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Ammonium Toxicity at High pH in a Marine Bioassay Using *Corophium volutator*

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Abstract. Two forms of ammonium exist in water: un-ionized ammonia NH_3 and ionized ammonium NH_4^+. The toxicity to many aquatic organisms is primarily attributed to the NH_3 (un-ionized) species, with the NH_4^+ ion (ionized) species being relatively less toxic. The pH level influences the degree of ionization. It is therefore very important that quality criteria be derived for total ammonium levels at several pH values in order to allow correct interpretation of the sediment bioassay with *Corophium volutator*. The responses of *Corophium* to total ammonium were studied in a series of pH-controlled experiments. The LC50 of total ammonium showed a significant decrease with increasing pH, in both water-only and sediment experiments. The results indicated a combined NH_4^+ and NH_3 toxicity at pH levels less than 8.3. The results can be used to set pH-dependent water quality criteria for total ammonium in overlying water in a 10-day sediment bioassay with *Corophium volutator*.

Two forms of ammonium exist in the aquatic environment: un-ionized ammonia NH_3 and ionized ammonium NH_4^+. The toxicity to many aquatic organisms is primarily attributed to the NH_3 (un-ionized) species, with the NH_4^+ ion (ionized) species being relatively less toxic (Williams *et al.* 1986; Kohn *et al.* 1994; Borgmann 1994). Factors such as pH (Erickson 1985), temperature (Erickson 1985), and oxygen saturation (Wasjbrot *et al.* 1990), which influence the degree of ionization, are therefore of critical importance in determining total ammonium toxicity. However, some authors state that the toxicity of total ammonium at low pH (< 8.2) levels is due not only to un-ionized NH_3, but also to ionized ammonium NH_4^+ (Armstrong *et al.* 1978; Landau and Sanchez 1991; Schubauer-Berigan *et al.* 1995).

The marine amphipod *Corophium volutator* is used as a bioassay species to assess the toxicity of marine sediments and dredged materials in several European countries, including the Netherlands (Stronkhorst *et al.* 2003), the United Kingdom (Bat and Raffaelli 1998), Germany (Peters *et al.* 2002), and Denmark (Pedersen *et al.* 1998). High levels of (total) ammonium in these toxicity tests can potentially confound bioassay results (Moore *et al.* 1997). In these circumstances, the contribution of priority pollutants to toxicity cannot be accurately quantified unless the potential contribution of total ammonium to toxicity is first ruled out (Borgmann and Borgmann 1997). In a *Corophium* bioassay on sediments originating from a harbor or another field location, temperature and salinity are kept constant and the overlying water is saturated with oxygen. The pH level depends on the sediment, however, and is less easy to control in the bioassay. This makes it very important that quality criteria be derived for total ammonium levels at several pH values in sediment bioassays such as the *Corophium* bioassay. These quality criteria should preferably address concentrations in the overlying water, because it is difficult to perform routine measurements in pore water during the bioassay without disturbing both sediment and bioassay.

Armstong *et al.* (1978) have hypothesized that, at pH levels less than 8.3, both NH_3 and NH_4^+ play a role in total ammonium toxicity. The goal of the present research was to determine whether additional toxicity of the ionized form NH_4^+ can be detected at pH values greater than 8, to aid setting of water quality criteria for this confounding factor in *Corophium* sediment bioassays. Ammonium tests mostly take the form of 24-hour to 96-hour water-only tests (*e.g.*, Arthur *et al.* 1987; Kohn *et al.* 1994; Richardson 1997). Sometimes water-only tests using an ammonium concentration range are performed over a longer period: one week (Borgmann and Borgmann 1997), 2 weeks (Williams *et al.* 1986) or even 10 weeks (Borgmann 1994). However, the absence of sediments might easily constitute an extra stress factor, biasing the result, especially for sediment-inhabiting organisms like *Corophium*. Therefore, next to water-only experiments, organisms were exposed in a pH-controlled sediment-water test system.

