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The toxicity of metal mixtures to the estuarine mysid *Neomysis integer* (*Crustacea*: *Mysidacea*) under changing salinity

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Abstract

Water quality criteria are mainly based on data obtained in toxicity tests with single toxicants. Several authors have demonstrated that this approach may be inadequate as the joint action of the chemicals is not taken into account. In this study, the combined effects of six metals on the European estuarine mysid *Neomysis integer* (Leach, 1814) were examined. Acute 96-h toxicity tests were performed with mercury, copper, cadmium, nickel, zinc and lead, and this as single compounds and as a mixture of all six. The concentrations of the individual metals of the equitoxic mixtures were calculated using the concentration–addition model. The 96-h LC50's for the single metals, at a salinity of 5‰, ranged from 6.9 to 1140 μg/l, with the following toxicity ranking: Hg > Cd > Cu > Zn > Ni > Pb. Increasing the salinity from 5 to 25‰ resulted in lower toxicity and lower concentrations of the free ion (as derived from speciation calculations) for all metals. This salinity effect was strongest for cadmium and lead and could be attributed to complexation with chloride ions. The toxicity of nickel, copper and zinc was affected to a smaller extent by salinity. The 96-h LC50 for mercury was the same for both salinities. In order to evaluate the influence of changing salinity conditions on the acute toxicity of metal mixtures, tests were performed at different salinities (5, 10, 15 and 25‰). The 96-h LC50 value (1.49 T.U.) of the metal mixture, at a salinity of 5‰, was clearly lower than the expected value (6 T.U.) based on the non-additive hypothesis, thus confirming the additive effect of these metals in the marine/estuarine environment. Changing salinity had a profound effect on the toxicity of the mixture. The toxicity clearly decreased with increasing salinity until 15‰. Higher salinities (25‰) had no further influence on the 96-h LC50 of the mixture which is situated at a value between 4.4 and 4.6. Finally, the relative sensitivity to the selected metals was compared with the relative sensitivity of the commonly used mysid *Americamysis* (= *Mysidopsis*) *bahia*.

Keywords: Combined effects; Metal toxicity; Mysid; *Neomysis integer*; Salinity; Speciation

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1. Introduction

Although aquatic organisms are usually exposed to a broad spectrum of toxicants in the environment, water quality criteria are generally derived for single compounds only, neglecting possible joint effects as synergism, antagonism and additivity. Attempts to model the joint action of chemicals have already been made for the freshwater environment (Altenburger et al., 1990; Drescher and Boedeker, 1995; Spehar and Fiandt, 1986). The toxicity of a mixture of chemicals with a similar mode of action can be predicted by the concentration–addition model as proposed by Anderson and Weber (1975). Different studies indicate additive effects of metals and organic compounds when occurring in a mixture (Altenburger et al., 1996; Sharma et al., 1999; Warne and Hawker, 1995). For *Daphnia magna* for example, Enserink et al. (1991) found that a mixture of metals present in a concentration well below their individual chronic values can still elucidate chronic effects on growth and reproduction. For the marine environment, however, information on this subject is limited.

This study examines the applicability of the concentration–addition model for assessing metal mixture toxicity in the marine/estuarine environment using the estuarine mysid *Neomysis integer* (Leach, 1814) and evaluates the effects of changing salinity conditions on the acute toxicity of metal mixtures. The choice of *N. integer*, a brackish water species indigenous to the European coastal waters, instead of the commonly used mysid *Americamysis* (= *Mysidopsis*) *bahia* (US-EPA, 1987) allows a more complete and realistic assessment of pollutant effects in European estuarine environments (Emson and Crane, 1994; Lussier et al., 1988; Roast et al., 1999; Wildgust and Jones, 1998). To explore the possible use of *N. integer* as an alternative to *A. bahia*, the sensitivity of these species were compared.

2. Biology, distribution and life history

N. integer, belonging to the genus *Neomysis czerniavski*, is one of the most common mysids inhabiting estuaries along the European coastlines. It is a hyperbenthic, euryhaline and eurythermic species, typically occurring in high numbers in estuarine and brackish water bodies (Tattersall and Tattersall, 1951). *N. integer* also inhabits closed oligohaline and freshwater bodies that were previously connected with the sea (Bremer and Vijverberg, 1982).

