



First experimental assessment of copper and cadmium toxicity and sublethal responses in *Clibanarius erythropus* (Latreille, 1818)

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ABSTRACT

Trace elements are a persistent threat to Mediterranean coastal ecosystems, where anthropogenic pressures continue to increase. Although biomarker-based approaches are widely used in marine biomonitoring, species-specific ecotoxicological data remain limited. The hermit crab *Clibanarius erythropus* (Latreille, 1818), a widespread Mediterranean detritivore chronically exposed to sediment-associated trace elements, has never been ecotoxicologically investigated. In this study, the first integrated assessment of the sensitivity of *C. erythropus* to copper (Cu) and cadmium (Cd) was conducted. Acute toxicity (96-h median lethal concentration, LC₅₀) was assessed along with inductively coupled plasma mass spectrometry (ICP-MS) quantification of hepatopancreatic bioaccumulation. Sublethal responses were evaluated in the entire soft tissues of the abdomen, including the hepatopancreas, after 24, 48, and 96 h of exposure to ½ LC₅₀ (high), ¼ LC₅₀ (medium), and 1/8 LC₅₀ (low). This study provides a specie-specific assessment in *C. erythropus*, confirming the expected higher toxicity of Cd over Cu as demonstrated by significantly lower Cu 96-h LC₅₀ values and greater bioaccumulation (LC₅₀ = 4.96 mg L⁻¹ for Cd vs. 46.3 mg L⁻¹ for Cu; 914 ± 75.0 mg kg⁻¹ for Cd vs. 421 ± 66.8 mg kg⁻¹ for Cu). With both metals, sublethal exposure triggered a significant increase in reactive oxygen species (ROS) and glutathione depletion, accompanied by the induction of alkaline phosphatase, esterase, and peroxidase activities. These findings establish the first species-specific ecotoxicological baseline for *C. erythropus* and support its potential use as a bioindicator in coastal monitoring programs under the Water Framework Directive (WFD) and Marine Strategy Framework Directive (MSFD).

1. Introduction

In recent decades, population growth, urbanization, and industrial expansion have significantly increased the input of contaminants into aquatic ecosystems (El-Sharkawy et al., 2025; Pauly et al., 2025). This anthropogenic pressure, largely due to the discharge of untreated industrial effluents and domestic wastewater, has progressively deteriorated coastal water quality, compromising ecosystem resilience and threatening marine biodiversity (Schweitzer and Noblet, 2018; Speight, 2020). In this context, water pollution emerges as one of the major environmental challenges of the 21st century (Orebiyi and Awomeso, 2008).

Among aquatic contaminants, trace metals have received sustained attention since the 1970s due to their persistence, non-biodegradability, and potential for bioaccumulation and biomagnification across trophic levels (Ahmed et al., 2019; Edo et al., 2024). Specifically, copper (Cu) and cadmium (Cd) are of particular concern in coastal environments due to their widespread occurrence and well-documented biological effects. Copper is widely used in aquaculture as copper sulfate (CuSO₄) –a therapeutic agent against parasitic infections and an algicide for controlling cyanobacterial blooms (Noga, 2010; Gao et al., 2022; Watson et al., 2024). Additionally, Cu enters aquatic systems through mining, industrial discharges, and agricultural runoff containing copper-based fungicides (Comber et al., 2023). Although Cu is an essential trace

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element for physiological processes such as respiration and antioxidant defense, excessive concentrations can induce toxicity by generating reactive oxygen species (ROS), promoting lipid peroxidation, and disrupting cellular homeostasis (Jiang et al., 2014; Gaetke et al., 2014). In contrast, cadmium is a non-essential element with no known biological function. It is a highly toxic priority pollutant, being recognized for its persistence and mobility (ATSDR, 2012), and exerts toxicity via ionic mimicry — displacing essential metals like zinc and calcium — and binding to sulfhydryl groups, thereby impairing enzymatic and mitochondrial functions, causing DNA damage, and triggering immunotoxic effects (Cuypers et al., 2010; Thévenod and Lee, 2013; Genchi et al., 2020). Major anthropogenic sources include industrial effluents, phosphate fertilizers, and mining (Xu et al., 2025). Both metals tend to accumulate in coastal sediments, which serve as both sinks and secondary sources of contamination. Their remobilization under changing environmental conditions can result in chronic exposure for benthic organisms, leading to tissue damage, immune dysregulation, and long-term ecological disturbances (Briffa et al., 2020; Zahran et al., 2025). This is particularly critical in the Mediterranean Sea, a semi-enclosed basin characterized by restricted water circulation and intense coastal urbanization, which promotes metal retention (Lotze et al., 2006; Alimba and Faggio, 2019). Furthermore, as a climate change “hotspot,” rising temperatures and acidification in the Mediterranean may further alter metal speciation and bioavailability (Noyes et al., 2025). Together, these factors increase the region's vulnerability to metal contamination and highlight the need for monitoring approaches that integrate chemical and biological responses.

In this context, traditional methods that focus solely on physico-chemical concentrations in water and sediment often fail to reflect chronic, sublethal impacts on biota. This limitation is especially evident in complex coastal environments, where the interaction of multiple stressors reduces the ecological relevance of single-parameter measurements (Islam and Tanaka, 2004; Adams, 2005). To bridge this gap, biomonitoring is increasingly used to complement chemical analyses, enabling the detection of early biological responses to contaminant exposure (Linton and Warner, 2003; Desrosiers et al., 2013). Among potential biomonitoring organisms, filter-feeding animals and benthic crustaceans are particularly informative due to their niche specialization and direct contact with sediment-bound trace metals (Navarro-Barranco et al., 2020). Consequently, in the last decades, extensive research has documented how metal exposure in bivalves alters feeding activity, respiration, and growth rates (Elfwing and Tedengren, 2002; Anandraj et al., 2002; Duquesne et al., 2004; Saidani et al., 2023). Similarly, in crustaceans, metals can disrupt essential processes such as molting, reproduction, and osmoregulation, while inducing oxidative stress and immune suppression (Frias-Espicueta et al., 2022; Banaee et al., 2024; Waqas et al., 2024). However, to date, many studies assessing the impact of metal contaminants using decapod crustaceans have focused primarily on established or commercially valuable model species. As a result, many taxa remain toxicologically uncharacterized in terms of comparative sensitivity. However, it is crucial to emphasize that regulatory toxicity thresholds derived from standard species often fail to accurately predict the resistance of local macroinvertebrates, whose sensitivity profiles can vary significantly due to specific physiological adaptations or synergistic environmental stresses. Therefore, comparing the toxicological sensitivity of new species with these established baselines is a necessary step to validate their environmental relevance and monitoring effectiveness.

Among these, the hermit crab *Clibanarius erythropus* (Latreille, 1818) is a common and widespread species along the coasts of the Mediterranean and northeastern Atlantic Ocean, with a geographic range extending from the Macaronesian archipelagos (Azores, Canary Islands, Madeira) and the British Isles to the Black Sea (GBIF Secretariat, 2024). It is a diogenid decapod crustacean, a diverse group that includes hermit crabs, crabs, lobsters, crayfish, and shrimps. Inhabiting shallow intertidal and subtidal zones, *C. erythropus* is regularly exposed to fluctuating

environmental conditions and anthropogenic inputs (Terlizzi et al., 2002; Widdows and Brinsley, 2002). Furthermore, its vital role in recycling organic matter as an opportunistic scavenger, its close and continuous interaction with sediments, its high abundance, limited dispersal, and site fidelity, make this species an ideal candidate for biomonitoring (Tran, 2014; Wang et al., 2015). However, no systematic investigations have yet addressed its sensitivity or bioaccumulation capacity.

Thus, this study aimed to address existing knowledge gaps by the first ecotoxicological profile of *C. erythropus* through the assessment of acute toxicity, metal bioaccumulation, and sublethal biomarker responses. This integrated approach provides the first species-specific characterization of the sensitivity of *C. erythropus* to Cu and Cd exposure, supporting its potential application in Mediterranean coastal monitoring programs. In conclusion, this study provides a framework for the interpretation of sublethal biomarker responses and highlights their relevance for environmental monitoring of this species, particularly in localized or episodic contamination scenarios in the Mediterranean.

2. Materials and methods

2.1. Study area and sampling

Specimens of *Clibanarius erythropus* were collected between March and May 2025 along the northwestern coast of Sicily (Italy) (Fig. 1A). Sampling was performed across two adjacent intertidal rocky-shore sites to represent the local population: Magaggiari Beach (Cinisi; 38°09'29" N, 13°05'01" E), a mixed rocky–sandy shore (Fig. 1B), and Cala Rossa–Capo Rama (Terrasini; 38°08'31" N, 13°04'20" E), located within a Special Area of Conservation (SAC, ITA020009, Natura 2000 network) (Fig. 1C). Intertidal specimens were collected manually during low tide along linear transects across tide pools and rock crevices (Stirn, 1981). Organisms were transported in aerated seawater to the laboratory and acclimated for 7 days in 5 L aerated glass aquaria under standardized laboratory conditions: temperature 18 ± 2 °C, salinity 33 ± 2 , and a 12:12 h light:dark photoperiod. Animals were fed ad libitum with a commercial pelleted diet twice weekly. Feeding was terminated 24 h prior to experimental exposures to standardize metabolic status. No mortality was observed in the control groups throughout either the acute toxicity or the sublethal exposure experiments.

