

## Effects of copper and other metals on fertilization, embryo development, larval survival and settlement of the polychaete *Nereis (Neanthes) virens*

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### Summary

Nectochaete larvae of the ecologically and economically important ragworm, *Nereis virens*, were exposed to cadmium, chromium, copper, lead and zinc dissolved in seawater to nominal concentrations ranging from 0 to 5000  $\mu\text{g l}^{-1}$ . Copper was the most toxic (mean  $\text{LC}_{50}$  of 76.5  $\mu\text{g l}^{-1} \pm 95\%$  CI 73.8–79.2 after 96 h exposure) and so was used for subsequent experiments. Exposure of gametes to greater than 500  $\mu\text{g l}^{-1}$  copper for 2 or 4 h at 10°C prior to fertilization, or a 10 min exposure during fertilization, significantly reduced embryo developmental success. The effect of copper on larval settlement was also assessed using sediment spiked to a range of concentrations (0, 50, 250, 500, 1000  $\text{mg kg}^{-1}$  dry weight). Significantly fewer larvae were found in sediment of  $\geq 250 \text{ mg kg}^{-1}$  in comparison to the control or the 50  $\text{mg kg}^{-1}$  treatment. Assessment of living larvae also confirmed a significant reduction in settlement, but in all treatments compared to the control, although the number of dead larvae also increased as the concentrations increased. These effects may have important implications for reproductive success and recruitment of *N. virens* to polluted sediments.

**Key words:** *Nereis virens*, polychaete, rag worm, copper, fertilization, zinc, lead, cadmium, metal

### Introduction

For several decades polychaetes have been used extensively as bioindicators of marine pollution and as ecotoxicological test species to assess the effects of contaminants (for review see Reish and Gerlinger, 1997). Metals vary considerably in their toxicities as do

the responses of different species. Reish and Gerlinger (1997) indicated that copper was the most toxic to polychaetes, however, other metals, including lead, cadmium, zinc and chromium can be found in relatively high concentrations in coastal regions (Bryan and Langston, 1992). All have been shown to have

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significant impacts on a variety of life stages in polychaetes, but the sensitivity of stages and species can differ markedly (e.g. Conner, 1972; Eisler and Hennekey, 1977; Oshida et al., 1981; Reish and Gerlinger, 1984; Mauri et al., 2003; Mendez and Green-Ruiz, 2006; Gopalakrishnan et al., 2007). It is, therefore, important to assess the relative toxicities of a variety of metals on one species and one life-stage.

Reish and Gerlinger's (1997) review counted 48 species and 20 families of polychaetes that have been used to assess metal toxicity and this list continues to grow. In Europe, *Nereis (Hediste) diversicolor* has been one of the most comprehensively studied (e.g. recent articles include: Rainbow et al., 2006; Burlinson and Lawrence, 2007). However, in fully saline areas this species is replaced by the larger *Nereis virens* (Kristensen, 1984) and is one of the dominant species in the harbours of the Solent area on the south coast of England. *N. virens* itself is an important prey item (McIntosh 1908–1910), but also feeds on a wide range of other invertebrates (Kay, 1972). It is also collected for bait for sea angling and is grown commercially (Olive, 1994; Watson et al., 2007). Despite the importance of understanding the response of all life-stages of this species to pollution, studies on the impacts of metals have been limited to the adults (Raymont and Shields, 1963).

Studies investigating the direct exposure of gametes to metals on fertilization and developmental success (Warnu et al., 1996; Reichelt-Brushett and Harrison, 1999; Au et al., 2000; Reichelt-Brushett and Michalek-Wagner, 2005) have all used broadcast spawning species that release gametes into a large volume of water. Bass and Brafield (1972) and Desrosiers et al. (1994) suggested that *N. virens* is also a typical broadcast spawner. However, Watson et al. (2003) have shown that both sexes release gametes in to shallow (a few centimetres deep) pools in inter-tidal areas at low tide. Intertidal mud flats are often exposed to human perturbations such as digging of the sediment for bait collection. Changes in conditions in the sediment can result in the desorption from sediment of loosely bound metals and these could be released into the overlying water (Furness and Rainbow, 1990). This may mean that the spawned gametes may be exposed to elevated levels prior to and during fertilization until tidal inundation. The consequent impact on fertilization and subsequent embryo development of metals may, therefore, be significant.

The effects on early larval stages have also been extensively studied in polychaetes (see Reish and Gerlinger, 1997), but again data are lacking for *N. virens*. Nectochaete larvae of this species spend a

number of days in the water column before settling on the sediment and so may be exposed directly to metals dissolved in the water (Bass and Brafield, 1972). In addition, high levels of metals within the sediment may have an adverse effect on larval settlement choice. This would change the interaction between competent larvae and substratum which could lead to the rejection of a potential settlement site and may have important implications for post-larval settlement mortality (Qian, 1999; Ng and Keough, 2003; Bellas et al., 2004).

To assess the toxicity of a suite of metals, we exposed nectochaete larvae to a range of concentrations of copper, zinc, lead, chromium and cadmium in 96-hour static mortality tests. From these results we focused on copper to assess the effect of pre-fertilization exposure of gametes on fertilization and early embryo developmental success. We also briefly (10 min) exposed gametes to copper during fertilization and the early embryo stages to assess the direct effects on these processes. Finally, to assess the impact of copper on settlement, nectochaete larvae were allowed to settle on sediment spiked to a range of nominal concentrations before their settlement position was assessed.

