Accepted Manuscript

Title: Hydrostatic pressure and temperature affect the tolerance of the free-living marine nematode *Halomonhystera disjuncta* to acute copper exposure

Authors: Lisa Mevenkamp, Alastair Brown, Chris Hauton, Anna Kordas, Sven Thatje, Ann Vanreusel

PII: S0166-445X(17)30264-3

DOI: http://dx.doi.org/10.1016/j.aquatox.2017.09.016

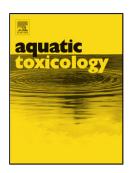
Reference: AQTOX 4748

To appear in: Aquatic Toxicology

Received date: 31-7-2017 Revised date: 13-9-2017 Accepted date: 19-9-2017

Please cite this article as: Mevenkamp, Lisa, Brown, Alastair, Hauton, Chris, Kordas, Anna, Thatje, Sven, Vanreusel, Ann, Hydrostatic pressure and temperature affect the tolerance of the free-living marine nematode Halomonhystera disjuncta to acute copper exposure. Aquatic Toxicology http://dx.doi.org/10.1016/j.aquatox.2017.09.016

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



Hydrostatic pressure and temperature affect the tolerance of the free-living marine nematode *Halomonhystera disjuncta* to acute copper exposure

Lisa Mevenkamp^{a*}, Alastair Brown^b, Chris Hauton^b, Anna Kordas^a, Sven Thatje^b, Ann Vanreusel^a

- ^a Marine Biology Research Group, Ghent University, Krijgslaan 281 S8, 9000 Ghent, Belgium
- ^b University of Southampton, Ocean and Earth Science, National Oceanography Centre Southampton, European Way, Southampton, SO14 3ZH, UK
- *Corresponding author: Lisa Mevenkamp, <u>lisa.mevenkamp@ugent.be</u>

Highlights

- Toxicity assessment in the deep sea requires a mechanistic understanding of metal toxicity under high pressure and low temperature
- LC₅₀ tests with varying pressure and temperature were performed on the intertidal nematode *Halomonhystera disjuncta*
- High hydrostatic pressure (10 MPa) and low temperature (10 °C) reduced acute copper toxicity in *H. disjuncta*

Abstract

Potential deep-sea mineral extraction poses new challenges for ecotoxicological research since little is known about effects of abiotic conditions present in the deep sea on the toxicity of heavy metals. Due to the difficulty of collecting and maintaining deep-sea organisms alive, a first step would be to understand the effects of high hydrostatic pressure and low temperatures on heavy metal toxicity using shallow-water relatives of deep-sea species. Here, we present the results of acute copper toxicity tests on the free-living shallow-water marine nematode *Halomonhystera disjuncta*, which has close phylogenetic and ecological links to the bathyal species *Halomonhystera hermesi*. Copper toxicity was assessed using a semi-liquid gellan gum medium at two levels of hydrostatic pressure (0.1 MPa and 10 MPa) and temperature (10 °C and 20 °C) in a fully crossed design. Mortality of nematodes in each treatment was assessed at 4 time intervals (24 and 48 h for all experiments and additionally 72 and 96 h for experiments run at 10 °C). LC₅₀ values ranged between 0.561 and 1.864 mg Cu²⁺ L⁻¹ and showed a decreasing trend with incubation time. Exposure to high hydrostatic pressure significantly increased sensitivity of nematodes to copper, whereas lower

temperature resulted in an apparently increased copper tolerance, possibly as a result of a slower metabolism under low temperatures. These results indicate that hydrostatic pressure and temperature significantly affect metal toxicity and therefore need to be considered in toxicity assessments for deep-sea species. Any application of pollution limits derived from studies of shallow-water species to the deep-sea mining context must be done cautiously, with consideration of the effects of both stressors.

Keywords: Hydrostatic pressure; LC₅₀; Copper toxicity; *Halomonhystera disjuncta*; Deep-sea mining; Gellan Gum

1. Introduction

Economically valuable mineral deposits can be found in a variety of deep-sea habitats such as abyssal plains (polymetallic nodules and deep-sea muds), active and extinct hydrothermal vents (seafloor massive sulphides) or seamounts (ferromanganese crusts) (Hein et al., 2013; Petersen et al., 2016; Sterk and Stein, 2015). The extraction of these mineral deposits may cause significant disturbances of these remote and ecologically valuable habitats, threatening their biological communities (Vanreusel et al., 2016). Despite significant international attention, ecosystems of these areas are poorly studied and mechanisms of resilience and recovery of the benthic fauna are largely unknown (Gollner et al., 2017; Wedding et al., 2015). One major concern is the mobilization and release of elevated concentrations of potentially toxic elements during extraction, transport in riser systems, or after processing of the minerals (e.g. the release of extraction water or tailings to the water column) (Boschen et al., 2013; Koschinsky et al., 2001a, 2001b; Thiel, 2001). Heavy metal concentrations are usually higher within marine sediments than in the overlying water column as heavy metals bind to small particles, organic matter and different hydroxides (Pempkowiak et al., 1999). Infaunal organisms are, therefore, particularly vulnerable to metal exposure if conditions in sediment or surrounding seawater change (e.g. pH, oxygen saturation) and bioavailability of those metals increases. The development of appropriate measures to identify risk requires knowledge of the impacts of heavy metal contamination on deep-sea benthic organisms. However, the acquisition and maintenance of deep-sea organisms is challenging, hampering their use in controlled laboratory experiments. As a first step towards understanding heavy metal toxicity in the deep sea researchers are advised to uncover the effects of abiotic factors such as high hydrostatic pressure and low temperatures on the sensitivity of marine species (Mestre et al., 2014). These two factors play major roles in determining the distribution of marine organisms (Brown and Thatje, 2011; Clarke, 2003; Pörtner, 2002; Pradillon and Gaill, 2007). Knowledge of pressure and temperature effects on metal toxicity would help us to better understand underlying mechanisms and possibly predict potential toxic effects in deep-sea species.

