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Com-Phy-Lead-Part I

Validating the chronic Pb algae bioavailability model at high pH: Single-species evaluation

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1. Introduction

The following two main issues currently hamper environmental risk assessment procedures for Pb. First, freshwater algae become the most sensitive organisms and are the main drivers of the HC5-level safe threshold of Pb at pH levels >7.4 (De Schamphelaere et al., 2014), but the available chronic algae bioavailability model has only been validated up to a pH boundary of 8.0. Yet, a significant portion of freshwater bodies in Europe have pH values above 8. In order to increase the level of confidence in bioavailability model-based toxicity predictions and related water quality criteria calculations (e.g. HC5 using the BLM-SSD tool, www.leadblm.com) in such waters, targeted experimental work with phytoplankton species is needed (Van Sprang et al., 2016). Second, there is currently no relevant, reliable, high-quality multi-species toxicity study with Pb available in freshwater. Indeed a community-level validation of whether or not the HC5 level is conservative to protect freshwater ecosystems has not been performed. This may result in additional arbitrary safety factors being applied to bioavailability-based HC5 values to derive PNECs, WQC and EQS. The Com-Phy-Lead project aims to address both aims simultaneously by performing community-level ecotoxicity tests with freshwater phytoplankton communities at two pH levels (~i.e. about 7.2 ± 0.2 and about 8.4±0.2) and by evaluating community-level endpoints related to ecosystem functioning and biodiversity. Furthermore, the trophic status (e.g. oligotrophic vs eutrophic) in terms of the PO_4^{3-} is hypothesized to play an important role in Pb effects on natural communities. Therefore, it is important to also evaluate the effect of different P-levels in the system (e.g. 10 µg/L and 100 µg/L) on Pb toxicity to algae (communities).The Com-Phy-Lead project aims to investigate the effects of pH and phosphor conditions on Pb toxicity to algae (communities). More specifically, the project has the following three main research objectives:

I) Validating the single-species algae bioavailability model to higher pH levels (pH 8.4)
II) Validating the algae BLM to predict the effect of pH on Pb toxicity (from pH 7 to pH 8.4) at the community level
III) Evaluating if the calculated $HC5_{Pb}$ is conservative with respect to algae community-level endpoints

In the present report, we focussed on the first research objective. We investigated toxicity of Pb to *Pseudokirchneriella subcapitata* using the standard 72h-growth inhibition assays at 2 pH levels (pH 7.2 & pH 8.4) and 2 P (P 10 µg/L & P 100 µg/L) levels, i.e. at 4 treatments in total. To test whether the existing algae Pb bioavailability model developed for pH≤8.0 (De Schamphelaere et al. 2014), can be used at higher pH>8.0, Pb toxicity at pH 8.4 was predicted based on the Pb toxicity observed at pH 7.2.

2. Material & Methods

2.1 General test design

The toxicity of Pb was investigated using the chronic 72h-*P. subcapitata* growth inhibition test (OECD 2011). Toxicity experiments were performed at two pH levels (i.e. pH 7.2 & pH 8.4) and two P levels (i.e. P 10 µg/L and P 100 µg/L). Resulting in the following four experimental treatments: I) pH 7.2, P 10 µg/L, II) pH 8.4, P 10 µg/L, III) pH 7.2, P 100 µg/L and IV) pH 8.4, P 100 µg/L. Pb toxicity in every experimental treatment was evaluated using 6 Pb concentrations and a control. For the pH 7.2, P 10 µg/L & pH 8.4, P 10 µg/L treatments, the tested nominal Pb concentration range was 32-1800 µg Pb/L. For the pH 7.2, P 100 µg/L & pH 8.4, P 100 µg/L treatments the tested nominal Pb concentration range was 18-1000 µg Pb/L. Each Pb concentrations included 3 replicates. In addition to this, 5 control replicates per treatment were also tested. Additionally, for every Pb concentration in all treatments a particle correction was tested. The particle controls contained a series of experimental units which contained the experimental medium but that were not inoculated with algae (see further). These particle controls allowed to I) correct the measured cell densities in algae exposures for background particle concentrations, and II) to more closely monitor the distribution of Pb species between the total and filtered fraction. All experimental units were run simultaneously.

2.2 Preparation of test media

The test water (OECD growth medium) was prepared according to the standard protocol 201 of the Organization of Economic Cooperation and Development for testing with *P. subcapitata* (OECD 2011). However, Na-EDTA was omitted from Stock 2 as EDTA is known to be a very strong metal complexing ligand and is therefore less relevant for the natural situation. Instead, Stock 2 was prepared by replacing EDTA with Natural Organic Matter (NOM; International Humic Substances Society, Suwannee River NOM; Reference 2R101N) at a nominal concentration of 10 mg NOM/L in the test medium. This represents about 5 mg Dissolved Organic Carbon/L, which is the median DOC concentration in European surface waters (FOREGS 2005). Additionally, KH_2PO_4 was omitted from Stock 1 because preliminary testing showed that Pb-PO_4 precipitation decreased when PO_4 was added after Pb had been allowed to equilibrate with the test medium. Test solutions of the pH 7.2 treatments were adjusted to the required pH adding 3.6 mmol 3-N-morpholinopropanesulfonic acid (MOPS)/L and the required amount of NaOH or HCl. MOPS has the property not to affect metal speciation (Kandegedara & Rorabacher 1999), nor is it toxic or does it affect metal toxicity to *P. subcapitata* (De Schamphelaere et al. 2004). 3 mmol $NaHCO_3$/L was added to the test solutions of the pH 8.4 treatments. Na levels were set equal between treatments using NaCl. The basic medium (i.e. no Pb added) was left to aerate for 1 day, after which Pb was spiked using

$PbCl_2$ and left to equilibrate for two days. Finally, PO_4 was added in the required volume by adding KH_2PO_4. The medium was left to equilibrate for one more day before test initiation. Test media were left to equilibrate at 25°C in total darkness.

2.3 Ecotoxicity testing

The chronic toxicity tests with *P. subcapitata* were conducted following the OECD Guideline (OECD 2011). *P. subcapitata* (CCAP 278/4) was obtained from the Culture Collection of Algae and Protozoa (CCAP, at the Scottish Association for Marine Science, Argyll, Scotland, United Kingdom). A culture of the algae was set up 4 days prior to testing in aerated tap water (Gent, Belgium) which was first passed sequentially through an activated carbon filter and a 0.45 µm filter and to which we then added the modified Provasoli's ES enrichment (Bold and Wynne, 1978) at 1/2 strength and, additionally, 1.4 mg/L $FeSO_4.7H_2O$, 15 mg/L $NaH_2PO_4.2H_2O$, 150 mg/L $NaNO_3$ and 2.35 mg/L $MnCl_2.4H_2O$. The flasks containing the algae were placed on a shaking device under continuous light (120 µmol photons m^{-2} s^{-1}) at 25°C. Algal tests were performed in 100 mL Erlenmeyer flasks containing 50 ml of test medium. Each replicate was inoculated with 10^4 cells/mL (= cell density N_0 at the start (t_0) of testing). Afterwards, all Erlenmeyer flasks were incubated at 24 °C on a light table (24 h light, 120 µmol photons m^{-2} s^{-1}) and were manually shaken two times per day. Cell densities (N_1, N_2 and N_3) were measured using a particle counter (Coulter Counter Z1, Beckman) after 24 (t_1), 48 (t_2) and 72 (t_3) hours. During the test, the pH was adjusted daily to the target pH by adding NaOH or HCl.