N. integer is an omnivorous species. As a predator on zooplankton it can structure zooplankton populations and as a detritivore it can also, to a certain extent, affect the detrital food chain (Mees et al., 1994). Due to its abundance, *N. integer* forms a major component in the marine food web. They are important prey for demersal and pelagic fish and larger epibenthic crustaceans (Mauchline, 1971; Mees et al., 1994; Tattersall and Tattersall, 1951).

Field observations on the hyperbenthic community of different European estuaries indicate that the *N. integer* dominates the community throughout the year with seasonal fluctuations. Peak abundances occur in spring and summer and lower abundances in winter (Mees et al., 1994, 1995).

3. Materials and methods

3.1. Culturing procedures

Initial *N. integer* populations were collected by handnet in the Galgenweel, a brackish water on the left bank of the river Scheldt near Antwerp, Belgium. Organisms were transferred to 60 l plastic shallow holding tanks (1.5 × 0.4 m) with a water depth of 10 cm. Culture medium was filtered (1.2 μm) natural seawater, diluted with aerated tap water to a salinity of 5‰. The laboratory culture was maintained under a 12-h light:12-h dark photoperiod. Holding tanks were aerated continuously and water quality was maintained using a gravel bottom filter and through renewal of 50% of the culture medium every week. Ammonia and ammonium concentrations were checked twice a week. Cultures were fed ad libitum twice daily with 24–48-h old *Artemia* nauplii to prevent adult mysids from cannibalizing their young. A feeding rate of 150 nauplii/mysid/day, as recommended for

N. mercedis by Brandt et al. (1993), was applied. Hatching of the *Artemia* cysts was performed in 1 l cylindro-conical vessels under vigorous aeration and continuous illumination at 25 °C. Culture density was kept at 20 mysids/l. Due to their epibenthic behavior the number of mysids per unit bottom volume may be more limiting than the number of mysids per unit water volume (Brandt et al., 1993).

3.2. Collection of test organisms

Although *N. integer* has been occasionally used as a test organism in toxicity tests (Emson and Crane, 1994; Laughlin and Lindén, 1983; Roast et al., 1999, 2000, 2001; Von Oertzen et al., 1988) so far no attempt has been made to develop culturing procedures and consequently organisms of mixed age (adults) have been used in previous studies. Sensitivity, however, can differ from one life stage to another and the use of juveniles not only increases sensitivity, but also allows standardization. Juveniles were separated from adult females by using a netted brood chamber (Breteler et al., 1982). Depending on the age of the female, brood size ranged from 7 to 17 juveniles. To ensure sufficient test juveniles, we used—as a rule of thumb—one gravid female for each required juvenile. After a 24-h period, newly released neonates were collected. This procedure was followed during a 5-day period until a sufficient number of juveniles were available to perform the toxicity tests.

3.3. Toxicity test procedures

3.3.1. Acute toxicity of six metals

Acute 96-h toxicity tests were carried out with mercury ($HgCl_2$), cadmium ($CdCl_2 \cdot 2.5H_2O$), copper ($CuCl_2$), zinc ($ZnCl_2$), nickel ($NiCl_2$) and lead ($Pb(NO_3)_2$) in order to determine the LC50 of these metals. All metals were of analytical grade and were purchased from UCB (Belgium). Toxicity tests were conducted in similar conditions as those recommended for the acute *A. bahia* toxicity test (US-EPA, 1987) at two different salinities, 5 and 25‰. Ten juveniles between 1- and 5-days old were randomly distributed to 200-ml test vessels containing 180 ml of the toxicant solution. Two replicates for each of the five toxicant concentrations and a control were tested. For each metal two definitive tests were performed. Toxicant stock solutions were prepared in deionized water at 100 times the desired concentration. Actual test concentrations were determined by atomic absorption spectrometry (flame-, graphite furnace-, or cold vapor AAS depending on the metal) and were within 10% of the nominal values. The required serial dilutions were made from the stock solutions with filtered (1.2 μm) natural seawater and adjusted to the desired salinity with deionized water. Test organisms were fed daily at a rate of 150 *Artemia* nauplii/mysid/day. All tests were conducted at 20 ± 1 °C in a temperature controlled room and a 12-h light:12-h dark photoperiod was maintained. Test solutions were renewed daily and the number of surviving mysids was noted. Dead organisms and debris were removed. Acute effects are reported as 96-h LC50 values (expressed as microgram per liter of the metal) and were calculated by the moving average method (Stephan, 1977). Metal speciation calculations were performed using the geochemical speciation model visual MINTEQ (download from http://www.a-mov.ce.kth.se/people/gustafjp/vminteq.htm) on the base of an average seawater composition as derived from Sadiq (1992).