Copper is a trace element that is essential to the health of most organisms (Mertz, 1981). It plays a role in multiple physiological pathways (e.g. in regulating oxidative stress), as a cofactor of several enzymes or structural components and is also associated with biological processes such as responses to hypoxia (Karlin and Tyeklár, 2012; Scheiber et al., 2013). However, an excess of copper can induce severe toxicity leading to metabolic dysfunction and ultimately to the death of an organism (Gaetke and Chow, 2003; Scheiber et al., 2013). Deep-sea minerals contain relatively high concentrations of copper (Hein et al., 2013) and it has been demonstrated that the potential for copper leaching from deep-sea minerals such as chalcopyrite is high (Fallon et al., 2017; Knight and Roberts, 2016). However, Simpson and Spadaro (2016) have recently reported limited toxicity of chalcopyrite-induced, copper-associated mortality in bivalves and amphipods. The relative high importance of copper in deep-sea mineral extraction and its important role in animal physiology support the need to explore the effects of hydrostatic pressure and temperature on copper toxicity.

Intermediate in size between micro- and macrofauna, metazoan meiobenthos play a major role in the benthic ecosystem as an important component of the benthic food-web, but also through facilitating mineralization and nutrient turnover (Bonaglia et al., 2014; Coull, 1999; Moens et al., 2013). Nematodes are the dominant taxon within this group of organisms and their short life span and high fecundity also make them suitable for laboratory experiments and short-term ecotoxicological research in particular (Beyrem et al., 2011; Kennedy and Jacoby, 1999). The tolerance of nematodes to metal toxicity, hypoxia and changing environmental conditions can be very variable and species-dependent (Bongers and Ferris, 1999; Gyedu-Ababio and Baird, 2006). Halomonhystera disjuncta is a free-living, bacterivorous shallow-water marine nematode which is known for its tolerance to temperature changes and high concentrations of heavy metals (Vranken et al., 1989, 1988, 1985, 1984). The intertidal, cryptic species Halomonhystera disjuncta GD1 (Derycke et al., 2007) is phylogenetically closely related to the species *H. hermesi* (Tchesunov et al., 2014) which inhabits cold-seep ecosystems in the deep sea, e.g. the Nyegga pockmark at 730 m on the Nordic Norwegian margin and the Håkon Mosby mud volcano at 1280 m depth in the Barents Sea (Van Campenhout et al., 2015, 2013; Van Gaever et al., 2006). Interestingly, H. disjuncta GD1 also shows higher tolerance towards bathyal seep conditions (high sulphide concentrations, low temperature) than other species in the cryptic species complex (Van Campenhout et al., 2014). The close phylogenetic relationship and H. disjuncta GD1's environmental tolerances suggest that *H. disjuncta* and *H. hermesi* share a recent common ancestor (Van Campenhout et al., 2015, 2014, 2013), making H. disjuncta a relevant species with which to investigate the effects of bathyal environmental conditions on copper toxicity.

In this study, we performed the first acute copper toxicity tests on the free-living marine nematode *H. disjuncta* incorporating different hydrostatic pressure and temperature regimes. The use of gellan gum as a medium for nematode toxicity testing has been described by Brinke et al. (2011) and was chosen for this study to facilitate the use of pressure chambers under the exclusion of air cavities. In comparison to water, gellan gum provides the advantage that the sediment dwelling nematodes are still able to move through the medium by body undulations but with lower activity and stress than would result from constant swimming in water. We investigated the acute effects of bathyal pressure experienced by H. hermesi on copper toxicity in H. disjuncta by including two pressures (0.1 MPa= surface pressure, and 10 MPa ≈ 1000 m water depth) and two temperatures (20°C and 10°C). Here, 20°C represents a standard temperature for toxicity testing that has been applied in previous acute toxicity studies on marine nematodes including H. disjuncta (Austen and McEvoy, 1997; Vranken et al., 1984; Vranken and Heip, 1986) whereas 10 °C is at the lower end of the optimal temperature range of the species, thus allowing normal growth and development (Van Campenhout et al., 2014). With this study we aim to investigate 1) the effect of high hydrostatic pressure on the survival of a shallow-water nematode and 2) the extent to which temperature and hydrostatic pressure affect copper toxicity in the shallow-water nematode.

2. Material and Methods

2.1. Nematode cultures

Monospecific cultures of *H. disjuncta* cryptic species GD1 were cultivated at 16 °C on petri dishes filled with 0.8 % nutrient:bacto agar in a ratio of 1:7 prepared in artificial seawater (Moens and Vincx, 1998) with a salinity of 25. The cultures were incubated at the respective experimental temperature one week prior to the experiment. An excess of frozen-and-thawed *Escherichia coli* K12 were added as a food source. A full description of species acquisition for the cultures is given in Van Campenhout et al. (2014).

2.2. Experimental setup

Nematodes of the species *H. disjuncta* GD1 were exposed to five different copper (Cu²+) concentrations at two different temperatures (10 °C and 20 °C) and two different pressures (0.1 MPa and 10 MPa) for 2 time intervals (24 h, 48 h) with 3 replicates per time interval and treatment. In addition, experiments at 10 °C were also run for 72 h and 96 h. Selection of dissolved copper concentrations at 10 °C (0, 0.5, 1, 2, 4, 6 mg Cu²+ L⁻¹) and 20 °C (0, 0.2, 0.5, 1, 2, 5 mg Cu²+ L⁻¹) were based on preliminary ranging experiments at atmospheric pressure. Survival was the chosen endpoint.