2.2. Acute toxicity test: LC_{50} determination

Acute toxicity tests (96 h) for copper (Cu) and cadmium (Cd) were conducted using a static-renewal experimental design, with full replacement of test solutions every 24 h to preserve water quality and nominal metal concentrations. Temperature and salinity were maintained under controlled conditions throughout the exposure period (18 ± 2 °C and 33 ± 2 , respectively), as established during the acclimation phase. Exposures were carried out in 5 L aquaria at a standardized density of 15 individuals per tank (3 ind L^{-1}).

For each metal, test groups ($n = 15$ per group, in triplicate) were exposed to nominal concentration series: 0, 20, 40, 60, and 80 mg L^{-1} for $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 0, 10, 20, 30, and 40 mg L^{-1} for $\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$ (Sigma-Aldrich, St. Louis, MO, USA). Reagent salts were weighed using an analytical balance (Bel Engineering M214AiH, Italy; $\pm 0.1 \text{ mg}$) and dissolved in ultrapure water before addition to natural seawater. Mortality was checked every 24 h; organisms were recorded as dead upon failing to retract into their shell following mechanical stimulation or displaying complete absence of appendage movement during a 4-min observation (Otítolaju and Don-Pedro, 2006). Median lethal concentrations (96-h LC_{50}) were calculated via Finney's Probit analysis (Finney, 1971).

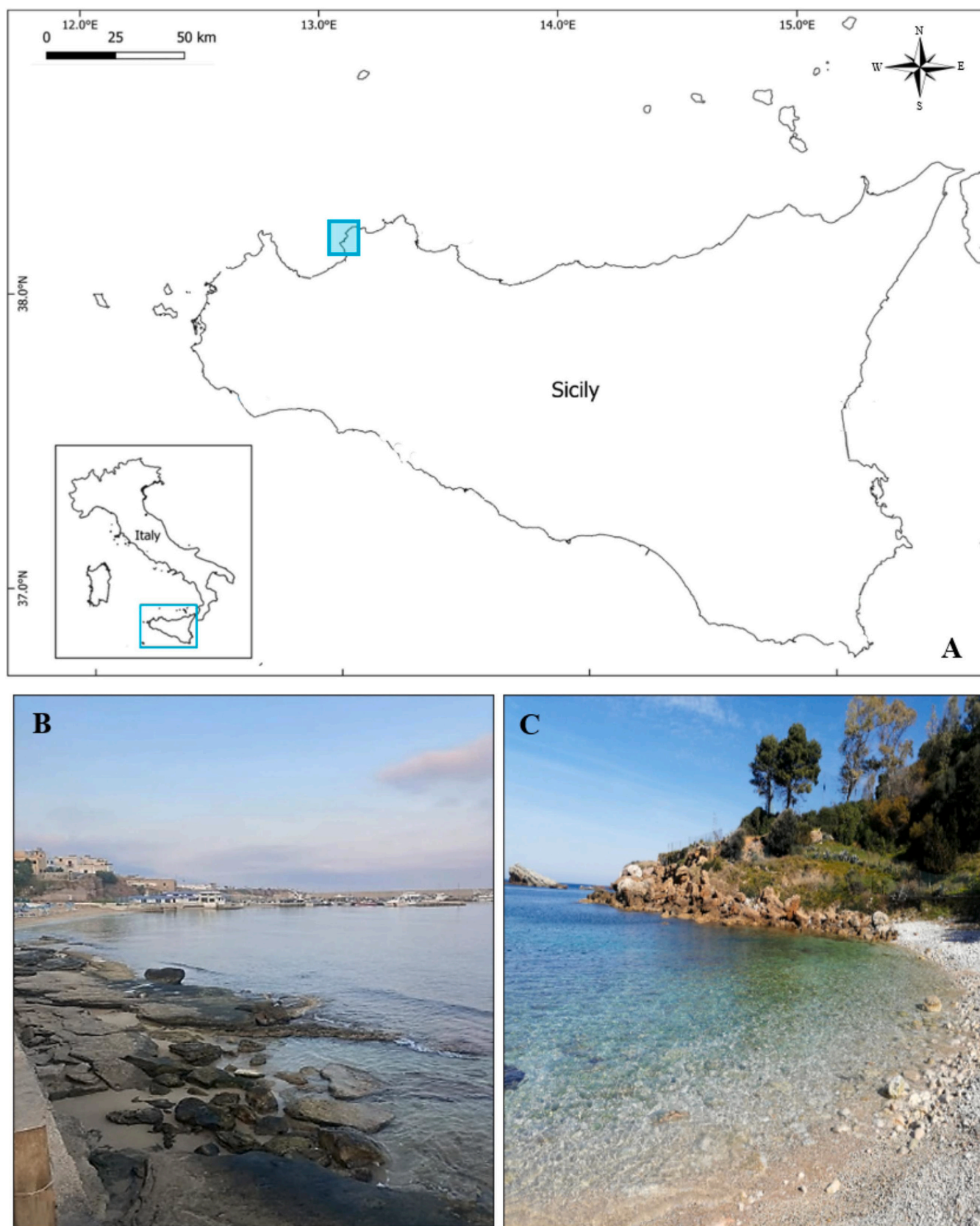


Fig. 1. (A) Location of the study area along the northwestern coast of Sicily, Italy. The blue box indicates the coastal sector where sampling activities were conducted. (B) First sampling site, *Magaggiari Beach* (Cinisi). (C) Second sampling point, *Cala Rossa-Capo Rama* (Terrasini). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Metal Bioaccumulation Assessment (ICP-MS)

Hepatopancreas tissues were excised from surviving individuals of both treatment and corresponding control groups at the end of the 96-h acute toxicity tests using ceramic and titanium tools to eliminate metallic contamination and stored at -20°C . Approximately 100 ± 10 mg (wet weight) of tissue was weighed (Sartorius Quintix®, Germany) and transferred into polytetrafluoroethylene (PTFE) vessels with 3 mL of ultrapure HNO_3 (Sigma-Aldrich). Samples underwent microwave-assisted digestion using an ultraWAVE® system (Milestone, Italy). Digests were cooled, diluted to 50 mL with ultrapure water (Milli-Q, $18.2\text{ M}\Omega\text{ cm}$), and filtered through $0.45\text{ }\mu\text{m}$ PTFE syringe filters.

Trace element concentrations were quantified using an Agilent 7700× ICP-MS (Agilent Technologies, USA) equipped with a collision/reaction cell (He/H_2 mode) to minimize polyatomic interferences. Instrument calibration employed multi-element standards with known analyte concentrations. Operating conditions included: carrier gas 1.2 mL min^{-1} , RF power $1100\text{--}1500\text{ W}$, plasma gas flow 15 mL min^{-1} , auxiliary gas flow 0.9 mL min^{-1} , spray chamber temperature $+2^{\circ}\text{C}$, lens voltage 4.5 V . Helium and hydrogen were used in the collision/reaction cell to reduce spectral interferences. A reagent blank (HNO_3 + ultrapure water) was processed identically for each digestion and analytical batch. A certified reference material (DORM-5, fish protein; National Research Council Canada) was used for the quality control. Final metal concentration (C_f) were expressed as mg kg^{-1} wet weight, calculated using the following formula:

$$C_f = \frac{C_s \times W \times 1000}{50}$$

where C_s is the metal concentration in the digested solution (mg L^{-1}), V is the final volume of the extract (50 mL), W is the wet weight of the hepatopancreas sample (g), and 1000 is the conversion factor to kg.

2.4. Sub-lethal exposure experiment

To assess sublethal biomarker responses, exposure concentrations were selected as fractions of the experimentally determined 96-h LC_{50} values, following Sprague (1971). Sublethal exposures were conducted for 24, 48, and 96 h using three fractional concentrations based on the calculated 96-h LC_{50} : $\frac{1}{2}\text{ LC}_{50}$ (high), $\frac{1}{4}\text{ LC}_{50}$ (medium), and $\frac{1}{8}\text{ LC}_{50}$ (low) for both Cu and Cd. Exposures were maintained in triplicate under identical conditions to the acclimation phase, with total solution renewal every 24 h. Temperature and salinity were monitored and maintained within the same ranges used during acclimation ($18 \pm 2^{\circ}\text{C}$ and 33 ± 2 , respectively). At each time point (24, 48, and 96 h), five individuals per replicate ($n = 15$ per treatment group) were randomly

sampled, cryo-anesthetized on ice, and dissected immediately. The entire soft tissues of the abdomen, including the hepatopancreatic tissue, were collected and stored at -20°C until analyses of cellular biomarkers.

2.5. Biometric measurements

At the conclusion of exposure period, individuals were collected and measured before soft-tissue extraction. First, the size of the occupied gastropod shell was determined by measuring its total shell length (TSL) along the main longitudinal axis (Fig. 2A) using a dial caliper ($\pm 0.01\text{ mm}$). Shells were then carefully fractured with a bench vise to extract the animals. Fresh body weight was determined using a precision analytical balance (Bel Engineering M214AiH, Monza, Italy). Recorded morphometric parameters followed standard protocols for anomuran decapods (Melo, 1999). These included cephalothorax length (CL)—measured dorsally with dial calipers ($\pm 0.01\text{ mm}$) from the rostrum tip to the posterior margin of the carapace shield (the entire calcified dorsal piece)—and carapace width (CW), taken at the maximum transverse axis of the carapace (Fig. 2B). Additionally, Propodus length, the maximum length of the left and right chelae, were measured from a ventral perspective (Fig. 2C).

