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Comparison of the Effect of Different pH Buffering Techniques on the Toxicity of Copper and Zinc to *Daphnia Magna* and *Pseudokirchneriella Subcapitata*

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Abstract. During the time-course of ecotoxicity tests with algae and chronic (reproductive) toxicity tests with daphnids, in which algae are present as a food source, pH can dramatically increase due to photosynthetic activity. As pH changes can significantly affect metal speciation and thus its bioavailability, it may be necessary to buffer the pH of the exposure medium. One class of buffers (Good's *N*-subtituted aminosulfonic acids) are increasingly being used in biological and chemical applications, including ecotoxicity testing. However, the potential effect of these buffers on metal toxicity has, so far, scarcely been examined. In this study we investigated if MOPS (3-*N* morpholino propane sulfonic acid) affected the toxicity of copper and zinc to two standard test organisms: the cladoceran *Daphnia magna* and the green alga *Pseudokirchneriella subcapitata*. First, we demonstrate that up to a concentration of 750 mg l^{-1} (which proved to be sufficient for pH buffering) MOPS did not affect 21-day net reproduction of *D. magna* or the 72-h population growth of *P. subcapitata*. Second, we conducted bioassays in copper and zinc spiked standard media for the pH range 6 – 8. For *D. magna* the possible effect of 750 mg l^{-1} MOPS on acute copper and zinc toxicity was investigated by performing parallel 48-h toxicity tests in $NaHCO_3$ and MOPS buffered test media. Seventy-two hour growth inhibition assays with *P. subcapitata* were performed in parallel in MOPS and $NaHCO_3$ buffered test media and in test media with daily manual pH adjustment with HCl. For daphnids no significant differences in copper and zinc toxicity were observed between MOPS or $NaHCO_3$ buffered test media. For algae no significant differences in metal toxicity were observed between MOPS and HCl buffered media, but in test media buffered with $NaHCO_3$ an increased copper and zinc toxicity was observed as a consequence of pH increases during the test. Clearly, the results of this study demonstrate the importance of pH buffering in metal toxicity testing and the suitability of the MOPS buffer for that purpose.

Keywords: copper and zinc toxicity; bioavailability; MOPS; pH buffering

Introduction

One of the major problems with the correct assessment of the toxicity of metals to key test species is the control of pH during the ecotoxicity assay. In algal tests for example, pH tends to increase during the time-course of the experiment due to the photosynthetic activity of the algae (Nyholm and Källqvist, 1989; Stumm and Morgan, 1996). This phenomenon can also occur in standard waters in chronic toxicity tests with daphnids, as algae are used as a food source. pH changes can also occur when using natural surface

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waters as test medium, during the ecotoxicity assay but also on transportation (due to the loss of chemical equilibrium at the time of sampling). Since pH is generally regarded as a major factor controlling metal toxicity to aquatic organisms (Meador, 1991; Macfie et al., 1994; Nalejawko et al., 1997; Franklin et al., 2000; De Schamphelaere and Janssen, 2002; Heijerick et al., 2002), pH changes can strongly affect the results of metal toxicity studies. Despite this knowledge, little has been done to incorporate this information into standard toxicity testing procedures for regulatory use. For example, both ISO (1989) and OECD (1984a) guidelines consider changes of up to 1 pH unit as acceptable and do not prescribe the use of a pH buffer. As a consequence, a large number of toxicity data for metals found in literature may be biased by such pH changes. Thus, to adequately assess the toxicity of metals, one should control pH during the experiment and one way to do this is with pH buffers. However, the selection of an appropriate buffer is not easy and should be based on extensive testing to ensure that the selected buffer does not alter metal toxicity itself.

