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The effects of temperature and hydrostatic pressure on metal toxicity: Insights into toxicity in the deep sea

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Abstract

Mineral prospecting in the deep sea is increasing, promoting concern regarding potential ecotoxicological impacts on deep-sea fauna. Technological difficulties in assessing toxicity in deep-sea species has promoted interest in developing shallow-water ecotoxicological proxy species. However, it is unclear how the low temperature and high hydrostatic pressure prevalent in the deep sea affect toxicity, and whether adaptation to deep-sea environmental conditions moderates any effects of these factors. To address these uncertainties we assessed the effects of temperature and hydrostatic pressure on lethal and sublethal (respiration rate, antioxidant enzyme activity) toxicity in acute (96-hour) copper and cadmium exposures, using the shallow-water ecophysiological model organism *Palaemon varians*. Low temperature reduced toxicity in both metals, but reduced cadmium toxicity significantly more. In contrast, elevated hydrostatic pressure increased copper toxicity, but did not affect cadmium toxicity. The synergistic interaction between copper and cadmium was not affected by low temperature, but high hydrostatic pressure significantly enhanced the synergism.

Differential environmental effects on toxicity suggest different mechanisms of action for copper and cadmium, and highlight that mechanistic understanding of toxicity is fundamental to predicting environmental effects on toxicity. Although results infer that sensitivity to toxicants differs across biogeographic ranges, shallow-water species may be suitable ecotoxicological proxies for deep-sea species, dependent on adaptation to habitats with similar environmental variability.

Keywords

adaptation, cadmium, copper, deep-sea mining, ecology, *Palaemon varians*, physiology

Introduction

Exploitation of low-grade terrestrial mineral deposits requires mining, processing, and disposal of large volumes of rock materials, posing a significant impediment to achieving environmentally acceptable operations. Consequently, the mining industry is developing alternative sources of metals. The deep-sea represents a large mineral resource, with economically and socially important metals and rare-earth elements represented in massive polymetallic sulphides, polymetallic manganese nodules, and cobalt-rich ferromanganese crusts[1], and even in deep-sea muds[2]. However, it remains unclear whether it is possible to achieve deep-sea mineral extraction in an environmentally sustainable way[3].

The logistical difficulties of accessing deep-sea ecosystems makes them challenging to study experimentally[4], but the drive to exploit deep-sea mineral resources demands rapid policy-making. Consequently, policy-makers may be tempted to apply evidence from shallow-water ecosystem to develop deep-sea mining regulation. Whether shallow-water taxa are suitable proxies for deep-sea taxa remains uncertain: the only comparative exploration of toxicity in phylogenetically related shallow-water and deep-sea species suggests that shallow-water holothurians may be suitable ecotoxicological proxies for deep-sea holothurians, but with significant caveats[4]. Adaptation to deep-sea environmental conditions may affect sensitivity to potential stressors[4] but interactions between stressors and environmental factors such as temperature, hydrostatic pressure, and oxygen concentration[5-7] may be fundamental to accurately constraining the impacts of deep-sea mineral exploitation across the temperature and hydrostatic pressure range individual species occupy. However, such interactions are unexplored and are typically ignored[8].

The extraction of mineral deposits in the deep sea may present significant ecotoxicological risk to the deep-sea fauna. For example, seafloor massive polymetallic sulphides, which form at hydrothermal vents, comprise high concentrations of potentially toxic metals as minerals such as chalcopyrite ($CuFeS_2$)[9]. Marine metal toxicity is dependent on organismal physiology[10] and closely related species inhabiting similar environments are expected to display similar physiologies and tolerances to toxicants[11-14]. Consequently, assessing the effects of temperature and hydrostatic pressure on sensitivity to toxicants in shallow-water species may also indicate whether deep-sea hydrothermal vent species are truly adapted to tolerate the toxic hydrothermal vent habitat. However, assessing the extent of physiological adaptation to toxicants in hydrothermal vents species is dependent upon the selection of appropriate phylogenetically related shallow-water model taxa.

The recent shallow-water ancestry suggested for bresiliid hydrothermal vent shrimp[15-18] makes them an ideal case study of physiological adaptation to the deep-sea hydrothermal vent setting. Whilst these species may have adapted to the relatively rich metal ion concentrations in the vicinity of hydrothermal systems[19-21], more ancient hydrothermal vent taxa may be expected to have diverged further from shallow-water taxa and may therefore be genetically more distant[22]. Indeed, hydrothermal vent shrimp demonstrate varying degrees of adaptation to hydrothermal conditions with adaptations ranging from few and inconspicuous in *Alvinocaris* spp., which are similar to non-vent deep-sea shrimp, to numerous and prominent adaptations in *Rimicaris* spp.[23].

Although *Rimicaris* and *Mirocaris* spp. appear to demonstrate higher maximum critical temperatures than shallow-water shrimp[24-26], this may be an artefact of acclimation to different temperature: temperature acclimation is an extended process which affects tolerance to a range of stressors[27,28]. Indeed, the critical thermal maximum reported for *Mirocaris fortunata* matches the modelled critical thermal maximum of shallow-water shrimp acclimated to 19°C[27], *M. fortunata*'s preferred temperature[29]. However, *M. fortunata* does appear to have evolved metabolic adaptations to the acute spatial and temporal scale of temperature changes inherent in the vent environment, demonstrating reduced metabolic sensitivity to acute temperature shifts[27,29,30]. Deep-sea hydrothermal vent shrimp appear adapted to high hydrostatic pressure too: the bathymetric distribution of the hydrothermal vent shrimp extends beyond the hydrostatic pressure tolerance of shallow-water shrimp at *in situ* temperatures[26,28], with some species thought to be excluded from shallow vent fields by relatively low hydrostatic pressure[31]. Whilst bresiliid shrimp constitute a key component of many active hydrothermal vent communities[32], other phylogenetically related carideans[18] are also an important constituent of inactive vent and wider deep-sea communities[33,34], strengthening the case for utilising shrimp in a comparative experimental approach.