2.6. Cellular stress biomarker analyses

The collected abdominal soft tissues, including the hepatopancreatic tissue, were homogenized 1:10 (w/v) in ice-cold 1X RIPA buffer (Millipore, USA) supplemented with a protease inhibitor cocktail (1:200; Sigma-Aldrich). Homogenization was executed using a rotor-stator device (Ultra-Turrax T25, IKA, Germany) for 2 min, followed by ultrasonic cell disruption (Vibra-Cell VC375, 8% amplitude, 50% pulse; Sonics & Materials, USA). Lysates were centrifuged at $12,000 \times g$ for 10 min at 4°C , and supernatants were collected. Total protein content was determined via the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard on a GloMax-Multi® microplate reader (Promega, USA).

Esterase and alkaline phosphatase (ALP) activities were determined according to Ross et al. (2000). Supernatants (50 μL) were incubated with 50 μL substrate solution containing 0.4 mM *p*-nitrophenyl myristate (for esterase) or 4 mM *p*-nitrophenyl phosphate with 1 mM MgCl_2 (for ALP) in 100 mM ammonium bicarbonate buffer (pH 7.8). Absorbance was monitored continuously for 60 min. Specific enzymatic activity ($\text{U }\mu\text{g}^{-1}\text{ protein}$) was calculated as:

$$\text{Activity} = \frac{\text{Abs}_{\text{min}}}{\text{Eb}} \times \frac{1000}{\text{Eb}} \times \frac{\text{Vf}}{\text{Vi}}$$

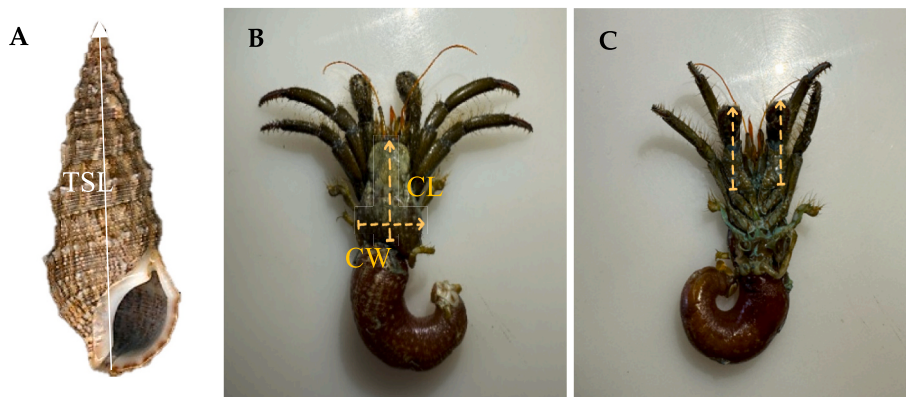


Fig. 2. Morphometric measurements of *Clibanarius erythropus* and associated gastropod shell. (A) Total shell length (TSL) of the gastropod shell measured along the main longitudinal axis. (B) Dorsal view of the hermit crab showing cephalothorax length (CL) and carapace width (CW). (C) Ventral view showing the measurement of the left and right chelae (propodus length). Arrows indicate the specific morphometric axes recorded.

where Abs/min is the absorbance value obtained for each sample divided by the total measurement time (60 min); Vf is the final volume in the microplate well; Vi is the initial volume in the well; Eb is the experimental constant (16.4 for esterase activity and 18.4 for ALP); One unit (U) corresponds to the amount of enzyme required to release 1 millimole of *p*-nitrophenol per 60 s.

Peroxidase activity was quantified as described by Quade and Roth (1997). Samples (50 µL) were incubated with 100 µL TMB solution (Sigma-Aldrich, St. Louis, MO, USA) for 30 min, and the reaction was stopped with 50 µL 2 M H₂SO₄. Absorbance was recorded at 450 nm.

Reactive oxygen species (ROS) and reduced glutathione (GSH) were measured using fluorescent probes as described by Bjedov et al. (2021). Reactive oxygen species (ROS) levels were assessed with CM-H₂DCFDA, and GSH was measured using CellTracker Green CMFDA (Thermo Fisher Scientific, Waltham, MA, USA) with excitation/emission at 485/530 nm on a GloMax-Multi® system. All fluorescence measurements were performed in triplicate, with blank and autofluorescence corrections applied.

2.7. Data analysis

All statistical analyses were performed using R software (v4.5.1, R Core Team). Normality of data distribution and homogeneity of variance were verified using the Shapiro–Wilk test and Levene's test, respectively. Differences among treatments were evaluated independently at each exposure time point (24, 48, and 96 h) using one-way Analysis of Variance (ANOVA), followed by Tukey's Honest Significant Difference (HSD) post-hoc test for pairwise comparisons. Statistical significance was defined at $p < 0.05$ (with significance levels reported as $p < 0.05$, $p < 0.01$, and $p < 0.001$). Probit analysis was executed to derive 96-h LC₅₀ values with 95% confidence intervals. Data visualizations were generated using the ggplot2 package, overlaying individual data points onto boxplots.

3. Results

3.1. Acute toxicity and LC₅₀ values of cu and cd

Acute 96-h exposure revealed an LC₅₀ of 46.3 mg L⁻¹ for Cu and 4.96 mg L⁻¹ for Cd, indicating an approximate 10-fold higher sensitivity to Cd (Table 1; Probit fit $p > 0.05$). Tissue bioaccumulation for both metals increased linearly with water concentration ($R^2 > 0.82$, $p < 0.0001$; Table 1). However, Cd accumulated at more than double the rate of Cu, as demonstrated by its steeper regression slope (10.14 vs. 4.62 mg kg⁻¹ per mg L⁻¹; Table 1).

3.2. Metal bioaccumulation

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analyses revealed a significant, dose-dependent accumulation of both copper and cadmium in the hepatopancreas of *Clibanarius erythropus* (Fig. 3). Baseline Cu concentration in control organisms was 51 ± 17 mg kg⁻¹.

Hepatopancreatic Cu levels increased significantly ($p < 0.001$) across all exposure groups, reaching 421 ± 67 mg kg⁻¹ at the highest exposure level (Fig. 3A). Cadmium concentration rose from 0.381 ± 0.237 mg kg⁻¹ in control individuals to 914 ± 75 mg kg⁻¹ at the maximum concentration ($p < 0.001$) (Fig. 3B). Post-hoc pairwise comparisons (Tukey's HSD test) confirmed statistically significant differences among treatment groups for both metals.

3.3. Biometric characteristics of experimental organisms

Specimens displayed uniform biometric traits, ensuring experimental groups were comparable. The experimental population showed a mean cephalothorax length (CL) of 0.99 ± 0.06 cm, a mean cephalothorax width (CW) of 0.53 ± 0.04 cm, and an average gastropod shell length of 3.63 ± 0.091 cm. Right and left chela lengths averaged 0.92 ± 0.03 cm and 0.86 ± 0.03 cm, respectively, while the fresh body weight averaged 0.76 ± 0.06 g.

3.4. Effects of sublethal copper and cadmium exposure on enzymatic activities and oxidative stress biomarkers

The effects of exposure to sublethal concentrations of Cu and Cd over different time periods (24–96 h) were investigated to assess metal-induced redox alterations. Animals were exposed to three sublethal concentrations corresponding to ½ LC₅₀ (high), ¼ LC₅₀ (medium), and ⅛ LC₅₀ (low) for both Cu and Cd. Specifically, for Cu, the exposure concentrations were 5.79, 11.6, and 23.2 mg L⁻¹, while for Cd, the concentrations were 0.62, 1.24, and 2.48 mg L⁻¹ (Table 2).

Reactive oxygen species (ROS) production, glutathione (GSH) levels, and peroxidase activity, were measured as indicators of changes in cellular redox status. In addition, enzymatic markers such as alkaline phosphatase (ALP) and esterase activity were assessed to evaluate potential alterations in hepatopancreatic function and general metabolic responses.

Reactive oxygen species (ROS) levels increased significantly across all experimental time points and treatments compared to the control for both metals ($p < 0.001$; Fig. 4A, B). Peak fluorescence was consistently recorded in the highest exposure groups (Cu3 and Cd3, respectively). Specifically, Cu3 values reached 13,005.10 ± 879.00 at 24 h, 14543.85 ± 692.29 at 48 h, and 13,247.86 ± 1143.10 at 96 h (Fig. 4A), whereas Cd3 values peaked at 12194.97 ± 705.76 at 24 h, 13336.57 ± 822.19 at 48 h, and 12,420.35 ± 716.30 at 96 h (Fig. 4B). Statistically significant pairwise comparisons among treatments for both metals are detailed in Table 3.

Glutathione (GSH) levels displayed an opposite pattern to ROS, decreasing significantly across all experimental time points and treatments compared to the control for both metals ($p < 0.001$; Fig. 5A, B). The most pronounced depletion was consistently recorded in the highest exposure groups (Cu3 and Cd3, respectively). Specifically, Cu3 values reached 3716.79 ± 48.83 at 24 h, a global minimum of 3216.08 ± 53.15 at 48 h, and 3987.35 ± 33.35 at 96 h (Fig. 5A), whereas Cd3 values dropped to 3583.19 ± 53.68 at 24 h, 3298.45 ± 40.54 at 48 h, and

Table 1

Acute toxicity (96-h LC₅₀) and bioaccumulation linear regression parameters for copper Cu and cadmium Cd in *Clibanarius erythropus*.