3.3.2. Mixture toxicity experiments

The joint effect of the six metals was evaluated using the concentration–addition model. In this approach the concentration of each single toxicant is expressed as a fraction of its LC50 (toxic unit, T.U.). Based on the 96-h LC50 values of the single metals at a salinity of 5‰, mixtures of these metals were prepared in equitoxic concentrations, i.e. each metal is present in the same fraction of its effective concentration (96-h LC50). The expected toxicity (toxic strength) of the mixtures (based on the non-additive hypothesis) was expressed as toxic units (T.U.), i.e. the sum of the ratios of actual metal concentration to their effective concentrations (LC50):

$$\text{Expected toxicity (T.U.)} = \sum \mathrm{Cm_i, a}/\mathrm{Cm_i, e}$$

where Cm_i,a: actual metal concentration; Cm_i,e: effective metal concentration (96-h LC50).

In case of complete concentration addition the 50% response of a mixture of chemicals is obtained when the sum of T.U. of all constituents equals unity. More than addition gives ΣT.U. < 1 and less than addition ΣT.U. > 1.

Nine equitoxic mixtures of the six metals were tested (0.5, 1, 1.5, 2, 2.5, 3, 5, 7 and 9 T.U.). Note that these sums function as a unit of additive toxicity, except that values greater than 1 are not symmetrical with values less than 1 (Spehar and Fiandt, 1986). For example, in our tests a value of 6 would indicate that there would be no interaction because six toxicants made up the mixture; however, values of 0.5 and 3.0 would be equally more than additive and less than additive, respectively, since they are midway between 0 and 1, and 0 and 6.

3.3.3. Effect of salinity on the acute mixture toxicity

The acute toxicity of the nine equitoxic mixtures was determined at different salinities: 5, 10, 15 and 25‰. Test organisms were taken from the culture (culturing salinity is 5‰) and acclimated to the specific salinity of the test conditions, 2 days prior to the toxicity test. This acclimation period was considered to be sufficient, since a previous study has demonstrated that mysids are extremely efficient osmoregulators, attaining osmotic balance within 2-h of exposure to a change in salinity (Moffat, 1996).

4. Results

4.1. Acute toxicity of six metals

Control survival in all 96-h tests ranged from 90 to 100%. Acute toxicity data for *N. integer* and *A. bahia*, the latter taken from literature data, are summarized in Table 1. At a salinity of 5‰, mercury was the most toxic to *N. integer* juveniles (96-h LC50 = 6.9 μg/l) and lead (96-h LC50 = 1140 μg/l) proved to be the least toxic among the metals tested. Based on these 96-h LC50 values, the following decreasing toxicity order was obtained: Hg > Cd > Cu > Zn > Ni > Pb. Increasing the salinity from 5 to 25‰ slightly alters this sequence. Copper replaces cadmium and the following ranking is obtained: Hg > Cu > Cd > Zn > Ni > Pb. When expressing toxicity on a micromole per liter scale, the order is also slightly altered with copper replacing cadmium at 5‰ and nickel replacing zinc at 25‰.

Table 1
Comparison of the 96-h LC50 values of six metals for *N. integer* and *A. bahia*. Free ion concentrations and activities of the six metals were calculated for two tested salinities (5‰ and 25‰)

Salinity	*N. integer*		*A. bahia*
	5‰	25‰	30‰
Mercury (Hg)	6.9[a]	7.0	3.5[b,c]
	(3.1–10.7)	(3.4–11.1)	(2.7–4.8)
$[Hg^{2+}]$[d]	< 1%	< 1%	
(Hg^{2+})[e]	1.68×10^{-20}	1.00×10^{-22}	
Cadmium (Cd)	45	318	110[b,f]
	(41–49)	(262–416)	(102–118)
$[Cd^{2+}]$	19.7%	3.4%	
(Cd^{2+})	2.87×10^{-8}	2.86×10^{-8}	
Copper (Cu)	46	68	181[b]
	(41–51)	(57–83)	(146–250)
$[Cu^{2+}]$	17.3%	20.5%	
(Cu^{2+})	4.56×10^{-8}	6.46×10^{-8}	
Zinc (Zn)	540	1037	499[b]
	(318–762)	(841–1291)	(350–600)
$[Zn^{2+}]$	74.0%	49.2%	
(Zn^{2+})	2.22×10^{-6}	2.30×10^{-6}	
Nickel (Ni)	765	1042	508[b]
	(435–1095)	(870–1263)	(387–635)
$[Ni^{2+}]$	82.8%	65.5%	
(Ni^{2+})	3.92×10^{-6}	3.43×10^{-6}	
Lead (Pb)	1140	4274	3130[b]
	(840–1440)	(3540–5710)	(2350–∞)
$[Pb^{2+}]$	17.9%	7.3%	
(Pb^{2+})	3.58×10^{-7}	4.43×10^{-7}	

