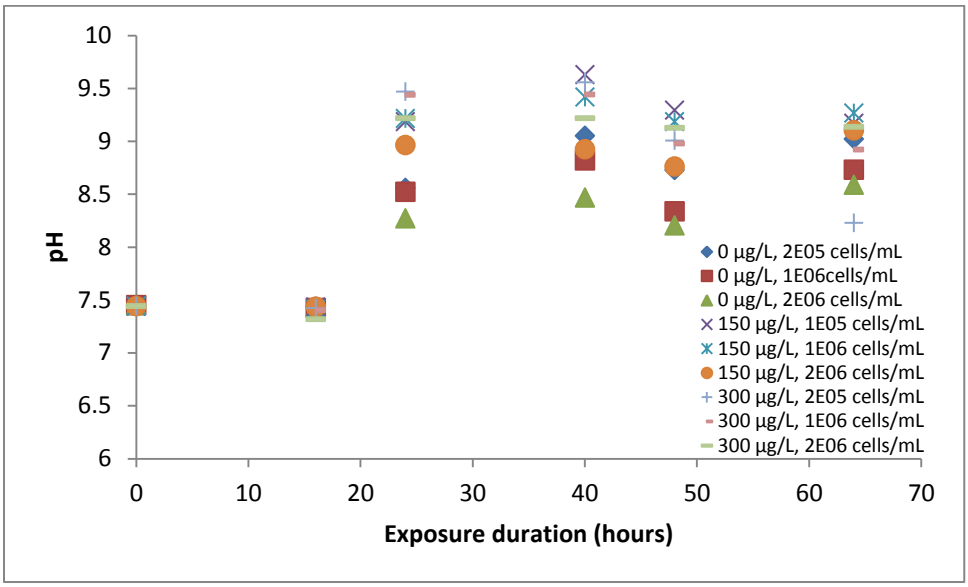

Figure A5 pH in the medium during the Pb exposure.

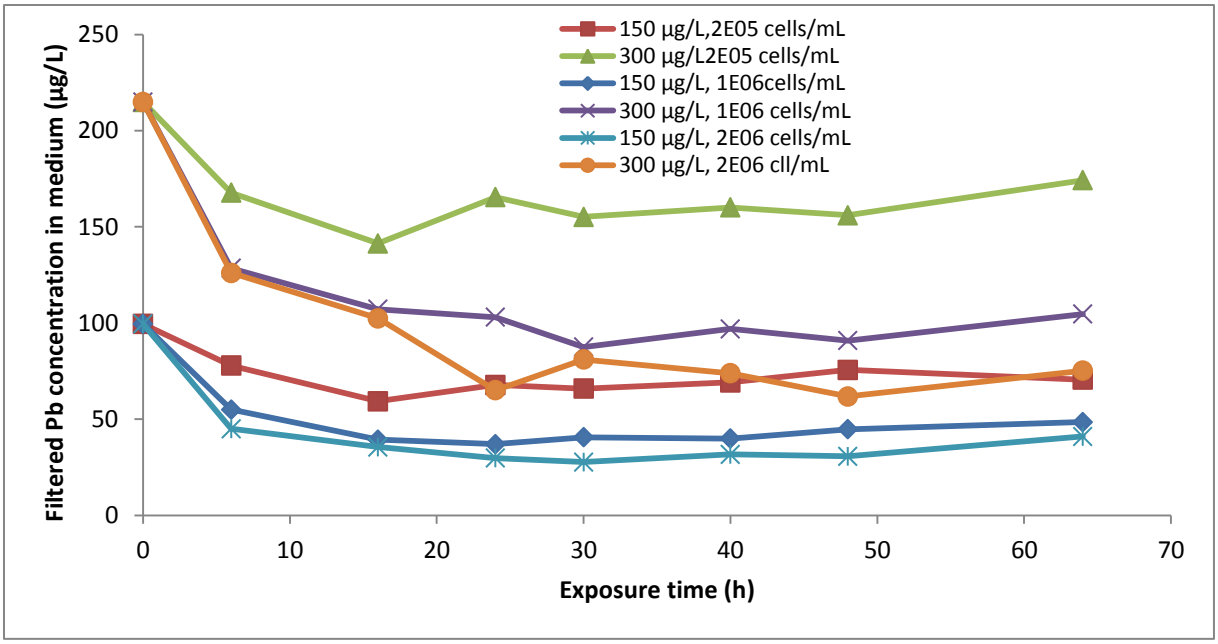

Figure A6 Filtered Pb concentrations in function of exposure time for the different Pb & cell density treatments