Screw top vials of 5 mL volume with a rubber septum were half filled with Cu²⁺-contaminated gellan gum and 20 adult and preadult nematodes were placed in the vials. The vials were

then filled up with the Cu²⁺-contaminated gellan gum medium and closed, ensuring that no air bubbles were trapped. One vial (empty control) without nematodes was added to each replicate measurement. Vials were placed in a pressure vessel, acutely pressurised, and incubated at the respective pressure and temperature for the respective time intervals (24, 48, 72 and 96 hours). A detailed description of the pressure vessels can be found in Mestre et al. (2009). Vials of all treatments, including those at surface pressure, were placed in pressure vessels to avoid any experimental artefacts arising from enclosure in the pressure vessel.

The semi-liquid gellan gum medium was made with 1.5 g L⁻¹ gelrite (Merck & Co., Kelco Division) solution prepared in MilliQ water and artificial seawater (Moens and Vincx, 1998) with a salinity of 34. The two components were autoclaved and the gellan gum solution was slowly added to the seawater in a 1:3 ratio under continuous stirring to obtain the required fluidity and salinity of 25. Sufficient volumes of medium were spiked with different dissolved copper (Cu²⁺) concentrations by adding the appropriate amount of CuSO₄ stock solution to the medium under continuous stirring for ~2 minutes. The stock solution was composed of 0.10155 g CuSO₄ and 250 mL MilliQ water resulting in a dissolved Cu²⁺ concentration of 259.66 mg L⁻¹.

At the end of each experiment, hydrostatic pressure vessels were immediately depressurised and oxygen levels in the middle of the vials were measured with an oxygen optode connected to a PreSens Microx TX3 array. Nematode mortality was assessed by observing movement through a stereo-microscope and/or response to physical stimulation with a needle.

Unavoidable bacterial contamination of the medium and nematode respiration led to a decrease of oxygen concentrations in the vials, especially at high temperatures. Based on the oxygen measurements we adjusted our experimental setup and only conducted 24 h and 48 h treatments at 20 °C, however, these particular treatment combinations were repeated once with a full set of 3 replicates. Furthermore, data analysis was adjusted by removing treatments where very low oxygen concentrations (<5 %) persisted in most vials at low copper concentrations in combination with an increased mortality of animals in those vials (Tab. S1). Potential oxygen-associated background mortality at zero copper concentration was accounted for in the data analysis. Oxygen deficiency and mortality occurred in all replicates of the following treatments: 20 °C, 0.1 MPa, 24 h (first measurement); 20 °C, 10 MPa, 24 h (first measurement); 20 °C, 0.1 MPa, 48 h (second measurement) and 20 °C, 10 MPa, 48 h (second measurement) (Tab. S1). Therefore, one set of replicates at each pressure at 24 and 48 h was retained in the analysis.

2.3. Data analysis

LC₅₀ values and their confidence intervals were estimated from concentration response curves based on the three replicates of each treatment, fitted with a binomial regression using a probit link and adjusting for background mortality as explained by Proctor et al. (2017). The models of the concentration-response curves were then compared by Analysis of Deviance (chi-squared) which allows comparison of generalized linear models comparable to variance testing in ANOVA (Nelder and Wedderburn, 1972). Additionally, concentration-response curves were fitted with the very similar log-normal function with fixed upper (1) and lower (0) limits (LN.2) using the *drc* package. This package allows for comprehensive visualization of the models. All data were analysed with the statistical software R version 3.4.0 Patched (R Core Team, 2013) and RStudio version 1.0.136 (RStudio Team, 2015) using the packages *LC50* (Proctor and Wotherspoon, 2015) and *drc* (Ritz et al., 2015). A significance level of α=0.05 was chosen for all tests.

3. Results

At the end of each experiment, nematode behaviour and motility in treatments without Cu²⁺ contamination appeared unaffected by compression and decompression. Surviving animals from high pressure treatments appeared and behaved similarly to those under atmospheric pressure.

 LC_{50} values ranged between 0.561 μ g L^{-1} (10 MPa, 10 °C after 72 h) and 1.864 mg L^{-1} (0.1 MPa, 10 °C, after 24 h) and showed a decreasing trend with incubation time (Fig. 1). Pressure and temperature significantly affected nematode sensitivity towards copper at the 24 h and 48 h time intervals, without an interaction effect (Tab. S2): nematode sensitivity in terms of LC_{50} was greater at 10 MPa than at 0.1 MPa and was greater at 20 °C than at 10 °C (Fig. 1). The negative effect of high pressure was still visible after 72 h of incubation but not at 96 h (Tab. 1).

Table 1 Results of the Analysis of Deviance comparing dose-response models of varying factors pressure (0.1 and 10 MPa) and temperature (10 and 20 °C). DF= degrees of freedom, NULL= null model (no variables included), Pr(>Chi)= probability of the model being different from the null model.

| | | DF | Deviance | Residual DF | Residual Deviance | Pr (>Chi) | |
|------|----------------------|----|----------|----------------|----------------------|-----------|-----|
| 24 h | NULL | | | 63 | 233.8 | | |
| | Temperature | 1 | 66.838 | 62 | 166.96 | 2.95E-16 | *** |
| | Pressure | 1 | 24.956 | 61 | 142 | 5.87E-07 | *** |
| 1 | Temperature*Pressure | 1 | 0.408 | 60 | 141.6 | 0.5231 | |
| 48 h | NULL | | | 63 | 156.3 | | |
| | Temperature | 1 | 30.683 | 62 | 125.62 | 3.04E-08 | *** |
| | Pressure | 1 | 12.564 | 61 | 113.05 | 3.93E-04 | *** |

| | Temperature*Pressure | 1 | 0 | 60 | 113.06 | 1 | |
|------|----------------------|---|--------|----|---------|----------|-----|
| 72 h | NULL | | | 31 | 143.838 | | |
| | Pressure | 1 | 79.983 | 30 | 63.855 | 2.20E-16 | *** |
| 96 h | NULL | | | 31 | 55.105 | | |
| | Pressure | 1 | 1.1235 | 30 | 53.982 | 0.2892 | |