Element	96-h LC ₅₀ (mg L ⁻¹)	Probit Model Fit (χ ² , <i>p</i> -value)	Bioaccumulation Slope b ± SE	Intercept a ± SE	Linear Fit Stats (R ² , F _(1,73) , χ ²)
Cu	46.3 (39.1–53.6)	χ ² = NS ($p > 0.05$)	4.62 ± 0.23 (4.16–5.08)	84.46 ± 11.32 (61.90–107.02)	R ² = 0.846 F = 399.80* χ ² = 140.11*
Cd	4.96 (1.25–9.80)	χ ² = NS ($p > 0.05$)	10.14 ± 0.55 (9.04–11.24)	– 64.93 ± 26.92 (–118.58 to –11.28)	R ² = 0.823 F = 340.41* χ ² = 130.04*

Note. Values are estimates with 95% confidence limits or ±SE. b: slope; a: intercept; * $p < 0.001$; NS: not significant ($p > 0.05$, goodness-of-fit to probit).

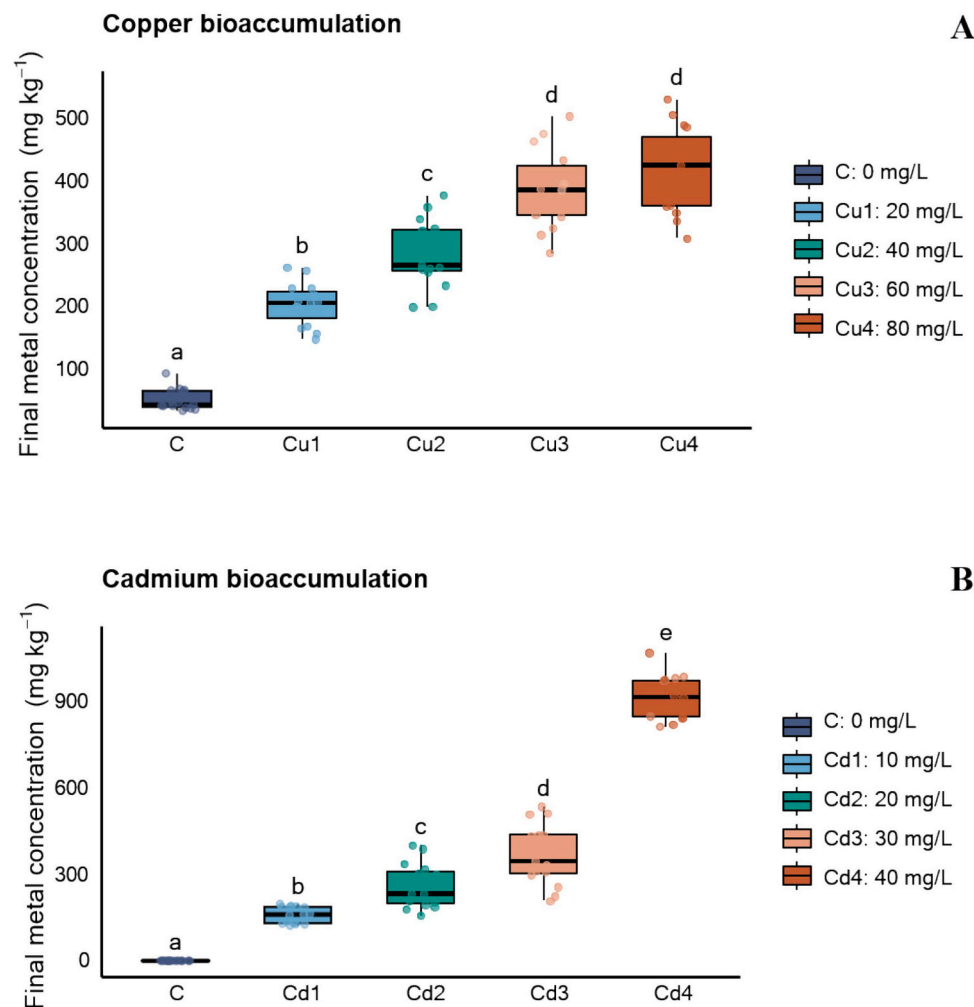


Fig. 3. Copper (A) and cadmium (B) bioaccumulation (mg kg⁻¹) in the hepatopancreas of *Clibanarius erythropus* following exposure to increasing metal concentrations. Boxes represent the interquartile range, the central horizontal line indicates the median, and dots represent individual observations ($N = 12$ per treatment). Different lowercase letters indicate statistically significant differences among treatments (Tukey's HSD test, $p < 0.05$).

Table 2

Sub-lethal concentrations of copper (Cu) and cadmium (Cd) (mg L⁻¹) selected for cellular biomarker analyses and calculated as fractions ($\frac{1}{8}$, $\frac{1}{4}$, and $\frac{1}{8}$) of the respective 96-h LC₅₀ values.

Element	96 h-LC ₅₀ (mg L ⁻¹)	Fractions	Sublethal concentration (mg L ⁻¹)
Cu	46.34	$\frac{1}{8}$ LC ₅₀ - Cu1	5.79
		$\frac{1}{4}$ LC ₅₀ - Cu2	11.6
		$\frac{1}{2}$ LC ₅₀ - Cu3	23.2
Cd	4.96	$\frac{1}{8}$ LC ₅₀ - Cd1	0.62
		$\frac{1}{4}$ LC ₅₀ - Cd2	1.24
		$\frac{1}{2}$ LC ₅₀ - Cd3	2.48

3403.98 $\pm$ 56.40 at 96 h (Fig. 5B). Statistically significant pairwise comparisons among treatments for both metals are detailed in Table 4.

Peroxidase activity increased significantly across all experimental time points and treatments compared to the control for both metals ($p < 0.001$; Fig. 6A, B). Peak activity was consistently recorded in the highest exposure groups (Cu3 and Cd3, respectively), reaching a maximum at 48 h followed by a moderate attenuation at 96 h. Specifically, Cu3 values reached 0.3176 $\pm$ 0.0070 at 24 h, a global maximum of 0.3562 $\pm$ 0.0086 at 48 h, and 0.3236 $\pm$ 0.0091 at 96 h (Fig. 6A), whereas Cd3 values peaked at 0.1785 $\pm$ 0.0163 at 24 h, 0.2262 $\pm$ 0.0647 at 48 h, and 0.1899 $\pm$ 0.0432 at 96 h (Fig. 6B). Statistically significant pairwise comparisons among treatments for both metals are

detailed in Table 5.

Alkaline phosphatase (ALP) activity increased significantly across all experimental time points and treatments compared to the control for both copper and cadmium ($p < 0.001$; Fig. 7A, B). Peak activity was consistently recorded in the highest exposure groups (Cu3 and Cd3, respectively). Specifically, Cu3 values reached 0.2511 $\pm$ 0.0065 at 24 h, 0.2863 $\pm$ 0.0093 at 48 h, and 0.2409 $\pm$ 0.0090 at 96 h (Fig. 7A), whereas Cd3 values reached 0.2482 $\pm$ 0.0119 at 24 h, 0.2513 $\pm$ 0.0125 at 48 h, and 0.2213 $\pm$ 0.0069 at 96 h (Fig. 7B). Statistically significant pairwise comparisons among treatments are detailed in Table 6.

Regarding esterase activity (Fig. 8A, B), in line with the ALP response, measured levels in metal-exposed individuals increased significantly across all experimental time points and treatments compared to the control ($p < 0.001$). Peak values were consistently recorded in the highest exposure groups (Cu3 and Cd3). Specifically, at 24 h, values reached 3.257 $\pm$ 0.080 for Cu3 and 3.330 $\pm$ 0.073 for Cd3. Levels peaked at 48 h, reaching 3.559 $\pm$ 0.070 in Cu3 and 3.571 $\pm$ 0.063 in Cd3, before being recorded at 3.248 $\pm$ 0.071 (Cu3) and 3.295 $\pm$ 0.065 (Cd3) at 96 h. Statistically significant pairwise comparisons among treatments are detailed in Table 7.

4. Discussion

Trace metal contamination remains a critical threat to Mediterranean coastal ecosystems due to its persistence and toxicity (Pachés et al.,