Good's *N*-subtituted aminosulfonic acids (Good et al., 1966; Good and Izawa, 1972) are a class of potentially suitable buffers, since these buffers were specially designed not to complex metals (Kandegedara and Rorabacher, 1999). Although "these buffers increasingly are being used in numerous biological and chemical applications" (Vasconcelos et al., 2000), there have been increasing reports of complexation of metals (especially copper) with these buffers (Kandegedara and Rorabacher, 1999). In fact, of Good's 20 buffer compounds, only three are reported to be completely non-complexing, i.e. 2-[*N*-morpholino]ethanesulfonic acid (MES), 3-[*N*-morpholino]propanesulphonic acid (MOPS) and Piperazine-*N*, *N*′-bis(ethanesulfonic acid) PIPES (Kandegedara and Rorabacher, 1999). However, when studying metal bioavailability and toxicity, the non-complexing property of the applied buffer should not be the only consideration made. Ferguson (1980) stressed that a buffer can also have physiological effects on an organism and can thus alter metal toxicity indirectly. It is clear that synthetic buffers that alter metal toxicity in this way should not be used. Until now, at least some buffers have been tested for their direct and/or indirect effects on metal toxicity. *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 3-[*N*,*N*-bis(hydroxyethyl)amino]-2-hydroxypropanesulfonic acid (DIPSO) *N*-(2-hydroxyethyl)piperazine-*N*′-*2*-hydroxypropanesulfonic acid (HEPPSO) and piperazine-*N*,*N*′-bis(2-hydroxypropanesulfonic acid) (POPSO) all affected copper toxicity for fresh water and marine organisms (Lage et al., 1996; Vasconcelos et al., 2000). To our knowledge, however, no evidence of a suitable buffer (i.e. not affecting metal toxicity) for metal toxicity testing with daphnids and algae has been reported.

The purpose of this study was to examine the suitability of MOPS as a pH buffer for metal toxicity testing with *Daphnia magna* and *Pseudokirchneriella subcapitata*. The choice of MOPS was based on its non metal complexing property, its useful pH range (6–8, pKa = 7.2 at 25 °C) and its recommended use in sediment pH buffering (US EPA, 1991).

Methods

Preparation of exposure media and chemical measurements

All exposure media, as described below, were prepared using carbon-filtered, deionised water. All chemicals were purchased from Merck-Eurolab (Leuven, Belgium) and were reagent grade.

Dissolved copper and zinc concentrations (samples filtered through 0.45 μm filters, Gelman Sciences, Ann Arbor, Michigan, USA) in the test media were determined at the start of each test (i.e. 24 after spiking the solutions with metal, see further for spiking procedure) using a graphite furnace atomic absorption spectrophotometer (SpectrAA800 with Zeeman background correction, Varian, Mulgrave, Australia) for copper and a flame atomic absorption spectrophotometer (SpectroAA100, Varian, Mulgrave, Australia) for Zinc. Calibration standards (Sigma-Aldrich, Steinheim, Germany) and a reagent blank were analysed with every ten samples.

pH (pH meter P407, Consort, Turnhout, Belgium) was measured before and after the test for acute daphnid experiments, before and after each renewal for chronic daphnid experiments and daily for the algae experiments. The pH glass

electrode was calibrated before each use, using pH 4 and pH 7 buffers (Merck, Darmstadt, Germany) and performance at higher pH was checked using a pH 10 buffer.

Effect of MOPS on D. magna *reproduction*

In a first experiment a 21-day chronic bioassay with *D. magna* was conducted according to test guideline no. 211 (OECD, 1998) to determine the no observed effect concentration (NOEC) of MOPS. Test organisms originated from a healthy *D. magna* clone which has been cultured under standardized conditions in M4 medium for about 20 years (Elendt and Bias, 1990). The test was conducted in standard ISO-medium (ISO, 1996) containing 2 mM $CaCl_2$, 0.5 mM $MgS0_4$, 0.77 mM $NaHCO_3$ and 0.078 mM KCI. Together with a control, following MOPS concentrations were tested: 0.5, 0.75, 1, 1.25 and 1.5 mg l^{-1}. For each of these MOPS concentrations, pH was adjusted to the same pH as the control (pH $\sim$ 7.6) with NaOH. Per concentration 10 replicates were tested. One juvenile/replicate ($<$ 24 h old) /replicate was exposed in 50 ml of the test solutions at 20 °C and with a 12 h photoperiod. The organisms were fed daily with an algal mix of *P. subcapitata* and *Chlamydomonas reinhardtii* in a 3: 1 ratio. Each organism received 8×10^6 cells/day in the first week, 12×10^6 cells/day in the second week and 16×10^6 cells/day in the third week of the 21-day assay. Every 2 days, the medium was renewed and parent mortality and the number of offspring was counted. At the end of the test, net reproduction (mean number of juveniles produced per parent animal) was calculated and MOPS treatments were compared with the control using ANOVA, followed by the post hoc Duncan's multiple range test (Statistica software, Statsoft, Tulsa, OK, USA).

