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Research paper

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

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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). LC_{50} values ranged between 0.561 and 1.864 mg Cu^{2+} 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.

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

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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 co-factor 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 (Cu^{2+}) 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 Cu^{2+} L^{-1}) and 20 °C (0, 0.2, 0.5, 1, 2, 5 mg Cu^{2+} 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 Cu^{2+}-contaminated gellan gum and 20 adult and preadult nematodes were placed in the vials. The vials were then filled up with the Cu^{2+}-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 h). 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 (Cu^{2+}) concentrations by adding the appropriate amount of $CuSO_4$ stock solution to the medium under continuous stirring for ~2 min. The stock solution was composed of 0.10155 g $CuSO_4$ and 250 mL MilliQ water resulting in a dissolved Cu^{2+} 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