2.4 Analytical chemistry

Samples of test media of all Pb concentrations of all treatments were collected regularly for analysis of total (test initiation) and filtered (0.45 µM, Acrodisc, PALL Life Sciences; at test initiation and after 48h and 72h of exposure) metal. For *P. subcapitata* tests, samples for analysis of OC (organic carbon) were taken before addition of MOPS, as it is not possible to do so afterwards. Samples for analysis of IC (inorganic carbon) were taken at the start of the test and after 72 hours. Pb, Ca, Mg, Na, K and P cconcentrations were measured using inductive coupled plasma-optical emission spectroscopy (iCAP 7000 series, Thermo Scientific, Limit of Quantification for Pb & P=15 µg/L and Limit of Detection for Pb & P=5 µg/L).

DOC (Dissolved Organic Carbon) and DIC (Disolved Inorganic Carbon) were measured with a Total Organic Carbon analyser following the NPOC method (TOC-5000, Shimadzu, Duisburg, Germany; Limit of Quantification 1.5 mg DOC/L; Method Detection Limit 0.5 mg DOC/L). Chloride, sulphate, nitrate and phosphate 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). The pH of fresh and old media were measured daily with a pH glass electrode (Hanna Instruments, Temse, Belgium).

2.5 Concentration response analysis

Growth rate μ (d^{-1}) was determined in each replicate of each treatment as the slope of the linear regression of the natural logarithm of cell density versus time (in days). Concentration response analysis was conducted on relative growth rates (relative to the mean control growth rate, %) as endpoint. 50%, 20% and 10% effective concentrations, expressed in μg filtered Pb/L ($EC50_{Pbfilt}$, $EC20_{Pbfilt}$ and $EC10_{Pbfilt}$, respectively), were calculated based on the measured filtered Pb concentration at test initiation. Concentration response curves were fitted using the log-logistic concentration-response model with 2 parameters in Statistica 7 (StatSoft) as described in Nys et al. (2015).

2.6 Chemical speciation calculations

Chemical speciation was calculated using Visual Minteq 3.0 (KTH, Stockholm, Sweden). Speciation was calculated at the $EC50_{Pbfilt}$ and $EC20_{Pbfilt}$. Complexation of Pb with dissolved organic matter (DOM), i.e., fulvic acid (FA), was modeled according to the NICA-Donnan formulation (as embedded in Visual Minteq 3.0). All default parameter values as described in Milne et al. (2003) were used. The assumption was made that DOM contains 50% carbon on a weight basis (Ritchie & Perdue 2003). Additionally, we assumed that 65% of the DOM is reactive and behaves as isolated FA. Previous research has shown that assumptions between 60% and 70% active FA typically work best for predicting metal toxicity in natural waters (Tipping 2002). Accordingly, the measured DOC content (mg C/L) for natural sources was multiplied by a factor of 1.3 to obtain the amount of FA (mg FA/L) to be used as the speciation modelling input.

2.7 Bioavailability model validation

Predictions of Pb toxicity were made using the chronic Pb *P. subcapitata* bioavailability model (De Schamphelaere et al. 2014; Eq. 1).

$$ECx_{Pb^{2+},pred} = 10^{(Q_x - S_{pH} \times pH_i)} \quad (1)$$

In Equation 1, $ECx_{Pb2+,pred}$ is the predicted x% effective concentration in test solution *i*, expressed as Pb^{2+} activities (mol/L). Q_x is the intrinsic sensitivity of *P. subcapitata*. This intrinsic sensitivity is the intercept of the linear relationship between the negative logarithm of the ECx_{Pb2}, and pH (De Schamphelaere et al. 2014). S_{pH} is the slope of this relationship (S_{pH}=1.509 for pH 6.0 to 8.0 as determined previously; De Schamphelaere et al. 2014). pH_i is the pH of test solution *i*.

The intrinsic sensitivity can be calculated based on the observed ECx_{Pb2+} using Equation 2 (De Schamphelaere et al. 2014):

$$Q_x = log_{10}(ECx_{Pb^{2+},obs}) + S_{pH} \times pH_i \quad (2)$$

In Equation 2, $ECx_{Pb2+,obs}$ is the observed x% effective concentration in test solution *i*, expressed as Pb^{2+} activity (mol/L).

The *P. subcapitata* bioavailability model was validated for high pH (~pH 8.4), by first calibrating the intrinsic sensitivity in Equation 2 based on the observed Pb^{2+} toxicity of the pH 7.2 treatment and subsequently using these parameters in Equation 1 to predict ECx_{Pb2+} at pH 8.4. Predicted ECx_{Pb2+} were translated to $ECx_{Pbfilt,pred}$ with Visual Minteq 3.0 and compared with the observed $ECx_{Pbfilt,obs}$.

A pH slope (S_{pH}) for the pH 7.2- pH 8.4 range was determined based on the regression equation of the linear relationship between the logarithm of $EC50_{Pb2+}$ and the pH at 7.2 and 8.4. The standard deviation of S_{pH} was determined through bootstrapping (Nys et al. 2016). 5000 random samples of the EC50s at both pH levels were drawn from a normal distribution defined by the estimated mean and standard error of the hardness corrected EC50s. These 5000 sampled EC50 pairs were then used to calculate 5000 values of S_{pH}, from which the standard deviation of S_{pH} was calculated. Five thousand sample pairs were taken as this number resulted in an adequate estimation of the variability of the pH slopes. The S_{pH} of the present study and the S_{pH} of the original algae bioavailability model were statistically compared using the method described by Sokal and Rohlf (1981).

3. Results

3.1 Chemistry in exposure media

The average chemical composition measured in the test media is given in Table 1. Filtered P concentrations in the test media measured during the exposure period are visualised in Figure 1. For both P levels, low concentrations of Pb lead to a decrease in P concentrations, most likely as a results of algae growth. However, at higher Pb concentrations (>$EC50_{Pb}$) decreases in P concentrations in the test media become minimal. This suggests that Pb-PO_4 precipitation in the test media is insignificant (unless precipitates are smaller than 0.45µm). In the pH 8.4, P 10 µg/L treatment there was a slight increase in P concentration compared to test initiation, although it has to be kept in mind that P concentrations in this treatment were below or close to the limit of quantification.

At test initiation, 90% of the total Pb concentration was present in the filtered Pb fraction (Figure 2). Filtered Pb concentrations in the algae exposures decreased on average 20% (pH 7.2) and 30% (pH 8.4) after 48h of exposure compared with filtered Pb concentrations at test initiation (Figure 2 & Figure 3).

There was no obvious difference in the fraction of filtered Pb between the low P and high P treatment in the pH 8.4 treatments, suggesting that Pb-PO_4 precipitation was minimal.

Table 1. Chemical composition[a] of all media used for investigating Pb toxicity to *P. subcapitata*. The chemical composition was used as input for speciation modelling with Visual Minteq.

Test ID	pH	DOC (mg/L)	Na (mg/L)	Mg (mg/L)	K[b] (mg/L)	Ca (mg/L)	SO_4 (mg/L)	Cl (mg/L)	DIC (mg/L)	MOPS[b] (mmol/L)
pH 7.2, P10	7.22±0.02	6.6±1.0	95±10	2.7±0.1	0.013	4.7±0.3	7.5±0.4	80±10	2.7±0.2	3.6
pH 8.4, P10	8.40±0.07	6.6±1.0	95±9	2.7±0.1	0.126	4.7±0.3	7.7±0.2	87±23	36±5	0
pH 7.2, P100	7.22±0.02	6.6±1.0	95±10	2.6±0.1	0.013	4.6±0.3	8.1±0.4	109±26	2.7±0.2	3.6
pH 8.4, P100	8.41±0.09	6.6±1.0	95±10	2.6±0.1	0.126	4.6±0.3	7.7±0.4	69±6	35±5	0

[a] Measured values±standard deviation are reported

[b] K and MOPS concentrations were not measured, nominal added concentrations are given.

DOC=dissolved organic carbon, DIC=dissolved inorganic carbon, MOPS:3-N-morpholino-propane-sulfonic acid.