^{***} Significant at the p ≤ 0.001 probability level

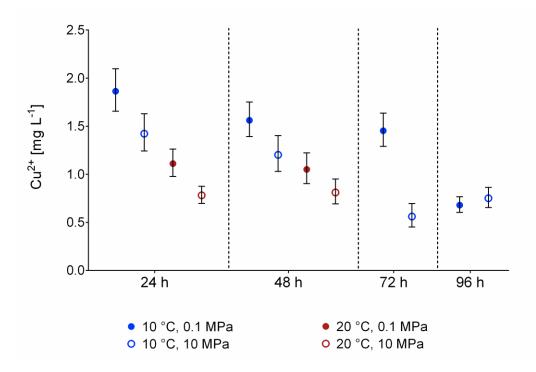


Figure 1 Mean LC_{50} values with upper and lower confidence intervals (error bars) for all experiments conducted in a fully crossed design with two varying factors (temperature and pressure), N=3.

In addition to differences in the LC_{50} , the slope of the concentration-response curve was slightly flattened when high hydrostatic pressure was applied (Fig 2., Tab. S2). A flattened slope indicates that the range of concentrations that is harmful for part of the population is larger whereas a steep slope is an indication for a sharp threshold level where passing the threshold leads to a strong increase in toxicity producing lower survival.

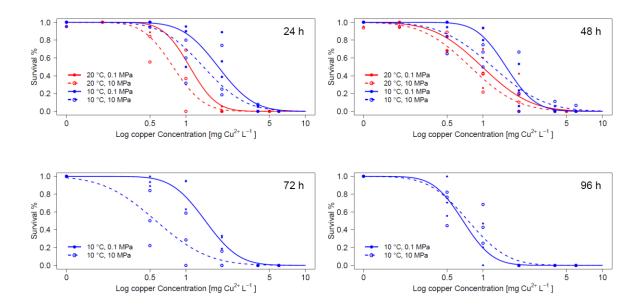


Figure 2 Concentration-response curves (log-normal models) of the copper toxicity tests at differing pressure (0.1 and 10 MPa) and temperature (10 and 20 °C) for 4 different time intervals. Symbols represent the individual replicate measurements of nematode survival.

4. Discussion

4.1. Effects of hydrostatic pressure

Hydrostatic pressure strongly determines the depth distribution of marine species (Brown and Thatje, 2014). This is a result of evolutionary adaptation of marine organisms to the adverse effects of high hydrostatic pressure on molecular interactions, e.g. with regard to electrochemical and hydrophobic interactions in biological systems (Brown and Thatje, 2014; Pradillon and Gaill, 2007). Nevertheless, shallow-water marine invertebrates seem to have a wider tolerance to increased hydrostatic pressure than might be predicted *a priori* (Brown and Thatje, 2014; Mestre et al., 2013, 2009; Smith et al., 2015) and are able to increase their tolerance after a short acclimation period (New et al., 2014). Similarly, in this study, mortality and activity of *H. disjuncta* in treatments without Cu²⁺ contamination remained unaffected by elevated pressure. Therefore, mortality associated with pressurization and depressurization was not an interfering factor in the assessment of copper toxicity. However, while the nematodes were tolerant in the short-term, negative effects of high hydrostatic pressure on the nematodes may arise with longer exposures.

In this study, high hydrostatic pressure reduced the tolerance of *H. disjuncta* to copper exposure at both 10 and 20 °C, as evidenced by lower LC₅₀ values and a flattened slope compared to surface pressure (Fig. 2). The flattened slope of the concentration-response curves at high hydrostatic pressure suggests that a wider range of concentrations adversely affects part of the population. Therefore, even low copper concentrations led to a weakening of the nematodes and mortality of several individuals at 10 MPa. Interestingly, the effect of

pressure was not evident after an incubation time of 96 h which may be caused by interfering factors such as starvation, reducing the organisms' tolerance to additional stress. Research has indicated that starvation effects can become evident after 72-120 h in laboratory experiments with marine nematodes (Ott and Schiemer, 1973; Wieser et al., 1974).

Previous research on copper uptake in nematodes has found a strong association of increased amounts of copper concentrations in the cuticle and hypodermis, supporting an uptake via the body wall rather than the digestive tract (Howell, 1983; Sávoly et al., 2013). Changes in hydrostatic pressure lead to a shift in biochemical reaction rates and in the fluidity of membranes (Brown and Thatje, 2014; Pradillon and Gaill, 2007) which may in turn affect uptake rates of copper from the environment. Copper toxicity is mainly caused by accumulation of oxidative damage resulting from reactive oxygen species in the cells, and organisms may respond to this by enhancing antioxidant enzyme expression (Song et al., 2014). This, however, will increase basal metabolic rate and may have consequences for the energy allocation of the organisms (Sokolova and Lannig, 2008).

Brown et al. (2017a) assessed the effects of low temperature and high hydrostatic pressure on acute (96 h) lethal and sublethal (respiration rate, antioxidant enzyme activity) copper and cadmium toxicity in the shallow-water shrimp *Palaemon varians*. In this study the researchers report that oxygen consumption and antioxidant enzyme activity (superoxide dismutase, glutathione peroxidase) were significantly increased at lower copper concentrations when a pressure of 10 MPa was applied (100 µg L⁻¹) than when at surface pressure (1000 µg L⁻¹). The exact mechanisms of the effect that pressure has on copper toxicity (e.g. enhanced copper uptake through membranes, inhibition of gene expression) still remain to be investigated, but the results of Brown et al. (2017a) suggest that enzyme expression and activity were not suppressed by increased hydrostatic pressure.

Deep-sea organisms have been shown to possess a series of adaptations to counteract the negative effects of hydrostatic pressure on their metabolism (Pradillon and Gaill, 2007; Somero, 2003; Yancey et al., 2004). Nevertheless, it remains to be investigated if these adaptations also enable them to counteract the negative effect of pressure on copper toxicity. This may be answered once our mechanistic understanding of copper toxicity in marine invertebrates and, more specifically in deep-sea invertebrates, improves.