## Materials and Methods

### Collection and maintenance of specimens

Mature *N. virens* were collected at low water during spring tides from Cobnor Point, Chichester Harbour, on the southeast coast of England (50°48'94"N, 36° W). Worms were collected by turning over the sediment with a fork and were placed in clean buckets and transported back to the laboratory on the same day. Damaged worms were discarded and the remaining worms were placed into flow-through tanks kept under ambient conditions. Gametes were removed from these individuals using a 25 g needles, and hypodermic syringe at the time of natural spawning (early March) and when individuals were also spawning spontaneously within the laboratory. For all LC<sub>50</sub> tests, batches of larvae were purchased from Seabait (Ashington, UK) and posted to the laboratory as trochophores or nectochaetes. For the settlement tests larvae were either purchased from Seabait or were produced from *in vitro* fertilizations within the laboratory from the locally collected adults at the time of natural spawning. Oocytes were pooled from a number of individuals prior to washing in twice filtered seawater, and sperm was collected and kept on ice until required. Fertilizations were performed in glass crystallising dishes, with a total volume of 20 ml using the optimum conditions (sperm concentration of  $2.5 \times 10^6$

sperm  $\text{ml}^{-1}$ , 10 min contact time and sperm/egg ratio of 1000:1 as determined by Williams and Bentley (2002). Dishes were loosely covered with pierced Parafilm™ and then placed at 10°C for 24 h before larvae were transferred to 500 ml conical flasks and aerated at 10°C under a photoperiod of LD 12:12.

### ***Metal solutions***

All experiments were performed with analytical grade salts:  $\text{K}_2\text{Cr}_2\text{O}_7$ ,  $\text{CdCl}_2$ ,  $\text{ZnCl}_2$ ,  $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$  and  $\text{PbCl}_2$  purchased from Sigma and Fisher Scientific and dissolved directly in pasteurized Langstone harbour seawater and then serially diluted to obtain the nominal concentrations. All experiments included a control of pasteurized seawater only. All concentrations are reported as  $\mu\text{g l}^{-1}$  except the spiked sediment which is in  $\text{mg kg}^{-1}$  of dry sediment. Glassware and equipment were acid washed before use and Petri dishes, repli dishes and Eppendorf tubes were allowed to equilibrate with the respective metal solutions overnight prior to all experiments. In each experiment the term ‘nominal concentration’ refers to the dose concentration of the metal ion and not the actual concentration available to the larvae or gametes. However, analysis of the spiked sediment was performed and is detailed below.

### ***LC<sub>50</sub> tests for nectochaete larvae***

Experiments to determine the  $\text{LC}_{50}$ s of all five metals on nectochaetes were performed in June 2001 (nominal concentrations of 0.1, 1, 10, 100, 1000  $\mu\text{g l}^{-1}$ ), April 2002 (0.1, 1, 10, 50, 100, 250, 1000, 5000  $\mu\text{g l}^{-1}$ ) and with copper only in May 2002 (0.1, 1, 10, 25, 50, 100, 200, 250, 500, 1000, 5000  $\mu\text{g l}^{-1}$ ). In each experiment, one batch of three or four segment stage larvae was used. For the experiment in June 2001, 20 larvae were added to individual glass Petri dishes and 10 ml of the respective metal solution added (three replicate dishes per concentration per metal). In April and May 2002, six-compartment plastic repli-dishes were used (six replicate wells per concentration per metal). In each well 10 larvae and 10 ml of the respective metal solution were added. All dishes were incubated at 10°C (LD12:12) for a total of 96 h. The numbers of dead larvae were recorded using a dissecting microscope at 24 and 96 h and larvae were classed as dead when no movement was observed after gentle probing with a glass rod and all dead larvae were removed.

### ***Pre-fertilization incubation of oocytes in copper***

Oocytes were extracted from two females using a

25-gauge needle and 1 ml disposable syringe and placed in Eppendorf tubes. Eggs were initially washed with pasteurized seawater (at 10°C) to remove coelomic fluid before oocytes were counted and pooled. 2500 eggs were incubated in 1 ml of the copper solutions (0.01, 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000  $\mu\text{g l}^{-1}$  plus a control) in plastic Eppendorf tubes for 2 and 4 h at 10°C. After incubation eggs were washed three times with clean pasteurized seawater. Oocytes were then transferred into Petri dishes and pooled sperm from three males added to give a final concentration of  $2.5 \times 10^6$  sperm  $\text{ml}^{-1}$ , 10 min contact time and sperm/egg ratio of 1000:1 (Williams and Bentley, 2002). To verify that no spontaneous fertilization or inadvertent fertilization of oocytes was occurring prior to exposure, a no-sperm control was also included at this stage. After 10 min, 7 ml of the overlying solution was replaced three times with 7 ml of pasteurized seawater to reduce the chances of polyspermy. Petri dishes were then covered and maintained at 10°C (LD12:12) for 24 h. The contents of each dish were then fixed with 4% formalin solution in seawater and developmental success was assessed in 50 oocytes as described by Lewis et al. (2003).

### ***Pre-fertilization incubation of sperm in copper***

Concentrated ‘dry’ sperm was extracted from three males using a 25-gauge needle, counted and pooled. In this state the sperm can be carefully pipetted, retaining its integrity and does not become diluted and, therefore, activated. Enough dry sperm for a final concentration of  $2.5 \times 10^6$   $\text{ml}^{-1}$  was incubated in 1 ml of the copper solutions (0.01, 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000  $\mu\text{g l}^{-1}$  plus control) in Eppendorf tubes for 2 and 4 h. After incubation, the sperm was carefully washed three times with pasteurized seawater and then transferred into glass Petri dishes containing 2500 per dish of pooled oocytes (from two females); a no-sperm control was also included at this stage. These were stirred, and after 10 min contact time, oocytes were rinsed, incubated and then fixed as described above.

### ***Incubation of oocytes and sperm in copper at fertilization***

A concentration of  $2.5 \times 10^6$   $\text{ml}^{-1}$  of sperm and 2500 eggs was incubated in 1 ml of the respective copper solution (0.01, 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000  $\mu\text{g l}^{-1}$  plus a pasteurized seawater and a no-sperm control) in pre-equilibrated glass Petri dishes for 10 min and then washed as described above. Petri dishes were then covered and placed on a shaker for 24 h at 10°C and then fixed as described above.

