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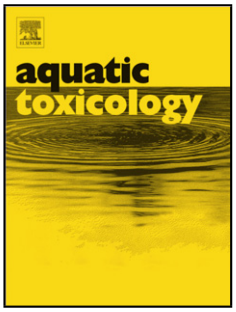

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A comparative experimental approach to ecotoxicology in shallow-water and deep-sea holothurians suggests similar behavioural responses

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Highlights

- Shallow-water and deep-sea holothurians avoided copper-contaminated sediment.
- Shallow-water taxa may be suitable ecotoxicological proxies for deep-sea taxa.
- Avoidance behaviour may have bioenergetic consequences.

Abstract

Exploration of deep-sea mineral resources is burgeoning, raising concerns regarding ecotoxicological impacts on deep-sea fauna. Assessing toxicity in deep-sea species is technologically challenging, which promotes interest in establishing shallow-water ecotoxicological proxy species. However, the effects of temperature and hydrostatic pressure on toxicity, and how adaptation to deep-sea environmental conditions might moderate these effects, are unknown. To address these uncertainties we assessed behavioural and physiological (antioxidant enzyme activity) responses to exposure to copper-spiked artificial sediments in a laboratory experiment using a shallow-water holothurian (*Holothuria forskali*), and in an *in situ* experiment using a deep-sea holothurian (*Amperima* sp.). Both species demonstrated sustained avoidance behaviour, evading contact with contaminated artificial sediment. However, *A*. sp. demonstrated sustained avoidance of 5 mg l^{-1} copper-contaminated artificial sediment whereas *H*. *forskali* demonstrated only temporary avoidance of 5 mg l^{-1} copper-contaminated artificial sediment, suggesting that *H*. *forskali* may be more tolerant of metal exposure over 96 h. Nonetheless, the acute behavioural response appears consistent between the shallow-water species and the deep-sea species, suggesting that *H. forskali* may be a suitable ecotoxicological proxy for *A*. sp. in acute (≤24 h) exposures which may be representative of deep-sea mining impacts. No antioxidant response was observed in either species, which was interpreted to be the consequence of avoiding copper exposure. Although these data suggest that shallow-water taxa may be suitable ecotoxicological proxies for deep-taxa, differences in methodological and analytical approaches, and in sex and reproductive stage of experimental subjects, require caution in assessing the suitability of *H. forskali* as an ecotoxicological proxy for *A.* sp.. Nonetheless, avoidance behaviour may have bioenergetic consequences that affect growth and/or reproductive output, potentially impacting fecundity and/or offspring fitness, and thus influencing source-sink dynamics and persistence of wider deep-sea populations.

Keywords

bathymetric; deep-sea mining; ecology; hydrostatic pressure; physiology; temperature; toxicology

Introduction

Interest in exploiting deep-sea mineral resources is increasing, driven by rising metal prices and the challenges of achieving environmentally and socio-economically acceptable solutions for extraction, processing, and disposal of materials from low-grade rock deposits on land. Economically important metals and rare-earth elements are present in deep-sea massive polymetallic sulphides, polymetallic manganese nodules, and cobalt-rich ferromanganese crusts (Petersen et al. 2016), and even muds (Kato et al. 2011). However, despite great international attention the responses of targeted ecosystems to mineral extraction remain largely unknown. The remoteness of deep-sea ecosystems makes experimental assessment of deep-sea mining impacts challenging, and the urgency to exploit these resources may drive researchers to use shallow-water ecosystems as proxies in assessments that influence policy-making. The extensive data available for shallow-water taxa make this an attractive approach, but the potential synergies that may arise from the interaction of stressors with environmental factors, such as temperature, hydrostatic pressure, and oxygen concentration (Pörtner 2010; Sokolova 2013; Brown and Thatje 2015), are typically ignored (Mestre et al. 2013). Nevertheless, these synergies may be crucial to accurately project the impacts of deep-sea mineral exploitation throughout species' thermal and baric distributions (Brown et al. in revision). Further, whether shallow-water taxa are suitable proxies for deep-sea taxa remains to be revealed; adaptation to environmental conditions in the deep sea may invalidate such an approach by affecting sensitivity to potential stressors.