Effect of MOPS on acute toxicity of copper and zinc to D. magna

For both copper and zinc six acute 48-h immobilization assays with juvenile *D. magna* ($<$ 24 h old) were performed following OECD test guideline 202 (OECD, 1984b). Tests were conducted at pH 6, 7 and 8 both with $NaHCO_3$ and MOPS buffering. In this, $NaHCO_3$ buffering is used as a control since it is the buffer in the generally applied ISO-medium (ISO, 1996). All tests were performed with pH adjusted ISO-medium containing 2 mM $CaCl_2$, 0.5 mM $MgS0_4$ and 0.078 mM KCI. For each pH, 2 l medium was buffered with $NaHCO_3$ and 2 l was buffered with 750 mg MOPS l^{-1}. For $NaHCO_3$ buffering, depending on the desired pH, different concentrations of $NaHCO_3$ were added: 0.02 mM for pH 6, 0.15 mM for pH 7 and 1.5 mM for pH 8. For MOPS buffering pH was adjusted to 6, 7 or 8 with NaOH. Since Na has been shown to decrease copper and zinc toxicity to *D. magna* (De Schamphelaere and Janssen, 2002; Heijerick et al., 2002) the Na content of $NaHCO_3$ and MOPS buffered media was equalled for each pH through the addition of NaCl. The final Na concentration for pH 6, 7 and 8 were 1.27, 2.52 and 4.02 mM, respectively. These test media were then used as the dilution water to make a logarithmic concentration series (1 control + 5 concentrations) of copper and zinc, added as their chloride salt. For each concentration, three replicates of 10 organisms in 50 ml test cups were tested at 20 °C with a 12 h photoperiod. In order to obtain near-equilibrium situations, all media were stored in the test cups at 20 °C for 1 day prior to testing. After 48 h the number of immobilized animals was recorded. 48-h EC50s were calculated based on measured dissolved metal concentrations with the Trimmed Spearman-Karber method (Hamilton et al., 1977).

Effect of MOPS on growth of P. subcapitata

All tests with *P. subcapitata* were conducted in accordance with OECD Guideline No. 201 (OECD, 1984a). *P. subcapitata* starter cultures were obtained from the Culture Collection of Algae and Protozoa (CCAP 278/4, Ambleside, UK) and were grown in standard OECD-medium (OECD, 1984a, which has the same composition as described by ISO, 1989) at 20 $\pm$ 1 °C with continuous light (5000 lux) and continuous aeration (filtered air). Each week, cultures were visually inspected for contamination using a light microscope. 72-h toxicity assays were conducted in OECD-medium (pH $\sim$ 7.4) to assess the effect of 0.5, 0.75 and 1 g MOPS l^{-1} on the growth of *P. subcapitata*. pH was adjusted daily to pH 7.4 with HCl. Tests were conducted in triplicate in 100 ml; erlenmeyers. At the start of each experiment

10,000 cells ml^{-1} (N_0) were added to 50 ml of test medium and the erlenmeyers were capped with an air-permeable stopper. The erlenmeyers were then kept for 72 h under 25 °C under continuous illumination (4000 lux) and were manually shaken daily. The number of cells after 24, 48 and 72 h of exposure (N_{24}, N_{48} and N_{72}) was determined using a coulter counter (Coulter Counter model DN, Harpenden, Herts, UK). Algal biomass growth was calculated using the area under the growth curve (A) method as described by OECD (1984a):

$$\begin{aligned} A = &\{(N_1 - N_0)t_1\}/2 \\ &+ \{(N_1 + N_2 - 2N_0)(t_2 - t_1)\}/2 \\ &+ \{(N_2 + N_3 - 2N_0)(t_3 - t_2)\}/2 \end{aligned} \quad (1)$$

with $N_0 =$ initial cell concentration (1 × 104 cells / mL), $N_x =$ cell concentration after t_x hours after the start of the test (measurements made after 24, 48 and 72 h of exposure). Treatment means of the area under the growth curve were statically compared using ANOVA, followed by the post-hoc Duncan's multiple range test (Statistica software, Statsoft, Tulsa, OK, USA).

