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Mesocosm validation of the marine No Effect Concentration of dissolved copper derived from a species sensitivity distribution

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HIGHLIGHTS

- Ecological impact of dissolved copper was investigated in outdoor marine mesocosms.
- Six, triplicated, exposure concentrations were actively maintained for 82 days.
- Development on the plankton and benthic community was followed.
- Bivalve reproduction formed the most sensitive endpoint.
- NOEC was comparable with PNEC from SSD based on single species lab studies.

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ABSTRACT

The Predicted No Effect Concentration (PNEC) for dissolved copper based on the species sensitivity distribution (SSD) of 24 marine single species tests was validated in marine mesocosms. To achieve this, the impact of actively maintained concentrations of dissolved copper on a marine benthic and planktonic community was studied in 18 outdoor 4.6 m^3 mesocosms. Five treatment levels, ranging from 2.9 to 31 μg dissolved Cu/L, were created in triplicate and maintained for 82 days. Clear effects were observed on gastropod and bivalve molluscs, phytoplankton, zooplankton, sponges and sessile algae. The most sensitive biological endpoints; reproduction success of the bivalve *Cerastoderma edule*, copepod population development and periphyton growth were significantly affected at concentrations of 9.9 μg Cu/L and higher. The No Observed Effect Concentration (NOEC) derived from this study was 5.7 μg dissolved Cu/L. Taking into account the DOC concentration of the mesocosm water this NOEC is comparable to the PNEC derived from the SSD.

1. Introduction

Copper is an essential element and enables multiple metabolic functions in all life forms when present at low levels. At elevated concentrations copper has been shown to trigger a number of adverse physiological, histological and behavioural responses, and it is in the dissolved cupric ion (Cu^{2+}) form that copper is the most bioavailable, although labile forms of copper are also suggested as contributing to observed toxicity (Brooks et al., 2007, 2008).

The bioavailability of metals in the aquatic environment can be influenced by many factors, but is especially affected by pH (Hyne et al., 2005; De Schamphelaere and Janssen, 2004), and dissolved organic carbon (DOC) concentrations (DePalma et al., 2011a). The influence of these factors can be very metal specific (e.g. Sánchez-Marin et al., 2010), but for dissolved copper in the marine environment the DOC concentration is the most significant environmental factor controlling the toxicity for pelagic organisms. When incorporated into a complex with DOC, the bioavailability of dissolved copper is strongly reduced. This has been demonstrated for a wide range of marine species, covering macroalgae (Brooks et al., 2008), rotifers (Arnold et al., 2010b), echinoderms (e.g. Lorenzo et al., 2002; Arnold et al., 2010a), bivalves (e.g. Zamuda and Sunda, 1982; Arnold et al., 2006, 2010a; Brooks et al., 2007) and fish (Gheorghiu et al., 2010). DOC concentrations in natural marine and estuarine waters show large variations, as illustrated by the analysis of 72 water samples from coastal marine and estuarine sites in the USA and Canada, with DOC levels ranging between 0.8 and 21 mg C/L (DePalma et al., 2011b). Therefore, the comparability of copper toxicity data from experiments performed with seawater from a different origin is strongly improved after normalisation of the effect concentration on DOC (Arnold et al., 2010a). By following this approach a Predicted No Effect Concentration (PNEC) for dissolved copper was calculated from

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a species sensitivity distribution (SSD; see Posthuma et al., 2002) by Van Sprang and co-workers (Van Sprang et al, 2008). This calculation was based on No Observed Effect Concentrations (NOECs) from 24 high quality marine single species laboratory tests covering 8 taxonomic groups, normalised for a DOC concentration of 2 mg/L, which can be considered as typical for coastal marine areas. The PNEC was 5.2 μg dissolved Cu/L and was calculated as the median Hazard Coefficient 5-50 (HC5-50) being the lower 50th percentile of the 95% protection level. It thus predicts that 95% of the species in a marine environment with 2 mg/L DOC will not be affected by a dissolved copper concentration that does not exceed 5.2 μg/L.

The aim of the study presented in this paper was to evaluate the robustness of this PNEC in a marine mesocosm study, mimicking a marine, soft sediment, near shore European ecosystem that is chronically (>80 days) exposed to a concentration series of dissolved copper ranging from 1 to 31 Cu μg/L.

2. Materials and methods

2.1. Mesocosms

In total 18 mesocosms were used, each consisting of a circular glass-fibre tank located on land, partly buried in the ground, with a volume of 4.6 m^3 (diameter 190 cm, depth 180 cm). The mesocosms were installed with about 20 cm of natural sandy sediment that was collected from the coastal North Sea and a 150 cm deep water column of natural seawater collected from the Oosterschelde, a relatively pristine tidal bay in direct connection with the North Sea, often used as a reference site in marine ecotoxicological studies in the Netherlands (e.g. Kuiper et al., 2007; Foekema et al., 2008, 2012). Concentrations of selected metals measured in the batch of water used for this study were 0.2 μg Cd/L, 1.26 μg Ni/L, 0.5 μg Pb/L, 59 μg Zn/L and 1.1 μg Cu/L.

The water level was not manipulated to simulate a tidal cycle. The water column of each mesocosm was continuously mixed by aeration at about 10 cm above the sediment. Each mesocosm was covered with a transparent lid as a defence against rainfall, birds and litter. Evaporation losses were replenished with demineralised water so that salinity was maintained at 32 ± 1 throughout the study. Prior to the first application of dissolved copper, the water was circulated through the hydraulically connected mesocosms for 33 days to ensure a similar development of the plankton community and water characteristics in all mesocosms. On day 0, before dosing commenced, the continuous circulation was stopped and the mesocosms were isolated.

2.2. Test substance and dosing

Copper(II) sulphate pentahydrate ($CuSO_4.5H_2O$, Sigma-Aldrich, purity 99.995%) dissolved in 0.45 μm-filtered seawater was used to achieve and maintain the concentration of dissolved copper in the mesocosm water at the appropriate level. For each mesocosm the stock solution was prepared at the appropriate concentration in 10 l aliquots of water in a polyethylene tank, which was then added to the mesocosm with continuous flow of 1 to 8 mL/min starting on April 29, 2009 (Day 0). Within a week the concentration of dissolved copper in the water column of each mesocosm was built-up until the intended (nominal) copper concentration was reached. These concentrations were, in addition to the controls, 2.6, 5.2, 9.0, 15 and 27 μg Cu/L. From then on, a constant concentration of dissolved copper was actively maintained by adjusting the dosing rate for each individual mesocosm, based on the analysis of dissolved copper (three times per week). All treatments and untreated controls were triplicated in a randomised block design. Filtered seawater without added copper was added to the control mesocosms in amounts comparable to the treated mesocosms. As somehow similar volumes of water were removed from the mesocosms during each sampling event (see below) the additions of the dosing solutions did not result in substantial increase of water levels.

The mesocosms were installed on March 24, 2009, exposure started on April 29, 2009 and lasted until the final sampling on July 22, 2009. The total duration of the exposure period was 82 days. The start of the exposure period is defined as Day 0 and the pre-exposure period is indicated by negative day numbers throughout this publication.