Extraction of deep-sea mineral deposits may have significant ecotoxicological impacts on the deep-sea fauna. For example, the polymetallic manganese nodules which form on abyssal plains comprise high concentrations of potentially toxic metals such as copper (Ramirez-Llodra et al. 2011) which may be released during extraction processes (Levin et al. 2016;

Gollner et al. 2017). Few studies have employed a comparative approach to explore the effects of deep-sea conditions on sensitivity to toxicants, and these have predominantly focused on adaptation to toxic challenges presented by hydrothermal vent environment. However, these studies have assessed *in situ* biomarker expression (metallothioneins, antioxidant enzymes, lipid peroxidation) and/or metal accumulation in hydrothermal vent and coastal shrimp (Geret et al. 2002; Kadar et al. 2006; Gonzalez-Rey et al. 2007, 2008). Whilst this approach identifies *in situ* physiology at ambient toxicant concentrations, it has yielded limited information regarding similarities and/or differences in responses and sensitivity to elevated toxicant concentrations among species from different environments. Such information may only be revealed through a comparative experimental approach, where individuals from different phylogenetically related shallow-water and deep-sea species are exposed to similar challenges under native environmental conditions (e.g. hydrostatic pressure, temperature).

Recent experimental assessment of the effects of hydrostatic pressure and temperature on sublethal responses to copper exposure in the deep-sea hydrothermal vent shrimp *Rimicaris exoculata* at 10°C and 30.0 MPa (Auguste et al. 2016) and the shallow-water shrimp *Palaemon varians* provided the first opportunity to develop significant insight into caridean adaptation to toxicity in a hydrothermal vent environment (Brown et al. in revision). Comparisons suggest that sensitivity and responses to toxicants may not differ between these species at a common temperature at native hydrostatic pressure, suggesting that shallow-water taxa may be suitable ecotoxicological proxies for deep-sea taxa (Brown et al. in revision). However, *P. varians* is significantly more tolerant of both copper and cadmium than other shallow-water caridean shrimp species, perhaps as a result of adaptations to highly variable environmental temperature, salinity, and oxygen concentration in estuarine, salt marsh, and saline lagoon habitat (Brown et al. in revision). Consequently, matching the environmental variability experienced by deep-sea species may be a key criterion for the selection of shallow-water ecotoxicological proxy species (Brown et al. in revision).

Whilst bathyal hydrothermal vent taxa may be affected by the exploitation of seafloor massive sulphides, extraction of polymetallic manganese nodules will impact abyssal fauna (Levin et al. 2016; Gollner et al. 2017). Adaptations to the environmental conditions in the abyssal deep-sea may be significantly different from adaptations to the bathyal hydrothermal vent environment (Childress and Fisher 1992; Brown and Thatje 2014). For example, temperature- and mass-normalised metabolic rate declines significantly with depth in holothurians (Brown et al. under review). Consequently, ecotoxicological comparisons between shallow-water species and bathyal hydrothermal vent species may not be representative of other deep-sea taxa. To address this uncertainty, we assessed responses to toxicant exposure in the temperate shallow-water holothurian *Holothuria forskali* and the abyssal holothurian *Amperima* sp..

H. forskali occurs along the Atlantic coasts of northwest Europe and in the Mediterranean Sea, from the intertidal to 100 m depth (OBIS, 2015). *H. forskali* can grow to 49 cm length (Tuwo and Conand 1992). *H. forskali* is benthic and has a tough body wall, typically occuring on boulders and rocks and moving using locomotory podia located on the ventral sole (personal observation). In contrast, *Amperima* species occur widely in the abyssal deep sea (>4000 m depth) and have been reported in the North and South Atlantic, Southern, Indian, and South Pacific (OBIS, 2015). *A.* sp. can grow to 13 cm and is capable of swimming by undulating its body, typically make brief excursions few metres into the water column before drifting back to the seabed (personal observation). Both *H. forskali* and *A.* sp. are detritivores (personal observation).

Copper was selected as the experimental toxicant based on representation in deep-sea mineral deposits (see Ramirez-Llodra et al. 2011), and the demonstration that copper has the greatest potential for rapid release during seafloor massive sulphide extraction (Knight and Roberts 2016).

Materials and Methods

Shallow-water exposures

Specimens of *Holothuria forskali* were sampled by divers (depth range 16 m to 19 m) on 27th May 2015 from ~50 m^2 of rocky reef with sand gullies immediately south of Plymouth Mewstone (50°18.158 N, 004°06.555 W) (indicative salinity 35.3, temperature 11.2°C, dissolved oxygen 260.9 µmol l^{-1} from 16 m to 19 m at the Western Channel Observatory L4 station at 50°25.00 N, 004°21.70 W on the 27th May 2015). Holothurians were transported to the Marine Biology and Ecology Research Centre at the University of Plymouth, and maintained for 1 week in a recirculating aquarium (salinity ~32, temperature 10°C, dissolved oxygen concentration >245 µmol l^{-1}). Subsequently, holothurians were transported to the National Oceanography Centre Southampton (NOCS) by temperature-controlled vehicle (10°C, <6 hours), and transferred to a recirculating aquarium (salinity ~32, temperature 9.8°C decreased by 1°C weekly to 4°C, pH 8.0, ammonia <0.1 mg l^{-1}, light:dark cycling following seasonal changes). Holothurians were fed approximately 15 g of Marine Cuisine (Tropical Marine Centre) three times each week. Holothurians were maintained for ≥1 weeks at 4°C prior to experimental treatments. Treatments were performed concurrently to minimise potential temporal influences on responses, but were logistically limited to 1 replicate of each treatment per week by the availability of incubators. Experimental exposures were conducted between 17th July and 28st August 2015 to reduce potential variability in biochemical responses resulting from annual reproductive cycles (Santos et al., 2016).