[a] Tabulated 96-h LC50 values are expressed in microgram per liter with 95% confidence limits in parentheses.
[b] Lussier et al. (1985).
[c] Gentile et al. (1983).
[d] % metal as the free ion.
[e] Free metal ion activity.
[f] Gentile et al. (1982).

The coefficient of variation (S.D. × 100/mean) of the performed tests ranged from 2.8 to 27.5%, indicating good repeatability.

4.2. Metal mixture toxicity

The concentration–response curve for the equitoxic mixtures of six metals tested at different salinities is presented in Fig. 1. The nominal concentration of each metal in the different mixtures is presented in Table 2. In absence of additive effects, the 96-h LC50 of the mixture is expected to be found in a mixture where each metal is present in a concentration equal to its individual 96-h LC50. For example, each metal contributes 1 T.U. to the toxic strength of the mixture resulting in an expected toxic strength of 6 T.U. The results, however, show a 50% mortality in a mixture with a toxic strength of 1.49 T.U. where each metal is present at 1/4 of its individual LC50.

As shown in Fig. 1, changing the salinity has a profound effect on the toxicity of the mixture. The toxicity clearly decreases with increasing salinity until 15‰. Higher salinities had no further influence on the 96-h LC50 of the mixture which is situated at a value between 4.4 and 4.6 T.U. (Table 3).

5. Discussion

Correlation analysis revealed a positive and significant correlation ($r^2 = 0.90$; $P = 0.0037$) between the mean 96-h LC50 values, obtained at a salinity of 25‰, for *N. integer* and those of *A. bahia* (at 30‰). The mean 96-h LC50 values (at 25‰) for mercury (7 μg/l), cadmium (318 μg/l), zinc (1037 μg/l), nickel (1042 μg/l) and lead (4274 μg/l) for *N. integer* are higher than the reported literature values for *A. bahia* as shown in Table 1. The mean 96-h LC50 value for copper (68 μg/l) is markedly lower than the value obtained by Lussier et al. (1985) for the marine mysid *A. bahia* (181 μg/l). The 96-h LC50 obtained for cadmium (318 μg/l) is higher than the value (151 μg/l) reported by Roast et al. (2001) at a salinity of 30‰. Emson and Crane (1994) found an extremely high sensitivity for *N. integer* to cadmium and found a 96-h LC50 between 1 and 3 μg/l. These values do not corroborate our findings.

Decreasing the salinity to 5‰ generally resulted in lower 96-h LC50 values of the tested metals for *N. integer*. This salinity effect becomes clearer, when the results of the combined toxicity experiments are considered. At a salinity of 5‰, 50% mortality was observed in a mixture with a toxic strength of 1.49 T.U., i.e. each metal is present at 1/4 of its individual LC50, suggesting a nearly strictly additive joint action. This result is compar-