2.3. Flora and fauna

Phyto- and zooplankton and small benthic invertebrate species were introduced with the water and sediment during installation. A selection of macroinvertebrates was introduced in known numbers during the first days of the establishment phase (Table 1). The introduced species are commonly present in shallow soft sediment coastal ecosystems of the North Sea and representatives from various taxonomic classes: sponges, crustaceans, molluscs and annelids. All introduced fauna were collected from relatively pristine field locations and were introduced in the mesocosms following a random table. Small parts of larger specimens of the sponge *Halichondria panicea* were suspended about 20 cm below the water surface.

2.4. Biota sampling and analyses

Phytoplankton was sampled by submerging a bottle in the mesocosm ca. 30 cm below water surface. Phytoplankton biomass was measured in these samples as chlorophyll-a concentration twice a week by means of a 1Hz-kuvetten Fluorimeter (BBE-Moldaenke). Subsamples of 100 mL were collected on days −2, 12, 26, 54 and 82, preserved with lugol and stored to be analysed by visual microscopic determination and counting of the various taxa.

On a weekly basis five water samples of about 1.5 L each were collected per mesocosm for determination of the zooplankton community by means of a core sampler covering the entire water column depth. The zooplankton from these samples was collected using a 55 μm plankton net and the composite sample was preserved in a formaldehyde solution. Visual microscopic analyses were performed on the samples collected on days −9, 12, 26, 54 and 82.

The development of periphyton (sessile algae) was monitored on three glass microscope slides (76 × 26 mm) that were placed in each mesocosm in vertical position facing south at ca. 10 cm below the water surface, out of reach of gastropods. At 28 days intervals the development of the periphyton on the slides was determined by measuring the chlorophyll-a fluorescence using a microtiter plate reader (BioteK FLx800).

The development of the sponges was monitored by determining the wet weight of the individual sponges with 28 day intervals.

In order to avoid disturbance of the system periwinkles, cockles, and lugworms were only sampled at the end of the study, after the water was pumped off. Survival and growth (biomass and where appropriate shell length) of the introduced individuals were determined. The density of small macroinvertebrates, including mudshrimps and juveniles of introduced species, was also determined only at the end of the study. For this, two rings (30 cm diameter each) were pressed in the sediment surface before the water was fully pumped off. The top 5 cm sediment layer within each ring was collected and sieved (500 μm). All macroinvertebrates that were recovered were stored in formaldehyde for taxonomic identification and counting.

2.5. Physico-chemical measurements

Water temperature and dissolved oxygen concentration (Hach LDO101), salinity (Hach CDC401), and pH (Hach PHC101) were determined twice a week by submerging electrodes at half water depth.

Sampling for the determination of the concentration of nutrients, Mg, Ca (bi weekly) and DOC (weekly) was performed by immersing

Table 1
Species that were introduced in known numbers in the mesocosms. Zoo- and phytoplankton and other macroinvertebrates were introduced with the water and sediment.

Phylum/group	Class	Species	Common name	N per mesocosm
Sponge	Porifera	*Halichondria panicea*	Bread-crumb sponge	2
Crustacean	Amphipoda	*Corophium volutator*	Mudshrimp	300
Mollusc	Gastropoda	*Littorina littorea*	Common periwinkle	40
Mollusc	Bivalvia	*Cerastoderma edule*	Cockle	25
Annelid	Polychaeta	*Arenicola marina*	Lugworm	20

pre-rinsed sample bottles while avoiding the collection of the surface micro-layer. Total hardness was calculated based on the analyses of dissolved calcium and magnesium, using inductively coupled plasma atomic emission spectrometry (ICP-AES), following Netherlands standard NEN-6966 (NEN, 2005). For the analyses of nutrients and DOC the water sample was filtered over a 0.45 μm cellulose acetate filter (Whatman Puradisc™). Analyses of the nutrient nitrite, ammonia, silicate and ortho-phosphate were carried out by an Autoanalyzer (Aquakem) with spectrophotometric detection, following Netherlands standard NEN-6604 (NEN, 2007). The concentration of nitrate was determined using a flow-through analysis system (Skalar) and spectrophotometric detection, following standard ISO-13395 (ISO, 1996). The water samples for DOC analysis were acidified to pH 2–3 with HCl (Merck Suprapur) and oxygen-purged to remove inorganic carbon. The remaining DOC was analysed by means of a TOC-V analyser (Shimadzu) following standard ISO-8245 (ISO, 1999).

2.6. Copper analyses

Sampling for the copper concentration in water was performed three times per week using new 60 mL polypropylene syringes in each mesocosm. Immediately after sampling the water was pushed through an acid washed 0.45 μm pore size cellulose acetate membrane filter (25 mm Ø, Whatman). The filtrate was collected in 50 mL LDPE acid washed polythene bottles and acidified to pH < 2 (HCl, Suprapur, Merck). Samples were transported within 1 h to the laboratory. Here, the samples were diluted ten times with a 0.1 M nitric acid (HNO_3 Suprapur, Merck) solution in demineralised (Milli-Q) water. The diluted solutions were than analysed for copper content using inductively coupled plasma atomic mass spectrometry (ICP-MS) at a mass of 63 m/z. The measurements were also performed at an alternative mass of 65 m/z. The difference between the results at these two masses was not considered to be significant (<10%). Peak intensity of rhodium was used as internal standard at the mass of 103 m/z, for calculation of the amount of copper. For the quality control (QC) of the analyses a sample of mesocosm water spiked at 10 μg Cu/L at the beginning of the study was included in each of the 12 batches of copper analysis. The average concentration determined in these QC-samples was 9.73 μg Cu/L with a standard deviation of 0.28 μg/L confirming the correctness and reproducibility of the analyses.

Sediment samples were collected only at the end of the study in order to avoid re-suspension of bottom material into the water column. By means of a core sampler (4 cm Ø) five samples from each mesocosm were collected and divided into a top (0–2 cm) and the deeper part. From both depths, composite samples were prepared per mesocosm and stored at −20 °C until analysis of total copper concentration. Samples for the copper analysis of selected macroinvertebrates were collected at the end of the study. Sponges were rinsed in clean seawater and stored at −20 °C in pre-cleaned glass containers. Lugworms were kept overnight in a temperature controlled room in water collected from their respective mesocosm for depuration of the gut and were then stored in pre-cleaned glass containers at −20 °C. Cockles and periwinkles were collected in polyethylene bags and stored frozen directly after sampling (−20 °C). Before analyses, after thawing the soft tissue was collected from the shells by means of titanium tools. A titanium Ultraturrax was used to homogenise all biota samples before analysis. Sample manipulations in the laboratory were performed inside a laminar flow hood using acid washed materials.

Analyses of total copper in sediment and biota were performed in duplicate following a similar procedure based on Netherlands standard NEN-EN 13805 (NEN, 2014). Samples were digested with concentrated nitric acid (65–67% HNO_3 Suprapur, Merck) using a heating block (100 °C) for 1 h. The digested samples were analysed for copper using high resolution inductively coupled plasma atomic emission spectrometry (ICP-AES) at a wavelength of 324 nm. Those samples that revealed copper concentrations below 2.5 mg/kg were further analysed using inductively coupled plasma mass spectrometry (ICP-MS) as described above. Quantitation was obtained by comparing the results of the samples with those of external standard solutions containing known amounts of copper in the range of the concentrations.