Static 96-hour experimental exposures were conducted in plastic aquaria (32.5 × 20 × 20 cm) filled with 10 l aquarium water (salinity 32, dissolved oxygen concentration >245 μmol l^{-1}, pH 8.0, ammonia <0.1 mg l^{-1}) at 4°C, covered with vented plastic lids allowing air circulation, with 12:12 photoperiod. The experimental temperature was selected to represent the lowest temperature occurring within the species' biogeographic range (Locarnini et al. 2013; OBIS, 2016). Adult holothurians were used in all treatments (size range 18 cm to 24 cm). Identification of *H. forskali* sex requires dissection. Post-treatment dissection indicated that both male and female holothurians were used in all treatments (1 or 2 males in each treatment), and that both male and female holothurians were in stage II (developing oocytes or sperm) or stage III (mature oocytes or sperm) in the reproductive cycle (determined according to Santos et al., 2016). Each holothurian was assigned to either a control, artificial sediment addition, or copper-spiked artificial sediment addition treatments (n = 5). 500 g of artificial sediment was prepared for each addition, with composition designed to reflect particle-size distribution at the DISturbance and reCOLonisation (DISCOL) experimental area in the abyssal Peru Basin (Grupe 2001), whilst incorporating OECD guidance (OECD 2004). Artificial sediment comprised 425 g white quartz sand (99% < 90 μm), 70 g kaolin (96% < 45 μm), and 5 g peat (100% < 500 μm). Artificial sediment was spiked with copper by 72 h incubation at 20°C in 250 ml of deionised water spiked with copper by the addition of stock solution of $CuSO_4{\cdot}5H_2O$, according to OECD guidance (OECD 2004). The stock solution was prepared using deionised water and analytical grade $CuSO_4{\cdot}5H_2O$, and incubation concentrations were 0, 0.1, 1, 5, 10, and 20 mg l^{-1}. Artificial sediment and copper-spiked artificial sediment was drained and released into the aquaria 24 hours prior to introduction of the holothurian. Holothurians were gently detached from the maintenance aquarium and transferred to the floor of the experimental aquarium with minimal aerial exposure. Position within the aquarium (i.e. anterior and posterior height above aquarium floor) and behaviour (e.g. contraction, ejection of cuvierian tubules, movement, feeding) were monitored for 15 minutes immediately following introduction to experimental conditions, and at 24-h intervals thereafter.

After 96 hours of experimental exposure, holothurians were removed from experimental aquaria and dissected immediately. Individuals were not anaesthetised prior to dissection to minimise potential for biochemical artefacts (Arafa et al. 2008). Feeding tentacle, body wall, longitudinal muscle, gonad, respiratory tree, rete mirabile, digestive tract, and digestive tract contents were sampled. Tissue was transferred to a 1.5 ml centrifuge tube, and snap frozen in liquid nitrogen within 10 minutes of removal from experimental aquarium. Once frozen, tissue was preserved at -80°C until analysed. Holothurians typically uptake heavy metals and demonstrate antioxidant responses to heavy metals in the body wall, respiratory tree, and digestive tracts (e.g. Wang et al. 2015, Li et al. 2016, Wang et al. 2016). *Holothuria forskali* digestive tract contents did not include the artificial sediment and therefore biochemical responses were only examined in respiratory tree and body wall tissues.

Deep-sea exposures

The *in situ* toxicology experiment was deployed from the RV *Sonne* during cruise SO242-2 of the Joint Program Initiative Oceans – Ecological Aspects of Deep-Sea Mining. Deployments were made at the southern reference site (007°07.5 S, 088°27.0 W) outside the DISCOL experimental area in the abyssal Peru Basin (see Borowski 2001) (salinity 35.96, temperature 1.85°C, dissolved oxygen 120.83 µmol l^{-1}). Experimental exposures were conducted between 12th September and 16th September 2015. Reproductive cycles in other

Amperima species appear linked to changes in the flux or organic matter to the seafloor (Wigham et al. 2003). However, the DISCOL region is a site with low organic matter flux (Weber et al. 2000) suggesting that populations here may be sink populations rather than source populations.