Effect of MOPS on toxicity of copper and zinc to P. subcapitata

For both copper and zinc six 72-h algal growth inhibition tests were conducted in OECD medium (OECD, 1984a) in OECD standard test medium (OECD, 1984a) (cf. above for exact test procedure). Tests were conducted at pH 6, 7 and 8 both with $NaHC0_3$ buffering, MOPS buffering (750 mg l^{-1}) and "buffering" by daily manual pH adjustment with HCl (further termed HCl buffering). Although we are aware that the latter is not a real pH buffer, we will adhere to this term throughout the text to increase readability. For $NaHCO_3$ buffering and HCl-buffering, OECD medium without $NaHCO_3$ was prepared and, depending on the desired pH, different concentrations of $NaHCO_3$ were added: 0.02 mM for pH 6,0.15 mM for pH 7 and 1.5 mM for pH 8. In the $NaHCO_3$ buffering experiment pH was not adjusted, while for the HC1 buffering experiment, pH was adjusted daily to its initial value with HCI. For MOPS buffering, OECD medium (with $NaHCO_3$) pH was adjusted to 6, 7 or 8 with NaOH. Here too, Na content of $NaHCO_3$, MOPS and HCl buffered test media was normalized to the same level by NaCl addition. The final Na concentrations for pH 6, 7 and 8 were 1.36 2.72 and 4.29 mM, respectively. These test media were then used as the dilution water to make a logarithmic concentration series (1 control + 5 metal concentrations) of copper and zinc, added as their chloride salt. In order to obtain near-equilibrium situations, all media were stored in the erlenmeyers at 25 °C for one day prior to testing. After 24, 48 and 72 h of exposure the number of algal cells was counted using a coulter counter. EC50s based on biomass (E_bC50s) were calculated with a logistic model (Haanstra et al., 1985) fitted to the observed area under the growth curve (A) versus measured dissolved metal concentrations (fitting performed with Sigmaplot software, SPSS Inc., Chicago, IL, USA).

Results

Effects of MOPS on daphnid reproduction and algal growth

All conditions for the validity of the toxicity tests with MOPS were fulfilled as prescribed in the standard test procedures (OECD, 1984a; OECD, 1998). Mean control offspring was 70.1 ± 14.6 juveniles/parent daphnid and algal cell densities in control exposures increased with a factor 20.3 ± 0.4 over the 72-h test period. Fig. 1 shows the performance of *D. magna* in a 21-day reproduction test and of *P. subcapitata* in a 72-h growth inhibition test with MOPS. Reproduction of *D. magna* gradually decreased with increasing MOPS concentrations. A NOEC of 750 mg MOPS l^{-1} and a LOEC of 1000 mg MOPS l^{-1} was derived. Up to the highest tested MOPS concentration of 1000 mg l^{-1}, *P. subcapitata* growth was not significantly affected. This means that for both species, 750 mg MOPS l^{-1} can be regarded as a buffer concentration not affecting these species. pH measurements also indicated that this MOPS concentration was able to maintain pH within 0.1 pH-unit, whereas adding no MOPS to the test media resulted in a pH variation of about 0.7 pH-unit (for *D.magna)* to 0.9 pH-units (for *P. subcapitata*) (Table 1). In tests with a MOPS concentration of

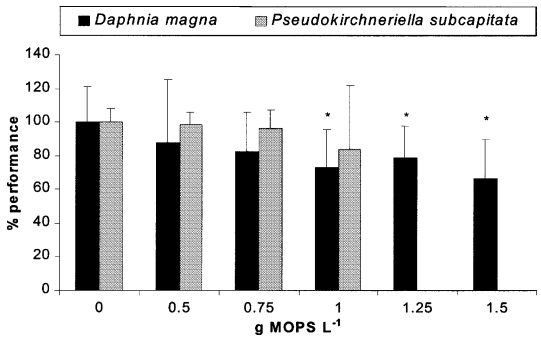

Figure 1. Performance of *Daphnia magna* in a 21-day reproduction test and *Pseudokirchneriella subcapitaia* in a 72-h growth inhibtion test with MOPS as toxicant. For *Daphnia magna* % performance was calculated for each treatment as number of offspring in MOPS exposure/number of offspring in control. For *P. subcapitata* it was calculated as the area under the growth curve for a MOPS-treatment divided by the area under the growth curve for the control treatment (See equations 1 in text). MOPS treatments of 1.25 and 1.5 g l^{-1} were not performed for *P. subcapitata*. Error bars represent 95% confidence interval. * = performance is significantly different from control ($p < 0.05$).