2.7. Data treatment

The significance of differences between the controls and the treated mesocosms for single species and endpoints was tested by using the Dunnett's multiple comparison test. Differences were considered statistically significant at $p < 0.05$. All statistical analyses were performed with the software package GraphPad Prism™ version 4.03 (January 21, 2005).

For the evaluation of the response of the plankton community to the treatment, Principal Response Curve (PRC) analyses were performed, as described in (van den Brink et al., 2009; Van den Brink and Ter Braak, 1999). The dominant plankton species were selected and the less abundant species were combined in taxonomic groups. Then observations below the detection limit were replaced by an arbitrary value of 25% of the detection limit and the data were log transformed. The results of the PRC analysis were summarised in a PRC plot, showing the canonical coefficient for each treatment level in time relative to the controls, thus visualising the response of the plankton community to the treatment. The significance of the deviation from the controls in the PRC analysis was tested by permuting all time series randomly over the treatment in a Monte Carlo simulation with 9999 runs, and testing whether the observed distribution departed significantly from a random distribution (Van den Brink and Ter Braak, 1999).

3. Results

3.1. Chemistry

The intended concentrations of dissolved copper were reached five days after the start of the copper dosing in most of the mesocosms. For the highest treatment levels nine days were required (Fig. 1). During the whole study copper had to be added to the mesocosms to maintain these levels and the total amount of copper added at the end of the study ranged between 40 and 900 mg for the lowest and the highest treatment levels respectively (Fig. S1 in supporting information). The dissolved copper concentrations were highly comparable between triplicate treatments and the respective exposure concentrations did not overlap. The actual mean exposure concentrations are used in this paper to address the treatment levels and the control (Table 2).

The background copper concentration in the sediment was 0.6 mg/kg dw. At the end of the study this was still the concentration

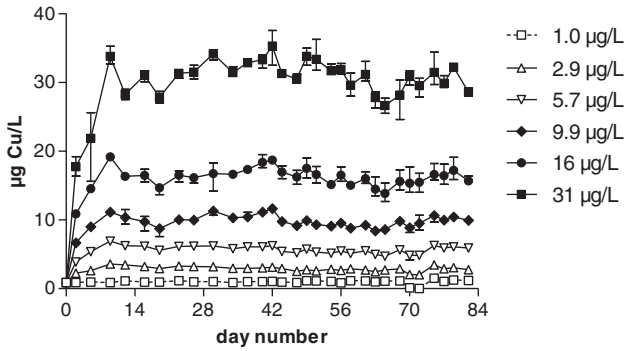


Fig. 1. Total dissolved copper concentrations in the water column of the mesocosms.

in the deeper sediment sampled below the top 2 cm in all mesocosms. In the top 2 cm concentrations were significantly increased in the 5.7 µg Cu/L treatments and above, with a maximum of 2.6 mg/kg dw at the highest treatment level (Table 3).

Copper concentrations in the tissue of all biota selected for chemical analyses showed a clear relation with treatment levels, with significantly increased tissue concentrations at treatment levels 5.7 µg Cu/L and above (Table 3). In periwinkles the copper concentrations were already significantly elevated at the lowest treatment level. Chemical analyses revealed unexpected high copper concentrations in cockles' tissue at introduction in the mesocosms. At the end these concentrations had decreased in treatments levels 5.7 µg Cu/L and below, and showed a clear correlation with treatment levels. Within treatment levels lugworms and cockles had the lowest tissue concentrations, and periwinkles the highest.

3.2. Water parameters

During the study water temperature in all mesocosms increased from about 10 to 22 °C. Concentrations of dissolved oxygen were never below 90% of the saturation level. Ca-concentrations increased from 338 to 398 mg/L and the Mg-concentrations from 1173 to 1363 mg/L. Concentrations of nitrate (NO_3) and nitrite (NO_2) in the water of all mesocosms never exceeded the analytical detection limits of 0.07 and 0.0035 mg/L respectively. Ammonium (NH_4) concentrations ranged between <0.6 and 1.1 mg/L. None of these parameters showed a relation with the treatments.

Ortho-phosphate and silicate concentrations were <0.03–0.78 mg PO_4/L and <0.25–2.9 mg SiO_2/L respectively. Concentrations of both these nutrients were near the detection limits until day 28 and increased from then on in most mesocosms. At the end of the study PO4 and SiO2 were in general somewhat higher in the highest treatment levels (Fig. S2 in supporting information).

The concentration of dissolved organic carbon (DOC) in the water of controls increased from 2.8 to 4.2 mg/L during the study (Fig. 2A). A comparable development was observed in all mesocosms up to the 9.9 µg Cu/L treatment. The two highest treatments showed a strong increase of the DOC concentration that was statistically significant from days 19 to 43 onwards for the 31 and 16 µg Cu/L treatments respectively (Table S1 supporting information). This situation continued until the end of the study, and resulted in statistically significant higher DOC concentrations in these mesocosms compared to the lower treatment levels and the controls (Fig. 2C).

Also the pH of the mesocosm water showed a relation with treatment (Fig. 3B), indicated by an increase in the 31 and 16 µg Cu/L treatments after the first week of the exposure phase, becoming statistically significant on days 26 and 47 respectively (Table S1 supporting information).

Table 2
Mean concentrations ± standard deviation (n = 28) of total dissolved copper as measured in the water column of the mesocosms from 5 days after the start of the application until the end of the study. Based on these data, the average actual treatment concentration was calculated that is used to address the treatment level throughout this paper.

Treatment level	Replicate I µg Cu/L	Replicate II µg Cu/L	Replicate III µg Cu/L
Control (1.0 µg Cu/L)	1.0 ± 0.34	1.0 ± 0.30	1.0 ± 0.31
2.9 µg Cu/L	2.9 ± 0.36	2.9 ± 0.41	2.9 ± 0.41
5.7 µg Cu/L	5.7 ± 0.58	5.7 ± 0.58	5.7 ± 0.58
9.9 µg Cu/L	9.8 ± 0.82	9.7 ± 1.06	10.0 ± 0.98
16 µg Cu/L	15.9 ± 1.55	16.3 ± 1.36	16.9 ± 1.54
31 µg Cu/L	31.0 ± 2.31	31.0 ± 2.90	31.0 ± 2.14

3.3. Response of the plankton community

The chlorophyll-a concentration in the mesocosm water indicates relatively low phytoplankton densities at the start of the exposure phase in all mesocosms. In the control mesocosms and treatments 2.9 µg Cu/L and 5.7 µg Cu/L, the chlorophyll-a concentrations never exceeded 10 µg/L (Fig. 3). In the higher treatments an increase of the chlorophyll-a concentration was observed. From day 16 onwards the chlorophyll-a concentrations in the 16 and 31 µg Cu/L treatments were frequently significantly higher than the controls (Table S1 in supporting information). In the 9.9 µg Cu/L treatments, chlorophyll-a concentrations started to increase around day 60; however this did not lead to significantly differences with the controls.

