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Assessing combined effects of long-term exposure to copper and marine heatwaves on the reef-forming serpulid *Ficopomatus enigmaticus* through a biomarker approach

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ABSTRACT

Sessile benthic organisms can be affected by global changes and local pressures, such as metal pollution, that can lead to damages at different levels of biological organization. Effects of exposure to marine heatwaves (MHWs) alone and in combination with environmentally relevant concentration of copper (Cu) were evaluated in the reef-forming tubeworm *Ficopomatus enigmaticus* using a multi-biomarker approach. Biomarkers of cell membrane damage, enzymatic antioxidant defences, metabolic activity, neurotoxicity, and DNA integrity were analyzed. The exposure to Cu alone did not produce any significant effect. Exposure to MHWs alone produced effects only on metabolic activity (increase of glutathione *S*-transferase) and energy reserves (decrease in protein content). MHWs in combination with copper was the condition that most influenced the status of cell homeostasis of exposed *F. enigmaticus*. The combination of MHWs plus Cu exposure induced increase of protein carbonylation and glutathione *S*-transferase activity, decrease in protein/carbohydrate content and carboxylesterase activity. This study on a reef-forming organism highlighted the additive effect of a climate change-related stressor to metals pollution of marine and brackish waters.

1. Introduction

Heatwaves (MHWs) are transient periods of anomalous and extreme ocean warming related to global climatic changes which can occur at the regional level (Sen Gupta et al., 2020). In particular, MHWs can be described as distinct periods characterized by abnormally warm water persisting at a specific location. Quantitative definitions of MHWs rely on ocean temperatures surpassing a set threshold, which may be fixed (Frölicher and Laufkötter, 2018), seasonally adjusted (Hobday et al., 2016), or cumulative (Eakin et al., 2010).

There is a growing concern with the rapid increase in frequency, magnitude, and severity of these events worldwide and during the last century an increase higher than 50 % in the annual number of marine MHWs days occurred (Laufkötter et al., 2020). The rising interest in this rapidly developing threat is also due to the high potential to exacerbate the degradation of ecosystems and associated biodiversity (Smale et al., 2019). Furthermore, in the Mediterranean Sea, the situation could be worse than expected. Due to its semi-enclosed nature, warming could occur very quickly (Kuglitsch et al., 2010). Shallow-water communities, such as biogenic reefs, are the first to experience these impacts, with effects such as tissues necrosis, reduced fertility, changes in feeding behavior, more susceptibility to diseases, bleaching, reduced growth and fitness due to starvation, oxidative stress and can even lead to death (Dias et al., 2019a; Bezuidenhout, 2021). Several studies have reported episodes of mass mortality of these organisms associated with high temperatures during summers in the Mediterranean since the 90s, with mortality triggered by increases of 1–2 °C above the average sea temperature (Kružić, 2014). Consequently, these events are expected to become more frequent, causing significant changes in the species composition and structure of marine communities (Rivetti et al., 2014).

In addition to global climate changes, sessile invertebrates face threats from local factors like chemical pollutants, including metals

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(Marangoni et al., 2017). Copper (Cu), a vital trace metal for cell and organism functions such as enzyme activity (Rainbow, 2002), is also known as a highly toxic cation and aquatic contaminant (Viarengo et al., 2002). Cu contamination stems from sources like agricultural runoff, mining, antifouling paints, and industrial discharges, contributing to widespread aquatic copper pollution with detrimental effects on aquatic life and ecosystems (Maria and Bebianno, 2011). High copper concentrations act as a significant physiological stressor, disrupting key metabolic processes, inducing oxidative stress, DNA damage, and other toxic effects in aquatic invertebrates (van Dam et al., 2011; Schwarz et al., 2013).