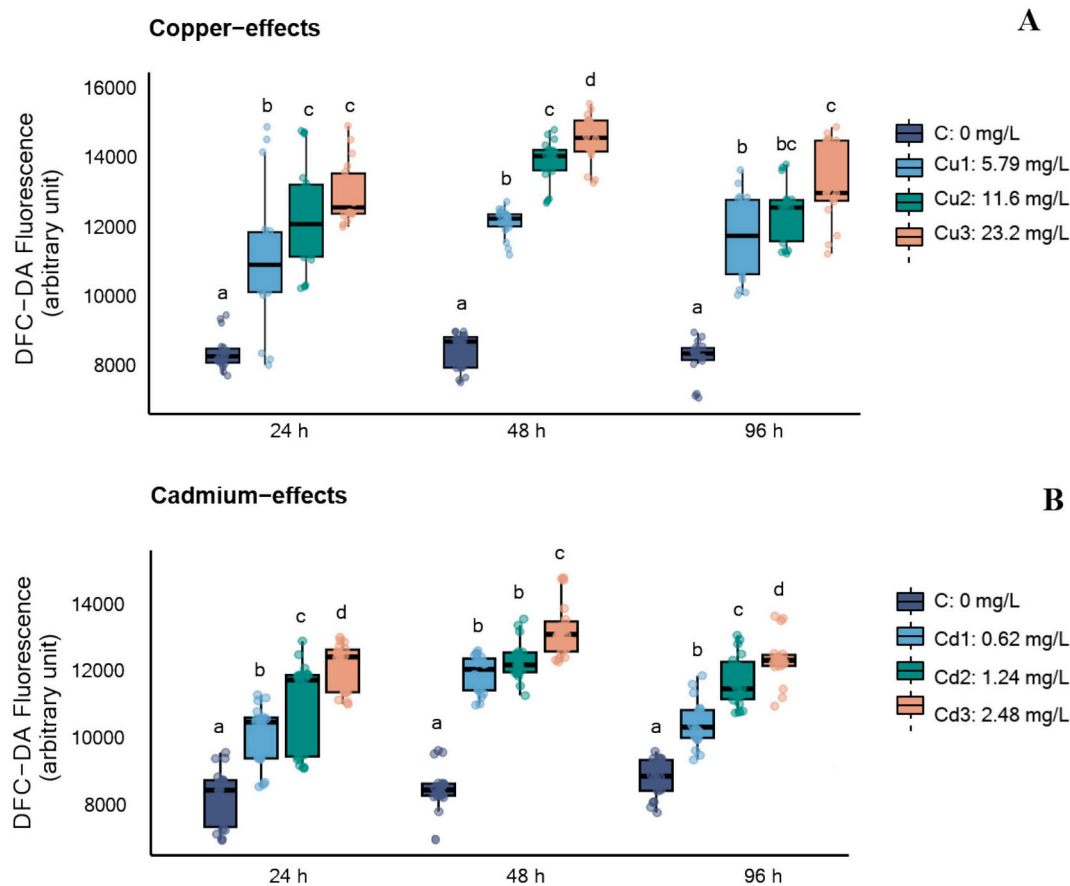


Fig. 4. Reactive oxygen species (ROS), expressed as DCF-DA fluorescence (arbitrary units), in individuals ($N = 5$, with three replicates per treatment) exposed to sublethal concentrations of copper (A) and cadmium (B) for 24, 48, and 96 h. Boxes represent the interquartile range, and the central line indicates the median. Dots represent individual observations ($N = 15$ per treatment). Different letters indicate statistically significant differences among treatments ($p < 0.05$).

Table 3
Pairwise statistical comparisons (p -values) of ROS among experimental treatments across exposure time points (24, 48, and 96 h).

24 h	Cu1	Cu2	Cu3	Cd1	Cd2	Cd3
C	<0.001	<0.001	<0.001	C	<0.001	<0.001
Cu1	–	<0.05	<0.001	Cd1	–	<0.05
Cu2	–	–	n.s.	Cd2	–	<0.01
48 h	Cu1	Cu2	Cu3	Cd1	Cd2	Cd3
C	<0.001	<0.001	<0.001	C	<0.001	<0.001
Cu1	–	<0.001	<0.001	Cd1	–	n.s.
Cu2	–	–	<0.01	Cd2	–	<0.001
96 h	Cu1	Cu2	Cu3	Cd1	Cd2	Cd3
C	<0.001	<0.001	<0.001	C	<0.001	<0.001
Cu1	–	n.s.	<0.001	Cd1	–	<0.001
Cu2	–	–	n.s.	Cd2	–	<0.05

Note. Significance thresholds: $p < 0.05$, $p < 0.01$, $p < 0.001$; n.s.: not significant.

2023; Edo et al., 2024; El-Sharkawy et al., 2025). Copper and cadmium, in particular, are of concern given their environmental prevalence worldwide, as well as their known sublethal effects on crustacean physiology (Manyin and Rowe, 2009; White and Briffa, 2017). In this context, biomonitors offer a powerful approach for early detection of these environmental stressors. Among them, hermit crabs are especially suitable due to their benthic lifestyle and sensitivity to sediment-bound contaminants (Sant’Anna et al., 2012; White et al., 2013; Matin et al., 2019; Briffa et al., 2024). Based on these premises, this study investigated for the first time the effects of Cu and Cd exposure in the hermit

crab *Clibanarius erythropus* to assess its potential as a biomonitor of coastal contamination. Overall, this study confirms the higher toxicity of cadmium compared with copper, while providing a species-specific assessment of this pattern in *Clibanarius erythropus*. The main contribution lies in the characterization of its sensitivity profile through acute toxicity data and a panel of sublethal biomarkers.

To obtain a comprehensive assessment of the effects of metals, an integrated approach was adopted, combining 96-hour acute toxicity tests to determine LC_{50} values, analysis of metal bioaccumulation in the hepatopancreas, and evaluation of biomarkers associated with redox homeostasis, antioxidant responses and cellular metabolism. The toxicity gradient observed in this study ($Cd > Cu$), evidenced by significantly lower LC_{50} values for Cd (4.96 mg L^{-1}) compared to Cu (46.34 mg L^{-1}), is consistent with those reported in numerous studies conducted on various aquatic taxa. Similar trends have been documented in the estuarine crab *Chasmagnathus granulata*, where Cd exhibited the highest acute toxicity across both larval (early zoea) and juvenile stages, following the order $Cd > Zn > Cu > Pb$ (Greco et al., 2001). This ranking is further supported by studies on the freshwater ostracod *Stenocypris major*, which reported 96-h LC_{50} values of $13.1 \text{ }\mu\text{g/L}$ for Cd versus $25.2 \text{ }\mu\text{g/L}$ for Cu (Shuhaimi-Othman et al., 2011). These observations across different crustacean groups demonstrate a consistent sensitivity to Cd over Cu, a pattern that remains coherent even when compared to other aquatic groups, such as the freshwater fish *Cyprinus carpio* (Delahaut et al., 2019). Consistent with these observations, recent meta-analyses covering marine bivalves, fish, and crustaceans have identified Cd as one of the most persistent and hazardous metals in aquatic environments, primarily due to its high bioaccumulation capacity and the broad spectrum of toxic effects it can induce in organisms

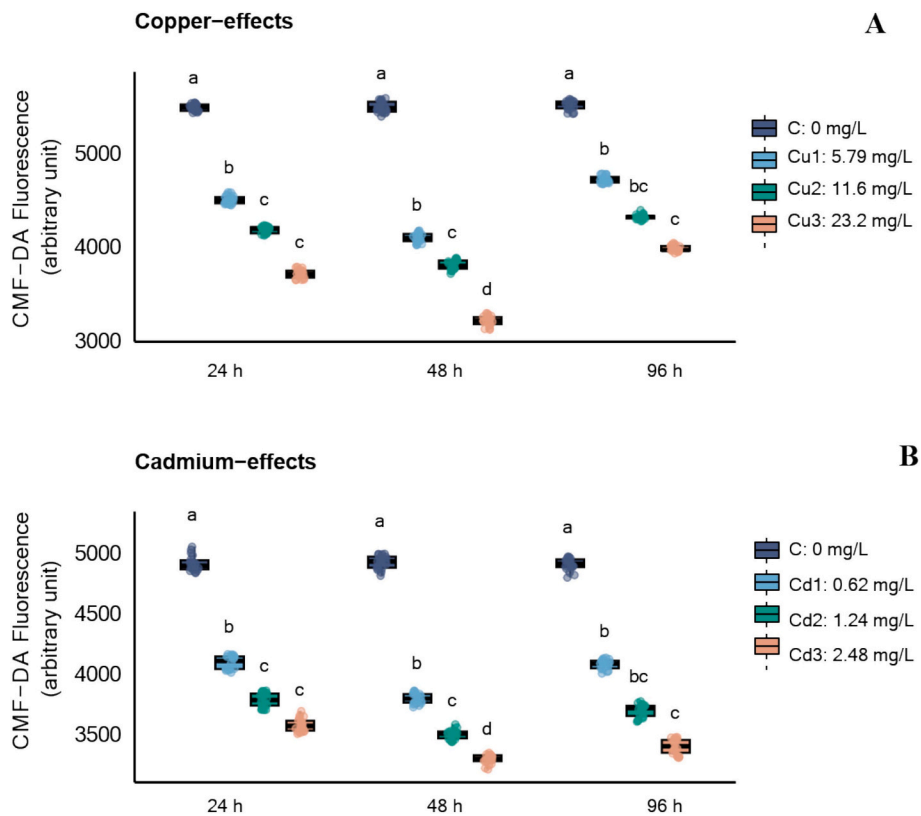


Fig. 5. Reduced glutathione (GSH), expressed as CMF-DA fluorescence (arbitrary units), in individuals ($N = 5$, with three replicates per treatment) exposed to sublethal concentrations of copper (A) and cadmium (B) for 24, 48, and 96 h. Boxes represent the interquartile range, and the central line indicates the median. Dots represent individual observations ($N = 15$ per treatment). Different letters indicate statistically significant differences among treatments ($p < 0.05$).

Table 4
Pairwise statistical comparisons (p -values) of GSH among experimental treatments across exposure time points (24, 48, and 96 h).

24 h	Cu1	Cu2	Cu3	Cd1	Cd2	Cd3
C	<0.001	<0.001	<0.001	C	<0.001	<0.001
Cu1	–	<0.01	<0.001	Cd1	–	<0.05
Cu2	–	–	n.s.	Cd2	–	n.s.
48 h	Cu1	Cu2	Cu3	Cd1	Cd2	Cd3
C	<0.001	<0.001	<0.001	C	<0.001	<0.001
Cu1	–	<0.01	<0.001	Cd1	–	<0.05
Cu2	–	–	<0.01	Cd2	–	<0.05
96 h	Cu1	Cu2	Cu3	Cd1	Cd2	Cd3
C	<0.001	<0.001	<0.001	C	<0.001	<0.001
Cu1	–	n.s.	<0.001	Cd1	–	n.s.
Cu2	–	–	n.s.	Cd2	–	n.s.

Note. Significance thresholds: $p < 0.05$, $p < 0.01$, $p < 0.001$; n.s.: not significant.