In situ treatments were conducted using corrals (Fig. 1). The internal dimensions of each corral tapered from 40 cm × 40 cm to 30 cm × 30 cm, and each corral was covered by a fine mesh. The corrals were deployed to the seafloor at 4196.5 m depth on the GEOMAR Remote Operated Vehicle (ROV) elevator platform. Subsequently, the GEOMAR ROV *Kiel 6000* collected each corral, located an *Amperima* sp. individual, and carefully placed the corral over the holothurian. *A*. sp. was selected as the test species based on high abundances observed during ROV dives on preceeding days. Adult holothurians were used in all treatments (size range 8 cm to 10 cm). Whilst post-treatment dissection did not resolve *A*. sp. sex (all *A*. sp. were in stage I in the reproductive cycle without identifiable gonad), *A*. spp. reach maturity at small size, e.g. 10 mm in *Amperima rosea* (Wigham et al. 2003). Each holothurian was assigned to either a control, artificial sediment addition, or copper-spiked artificial sediment addition treatment (n = 2). Control treatments remained undisturbed, whereas artificial sediment or copper-spiked artificial sediment was released into each corral using the ROV manipulator. Artificial sediment was transported to the seafloor aboard the ROV, in 1 litre plastic bottles adapted to facilitate opening using the ROV manipulator. The appropriate plastic bottle was inverted immediately above the mesh covering the corral, and inserted through a flap in the mesh to release the artificial sediment. Preparation of artificial sediments and copper-spiking were performed as described previously, but incubation concentrations were limited to 0 and 5 mg l^{-1} due to logistical constraints of operating in the deep sea. Incubation concentrations were selected based on behavioural data from the *Holothuria forskali* exposures. After ~94 hours holothurians were sampled using the ROV suction pump, which was inserted through the flap in the mesh. Additional holothurians were sampled to assess the effect of corral enclosure (n = 3). Holothurians were isolated in the ROV suction pump chambers and recovered to the surface where they were dissected in a temperature controlled laboratory (2°C). As with *Holothuria forskali,* individuals were not anaesthetised prior to dissection. The anatomy of *Amperima* sp. differed from that of *Holothuria forskali* and separation of different tissues was challenging. Consequently, only bulk tissue samples (predominantly body wall) were taken from *Amperima* sp. individuals. Tissue was transferred to a 1.5 ml centrifuge tube, and snap frozen in liquid nitrogen. Once frozen, tissue was preserved at -80°C until analysed.

Biochemical response

Tissue samples were removed from -80°C and maintained on ice. A ~10 mg subsample was dissected for biochemical analysis. Total protein and enzyme activities were determined by absorbance using a microplate reader (FLUOstar OPTIMA, BMG Labtech). Total protein was assessed at 584 nm absorbance using an assay kit employing the Lowry method with Peterson's modification (Sigma-Aldrich TP0300). Superoxide dismutase (SOD) activity was assessed at 450 nm absorbance using an assay kit employing inhibition of xanthine oxidase activity (Sigma-Aldrich 19160). Glutathione peroxidase (GPx) activity was assessed at 340 nm absorbance using an assay kit employing NADPH oxidation in the presence of excess glutathione reductase, reduced glutathione, and corresponding peroxide (Abcam ab102530).

Statistical analysis

H. forskali position and biochemical data were homoscedastic (Levene's test, $p > 0.05$). Consequently, the effect of exposure to copper-spiked artificial sediment on *H. forskali* position was assessed using two-way repeated measures analysis of variance (ANOVA), with treatment (control, copper-spiked artificial sediment additions) and exposure duration as fixed factors ($\alpha = 0.05$). The *post-hoc* Holm-Sidak multiple comparisons test was used to determine significant differences from initial positions within treatments. Statistical inferences were confirmed using the repeated measures ANOVA on ranks employing Dunn's test to assess to assess differences in holothurian anterior and posterior position within each treatment over exposure duration. The effect of exposure to copper-spiked artificial sediment on *H. forskali* biochemistry was assessed using two-way ANOVA, with tissue (body wall, respiratory tree) and treatment (control, copper-spiked artificial sediment additions) as fixed factors ($\alpha = 0.05$). The effects of exposure to copper-spiked artificial sediment on *Amperima* sp. position and biochemistry were not assessed statistically because of the low replication (Vaux 2012).