The exposure of the mussel *Mytilus galloprovincialis* to Cu increased metallothionein (MT) gene expression (Dondero et al., 2005; Zorita et al., 2007) and lipid peroxidation (LPO) levels (Maria and Bebianno, 2011). *Patella vulgata* limpets showed high sensitivity to copper, with significant effects at 6.1 μg Cu/L, while *Carcinus maenas* crabs were affected significantly only at 68.1 μg/L Cu (Brown et al., 2004). Regarding early life stages, spermiotoxic effects in the polychaete *Ficopomatus enigmaticus* (Fauvel, 1923) were detected after short-term exposure to 40 μg/L Cu (Cuccaro et al., 2021).

Copper concentrations in the Mediterranean Sea can vary significantly depending on the location, from 0.2 to 50 μg/L in the proximity of point sources (UNEP, 1996). In the present work, it was decided to expose the organisms to a copper concentration of 50 μg/L to evaluate whether there were any effects of this contaminant at an environmentally relevant concentration and based on the bibliographic research on impacts on invertebrate organisms as described above. The choice to select the highest copper concentration, remaining within the environmental ranges, was driven by our specific research objective, which is to investigate the influence of heat waves on the toxicity of metals.

In this study, we evaluated whether any effects of copper were influenced by heatwaves. The combined effects of global climate change and local impacts on aquatic organisms are undergoing increased interest recently (Sokolova and Lannig, 2008), since in their natural environments' organisms are exposed to numerous and combined other local human-related stressors that can determine additive or multiplicative effects, larger than those caused by each stressor alone (Halpern et al., 2008, 2015). Specifically for the two stressors considered in this study (increasing temperature and copper contamination), Fonseca et al. (2017) showed that the combination of them reduced the photosynthetic capacity, displayed an unbalanced enzymatic activity, and led to a reduction in LPO in the south Atlantic reef-builder coral *Mussismilia harttii* (Verrill, 1868), presenting a synergistic effect. A similar effect was described in the same species by Fonseca et al. (2019), who found a severe inhibition of the activity of energy metabolism enzymes. Fonseca et al. (2021a), found that the increased temperature, compared with Cu exposure, was the factor that most influenced the trophic behavior of *M. harttii*. Exposure to high temperature and to Cu combined induced in this coral oxidative damage (Fonseca et al., 2017; Fonseca et al., 2021a, 2021b).

F. enigmaticus, a tubeworm serpulid capable of forming reef-like structures in brackish environments, has been used as a model organism in ecotoxicological studies due to its sensitivity to pollutants and metals (Oliva et al., 2018, 2019; Vieira Sanches et al., 2020; Cuccaro et al., 2021, 2022). Most research on this species has focused on its early life stages and short-term exposure (Oliva et al., 2018, 2019; Vieira Sanches et al., 2020; Cuccaro et al., 2021), with fewer studies addressing chronic exposure (28 days) in adults (De Marchi et al., 2019; Cuccaro et al., 2022). To date, only one study on adults was carried out considering the effects of global climate changes: Bezuidenhout (2021) exposed for 3 days *F. enigmaticus* to decreased pH and increased temperature, to test the feeding behavior. There is an existing lack of knowledge on this topic, emphasizing the needing for investigations that encompass both global and local stressors within the context of climate change. This study aimed to examine the synergistic impacts of rising temperatures (MHWs) and copper exposure on the health status of adult reef-building tubeworms (*F. enigmaticus*). A multi-biomarker approach under different conditions was used for investigating effects in term of oxidative status, cellular damage, biotransformation activities, energy reserves, neurotoxicity and DNA integrity.