The shallow-water shrimp *Palaemon varians* (recently identified as the senior synonym of *Palaemonetes varians*[35]) is a brackish-water shrimp which inhabits salt marsh, lagoon and estuarine environments throughout North-West Europe and the western Mediterranean[36]. *P. varians* has a close phylogenetic relationship to both hydrothermal vent shrimp species and non-vent deep-sea species[17,18], making it an excellent comparator for deep-living shrimps[29,37,38]. Indeed, the physiology of *P. varians* with respect to temperature and hydrostatic pressure is well studied[26-30,39-42]. The temperature regimes experienced by *P. varians* and hydrothermal vent shrimp are of similar range, and the acute temperature tolerance of the shallow-water shrimp and the hydrothermal vent shrimp are similar at native hydrostatic pressures[24-27]. Further, *P. varians*' sustained hyperbaric tolerance extends to the depth of some hydrothermal vents inhabited by vent shrimp[28,39,40]. Therefore, the aim of this study was to utilise *P. varians* as a model organism to determine the effects of temperature and hydrostatic pressure on metal toxicity.

To explore the possibility of variation in the effects of temperature and pressure on sensitivity to different toxicants, two metals were selected for experimental treatments. Copper and cadmium were selected as the experimental toxicants based on representation in deep-sea mineral deposits[1], elevated concentrations in the hydrothermal vent environments and shrimp habitat[19,43-45], and the demonstration that copper has the greatest potential for rapid release during seafloor massive sulphide extraction[46]. Copper and cadmium were also selected for the extent of existing toxicological knowledge regarding these metals, and the putative similarities in their modes of toxicity, i.e. ionoregulatory, osmoregulatory, and circulatory impairment[47]. Both lethal and sublethal (respiration rate, antioxidant enzyme activity) responses to acute exposures were assessed to deliver a holistic comparative approach[48]. Lethal responses provide an indication of acute toxicological tolerance. Sublethal responses provide an indication of chronic toxicological tolerance: metal-induced formation of reactive oxygen species results in increase in antioxidant enzyme activity (e.g. superoxide dismutase and glutathione peroxidase) which increases basal metabolic demand (reflected in increased respiration rate) and indicates an ecological impact[49]. Demonstrating consistent effects of temperature and hydrostatic pressure on sensitivity to both copper and cadmium may identify predictability of environmental effects on toxicity; demonstrating contrasting effects may elucidate mechanisms of toxicity.

Experimental Materials and Methods

Sampling and maintenance

Adult specimens of *Palaemon varians* (4 to 5 cm in total length) were collected by hand-netting from Lymington salt marshes (Hampshire, England: 50°45’N, 1°32’W) between May 2014 and May 2015, and transported to the National Oceanography Centre Southampton

(NOCS) in 10 l buckets containing water from the sampling location. Upon arrival at NOCS shrimp were transferred to a 185 l flow-through holding aquarium within the recirculating NOCS research aquarium and maintained at ambient salinity (~32), with stable temperature (~15°C) and light:dark cycling following seasonal changes. Shrimp were fed to excess three times per week with Tetra Goldfish flakes. Shrimp were maintained for > 4 weeks but < 8 weeks prior to experimental treatment to minimise the potential for influences of seasonal temperature acclimation[28]. 7 days prior to experimental treatments animals were transferred to 10 l PVC plastic tanks filled with continuously aerated artificial seawater (salinity 32; pH 8.1; dissolved organic carbon 0.3 mg l^{-1}) made using Tropic Marin Sea Salt Classic (elemental composition similar to seawater[50]) according to manufacturer's instructions and acclimated to 15°C using a water bath (controlled by a Haake EK20 chiller and a Haake DC10 heater) (12:12 photoperiod). The water bath was adjusted to experimental temperature to achieve a continuous gradual temperature shift over ~5 hours, and experimental temperature was subsequently maintained until experimental treatments (7 days). Although thermal acclimation in *P. varians* may take significantly longer than 7 days[28], logistical constraints (size and number of water baths) prevented longer acclimation of organisms to experimental temperature. Water changes (50%; artificial seawater at experimental temperature) were conducted three times per week. Shrimp were starved for 3 days prior to experimental treatments to reduce potential effects of differences in digestive state on variability in sensitivity to toxicants due to temperature and hydrostatic pressure[51].

Standard and hyperbaric methods

Treatments exploring the effect of temperature on copper and cadmium toxicity were performed in 10 l PVC plastic tanks incubated within LMS model 230 series 2 cooled incubators at experimental temperature with 12:12 photoperiod (hereafter referred to as standard method). Treatments were conducted at 20°C and 10°C, respectively representing ecologically relevant shallow-water and hydrothermal vent temperatures. Treatments exploring the effect of hydrostatic pressure on copper and cadmium toxicity were performed in 6 l PVC plastic barrels incubated within the IPOCAMP hydrostatic pressure system[25] at 10°C with 24-h darkness (hereafter referred to as hyperbaric method). Treatments were conducted at 10°C and 0.1 or 10.0 MPa, respectively representing ecologically relevant shallow-water and hydrothermal vent hydrostatic pressures. Pressurisation of the experimental vessel was continuous and acute, taking less than 10 seconds. Performing treatments at 10°C and 0.1 MPa using both standard and hyperbaric methods allowed exploration of methodological effects.

Lethal toxicity

96-h copper and cadmium LC_{50} (concentration lethal to 50% of test individuals) were assessed by static renewal exposures. 10 individuals were exposed to each treatment, and treatments were performed in triplicate (n per treatment = 30). Individuals were exposed to artificial seawater or artificial seawater spiked with metal by the addition of stock solutions of $CuSO_4 \cdot 5H_2O$ or $CdCl_2 \cdot 2H_2O$ and equilibrated for 24 hours. Stock solutions were prepared using deionised water and analytical reagent grade compounds. Exposure concentrations (dissolved Cu and Cd addition: 0, 0.1, 1, 5, 10, 20, 30, 40 mg l^{-1}) were selected based on available copper lethal toxicity data in palaemonids (96-h LC_{50} 37.0 mg l^{-1} in *Palaemonetes pugio* at 22°C[52], preliminary ranging treatments at 20°C and 0.1 MPa, and available hydrothermal vent environmental data.