Results

Positional response

Holothuria forskali responded to copper-spiked artificial sediment exposure by moving both anterior and posterior away from ≥5 mg l^{-1} copper-contaminated artificial sediment (Fig. 2) (respectively, $F_{20,96} = 1.726$, $p = 0.042$ and $F_{20,96} = 3.391$, $p < 0.001$, with *post-hoc* test $p < 0.05$). However, only anterior position in the 5 mg l^{-1} treatment differed significantly from starting position and only after 24 hours exposure duration.

Amperima sp. in the control treatment remained on the seafloor. In contrast, individuals exposed to uncontaminated or copper-spiked artificial sediment immediately engaged in swimming. After ~94 hours, individuals in both the control treatment and the uncontaminated artificial sediment addition treatment were on the seafloor. However, individuals exposed to copper-spiked artificial sediment were attached inverted to the mesh covering the corral (Fig. 1).

Biochemical response

Exposure to copper-spiked artificial sediment had no effect on body wall and respiratory tree superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity in *H. forskali* (Fig. 3) (respectively, $F_{5,48} = 0.541$, $p = 0.744$ and $F_{5,48} = 1.188$, $p = 0.329$). Exposure to copper-spiked artificial sediment appeared to have no significant effect on bulk tissue (predominantly body wall) SOD or GPx activity in *Amperima* sp. (Fig. 4).

Discussion

Differences in methodological (static laboratory exposures compared with *in situ* exposures) and analytical approaches (tissue-specific analysis compared with bulk tissue analysis), and in sex (male and female compared to undetermined) and reproductive stage (II or III compared with I) of experimental subjects, require caution in assessing the suitability of *Holothuria forskali* (a shallow-water species) as an ecotoxicological proxy for *Amperima* sp. (a deep-sea species): these factors may affect results. Further, the low replication achieved during *in situ* experimentation allows only extremely limited confidence in inferences. Nonetheless, *A*. sp. demonstrated sustained avoidance of 5 mg l^{-1} copper-contaminated artificial sediment whereas *H. forskali* demonstrated only temporary avoidance of 5 mg l^{-1} copper-contaminated artificial sediment, suggesting that *H. forskali* may be more tolerant of metal exposure over 96 h. However, the acute behavioural response appears consistent between the shallow-water species and the deep-sea species, suggesting that *Holothuria* *forskali* may be a suitable ecotoxicological proxy for *A*. sp. in acute (≤24 h) exposures, which may be representative of deep-sea mining impacts.

The positional response to exposure to copper-spiked artificial sediment displayed by both the shallow-water and deep-sea holothurians likely represents an adaptive behavioural response, contributing directly to individual survival (Dill 1987). Holothurians putatively possess chemosensing capability, detecting sediment patches with higher organic carbon content (Slater 2010) and selecting organic carbon-rich particles (Paltzat et al. 2008). The avoidance behaviour observed in both shallow-water and deep-sea species suggests that the chemosensory ability extends to potential toxicants. The absence of any antioxidant response in either species may be a consequence of avoiding sustained copper exposure through behavioural modification: analysis of copper concentrations or metallothionein concentrations would be required to confirm this. However, sediment plume avoidance or avoidance of redeposited contaminated sediments in deep-sea holothurians may be ineffective in the mining context. Individuals may engage in swimming, but will may drift with the same prevailing current that will disperse the potentially toxic sediment plume (Levin et al. 2016; Gollner et al., 2017). Individuals may subsequently settle in areas with contaminated sediment redeposition, thus maintaining metal exposure and stimulating further avoidance behaviour. Bioenergetic consequences of avoidance behaviour or responses to sustained sublethal metal exposures may be critical (Sokolova et al. 2012). Avoidance behaviour increases energy allocation to activity through energetic trade-offs: increased allocation of energy to activity during stress diminishes energy available for other functions, and consequently reduces scope for growth and reproductive output (Kooijman 2010; Sokolova et al. 2012). Responses to sustained sublethal metal exposures may have similar energetic impacts (Sokolova et al. 2012).

The potential ecological implications of holothurian avoidance behaviour extend beyond individual holothurian species. Holothurians are keystone deep-sea species (Gage & Tyler 1991; Kuhnz et al. 2014), deepening the oxic sediment horizon through bioturbation and therefore influencing the distribution of infaunal species (Iken et al. 2001; Soto et al. 2010). Further, bioturbation by holothurians can moderate toxicity to both epi- and infauna by distributing toxicants within sediments (Storelli et al. 2001; Yuan et al. 2016). Reduced bioturbation resulting from behavioural avoidance may therefore also affect the persistence of both epi- and infaunal species (Mermillod-Blondin 2011) in areas impacted by sediment plumes incorporating toxicants. Sediment re-deposition may occur over scales of 10-100 km from mining sites (see Wedding et al. 2013), therefore the behavioural avoidance reported here may have significant farfield ecosystem scale effects that require consideration in environmental management of deep-sea mineral exploitation.