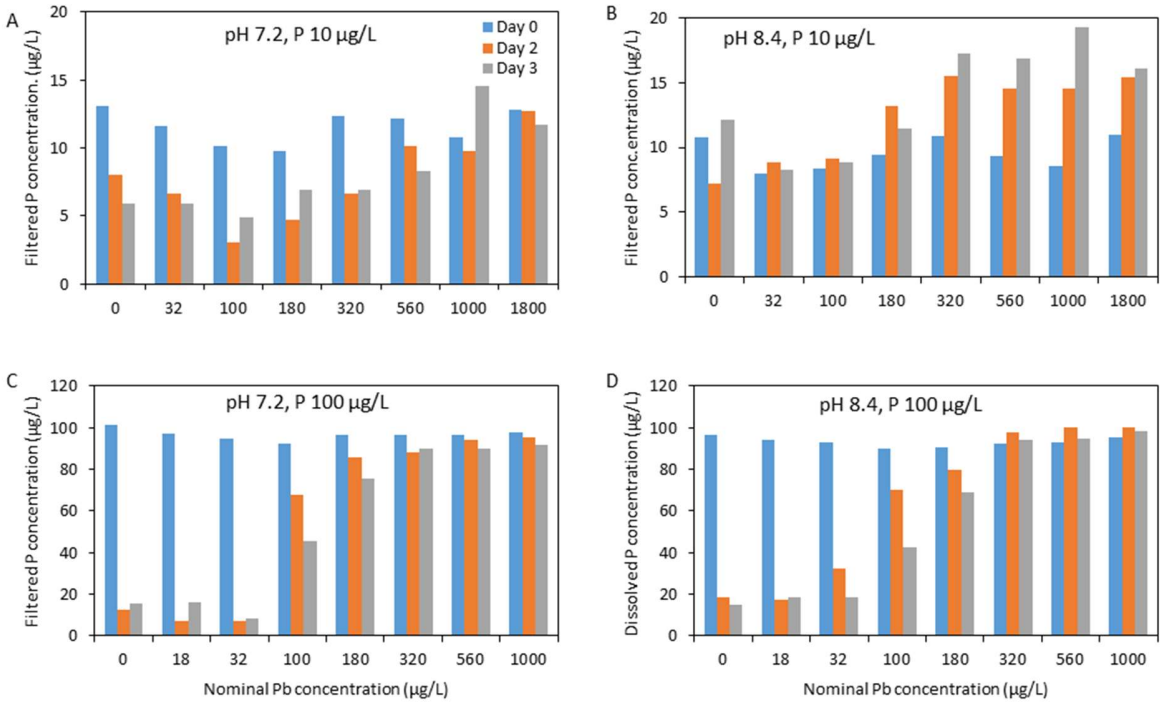

Figure 1. Dissolved phosphorous (P) concentrations in the algae exposure media of the different Pb concentrations of the pH 7.2, P 10 µg/L (A), pH 8.4, P 10 µg/L (B), pH 7.2, P 100 µg/L (C), pH 8.4, P 100 µg/L (D) treatment. Blue, orange and grey bars are measured P concentrations at 0h, 48h and 72h, respectively.

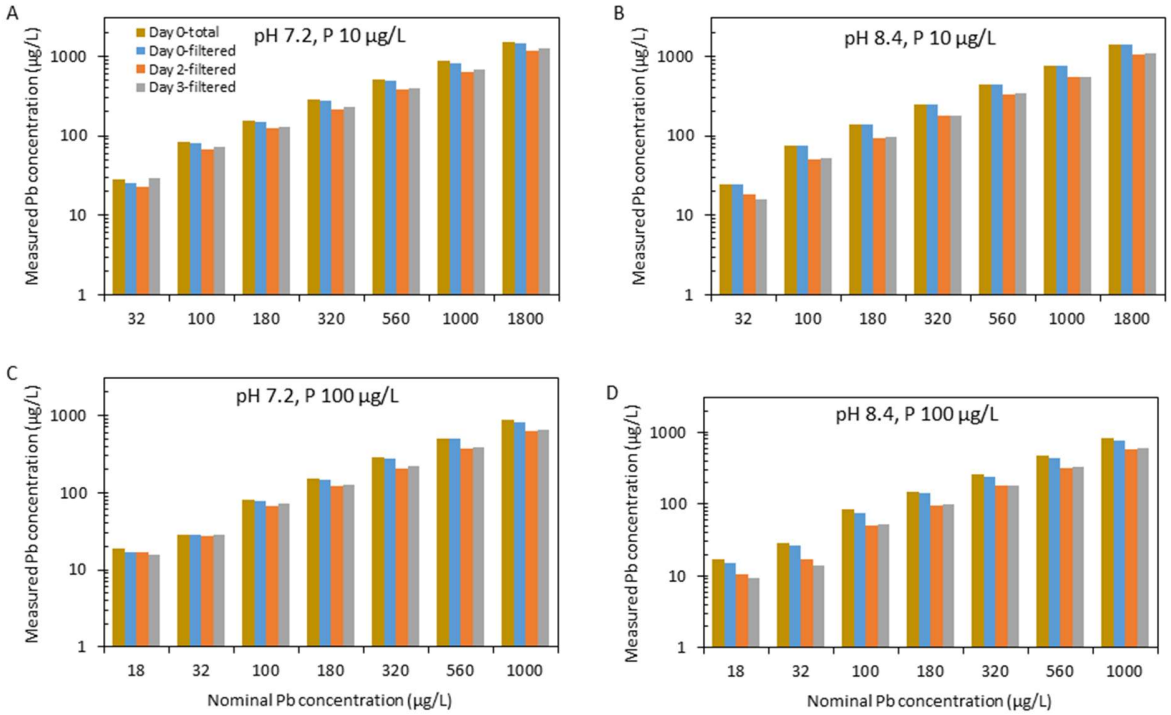

Figure 2. Measured total Pb concentrations on day 0 (brown bars), and filtered Pb concentrations on day 0 (blue bars), day 2 (orange bars) and day 3 (grey bars) in the algae exposure media of the different Pb concentrations of the pH 7.2, P 10 µg/L (A), pH 8.4, P 10 µg/L (B), pH 7.2, P 100 µg/L (C), pH 8.4, P 100 µg/L (D) treatment.