Total copper concentrations measured near hydrothermal vent shrimp habitats range between 0.03 mg l^{-1} at Rainbow vent and 0.04 mg l^{-1} at Lucky Strike vent, with 0.45µm filter-passing fraction ranging between 26 and 57%[19]. However, it is unlikely that these concentrations represent the range experienced within hydrothermal vent shrimp habitat: the proximity of sampling to hydrothermal vent shrimp habitat was unspecified, but the sample seawater temperature (4.3-8°C[19]) does not represent the range experienced within hydrothermal vent shrimp habitat (3-40°C[24,25,27,30,53,54]). Dissolved copper concentrations may be estimated based on end-member mixing using trace metal content at hydrothermal vents reported by Douville et al.[45] and ambient seawater temperatures reported by Desbruyeres *et al.*[44]. Estimated dissolved copper concentrations at 40°C, the proposed upper temperature experienced by hydrothermal vent shrimp[24,25,27,30,53], and at 18°C, the maximum temperature within *Rimicaris exoculata* swarms[54], are respectively 0.9 mg l^{-1} and 0.4 mg l^{-1} at the Rainbow vent, and 0.2 mg l^{-1} and 0.1 mg l^{-1} at the Lucky Strike vent, on the Mid-Atlantic Ridge. Dissolved copper concentrations of 0.1 and 1 mg l^{-1} are therefore likely to be ecologically relevant to hydrothermal vent shrimp habitat.

Exposure concentrations were matched in copper and cadmium treatments to provide material for comparison of sublethal responses to exposure concentrations. However, the magnitude of the low temperature effect on Cd toxicity was unexpected and additional treatments were required to effectively constrain the LC_{50} at low temperature (dissolved Cd addition: 50, 60, 70, 80 mg l^{-1}).

Dissolved copper and cadmium concentrations were measured in a representative subset of experimental seawater samples (n = 5 per target concentration) using ICP-OES (Optima 4300DV, Perkin Elmer), calibrated using synthetic standards. Instrumental drift was assessed every 10 samples and corrections were applied subsequently. Dissolved copper and cadmium concentrations were within 3% of target concentrations.

Mortality was assessed every 24 hours during a 100% water change performed with artificial seawater preparations acclimated to experimental temperature. No mortalities were observed in control treatments. Seawater oxygen saturation was determined at the end of each 24 hour incubation using an oxygen micro-optode connected to a PreSens Microx TX3 array, calibrated according to manufacturer's instructions. Oxygen saturation did not decrease below 70% in any treatment reducing the potential for influences of hypoxia[55]. Treatments exploring temperature and hydrostatic pressure effects were performed concurrently to minimise potential temporal influences on responses, but were logistically limited to 2 concentrations per week by the availability of incubators and hydrostatic pressure systems: concentration order in each replicate was randomised.

Probit analysis of pooled mortality data from replicate treatments was used to model the lethal effects of metal exposures and determine the 96 h LC_{50}, assuming a logistic distribution based on the sigmoidal data pattern: $\ln\left[\frac{Y}{1-Y}\right] = a + bX$, where X is the exposure metal concentration and Y is the proportion of individuals suffering mortality. Subsequently, probit analysis was used to make pairwise comparisons of lethal effects of metal exposures in different treatments, thus incorporating an assessment of the effect of the different experimental methods at 10°C and 0.1 MPa. The Holm-Bonferroni correction was used to maintain the family-wise error rate during multiple comparisons.

Interacting toxicity

The effect of temperature and hydrostatic pressure on the interaction of copper and cadmium was explored by testing deviation from the independent addition reference model (IA) predictions[56,57]. 96 h lethal toxicity was assessed in a mixture of copper and cadmium with LC_{29} of both metals (derived from the modelled mortality responses to individual metals in each temperature/hydrostatic pressure treatment) following the previously described protocol. Mortalities were compared to a control treatment and to individual exposures to copper and cadmium LC_{29}. IA predicted mortality in a binary mixture with LC_{29} of both constituents is 50% according to Faust et al.'s[58] equation:

$$E_{(c_{mix})} = 1 - \prod_{i=1}^{n} (1 - E_{(c_i)}$$

where $E_{(c_{mix})}$ is the calculated effect of the mixture, ci the exposure concentration of substance i, and $E_{(c_i)}$ the effect of substance *i* at concentration c_i expressed as a fraction of a maximum possible effect. Copper and cadmium LC_{29} was respectively 10.7 mg l^{-1} and 7.8 mg l^{-1} at 20°C and 0.1 MPa using standard method, 21.8 mg l^{-1} and 51.2 mg l^{-1} at 10°C and 0.1 MPa using standard method, 19.4 mg l^{-1} and 27.7 mg l^{-1} at 10°C and 0.1 MPa using hyperbaric method, and 12.5 mg l^{-1} and 44.7 mg l^{-1} at 10°C and 10.0 MPa using hyperbaric method (Figure 1). Deviation from expectations was assessed using established criteria: combination in a mixture caused a more severe (synergism) or a less severe (antagonism) effect than predicted by simple addition[56,57]. Temperature and hydrostatic pressure treatments were performed concurrently with replicate treatments performed in consecutive weeks due to logistical limitations. The effect of temperature and hydrostatic pressure on the interaction of copper and cadmium was analysed by ANOVA using arcsine square-root transformed proportion data: data were normal (Shapiro-Wilk test) and homoscedastic (Levene's test) ($p > 0.05$).

Respiratory response to sublethal toxicity

Oxygen consumption rates were measured to assess the effect of temperature and hydrostatic pressure on the respiratory response to copper or cadmium. Oxygen consumption rates were measured using an adaptation of established protocols[59]. In brief, 5 individuals were exposed to 0, 0.1, or 1 mg l^{-1} dissolved Cu or Cd additions for 96 hours as described previously. Shrimp were subsequently transferred to 33 ml plastic vials filled with water from their incubation. Vials were closed underwater to ensure the absence of air bubbles and each vial was placed inside a temperature-acclimated hydrostatic pressure vessel filled with temperature-acclimated freshwater. Hydrostatic pressure vessels containing individuals from the 10.0 MPa hydrostatic pressure treatment were pressurised to 10.0 MPa: pressurisation of the experimental vessel was continuous and acute, taking less than 10 seconds, and was achieved using a Maximator M72 manual air-driven liquid pump. Hydrostatic pressure vessels were placed within LMS model 230 series 2 cooled incubators to maintain experimental temperature. Isolation periods differed between 20°C and 10°C to compensate for temperature-dependent metabolic rate: animals were isolated for 45 minutes at 10°C and 30 minutes at 20°C. Oxygen concentration within the vial did not fall below 50% oxygen saturation, reducing the potential for hypoxic metabolic influences in *P. varians*[55]. For each treatment, 5 control vials containing only seawater from the 96 hour incubation were isolated using an identical procedure, to control for microbial respiration within the seawater.