2. Materials and methods

2.1. Experimental set up

Colonies of *Ficopomatus enigmaticus* were collected at the S. Rossore-Migliarino Massaciuccoli Regional Park in Pisa, Italy. Samples were scraped out from a wharf. The serpulid colonies were then collected in 1-l polypropylene jars filled with local water and transported to the laboratory. The colonies were rinsed with natural filtered (0.45 μm) seawater (NSW) and placed in a glass aquarium containing seawater (80 L), with salinity adjusted to match that of the original sampling site. Throughout the acclimation period, the polychaetes were fed with a suspension of *Isochrysis galbana* (3×10^5 cells/mL). Salinity was gradually increased each day (up to a maximum of 3 units per day) using NSW until reaching a salinity of 30, while other environmental parameters were maintained to mimic conditions at the sampling location (dissolved oxygen >90 % saturation, temperature 22 ± 1 °C, pH maintained at 8.1 ± 0.1, and a photoperiod of 10:14 h - light: dark cycle. Following the acclimation phase, approximately 100 g of calcareous colonies (equivalent to ~200 individuals, with 3 replicates per condition) were distributed into separate 1-l glass containers and subjected to different conditions over a period of 28 days as outlined in Table 1.

Pure copper from a certified material Copper AA Standard (Agilent) was used. The experiment was semi-static as the water and the contaminant were renewed every week.

Colonies were submitted to MHWs, following a ramp described in Fig. 1. To mimic the ephemeral and intermittent nature of MHWs occurring in the sea, two unique scenarios of short-lived, repeatedly occurring MHWs were created. Two MHWs lasting 4 days each were simulated with a temperature increase of 5 °C compared to the control temperature (20 °C). Each temperature increase/decrease ramp (1 °C/day) lasted for 5 days.

Tubeworms from CTRLs and treated conditions were sampled and extracted from the calcareous tubes by breaking them at the end of the exposure. Next, the individuals were collected, pooled (approximately 70 individuals, totaling around 2 g), and thoroughly mixed in a 1:2 ratio (weight to volume) with ice-cold phosphate buffer (50 mM, pH 7) using a Potter Elvejem homogenizer. The resulting homogenized samples were then subjected to centrifugation at 10,000 ×*g* for 30 min. The obtained supernatants were subsequently portioned into aliquots and preserved at −80 °C until they were utilized for the determination of biomarkers. Additionally, a portion of whole individuals (0.5 g) was assembled into pools for chemical analyses.

Table 1
Experimental design: CTRL (control unexposed), Cu (copper exposed), MHWs (marine heatwaves exposed as shown in Fig. 1), Cu + MHWs (exposure to combination of Cu + MHWs). The experiment was semi-static as the water and the contaminant were renewed every week.

Condition	Copper concentration (μg/L)	Temperature (°C)	Time of Exposure (days)
CTRL	0	20	28
Cu	50	20	28
MHWs	0	MHWs as in Fig. 1	28
Cu + MHWs	50	MHWs as in Fig. 1	28