(Zhan et al., 2022; Das et al., 2023; Banaee et al., 2024). The increased toxicity of Cd is generally attributed to its nonessential nature and lack of a known biological function (Genchi et al., 2020), characteristics that allow it to interfere with numerous physiological processes even at relatively low concentrations. Indeed, at the cellular level, Cd can bind to the sulfhydryl proteins, replace essential metals in metalloenzymes, and alter enzymatic activity, thereby compromising metabolic homeostasis (Godt et al., 2006; Järup and Åkesson, 2009; Matović et al., 2011). In crustaceans, these mechanisms are often associated with the induction of oxidative stress, suppression of immune responses, and alterations in hemocyte parameters and antioxidant systems (Wu and Chen, 2004; Bjerregaard et al., 2005; Lei et al., 2011; Das et al., 2019; Duan et al., 2021). Furthermore, Cd exposure has also been linked to

additional physiological disorders in decapod crustaceans, including changes in gut microbial communities and dysfunctions in digestive processes, highlighting the potential multifactorial impact of this metal on organism health (Bjerregaard et al., 2005; Duan et al., 2021). Conversely, Cu is an essential micronutrient involved in numerous enzymatic reactions and the formation of respiratory pigments, such as hemocyanin in crustaceans. Most aquatic organisms possess physiological mechanisms that regulate Cu absorption, storage, and excretion, thereby maintaining cellular and systemic homeostasis (Brouwer et al., 2003; Truchet et al., 2023; Chen et al., 2025). However, when environmental concentrations exceed tolerable physiological thresholds, Cu can also exert toxic effects, interfering with ion transport, altering osmoregulatory processes, and promoting the production of reactive oxygen species, resulting in oxidative stress (Paila and Yallapragada, 2010; Yang et al., 2022). In the present study, the lower acute toxicity of Cu compared to Cd in *Clibanarius erythropus*, as evidenced by higher LC_{50} values, suggests that this species possesses relatively efficient regulatory mechanisms for managing exposure to this essential metal. These results indicate that *C. erythropus* can tolerate moderate increases in environmental Cu concentrations without exhibiting immediate lethal effects, likely due to physiological regulatory and detoxification processes already described in other crustaceans (Taylor and Anstiss, 1999; Zhao et al., 2019). Conversely, the greater sensitivity observed to Cd may reflect a more limited capacity for detoxification or rapid excretion of this nonessential metal. This finding is consistent with reports from several studies on decapods and bivalves, in which Cd tends to persist in tissues longer than essential metals, causing toxic effects even at relatively low concentrations (Bordon et al., 2023; Zhan et al., 2023). It is also important to consider that studies have shown that even within decapod crustaceans, sensitivity can be highly species-specific due to variations in metallothionein induction and hemocyte-mediated sequestration (Ahearn et al., 2004; Lima et al., 2019). In this

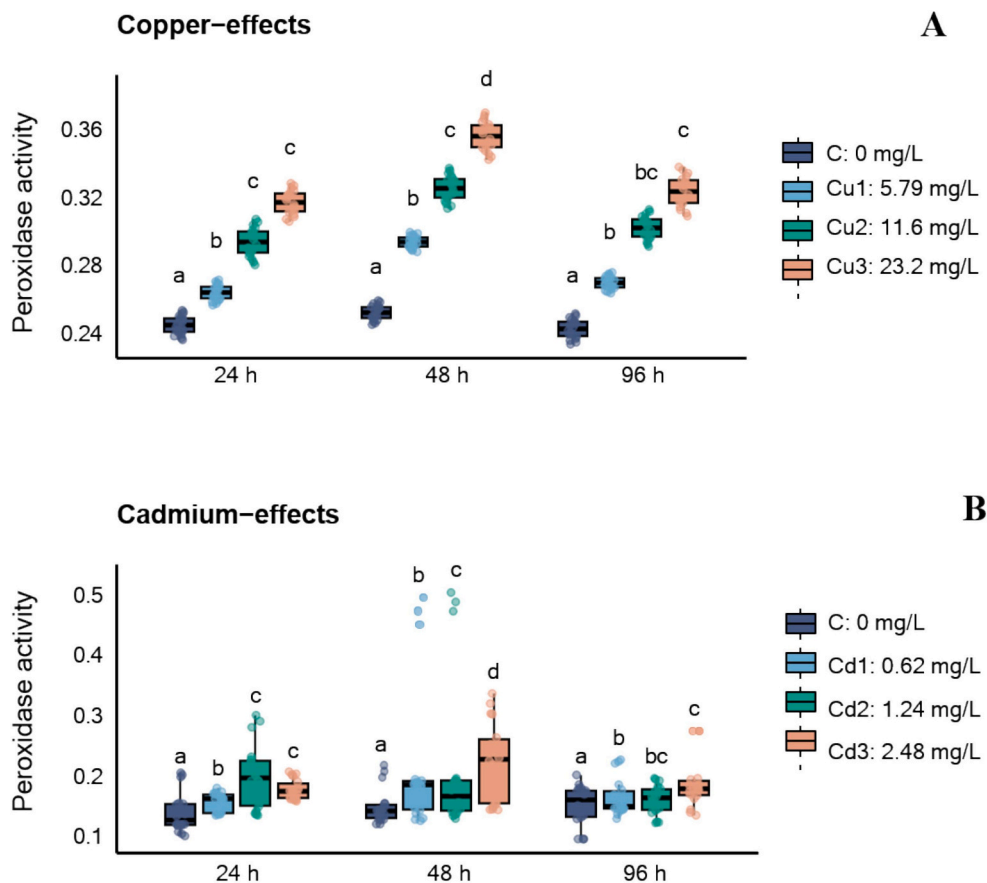


Fig. 6. Peroxidase activity in individuals ($N = 5$, with three replicates per treatment) exposed to sublethal concentrations of copper (A) and cadmium (B) for 24, 48, and 96 h. Boxes represent the interquartile range, and the central line indicates the median. Dots represent individual observations ($N = 15$ per treatment). Different letters indicate statistically significant differences among treatments ($p < 0.05$).

Table 5
Pairwise statistical comparisons (p -values) of peroxidase activity among experimental treatments across exposure time points (24, 48, and 96 h).

24 h	Cu1	Cu2	Cu3	Cd1	Cd2	Cd3
C	< 0.001	< 0.001	< 0.001	C	< 0.001	< 0.001
Cu1	–	< 0.01	< 0.001	Cd1	–	< 0.001
Cu2	–	–	n.s.	Cd2	–	n.s.
48 h	Cu1	Cu2	Cu3	Cd1	Cd2	Cd3
C	< 0.001	< 0.001	< 0.001	C	< 0.001	< 0.001
Cu1	–	< 0.001	< 0.001	Cd1	–	< 0.05
Cu2	–	–	< 0.01	Cd2	–	< 0.05
96 h	Cu1	Cu2	Cu3	Cd1	Cd2	Cd3
C	< 0.001	< 0.001	< 0.001	C	< 0.001	< 0.001
Cu1	–	n.s.	< 0.001	Cd1	–	n.s.
Cu2	–	–	n.s.	Cd2	–	n.s.

context, the LC_{50} values obtained in the present work suggest that *C. erythropus* may show a relatively high tolerance to both Cu and Cd compared to some congeneric species (e.g., *Clibanarius africanus* and *C. longitarsus*) or other previously studied decapods (e.g., *Pagurus bernhardus*) (Otitoloju and Don-Pedro, 2002, 2006; Lyla and Khan, 2010; White et al., 2013). These differences could be attributed to specific physiological characteristics of the species, ecological adaptation strategies, or biological factors such as body size and developmental stage of the tested individuals, factors known to influence metal uptake and detoxification capacity (Liu et al., 2010; Crémazy et al., 2017; Delahaut et al., 2020).

Consistent with the observed toxicity patterns, ICP-MS analyses revealed a clear dose-dependent accumulation of both metals in the hepatopancreas of exposed individuals, with Cd reaching particularly

high concentrations at the highest exposure levels. This finding confirms the hepatopancreas as the primary organ for metal accumulation and detoxification in decapod crustaceans (De Almeida-Rodrigues et al., 2021; Banaee et al., 2024). The high accumulation of Cd observed in this study likely reflects its strong affinity for intracellular binding proteins, such as metallothioneins, which sequester metals within hepatopancreatic tissues to limit their distribution to other vital organs (Matović et al., 2011; Cuypers et al., 2010). In contrast, essential metals like Cu may be partially mobilized for metabolic functions or more efficiently excreted through established physiological regulatory pathways, in line with what has been discussed so far (Chen et al., 2025). Similar accumulation patterns have been documented in other hermit crab species. For instance, Oyewo and Don-Pedro (2004) reported higher concentrations of Cu in the hepatopancreas of *Clibanarius africanus* compared to co-occurring gastropods, supporting the premise that hermit crabs are effective biomonitors of coastal metal pollution. Ultimately, the robust hepatopancreatic accumulation observed in *C. erythropus*, combined with its high abundance and relatively sedentary behavior in intertidal habitats, reinforces this species' potential for long-term biomonitoring programs in coastal ecosystems. It is important to acknowledge a potential limitation of this assessment, namely that the organisms may have already bioaccumulated metals at their respective collection sites prior to the experimental exposures. However, this potential confounding factor was addressed by performing bioaccumulation analyses on the control individuals as well, allowing us to determine the baseline metal burden in the absence of experimental treatments and to interpret the results accordingly.

Beyond acute toxicity and tissue accumulation, significant cellular alterations were also revealed in response to sublethal metal exposure.