Following the isolation period, hydrostatic pressure vessels were immediately depressurised, and the vial was removed and inverted three times to ensure homogeneity of seawater oxygen within the vial. The vial lid was removed and the oxygen saturation of the seawater was determined using an oxygen micro-optode connected to a PreSens Microx TX3 array, calibrated according to the manufacturer's instructions. The animal was then removed from the vial, gently blotted on tissue paper, transferred to a 1.5 ml centrifuge tube, and flash frozen in liquid nitrogen within 10 minutes of departure from experimental condition. Samples were stored at -80°C for subsequent biomass and biochemical analysis. Molar oxygen consumption (MO_2, μmol O_2 mg^{-1} h^{-1}) was calculated from the difference between the oxygen saturation in the control vial and the oxygen saturation in the treatment vials, following established methods for determining oxygen concentration in air-saturated seawater[60]. Shrimp total wet mass was 179.3 ± 24.0 mg (mean ± SD).

The effect of temperature and hydrostatic pressure on the respiratory response to copper and cadmium was analysed using two-way ANOVAs: data were normal (Shapiro-Wilk test) and homoscedastic (Levene's test) ($p > 0.05$). The post-hoc multiple comparison Holm-Sidak test was used to determine which treatments produced the differences.

Antioxidant response to sublethal toxicity

Tissue from individuals used to assess respiratory response to sublethal toxicity was homogenised at 4°C in Tris-HCl pH 7.6 buffer. Following 10 mins incubation at 4°C, the homogenates were centrifuged at 1000*g* for 10 min at 4°C and supernatant was retained for analysis. Total protein and enzyme activities were determined using a microplate reader (FLUOstar OPTIMA, BMG Labtech). Total protein was assessed at 584 nm absorbance employing the Lowry method with Peterson's modification using an assay kit (Sigma-Aldrich TP0300). Superoxide dismutase (SOD) activity was assessed at 450 nm absorbance employing inhibition of xanthine oxidase activity using an assay kit (Sigma-Aldrich 19160). Glutathione peroxidase (GPx) activity was assessed at 340 nm absorbance employing

NADPH oxidation in the presence of excess glutathione reductase, reduced glutathione, and corresponding peroxide using an assay kit (Abcam ab102530).

The effects of temperature and hydrostatic pressure on biochemical responses to copper and cadmium were analysed using two-way ANOVAs: data were normal (Shapiro-Wilk test) and homoscedastic (Levene's test) ($p > 0.05$). The post-hoc multiple comparison Holm-Sidak test was used to determine which treatments produced the differences.

Results

Temperature and hydrostatic pressure effects on lethal toxicity

Copper and cadmium LC_{50} was respectively 13.7 mg l^{-1} and 9.8 mg l^{-1} at 20°C and 0.1 MPa using standard method, 26.9 mg l^{-1} and 61.4 mg l^{-1} at 10°C and 0.1 MPa using standard method, 24.3 mg l^{-1} and 58.4 mg l^{-1} at 10°C and 0.1 MPa using hyperbaric method, and 15.9 mg l^{-1} and 55.6 mg l^{-1} at 10°C and 10.0 MPa using hyperbaric method (Figure 1). Both copper and cadmium LC_{50} were significantly reduced at low temperature ($p < 0.001$) and there was no significant difference between the different incubation methods at 10°C and 0.1 MPa ($p > 0.05$) (Figure 1). However, whilst copper LC_{50} was significantly lower at high hydrostatic pressure ($p = 0.003$), cadmium LC_{50} was not significantly affected ($p > 0.05$) (Figure 1).

The interaction between copper and cadmium was potentiating (Figure 2). Low temperature did not significantly affect the interaction ($F_{1,4} = 0.500$, $p = 0.519$) and there was no significant difference between standard treatment and hyperbaric aquaria treatment at 10°C and 0.1 MPa ($F_{1,4} = 0.002$, $p = 0.967$). However, high hydrostatic pressure significantly increased the potentiation effect ($F_{1,4} = 11.768$, $p = 0.027$).

Respiratory response to sublethal toxicity

Exposure to sublethal concentrations of both copper and cadmium significantly affected respiratory rate, but the effect depended on environmental conditions (Figure 3) (respectively, $F_{6,48} = 4.521$, $p = 0.001$ and $F_{6,48} = 5.180$, $p < 0.001$). Respiratory sensitivity to both copper and cadmium exposures was reduced at low temperature ($p < 0.05$), but the effect of high hydrostatic pressure on sensitivity to copper and cadmium differed: high hydrostatic pressure increased respiratory sensitivity to copper ($p < 0.05$), but not to cadmium ($p > 0.05$).

Antioxidant response to sublethal toxicity

Exposure to sublethal concentrations of both copper and cadmium significantly affected SOD activity and GPx activity, but the effect depended on environmental conditions (Figures 4 and 5) (respectively, $F_{6,48} = 2.463$, $p = 0.037$ and $F_{6,48} = 2.682$, $p = 0.025$, and $F_{6,48} = 3.391$, $p = 0.007$ and $F_{6,48} = 5.350$, $p < 0.001$). SOD and GPx activities were significantly elevated in response to 0.1 and 1.0 mg l^{-1} dissolved copper and cadmium exposures at 20°C ($p < 0.05$). Sensitivity to both copper and cadmium exposures was reduced at low temperature ($p < 0.05$). SOD and GPx activities were significantly elevated in response to 1.0 mg l^{-1} dissolved copper exposure at 10°C, whereas there was no significant effect of 1.0 mg l^{-1} dissolved cadmium exposure on SOD and GPx activities at 10°C. In contrast, the effect of high hydrostatic pressure on sensitivity to copper and cadmium differed: high hydrostatic pressure increased biochemical sensitivity to copper ($p < 0.05$), but not to cadmium ($p > 0.05$). SOD and GPx activities were significantly elevated in response to 0.1 and 1.0 mg l^{-1} dissolved copper at 10°C and 10.0 MPa, whereas there was no significant effect of 1.0 mg l^{-1} dissolved cadmium on SOD and GPx activities at 10°C and 10.0 MPa.

