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Differentiating Metal from Ammonia Toxicity in Toxicity Identification Evaluations

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Ammonia is a common nutrient and originates from sewage, industrial and farm wastes, fertilisers, and from natural decomposition processes (Sarda and Burton 1995). In water ammonia exists as un-ionised ammonia (NH_3) and as the ammonia ion (NH_4^+). The toxicity of ammonia solutions to freshwater macroinvertebrates is primarily attributed to the NH_3 (the un-ionised species), with the ammonium ion (ionised species) being relatively less toxic (Kendall 1986). High concentrations of ammonia often co-occur with high levels of heavy metals and organic pollutants. This can lead to confounding toxicity test results (Ankley et al. 1990; Ankley and Schubauer-Berigan 1995; Ferretti et al. 2000). Distinguishing ammonia toxicity from toxicity caused by metals or organic pollutants is therefore important in determining appropriate methods for ecological risk assessment. Toxicity Identification Evaluation (TIE) studies have indicated that ammonia is often partly responsible for the toxicity of sediment samples (Ankley et al. 1990; Schubauer-Berigan and Ankley 1991; Burkhard and Jenson 1993). Within standard TIE procedures, the graduated pH test and EDTA-chelation are treatments that have been proposed as methods to differentiate between ammonia and metal toxicity (Norberg-King et al. 1991). EDTA-chelation is used as an indicator of metal toxicity (Norberg-King et al. 1991; Hockett and Mount 1996). However, it is not clear from literature whether EDTA can also influence ammonia toxicity. Most positively charged ions will interact with EDTA to some extent, but especially divalent transition metals, including cadmium, copper, nickel, lead and zinc, have high affinities (Garvin 1964). The toxicity of ammonia is pH dependent because of the pH-dependent equilibrium between ionised and unionised ammonia. However, the toxicity of metals and organic acids is also dependent on the pH (Norberg-King et al. 1991; Schubauer Berigan et al. 1993). Therefore, toxicity changes after pH adjustments can not be interpreted as resulting exclusively from ammonia and changes in toxicity after addition of EDTA can not be exclusively related to metals. As alternatives, the equitoxic solution test and the zeolite test can be used (Mount et al. 1989). The equitoxic solution test is difficult to perform because pH adjustments and pH control within 0.1 pH unit is required. The zeolite test has the following disadvantages: both ammonia and metals are removed from porewater and most important, the material can leach toxic artefacts (Mount et al. 1989).

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Therefore these techniques are not advised for use in TIE phase I. The present paper describes a number of observations from different experiments that are related to the problem of distinguish metal and ammonia toxicity. First the influence of EDTA on ammonia toxicity is evaluated. Moreover, three alternative methods were investigated to distinguish metal toxicity from ammonia toxicity. In the first method water plants were used to remove ammonia, as described previously by Ho et al. (1999). The second method comprised of removing ammonia by air stripping at high pH and subsequent toxicity testing at the initial pH of the sample. This technique is based on the EPA TIE-manipulation to remove volatile toxicants (Norberg-King et al. 1992). The third method is based on the use of an ion-exchange material. This type of material can be used to remove metals and ammonia from aquatic samples (Burgess et al. 1997). The three methods were compared using porewater from contaminated sediment or porewater from reference sediment that had been spiked. Toxicity before and after TIE manipulations was measured using the bioassay with *Daphnia magna.*

MATERIALS AND METHODS

Ammonium chloride, zinc chloride, nitric acid, NaOH and EDTA was supplied by J.T. Baker (Deventer, Netherlands). All chemicals were purchased in analysed grade. Metals were obtained from Merck (Titrisol® standard stock solutions). The ion-exchange material used was Chelex 100, 200-400 mesh, nr. 142-2842 (Bio-Rad®). The POPSO buffer was from Sigma (nr p3405). Ammonium was measured with a spectrophotometric method using a LASA® 20 sensor array photometer and LCK302 cuvets from DRLANGE, Düsseldorf, Germany. Electrical conductivity and pH were measured with WTW, Multiline F/set andTetraCon® 325 or SenTix 41 electrode. Photosynthetically active radiation (PAR) was measured using light Meter Li-cor-250 and Li-cor 190SA Quantum Sensor. *Enteromorpha intestinalis* was obtained at Lake Volkerrak (southern part of the Netherlands) in September 1998. The plants were transported at the same day to the laboratory. The *Enteromorpha intestinalis* plants were placed in an aquarium with lake Volkerrak water and illuminated (Philips HPI-T 400W lamp) with 16 h light ((PAR) approximately 200 $\mu mol\ s^{-1}\ m^{-2}$) and 8 h darkness. Plants with white, brown or yellow "leaves" were removed. Water temperature was approximately 20 °C. *Elodea nuttallii* was purchased from a local aquarium store and kept in an aquarium containing tap water. Illumination and temperature were the same as with *Enteromorpha intestinalis.*