The significance test in the PRC analysis on the plankton community data set showed high significance ($p = 0.0001$) with the first canonical axis. This first axis representing the treatment, explained about 38% of the variance in the plankton data. For the zooplankton and phytoplankton individually this was 64% and 34% respectively. In Fig. 4 the PRCs visualise the impact of the treatment on the plankton community in the 16 and 31 µg Cu/L treatments. The calculated species weight for the individual taxa show in general a 'positive' response of the phytoplankton community to the treatment. It indicates that the small and medium sized flagellates that dominated the phytoplankton community, and the cyanobacteria *Chroococcus turgidus* showed the strongest response to the treatment.

Between days 26 and 54 the cell density of the total flagellates was significantly higher in the 31 µg Cu/L treatment than in the controls. This was due to a decreasing density in the controls during that period that was not observed at the highest treatment level (Fig. 5A). For the cyanobacteria *C. turgidus* the treatment also resulted in higher cell densities that were significant around day 54 in the 16 and 31 µg Cu/L treatments (Fig. 5B). In contrast to the flagellates, this was the result of increasing densities at the higher treatment levels, while the density in the controls declined. The negative species weight (Fig. 4) of the diatoms indicates that this group was negatively affected by the treatment, but this did not lead to significant differences with the controls.

The zooplankton community was strongly dominated by copepods with *Acartia clausi*, *Temora longicornis* and *Centrophagus hamatipes* as the dominant species. The species weight for the individual taxa calculated in the PRC analyses shows for all these species a negative response to the treatment (Fig. 4). The population density of *A. clausi* (Fig. 6A) and *T. longicornis* (Fig. 6B) in the control mesocosms varied around 10 ind./L during most of the study, but with a strong decline at the end. The effect of the treatment was evident with significantly lower densities in the higher treatment levels between days 12 and 54. The lowest treatment levels that caused significant effects were 9.9 µg Cu/L and 16 µg Cu/L for *A. clausi* and *T. longicornis* respectively (Table S1 in supporting information). After day 28 an indication of recovery of the *Acartia* population in one of the 9.9 µg Cu/L replicates was observed, resulting in high densities up of to 70 ind./L at the final sampling event. In the other two replicates about 7 individuals per litre were present at that time, comparable to the controls.

Table 3
Total copper concentrations (mean ± standard deviation) at the start (initial) and at the end of the study in sediment (top 2 cm and deeper layers) and selected biota. 'n.d' indicates that no sample was available due to high mortality. Values printed in bold are statistically significantly ($p < 0.05$) different from the untreated systems (1 μg/L).

Treatment level	Sed. >2 cm (mg/kg dw)	Sed. 0–2 cm (mg/kg dw)	Cockle (mg/kg afdw)	Sponge (mg/kg afdw)	Lugworm (mg/kg afdw)	Periwinkle (mg/kg afdw)
Initial	0.6	0.6	38.3	17.1	11.8	127
1.0 μg Cu/L	0.55 ± 0.04	0.55 ± 0.06	17.4 ± 1.88	17.3 ± 0.49	8.27 ± 0.47	106 ± 9.65
2.9 μg Cu/L	0.60 ± 0.02	0.62 ± 0.06	19.8 ± 2.49	32.6 ± 7.08	9.87 ± 0.23	**204 ± 30.8**
5.7 μg Cu/L	0.62 ± 0.06	**0.85 ± 0.02**	**28.1 ± 6.14**	**84.6 ± 22.0**	**17.3 ± 2.00**	**227 ± 22.6**
9.9 μg Cu/L	**0.65 ± 0.02**	**1.18 ± 0.06**	**85.4 ± 8.36**	**289 ± 22.3**	**39.7 ± 3.18**	**411 ± 66.8**
16 μg Cu/L	0.64 ± 0.04	**1.83 ± 0.55**	n.d	**523 ± 123**	**92.7 ± 30.0**	**470 ± 60.2**
31 μg Cu/L	0.66 ± 0.10	**2.55 ± 0.88**	n.d	n.d	**401 ± 9.75**	**749 ± 63.5**

C. hamatipes reached maximum densities of 20–50 individual per litre in the controls around day 12, after a strong population development during the first two weeks of the exposure phase (Fig. 6C). Similar population development was observed in the other mesocosms except for the 16 and 31 μg Cu/L treatments which had significantly lower densities. Since the populations in all other mesocosms, including the controls, declined rapidly from then on, the differences between treatments disappeared. The densities of the first larval stage (nauplii) of the copepods, not determined to the species level, were significantly higher than the controls on day 28 in treatments 9.9 and 16 μg Cu/L (Fig. 6D). At the highest treatment levels such an indication of treatment induced reproduction was not seen.

3.4. Response of sponges, periphyton and macroalgae

During the first four weeks of the exposure phase the wet weight of the individual sponges increased between 25 and 50% in the all mesocosms. From then on the biomass more or less stabilised until the end of the study in the treatments up to 9.9 μg Cu/L (Fig. 7A). At the higher treatment levels the sponges started to lose weight after day 28 and from day 54 until the end of the study sponge biomass development was statistically significantly reduced in the 16 and 31 μg Cu/L treatments (Table S1 in supporting information). During the final sampling no sponges were alive in the 31 μg Cu/L mesocosms.

The chlorophyll-a fluorescence, indicative of the development of periphyton on the glass slides, steadily increased in the control mesocosms during the study (Fig. 7B). At higher treatment levels this development was reduced, indicating significantly lower chlorophyll-a concentrations in the 9.9, 16 and 31 μg Cu/L treatments on day 48. During the final sampling event the differences between treatments and controls were no longer significant. This was due to a relatively strong increase of the periphyton biomass in the treated mesocosms compared to the controls after day 48, in combination with increasing variation between replicates.

3.5. Response of macroinvertebrates

At the end of the study about 50% of the introduced cockles were recovered alive in the controls and treatments up to 9.9 μg Cu/L. Significant mortality had appeared at treatments 16 and 31 μg Cu/L, with