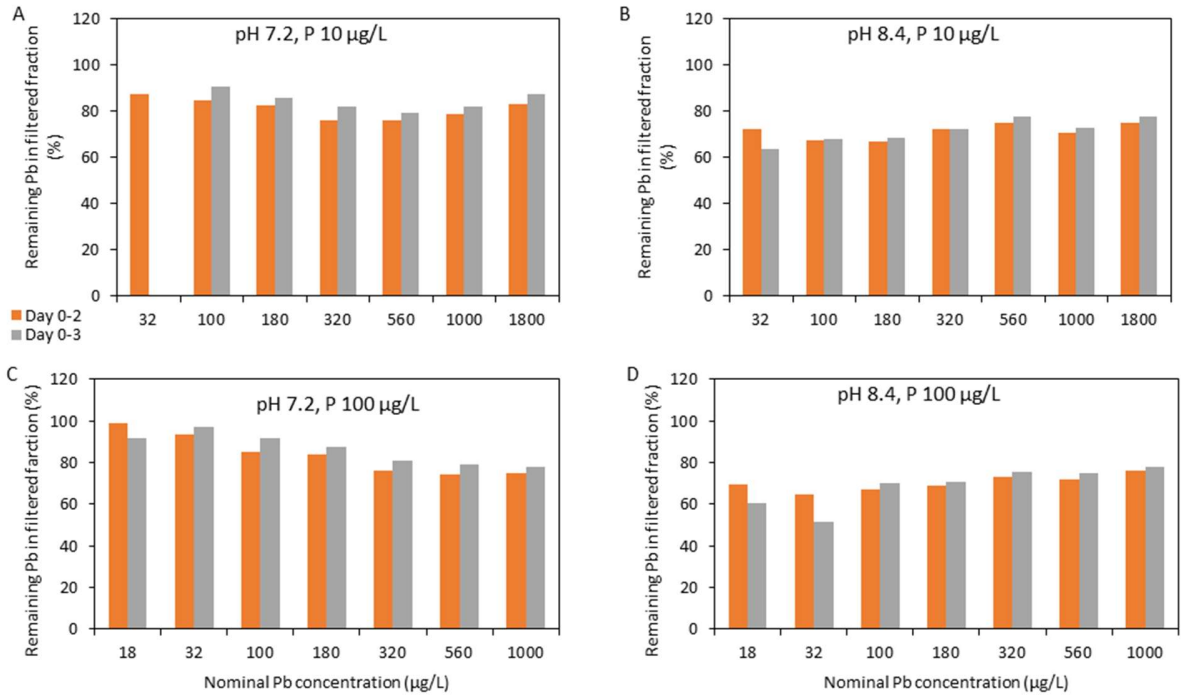

Figure 3. Remaining Pb in filtered fraction (%) during day 0 until 2 (blue bar) and day 0 until 3 (orange bar) compared to day 0 in the algae exposure media of the different Pb concentrations of the pH 7.2, P 10 µg/L (A), pH 8.4, P 10 µg/L (B), pH 7.2, P 100 µg/L (C), pH 8.4, P 100 µg/L (D) treatment.

To have a better idea about the speciation processes occurring in the test medium, particle controls (i.e. test medium not-inoculated with algae) were run simultaneously with the algae exposures for every Pb exposure concentration in all pH & P treatments. In the P 100 µg/L treatments almost all P was present in the filtered fraction at test initiation (~98%; Figure 4.C & 4.D). The percentage of P in the filtered fraction decreased slightly on day 2 and 3 in all Pb concentrations of the pH 7.2, P 100 µg/L and in the intermediate Pb concentrations of the pH 8.4, P 100 µg/L treatment (i.e. ~85-90% of P in filtered fraction). At the high Pb concentrations of the pH 8.4, P 100 µg/L treatment almost all P was present in the filtered fraction. The latter suggest that Pb-PO_4 precipitation at high pH was minimal during the exposure period.

In most Pb concentrations of the particle corrections, at least 90% of the total Pb was present in the filtered fraction (Figure 5). There was no substantial difference between the P treatments in % of total Pb in filtered fraction.

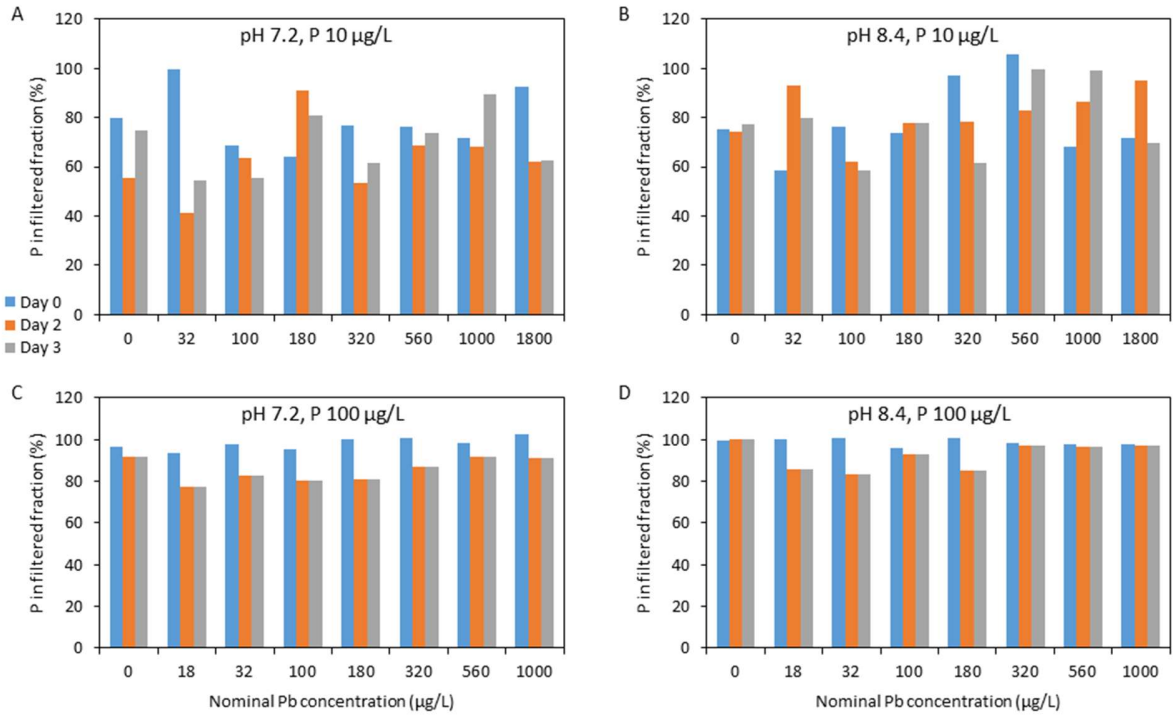

Figure 4. Percentage of total P in filtered fraction (%) on day 0 (blue bar), day 2 (orange bar) and day 3 (grey bar) compared to day 0 in the particle controls of the different Pb concentrations of the pH 7.2, P 10 µg/L (A), pH 8.4, P 10 µg/L (B), pH 7.2, P 100 µg/L (C), pH 8.4, P 100 µg/L (D) treatment.

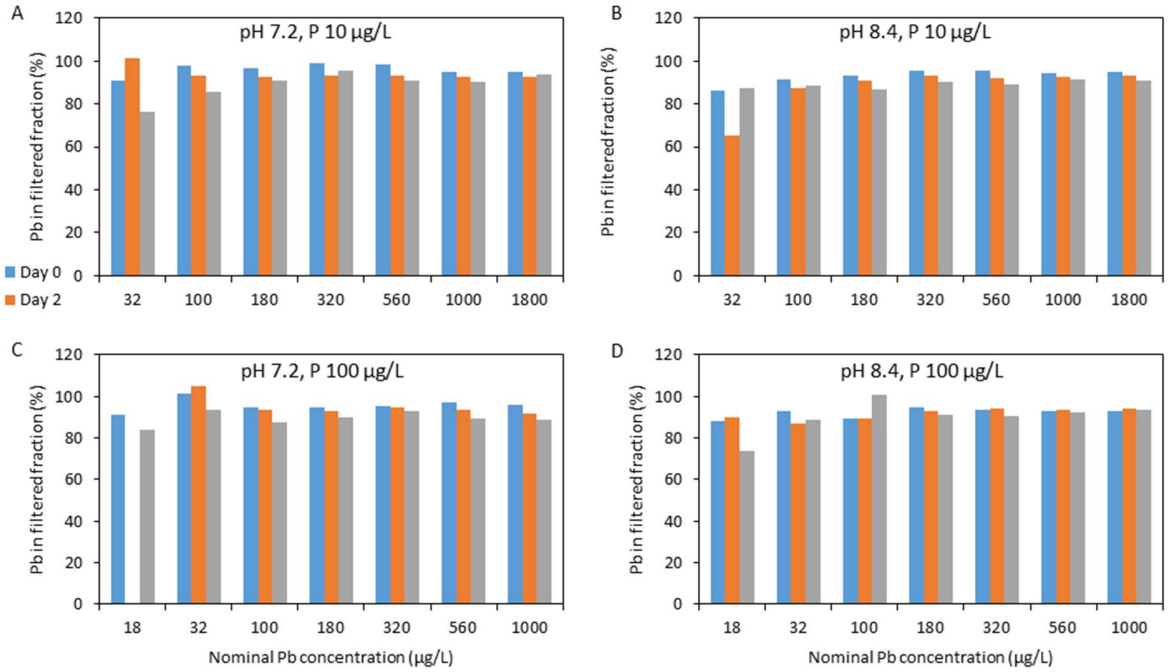

Figure 5. Percentage of total Pb in filtered fraction (%) on day 0 (blue bar), day 2 (orange bar) and day 3 (grey bar) compared to day 0 in the particle controls of the different Pb concentrations of the pH 7.2, P 10 µg/L (A), pH 8.4, P 10 µg/L (B), pH 7.2, P 100 µg/L (C), pH 8.4, P 100 µg/L (D) treatment.