### Effects of copper-spiked sediment on larval settlement

Sediment was collected next to the animal collection site at low water and placed into clean high-density polyethylene (HDP) boxes for transport back to the laboratory and frozen at  $-20^{\circ}\text{C}$  until ready to be spiked. Sediment was defrosted for 48 h before being spiked with cupric nitrate ( $\text{Cu}(\text{NO}_3)_2 \cdot n\text{H}_2\text{O}$ ) solutions to give nominal concentrations of 50, 250, 500 and 1000  $\text{mg kg}^{-1}$  (dry weight) and non-spiked sediment was also included as a control (nominal concentration of 0  $\text{mg kg}^{-1}$ ). Percentage water content (approximately 49%) of the sediment was taken into account when spiking and appropriate amounts of the cupric nitrate were dissolved in 10 ml of distilled water and then diluted to 100 ml with seawater before being added to 400 g of sediment and then gently mixed with a spatula and then a mechanical mixer. Once mixed, the solution was left in the fridge to settle for 30 min before being frozen at  $-20^{\circ}\text{C}$ .

Sediment to be used for larval settlement was defrosted and 1 g of the appropriate sediment was placed in a compartment (2 cm $\times$ 2 cm) of a 25 compartment repli-dish (10 cm $\times$ 10 cm). Dishes were left at  $4^{\circ}\text{C}$  to allow the sediment to settle overnight and then any surface water was removed. Filtered seawater was then added carefully to each compartment and once all compartments were full, a piece of filter paper was used to remove sediment floating on the water's surface.

Each repli dish was then placed in a perspex settlement chamber (12 $\times$ 12 $\times$ 6 cm) and this was filled with approximately 500 ml of seawater. Two hundred and fifty actively swimming nectochaete larvae (three or four segments/6–10 days old) were added to each chamber and then each chamber was maintained at  $10^{\circ}\text{C}$  (LD12:12). Chambers were gently aerated for 1 h each day and experiments were terminated when no larvae could be seen swimming in the water column (between 4 and 7 days). Once terminated, the surface water and water from each compartment was removed and passed through the 100  $\mu\text{m}$  sieve to count any larvae that had not settled. The remaining contents of each compartment were subsequently fixed by adding 2 ml of 10% formalin in seawater. To assess the numbers of settled larvae the fixed sediment were passed through the sieve, the sediment and larvae retained on the sieve stained with a small amount of Rose Bengal before counting.

For the visualization of living larvae, water was removed from all compartments as described above and 10  $\mu\text{l}$  of 0.01% Neutral red dye was added to each compartment and left for 1 h to ensure that larvae were stained. Lewis et al. (2003) found this protocol to have no impact on survivorship. Sediment was passed through the 100  $\mu\text{m}$  sieve and the settled larvae were recorded as dead or alive as described above.

Table 1. Details of the site used for the collection of sediment for the settlement experiments including actual measured concentrations (see Materials and Methods for description) of the copper spiked sediment (percentages in brackets are the differences between actual and nominal). Cobnor Point Site 1 and 2 are located approximately 300 m away from the sediment collection site, but closer to the main channel. These sites were analysed for a suite of contaminants by the Environment Agency in 2002 (data supplied by S. Rees-Jones, Environment Agency). These EA data are single samples using the  $<63 \mu\text{m}$  fraction of sediment. All concentrations are expressed as  $\text{mg kg}^{-1}$  dry weight of sediment

	Cobnor Point (sediment collection site)	Cobnor Point Site 1 (EA data)	Cobnor Point Site 2 (EA data)
OS grid ref	SU 794 024	SU 796 019	SU 795 016
Latitude and longitude	50° 48' 57.73" N 0° 52' 22.02" W	50° 48' 41.13" N 0° 52' 10.16" W	50° 48' 31.61" N 0° 52' 16.52" W
Percent organic content, %	5.12 $\pm$ 0.136	1.43	1.35
Size particle analysis (% $<63 \mu\text{m}$ )	85 $\pm$ 1.01	73.4	59.79
Cu (background) nominal 0 $\text{mg kg}^{-1}$	15.6	41.9	78.6
Cu, nominal 50 $\text{mg kg}^{-1}$	56.6 (113%)	—	—
Cu, nominal 250 $\text{mg kg}^{-1}$	240 (96%)	—	—
Cu, nominal 500 $\text{mg kg}^{-1}$	484 (97%)	—	—
Cu nominal 1000 $\text{mg kg}^{-1}$	907 (91%)	—	—
As	—	17.3	13.4
Cd	—	0.266	0.515
Cr	—	81.9	129
Pb	—	18.7	26.3
Hg	—	0.039	0.190
Ni	—	25.4	22.8
Zn	—	78.9	88.1

### Metal analysis

Analysis of copper in the sediment for the settlement experiments was performed on sediment collected directly from the sampling site that had undergone the same protocol of spiking, but had not been used in the settlement experiments. Frozen spiked sediment was sent to the National Laboratory Service and analysed using Inductively coupled plasma/optical emission spectrometry (ICP/OES). Briefly, the extraction was performed on a dried (30°C) sample, sieved to 2 mm and extracted with *aqua regia*. Samples were left for a minimum of 8 h then placed in a heating block at 120°C for 150 min before analysis using the ICP/OES. This method and the laboratory is UKAS accredited to ISO/IEC 17025. The nominal and measured concentrations for the spiked sediment are presented in Table 1 and show that actual values range between 91 and 113% of the nominal concentrations stated. A Wilcoxon signed rank test confirms that these actual values are not significantly different from the nominal concentrations ( $W_4 = 9, p = 0.201$ ).

### Statistical analysis

Data were analysed using Minitab (V.14) and were tested for normality and heterogeneity of variances where necessary. Data for nectochaete mortality from each experiment were combined and an  $LC_{50}$  calculated using ToxCalc 5.0 using the Trimmed Spearman Karber method with smoothing and Abbott's adjustment (where necessary). It is a non-parametric procedure for estimating median  $LC_{50}$ s and the associated 95% confidence intervals and is recommended when partial mortalities occur and the data do not fit the probit model as is the case here (Hamilton et al., 1977). Percentage

embryo developmental success data for the gamete incubations were arcsine transformed before using one way ANOVA and subsequent Tukey's pairwise comparisons to compare between concentrations within each gamete and time incubation. Differences in the mean number of settled fixed larvae per compartment between treatments were tested using a General Linear Model and subsequent Tukey's pairwise comparisons. Differences in the mean number of settled living larvae per compartment between treatments were also tested using one-way ANOVA and subsequent Tukey's pairwise comparisons, but the data for dead larvae were square root transformed to meet the assumptions.