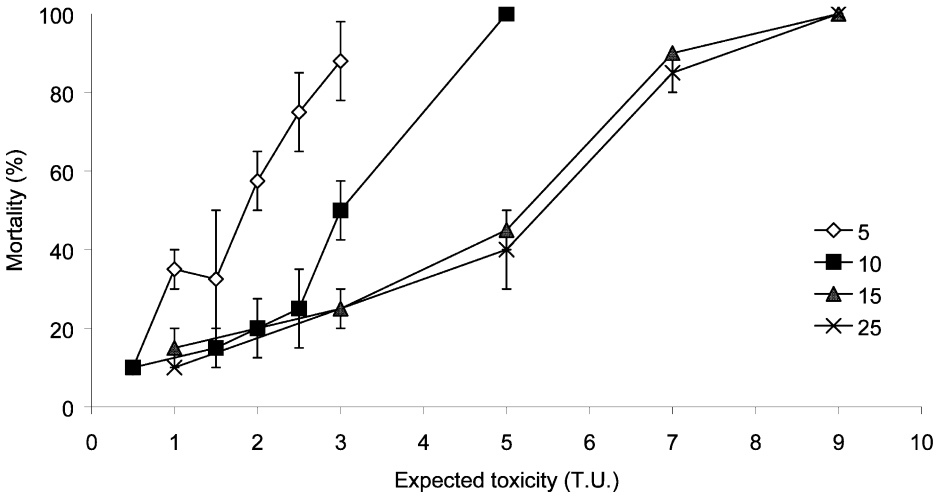

Fig. 1. The concentration–response curve of *N. integer* for a mixture of six metals at different salinities (5, 10, 15 and 25‰).

Table 2
Nominal concentrations (μg/l) of the six metals in an acute toxicity test with *N. integer*. Test organisms were exposed to dilutions of equitoxic mixtures

	Toxic unit (T.U.)								
	0.5	1	1.5	2	2.5	3	5	7	9
Hg	0.6	1.1	1.7	2.3	3.0	3.5	5.8	8.1	10.4
Cd	3.8	7.5	11.3	15	18.8	22.5	37.5	52.5	67.5
Cu	3.8	7.6	11.5	15.3	19.2	23	38.3	53.7	69
Zn	45	90	135	180	225	270	450	630	810
Ni	64	128	191	225	319	383	638	893	1148
Pb	95	190	285	380	475	570	950	1330	1710

Table 3
Toxicity (96-h LC50) for an equitoxic mixture of six metals to *N. integer* at four different salinities

Salinity	96-h LC50
5‰	1.49[a] (1.19–1.81)
10‰	3.07 (2.64–4.20)
15‰	4.61 (3.87–5.41)
20‰	4.37 (3.51–5.29)

[a] Tabulated 96-h LC50 values are expressed in toxic units with 95% confidence limits in parentheses.

able to the value found by Enserink et al. (1991) of 1.8 (0.89–3.1) T.U. at which a 50% mortality was observed in *D. magna* juveniles exposed to a mixture of eight metals. Similarly, Spehar and Fiandt found near complete addition (1.47 T.U.) in *Ceriodaphnia dubia* exposed to a mixture of six metals. Increasing the salinity in this study shifted the 96-h LC50 of the mixture from 1.49 T.U. to a value which is situated between 4.4 and 4.6 T.U.

The individual contribution of each metal to the toxic strength of the mixture at a salinity of 25‰ can be calculated by dividing the actual metal concentrations (i.e. the individual metal concentrations in the 4.37 T.U. mixture) with the 96-h LC50 values obtained for each individual metal at the same salinity (Table 4). Keeping in mind that we initially departed from an equitoxic mixture at a salinity of 5‰, it can be clearly shown that at a salinity of 25‰ the contribution of each metal is different (i.e. deviates from the expected contribution of 1 T.U.), indicating specific salinity effects for the investigated metals. Cadmium and lead which contribute 0.11 and 0.19 T.U. to the overall mixture toxicity are the most influenced by increasing salinity. The toxicity of mercury on the other hand shows little change (0.72 T.U.). Zinc, nickel and copper contribute in equal fractions to the toxicity of the mixture.

One possible mechanism that can explain the observed phenomenon is complexation of the metals with chloride ions (Wright, 1995). The most commonly accepted explanation for the increased toxicity of metals at low salinity is that the free metal ion—the most bioavailable form—is most abundant at low salinity because of reduced formation of chloro-complexes. Cadmium and lead speciation (in cases where total dissolved Pb remains the same) and bioaccumulation are primarily influenced by inorganic ligands as Cl^-. Both inorganic- and organic ligand concentrations determine the speciation of copper (Sadiq, 1992).