From the above it is likely that Pb-PO_4 precipitation in the test media was minimal. Additionally, in contrast with previous experiments at high pH (De Schamphelaere & Janssen 2008), in the present experiment a clear monotonic-concentration response was observed in the pH 8.4 treatments (see further). This all suggests that adding PO_4 to the test medium after allowing Pb to equilibrate with the PO_4-free medium can be considered as an appropriate method to investigate Pb toxicity at high pH.

3.2 Validity of the toxicity tests

Details on test performance in the controls of the different treatments are given in Table 2. None of the treatments was valid according the OECD test protocol (OECD 2011) after 72h of exposure. For all treatments, the coefficient of variation of the sectional growth rate in the controls (i.e. comparing growth rates between day 0-1, day 1-2 and day 2-3) was too large. Algal growth, expressed as cell densities, occurred mainly in the first day of exposure, and decreased over the following two days of exposure (Figure 6). This decrease in growth rate is probably the consequence of depletion of P in the growth medium (Figure 1).

However, the OECD protocol allows to shorten the exposure period to 48h if the validation criteria during this exposure period are met (OECD 2011). In the P 100 µg/L treatments, all validity criteria were met after 48h. As a consequence, all further data-analyses were done for the 48h exposure period. For the P 10 µg/L treatment, test validity criteria was also not met after 48h of exposure. To be able to compare Pb toxicity to *P. subcapitata* between oligotrophic (10 µg/L) and mesotrophic (100 µg/L) conditions concentration response analyses were also made for the low P treatments. However, since control growth validity criteria in these treatments were not met the toxicity results should not be used for conventional risk assessment applications.

Table 2. Details on test validity criteria[a] in the chronic toxicity tests with Pb with *P. subcapitata* in all test media after 72h and 48h of exposure.

Test ID	72h-μ_c (d^{-1})	72h-FI_c	72h-$CV_{\mu,c}$ (%)	72h-$CV_{s,\mu,c}$ (%)	48h-μ_c (d^{-1})	48h-FI_c	48h-$CV_{\mu,c}$ (%)	48h-$CV_{s,\mu,c}$ (%)
pH 7.2, P10	1.13	31.8	0.9	*81.6*	1.56	22.7	1.6	*56.2*
pH 8.4, P10	1.12	31.0	0.9	*90.6*	1.58	23.4	0.7	*64.2*
pH 7.2, P100	1.48	87.6	1.2	*54.9*	1.88	42.9	2.2	35.0
pH 8.4, P100	1.60	127.2	1.7	*42.3*	1.90	45.5	3.5	34.1

[a] Validity criteria based on control performance (OECD 2011): mean growth rate μ_c>0.92, mean factor cell increase FI_c>16, Coefficient of Variation (CV) of control growth rate $CV_{\mu,c}$<7%, CV of sectional growth rate $CV_{s,\mu,c}$≤35%. Underlined values fail the criterion

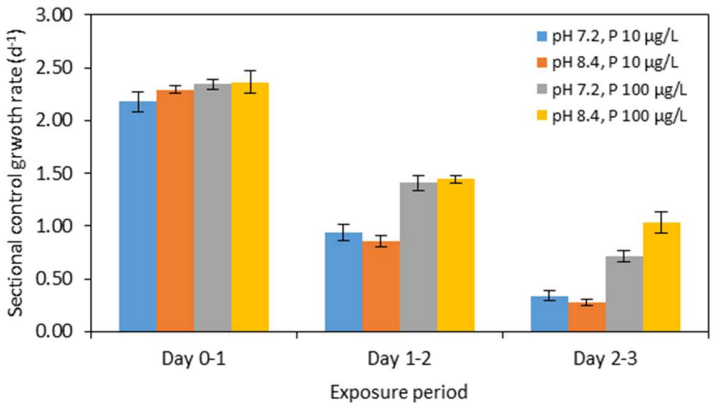

Figure 6. Section-by-section specific growth rates (d^{-1}, days 0-1, 1-2, 2-3) in the control exposures of the pH 7.2, P 10 µg/L (blue bars), pH 8.4, P 10 µg/L (orange bars), pH 7.2, P 100 µg/L (grey bars), pH 8.4, P 100 µg/L (yellow bars) treatment. Error bars indicate standard deviations.

3.3 Ecotoxicity of Pb to *P. subcapitata*

Concentration response data of the different P & pH treatments is given in Figure 7. Corresponding estimated effective concentrations are reported in Table 3. Overall, concentration responses were relatively similar between treatments. 48h-$EC50_{Pbfilt}$ differed only 1.5-fold over all treatments. Pb toxicity

was significantly higher in media with higher initial P concentrations (Table 4), except when the pH 7.2, P 10 µg/L and pH 7.2 P 100 µg/L were compared. At the EC20, Pb toxicity was significantly lower in the pH 8.4, P 100 µg/L treatment compared to all other treatments, while for all other treatments no significant differences were observed.

pH had minimal effects on filtered Pb toxicity: 48h-$EC50_{Pbfilt}$ differed only 1.2-fold between the pH treatments within both P-levels. The effect of pH on 48h-$EC50_{Pbfilt}$ was also different between the P 10 µg/L and P 100 µg/L treatment. 48h-$EC50_{Pbfilt}$ decreased (increasing toxicity) slightly with increasing pH at high P concentrations, while it increased (decreasing toxicity) slightly with increasing pH at low P concentrations. However, there were no significant differences in $EC50_{Pbfilt}$ between pH levels within a P level.

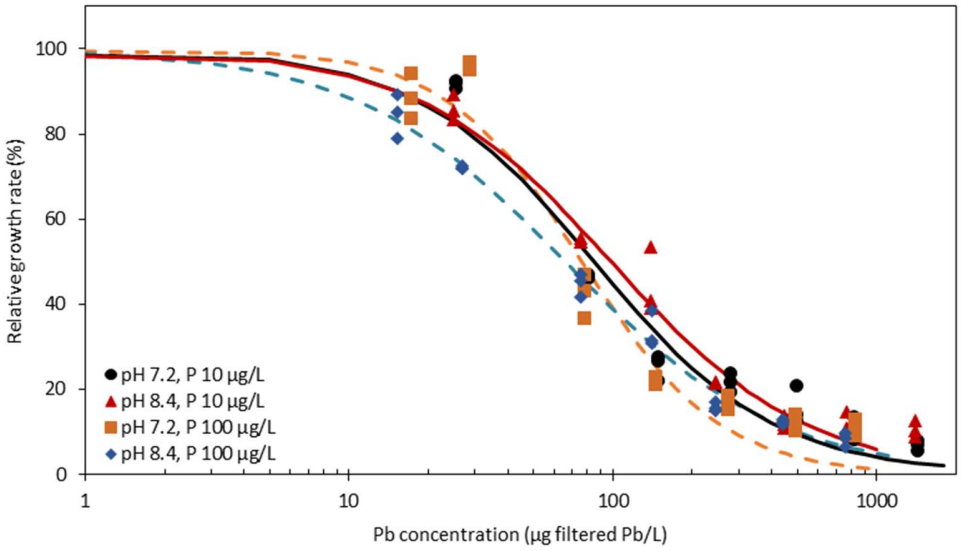

Figure 7. Comparison of concentration response data (points) and fitted log-logistic concentration response curves (lines) between the pH 7.2, P 10 µg/L (black circles, full black line), pH 8.4, P 10 µg/L (red triangles, full red line), pH 7.2, P 100 µg/L (orange squares, orange dashed line), pH 8.4, P 100 µg/L (blue diamonds, blue dashed line) treatments. Estimated effect concentrations are reported in Table 3.