Discussion

Mechanisms of toxicity

Both copper and cadmium lethal toxicity appear related to respiratory impairment[47]. Lethal toxicity in copper and cadmium exposures results from systemic disturbance in acid-base balance in decapods, caused by the inhibition of carbonic anhydrase[61,62]. Carbonic anhydrase facilitates acid-base regulation and respiratory exchange by promoting rapid equilibration between HCO_3^- and CO_2 and contributing to haemocyanin modulation and gas transport[63,64]. At high concentrations, copper and cadmium reduce respiratory capacity, leading to a mismatch in oxygen supply and demand and ultimately resulting in death[49]. However, exposure to sublethal copper concentration stimulates increases in cardiac activity in caridean shrimp, whereas cardiac activity is not significantly affected by exposure to sublethal cadmium concentration[65], highlighting that the toxicity of these metals manifests via different mechanisms.

Metal exposures also have a direct effect on cellular bioenergetics, interfering with ATP-producing pathways. Copper and cadmium adversely affect mitochondrial function and capacity for aerobic ATP production, resulting in reduced mitochondrial efficiency and coupling, elevated proton leak and, in severe cases, depolarization, which all increase oxidative stress[66]. Elevated oxidative stress in response to copper and cadmium exposures is apparent in increased SOD and GPx activity in *P. varians*. Whilst decreased sensitivity to copper and cadmium at lower temperature is likely the result of thermodynamic effects on toxicokinetics, the differential magnitude in the reduction of sensitivity likely results from contrasting mechanisms of action in these metals. The contrasting effects of hydrostatic pressure on copper and cadmium toxicity similarly likely results from differing mechanisms of toxicity: hydrostatic pressure is also a thermodynamic parameter, but the effects of hydrostatic pressure depend on the system volume change in individual reactions, rather than on molecular kinetic energy[67].

Indeed, the contrasting effects of hydrostatic pressure on sensitivity to copper and cadmium may reveal the different mechanisms of copper and cadmium toxicity. Hyperbaric tolerance is proposed to depend on reduced respiratory capacity resulting from diminished cardiac performance, itself mediated by depressed neurotransmission caused by the effects of hydrostatic pressure on cell surface receptors[68]. *N*-methyl-$_{\text{D}}$-aspartate (NMDA) receptors are ligand- and voltage-gated glutamate receptors, i.e. ion channels that mediate excitatory neurotransmission, predominantly regulated by Mg^{2+} blockade[69]. NMDA receptors are present in all major ganglia of decapods, demonstrating strong localisation in synaptosomal membranes[70]. Hyperbaric neurophysiological stress at ecologically relevant temperatures appears to result from increased NMDA receptor activity in *P. varians*[40-42], and also in the bathyal decapod *Lithodes maja*[68,71].

Copper and cadmium also modulate NMDA receptor activity and lead to neurophysiological stress, affecting synaptic function and consequently impacting cardiac performance[72-75]. However, copper and cadmium modulate NMDA receptor activity through contrasting actions; copper increases NMDA-receptor activity[72,73], whereas cadmium decreases NMDA-receptor activity[74,75]. The effects of hydrostatic pressure on NMDA receptor activity may therefore explain the contrasting effects of hydrostatic pressure on sensitivity to copper and cadmium; hydrostatic pressure may exacerbate increases in NMDA receptor activity caused by copper, but may mitigate decreases in NMDA receptor activity caused by cadmium. The significant effect of hydrostatic pressure on the synergistic interaction between copper and cadmium supports this hypothesis.

Ecological implications

Although acute lethal toxicity may be relevant to deep-sea mining scenarios, it may be the bioenergetic consequences of sublethal metal exposures that are critical to determining long-term survival[66]. Metabolic rate may be elevated during exposure to copper and cadmium due to elevated basal metabolic demand[66]. Cellular protective mechanisms respond to metal exposure with upregulated antioxidants, metallothioneins, glutathione, molecular chaperones, and/or cellular repair pathways, which increase basal metabolic demand. Indeed, elevated protein synthesis and turnover are amongst the most important ATP sinks in the cell[66]. Increased basal metabolic demand is met by increased energy allocation to basal maintenance, which comprises key cellular processes (e.g. ion regulation, protein turnover) and essential systemic activities such as ventilation and circulation[76]. Individual fitness is therefore reduced as additional homeostatic energy costs lead to energetic trade-offs: increased allocation of energy to maintenance during stress diminishes energy available for other functions, and consequently reduces scope for growth, activity and reproductive output [66,76]. Decreased sequestration of energy as reserves and/or a reallocation of energy reserves to maintenance, decreases capacity to buffer fluctuating food availability and ensure continuous metabolic energy supply[66]. This may crucially affect survival in the deep-sea, where energy constraints are potentially high[77], and may become even more constrained with climate change[78]. Individual bioenergetic responses also decrease capacity to allocate energy to provisioning offspring, potentially impacting fecundity and/or offspring fitness[66], and thus influencing source-sink dynamics of wider deep-sea populations[79].

The physiological effects of toxicity are hypothesised to integrate with the physiological effects of temperature, hydrostatic pressure, oxygen concentration, and carbon dioxide concentration in a matrix of oxygen-limited acute tolerance, limited by diminishing respiratory capacity, and energy-limited chronic tolerance[5-7]. Indeed, a stress addition model has been proposed to provide a tool that quantitatively predicts the synergistic direct effects of independent stressor combinations, including toxicity[80]. The interacting effects of temperature and hydrostatic pressure on sensitivity to copper indicates that the ecotoxicological risks posed by mobilisation of some toxicants during deep-sea mining will vary with the location of an individual within the species' biogeographic range. Predictions based on this hypothesis imply that toxic exposure reduces both acute and chronic thermal tolerance[49]. Similarly, hydrostatic pressure tolerance may also narrow in response to toxic exposures. Since marine ectotherms tend to fully occupy their thermal niches[81], and at least some species fully occupy their baric niches[68,82], this may result in both reduced temperature and depth range. Therefore, the effects of metal exposures associated with deep-sea mining processes may result in the erosion of deep sea populations at biogeographic range limits.

Insights into adaptation in deep-sea hydrothermal vent shrimp

Several studies have employed a comparative approach in hydrothermal vent and coastal shrimp, focusing on measuring *in situ* metal accumulation and/or biomarker expression (lipid peroxidation, antioxidant enzymes, metallothioneins) to explore adaptation to the toxic challenges presented by hydrothermal vent environment[20,37,38,83]. Although such an approach explores *in situ* physiology at ambient toxicant concentrations, it contributes relatively limited information about differences and/or similarities in sensitivity and responses to elevated toxicant concentrations among species. A comparative experimental approach is required to reveal such knowledge, with individuals from different phylogenetically related deep-sea and shallow-water species exposed to similar challenges under native environmental conditions (e.g. hydrostatic pressure, temperature)[4]. The recent experimental assessment of sublethal responses to copper exposure in *Rimicaris exoculata* at 10°C and 30.0 MPa[84] thus provides the first opportunity to develop significant insight into caridean adaptation to toxicity in the hydrothermal vent environment.