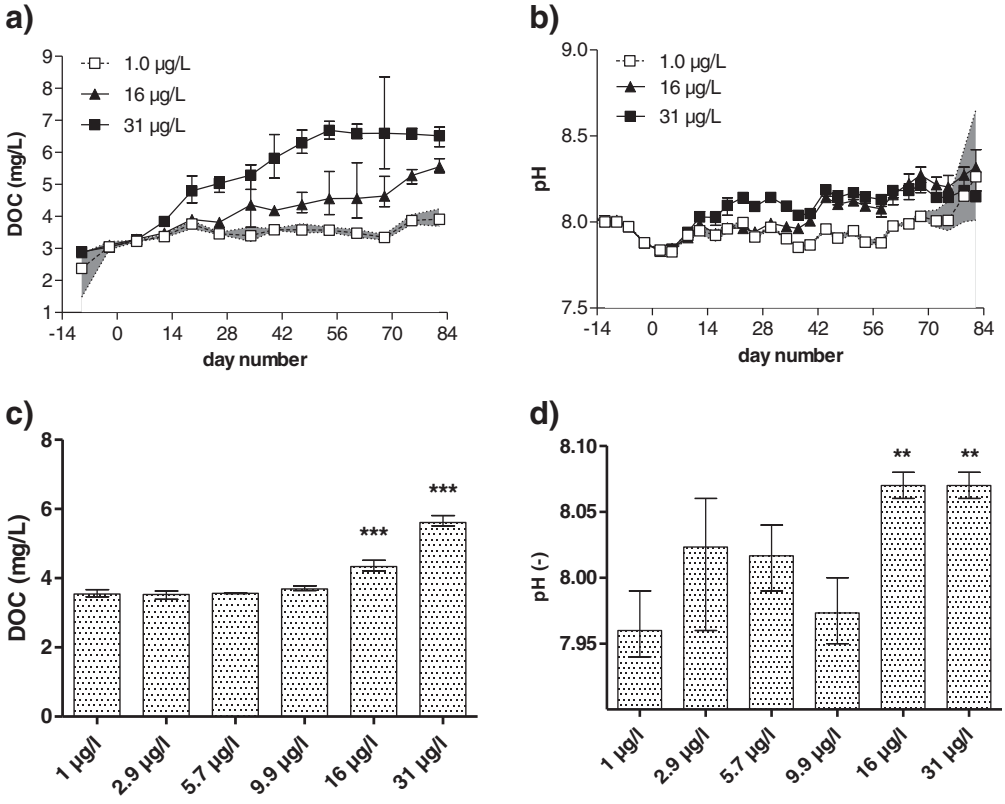


Fig. 2. Concentrations in time of a) dissolved organic carbon (DOC) and b) pH (Presented are mean values and the range of the replicates (error bars) for the controls and treatments showing statistically significant responses. The range of the controls is shown by the shaded area. The complete figures, showing all treatments are available as supporting information.), and mean values of the same parameters after day number 5 until the end of the study (c and d).

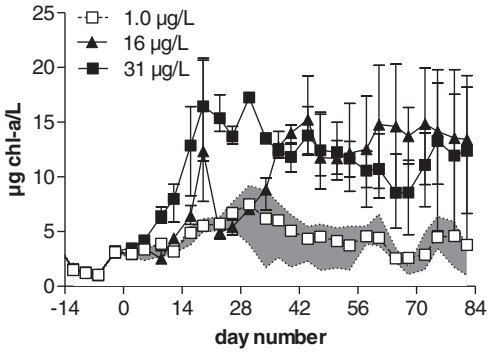


Fig. 3. Chlorophyll-a concentration in the water column during the study. Presented are mean values and the range of the replicates (error bars) for the controls and treatments showing statistically significant responses. The range of the controls is shown by the shaded area. The complete figure, showing all treatments is available as supporting information.

respectively 10 and 0% survival (Fig. 8A). Shell length increased from 24.5 mm at introduction to 26.3 ± 0.3 mm at the end of the study in the controls, and was comparable for all treatments up to 9.9 µg Cu/L. In controls and treatments up to 5.7 µg Cu/L the bivalves had reproduced successfully, as reflected by the presence of 40–60 juveniles per m^2 during the final sampling. At treatment 9.9 µg Cu/L the numbers of juveniles were significantly lower than the controls. At treatment 16 µg Cu/L juveniles were only present in one of the replicates in relatively low numbers, while at 31 µg Cu/L juveniles were completely absent (Fig. 8B).

The shell length of the juvenile cockles in the 2.9 and 5.7 µg Cu/L treatments ranged between 7 and 8 mm, and comparable to the controls. Smaller juveniles were found at higher treatments levels but due to large variation, differences with controls were not significant.

Of the 40 periwinkles introduced at the start of the experiment, between 50 and 75% were recovered alive at the end of the study. This was comparable for all treatments. At introduction the average shell length was 16.3 mm and in the controls and treatments up to 9.9 µg Cu/L, this increased to 17.5 ± 0.3 mm by the end of the study. In the 16 and 31 µg Cu/L treatments the shells were significantly smaller, with an average of 16.6 ± 0.1 mm (Fig. 8C).

No indications were found that the lugworms were affected by the treatments. In all mesocosms between 50 and 75% of the introduced individuals were recovered alive at the end of the study and average ash free dry weight was comparable between controls and treatments. In some of the mesocosms juvenile lugworms were found. However, as they were absent in at least one replicate of each treatment conclusions cannot be drawn. Moreover, the juveniles were only present in low numbers (1–3/m^2), with one exception; in one replicate of the highest treatment level more than 100 juvenile lugworms per m^2 were found.

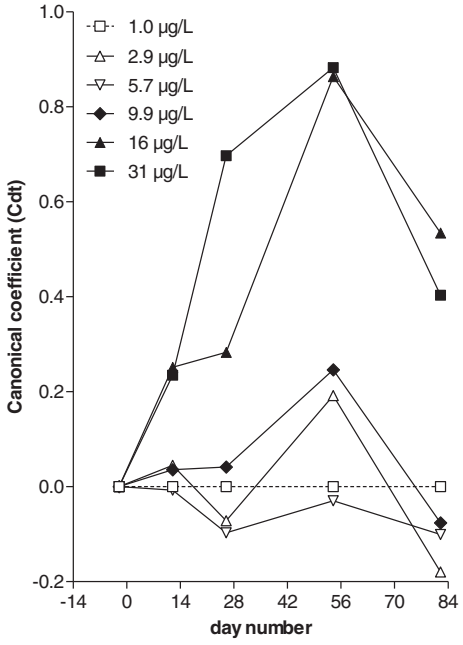
At the end of the study the mudshrimp population in the control mesocosms had reached a population density of over 1000 individuals per m^2 (Fig. 8D). Statistically comparable population densities were found in all treatments with exception of the 2.9 µg Cu/L treatment where the population density was significantly lower than in the controls. The relation with treatment of this observation is not clear since negative effects were not observed at higher exposure concentrations.

Other macroinvertebrates that were found with some frequency during the final sampling were the polychaete species: *Spio* sp., *Scoloplos armiger*, *Pygospio elegans*, *Heteromastus filiformis*, the molluscs *Peringia ulvae*, *Macoma balthica* and *Petricola pholadiformis* and some Isopods and Gammarids that were not further identified. All taxa present in the controls were also observed in the 31 µg Cu/L treatment. Biodiversity and species richness of the benthos community were not significantly affected by the treatments.