## Results

### $LC_{50}$ metal tests for nectochaete larvae

During the first 24 h of incubation with the five metals, only copper had a significant effect on nectochaete mortality. As three experiments using a different batch were performed using this metal (June 2001, April 2002, May 2002) it is possible to produce a 24 h median  $LC_{50}$  value for each batch and a combined value ( $143.2 \mu\text{g l}^{-1} \pm 95\%$  CI of 138.4–148.2), and these are presented in Table 2. No  $LC_{50}$ s could be calculated for any of the other metals at this stage as the mean mortality in all concentrations remained below 20%. After 96 h of exposure to copper the  $LC_{50}$ s can be recalculated and have a combined  $LC_{50}$  of  $76.5 \mu\text{g l}^{-1} \pm 95\%$  CI of 73.8–79.2. After 96 h incubation an  $LC_{50}$  is only possible for zinc and estimated graphically to be  $2236.1 \mu\text{g l}^{-1}$  for the combined value. These data indicate that copper is the most toxic to larvae by an order of magnitude from zinc with the others being less toxic.

Table 2. Median  $LC_{50}$  values for 24 h and 96 h exposure of *N. virens* nectochaete larvae (three–four segments) to heavy metals (copper, zinc, chromium, cadmium and lead) dissolved in pasteurized seawater. All values are nominal concentrations expressed as  $\mu\text{g l}^{-1}$  of the metal ion with 95% confidence intervals in parenthesis (if these could be calculated from the data). Those metals shown as >1000 or >5000 did not produce significant mortality at the maximum concentrations tested. Larvae were incubated at 10°C (12:12 LD) and classed as dead when no movement was observed after gentle probing with a glass rod

	Period (h)	Copper ( $\mu\text{g l}^{-1}$ ) median (95% CI)	Zinc ( $\mu\text{g l}^{-1}$ ) median (95% CI)	Chromium ( $\mu\text{g l}^{-1}$ ) median	Cadmium ( $\mu\text{g l}^{-1}$ ) median	Lead ( $\mu\text{g l}^{-1}$ ) median
June 01	24	316.2	>1000	>1000	>1000	>1000
	96	31.6	>1000	>1000	>1000	>1000
April 02	24	116.3 (112–120)	>5000	>5000	>5000	>5000
	96	63.6 (59.9–67.4)	2236.1	>5000	>5000	>5000
May 02	24	173.21	—	—	—	—
	96	89.2 (84.7–93.9)	—	—	—	—
Combined	24	143.2 (138.4–148.1)	>5000	>5000	>5000	>5000
	96	76.5 (73.8–79.2)	2236.1	>5000	>5000	>5000

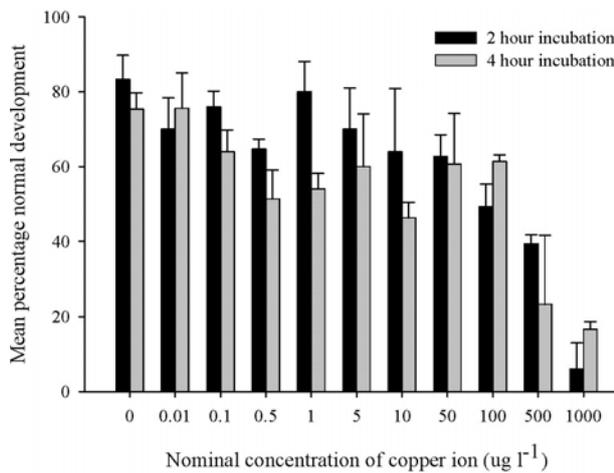


Fig. 1. Mean percentage ( $\pm$  SEM) of *N. virens* embryos developing normally 24 h post-fertilization after incubation of oocytes for either 2 or 4 h prior to fertilization. Oocytes were extracted from two females, washed and then pooled. 2500 were incubated in 1 ml of the copper solutions in three Eppendorf tubes for 2 and 4 h at 10°C. After incubation, oocytes were washed three times, the contents of each tube transferred into a Petri dish and pooled sperm (from three males) added to give a final concentration of  $2.5 \times 10^6$  sperm ml<sup>-1</sup>, 10 min contact time and sperm/egg ratio of 1000:1. After 10 min, oocytes were washed three times and then maintained at 10°C (LD12:12) for 24 h. All concentrations of copper are the metal ion in  $\mu\text{g l}^{-1}$ .

#### Incubation of gametes with copper

Mean developmental success of embryos after pre-fertilization incubation of oocytes for 2 or 4 h in increasing concentrations of copper are presented in Fig. 1. Mean developmental success in the control after 2 h was  $83.3 \pm 6.3\%$ , and after 4 h was  $75.3 \pm 8.3\%$ , but then developmental success was reduced over the concentrations to only  $6 \pm 6.3\%$  (2 h) and  $16.6 \pm 6.32\%$  (4 h) with exposure to  $1000 \mu\text{g l}^{-1}$ . One-way ANOVA confirms that there are significant differences between the treatments for the 2 h ( $F_{10,22} = 6.27$ ,  $p = 0.000$ ) incubation period; however, exposure to only the  $1000 \mu\text{g l}^{-1}$  reduced the developmental success when compared to the control and all other concentrations except the  $500 \mu\text{g l}^{-1}$ . For the 4 h incubation, one-way ANOVA confirms that there are also significant differences between the treatments ( $F_{10,22} = 5.16$ ,  $p = 0.001$ ), but both the 500 and the  $1000 \mu\text{g l}^{-1}$  are significantly different from the control and 0.01, 0.1, 5 and  $100 \mu\text{g l}^{-1}$  treatments.