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Figures

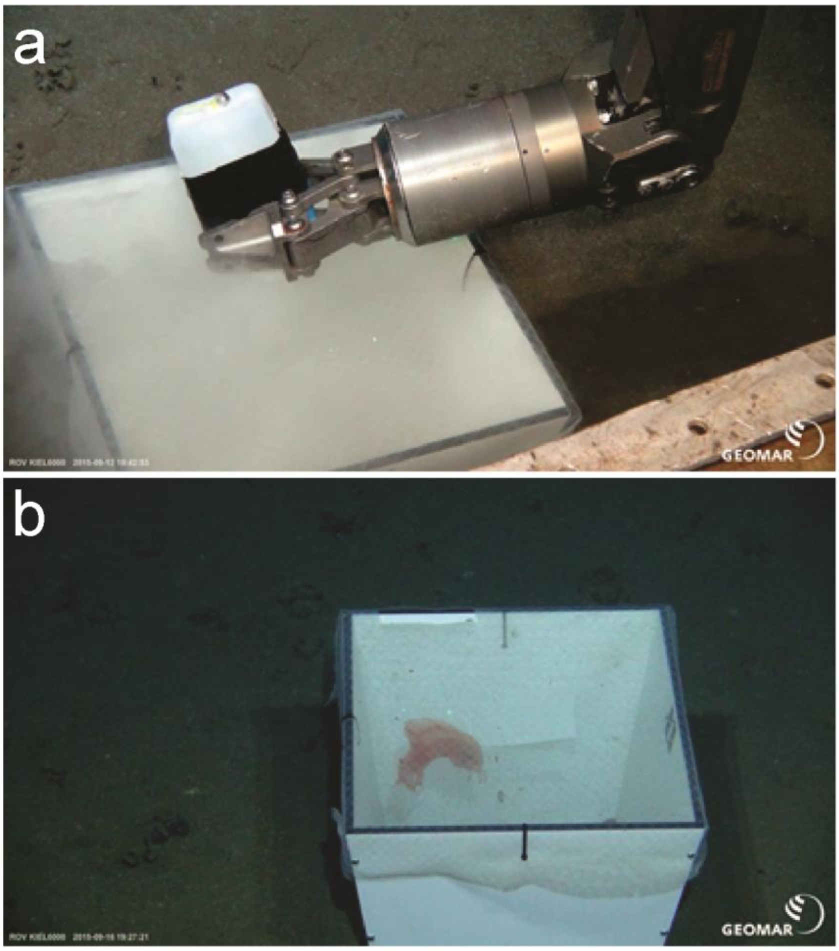
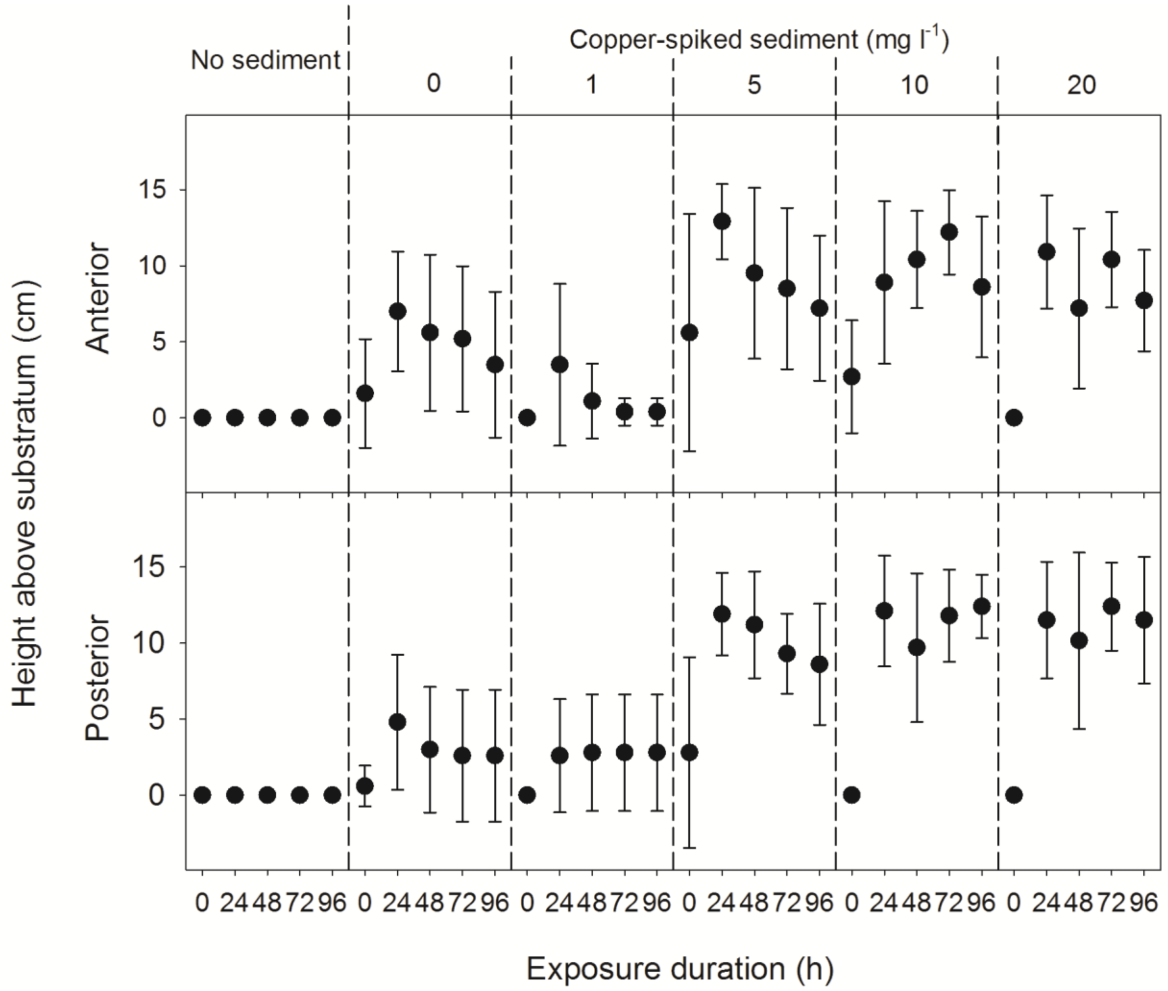