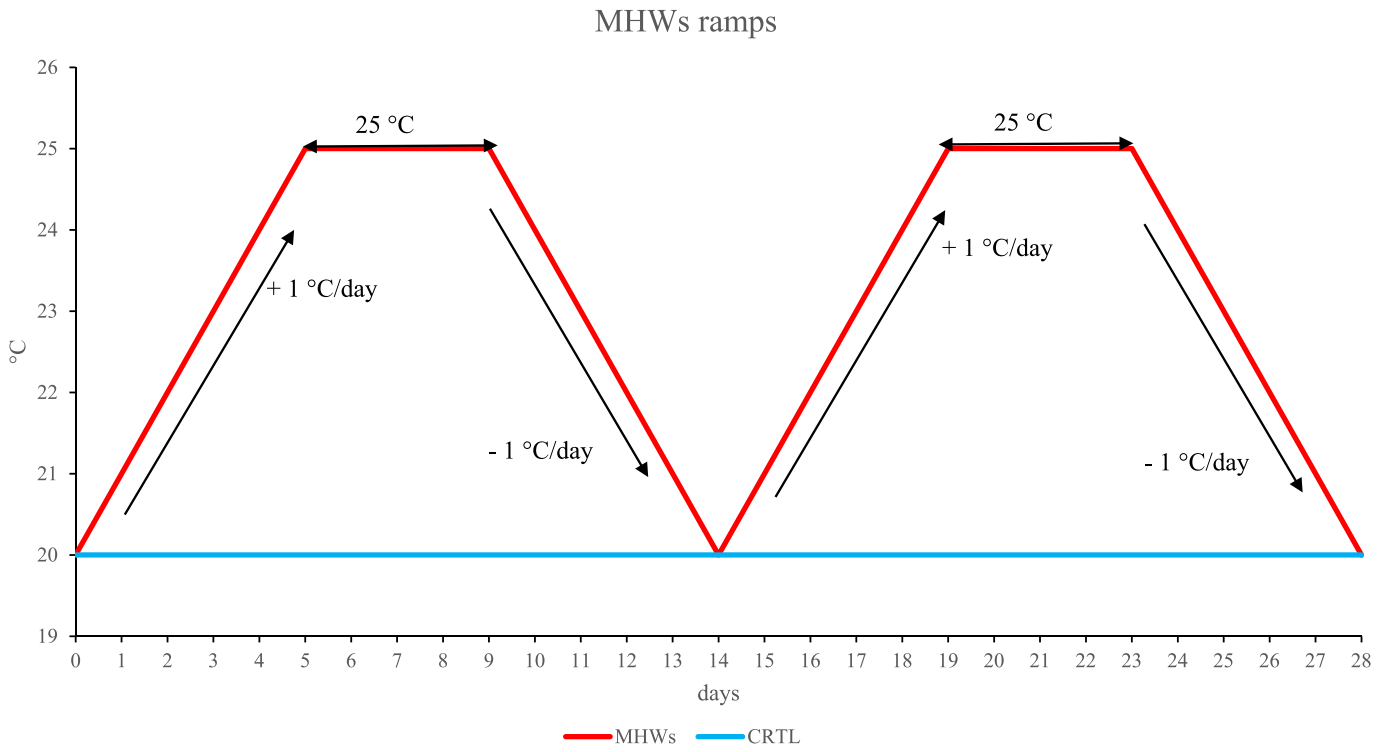

Fig. 1. MHWs ramps. Temperature spikes during 28 days of experimental trial.

2.2. Chemical analysis

Analyses of copper contents in waters were carried out following the method EPA 620B: 2014, by direct injection of ultrapure water-diluted samples (acidified at pH <2 with nitric acid) in an inductively coupled plasma mass spectrometer (ICP-MS) Agilent 7850. The copper content in worms was determined by the methods EPA 3051 A 2007 (digestion) and EPA 6010D 2018 (spectrometry analysis). Pools of whole worms (1 g corresponding ~35 individuals) were dried at 40 °C and the dried tissues samples were ground by porcelain mortar. Samples were then digested and mineralized in a microwave oven ETHOS easy FKV with 35 % hydrogen peroxide (1 mL), 65 % nitric acid (5 mL) and 2 mL ultrapure water. Digestions samples were diluted to 25 mL with ultra-pure water and analyzed for copper content in ICP-MS (tissue samples: recovery = 96.90 %, LOQ = 0.1 mg/Kg; water samples: recovery = 106.5 %, LOQ = 0.1 μg/L).

2.3. Biochemical parameters

Biochemical analyses were carried out in triplicate by spectrophotometric/spectrofluorometric methods using a micro-plate reader Bio-Tek Synergy HT. Biomarkers of cell membrane damage [lipid peroxidation (LPO) and protein carbonylation (PC)], enzymatic antioxidant defences [superoxide dismutase (SOD) and glutathione peroxidase (GPx)], metabolic activity [carboxylesterase (CE) and glutathione S-transferase (GST)], energy reserves [total protein(PROT) and carbohydrate (CARB) content], neurotoxicity [acetylcholinesterase (AChE)], and DNA integrity [DNA single strand breaks (DNAssb)] were analyzed.