The influence of EDTA on ammonium toxicity was investigated in the following experiment. The toxicity of an ammonium chloride-spiked porewater with and without EDTA was measured. The porewater for this experiment was collected from sediment of the reference location Oostvaardersplassen. The Oostvaarders-plassen are regarded as a clean reference area (Maas *et al.* 1993). Using this porewater, a dilution series of ammonium chloride was prepared ranging from 700 to 50 mg NH_4^+/L. To stabilise the pH of the tested solutions a POPSO buffer was used (concentration 4.5 g/L). Toxicity was determined in a 48h bioassays with *Daphnia magna* (see below).

To investigate if *Elodea nuttallii* has the ability to remove ammonia the following experiment was performed. Porewater was obtained by centrifugation (30 minutes 2500 x g) from river Nieuwe Merwede sediment (collected from 10-40 cm below the sediment surface). Before further use the porewater was filtered through a 0.45 µm membrane filter. To the freshly prepared porewater the plants were added (7.5 g/100 ml) and illuminated for 24 hours. The ammonia concentration and pH were measured before and after the incubation. The ammonia uptake by *Enteromorpha intestinalis* was studied using a solution of ammonium chloride (130 mg/L) in Dutch Standard Water (Maas *et al.* 1993). Metal uptake experiments were done with *Elodea nuttallii* and *Enteromorpha intestinalis.* A solution of metal salts (see Table 1) and 140 mg/L ammonium chloride in Dutch Standard Water (Maas *et al.* 1993) was prepared. The plants were rinsed with this metal solution before the experiment was started. To 1L ammonium solution 225 gram of *Elodea nuttallii* was added (in triplicate). Two controls were prepared without *Elodea nuttallii.* For *Enteromorpha intestinalis* 50 grams of plant material was added to 500 ml of the test solution. Two controls without *Enteromorpha intestinalis* were included. Before and after 24 hours of incubation (see above), the pH and the concentrations of the following metals were measured: cadmium (Cd), copper (Cu), nickel (Ni), zinc (Zn), lead (Pb) and arsenic (As). Metal analyses were done by OMEGAM Amsterdam, the Netherlands, with ICP-MS according to certified protocols. Before metal analysis, samples were preserved in glass bottles by addition of droplets of nitric acid.

Table 1. (Metal) composition of test solution for the incubation of plants

Metal salts	Metal ion concentration (µg/L)
$CdCl_2$	60
$CuSO_4$	36
$NiCl_2$	1400
$PbCl_2$	22.3
$ZnSO_4$	140
As_2O_3	413

For the aeration experiment porewater was obtained by centrifugating sediment (30 minutes 2500 x g) from the reference site Oostvaardersplassen. The ammonium concentration in the porewater was < 10 mg/L. The isolated porewater was spiked by addition of zinc chloride (2.4 mg Zn^{2+}/L) and ammonium chloride (645 mg NH_4^+/L). The pH of 3 liters of spiked porewater was raised to pH 9.0 by addition of NaOH. The TIE guidance document (Norberg-King et al. 1991) advises to use pH 11 for removing volatile components. However, the salinity of the sample increases more when this pH is used. Because the toxicity of metals changes when the salinity is altered aeration at pH 9.0 was used. After the pH adjustment the porewater was divided over three glass jars (1.5 L). Two controls were subjected to the same treatment without addition of zinc and ammonium chloride. The weight of each jar was measured with a maximum error of 0.1 g. All jars were aerated using glass pipets (air-flow approximately: 400 ml/min). Every 24 hours pH and un-ionised ammonia concentrations were measured. Changes in