4. Discussion

4.1. Mesocosm functioning and effects

During the pre-treatment period all 18 mesocosms showed a highly similar development as indicated by the lack of statistically significant



		species weight
Acartia clausi	Copepoda (zoopl)	-2.15
Copepodites	Copepoda (zoopl)	-1.30
Temora longicornis	Copepoda (zoopl)	-1.28
copepoda nauplii	Copepoda (zoopl)	-1.09
Centropagus hamatipes	Copepoda (zoopl)	-0.91
other diatoms	Diatoms (phytopl)	-0.71
Nitzsia closterium	Diatoms (phytopl)	-0.44
Gastropoda larvae	Gastropoda	-0.41
Polychaeta larvae	Polychaeta	-0.35
Bivalve larvae	Bivalvia	-0.33
macro flagellate	Green algae (phytopl)	-0.30
Nematodes	Nematoda	0.04
other green algae	Green algae (phytopl)	0.17
Harpactoide copepoda	Copepoda (zoopl)	0.17
Peridinium sp.	Green algae (phytopl)	0.56
other cyanobacteria	Cyanobact. (phytopl)	0.83
micro flagellate	Green algae (phytopl)	0.94
medium s. flagellate	Green algae (phytopl)	1.24
Chroococcus turgidus	Cyanobact. (phytopl)	2.09

Fig. 4. Principal Response Curves (PRCs) for the plankton community and the species weight for the different taxa.

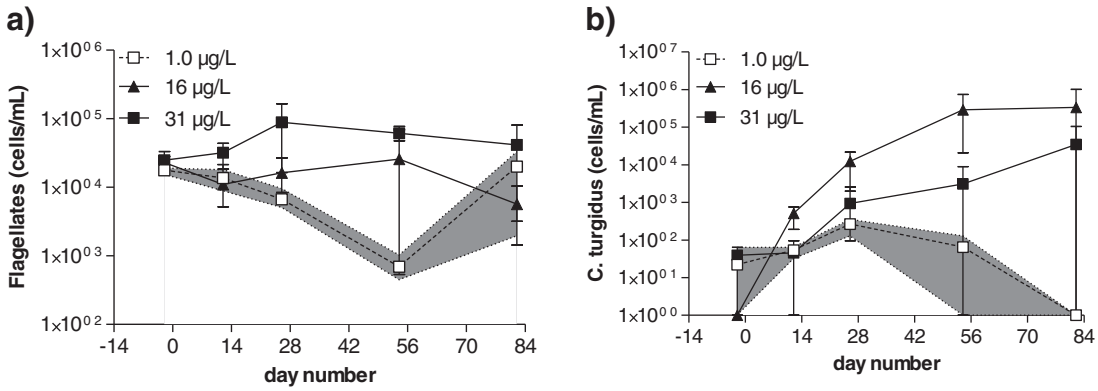


Fig. 5. Density of the a) total flagellates and b) the cyanobacteria *Chroococcus turgidus* in the mesocosms during the study. Presented are mean values and the range of the replicates (error bars) for the controls and treatments showing statistically significant responses. The range of the controls is shown by the shaded area. The complete figures, showing all treatments are available as supporting information.

differences in measured endpoints between the mesocosms at the start of the treatment. After disconnection the triplicate untreated controls developed very similar for the following 82 days. During the 82 days of the exposure phase the concentrations of dissolved copper were successfully maintained at levels that were well replicated within treatments and clearly separated between treatments, thus allowing a sound interpretation of the results obtained.

In the higher treatment levels copper had a clear negative effect on different copepod species, the (juveniles of the) bivalve *Cerastoderma edule*, and on the sponge *H. panicea*. This must have resulted in reduced grazing pressure on the phytoplankton, and hence explains the higher chlorophyll-a concentrations and densities of dominant phytoplankton species that were observed at the higher treatment levels. In particular the zooplankton community with its short generation time, is able to respond rapidly to changes in food (phytoplankton) availability and hence keep the phytoplankton community at a low density level. Toxic (or other) stress on the zooplankton community can thus result in increased phytoplankton densities (Jak et al., 1996).

That primary producers are not completely resistant to high copper levels was indicated by the development of the periphyton on the glass slides that was reduced at higher treatment levels. The periwinkles in these treatments also showed reduced growth rates which might reflect poorer feeding conditions for these gastropods that use periphyton as major food source.

The reduced population density of *Corophium volutator* in only the 2.9 µg Cu/L treatments is remarkable, especially since the population

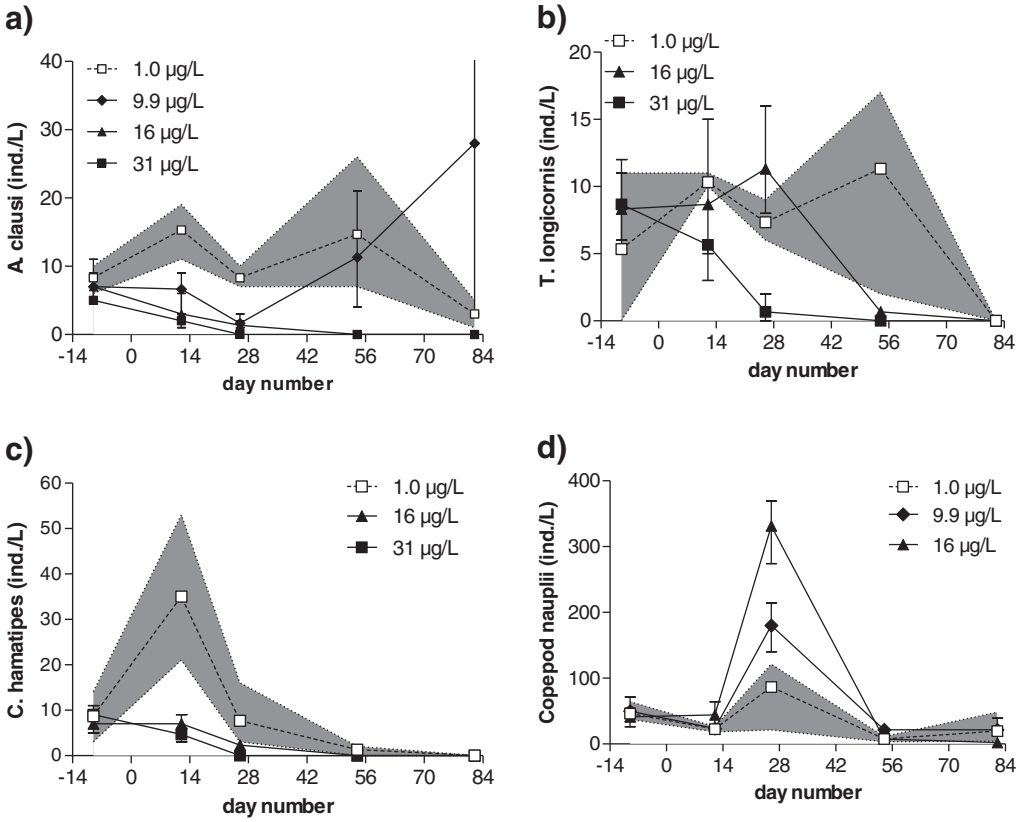


Fig. 6. Density of the dominant copepods in the mesocosms during the study: a) *Acartia clausi*, b) *Temora longicornis*, c) *Centrophagus hamaptipes* and d) copepod nauplii. Presented are mean values and the range of the replicates (error bars) for the controls and treatments showing statistically significant responses. The range of the controls is shown by the shaded area. The complete figures, showing all treatments are available as supporting information.