In all treatments including the control and for both exposure periods, less than 10% of the oocytes failed to fertilize and even in the 500 and  $1000 \mu\text{g l}^{-1}$  treatments, failure to fertilize was 0.6 and 3.3%, respectively. This indicates that the reduction in developmental success at

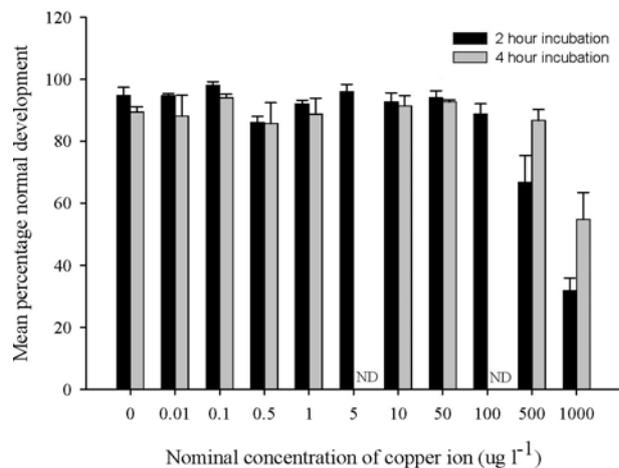


Fig. 2. Mean percentage ( $\pm$  SEM) of *N. virens* embryos developing normally 24 h post-fertilization after incubation of sperm for either 2 or 4 h prior to fertilization. Enough dry sperm for a final concentration of  $2.5 \times 10^6$  ml<sup>-1</sup> was extracted from three males, pooled and incubated in 1 ml of the copper solutions in three Eppendorf tubes for 2 and 4 h at 10°C. After incubation, sperm was washed three times, and the contents of each tube transferred into glass Petri dishes containing 2500 per dish of pooled oocytes (from two females). After 10 min, 7 ml of the overlying solution was replaced three times and then dishes were maintained at 10°C (LD12:12) for 24 h. ND: no data available. All concentrations of copper are the metal ion in  $\mu\text{g l}^{-1}$ .

these concentrations was because oocytes fertilized and then failed to develop normally rather than did not fertilize.

Developmental success in the controls of the pre-fertilization incubation of sperm (Fig. 2) is comparable to that in Fig. 1, with  $94.6 \pm 2.67\%$  of the embryos developing normally after sperm were exposed for 2 h and  $89.3 \pm 1.76\%$  after 4 h. One-way ANOVA confirms that there are significant differences between the treatments for both the 2 h ( $F_{10,22} = 15.87$ ,  $p = 0.000$ ) and 4 h ( $F_{8,18} = 3.91$ ,  $p = 0.008$ ) incubation periods. Pairwise comparisons confirm that developmental success is significantly reduced when compared to all other treatments after exposure to  $1000 \mu\text{g l}^{-1}$  for both incubation periods with mean developmental success being reduced to  $31.8 \pm 4\%$  after the 2 h exposure and  $54.7 \pm 8.6\%$  for 4 h. In addition, exposure to the  $500 \mu\text{g l}^{-1}$  treatment for 2 h also significantly reduced the developmental success to  $66.7 \pm 8.7\%$  when compared to the control, 0.01, 0.1, 5, 10 and  $50 \mu\text{g l}^{-1}$  treatments. The reduction in developmental success for all treatments, except the  $1000 \mu\text{g l}^{-1}$  is also attributable to a failure of the oocytes to develop normally rather than not being fertilized as less than 5% failed to fertilize in any treatment (data not shown). Failure to fertilize began to contribute to the reduction in

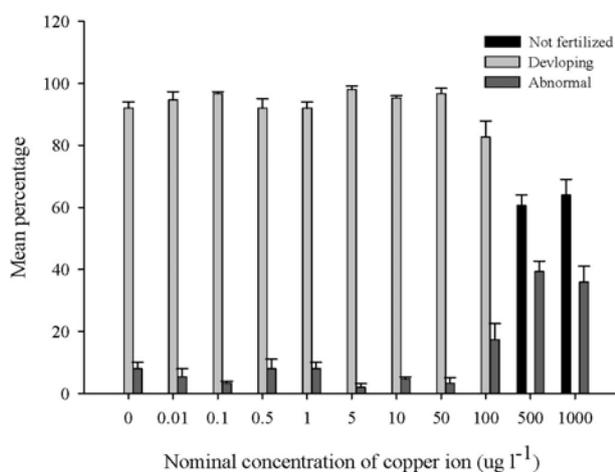


Fig. 3. Mean percentage ( $\pm$  SEM) of *N. virens* embryos developing normally 24 h post-fertilization after incubation for 10 min during fertilization with copper solutions. 2500 eggs were incubated in 1 ml of the copper solution with sperm (final concentration  $2.5 \times 10^6 \text{ ml}^{-1}$ ) in three pre-equilibrated glass Petri dishes for 10 min. Oocytes were washed three times and then maintained at  $10^\circ\text{C}$  (LD12:12) for 24 h. All concentrations of copper are the metal ion in  $\mu\text{g l}^{-1}$ .

developmental success in the  $1000 \mu\text{g l}^{-1}$  as the percentage of oocytes not fertilized increased to 12.5% for the 2 h and 14.6% for the 4 h exposure.

Exposure of both sperm and oocytes to copper for 10 min during the fertilization period (Fig. 3) resulted in significant differences in the developmental success of embryos from all the treatments ( $F_{10,22} = 108.87$ ,  $p = 0.000$ ), but pairwise comparisons confirm the significant differences are between all treatments and the 500 and  $1000 \mu\text{g l}^{-1}$  treatments only. In both, no oocytes were found to be developing normally; the majority ( $60.6 \pm 3.3\%$  for the  $500 \mu\text{g l}^{-1}$  and  $64 \pm 5\%$  for the  $1000 \mu\text{g l}^{-1}$  treatment) remained unfertilized whilst the remainder were classed as abnormal.