Fig. 1. *In situ* ecotoxicology experiments. (a) Introduction of artificial sediment to a corral using the ROV *Kiel 6000* manipulator. (b) *Amperima* sp. position ~94 hours after exposure to 5 mg l^{-1} copper-spiked artificial sediment. The internal dimensions of each corral tapered from 40 cm × 40 cm to 30 cm × 30 cm.

483 

484 **Fig. 2.** Effect of exposure to copper-spiked sediment on *Holothuria forskali* position above 485 the sediment surface (mean ± SD; n = 5). Error bars are absent where all individuals were 486 positioned on the floor of the aquarium.

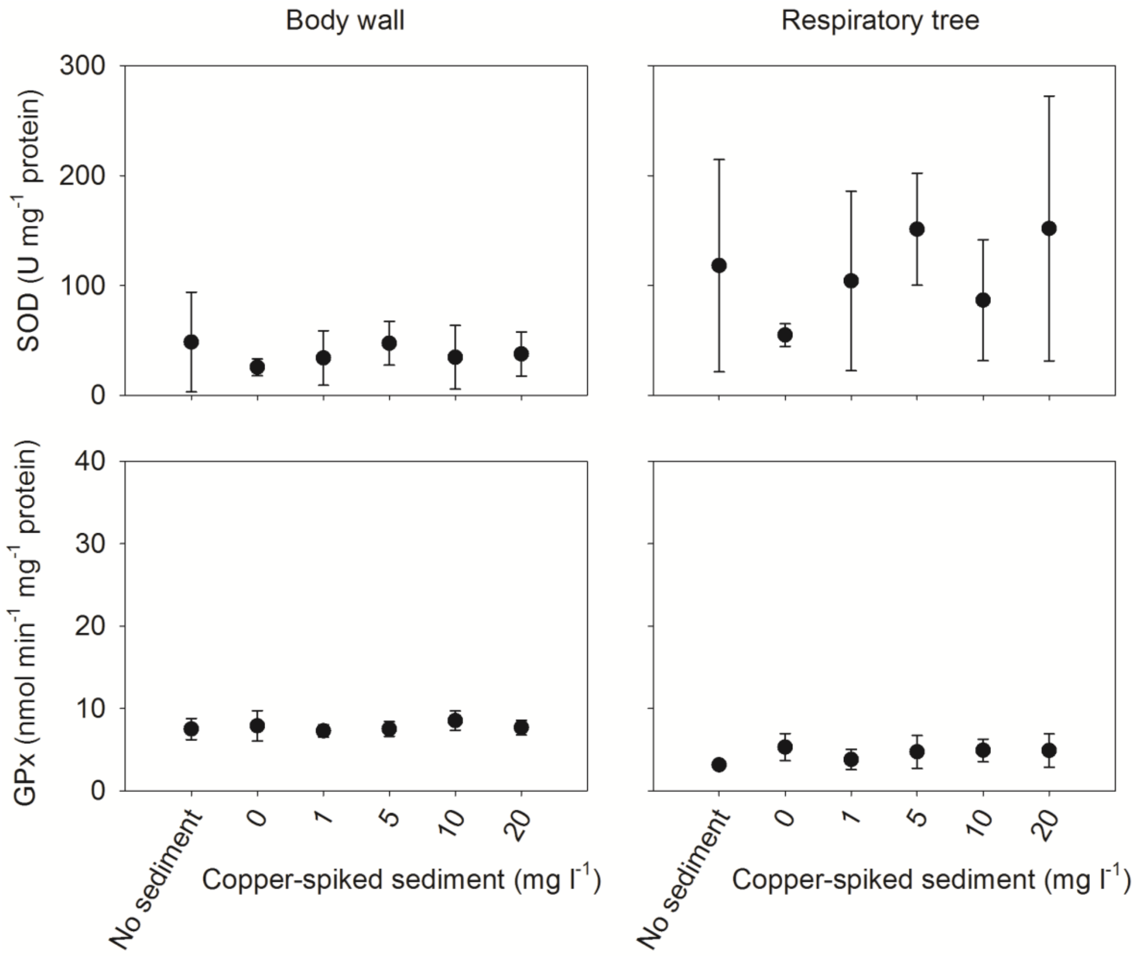


Fig. 3. Effect of exposure to copper-spiked sediment on body wall and respiratory tree superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity in *Holothuria forskali* (mean ± SD; n = 5). Error bars appear absent where SD is smaller than the data point.

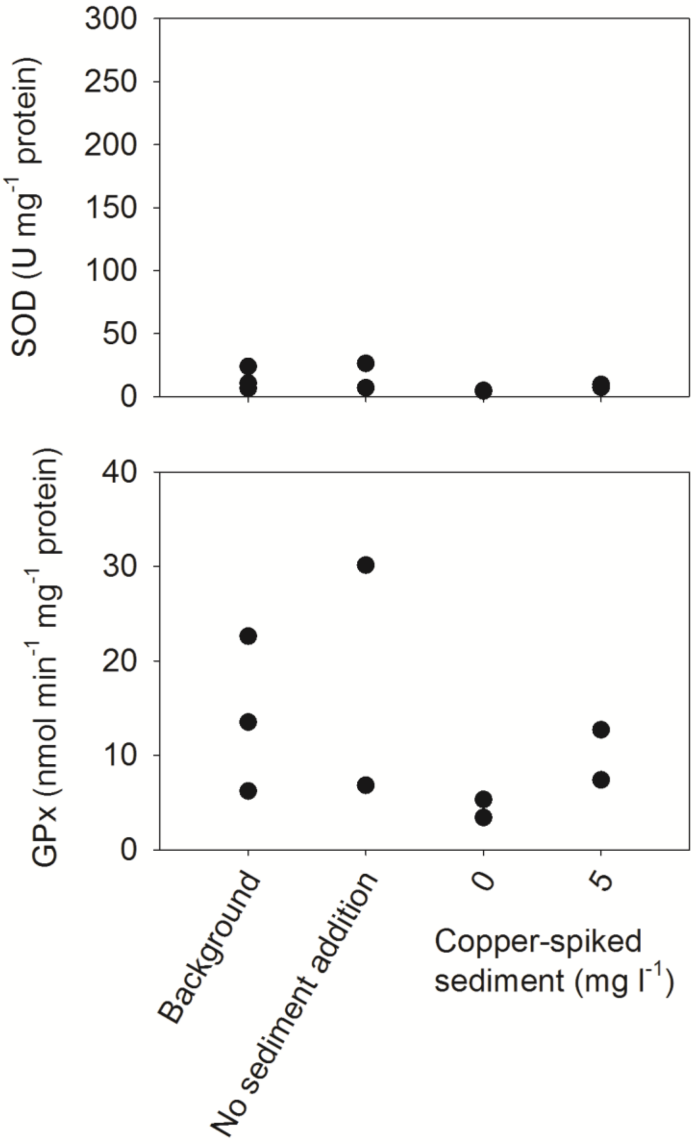


Fig. 4. Effect of exposure to copper-spiked sediment on bulk tissue (predominantly body wall) superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity in *Amperima* sp. (individual data).