Table 3. Calculated x% effective concentrations), expressed as µg filtered Pb/L (48h-ECx_{Pbfilt}), of the chronic toxicity tests with Pb with *P. subcapitata* in all media.

Test ID	48h-$EC10_{Pbfilt}$ (µg/L)	48h-$EC20_{Pbfilt}$ (µg/L)	48h-$EC50_{Pbfilt}$ (µg/L)
pH 7.2, P10	-[a]	*29 (22-38)*[b]	*85 (73-99)*[b]
pH 8.4, P10	-[a]	*31 (24-38)*[b]	*98 (88-111)*[b]
pH 7.2, P100	21 (15-29)	33 (26-43)	77 (66-89)
pH 8.4, P100	-[a]	18 (16-21)	66 (61-72)

[a] EC10 for the pH 7.2, P10; pH 8.4, P10, and pH 8.4, P100 treatments were extrapolated below the lowest test concentrations and were not further considered.

[b] Test validity criteria for the pH 7.2, P10 and pH 8.4, P10 treatment were not met, EC20 & EC50 are only given for comparison.

EC10=10% effective concentration; EC20=20% effective concentration; EC50=50% effective concentration

Table 4. Results of the Wheeler ratio-test comparing the 48h-EC50$_{Pbfilt}$ and 48h-EC20$_{Pbfilt}$ between treatments.

	pH 7.2, P10	pH 8.4, P10	pH 7.2, P100
EC20			
pH 7.2, P10	-	-	-
pH 8.4, P10	*p>0.05*	-	-
pH 7.2, P100	*p>0.05*	*p>0.05*	-
pH 8.4, P100	p=0.003	p<0.001	p<0.001
EC50			
pH 7.2, P10	-	-	-
pH 8.4, P10	*p*>0.05	-	-
pH 7.2, P100	*p*>0.05	*p*=0.02	-
pH 8.4, P100	*p*=0.005	*p*<0.001	*p*>0.05

3.4 Validation of the algae Pb bioavailability model at high pH

To evaluate the ability to extrapolate the algae Pb bioavailability model at high pH (pH 8.4), the intrinsic sensitivity of the bioavailability model was first calibrated on the pH 7.2 water using Equation 2. Pb toxicity to *P. subcapitata* at pH 8.4 was then predicted using this intrinsic sensitivity in Equation 1. The same approach has been previously used to validate the chronic Ni bioavailability models at high pH (Nys et al. 2016). Only the high phosphorous waters (P 100 µg/L) were considered for validation of the algae model, as in the low phosphorous waters test validity criteria were not met.

Table 5. Intrinsic sensitivity used for validation of the algae Pb bioavailability model at high pH

	Intrinsic sensitivity
Q50	1.70[a]
Q20	1.17

[a] Calibrated on Pb^{2+} toxicity in the pH 7.2, P 100 µg/L test water using Equation 2.

The intrinsic sensitivities based on the 48h-EC50$_{Pbfilt}$ and 48h-EC20$_{Pbfilt}$ are listed in Table 5. Using these intrinsic sensitivities, the 48h-EC50$_{Pbfilt}$ and 48h-EC20$_{Pbfilt}$ at pH 8.4 were predicted within 2.0- and 1.3-fold error, respectively (Figure 8). These prediction errors are in line with those earlier reported for predictions of Pb toxicity to *P. subcapitata*, expressed as 72h-EC50$_{Pbfilt}$, by the algae Pb bioavailability model in 14 test media with pH ranging between 6.0 and 8.0 (i.e. mean and maximum prediction error were 1.4 and 2.2, respectively; De Schamphelaere et al. 2014). However, Pb toxicity at pH 8.4 was slightly overestimated, i.e. 48h-EC50$_{Pbfilt}$ was underestimated by twofold (Figure 9, right panel). Figure 9 shows that the predicted effect of pH on filtered Pb toxicity, expressed as EC50$_{Pbfilt}$, was more steep than the observed effect of pH. Indeed, the observed slope of the linear relationship between pH and $\log_{10}$EC50$_{Pb2+}$ was less steep than the pH slope of the algae bioavailability model, i.e. 1.138±0.045 vs. 1.509±0.038, respectively (Figure 10, right panel). However, the difference in slopes was not significant (p=0.06). At the EC20 level, the difference between the observed and predicted effect of pH on filtered Pb

was minimal (Figure 8) and the observed slope of the linear relationship between pH and $\log_{10}EC20_{Pb2+}$ approached that of the algae bioavailability model (Figure 9, left panel).

The algae bioavailability model resulted in reasonable predictions of Pb toxicity at pH 8.4. This suggests that the chronic algae Pb bioavailability model can be extrapolated to predict Pb toxicity for pH up to 8.4. However, additional toxicity tests at more pH levels using the new PO_4 addition method may further corroborate this conclusion.

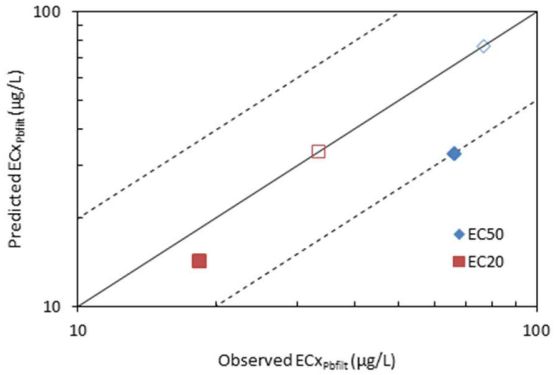

Figure 8. Observed vs. predicted 48h-$EC50_{Pbfilt}$ (blue diamonds) and 48h-$EC20_{Pbfilt}$ (orange squares), expressed as µg filtered Pb/L, of *Pseudokirchneriella subcapitata* at pH 7.2 (open symbols) and pH 8.4 (filled symbols) at 100 µg P/L. Predictions were made using the algae Pb bioavailability model (Eq. 1) linked to Visual Minteq. The dashed line represents a difference of a factor of two between the observed and predicted data. The full line represents a perfect fit between observed and predicted data. Please note that the position of the pH 7.2 on the perfect prediction line results from the calibration of the bioavailability model on this data point.

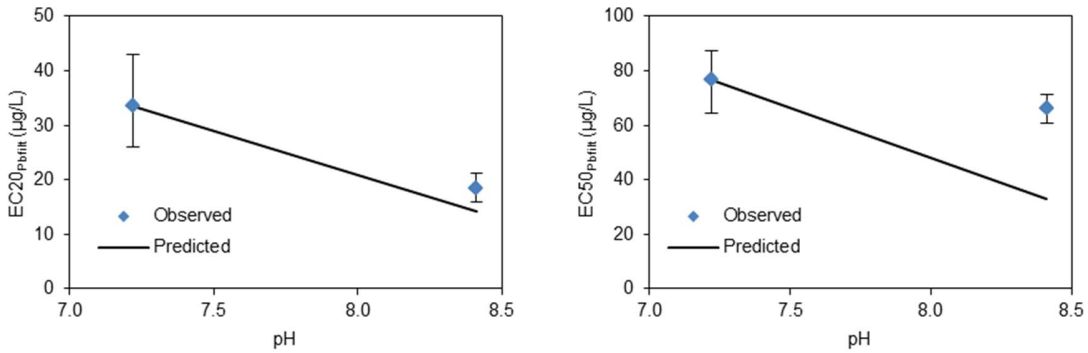

Figure 9. Observed (symbols) and predicted (line) 48h-$EC20_{Pbfilt}$ (left) and 48h-$EC50_{Pbfilt}$ (right) of *Ceridoaphnia dubia* as a function of pH. Plotted error bars denote 95% confidence intervals on observed ECx_{Pbfilt}. Predictions were made using the algae Pb bioavailability model (Eq. 1 linked to Visual Minteq) calibrated on the Pb toxicity at pH 7.2.