#### Effects of copper-spiked sediment on settlement

Four separate experiments with three chambers each were used to investigate the settlement of larvae in the presence of sediment spiked with various concentrations of copper, but there are no significant differences between the experimental runs. No larvae were found swimming within the water column, but larvae were found in the sediment and the mean number ( $\pm$ SEM) of larvae in sediment of each compartment is presented in Fig. 4. In the control sediment a mean of  $10.4 \pm 1.13$  larvae per compartment were found, but increasing concentrations of copper reduced this until only  $4.86 \pm 0.46$  larvae were present in sediment spiked to

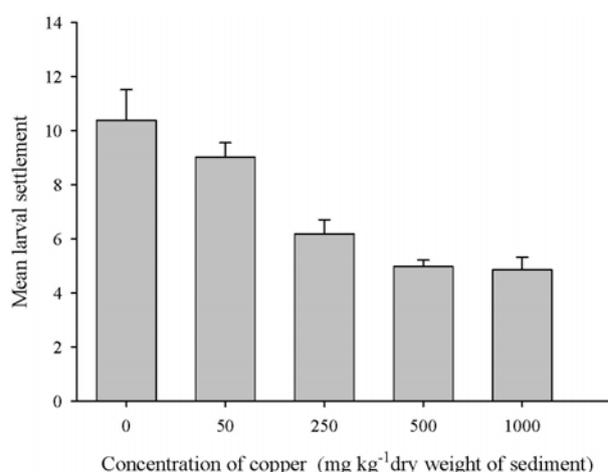


Fig. 4. Mean number of larvae settled in each compartment ( $2 \text{ cm} \times 2 \text{ cm}$ ) of a 25 repli-dish with each compartment containing 1 g of spiked sediment (nominal concentrations of 0, 50, 250, 500 and  $1000 \text{ mg kg}^{-1}$  of dry weight of sediment) collected from Chichester Harbour. Two hundred and fifty actively swimming nectochaete larvae (three or four segments) were added and chambers were maintained at  $10^\circ\text{C}$  (LD 12:12). Chambers were aerated for 1 h each day and experiments were terminated when no larvae could be seen swimming in the water column. The contents of each compartment were fixed by adding 10% formalin. The experiment was repeated with four batches of larvae.

$1000 \text{ mg kg}^{-1}$ . Analysis shows that these differences between treatments are significant ( $F_{4,42} = 15.37$ ,  $p = 0.000$ ). Pairwise comparisons show that the control and  $50 \text{ mg kg}^{-1}$  treatments are not significantly different from each other, but both have significantly more larvae in the sediment than the other three, which are not significantly different from each other.

A subsequent experiment using one batch and three chambers investigated the number of larvae in the sediment of each compartment, but did not use fixative, enabling the status (alive or dead) of larvae to be recorded (Fig. 5). The data indicate a significant reduction in the number of living larvae as the concentration of copper in the sediment increased ( $F_{4,10} = 17.92$ ,  $p = 0.000$ ) and significantly more are found in the control compartments ( $13.3 \pm 2$ ) than any of the other treatments. Of these, only the  $50 \text{ mg kg}^{-1}$  treatment has significantly more living larvae than the  $1000 \text{ mg kg}^{-1}$  treatment. Fig. 5 also presents the mean number of dead larvae found per compartment. As the concentration of copper is increased the number of dead larvae increases resulting in significant differences between the treatments ( $F_{4,10} = 8.73$ ,  $p = 0.003$ ). There are no significant differences between the control, 50 and  $250 \text{ mg kg}^{-1}$  treatments, but there are significantly more dead larvae in the  $500 \text{ mg kg}^{-1}$  treatment than the control and

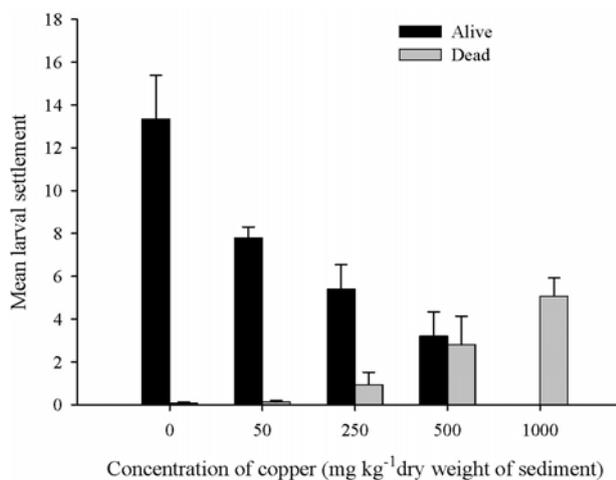


Fig. 5. Mean number of larvae settled in each compartment (2 cm × 2 cm) of a 25 repli-dish with each compartment containing 1 g of spiked sediment (nominal concentrations of 0, 50, 250, 500 and 1000 mg kg<sup>-1</sup> of dry weight of sediment) collected from Chichester Harbour. Two hundred and fifty actively swimming nectochaete larvae (three or four segments) were added and chambers were maintained at 10°C (LD 12:12). Chambers were aerated for 1 h each day and experiments were terminated when no larvae could be seen swimming in the water column. Neutral red dye was added to stain the larvae and sediment was passed through the 100 µm sieve and the settled larvae were recorded as dead or alive.

50 mg kg<sup>-1</sup>, and the 1000 mg kg<sup>-1</sup> has significantly more than the control, 50 and 250 mg kg<sup>-1</sup> treatments.