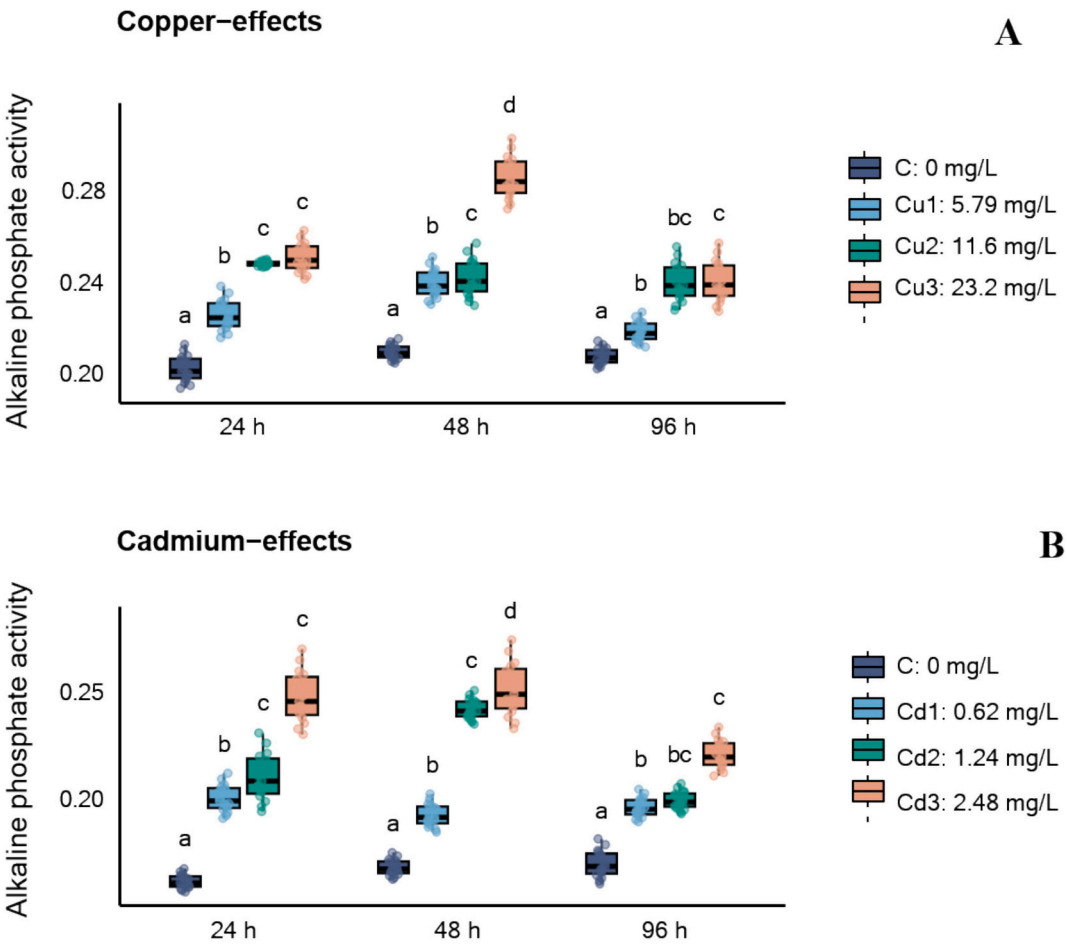


Fig. 7. Alkaline phosphatase (ALP) activity in individuals ($N = 5$, with three replicates per treatment) exposed to sublethal concentrations of copper (A) and cadmium (B) for 24, 48, and 96 h. Boxes represent the interquartile range, and the central line indicates the median. Dots represent individual observations ($N = 15$ per treatment). Different letters indicate statistically significant differences among treatments ($p < 0.05$).

Table 6
Pairwise statistical comparisons (p -values) of ALP activity among experimental treatments across exposure time points (24, 48, and 96 h).

24 h	Cu1	Cu2	Cu3	Cd1	Cd2	Cd3
C	<0.001	<0.001	<0.001	C	<0.001	<0.001
Cu1	–	<0.05	<0.01	Cd1	–	<0.05
Cu2	–	–	n.s.	Cd2	–	n.s.
48 h	Cu1	Cu2	Cu3	Cd1	Cd2	Cd3
C	<0.001	<0.001	<0.001	C	<0.001	<0.001
Cu1	–	<0.05	<0.001	Cd1	–	<0.01
Cu2	–	–	<0.001	Cd2	–	<0.05
96 h	Cu1	Cu2	Cu3	Cd1	Cd2	Cd3
C	<0.001	<0.001	<0.001	C	<0.001	<0.001
Cu1	–	n.s.	<0.01	Cd1	–	n.s.
Cu2	–	–	n.s.	Cd2	–	n.s.

Note. Significance thresholds: $p < 0.05$, $p < 0.01$, $p < 0.001$; n.s.: not significant.

Biomarker-based approaches are increasingly recognized as powerful tools for linking contaminant exposure to physiological effects (Vlahogianni et al., 2007; El-Sharkawy et al., 2025; Simonato et al., 2015; Edo et al., 2024). The biomarkers investigated in the present study represent key components of cellular redox homeostasis, antioxidant defense and metabolic regulation pathways commonly affected by metal exposure in aquatic invertebrates. Indeed, it is well established that many of the toxic effects of metals are associated with disturbances

in cellular redox balance and increased production of reactive oxygen species (ROS) (Banaee et al., 2024). The results of the present study demonstrate that both Cu and Cd exposure induced significant dose- and time-dependent increases in ROS levels in *C. erythropus*. This elevation indicates a marked alteration of cellular redox balance, suggesting that even at sublethal concentrations, these metals trigger a rapid failure of the crab's primary homeostatic controls. Similar increases in ROS production have been reported in a variety of aquatic organisms exposed to heavy metals, including crustaceans, mollusks, and fishes (Sun et al., 2022; Brix et al., 2022). Mechanistically, these two metals disrupt the redox equilibrium through different pathways. Copper can directly participate in Fenton-like redox cycling reactions to generate highly reactive hydroxyl radicals (Matović et al., 2011; Sun et al., 2022). In contrast, although Cd is not redox-active, it indirectly stimulates massive ROS production by disrupting mitochondrial electron transport chains and interfering with thiol-dependent redox systems (Stohs and Bagchi, 1995; Cuypers et al., 2010; Zhou et al., 2017). To counteract this initial burst of ROS, organisms rely on the glutathione system as a primary, non-enzymatic defense. Reduced glutathione (GSH) acts as a critical intracellular redox buffer, directly scavenging reactive species and binding to metals (Cuypers et al., 2010). In this study, the sharp rise in ROS levels was accompanied by a severe depletion of intracellular GSH, which became particularly evident after 48 h. This simultaneous drop in GSH and peak in ROS is not merely a temporal coincidence but rather reflects the active and massive consumption of glutathione reserves to neutralize the oxidative challenge. Consequently, the depletion of this primary cellular defense may increase cellular susceptibility to

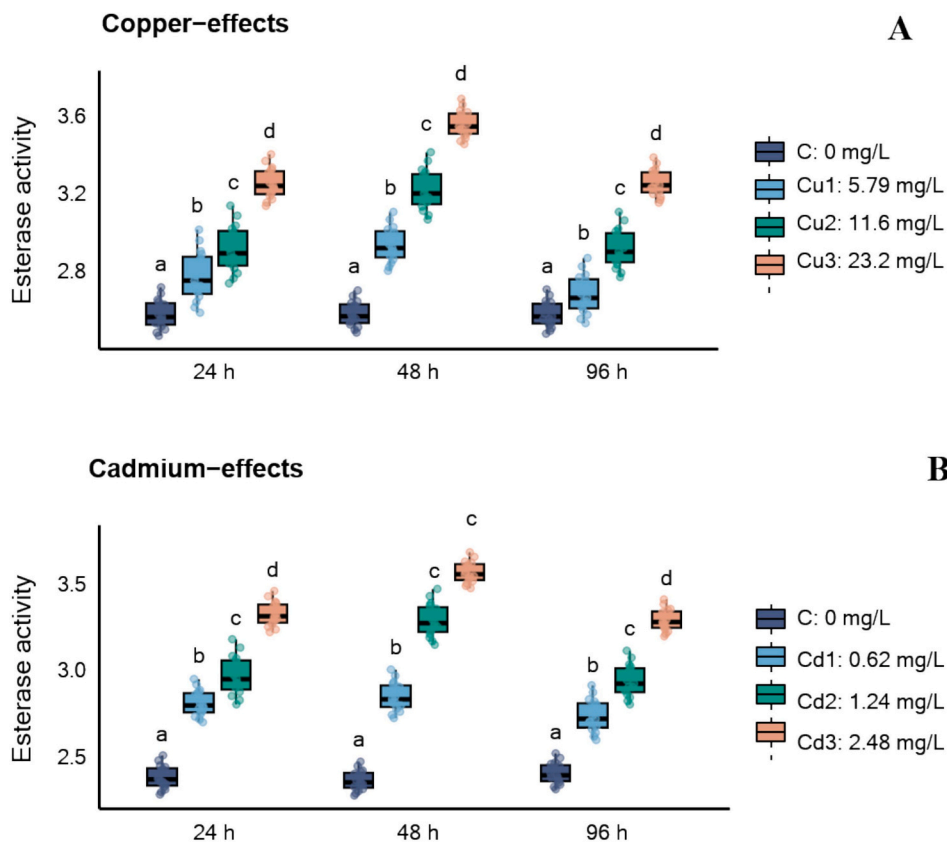


Fig. 8. Esterase activity in individuals (N = 5, with three replicates per treatment) exposed to sublethal concentrations of copper (A) and cadmium (B) for 24, 48, and 96 h. Boxes represent the interquartile range, and the central line indicates the median. Dots represent individual observations (N = 15 per treatment). Different letters indicate statistically significant differences among treatments ($p < 0.05$).