total weight caused by evaporation were corrected by addition of purified water. If necessary, pH was adjusted to 9.0. After five days, from each jar 0.5 L porewater was transferred to a glass bottle and mixed with a small volume of nitric acid for preservation and analysis of the zinc concentration. In addition, porewater was sampled for testing in the bioassays. The toxicity of the aerated and untreated porewater was determined in a 48h-bioassay with *Daphnia magna* using a method that was described earlier (Den Besten *et al.* 1995) and modified with regard to the following points. Porewater samples from the aeration experiment were tested in a dilution series (diluted with the original porewater from sediment of the Oostvaardersplassen to give concentrations (v/v) of 100%, 56%, 32%, 18%, 10% and 0% spiked porewater). 30 ml of these dilutions was divided over three 15 ml test tubes. Five *Daphnia magna* were transferred to each test tube with a minimum amount (±0.1 ml) of culture water. The daphnids (age <24 hour) were cultured according to Maas *et al.* (1993). All tests were conducted in a temperature-controlled room at 20 °C ±1. After 48 hours survival was determined. Toxicity tests were also performed after addition of 50 mg/L EDTA to the porewater samples. Toxicity values were calculated as the dilution of porewater (% spiked porewater) causing 50% mortality (LC_{50}). Subsequently Toxic Units were calculated as follows: $TU= 100/LC_{50}$. For the statistical evaluation of test results the Toxstat program (version 3.3) was used to run the Tukey method of multiple comparisons.

To investigate the third method, which makes use of the ion-exchange material, porewater that was toxic for *Daphnia magna* and that contained high amounts of ammonia (95 mg/L) was obtained from deeper layers (10-40 cm) of sediment from the river Nieuwe Merwede. This river in part of the lower Rhine delta is polluted with metals and organic pollutants. EDTA-chelation, pH adjustment and C18-column extraction were performed to characterise the toxicity. In addition, ionised ammonia and metals were extracted using a chelex 100 column according to the column method described in the instruction manual. The column method involves preparing a column with the chelex resin and conditioning this column with purified water (no buffer was used). 400 ml of porewater was passed through a column of 20 gram chelex resin. Ammonium chloride was added to the column effluent in order to restore the original ammonium concentration. The hypothesis here was that if the original toxicity had been caused by ammonia, this procedure would restore the toxicity. Changes in the pH during the experiment were measured. Toxicity of the different fractions was measured in the bioassay with *Daphnia magna.* The bioassay was carried out as follows: porewater dilutions were prepared with Dutch Standard Water to give concentrations of 100%, 56%, 32%,18%, 10% and 0%. 150 ml of each dilution was divided over three glass jars (50 ml), after which 10 daphnids were transferred to each jar. Other conditions and the calculation of LC_{50}s and Toxic Units were performed as described above.

RESULTS AND DISCUSSION

The effect of EDTA on ammonia toxicity was evaluated in the first experiment. Since a POPSO buffer was used in order to stabilise the pH, no shift in pH-was

observed after EDTA addition. Addition of 50 mg/L EDTA to a dilution series of ammonium chloride solution showed a small reduction in the toxicity measured with *Daphnia magna* (significant at $p<0.05$). By contrast, 100 mg/L EDTA did not change the toxicity significantly. The results of this experiment are outlined in Table 2.

Table 2. Effect of EDTA on the toxicity of ammonia in the bioassay with *D. magna*

Treatment	Toxicity to *Daphnia magna* (mean 48h-LC_{50} ± SD expressed in mg/L of ionised ammonia)
Porewater spiked with ammonium chloride pH=8.0	84 ±6
Porewater spiked with ammonium chloride and 50 mg/L EDTA pH=8.0	102 ± 1*
Porewater spiked with ammonium chloride and 100 mg/L EDTA pH=8.0	87 ± 5

*=significant difference (p=0.05) with Tukey method of multiple comparisons

The results of this experiment indicate that toxicity changes after the addition of EDTA can not exclusively be related to metals. Based on this information three methods were investigated to confirm metal or ammonia toxicity.