Methodological and analytical differences between these studies demand caution; identical methodological and analytical approaches are required for unequivocal conclusions. Nonetheless, significant up-regulation of antioxidant enzymes in *P. varians* in response to both 0.1 mg Cu^{2+} l^{-1} and 1.0 mg Cu^{2+} l^{-1} at 20°C and 0.1 MPa (this study) differs from the absence of significant responses to both ~0.025 mg Cu^{2+} l^{-1} and ~0.25 mg Cu^{2+} l^{-1} (0.4 µM Cu^{2+} and 4.0 µM Cu^{2+}) in *R. exoculata* at 10°C and 30.0 MPa[84]. In contrast, the absence of a response to 0.1 mg Cu^{2+} l^{-1} at 10°C and 0.1 MPa in *P. varians* is consistent with the responses reported in *R. exoculata*. These comparisons suggest that sensitivity and responses to toxicants may not differ between these species at a common temperature at native hydrostatic pressure, and therefore that shallow-water taxa may be suitable ecotoxicological proxies for deep-sea taxa. However, *P. varians* is significantly more tolerant of both copper and cadmium than other shallow-water caridean shrimp species[85-91], indicating that shallow-water ecotoxicological proxy species cannot be chosen arbitrarily. High metal tolerance in *P. varians* may result from adaptation to environmental exposure levels, which vary significantly in estuarine, salt marsh, and lagoon habitats[92]. Alternatively, high metal tolerance in *P. varians* may result from adaptation to highly variable environmental temperature, salinity, and oxygen concentration. Other caridean shrimp inhabiting fully marine or freshwater environments demonstrate significantly lower tolerance to temperature, salinity, and oxygen concentration[93]. Consequently, matching environmental exposure levels and environmental variability experienced by deep-sea species may be a key criterion for the selection of shallow-water ecotoxicological proxy species. For example, non-vent deep-sea taxa may be more sensitive to metal disrupted acid-base regulation than shallow-water taxa from temperate latitudes due to lower capacities for pH buffering and low concentrations of ion-transport proteins arising from adaptations to stable environmental conditions in the wider deep-sea[94]. In contrast, adaptations to cold stenothermal environments suggest that polar shallow-water fauna may be physiologically more similar to deep-sea fauna than temperate or tropical shallow-water fauna[95], and thus may be more suitable ecotoxicological proxies.

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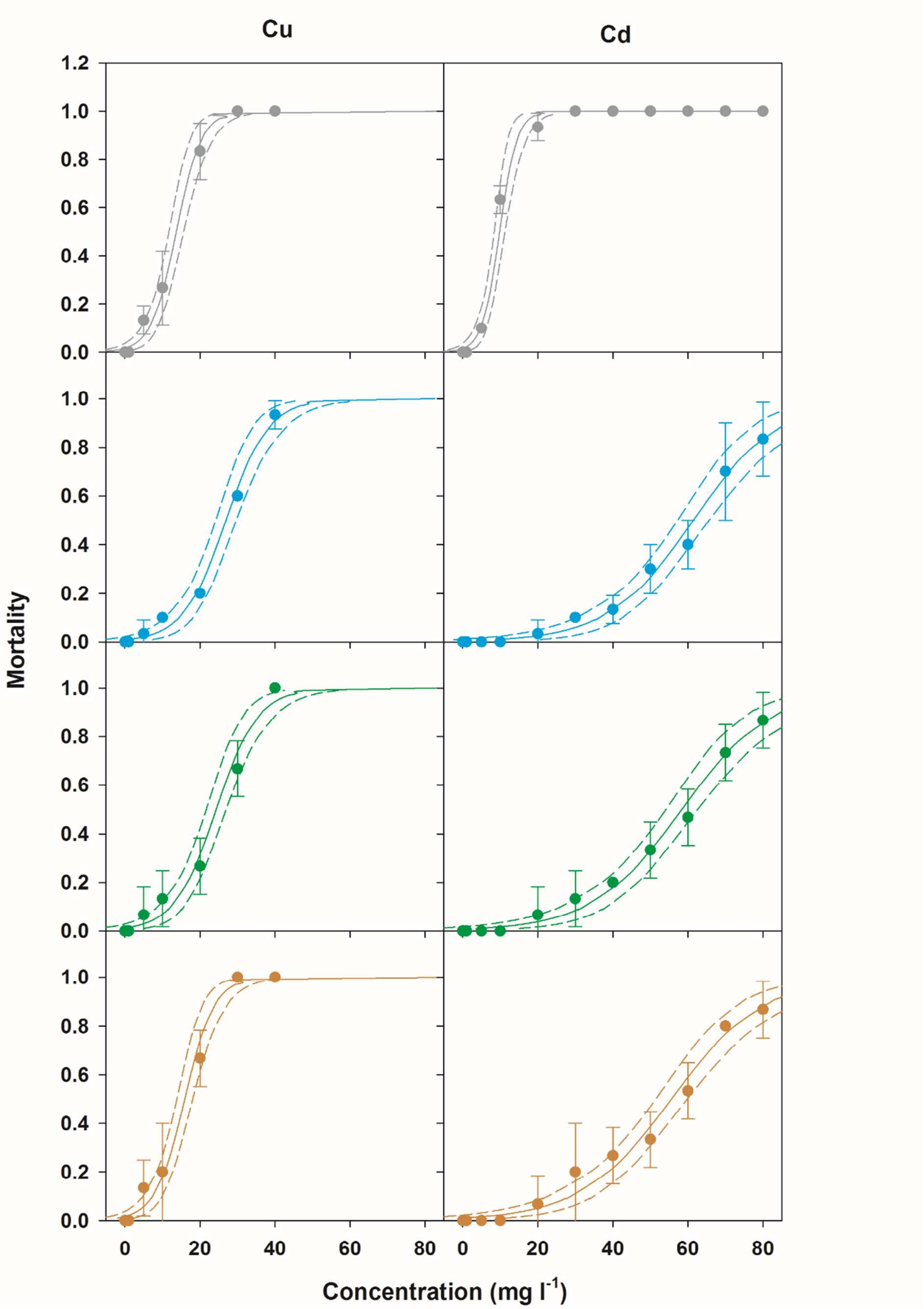
475 **Figures**



476

Figure 1. Effect of temperature, exposure method, hydrostatic pressure, and exposure duration on lethal copper and cadmium toxicity in *Palaemon varians*. Filled circles represent mean mortality and error bars represent ± standard deviation (n = 3) at 20°C and 0.1 MPa using standard method (grey), 10°C and 0.1 MPa using standard method (blue), 10°C and 0.1 MPa using hyperbaric method (green), and 10°C and 10.0 MPa using hyperbaric method (orange). The solid line and dashed lines represent mortality modelled using probit analysis of pooled mortality data (n = 30), assuming a logistic distribution.