500 mg l^{-1}, pH increased by more than 0.5 pH unit. Therefore, 750 mg MOPS l^{-1} was chosen as the buffer concentration for the further experiments, as the lowest concentration with a sufficient buffering capacity while not exerting toxic effects on the test species.

Effects of MOPS on acute copper and zinc toxicity to D. magna

The 48-h EC50s of copper to *D. magna* increased with increasing pH and were, in order of increasing pH (pH 6, 7 and 8) 14.1, 21.6 and 67.3 μg l^{-1} in $NaHCO_3$-buffered media and 14.7, 19.4 and 68.4 μlg l^{-1} in MOPS-buffered media (Fig. 2a). The 48-h EC50s of zinc to *D. magna* slightly increased with increasing pH and were, in order of increasing pH (pH 6, 7 and 8) 1.86, 1.99 and 2.12 mg l^{-1} in $NaHCO_3$-buffered exposure media and 2.18, 2.53 and 2.69 mg l^{-1} in MOPS-buffered media (Fig. 2b).

Table 1. pH changes during ecotoxicity tests with MOPS. Reported values are minimum and maximum recorded pH values

MOPS (g l^{-1})	*D. magna*	*P. subcapitata*
0	7.64–8.38	7.42–8.34
0.5	7.62–8.13	7.43–8.01
0.75	7.63–7.72	7.40–7.50
1	7.65–7.71	7.43–7.49
1.25	7.62–7.74	NP
1.5	7.61–7.70	NP

NP, tests not performed.

At each pH level, and both for copper and zinc, no significant differences could be observed between 48-h EC50s in $NaHCO_3$-buffered and MOPS-buffered media (based on overlapping 95% confidence limits). From Table 2 it is also clear that in MOPS-buffered media, pH remains more constant during the toxicity test than in $NaHCO_3$-buffere media. Indeed in MOPS-buffered media pH-variations during the tests were between 0.03 and 0.08 pH-units, whereas in $NaHCO_3$-buffered media, these variations were between 0.10 and 0.20 pH-units (Table 2).

Effects of MOPS on copper and zinc toxicity to P. subcapitata

The 72-h E_bC50s of copper and to *P. subcapitata* decreased with increasing pH. For copper 72-h E_bC50s were, in order of increasing pH (pH 6, 7 and 8) 49.6, 24.3 and 15.1 μg l^{-1} in HCI-buffered exposure media; 55.8, 22 and 14.7 μ g l^{-1} in MOPS-buffered media and 24.3, 20.5 and 13.7 μ g l^{-1} in $NaHCO_3$-buffered media (Fig. 2c). For zinc, 72-h E_bC50s were, in order of increasing pH (pH 6, 7 and 8) 191, 137 and 71.2 μg l^{-1} in HCI buffered exposure media; 215, 142 and 58.1 μg l^{-1} in MOPS-buffered media and 142, 85.0 and 62.3 μg l^{-1} in $NaHCO_3$-buffered media (Fig. 2d).

For both metals there was no significant difference between 72-h E_bC50s in HCl and MOPS-buffered media (based on overlapping 95% confidence limits). However, for copper tested at pH 6, a significantly lower E_bC50 was observed in $NaHCO_3$-buffered medium compared to that observed in HCl and MOPS-buffered media. Similarly, for zinc a significantly lower E_bC50 was observed in $NaHCO_3$-buffered medium was observed for tests performed at pH 6 and 7. From Table 3 it can be noted that the MOPS-buffering was the most effective buffering method (0.04–0.10 pH units variation), followed by HCl-buffering (0.12–0.37 pH units variation) and then $NaHCO_3$