Materials and Methods

Corophium volutator

The amphipods used in the present study were collected at Oesterput, a relatively unpolluted site located in the Eastern Scheldt (the Netherlands). The animals were collected by sieving (500-μm mesh)

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Table 1. Dates of sediment and water-only tests

	Winter		Summer
pH	Water-only	Sediment	Sediment
8.1		February 2001 March 2001	July 2001
8.3	November 2000	March 2001	September 2001
	February 2001	April 2001	
8.5	November 2000	February 2001	July 2001
	January 2001	September 2001	
8.6	November 2000		
8.7	November 2000	February 2001	August 2001
	February 2001		September 2001
9.0	January 2001		

sediment from the intertidal zone. The remaining fraction, containing the animals, was transported to the laboratory, where the *Corophium* were acclimatized to the standard temperature of 15°C for at least 2 days. They were not fed. Only animals not able to pass through a 500-μm mesh were used in the experiments. All experiments were performed within 10 days of collecting the animals in the field.

pH-Controlling

In all experiments, the pH was controlled using the carbonate buffer system. In this system, carbon dioxide is added to or extracted from the air used for aeration (to raise or lower the pH, respectively). This form of pH manipulation in marine toxicity tests is described by Mount and Mount (1992). The method has been adjusted for the testing of larger systems by using continuous aeration with a (carbon dioxide) manipulated mixture of air and carbon dioxide, using flow meters for regulation. Glass beakers containing a water or sediment–water system were placed in the experimental chamber, in which the desired pH level was reached after 24 hours. In all experiments, pH was controlled twice a day and adjusted if it differed from the desired pH by more than 0.1.

pH-Controlled Water-Only Tests

Toxicity tests with ammonium chloride (NH_4Cl; Acros Organics, Geel, Belgium) dissolved in sea water (32 psu) were performed to evaluate the response in water-only tests. *Corophium* were exposed for 72 hours to a series of concentrations, using three replicates of each concentration. Depending on expected sensitivity, nominal ammonium concentrations varied between 0 mg/l and 100 mg/l at low pH and between 0 mg/l and 45 mg/l at high pH. Ammonium chloride was diluted in seawater from the Eastern Scheldt filtered over a sand-bed filter to remove particles larger than 10 μm. Experiments were performed in 1-litre glass beakers. Twenty randomly selected *Corophium* were added to each beaker at the beginning of the test. Test solutions were aerated continuously during the experiment. The following parameters were measured: salinity and oxygen saturation at the beginning and end of each experiment, and temperature, pH, and total ammonium concentration on a daily basis. Total ammonium was measured using the indophenol blue spectrophotometric method (Dr. Lange GmbH, Düsseldorf, Germany). After 72 hours (70–76 hours), the number of surviving *Corophium* in each beaker was ascertained.

pH-controlled water-only tests were performed during the winter of 2000–2001. Table 1 gives an overview of the experiments performed and their dates. A water-only test without controlled pH was performed four times in winter and four times in summer to indicate any variation in the fitness of the batches of *Corophium* used.

pH-Controlled Sediment Tests

Sediment toxicity tests (10-day) with ammonium chloride (NH_4Cl; Acros Organics, Geel, Belgium) dissolved in sea water (32 psu) as overlying water were performed to evaluate the response in sediment bioassays. The sediment used in the experiments was collected at Oesterput, the location where the *Corophium* were collected. This sediment is known for its low natural ammonium content. Sediments were sieved over 500 μm in the field to remove organisms present and transported to the laboratory. Experiments were performed in 1-litre glass beakers, with three replicates per concentration. Depending on expected sensitivity, nominal ammonium concentrations varied between 0 mg/l and 320 mg/l at low pH and between 0 mg/l and 100 mg/l at high pH. Each beaker was filled with 200 ml sieved sediment, and 600 ml sea water with the desired total ammonium concentration. To obtain the desired concentrations, ammonium chloride was diluted in seawater from the Eastern Scheldt filtered over a sand-bed filter to remove particles larger than 10 μm. After 24 hours, 20 randomly selected *Corophium* were added to each beaker, and exposed to the concentration series for 10 days. The overlying water was aerated continuously during the experiment. The following parameters were measured: salinity and oxygen saturation at the beginning and end of each experiment, and temperature, pH, and total ammonium (for method see Water-Only Tests) on a daily basis. Total ammonium concentrations tend to decrease during the experiment; when measured total ammonium were more than 10% lower compared to the ammonium concentration on day 0, the ammonium concentration was adjusted. This was done by calculating which volume from a stock of 10 g NH_4CL/l was needed to adjust the ammonium concentration in the experimental water volume of 600 ml, and adding this volume. After 10 days, the number of surviving *Corophium* in each beaker was ascertained. pH-controlled sediment tests were performed during the winter of 2000–2001. Because *Corophium* is known for its seasonal variation in response to cadmium (Kater *et al.* 2000), a second series of pH-controlled sediment tests was performed in the summer of 2001. Table 1 gives an overview of the experiments performed and their dates.