The investigated plant species were able to reduce the concentration of ionised ammonia within 24 hours. During this period the ammonium concentration decreased from approximately 142 mg/L to 19 mg/L for *Elodea nuttallii* and from 130 mg/L to approximately 47 mg/L for *Enteromorpha intestinalis*. In the metal uptake experiment a small decrease of pH (from 7.4 to 7.0) was observed in the presence of *Elodea nuttallii*, whereas in the presence of *Enteromorpha intestinalis* the pH increased from 7.9 to 8.1. No precipitation occurred in the samples during the experiment and the plants remained in good condition (no yellow leaves were noticed). Figure 1 shows the metal concentrations in the metal/ammonia solution after 24h incubation with the two species of water plants. All metal concentrations measured after incubation with plants were considerably lower than in the controls without plants. The decrease in the presence of *Elodea nuttallii* is between 40% (zinc) and 88% (cadmium and copper). The calculated uptake of metals by *Elodea nuttallii* was approximately 17 µg/g plant (total metal uptake per 24 h). *Enteromorpha intestinalis* lowered the metal concentrations between 56% (arsenic) and 89% (zinc). The calculated total uptake of metals per 24h was approximately 19 µg/g *Enteromorpha intestinalis*.

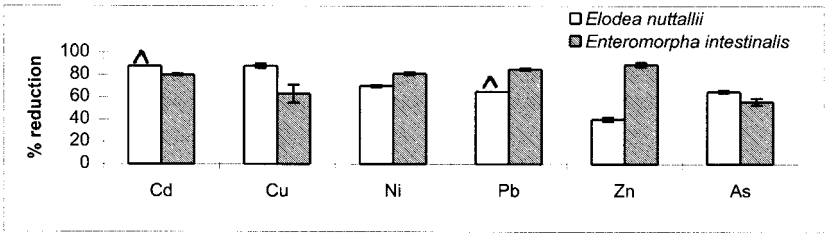

Figure 1. Changes in metal concentrations after incubation with *Elodea nuttallii* or *Enteromorpha intestinalis*. Values are means ± SD (n=3)
^ = minimal decrease (due to metal concentrations below the detection limit).

The results of the aeration experiment are shown in figure 2. In all jars the water remained clear during aeration. Just above the surface zone precipitation occurred on the glass wall. After every 24 hours this precipitation dissolved again when purified water was added in order to correct for evaporation. The ammonia concentration decreased continuously over the period of aeration. The initial concentration of 645 mg/L ammonia had decreased to approximately 90 mg/L ammonia after 6 days of aeration. Zinc analyses showed a small decrease in the metal concentration from 2300 µg/L until 2000 µg/L (Fig. 2). During the aeration period the conductivity of the sample decreased from 4.87 mS/cm to 3.89 mS/cm.

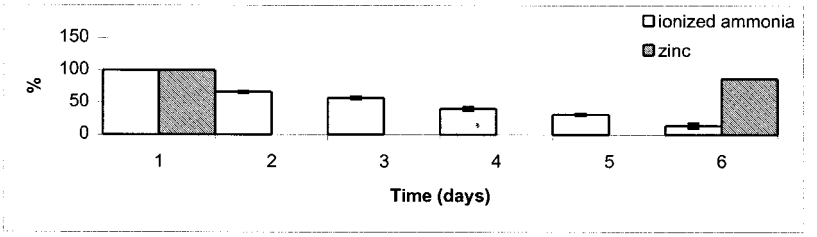

Figure 2. Changes in the concentrations of ionised ammonia and zinc in porewater before and after aeration at pH 9.0 (n=3).

Figure 3 shows the difference in toxicity before and after aeration with or without EDTA addition. The initial spiked porewater was highly toxic for *Daphnia magna*. After addition of EDTA the toxicity of the porewater before aeration was reduced by a factor of about two. After six days of aeration, toxicity of the porewater had decreased by a factor of nearly five. Addition of EDTA addition still resulted in a significant reduction of the toxicity. The porewater used to investigate the third method was toxic to *Daphnia magna.* The LC_{50} after 48 hours of exposure was equal to a dillution of 660 ml/L porewater (TU=1.5). The ammonia concentration was 94.4 mg/L. EDTA chelation resulted in a significant reduction in toxicity for *Daphnia magna.* After C18-extraction and pH manipulation to 5.5 no changes in the toxicity were observed (Figure 4A). The treatment with chelex 100 lowered the ammonia concentration of the pore-water to nearly 0 mg/L. This resulted in a decrease from TU=2.1 (or a LC_{50} of 476 ml porewater/L) to TU=1.1 (a LC_{50} of 880 ml/L; Figure 4B). After addition of ammonium chloride, the concentration of ionised ammonia was 93.4 mg/L.