4.2. Effects of temperature

Temperature also affected copper tolerance in *H. disjuncta* and survival of nematodes was higher at 10 °C than at 20 °C. *H. disjuncta*'s optimal growth temperature is approximately 16 °C, but most life history traits do not vary considerably when temperature is reduced to 10 °C (Van Campenhout et al., 2014). Therefore, a temperature of 10 °C can be considered to

lie within the species' thermal window. The metabolism of an organism is an interplay of different biochemical reactions performed by multiple enzymes (Brown et al., 2004). These metabolic reactions obey the laws of thermodynamics and increase exponentially with increasing temperatures inside the temperature range of normal activity (Brown et al., 2004; Clarke and Fraser, 2004). Therefore, the higher tolerance of *H. disjuncta* towards copper at 10 °C compared to 20 °C may be attributed to a slower metabolism at low temperatures leading to a decreased copper uptake, which delays reaching lethal systemic metal concentration. This is consistent with previous research stating that in most ectotherms (80% of N=118 investigated species) an increased temperature enhanced metal toxicity in terms of mortality and uptake (Sokolova and Lannig, 2008; and references therein). Indeed, chromium toxicity in *H. disjuncta* was increased when temperature was high (22 °C) compared to optimal temperature (17 °C) and mortality was reduced at low temperature (12 °C) (Vranken et al., 1989).

Although deep-sea animals have adapted to very stable and low temperatures (typically 4 °C), enzymatic reactions and, consequently, metabolic rate of stenothermal species living at <8 °C is lower than in eurythermal organisms (Pörtner, 2002). Therefore, the observed lower acute effects of copper toxicity at low temperature may be similar in deep-sea organisms. However, additional stress responses induced by copper exposure at low, sublethal concentrations may increase basal metabolic maintenance and reduce the amount of energy available for other functions, reducing the organisms' fitness (Brown et al., 2017a; Sokolova et al., 2012). This may especially be of relevance in the food and energy limited abyssal deep sea (Smith et al., 2008). Chronic effects considerably differ from acute exposures and sublethal copper concentrations have been shown to substantially affect life-cycle characteristics (Bechmann, 1994; Kwok et al., 2008). This, in turn, may have unknown consequences for ecosystem functioning and the assessment of chronic toxicity effects should, therefore, be considered in future studies.

Temperature and hydrostatic pressure have antagonistic properties with regard to kinetics and equilibria in biological systems e.g. as pressure increases, it reduces the flexibility of lipids and nucleic acids while the opposite is true for temperature increases (Pradillon and Gaill, 2007 and citations therein). However, the results of this study indicate that pressure and temperature act in different ways on the sensitivity of *H. disjuncta* to copper since a simultaneous increase in both, pressure and temperature lead to an increase in copper sensitivity.

4.3. Conclusion

Our research shows that increased hydrostatic pressure and temperature reduce the sensitivity of *H. disjuncta* to acute copper exposure in terms of mortality. An integrative

approach of laboratory experiments using shallow-water species under controlled conditions in combination with *in situ* deep-sea experiments using related species is, therefore, crucial to fully understand ecotoxicology in the deep sea (Brown et al., 2017b). Nevertheless, the use of shallow-water species helps to elucidate general mechanisms of both factors on copper toxicity in marine nematodes.

As an acute toxicity assessment with only one toxicant tested, these results are not intended to assess the specific risks of deep-sea mineral extraction, but they provide evidence for effects of hydrostatic pressure and temperature on copper toxicity that need to be considered in environmental risk assessment. *In situ* studies including realistic multiple metal exposures are needed to produce environmentally relevant data and enable proper risk assessment. Furthermore, experiments with longer exposures would enable investigations of effects of chronic exposures to toxicants, which may pose greater risks for organisms in the long term and which cannot be assessed in acute toxicity studies (Freitas and Rocha, 2014).

Acknowledgements

AV, CH, ST and AB supported LM in conceiving of the study and executing it. LM, AB and AK conducted the experimental work. LM analysed the data and wrote the manuscript with input from all authors. The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under the MIDAS project, grant agreement n° 603418. This publication reflects only the views of the authors; the EC is not liable for any use that may be made of the information contained herein. LM received funding by the Flemish BOF research fund (BOF.DC1.2016.0006) and the UGent - BOF GOA project "Assessing the biological capacity of ecosystem resilience" (BOFGOA2017000601). The authors thank Luana Da Costa Monteiro and Jelle Van Campenhout for their valuable input in designing the experiments.

References

- Austen, M.C., McEvoy, A.J., 1997. The use of offshore meiobenthic communities in laboratory microcosm experiments: response to heavy metal contamination. J. Exp. Mar. Biol. Ecol. 211, 247–261.
- Bechmann, R.K., 1994. Use of life tables and LC50 tests to evaluate chronic and acute toxicity effects of copper on the marine copepod *Tisbe furcata* (Baird). Environ. Toxicol. Chem. 13, 1509–1517. doi:10.1002/etc.5620130913
- Beyrem, H., Boufahja, F., Hedfi, A., Essid, N., Aïssa, P., Mahmoudi, E., 2011. Laboratory study on individual and combined effects of cobalt-and zinc-spiked sediment on meiobenthic nematodes. Biol. Trace Elem. Res. 144, 790–803.
- Bonaglia, S., Nascimento, F.J.A., Bartoli, M., Klawonn, I., Brüchert, V., 2014. Meiofauna increases bacterial denitrification in marine sediments. Nat. Commun. 5. doi:10.1038/ncomms6133