Table 7
Pairwise statistical comparisons (p -values) of esterase activity among experimental treatments across exposure time points (24, 48, and 96 h).

24 h	Cu1	Cu2	Cu3	Cd1	Cd2	Cd3
C	< 0.001	< 0.001	< 0.001	C	< 0.001	< 0.001
Cu1	–	< 0.05	< 0.01	Cd1	–	< 0.05
Cu2	–	–	< 0.05	Cd2	–	< 0.01
48 h	Cu1	Cu2	Cu3	Cd1	Cd2	Cd3
C	< 0.001	< 0.001	< 0.001	C	< 0.001	< 0.001
Cu1	–	< 0.01	< 0.001	Cd1	–	< 0.001
Cu2	–	–	< 0.01	Cd2	–	n.s.
96 h	Cu1	Cu2	Cu3	Cd1	Cd2	Cd3
C	< 0.001	< 0.001	< 0.001	C	< 0.001	< 0.001
Cu1	–	< 0.05	< 0.01	Cd1	–	< 0.05
Cu2	–	–	< 0.01	Cd2	–	< 0.01

Note. Significance thresholds: $p < 0.05$, $p < 0.01$, $p < 0.001$; n.s.: not significant.

ROS-mediated effects. As documented in other aquatic species, sustained ROS accumulation following depletion of GSH reserves has been associated with oxidative damage, including lipid peroxidation, DNA damage, and alterations of redox-sensitive proteins (Martinez-Finley et al., 2013; Wang et al., 2016). Although such endpoints were not directly assessed in the present study, the observed changes in ROS and GSH indicate a substantial disruption of redox homeostasis. However, it should be noted that GSH levels were assessed indirectly using Cell-Tracker Green CMFDA fluorescence. Although this probe provides a useful indication of intracellular glutathione status, the measured fluorescence may also be influenced by cellular esterase activity, probe uptake, and overall cell viability. In addition, when non-enzymatic

defenses such as GSH are strained, the cell mobilizes enzymatic systems to mitigate further damage. In *C. erythropus*, peroxidase activity increased significantly, mirroring the dynamics of ROS. Specifically, peroxidase activity spiked at 48 h in response to the highest metal concentrations, acting as a crucial compensatory mechanism to neutralize hydrogen peroxide before it could generate more toxic radicals (Raeeszadeh et al., 2023). However, by 96 h, enzymatic activity showed a decreasing trend. Although temporal comparisons between the different exposure times were not directly assessed by statistical analyses, this observational trend could indicate a stabilization or partial attenuation of the enzymatic antioxidant response following prolonged exposure, rather than a definitive exhaustion of the enzymatic capacity. This interpretation is consistent with what was reported by Sun et al. (2014) on decapods and by Vlahogianni et al. (2007) on bivalves, where prolonged exposure to contaminants has been associated with a reduction in antioxidant defenses and a progressive depletion of the antioxidant pool. Therefore, the pattern observed in *C. erythropus* aligns with the literature: an initial intense, but ultimately unsustainable, mobilization of the enzymatic antioxidant machinery in response to sustained ROS generation. Finally, in addition to antioxidant defense, alterations in redox homeostasis are often accompanied by broader metabolic adjustments aimed at maintaining cellular homeostasis and supporting detoxification processes. These responses frequently include modulation of enzymes involved in membrane transport, hydrolysis reactions, and energy metabolism (Liao et al., 2022; Brix et al., 2022; Ali et al., 2025). In this context, enzymes such as alkaline phosphatase (ALP) and esterases play key roles in membrane transport, phosphate ester hydrolysis, lipid metabolism, and xenobiotic detoxification processes. The results of this study show that both ALP and esterase activities increased significantly in a dose- and time-dependent manner in *C. erythropus*. In aquatic organisms, changes in these enzymes are widely

recognized as sensitive indicators of metabolic impairment and physiological adaptation to contaminants (Bonacci et al., 2004; Hannam et al., 2008; Jiang et al., 2012). Specifically, ALP is a key brush-border enzyme involved in membrane transport, phosphate ester hydrolysis, and the regulation of cellular energy processes. This enzyme is widely documented across a broad range of taxa, with its biochemical importance well-established in crustaceans. For instance, ALP has been characterized in the hepatopancreas of the red king crab *Paralithodes camtschatica* (Menzorova et al., 2008), as well as in the brachyuran crabs *Callinectes sapidus* and *Cardisoma carnifex* (Raimi et al., 2012). In these species, ALP serves as a critical functional marker for nutrient absorption and metabolic homeostasis, making its response to metals exposure a valuable indicator of physiological stress in *C. erythropus*. Its increased activity likely reflects an intensification of cell membrane turnover and energy-demanding repair processes. Such a response is aimed at maintaining structural integrity against metal-induced damage, as previously documented in the shrimp *Litopenaeus vannamei* following sublethal copper exposure (Prasath et al., 2019). Similarly, the rise in Esterase activity indicates activation of phase-I detoxification pathways and increased hydrolytic metabolism required to process xenobiotics (Dzobo and Naik, 2013; Ali et al., 2025). This metabolic shift is consistent with findings in other marine invertebrates and fish, in which the response to oxidative stress is coupled with upregulation of hydrolytic enzymes to support detoxification and cellular renewal (Banaee et al., 2024; Ali et al., 2025).

In *C. erythropus*, the synchronization of these biomarkers provides evidence of a robust physiological strategy: metal accumulation triggers a redox imbalance, which is followed by the mobilization of GSH and peroxidases, and finally by a broader metabolic adjustment to sustain long-term homeostasis. The concentration-dependent nature of these responses confirms the high sensitivity of these biomarkers in highlighting the physiological effects of metal exposure in this species. Furthermore, this integrated response shows that the hermit crab *C. erythropus* does not merely survive metal exposure but actively re-allocates its metabolic energy to preserve cellular integrity, confirming its robustness as a biomonitor. The integration of mortality, tissue accumulation, and biomarker responses therefore represents the principal contribution of this work, providing a species-specific ecotoxicological profile that can support the future application of *C. erythropus* in Mediterranean biomonitoring programs. Given the wide distribution and ecological importance of *C. erythropus* in rocky coastal environments of the Mediterranean and Atlantic, validating these biomarkers provides a solid basis for integrating this species into biomonitoring programs for coastal ecosystems.

5. Conclusions

In conclusion, the findings of this study are consistent with a growing body of literature emphasizing the role of crustaceans as biomonitors of metal contamination (De Almeida-Rodrigues et al., 2021; Zhan et al., 2022; Banaee et al., 2024) and support the integration of biomarker-based approaches into existing regulatory frameworks such as the Water Framework Directive (WFD) and the Marine Strategy Framework Directive (MSFD). These findings also complement evidence from other Mediterranean marine taxa occupying different trophic levels. Similar organ-specific patterns of trace element accumulation have also been reported in Mediterranean loggerhead sea turtles (*Caretta caretta*; Simsek et al., 2025), in which the liver and kidneys, although anatomically distinct from the crustacean hepatopancreas, perform comparable physiological functions in metal accumulation, detoxification, and metabolic regulation. Together, these observations highlight common biological strategies for coping with metal exposure across marine organisms and support the integration of complementary biomonitor species into Mediterranean monitoring programs. By coupling acute toxicity, bioaccumulation, and biomarker endpoints, this study provides the first integrated assessment into the response of *Clibanarius erythropus*

to Cu and Cd, reinforcing its potential as a biomonitor in Mediterranean coastal monitoring programs. Consistent with the established higher toxicity of Cd compared with Cu, the results of this study extend this evidence to *C. erythropus* and demonstrate that an integrated suite of metabolic biomarkers provides sensitive indicators of sublethal metal exposure in this species. The higher acute toxicity and stronger hepatopancreatic accumulation of Cd highlight the need for particular attention to this metal in risk assessment and management, especially in intertidal zones affected by urban, industrial, or mining-related inputs. At the same time, the sensitivity of oxidative stress and immune-related biomarkers indicates that sublethal cellular responses can be detected at exposure levels well below those that cause mortality, providing valuable early-warning signals of ecosystem deterioration. Moreover, the study of oxidative stress biomarkers, together with other detoxification and immunological pathways, proves crucial for the early detection of physiological alterations before lethal effects manifest, thereby reinforcing the value of integrating complementary biomonitor species, from invertebrates to higher trophic organisms, within Mediterranean biomonitoring programs. Building on this framework, future studies should extend this work by investigating chronic exposures, mixture effects, and field validation in populations inhabiting differently impacted sites along the Mediterranean coast to further refine the ecological relevance of *C. erythropus* within a multi-species biomonitoring framework for Mediterranean coastal ecosystems. In the context of increasing anthropogenic pressures on the Mediterranean, understanding the mechanisms by which marine organisms respond to contaminants is essential for effective monitoring and sustainable management of coastal ecosystems.

CRedit authorship contribution statement

Rosi De Luca: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Gaetano Cammilleri:** Methodology, Investigation, Formal analysis. **Francesco Longo:** Investigation. **Ignazio Restivo:** Methodology, Investigation, Formal analysis. **Vincenzo Ferrantelli:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis. **Luisa Tesoriere:** Writing – review & editing, Visualization, Supervision, Methodology, Investigation, Formal analysis. **Vinicius Queiroz:** Writing – review & editing, Investigation. **Mirella Vazzana:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Davide Alfonso Cammarata:** Investigation. **Manuela Mauro:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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