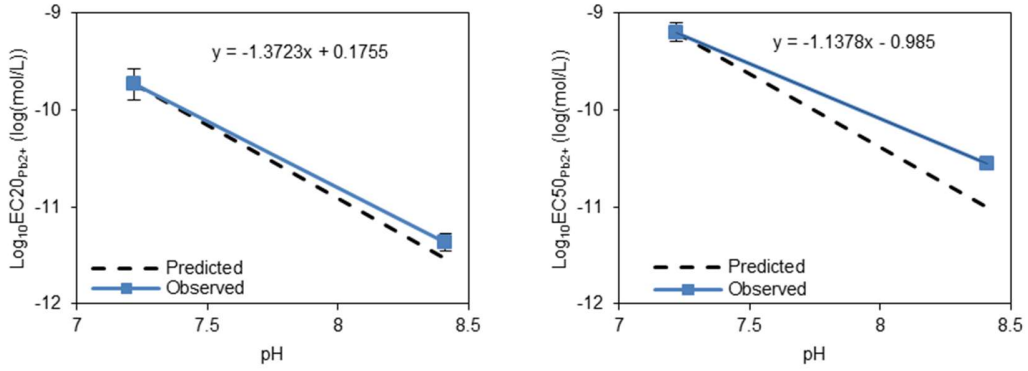

Figure 9. The effect of pH on log 48h-EC20$_{Pb2+}$ (left pannel) and log 48h-EC50$_{Pb2+}$ (right pannel) of *Pseudokirchneriella subcapitata*. Symbols denote observed 48h-ECx$_{Pb2+}$. Plotted error bars denote 95% confidence intervals on observed ECx$_{Pb2+}$. Full line represents observed effect of pH on log 48h-ECx$_{Pb2+}$. Dashed line represents the predicted effect of pH on log 48h-ECx$_{Pb2+}$ using the algae bioavailbility model (Eq. 1).

4. Conclusions

Measurements of the chemical composition of the exposure media suggest that precipitation of Pb-PO_4 was minimal during the testing period, even at the high pH treatments. Hence, adding PO_4 to the test medium after allowing Pb to equilibrate with the PO_4-free medium can be considered as a suitable method to prevent Pb-PO_4 precipitation in the exposure media.

Filtered Pb toxicity, expressed as EC50$_{Pbfilt}$, differed only 1.5-fold over all treatments. pH had no significant effect on filtered Pb toxicity at both P levels. P had only a significant effect at the high pH treatment.

The algae Pb bioavailability model calibrated on Pb toxicity at pH 7.2 (P 100 µg/L) predicted filtered Pb toxicity(EC50$_{Pbfilt}$ and EC20$_{Pbfilt}$) at pH 8.4 (P 100 µg/L) with reasonable accuracy, i.e. within 2-fold error. Although, the slope of the linear relationship between pH and log$_{10}$EC50$_{Pb2+}$ for the pH range between 7.2 and 8.4 (present study) was slightly lower than that of the pH range between 6 and 8, our study suggests that the chronic algae Pb bioavailability model can be extrapolated to predict Pb toxicity up to pH 8.4.

References

Borgmann U, Nowierski M, Dixon DG. 2005. Effect of major ions on the toxicity of copper to *Hyalella azteca* and implications for the biotic ligand model. *Aquat Toxicol* 73: 268-287.

De Schamphelaere KAC, Heijerick DG, Janssen CR. 2004. Comparison of the different pH buffering techniques on the toxicity of copper and zinc to *Daphnia magna* and *Pseudokirchneriella subcapitata. Ecotoxicology* 13:697-705.

De Schamphelaere KAC, Nys C, Janssen CR. 2014. Ecotoxicity of lead (Pb) to freshwater green algae: development and validation of a bioavailability model and inter-species sensitivity comparison. *Aquatic Toxicology* 155: 348-359.

FOREGS. 2005. Salminen, R. (ed.): Geochemical Atlas of Europe. Part 1: Background Information, Methodology and Maps. Espoo, Finland.

Gao C, De Schamphelaere KAC, Smolders E. 2016. Zinc toxicity to the alga *Pseudokirchneriella subcapitata* decreases under phosphate limiting growth conditions. *Aquatic Toxicology* 173: 74-82.

Kandegedara A, Rorabacher DB. 1999. Noncomplexing tertiary amines as 'better' buffers covering the range of pH 3-11. Temperature dependence of their acid dissociation constants. *Analytical Chemistry* 71: 3140-3144.

Milne CJ, Kinniburgh DG, Van Riemsdijk WH, Tipping E. 2003. Generic NICA-Donnan Model Parameters for Metal-Ion Binding by Humic Substances. *Environmental Science and Technology* 37: 958-971.

Nys C, Asselman J, Hochmuth JD, Janssen CR, Blust R, Smolders E, De Schamphelaere KAC. 2015. Mixture toxicity of nickel and zinc to *Daphnia magna* is non-interactive at low effect sizes, but becomes synergistic at high effect sizes. Environmental Toxicology and Chemistry 34: 1091-1102.

Nys C, Janssen CR, De Schamphelaere KAC. The effect of pH on chronic aquatic Ni toxicity is dependent on the pH itself: extending the chronic Ni bioavailability models. *Environmental Toxicology and Chemistry:* accepted manuscript: (DOI:10.1002/etc.3232).

Organization for Economic Co-operation and Development. 2011. Freshwater Alga and Cyanobacteria, Growth Inhibition Test: OECD Guideline 201. Paris, France.

Ritchie JD, Perdue EM. 2003. Proton-binding study of standard and reference fulvic acids, humic acids, and natural organic matter. *Geochimica et Cosmochimica Acta* 67 (1): 85-96.

Sokal RR, Rohlf FJ. 1981. Biometry. WH Freeman and Company New York, NY, USA.

Tipping E. 2002. Cation binding by humic substances. Cambridge environmental chemistry series. Campbell PGC, Harisson RM, de Mora SJ (Eds.). Cambridge University Press, New York.

Appendix: Raw concentration response data

Table A1: Raw concentration response data of the **pH 7.2; P 10 µg/L** treatment

	Nominal Conc.	Filtered conc at test initiation	Filtered conc day 0-2	Filtered conc day 0-3	Cell density (x10^4 cells/mL)			Growth rate (µ) d-1
	µg Pb/L	µg Pb/L	µg Pb/L	µg Pb/L	day 1	day 2	day 3	day 0-2
Control A	0.25	0.25	0.25	0	7.73	22.43	31.43	1.56
Control B	0.25	0.25	0.25	0	9.45	24.51	32.52	1.60
Control C	0.25	0.25	0.25	0	8.39	21.43	32.34	1.53
Control D	0.25	0.25	0.25	0	9.78	22.91	32.05	1.57
Control E	0.25	0.25	0.25	0	9.09	22.19	30.84	1.55
32 A	32	26	24	28	8.40	17.97	24.40	1.44
32 B	32	26	24	28	9.30	17.04	23.02	1.42
32 C	32	26	24	28	10.00	17.81	23.88	1.44
100 A	100	81	74	77	2.43	4.29	8.62	0.73
100 B	100	81	74	77	2.57	4.36	8.76	0.74
100 C	100	81	74	77	2.55	4.23	8.41	0.72
180 A	180	149	136	138	1.84	2.30	3.17	0.42
180 B	180	149	136	138	1.77	1.99	2.63	0.34
180 C	180	149	136	138	1.80	2.37	3.53	0.43
320 A	320	280	246	255	1.67	1.97	2.20	0.34
320 B	320	280	246	255	0.97	2.11	2.26	0.37
320 C	320	280	246	255	1.56	1.83	2.21	0.30
560 A	560	496	437	445	1.25	1.91	1.52	0.32
560 B	560	496	437	445	1.36	1.56	1.51	0.22
560 C	560	496	437	445	1.43	1.48	1.50	0.20
1000 A	1000	819	731	746	1.47	1.33	1.48	0.14
1000 B	1000	819	731	746	1.27	1.29	1.47	0.13
1000 C	1000	819	731	746	1.14	1.52	1.31	0.21
1800 A	1800	1429	1307	1340	1.07	1.25	1.13	0.11
1800 B	1800	1429	1307	1340	1.45	1.19	1.08	0.09
1800 C	1800	1429	1307	1340	1.13	1.28	0.98	0.12