2.4. Cell membrane damage

The assessment of Lipid Peroxidation (LPO) levels was conducted through the quantification of thiobarbituric acid reactive substances (TBARS), following the protocol outlined by Ohkawa et al. (1979). This method relies on the generation of malondialdehyde (MDA) as a degradation by-product of polyunsaturated lipids. MDA subsequently reacts with 2-thiobarbituric acid (TBA), forming TBARS. The amount of MDA generated was measured at 532 nm using an ε value of 1.56 × 105 M-1 cm-1. The levels of LPO were expressed as nanomoles of MDA per g of fresh weight (F.W.).

The assessment of Protein Carbonyl content (PC) was determined in accordance with the method described by Mesquita et al. (2014). This method is based on the reaction between 2,4-Dinitrophenylhydrazine (DNPH) and the carbonyl group. Absorbance readings were taken at both 450 nm and 750 nm, and the results were reported as nanomoles per mg of protein.

2.5. Enzymatic antioxidant defences

The activity of the SOD enzyme was measured following the method described by Magnani et al. (2000): it is based on the ability of copper-zinc SOD (Cu—Zn) to inhibit the autoxidation of pyrogallol, which is 50 % in the presence of ethylenediaminetetraacetic acid (EDTA) at pH 8.2. The absorbance was read at 420 nm, at the initial time (t0) before the addition of pyrogallol, and 1 min after its addition (final time t1). Results were expressed as U/mL, where U represents the amount of enzyme required to induce 50 % inhibition of pyrogallol autoxidation.

The activity of the GPx enzyme was determined using t-butyl hydroperoxide (TBH) as substrate by the method described by Badary et al. (2005). Results were expressed as nmol of NADPH oxidized per min per mg of protein.

2.6. Metabolic activity

The enzymatic activity of GST was determined according to Habig et al. (1974) by measuring the increase in absorbance at 340 nm due to the conjugation reaction of CDNB (1-Cl-2,4-dinitrobenzene) with reduced glutathione (GSH). Results were expressed as nmol/min/mg of protein.

CE activity was determined according to Hosokawa and Satoh (2001): the *p*-nitrophenyl butyrate (p-NPB) is a substrate for this esterase and its hydrolysis produces the chromophore 4-nitro-phenol.

Formation of 4-nitro-phenol was recorded for 3 min at 415 nm (with ε = 18 mM-1 cm-1). Carboxylesterase activity was expressed as nmol/min/mg of protein.

2.7. Energy reserves

Protein (PROT) content was assessed using the protocol outlined by Lowry et al. (1951), utilizing bovine serum albumin (BSA) as standard. Absorbance readings were taken at 750 nm, and the results were expressed in mg per gram of F.W.

Carbohydrate (CARB) content was determined using the sulfuric acid method as described by DuBois et al. (1956), employing glucose as standard at concentrations ranging from 0 to 2 mg/mL. Absorbance was measured at 540 nm, and the CARB content was expressed in mg per gram of F.W.

2.8. Neurotoxicity

The enzymatic activity of AChE was evaluated according to the Ellman's method (Ellman et al., 1961). AChE hydrolyzes the substrate acetylthiocholine iodide (ATChI) forming thiocholine iodide base, which reacts with 2,2′-dinitro-5,5′-dithiodibenzoic acid (DTNB) to generate 5-thio-2-nitrobenzoate (TNB, an anion), which is yellow in color. The enzymatic activity was recorded for 5 min at a wavelength of 412 nm, taking into consideration the molar extinction coefficient of TNB (ε) equal to 136,000 mM-1 cm-1 and the activity was expressed as nmol/min/mg of protein.