For cadmium, salinity is the overriding factor which can alter free Cd ion activity and hence bioavailability and toxicity of cadmium in marine systems (Sadiq, 1992; Sunda et al., 1978). Blust et al. (1992) investigated the effect of salinity on cadmium uptake with the brine shrimp *Artemia fransiscana* and found a decrease in the cadmium uptake with increasing salinity. De Lisle and Roberts (1988, 1994) and Roast et al. (2001) also earmarked Cd^{2+} as the primary toxic species for *A. bahia* and *N. integer*. When cadmium speciation in our study was modeled, free ion percen-

Table 4
Calculated contribution (A/B) of the six tested metals, based on the concentration in the mixture (A) and the individual 96-h LC50 (B), to the toxicity of the mixture to *N. integer* at a salinity of 25‰

Metal	Concentration in mixture (μg/l) (A)[a]	Individual 96-h $LC50_{(25‰)}$ (μg/l) (B)	Contribution (T.U.) (A/B)
Hg	5.1	7.0	0.72
Cd	32	318	0.11
Cu	34	68	0.49
Zn	393	1037	0.38
Ni	557	1042	0.53
Pb	830	4276	0.19

[a] Actual concentrations of the individual metals in a 4.37 T.U. mixture which caused 50% mortality at a salinity of 25‰.

tages (Cd^{2+}) were calculated to be 19.7 and 3.4, at 5 and 25‰, respectively (Table 1). These values are in agreement with earlier published speciation estimates (De Lisle and Roberts, 1994; Roast et al., 2001) and demonstrate that the toxicity difference observed at the two salinities are caused by differences in the free Cd ion.

The reduced contribution of lead to the toxicity of the mixture is probably due to precipitation. At the normal pH of seawater, most of the total amount of lead is precipitated as lead (II) chloride and about 10% is an active ion form (Byrne and Miller, 1984). Free ion percentages in our study were 17.9% (at 5‰) and 7.3% (at 25‰) for Pb^{2+}, which is in accordance with the observed lower toxicity at the highest salinity.

The 96-h LC50 of mercury for *N. integer* is the same for both tested salinities (5 and 25‰), respectively 6.9 and 7.0 μg/l. Speciation calculations for Hg resulted in similar very low free ion percentages ($< 1\%$ Hg^{2+}) at both salinities. Consequently, it can be concluded that salinity has a minor effect on the speciation and toxicity of mercury species to *N. integer*.

The toxicity of nickel, copper and zinc was affected to a smaller extent by salinity. According to Kushner (1993) nickel ions are not complexed by Cl^-, even in seawater and the concentrations of Cl^- that occur in seawater do not affect the toxicity of this metal. This is in contradiction with Nriagu (1980) who stated that considerable portions of the nickel ions were reported to exist in seawater as complexes with chloride and sulphate ions, leading to the depletion of free nickel ions. Our results support the latter statement if the free nickel ion can be considered as the most toxic form. With regard to copper one has to be cautious. Copper probably forms stronger complexes with organic ligands than any other trace metal at comparable concentrations. The distribution of Cu between ionic and complexed forms (both organic and inorganic) in seawater is complicated and many investigators have speculated on inorganic Cu speciation (Sadiq, 1992). It may, however, be generalized that Cu–Cl complexes will increase as the salinity increases and this will decrease bioaccumulation and toxicity. Cupric (Cu^{2+}) is the most biotoxic form and varied little in our study according to the speciation calculations; 17.3% (at 5‰) and 20.5% (at 25‰). The observed slight reduction in copper toxicity at higher salinities is therefore difficult to link to inorganic complexation behavior.

Finally, the lower zinc toxicity at higher salinities correlated well with the observed decrease in the free Zn ion; 74.0% (at 5‰) and 49.2 (at 25‰).

Since our speciation calculations did not account for the presence of organic carbon in the test medium, we were not able to account for sorption of the tested metals to these organic colloids. It has been demonstrated that these mechanisms affect metal speciation, primarily by reducing the dissolved concentrations, and ultimately influencing the bioavailability and toxicity of metals (Cantwell and Burgess, 2001). Unfortunately, data on modelling these mechanisms in seawater are very scarce and future research should focus on the quantitative understanding of these processes, which should allow a more fundamental understanding of the influence of salinity and organic content on

the speciation and toxicity of individual metals and metal mixtures in marine environments.

6. Conclusions

In this study, the combined effects of six metals on *N. integer* were examined at different salinities. At a salinity of 5‰, 50% mortality was observed in a mixture with a toxic strength of 1.49 T.U. suggesting a nearly strictly additive joint action. Higher salinities resulted in lower toxicity. This salinity effect could be partly attributed to complexation of the metals with chloride ions, i.e. lower free metal ion concentrations at a salinity of 25‰ resulted in lower toxicity. This effect was stronger for Cd and Pb than for Zn, Cu and Ni. Pb toxicity was unaffected by salinity.

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