Table A2: Raw concentration response data of the **pH 8.4; P 10 µg/L** treatment

	Nominal Conc.	Filtered conc at test initiation	Filtered conc day 0-2	Filtered conc day 0-3	Cell density (x10^4 cells/mL)			Growth rate (µ) d-1
	µg Pb/L	µg Pb/L	µg Pb/L	µg Pb/L	day 1	day 2	day 3	day 0-2
Control A	0.25	0.25	0.25	0	9.58	23.97	30.67	1.59
Control B	0.25	0.25	0.25	0	9.56	23.74	30.63	1.58
Control C	0.25	0.25	0.25	0	10.28	22.66	30.30	1.56
Control D	0.25	0.25	0.25	0	9.96	23.56	32.52	1.58
Control E	0.25	0.25	0.25	0	10.15	23.20	30.77	1.57
32 A	32	25	21	20	5.19	16.76	25.69	1.41
32 B	32	25	21	20	5.33	14.86	22.70	1.35
32 C	32	25	21	20	4.56	13.84	20.36	1.31
100 A	100	76	63	64	2.48	5.61	10.63	0.86
100 B	100	76	63	64	2.46	5.58	10.25	0.86
100 C	100	76	63	64	2.49	5.76	9.86	0.88
180 A	180	139	116	117	2.33	5.42	5.81	0.84
180 B	180	139	116	117	1.97	3.44	5.36	0.62
180 C	180	139	116	117	1.92	3.63	7.01	0.65
320 A	320	246	212	212	1.69	1.96	2.39	0.34
320 B	320	246	212	212	1.75	1.98	2.26	0.34
320 C	320	246	212	212	1.61	1.67	2.07	0.26
560 A	560	445	390	396	1.45	1.41	1.52	0.17
560 B	560	445	390	396	1.53	1.54	1.56	0.22
560 C	560	445	390	396	1.50	1.45	1.37	0.19
1000 A	1000	765	652	660	1.41	1.39	1.26	0.17
1000 B	1000	765	652	660	1.49	1.59	1.40	0.23
1000 C	1000	765	652	660	1.46	1.41	1.38	0.17
1800 A	1800	1399	1226	1245	1.35	1.49	1.25	0.20
1800 B	1800	1399	1226	1245	1.39	1.39	1.29	0.16
1800 C	1800	1399	1226	1245	1.36	1.32	1.15	0.14

Table A3: Raw concentration response data of the **pH 7.2; P 100 µg/L** treatment

	Nominal Conc.	Filtered conc at test initiation	Filtered conc day 0-2	Filtered conc day 0-3	Cell density (x10^4 cells/mL)			Growth rate (µ) d-1
	µg Pb/L	µg Pb/L	µg Pb/L	µg Pb/L	day 1	day 2	day 3	day 0-2
Control A	0.25	0.25	0.25	0	10.60	41.63	85.78	1.86
Control B	0.25	0.25	0.25	0	9.53	40.19	81.30	1.85
Control C	0.25	0.25	0.25	0	10.67	41.05	87.88	1.86
Control D	0.25	0.25	0.25	0	10.55	41.97	90.29	1.87
Control E	0.25	0.25	0.25	0	10.82	49.59	92.76	1.95
18 A	18	17	17	16	9.92	34.47	81.29	1.77
18 B	18	17	17	16	8.01	23.12	70.42	1.57
18 C	18	17	17	16	8.22	27.79	77.93	1.66
32 A	32	29	28	28	9.47	36.68	82.00	1.80
32 B	32	29	28	28	4.33	37.93	91.78	1.82
32 C	32	29	28	28	8.09	35.58	81.29	1.79
100 A	100	78	72	75	2.90	5.87	12.87	0.88
100 B	100	78	72	75	2.84	5.08	10.64	0.81
100 C	100	78	72	75	2.71	4.00	8.52	0.69
180 A	180	145	133	136	2.07	2.36	2.78	0.43
180 B	180	145	133	136	2.03	2.25	2.90	0.40
180 C	180	145	133	136	2.09	2.21	2.99	0.40
320 A	320	272	239	246	1.90	1.80	1.95	0.29
320 B	320	272	239	246	1.95	2.01	2.02	0.35
320 C	320	272	239	246	1.89	1.78	1.96	0.29
560 A	560	492	430	441	1.67	1.63	1.48	0.24
560 B	560	492	430	441	1.72	1.70	1.60	0.27
560 C	560	492	430	441	1.68	1.47	1.45	0.19
1000 A	1000	827	724	737	1.48	1.41	1.24	0.17
1000 B	1000	827	724	737	1.52	1.64	1.36	0.25
1000 C	1000	827	724	737	1.56	1.51	1.36	0.21

Table A4: Raw concentration response data of the **pH 8.4; P 100 µg/L** treatment

	Nominal Conc.	Filtered conc at test initiation	Filtered conc day 0-2	Filtered conc day 0-3	Cell density (x10^4 cells/mL)			Growth rate (µ) d-1
	µg Pb/L	µg Pb/L	µg Pb/L	µg Pb/L	day 1	day 2	day 3	day 0-2
Control A	0.25	0.25	0.25	0	9.34	37.91	110.86	1.82
Control B	0.25	0.25	0.25	0	10.16	43.97	134.26	1.89
Control C	0.25	0.25	0.25	0	12.29	54.79	128.86	2.00
Control D	0.25	0.25	0.25	0	11.13	46.21	135.86	1.92
Control E	0.25	0.25	0.25	0	10.52	44.41	126.26	1.90
18 A	18	15	13	12	9.45	30.10	101.86	1.70
18 B	18	15	13	12	8.99	25.68	93.89	1.62
18 C	18	15	13	12	7.04	20.39	87.73	1.51
32 A	32	27	22	20	4.77	15.46	61.27	1.37
32 B	32	27	22	20	5.34	15.55	61.68	1.37
32 C	32	27	22	20	5.51	15.94	61.95	1.38
100 A	100	75	63	64	2.64	5.98	13.62	0.89
100 B	100	75	63	64	2.53	4.92	12.35	0.80
100 C	100	75	63	64	2.61	5.70	14.98	0.87
180 A	180	140	119	120	2.25	4.33	7.83	0.73
180 B	180	140	119	120	1.98	3.24	5.00	0.59
180 C	180	140	119	120	2.05	3.33	3.61	0.60
320 A	320	244	211	215	1.70	1.93	2.13	0.33
320 B	320	244	211	215	1.73	1.77	1.85	0.29
320 C	320	244	211	215	1.73	1.81	1.85	0.30
560 A	560	443	380	387	1.19	1.65	1.66	0.25
560 B	560	443	380	387	1.50	1.60	1.36	0.24
560 C	560	443	380	387	1.61	1.57	1.45	0.22
1000 A	1000	761	669	677	1.48	1.29	1.14	0.13
1000 B	1000	761	669	677	1.53	1.39	1.34	0.17
1000 C	1000	761	669	677	1.50	1.45	1.30	0.18