- Bongers, T., Ferris, H., 1999. Nematode community structure as a bioindicator in environmental monitoring. Trends Ecol. Evol. 14, 224–228.
- Boschen, R.E., Rowden, A.A., Clark, M.R., Gardner, J.P.A., 2013. Mining of deep-sea seafloor massive sulfides: A review of the deposits, their benthic communities, impacts from mining, regulatory frameworks and management strategies. Ocean Coast. Manag. 84, 54–67. doi:10.1016/j.ocecoaman.2013.07.005
- Brinke, M., Heininger, P., Traunspurger, W., 2011. A semi-fluid gellan gum medium improves nematode toxicity testing. Ecotoxicol. Environ. Saf. 74, 1824–1831. doi:10.1016/j.ecoenv.2011.07.007
- Brown, A., Thatje, S., 2014. Explaining bathymetric diversity patterns in marine benthic invertebrates and demersal fishes: physiological contributions to adaptation of life at depth. Biol. Rev. 89, 406–426. doi:10.1111/brv.12061
- Brown, A., Thatje, S., 2011. Respiratory response of the deep-sea amphipod Stephonyx biscayensis indicates bathymetric range limitation by temperature and hydrostatic pressure. PloS One 6, e28562.
- Brown, A., Thatje, S., Hauton, C., 2017a. The effects of temperature and hydrostatic pressure on metal toxicity: Insights into toxicity in the deep sea. Environ. Sci. Technol. doi:10.1021/acs.est.7b02988
- Brown, A., Wright, R., Mevenkamp, L., Hauton, C., 2017b. A comparative experimental approach to ecotoxicology in shallow-water and deep-sea holothurians suggests similar behavioural responses. Aquat. Toxicol. doi:10.1016/j.aquatox.2017.06.028
- Brown, J.H., Gillooly, J.F., Allen, A.P., Savage, V.M., West, G.B., 2004. Toward a metabolic theory of ecology. Ecology 85, 1771–1789. doi:10.1890/03-9000
- Clarke, A., 2003. Costs and consequences of evolutionary temperature adaptation. Trends Ecol. Evol. 18, 573–581. doi:10.1016/j.tree.2003.08.007
- Clarke, A., Fraser, K.P.P., 2004. Why does metabolism scale with temperature? Funct. Ecol. 18, 243–251. doi:10.1111/j.0269-8463.2004.00841.x
- Coull, B.C., 1999. Role of meiofauna in estuarine soft-bottom habitats. Aust. J. Ecol. 24, 327–343. doi:10.1046/j.1442-9993.1999.00979.x
- Derycke, S., Backeljau, T., Vlaeminck, C., Vierstraete, A., Vanfleteren, J., Vincx, M., Moens, T., 2007. Spatiotemporal analysis of population genetic structure in *Geomonhystera disjuncta* (Nematoda, Monhysteridae) reveals high levels of molecular diversity. Mar. Biol. 151, 1799–1812. doi:10.1007/s00227-007-0609-0
- Fallon, E.K., Petersen, S., Brooker, R.A., Scott, T.B., 2017. Oxidative dissolution of hydrothermal mixed-sulphide ore: An assessment of current knowledge in relation to seafloor massive sulphide mining. Ore Geol. Rev. 86, 309–337. doi:10.1016/j.oregeorev.2017.02.028
- Freitas, E.C., Rocha, O., 2014. Acute and chronic toxicity of chromium and cadmium to the tropical cladoceran *Pseudosida ramosa* and the implications for ecotoxicological studies. Environ. Toxicol. 29, 176–186. doi:10.1002/tox.20784
- Gaetke, L.M., Chow, C.K., 2003. Copper toxicity, oxidative stress, and antioxidant nutrients. Toxicology, Environmental and Nutritional Interactions Antioxidant Nutrients and Environmental Health, Part C 189, 147–163. doi:10.1016/S0300-483X(03)00159-8
- Gollner, S., Kaiser, S., Menzel, L., Jones, D.O.B., Brown, A., Mestre, N.C., van Oevelen, D., Menot, L., Colaço, A., Canals, M., Cuvelier, D., Durden, J.M., Gebruk, A., Egho, G.A., Haeckel, M., Marcon, Y., Mevenkamp, L., Morato, T.,