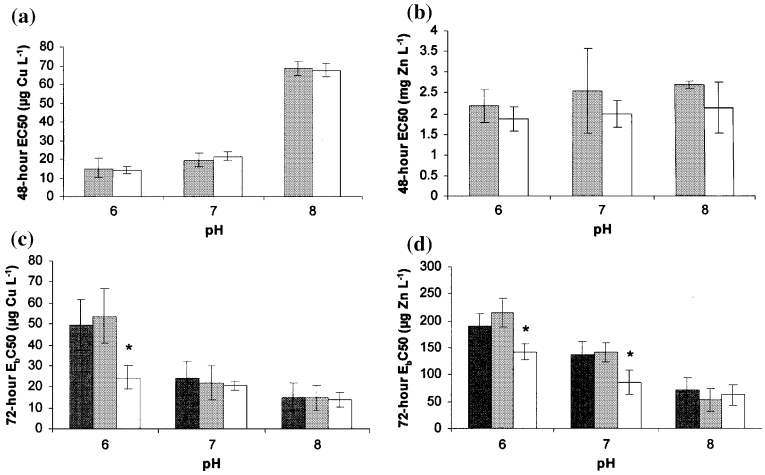

Figure 2. Effect of different pH "buffering" methods (dark grey bars = HCl-buffering, light grey bars = MOPS-buffering and white bars = $NaHCO_3$-buffering) on copper and zinc toxicity to *Daphnia magna* (a and b) and *Pseudokirchneriella subcapitata* (c and d). Error bars represent 95% confidence intervals. * = significantly different from HCl and MOPS buffering (based on non-overlapping 95% confidence intervals).

buffering (0.68–1.92 pH units variation). The lower E_bC50 in $NaHCO_3$-buffered media was accompanied by an increasing pH during the time-course of the experiment. This pH increase was observed to be larger at lower starting pH (i.e. 1.9 pH units at pH 6; 1.2pH units at pH 7; 0.7 pH units at pH 8).

Discussion

The purpose of this study was to examine the suitability of MOPS as a pH buffer for metal toxicity testing with daphnids and algae. Suitability was *a priori* defined by four criteria: non-metal-complexing, good pH buffering capacity in the pH range 6–8, no toxic effect on test species and no effect on metal toxicity.

Kandegedera and Rorabacher (1999) indeed report that MOPS is one of the three absolutely non-metal-complexing Good's buffers. The fulfilment of the second criterion is clearly demonstrated in this study (Tables 1, 2 and 3), as an addition of 750 mg MOPS l^{-1} to the test medium was sufficient to maintain pH within 0.1 pH units, while other buffering methods (HCl and $NaHCO_3$) were always less effective in preventing pH changes during the experiments.

The largest pH increases were observed in algal tests with $NaHCO_3$ as the only pH buffer (Table 1 and 3). These increases were more pronounced in exposure media with a lower initial pH. This observation conforms to the higher pH buffering capacity at higher alkalinities (more $NaHCO_3$ added) (Stumm and Morgan, 1996). This means that effects of not buffering algal exposure media on metal toxicity, will be more pronounced in test media with a lower pH.

The third criterion, and the main focus of this study, covers two aspects of the organisms'

Table 2. pH changes during acute toxicity tests with *D. magna* exposed to copper and zinc. Values refer to minimum and maximum recorded pH values before and after the test in the different replicates

	Copper		Zinc	
pH level	$NaHCO_3$	MOPS	$NaHCO_3$	MOPS
6	6.10–6.30	6.17–6.25	6.08–6.26	6.19–6.23
7	6.95–7.05	6.98–7.03	6.93–7.06	6.97–7.02
8	7.96–8.06	7.99–8.02	7.94–8.08	7.98–8.01

Table 3. pH changes during toxicity experiments with *P. subcapitata* exposed to copper and zinc

	Copper			Zinc		
pH	HCl	MOPS	$NaHCO_3$	HCl	MOPS	$NaHCO_3$
6	5.98–6.35	6.02–6.08	6.00–7.85	5.99–6.31	6.02–6.12	6.02–7.94
7	6.97–7.23	7.02–7.08	7.02–8.19	6.98–7.19	7.01–7.07	7.02–8.25
8	7.99–8.14	7.99–8.03	7.97–8.65	8.01–8.13	8.00–8.05	7.98–8.70

Values refer to minimum and maximum of daily recorded pH values in the different replicates.

sensitivity. First, MOPS should be applied to the test medium in a concentration that does not affect the organisms' performance in chronic toxicity testing. If applied at a higher concentration than the NOEC, MOPS might disturb the organisms physiology, which may result in an altered sensitivity to metals. It was demonstrated that for both species, 750 mg MOPS l^{-1}, did not result in a significant decrease of performance during chronic toxicity testing.