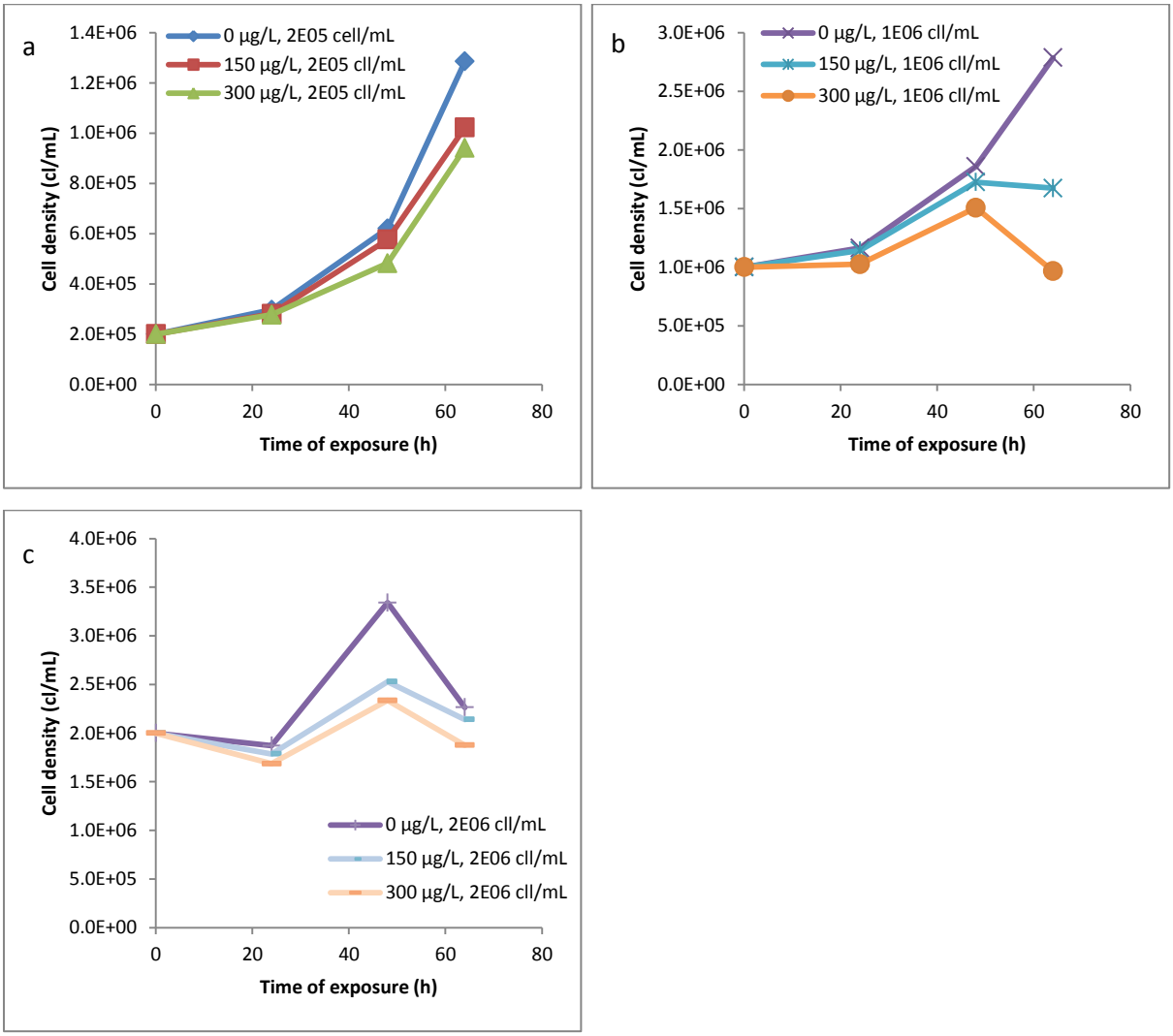

Figure A7 Cell density in function of exposure time for the 2E05 cells/mL (a), 1E06 cells/mL (b) and 2E06 cells/mL (c) treatments.

Tabel A6 Internal and external Pb burdens of the exposed algae

Cell density (cells/mL)	Nom. Pb conc (µg/L)	Pb_{extern} (µg/g dryweight)	Pb_{intern} (µg/g dryweight)
Pr Bl	**Pr BL**	-	-
1.00E+06	**0**	14	11
1.00E+06	**150**	690	238
1.00E+06	**300**	1126	707
2.00E+06	**0**	7	7
2.00E+06	**150**	491	155
2.00E+06	**300**	1221	683

C) Preliminary pH test

In the preliminary cell density test it was noticed that pH raised up to pH 9, and precipitation, likely Pb, at the bottom of the exposure jar was clearly visible at higher Pb exposure concentrations. In addition, precipitates were also noticed on the filter after filtering the algae for dry weight determination Figure A8. Therefore, we conducted a preliminary pH experiment to test which technique was most suitable to keep the pH more or less constant around pH 7 to avoid precipitation during the exposure.