Calculations and Statistics

For each water-only and sediment test, the LC50 was computed, after logarithmic transformation of the concentration data, using the trimmed Spearman Karber method (Hamilton *et al.* 1977) and TOXCALC (Tidepool 1995).

Possible differences in responses during the winter and summer were tested using a Kruskal-Wallis test.

Assuming only NH_3 and no NH_4^+ effect at a pH level greater than 8.3, all total ammonium concentrations of test performed at a pH > 8.3 were recalculated to NH_3 concentrations, using the following equation (Whitfield 1974):

$$\% \ NH_3 = 100 * (1 + \text{antilog}\ (\text{pKa}\ (= \textit{dissociation constant}) - \text{pH}) - 1)$$

All single NH_3 concentrations from all experiments and their effect data were combined. One dose–effect relationship was calculated using the trimmed Spearman-Karber method. The LC50 value of this overall dose–effect relationship was calculated. This LC50 value was used to calculate an expected total ammonium toxicity (based on single NH_3 toxicity), and confidence interval, at all pH levels between 8.0 and 9.0. The LC50 based on the individual sediment experiments was compared to this expected total ammonium toxicity.

Results and Discussion

General Performance of the Tests

In all experiments performed survival in control sediments (*i.e.*, sediments without ammonium addition) was more than 90%, even at a pH of 9.0. *Corophium* was found not to be sensitive to high pH levels. In control experiments, total ammonium concentrations were measured on a daily basis. The average total ammonium concentration in 10-day sediment experiments varied between 1.7 and 5.3 mg/l. The highest total ammonium concentration measured in a control was 7.5 mg/l (day 10 at pH = 8.1 sediment experiment). The average temperature in sediment experiments varied between 13.8 and 15.9°C, whereas the average oxygen saturation in sediment experiments varied between 86% and 99%, and average salinity in sediment experiments varied between 28.7 and 33.8 psu. The pH levels in the experiments were kept constant and never varied more than 0.1 unit. Total ammonium concentrations were measured on a daily basis. Total ammonium concentrations tended to decrease during the experiment, but daily adjustment of the concentration kept this variation less than 10%. All calculations were performed with measured total ammonium values.

pH-Dependent Toxicity in Water-Only Experiments

From a total of eight 3-day water-only pH-controlled experiments, it was possible to calculate LC50 values and 95% confidence intervals using the trimmed Spearman-Karber method. Figure 1 gives the results of these pH-controlled water-only experiments. After logarithmic transformation of LC50 values, they showed a significant decrease with increasing pH ($p < 0.001$). Other studies have shown that sensitivity to ammonium is affected by life-stage: the younger or smaller the individuals, the more sensitive the organisms are to ammonium (Young-Lai *et al.* 1991; Lin *et al.* 1993; Moore *et al.* 1997). Noack *et al*. (2003) showed that *Corophium* with different body lengths (4–8 mm) showed no significant differences in sensitivity to ammonium. In this study, possible age effects were reduced by selecting organisms on inability to pass through a 500-μm sieve, and were therefore assumed not to play an important role.