- Pham, C.K., Purser, A., Sanchez-Vidal, A., Vanreusel, A., Vink, A., Arbizu, P.M., 2017. Resilience of benthic deep-sea fauna to mining activities. Mar. Environ. Res. doi:10.1016/j.marenvres.2017.04.010
- Gyedu-Ababio, T.K., Baird, D., 2006. Response of meiofauna and nematode communities to increased levels of contaminants in a laboratory microcosm experiment. Ecotoxicol. Environ. Saf. 63, 443–450.
- Hein, J.R., Mizell, K., Koschinsky, A., Conrad, T.A., 2013. Deep-ocean mineral deposits as a source of critical metals for high- and green-technology applications: Comparison with land-based resources. Ore Geol. Rev. 51, 1–14. doi:10.1016/j.oregeorev.2012.12.001
- Howell, R., 1983. Heavy metals in marine nematodes: uptake, tissue distribution and loss of copper and zinc. Mar. Pollut. Bull. 14, 263–268.
- Karlin, K.D., Tyeklár, Z., 2012. Bioinorganic chemistry of copper. Springer Science & Business Media.
- Kennedy, A.D., Jacoby, C.A., 1999. Biological indicators of marine environmental health: meiofauna–a neglected benthic component? Environ. Monit. Assess. 54, 47–68.
- Knight, R., Roberts, S., 2016. Initial results of batch reactor experiments to determine sulphide oxidation rates and trace metal release under seafloor conditions. Appl. Earth Sci. 125, 88–89. doi:10.1080/03717453.2016.1166645
- Koschinsky, A., Fritsche, U., Winkler, A., 2001a. Sequential leaching of Peru Basin surface sediment for the assessment of aged and fresh heavy metal associations and mobility. Deep Sea Res. Part II Top. Stud. Oceanogr., Environmental Impact Studies for the Mining of Polymetallic Nodules from the Deep Sea 48, 3683–3699. doi:10.1016/S0967-0645(01)00062-5
- Koschinsky, A., Gaye-Haake, B., Arndt, C., Maue, G., Spitzy, A., Winkler, A., Halbach, P., 2001b. Experiments on the influence of sediment disturbances on the biogeochemistry of the deep-sea environment. Deep Sea Res. Part II Top. Stud. Oceanogr., Environmental Impact Studies for the Mining of Polymetallic Nodules from the Deep Sea 48, 3629–3651. doi:10.1016/S0967-0645(01)00060-1
- Kwok, K.W.H., Leung, K.M.Y., Bao, V.W.W., Lee, J.-S., 2008. Copper toxicity in the marine copepod Tigropus japonicus: Low variability and high reproducibility of repeated acute and life-cycle tests. Mar. Pollut. Bull., 5th International Conference on Marine Pollution and Ecotoxicology 57, 632–636. doi:10.1016/j.marpolbul.2008.03.026
- Mertz, W., 1981. The essential trace elements. Science 213, 1332–1338. doi:10.1126/science.7022654
- Mestre, N.C., Brown, A., Thatje, S., 2013. Temperature and pressure tolerance of larvae of *Crepidula fornicata* suggest thermal limitation of bathymetric range. Mar. Biol. 160, 743–750.
- Mestre, N.C., Calado, R., Soares, A.M., 2014. Exploitation of deep-sea resources: The urgent need to understand the role of high pressure in the toxicity of >chemical pollutants to deep-sea organisms. Environ. Pollut. 185, 369–371.
- Mestre, N.C., Thatje, S., Tyler, P.A., 2009. The ocean is not deep enough: pressure tolerances during early ontogeny of the blue mussel *Mytilus edulis*. Proc. R. Soc. Lond. B Biol. Sci. 276, 717–726.
- Moens, T., Braeckman, U., Derycke, S., Fonseca, G., Gallucci, F., Ingels, J., Leduc, D., Vanaverbeke, J., Van Colen, C., Vanreusel, A., Vincx, M., 2013. Ecology of free-living marine nematodes, in: Schmidt-Rhaesa, A. (Ed.), Handbook of

- Zoology: Gastrotricha, Cycloneuralia and Gnathifera, Vol. 2: Nematoda. De Gruyter, pp. 109–152.
- Moens, T., Vincx, M., 1998. On the cultivation of free-living marine and estuarine nematodes. Helgoländer Meeresunters. 52, 115–139. doi:10.1007/BF02908742
- Nelder, J.A., Wedderburn, R.W.M., 1972. Generalized linear models. J. R. Stat. Soc. Ser. Gen. 135, 370–384. doi:10.2307/2344614
- New, P., Brown, A., Oliphant, A., Burchell, P., Smith, A., Thatje, S., 2014. The effects of temperature and pressure acclimation on the temperature and pressure tolerance of the shallow-water shrimp *Palaemonetes varians*. Mar. Biol. 161, 697–709.
- Ott, J., Schiemer, F., 1973. Respiration and anaerobiosis of free living nematodes from marine and limnic sediments. Neth. J. Sea Res. 7, 233–243. doi:10.1016/0077-7579(73)90047-1
- Pempkowiak, J., Sikora, A., Biernacka, E., 1999. Speciation of heavy metals in marine sediments vs their bioaccumulation by mussels. Chemosphere, Matter and Energy Fluxes in the Anthropocentric Environment 39, 313–321. doi:10.1016/S0045-6535(99)00112-5
- Petersen, S., Krätschell, A., Augustin, N., Jamieson, J., Hein, J.R., Hannington, M.D., 2016. News from the seabed Geological characteristics and resource potential of deep-sea mineral resources. Mar. Policy 70, 175–187. doi:10.1016/j.marpol.2016.03.012
- Pörtner, H.O., 2002. Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 132, 739–761. doi:10.1016/S1095-6433(02)00045-4
- Pradillon, F., Gaill, F., 2007. Pressure and life: some biological strategies. Rev. Environ. Sci. Biotechnol. 6, 181–195.
- Proctor, A., Wotherspoon, S., 2015. LC50: Estimate LC50 in the presence of additional stressors, R package version 0.1.1.
- Proctor, A.H., King, C.K., Holan, J.R., Wotherspoon, S.J., 2017. Integrated modelling of survival data from multiple stressor ecotoxicology experiments. Environ. Sci. Technol. doi:10.1021/acs.est.7b02255
- R Core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
- Ritz, C., Baty, F., Streibig, J.C., Gerhard, D., 2015. Dose-response analysis using R. PLOS ONE 10, e0146021. doi:10.1371/journal.pone.0146021
- RStudio Team, 2015. RStudio: Integrated development for R. RStudio, Inc., Boston, MA.
- Sávoly, Z., Nagy, P., Varga, G., Havancsák, K., Hrács, K., Záray, G., 2013. A novel method for investigation of uptake and distribution of polluting microelements and nanoparticles in soil-inhabiting nematodes. Microchem. J. 110, 558–567. doi:10.1016/j.microc.2013.07.007
- Scheiber, I., Dringen, R., Mercer, J.F.B., 2013. Copper: Effects of deficiency and overload, in: Sigel, A., Sigel, H., Sigel, R.K.O. (Eds.), Interrelations between Essential Metal Ions and Human Diseases, Metal Ions in Life Sciences. Springer Netherlands, pp. 359–387. doi:10.1007/978-94-007-7500-8_11
- Simpson, S.L., Spadaro, D.A., 2016. Bioavailability and chronic toxicity of metal sulfide minerals to benthic marine invertebrates: Implications for deep-sea