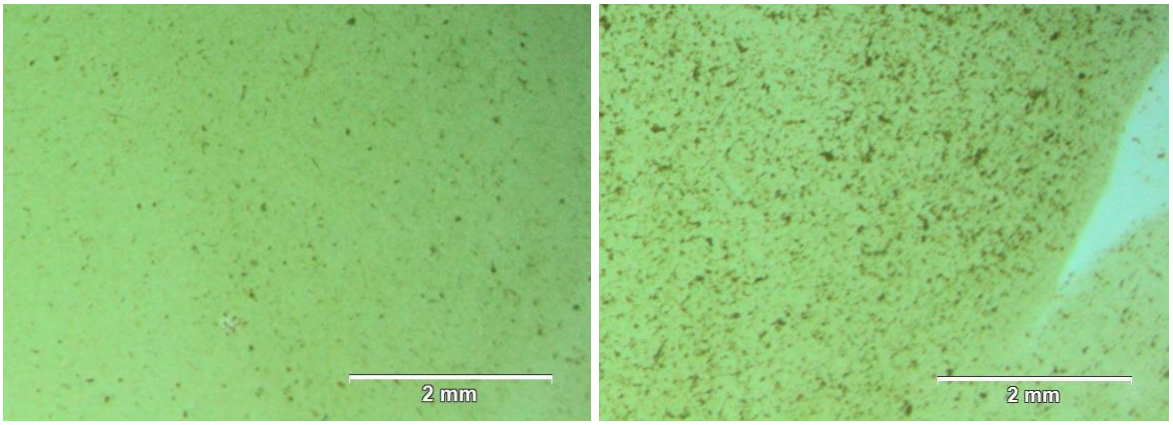

Figure A8 Close up showing precipitates remaining on the filter after filtering of the algae for dry weight determination, with control algae (left) and algae exposed to the highest Pb treatment (right).

Material & Methods:

The pH test was conducted in 500 mL of the modified Brisy medium (no Pb added) inoculated with 10^6 cells/mL, under the algae exposure conditions (25°C and 16h light-8h dark). Three different pH treatments (continuous aeration, 1 mM MOPS added and 3.6 mM MOPS added) and a control (no modifications) were tested. Every treatment had 3 replicates. The pH of the MOPS treatments were

adjusted with NaOH to pH 7. Start pH for all treatments was pH 7, pH was measured twice daily and adjusted with dilute HCl when needed. The pH test continued for 64h.

Results

The pH of the different treatments during the preliminary pH test is plotted in Figure A9. The control pH increased steadily up to 8.5. The aeration treatment made the increase in pH lower, but pH raised up to 7.8. The MOPS treatments kept the pH most stable during the preliminary test, pH did not raise above pH 7.4. There was almost no differences in measured pH between the MOPS 1 mM and MOPS 3.6 mM treatment. Based on these results it was decided to add 1 mM MOPS to the algae exposure medium for the final test.

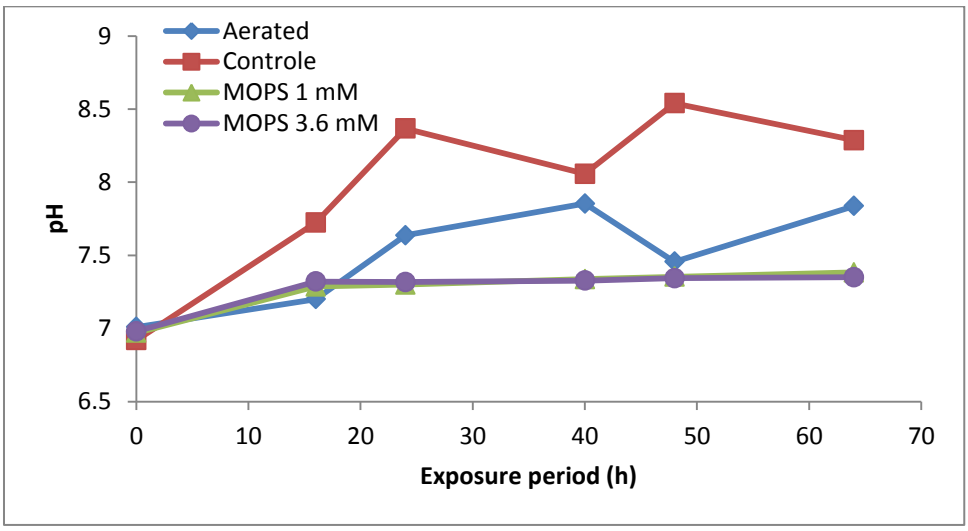

Figure A9 pH for the different pH treatments during the preliminary pH-test.

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