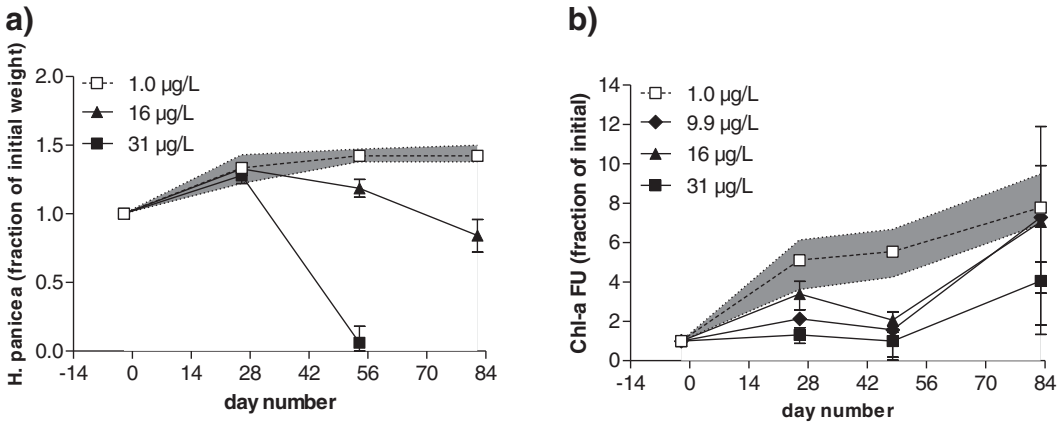


Fig. 7. Relative growth of a) sponges (*Halichondria panicea*; and b) the periphyton community in the mesocosms. Growth was determined as change in biomass (ww) for sponges and change in chlorophyll-a fluorescence op the introduced substrate for periphyton. Presented are mean values and the range of the replicates (error bars) for the controls and treatments showing statistically significant responses. The range of the controls is shown by the shaded area. The complete figures, showing all treatments are available as supporting information.

levels equalled that of the untreated control mesocosms at higher treatment levels. Acute EC_{50} values for *C. volutator* determined in tests with a duration of 96 to 168 h have been reported between 20 and 50 mg Cu/L (various references in Conradi and Depledge, 1998; McPherson and Chapman, 2000). In a 100 day life history experiment with a concentration series of 200 to 1000 µg Cu/L (McPherson and Chapman, 2000), the lowest concentration (200 µg/L) caused a reduction of the individual growth rate of only 2.4%, while at 800 µg/l this was 20%. At 200 µg Cu/L a higher juvenile mortality was observed, but the overall mortality rate of the population was reduced and as a result the life span at the end of the experiment was the same as the control populations. These observations were made at copper concentrations that were much higher than those tested in our mesocosm study and thus indicate that it is unlikely that the reduced mudshrimp population at the 2.9 µg Cu/L treatment is the direct result of copper. Indications that the mudshrimps were indirectly affected are also absent, as none of the endpoints followed in this study were significantly affected by the 2.9 µg Cu/L treatment. Known predators of mudshrimps were not present in the mesocosms and indications of reduced food availability in the 2.9 µg Cu/L treatment, as should be reflected by substantially lower periphyton production, were not found.

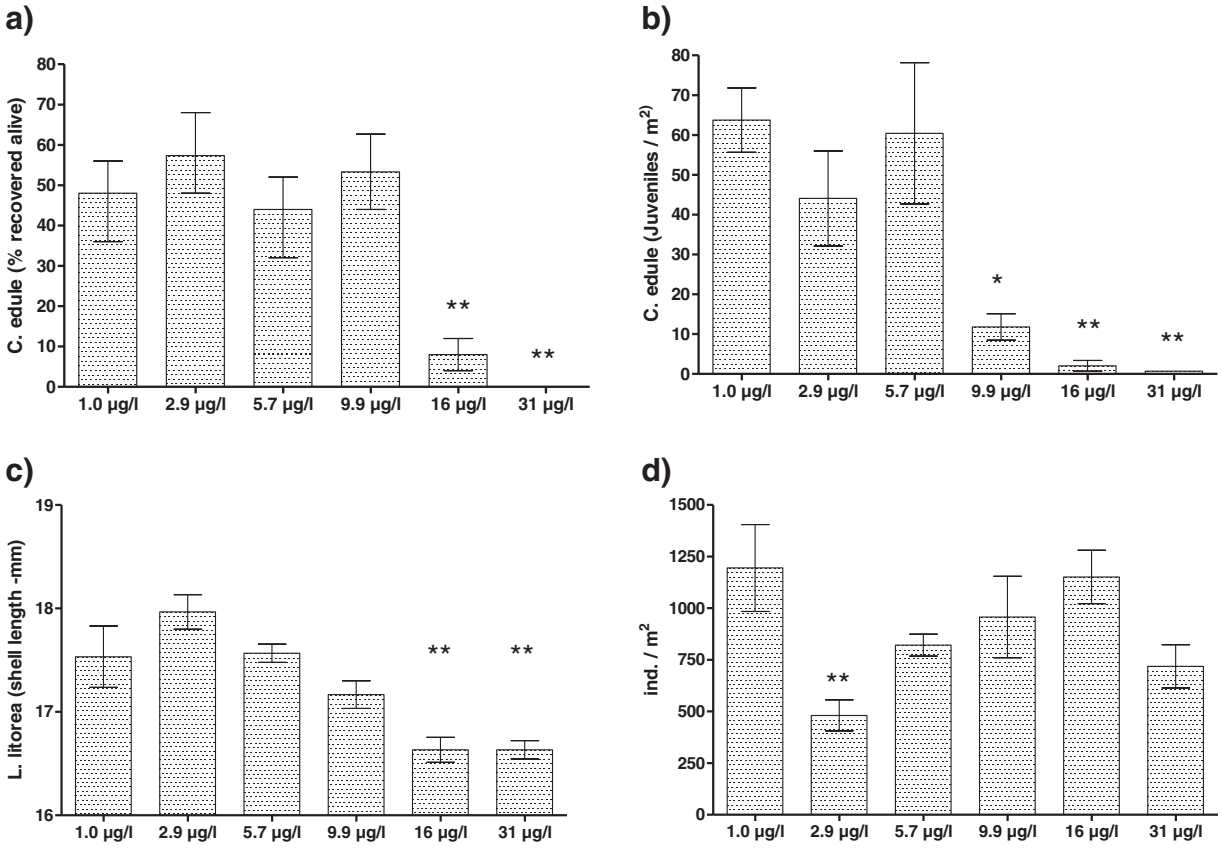


Fig. 8. Status of selected taxa at the end of the study. a) percentage of the introduced adult cockles (*Cerastoderma edule*; that were recovered alive, b) densities of juvenile cockles, c) shell length of periwinkles and d) densities of the mudshrimp Corophium volutator. Presented are the mean values (bar) and the range (error bars) of the measurements per treatment. '*' indicates a significance difference with the controls (1.0 µg/L) with $p < 0.05$; '**' $p < 0.01$.

For these reasons the significantly lower numbers of mudshrimps at the lowest treatment levels were not regarded as an effect of the 2.9 μg Cu/L treatment.