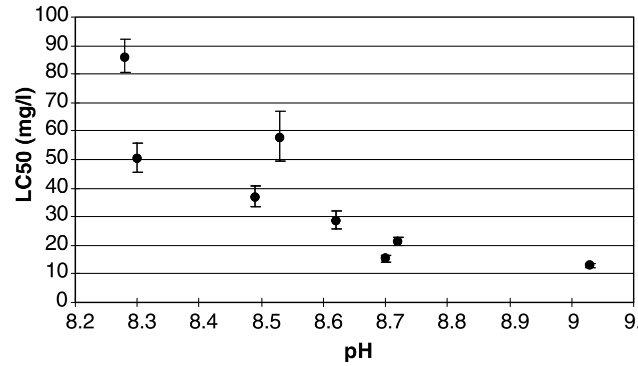

Fig. 1. pH-dependent response of *Corophium volutator* to total ammonium in eight pH-controlled water-only tests

pH-Dependent Toxicity in Sediment Experiments

The LC50 and 95% confidence interval were calculated from a total of six 10-day sediment pH-controlled experiments in the winter period, and six in the summer period, using the trimmed Spearman-Karber method. A Kruskal-Wallis test did not show a significant difference in response between winter and summer ($p = 0.631$; $n = 12$). Figure 2 gives the results of these pH-controlled sediment experiments. After logarithmic transformation of LC50 values, they showed a significant decrease with increasing pH ($p < 0.001$). LC50 values varied, depending on pH, between 30 and 80 mg/l, based on overlying water concentrations. At a pH of around 6.8, which is lower than the lowest pH of 8.1 in this study, the LC50 in a 10-day spiked sediment test was 43.3 mg/l for *Lumbriculus variegatus*, 87.0 mg/l for *Chironomus tentans*, and 9.7 mg/l for *Hyalella azteca* (Whiteman *et al.* 1996). Although the spiking method differs from the methods used in this study, *Corophium* seems less sensitive than the abovementioned species.

Toxicity in Sediment Tests and in Water-Only Tests

When the LC50 of 10-day sediment tests was comparable to 3-day water-only test responses, the quotient between LC50 in sediment and LC50 in water was 1.03 for pH = 8.3 and pH = 8.5. At a pH of 8.7, this quotient increased to 1.69. Parallel 10-day water-only and 10-day sediment tests showed a

Table 2. LC50 (mg NH3/l) of several marine and estuarine crustaceans

Species	Amphipod	48-h	72-h	96-h	Reference
Corophium volutator	Y		3.1		This study
Leptocheirus plumulosus	Y			0.70	Moore *et al*. 1997
Ampelisca abdita	Y			0.83	Kohn *et al*. 1994
Rhepoxynius abronius	Y			1.59	Kohn *et al*. 1994
Eohaustorius estuarius	Y			2.52	Kohn *et al*. 1994
Grandidierella japonica	Y			3.48	Kohn *et al*. 1994
Penaeus japonicus	N	3.5	3.2	3.1	Lin *et al*. 1993
Penaeus semisulcatus	N			1.43	Wasjbrot *et al*. 1990
Homarus americanus	N			5.12	Young-Lai *et al*. 1991

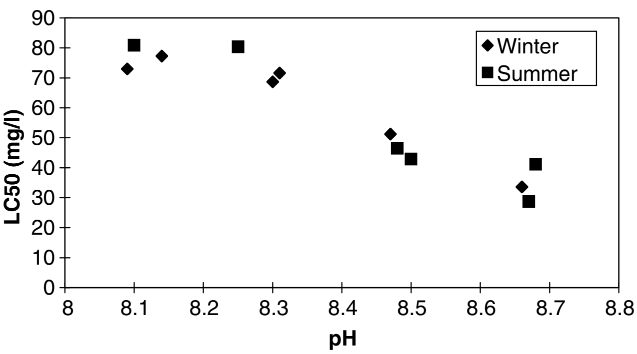

Fig. 2. pH-dependent response of *Corophium volutator* to total ammonium in 12 pH-controlled sediment tests