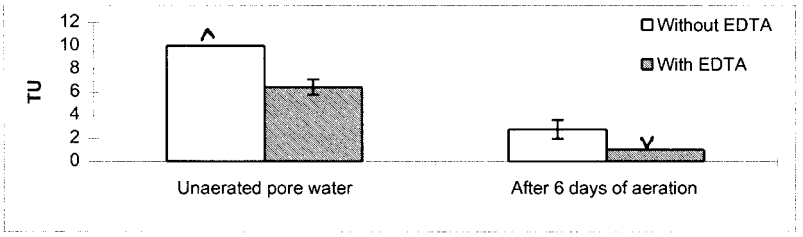

Figure 3. Toxic units, measured in the bioassay with *Daphnia magna*, of porewater before and after aeration at pH 9.0 (error bars represent standard deviation; n=3). ^ indicates toxicity is higher than TU=10, v indicates toxicity is lower than TU=1.

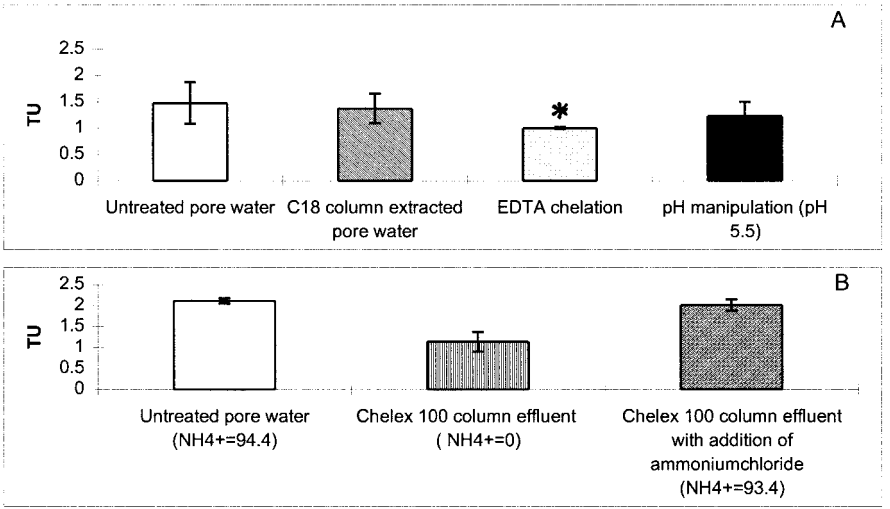

Figure 4. Changes in toxicity for *Daphnia magna* after TIE-phase 1 treatments (A) and chelex 100 treatment with and without addition of ammoniumchloride to the column effluent (B). *= significant difference ($p<0.05$) with Tukey method of multiple comparisons. Error bars represent the standard deviation (n=3).

This resulted in a toxicity of TU=2.0 (LC_{50}-48 hours of 500 ml/L (TU=2). After the column treatment with chelex 100 the pH changed from 8.1 to 8.0. Addition of ammonium chloride lowered the pH to 7.6. No metal analyses could be done to check for the metal concentrations in the treated porewater because not enough porewater was available.

The experiments described in the present paper show that the selected species of water plants have the ability to remove ammonia from the test solution within ranges that are relevant for the toxicity measured in the bioassay with *D. magna*. However, both *Elodea nutallii* and *Enteromorpha intestinalis* were found to accumulate metals to a degree that makes the usefulness of plants in TIE studies questionable. In experiments that were performed to determine metal uptake by *Ulva lactuca* only a small decrease of metal concentrations (10%) was noticed (Ho et al. 1999). In these experiments considerably higher metal concentrations

(100 mg/L Cu, Cd, Pb, Ni and Zn and 100 mg/L total ammonia in seawater) and a shorter incubation period (5 hours) were used than in the present study. While the metal uptake by plants was generally less than 10 %, the estimated metal uptake may have been as high as 600 µg/gram *Ulva* per 5 hours (calculated from data of Ho et al. (1999)). This uptake rate is much higher than those found in the present study *(*per 24h: 17 µg/g *Elodea nuttallii* and 19 µg/g *Enteromorpha intestinalis).* So, although the relative decrease in metal concentrations may have been small at the high metal concentrations used in the former study, there can be quite strong changes at lower metal levels, as shown in the present study. The metal concentrations used in the present study are more realistic with respect to the metal concentrations in porewater and industrial effluents as usually encountered in the Netherlands. Because the acute metal toxicity of most samples is often low, a reduction in this toxicity as a consequence of a treatment will further complicate attempts to distinguish ammonia from metal toxicity. Therefore, although it can not be excluded that shorter incubation periods can be used to minimize metal accumulation in water plants, this procedure does not seem promising.