The most sensitive treatment related endpoints in the mesocosms were significantly affected at treatment level 9.9 μg Cu/L (Table S1 in supporting information). Most of these responses were only affected by this treatment level for a short period, such as increased production of copepod nauplii, reduced population density of the copepod *A. clausi* and the reduced periphyton growth rate. Other responses might have a limited ecological impact as is the case for the slight increased nutrient availability and turbidity. However, the reduced recruitment of the cockle is a clear adverse ecological effect. The LOEC that was determined in this mesocosm study is thus 9.9 μg Cu/L, and is based on multiple endpoints at population level (e.g. copepods and cockles) and ecosystem functioning (periphyton growth, nutrient cycle, turbidity).

4.2. Impact on water characteristics related to copper toxicity

As mentioned in the Introduction the bioavailability of the cupric ion (Sunda and Guillard, 1976) is affected by water characteristics and especially by the presence of DOC. The DOC concentration in the 16 and 31 μg Cu/L treatments was significantly higher than the controls and the lower treatment levels. This was most likely related to the phytoplankton density (Sintes et al., 2010), that was elevated in the 16 and 31 μg Cu/L treatments and hence resulted in more DOC release from the phytoplankton. Together with the DOC released from decaying tissues of introduced cockles and sponges that suffered high mortality in the same mesocosms, this probably explains the steadily increasing DOC concentrations in the 16 and 31 μg Cu/L treatments. In addition, it cannot be excluded that the microbial community also suffered from the elevated copper concentrations resulting in reduced microbial carbon demand (Sintes et al., 2010), thus allowing a further building up of the DOC concentrations in the 16 and 31 μg Cu/L treatments.

As a result of the steadily increasing DOC concentration in 16 and 31 μg Cu/L mesocosms the bioavailability of the dissolved copper, must thus have been less here than at lower treatment levels, where DOC concentrations were comparable to the controls.

Besides the DOC concentration, also the pH of the mesocosm water was affected by the response of the phytoplankton community. The increased CO_2 consumption related to the higher phytoplankton density led to the increased pH level of the water column in the 16 and 31 μg Cu/L treatments that became significantly higher than in the controls. This might have further reduced the copper bioavailability, although relative to DOC the impact of pH must have been limited. It must be noted that static mesocosms are very sensitive in showing secondary effects of a stressor, and that the impact of the 16 and 31 μg Cu/L treatments on the water characteristics as mentioned will be less pronounced in a natural situation with water exchange.

In the treatment levels up to 9.9 μg Cu/L pH, DOC and other water characteristics were comparable to the controls. The critical endpoints that set the overall LOEC for the mesocosm study were found in the 9.9 μg Cu/L treatment, thus within this treatment range. Therefore, we conclude that the effect concentrations of these critical endpoints were not affected by exposure related changes in these water characteristics. The LOECs and NOECs derived from this study (Table S1 in supporting information) can thus be considered representative for the water characteristics in the controls and treatment levels up to 9.9 μg Cu/L, with a DOC concentration of 3.6 mg/L (range 3.2–4.5) (Table 4).

Table 4
Mean and range (min.–max.) of selected water characteristics that can affect the toxicity of copper, calculated using the data from individual mesocosms of the controls and treatment levels 2.9; 5.7 and 9.9 μg Cu/L during the exposure phase (days 0–83).

	Salinity (‰)	pH (−)	DOC (mg C/L)	Ca (mg/L)	Mg (mg/L)
Mean ± st.dev.	32.2 ± 0.7	8.0 ± 0.1	3.6 ± 0.3	370 ± 18	1252 ± 50
Range	30.9–33.0	7.8–8.4	3.2–4.5	330–431	1140–1360

4.3. Comparison mesocosm with SSD data

In the data set that was used for the calculation of the Species Sensitivity Distribution and the marine HC5-50 (being the lower 50th percentile of the 95% protection level) for dissolved copper (Van Sprang et al., 2008), two phytoplankton species were most sensitive with NOECs between 4.4 and 5.6 Cu μg/L (normalised at 2 mg DOC/L). In our mesocosms the phytoplankton community did not deliver the most sensitive endpoints; negative effects were only significant for flagellates at the highest treatment level, and total phytoplankton abundance (expressed as chlorophyll-a concentration) increased at higher treatment levels. It is likely that a single species algal growth test performed under optimal conditions in a laboratory is more accurate in determining the impact of a stressor than the more complex multi-species situation in (our) mesocosms. It cannot be excluded that in the mesocosms a net reduced growth rate of the phytoplankton (or specific species) occurred at a certain treatment levels, but that this did not result in lower phytoplankton densities as it was compensated by reduced grazing pressure from the affected zooplankton community.

The next sensitive end point in the SSD data set, concerns the growth of the larvae of the bivalve mollusc *Mytilus edulis* with a NOEC of 6.0 μg Cu/L (data from Redpath, 1985, normalised at 2 mg DOC/L) which is in agreement with the mesocosm results where reproduction success of a bivalve mollusc (Cockle) formed the most sensitive endpoint that sets the overall NOEC for this mesocosm study, being the highest tested concentration that caused no observable effects on any of the measured endpoints at 5.7 μg Cu/L.

The Predicted No Effect Concentration (PNEC) of 5.2 μg Cu/L that was calculated by Van Sprang et al., 2008 as the median HC5-50, was normalised for a DOC concentration of 2 mg/L. In order to compare this value to the effect concentrations from the mesocosm study, it was normalised to the average concentration of 3.6 mg DOC/L from the relevant mesocosms, by using the following formula (Van Sprang et al., 2008): $PNEC_{3.6\ mg\ C/L} = PNEC_{2\ mg\ C/L} * (3.6/2)^{0.6136}$. When normalised to this DOC level, the PNEC calculated by Van Sprang et al. is 7.5 μg Cu/L (range 6.9–8.6). This is an intermediate concentration between the No Observed Effect Concentration (NOEC: 5.7 μg Cu/L) and the Lowest Observed Effect Concentration (LOEC: 9.9 μg Cu/L) from the mesocosm study, which indicates that the protection level calculated from the SSD was also valid for the ecosystem tested in the mesocosms. This provides some confidence in the validity of the PNEC calculated from the Species Sensitivity Distribution for dissolved copper as presented by Van Sprang et al. (2008).

5. Conclusions

The overall NOEC from this mesocosm study with more than 80 days actively maintained dissolved copper exposure of a marine soft sediment benthic ecosystem was 5.7 μg Cu/L (LOEC 9.9 μg Cu/L) at a DOC concentration of 3.6 mg/L (range 3.2–4.5). This is in line with the marine PNEC (HC5-50) of 5.2 μg Cu/L, normalised for 2 mg DOC/L that is based on a species sensitivity distribution of 24 marine single species laboratory tests covering 8 taxonomic groups.

This study shows that (marine) mesocosms, where simple ecosystems are tested for a prolonged period, can be a useful tool for the validation (or correction) of permitted maximum concentrations (PMC) that are often derived from data obtained by relatively short term single species laboratory tests to which is applied a safety factor. In parallel with the risks assessments procedures for agricultural chemicals, results from (marine) mesocosm studies can help authorities in the reduction of the uncertainty when setting a PMC for other substances.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.03.086.

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