Second, we evaluated if 750 mg MOPS l^{-1} did not affect the metal sensitivity of the two test species, as it was shown that organic chemicals may alter metal toxicity through synergistic or antagonistic action. For example, Lage et al. (1996) have shown that 25 mM of HEPES buffer increased copper toxicity to the alga *Amphidinium cartera,* yet HEPES was not toxic itself. This may indeed be explained by either a toxic synergism of copper and HEPES or, alternatively, by an increased bioavailability of copper in the presence of HEPES.

The results of this study demonstrate that between pH 6 and pH 8, MOPS did not significantly increase or decrease acute copper or zinc toxicity to *D. magna*. The 5-fold increase of 48-h EC50 of copper with increasing pH from 6 to 8, however, demonstrates that in exposure media where pH is expected to change during the experiment (or as a consequence of sampling for natural surface waters), pH buffering should be applied. The 48-h EC50 of zinc only increased with about 20% with increasing pH from 6 to 8. Hence, the pH effect for Zn is not as pronounced as for copper and this was already observed by Heijerick et al. (2002). The observed trend of decreasing metal toxicity to daphnid species with increasing pH has previously been observed by a relatively large number of authors (e.g. Meador, 1991; De Schamphelaere and Janssen, 2002; Heijerick et al., 2002). Although some exceptions to this trend have been noted with respect to other invertebrates (e.g., Shubauer-Berigan et al., 1993), a large number of these (older) studies should be interpreted with care. Indeed, data interpretation and comparison are often complicated by confounding factors such as metal-hydroxide precipitation at higher pH, metal binding to present food particles and subsequent exposure via a dietary route next to the waterborne route (see for example Santore et al., 2002 for an overview of possible difficulties in interpreting older Zn toxicity data).

It should also be noted that the tests in this study were performed in media without organic matter additions. In test media with organic matter present, the effect of pH changes might be more important as metal complexation to organic matter is strongly influenced by pH (more binding sites become deprotonated at higher pH levels). It also needs to be noted that pH has shown to significantly affect chronic zinc toxicity to *D. magna*. (Heijerick et al., 2003). Therefore it is strongly recommended that pH buffering is applied whenever pH changes are expected to affect metal speciation and/or toxicity.

The results of this study also indicate the opposite trend of the effect of pH on metal toxicity to *P. subcapitata* as compared to *D. magna*. The increase of metal toxicity to algae species with increasing pH has also been shown previously (Macfie et al., 1994; Nalejawko et al., 1997; Franklin et al., 2000).

For algae too, however, there seemed not to be an effect of MOPS on both copper and zinc toxicity as compared to HCl-buffered media for any pH level, although in HCl-buffered media pH slightly varied during the experiments. However, when comparing MOPS and HCl-buffered media with $NaHCO_3$-buffered media, it was observed that $NaHCO_3$-buffering in the case of algal

toxicity testing is not appropriate to adequately control pH. In this case, pH increases were large enough to cause significant changes in metal toxicity, especially at the lower starting pH, where pH increases are largest (see above). This clearly demonstrates that in toxicity testing of metals to algae the control of pH is absolutely necessary and that literature data on metal toxicity to algae should be interpreted with caution and, if possible, assessed for pH variation. This also means that the requirements of existing guidelines for algal toxicity testing concerning pH changes (i.e. 1 pH unit is considered acceptable, OECD, 1984a; ISO, 1989) are not sufficient to obtain appropriate experimental data on metal toxicity. In conclusion, it can be stated that pH buffering should always be considered when performing toxicity tests with metals. Moreover, in this study and to our knowledge, MOPS is the first of Good's buffers that is demonstrated not to interfere in any way with copper and zinc toxicity to a daphnid and an algal species. However, whenever using MOPS (or any other buffer) in toxicity testing with other species than *D. magna* or *P. subcapitata,* it is advised to perform preliminary tests to ensure that this is also the case for the species to be tested and under the specific experimental conditions of the test.

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