The aeration experiment showed a reduction in the ammonia concentration used in combination with a slightly lowered zinc concentration. The observed toxicity was in line with these results. After aeration zinc-toxicity was still present, as indicated by the lower toxicity after EDTA addition. Whether EDTA has affected the toxicity caused by ammonia in this experiment is uncertain. These results indicate that the aeration treatment can be used to distinguish between zinc and ammonia toxicity. Further research should be pointed at determination of the applicability of the method used in combination with other metals. Confounding factor in this aeration technique may be the alterations in the porewater matrix caused by the prolonged aeration at pH 9.0 which may change metal toxicity. The decrease in conductivity indicated that some precipitation of ionics has occurred during the aeration period. The TIE-phase 1 manipulations on pore water from Nieuwe Merwede sediment indicate metal toxicity (change in the toxicity after EDTA manipulation). Since the C18-column extraction did not change the toxicity, the contribution of organic toxicants can be neglected. The manipulation to pH 5.5 did not show a significant reduction in the toxicity. However, during the experiment it was not possible to stabilise the pH at 5.5. The results from this treatment are therefore not valid.

Use of the chelex 100 column lowered the toxicity to *Daphnia magna*. It was found that this column can extract ionised ammonia very efficiently. Pilot experiments with chelex 100 showed a variable extraction efficiency of heavy metals from porewater from metal-contaminated sediment (Hg: 23%, As: 33%, Cu: 54%, Pb: 75%, Zn: 77%, Cd: 92%, Ni: 95%, and Cr: 99%). Therefore, the remaining toxicity (TU=1) after the chelex treatment may still have been caused by metals. Furthermore, it was demonstrated that restoration of the ammonia concentration after the chelex 100-extraction can provide more insight in the cause of porewater toxicity. It was observed that restoration of the ammonium chloride concentration resulted in the original toxicity. Theoretically, if the toxicant removal technique does not alter key water quality parameters, the spiked

sample should have the same toxicity as the unaltered sample. The approach followed is particularly attractive in that, as opposed to using laboratory control/dilution water for spiking and toxicity comparisons, data may be derived for suspected toxicants in a matrix that is similar to that of the original sample (Burgess et al. 1997). During the chelex-extraction step only a small decrease in the pH (8.1 to 7.6) occurred. This change can not be responsible for increased ammonia toxicity, because ammonia toxicity will decrease if the pH is lowered. Since a toxicity decrease was also observed after EDTA manipulation and because of the remaining toxicity after the chelex 100 treatment (TU=1.1 compared to TU=2.1 in the original sample) it is indicated that also metals played a role in the effects on *D. magna*. To our knowledge there is no published evidence that addition of EDTA can lower the toxicity caused by ammonia. The results from the first experiment of the present study, however, showed some decrease (about 15%) in ammonia toxicity after the addition of 50 mg/L EDTA. Therefore changes in toxicity after EDTA addition can not be regarded as being caused exclusively by metals. At the same time, the chelex treatment resulting in a column effluent that contained no detectable ammonium (and supposedly had reduced levels of metals) allows the estimation that at least 50% of the original toxicity was caused by metals and most likely less than 50% by ammonia. The role of metals was also concluded in earlier reports based on a comparison of contaminant levels in the sediment with toxicity data in literature: the effects of pore water from Nieuwe Merwede sediment on *D. magna* could be explained by the levels of especially cadmium, nickel and zinc, and to a lesser extend by copper, lead and mercury (Den Besten 1993; Den Besten *et al.* 1995).

It is clear that each of the three methods has its drawbacks en should therefore be used with great care. Our results indicate that the use of plants has no potential within TIE-studies because plants accumulated significant amounts of metal. Reduction in the concentration of ammonia can be achieved by aeration at high pH. However, a confounding factor may be the alterations in the porewater matrix caused by the prolonged aeration at pH 9.0. An important advantage of the cation-exchange material is its application to confirm ammonia toxicity in porewater and to estimate the relative contribution of contaminants in the observed toxicity.

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