## Discussion

### *LC<sub>50</sub> metal tests for nectochaete larvae*

Larval stages have often been shown to be more sensitive to metal pollution than adults for marine invertebrates (Rand et al., 1995) and the data presented here support this statement, but only for copper. Eisler and Hennekey (1977) reported 96 h LC<sub>50</sub>s for *N. virens* adults for cadmium, zinc and chromium as 9.3, 8.1 and 2 mg l<sup>-1</sup>, respectively. However, it is not possible to confirm that the larvae are more sensitive than the adults for cadmium as no median LC<sub>50</sub> could be calculated with the concentrations used here, although it would seem that they are less sensitive than the adults for chromium as they were still alive in the 5000 µg l<sup>-1</sup> concentration. The 96 h LC<sub>50</sub> obtained for zinc of 2236.1 µg l<sup>-1</sup> suggests that tolerance is very similar, if not slightly higher, than the adults. The sensitivity of adult *N. virens* to lead has not been tested, but the low toxicity of this metal here (>5000 µg l<sup>-1</sup>) is corroborated by studies on other nereids where the LC<sub>50</sub>s range from 7.7 to >10 mg l<sup>-1</sup> (Reish, 1978; Reish and LeMay, 1991). The

data presented in Table 2 support the findings that copper is one of the most toxic metals to polychaetes (Reish and Gerlinger, 1997). The individual LC<sub>50</sub> values of the experiments do vary, but personal observations by the authors have found that larval quality can differ between batches. Fewer concentrations were also used in June 2001, reducing the precision of the calculations for this experiment. Nevertheless, the overall 96 h pooled mean LC<sub>50</sub> of 76.55 µg ± 2.75 (95% CI) confirms that the nectochaete larvae are approximately one order of magnitude more sensitive than the adults as Raymont and Shields (1963) found concentrations exceeding 0.5 mg l<sup>-1</sup> were lethal after 96 h.

Many studies have investigated the toxicity of copper to polychaete larvae, yet most are difficult to compare directly due to dissimilar methodologies or developmental stage used. However, our study suggests that *N. virens* are more sensitive to copper than other species. Gopalakrishnan et al. (2007) calculated a 24 h LC<sub>50</sub> for *Hydroides elegans* of 122 µg l<sup>-1</sup> for incubation from fertilization to trochophore and a 96 h LC<sub>50</sub> of 180 µg l<sup>-1</sup> was calculated for *Capitella capitata* larvae (Reish et al., 1976). Our results confirm that copper is the most toxic to nectochaete larvae of the five metals tested, but to fully assess the effects all key developmental stages must be tested as responses to toxicants can be stage specific (Kobayashi, 1980).

It is not clear why *N. virens* larvae are less tolerant to copper than other species and that the nectochaete stage is much less tolerant than an adult. Polychaetes can excrete, sequester or detoxify significant concentrations of heavy metals using a number of mechanisms including binding metals to metallothioneins and myrohemythrins (Amiard et al., 2006; Demuyne et al., 2007). Differences among species in the efficacy of these mechanisms and that the larval stages may not have fully functioning systems may be responsible for the differences observed.

### *Incubation of gametes with copper*

The developmental success of embryos in the controls of all three gamete incubation experiments was between 83–95% and this is comparable with previous studies by Lewis et al. (2002) and Ushakopva and Sarantchova (2004), validating the methodology and confirming that gametes were competent. Spontaneous elevation of the fertilization membrane and jelly coat was not recorded in any of the oocyte-only controls confirming that all fertilizations and subsequent development were due to the addition of the sperm.

From the data presented in Figs. 1 and 2, it is clear that pre-fertilization exposure of gametes to increasing concentrations of copper for either 2 or 4 h reduces

developmental success of the embryos, although inspection of the data indicates that oocytes are less sensitive than sperm. When individual concentrations are compared with the controls the 4 h exposure is more toxic to oocytes. For sperm, developmental success is reduced after exposure to the  $1000 \mu\text{g l}^{-1}$  for both 2 and 4 h, but for only 2 h at the  $500 \mu\text{g l}^{-1}$ , although it is not clear why there are these slight contradictions in incubation times and toxicities for sperm. This may be due to using only two females for the pooled oocytes, however, if this was the case then differences would also be present in the other experiments and other concentrations.

Copper and other metals have been shown to damage gametes of other marine species through oxidative stress (Lloyd et al., 1997), cytological alterations including increased ionic permeability of organelles to calcium and phosphorus (Earnshaw et al., 1986), inhibition of gamete respiration (Akberali et al., 1986) and sperm swimming (Earnshaw et al., 1986; Kime et al., 1996) and all these may be implicated in the reduction in developmental success observed. The data for the 10 minute exposure to copper during fertilization (Fig. 3) are consistent with those of the pre-fertilization incubation in that a significant reduction in developmental success occurs only after exposure to concentrations of 500 and  $1000 \mu\text{g l}^{-1}$ . What is different is the extent to which developmental success is reduced after exposure compared to pre-fertilization incubation of sperm and oocytes. Exposure during fertilization resulted in no oocytes developing normally and the majority of these oocytes from both concentrations failed to fertilize compared with much higher levels for the pre-incubation of gametes. This may suggest that copper is having a specific effect on the mechanism of fertilization such as sperm/egg recognition or the acrosome reaction, but requires further investigation.

Copper has been shown to be highly toxic to gametes and during fertilization for many marine invertebrates with  $\text{EC}_{50}$  values ranging from 14.5 to  $45 \mu\text{g l}^{-1}$  for some corals and sea urchins (Ringwood, 1992; Reichelt-Brushett and Harrison, 1999; Victor and Richmond, 2005) and more recently *Galeolaria caespitosa* (Hollows et al., 2007). Williams and Bentley (2002) found that oocytes and undiluted sperm of *N. virens* are viable for up to 72 h, so it is possible that an extended period of exposure may lower the  $\text{EC}_{50}$  values considerably; however, *N. virens* gametes seem to be significantly more tolerant of copper in comparison to most other species. Few explanations have been proposed for these species-specific differences, but Reichelt-Brushett and Michalek-Wagner (2005) suggested that the relatively high tolerance of the soft coral *Lobophytum compactum* ( $\text{EC}_{50}$   $261 \mu\text{g l}^{-1}$ ) was due to an

extended gametogenic cycle allowing for an extended period of exposure to copper loads. Copper loads in the oocytes of *N. virens* were not measured, but determination of the mechanism of copper toxicity in gametes should begin to elucidate the reasons for their tolerance.