- exploration, mining and tailings disposal. Environ. Sci. Technol. 50, 4061–4070. doi:10.1021/acs.est.6b00203
- Smith, C.R., De Leo, F.C., Bernardino, A.F., Sweetman, A.K., Arbizu, P.M., 2008. Abyssal food limitation, ecosystem structure and climate change. Trends Ecol. Evol. 23, 518–528. doi:10.1016/j.tree.2008.05.002
- Smith, K.E., Brown, A., Thatje, S., 2015. The metabolic cost of developing under hydrostatic pressure: experimental evidence supports macroecological pattern. Mar. Ecol. Prog. Ser. 524, 71–82.
- Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G., Sukhotin, A.A., 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Marine Environmental Research 79, 1–15. doi:10.1016/j.marenvres.2012.04.003
- Sokolova, I.M., Lannig, G., 2008. Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms:: implications of global climate change. Clim. Res. 37, 181–201.
- Somero, G.N., 2003. Protein adaptations to temperature and pressure: complementary roles of adaptive changes in amino acid sequence and internal milieu. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 136, 577–591. doi:10.1016/S1096-4959(03)00215-X
- Song, S., Zhang, X., Wu, H., Han, Y., Zhang, J., Ma, E., Guo, Y., 2014. Molecular basis for antioxidant enzymes in mediating copper detoxification in the nematode *Caenorhabditis elegans*. PLoS One 9, e107685. doi:10.1371/journal.pone.0107685
- Sterk, R., Stein, J.K., 2015. Seabed mineral resources: A review of current mineral resources and future developments. Deep Sea Mining Summit. Aberdeen, Scotland.
- Tchesunov, A.V., Portnova, D.A., Van Campenhout, J., 2014. Description of two free-living nematode species of *Halomonhystera disjuncta* complex (Nematoda: Monhysterida) from two peculiar habitats in the sea. Helgol. Mar. Res. 1–29. doi:10.1007/s10152-014-0416-1
- Thiel, H., 2001. Evaluation of the environmental consequences of polymetallic nodule mining based on the results of the TUSCH Research Association. Deep Sea Res. Part II Top. Stud. Oceanogr., Environmental Impact Studies for the Mining of Polymetallic Nodules from the Deep Sea 48, 3433–3452. doi:10.1016/S0967-0645(01)00051-0
- Van Campenhout, J., Derycke, S., Moens, T., Vanreusel, A., 2014. Differences in lifehistories refute ecological equivalence of cryptic species and provide clues to the origin of bathyal *Halomonhystera* (Nematoda). PLoS One 9, e111889. doi:10.1371/journal.pone.0111889
- Van Campenhout, J., Derycke, S., Tchesunov, A., Portnova, D., Vanreusel, A., 2013. The *Halomonhystera disjuncta* population is homogeneous across the Håkon Mosby mud volcano (Barents Sea) but is genetically differentiated from its shallow-water relatives. J. Zool. Syst. Evol. Res. 52, 203–216. doi:10.1111/jzs.12054
- Van Campenhout, J., Vanreusel, A., Van Belleghem, S., Derycke, S., 2015.

 Transcription, signaling receptor activity, oxidative phosphorylation, and fatty acid metabolism mediate the presence of closely related species in distinct intertidal and cold-seep habitats. Genome Biol. Evol. 8, 51–69. doi:10.1093/gbe/evv242

- Van Gaever, S., Moodley, L., De Beer, D., Vanreusel, A., 2006. Meiobenthos at the Arctic Håkon Mosby Mud Volcano with a parental caring nematode thriving in sulphide-rich sediments. Mar. Ecol. Prog. Ser. 321.
- Vanreusel, A., Hilario, A., Ribeiro, P.A., Menot, L., Arbizu, P.M., 2016. Threatened by mining, polymetallic nodules are required to preserve abyssal epifauna. Sci. Rep. 6, 26808. doi:10.1038/srep26808
- Vranken, G., Brussel, D. van, Vanderhaeghen, R., Heip, C.H.R., 1984. The toxicity of mercury on the free-living marine nematode *Monhystera disjuncta* Bastian, 1865. Ecotoxicological Test. Mar. Environ. 2.
- Vranken, G., Heip, C., 1986. Toxicity of copper, mercury and lead to a marine nematode. Mar. Pollut. Bull. 17, 453–457. doi:10.1016/0025-326X(86)90834-9
- Vranken, G., Tiré, C., Heip, C., 1989. Effect of temperature and food on hexavalent chromium toxicity to the marine nematode *Monhystera disjuncta*. Mar. Environ. Res. 27, 127–136.
- Vranken, G., Tire, C., Heip, C., 1988. The toxicity of paired metal mixtures to the nematode *Monhystera disjuncta* (Bastian, 1865). Mar. Environ. Res. 26, 161–179. doi:10.1016/0141-1136(88)90025-6
- Vranken, G., Vanderhaeghen, R., Heip, C.H.R., 1985. Toxicity of cadmium to freeliving marine and brackish water nematodes (*Monhystera microphthalma*, *Monhystera disjuncta*, *Pellioditis marina*). Dis. Aquat. Organ. 1.
- Wedding, L.M., Reiter, S.M., Smith, C.R., Gjerde, K.M., Kittinger, J.N., Friedlander, A.M., Gaines, S.D., Clark, M.R., Thurnherr, A.M., Hardy, S.M., Crowder, L.B., 2015. Managing mining of the deep seabed. Science 349, 144–145. doi:10.1126/science.aac6647
- Wieser, W., Ott, J., Schiemer, F., Gnaiger, E., 1974. An ecophysiological study of some meiofauna species inhabiting a sandy beach at Bermuda. Mar. Biol. 26, 235–248. doi:10.1007/BF00389254
- Yancey, P.H., Rhea, M.D., Kemp, K., Bailey, D.M., 2004. Trimethylamine oxide, betaine and other osmolytes in deep-sea animals: depth trends and effects on enzymes under hydrostatic pressure. Cell. Mol. Biol. 50, 371–376.