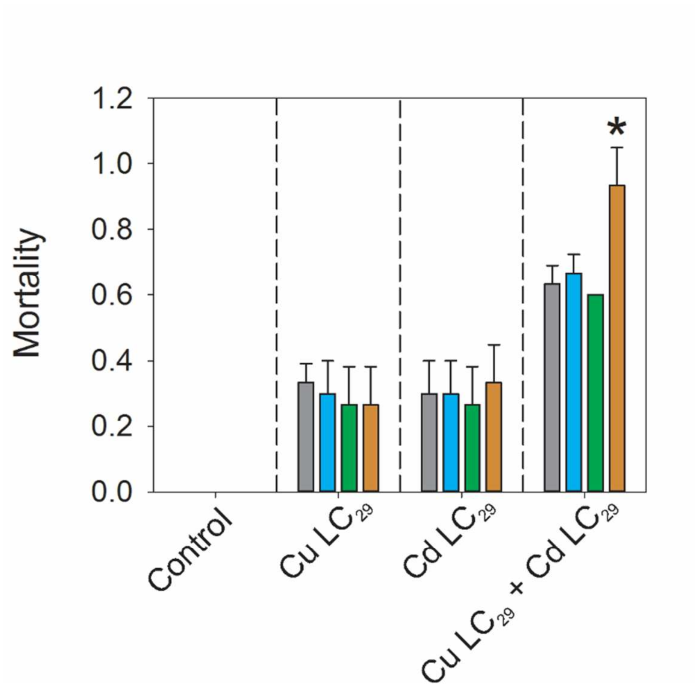


Figure 2. Effect of temperature, exposure method, and hydrostatic pressure on the interaction between 96 h copper and cadmium toxicity in *Palaemon varians*. Bars represent mortality (mean ± SD; n = 3) at 20°C and 0.1 MPa using standard method (grey), 10°C and 0.1 MPa using standard method (blue), 10°C and 0.1 MPa using hyperbaric method (green), and 10°C and 10.0 MPa using hyperbaric method (orange). Asterisks indicate significant differences from 20°C and 0.1 MPa within toxicant treatments.

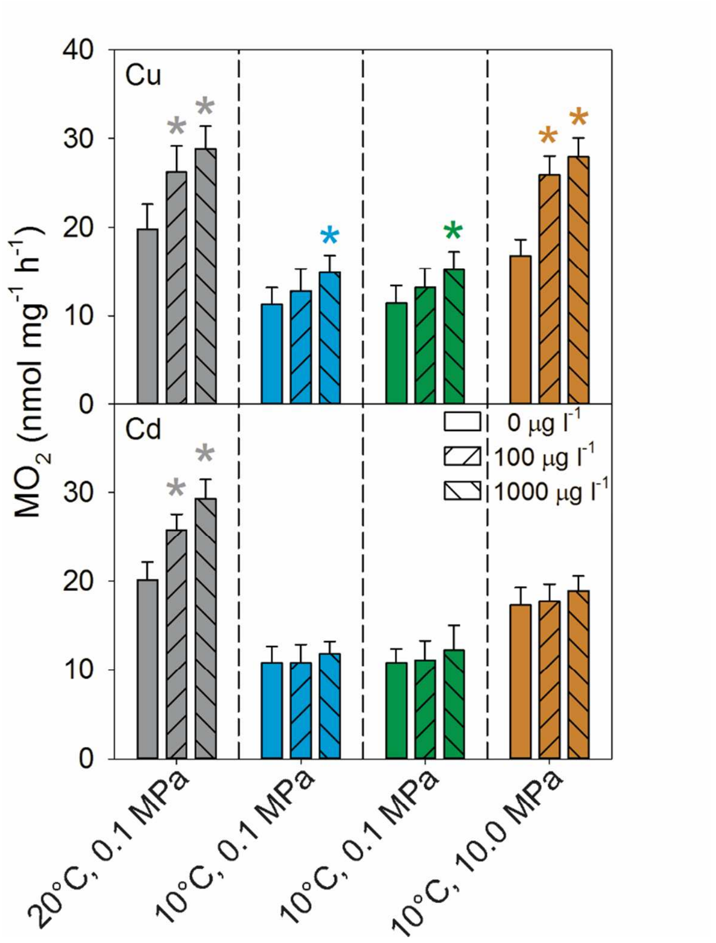


Figure 3. Effect of temperature, exposure method, and hydrostatic pressure on respiratory response to 96 h copper and cadmium exposure in *Palaemon varians*. Bars represent molar oxygen consumption (MO_2) (mean ± SD; n = 5) at 20°C and 0.1 MPa using standard method (grey), 10°C and 0.1 MPa using standard method (blue), 10°C and 0.1 MPa using hyperbaric method (green), and 10°C and 10.0 MPa using hyperbaric method (orange). Asterisks indicate significant differences from 0 µg l^{-1} treatment within experimental conditions.