Gametes of broadcast spawning invertebrates, which are spawned into large volumes of water, are exposed to relatively constant but low levels of toxicants preceding and during fertilization. Copper concentrations in coastal waters have been recorded in a number of studies and vary considerably (see Lewis and Cave, 1982), but it is highly unlikely that gametes would be exposed to coastal water concentrations approaching  $500 \mu\text{g l}^{-1}$  (the UK has an annual average Environmental Quality Standard of  $5 \mu\text{g l}^{-1}$  [Matthiessen et al., 1999]), although some sites close to point sources of copper inputs may have elevated concentrations, e.g. Restronguet Creek of up to  $176 \mu\text{g l}^{-1}$  (Bryan and Gibbs, 1983). Nevertheless, the shallow nature (approximately 10 cm deep  $\times$  5 m  $\times$  5 m) of the low tide pools in which intertidal *N. virens* spawn (Watson et al., 2003) may make them susceptible to environmental changes (such as turning over the sediment during bait collection [Watson et al., 2007]). This may provide conditions favourable for the release of copper from sediment during the low tide period. Direct measurements of copper in the pools under different environmental conditions combined with experimental manipulations are needed to elucidate the possible role of bait collection and other disturbances in mobilization and the bioavailability of metals during the spawning process.

#### *Effects of copper-spiked sediment on settlement*

Settlement is a critical stage in the life-cycle of many marine invertebrates and a range of environmental and other factors have been shown to influence this process in polychaetes (see Qian, 1999 for review). Laboratory-based studies on polychaete larval settlement have been limited to organic pollutants, but the results were equivocal. Chandler and Scott (1997) found no significant impact of sediment contaminated with PAHs on settlement of *Streblospio benedicti*, whilst Chandler et al. (1991) found the organochlorine pesticide endosulfan did reduce settlement in comparison to the control. Johnston and Keough (2000) investigated the effect of copper pulses from impregnated blocks on settlement of a range of marine invertebrates in the field but found serpulids to be insensitive, as was *G. caespitosa* when exposed to polluted waters (Moran and Grant, 1993). Our study is, therefore, the first to show a negative impact of a heavy metal on polychaete larval settlement. In the first experiments (Fig. 4) sediment

spiked to  $250 \text{ mg kg}^{-1}$  or above had a significant effect, but as the larvae were fixed it was not possible to distinguish the effects on the behavioural decision to settle from any direct effects on larval survivorship. Analyzing the living larvae confirms the significant effect of copper on settlement, but indicates an even greater sensitivity. The number of dead larvae in each treatment shows that the two highest concentrations are toxic compared to the control, but what is not clear is the time of death. Were the larvae dead or moribund before they reached the sediment or were they dying once settled? The 96 h  $\text{LC}_{50}$  calculated earlier of  $76.55 \mu\text{g l}^{-1}$  for nectochaetes of a similar stage suggests that larvae could be affected if copper is remobilized from sediment. Although concentrations in the seawater within the chambers were not measured, it is unlikely that large differences would be present between compartments as the chambers were aerated each day facilitating mixing of the water. It is likely that larvae died once they had settled in the sediment. The lack of significant differences in the number of dead larvae between 0, 50 and  $250 \text{ mg kg}^{-1}$  suggests that their contribution to the total number of larvae per compartment after fixing is negligible also supporting the differences highlighted in Fig. 4.

A number of studies of marine invertebrates have shown copper to negatively affect settlement at low concentrations with  $\text{EC}_{50}$  values ranging from 42 to  $67.8 \mu\text{g l}^{-1}$  (Reichelt-Brushett and Harrison, 2000; Bellas et al., 2004). Whilst some have also shown an enhancement of settlement at low concentrations of copper (Ng and Keough, 2003; Cebrian and Uriz, 2007), the data presented here show no evidence of hormesis and the effective concentrations are similar to a study on sub-lethal behavioural effects of copper on juvenile bivalves *Macomona liliana* by Roper et al. (1995). They found that juveniles crawled away from sediment spiked with  $10 \text{ mg kg}^{-1}$  and there was a significant reduction in the number of burying in sediment spiked with  $25 \text{ mg kg}^{-1}$ .

It is clear that *N. virens* larvae are not settling in sediment that is contaminated with copper above  $250 \text{ mg kg}^{-1}$ , although in Fig. 5 even the sediment spiked to  $50 \text{ mg kg}^{-1}$  was avoided. Sediment from Chichester Harbour was chosen as a relatively clean site with low background concentrations and this is confirmed by the Environment Agency data and the copper concentration in the control sediment of only  $15 \text{ mg kg}^{-1}$  (shown in Table 1). However, of the 19 UK estuaries reviewed by Bryan and Langston (1992), copper concentrations in eight exceeded  $50 \text{ mg kg}^{-1}$ , suggesting that in many locations elevated copper levels within sediment could affect settlement of *N. virens* larvae. Extrapolating laboratory-based spiking experiments to

the field is complex as the bioavailability of metals in sediments is dependent on a range of factors (for review see Luoma, 1990). The data presented in Table 1 show that the actual concentrations of copper are in good agreement with the nominal concentrations, but the bioavailable fraction could be quite different. For example, Phelps et al. (1985) found that sediment freshly dosed with copper caused abnormal burrowing in the bivalve *Protothaca staminea*, but after ageing the sediment for 1 day there was no effect. Sequential extraction would be needed to begin to understand how copper is partitioned between the different components, such as crystalline minerals, carbonates, hydrous metal oxides and organic substances and to confirm its bioavailability to the larvae.

*N. virens* is often thought of as being a tolerant species that can exploit contaminated sediments. It is clear from the data presented here that *N. virens* larvae are particularly susceptible to direct exposure of copper compared to other heavy metals and the adult stages. Although the concentrations required to affect gametes and fertilization directly are much higher than the vast majority of concentrations found in the field, the additional sub-lethal effects of copper on larval settlement behaviour should not be overlooked as a driver of population change. The effects on larval survival and settlement may have important implications for recruitment to polluted sediments; in particular this may be exacerbated through sediment disturbance which may increase the bioavailability of metals. Investigations are urgently needed to confirm if the effects on settlement can be transferred into the field and whether there is a genetic component to tolerance as has been shown for *N. diversicolor* (Grant et al., 1989).

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