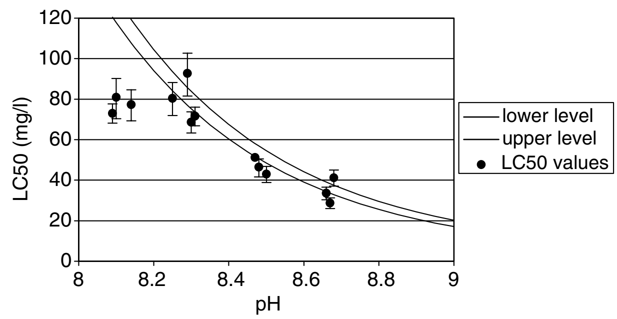

Fig. 3. NH_3-only toxicity of *Corophium volutator*: solid lines give upper and lower level of the 95% confidence interval of the expected LC50 values, dots give the measured LC50 values, including their 95% confidence intervals

higher LC50 for water-only tests compared to sediment tests with the oligochaete *Lumbriculus variegates* (LC50 sediment/ LC50 water = 7.3; Whiteman *et al.* 1996) and the midge *Chironomus tentans* (LC50 sediment/ LC50 water = 6.1; Whiteman *et al.* 1996). On the other hand, however, the amphipod *Hyalella azteca* showed a higher LC50 in the sediment test (LC50 sediment/LC50 water = 0.9; Whiteman *et al.* 1996). It seems that LC50 values in sediment tests are higher on the whole than in water-only tests. In the case of *Corophium*, this can be explained by the stress experienced by a benthic organism in a water-only test.

NH_4^+ Versus NH_3 Toxicity

All sediment experiments with a pH greater than 8.3 were used to estimate an overall LC50 for NH_3. The NH_3 concentration was calculated from each individual total ammonium concentration in sediment experiments with a pH greater than 8.3. The calculated NH_3 concentrations ranged from 0.5 to 9.6 mg/l. A dose–effect relationship was derived from this set of data. The LC50 of NH_3 to *Corophium* thus derived is 3.1 mg/l (3.0–3.2). As shown in Table 2, *Corophium* is less sensitive to NH_3 compared to other marine crustaceans. The LC50 value of 3.1 mg/l was used to calculate expected LC50 values (including confidence interval) for total ammonium at each pH, assuming toxicity is caused only by NH_3. The results, as upper and lower level of the confidence interval, are plotted in Figure 3, together with the LC50 calculated from pH-controlled sediment experiments. The graph shows that all LC50 values from individual sediment tests with a pH of 8.3 or greater fit in the confidence range of the expected toxicity, whereas at lower pH the toxicity is higher than expected, indicating a combined NH_4^+ and NH_3 toxicity at pH levels less than 8.3.

NH_4^+ toxicity at pH levels below 8.4 was also found when the ammonium responses of larvae of *Macrobrachium rosenbergii* were modeled (Armstrong *et al.* 1978). A contribution by the NH_4^+ ion has also been demonstrated for *C. tentans* and *L. variegatus* on the basis of the ratio between responses at the highest and lowest pH. The *C. tentans* test showed a more significant contribution by the NH_4^+ ion to the toxicity of total ammonium compared to *L. variegatus* (Schubauer-Berigan *et al.* 1995). For *Artemia franciscana*, total ammonium seems to be more important than NH_3 in determining toxicity (Landau and Sanchez 1991), thus indicating a form of NH_4^+ toxicity.

Quality Criteria for Sediment Bioassays

Ammonium problems are relevant in sediment bioassays, especially those involving dredged materials. Based on pore water NH_3 concentrations in dredged material, there is a 2% to 18% probability of seeing statistically significant toxicity in 10-day sediment toxicity tests with *Leptocheirus plumulosus* due to NH_3 alone (Moore *et al.* 1997). Well-developed water quality criteria are needed for total ammonium concentrations in sediment bioassays. The results of this study show that water quality criteria for total ammonium concentrations in overlying water in sediment bioassays with *C. volutator* should be pH dependent. At a pH greater than 8.3, this criterion can justifiably be expressed as an NH_3 concentration. At less than this pH, however, criteria should be expressed on a total ammonium basis, because of the combined NH_4^+ and NH_3 toxicity. The data derived in this study can be used to propose pH-dependent water quality criteria for total ammonium in overlying water of a 10-day sediment bioassay with *C. volutator*.

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