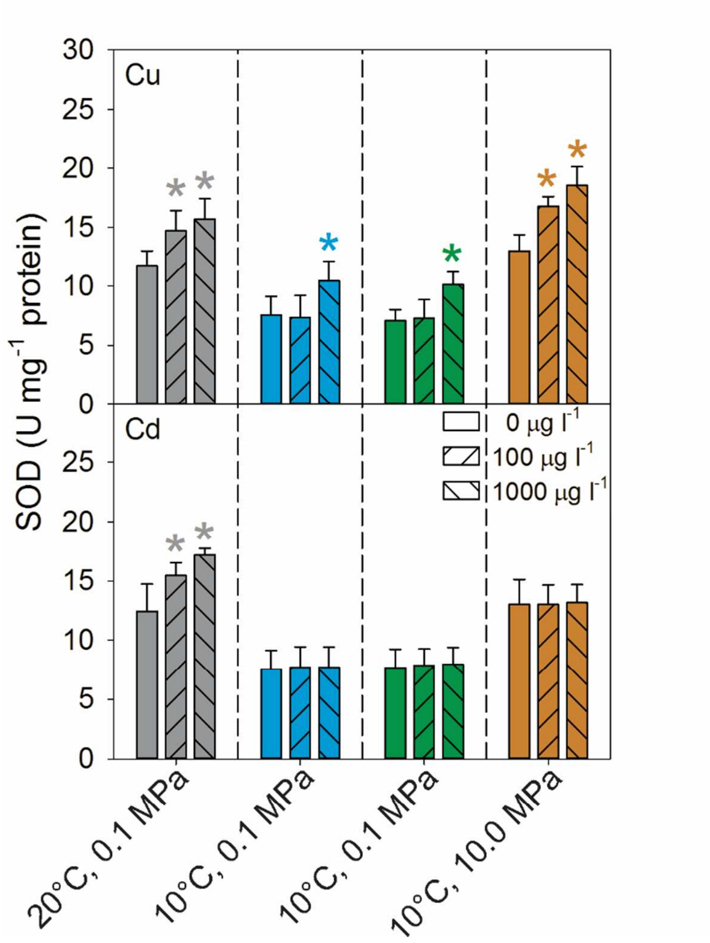


Figure 4. Effect of temperature, exposure method, and hydrostatic pressure on superoxide dismutase (SOD) response to 96 h copper and cadmium exposure in *Palaemon varians*. Bars represent SOD activity (mean ± SD; n = 5) at 20°C and 0.1 MPa using standard method (grey), 10°C and 0.1 MPa using standard method (blue), 10°C and 0.1 MPa using hyperbaric method (green), and 10°C and 10.0 MPa using hyperbaric method (orange). Asterisks indicate significant differences from 0 µg l^{-1} treatment within experimental conditions.

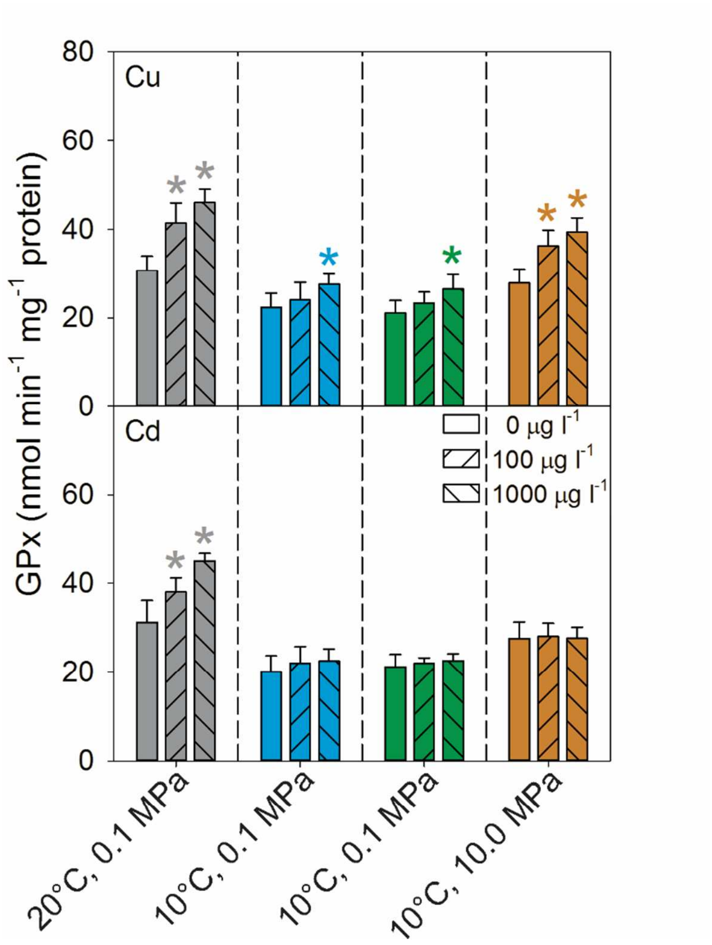


Figure 5. Effect of temperature, exposure method, and hydrostatic pressure on glutathione peroxidase (GPx) response to 96 h copper and cadmium exposure in *Palaemon varians*. Bars represent GPx activity (mean ± SD; n = 5) at 20°C and 0.1 MPa using standard method (grey), 10°C and 0.1 MPa using standard method (blue), 10°C and 0.1 MPa using hyperbaric method (green), and 10°C and 10.0 MPa using hyperbaric method (orange). Asterisks indicate significant differences from 0 µg l^{-1} treatment within experimental conditions.

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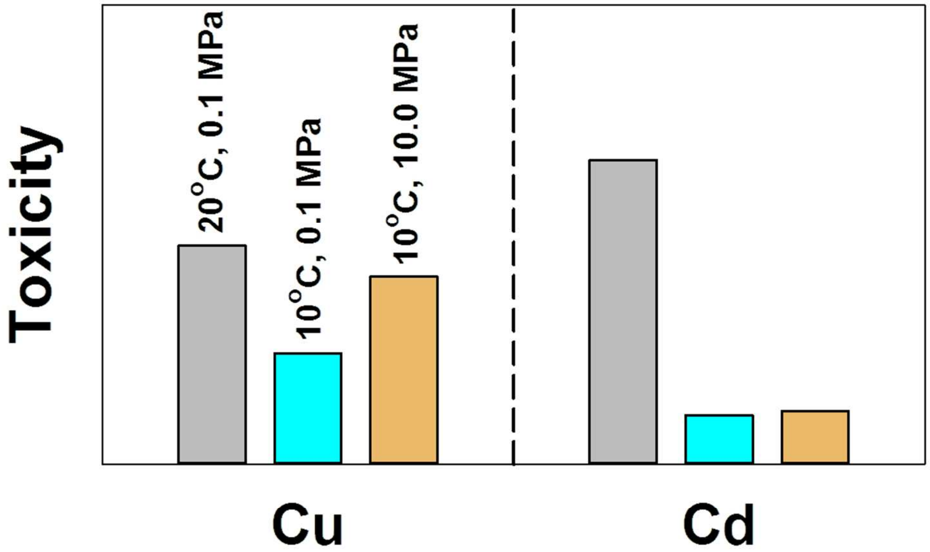

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