2.9. DNA integrity

DNA integrity as DNA single strand breaks (DNAssb) was evaluated by the fluorometric assay Fast-Micro-Method® (Schröder et al., 2006). The principle of the method is based on the ability of the specific fluorochrome Pico488 (Lumiprobe GmbH) to form a very stable complex with dsDNA (double-stranded DNA) in the presence of a highly alkaline medium (pH 12.4). The procedure consists of a cell lysis phase of the sample in the presence of urea, sodium dodecyl sulfate (SDS), and EDTA at pH 10 so that the interaction between the fluorochrome and the DNA can be generated, subsequently followed by a phase of denaturation of the DNA at pH 12.4 following the addition of NaOH. Samples were incubated in the dark for 1 h with lytic solution (0.1 % sodium dodecyl sulfate SDS, 9 M urea, 0.2 M ethylenediaminetetraacetic acid EDTA) at pH 10, containing the fluorochrome Pico488. Subsequently, 1 M NaOH and 20 mM EDTA solutions were added, favoring the denaturation of the DNA. The denaturation kinetics was determined by monitoring the decrease in fluorescence of the complex formed by the fluorochrome (already present in the lysis solution) and by the DNA, in a minimum period of 20 min (excitation 485 nm/emission 520 nm). Results are normally expressed as strand scission factors (SSF), calculated by the formula:

SSF = -log10(fluorescence units of samples)/(fluorescence units of control). For a simpler data representation, DNAssb was expressed as a percentage, where values below 100 % correspond to loss of DNA integrity.

2.10. Statistical analysis

The results were presented as mean ± standard deviations (SD). To ensure the validity of the statistical tests, normality and homogeneity of variances were assessed using Shapiro-Wilk and Bartlett's tests, respectively. In cases where necessary, data transformation was applied. Once these assumptions were met, comparisons were made to elucidate the effects of different conditions on *F. enigmaticus*. This was achieved through One-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons, using GraphPad Prism 7.00 software (Windows version, GraphPad Software, La Jolla California, USA, www.graphpad.com). The null hypotheses under investigation were: i) MHWs do not influence the biochemical status of *F. enigmaticus*; ii) Copper has no impact on the biochemical status of the species under examination; iii) The combined exposure to both MHWs and copper does not lead to any effects on the biochemical status of the tubeworm. A significance threshold level of $p = 0.05$ was set. Statistically significant differences in comparison to controls and among conditions were denoted by different letters in the figures.

In terms of multivariate analysis, a matrix encompassing all biochemical measurements (including LPO, PC, SOD, GPx, CE, GST, PROT, CARB, AChE, and DNAssb) for various conditions (CTRL, Cu, MHWs, MHWs+Cu) was utilized to generate a Euclidean distance similarity matrix using PRIMER v6 (Anderson et al., 2008). This similarity matrix was then simplified by calculating the distances among centroids based on the tested exposure conditions. Subsequently, multivariate analysis was carried out employing Principal Coordinates (PCO) analysis. Pearson correlation vectors corresponding to the biochemical descriptors (with $r > 0.75$) were included as supplementary variables superimposed on the PCO graph.

2.11. Integrated biomarker response (IBR index)

The Integrated Biomarker Response (IBR) index is a straightforward multivariate graphical method that enables the visual integration of a series of early warning responses assessed through biomarkers. The IBR index, along with its corresponding star plot, was determined for all biomarkers under consideration following the original methodology proposed by Beliaeff and Burgeot (2002), as well as the modifications outlined by Devin et al. (2014). In summary, the data processing steps were carried out as follows (Beliaeff and Burgeot, 2002): I) Computation of the overall mean (m) and standard deviation (s) for each biomarker using the values from all treatments and time points. II) Standardization of the data for each biomarker using the formula: Y = (X - m) / s, where Y represents the standardized value of the biomarker and X is the mean value of a biomarker from each treatment and time. III) Calculation of Z = -Y or Z = Y based on the expected biological effect (inhibition or activation, respectively). IV) Determination of an S score for each biomarker using S = Z + |Min|, where Min signifies the minimum value observed for each biomarker across all treatments and time points. V) Plotting of all Si values on a radar diagram, and computation of IBR as the total area enclosed by the diagram (sum of the areas of all triangles). The expressions employed for IBR calculation were as follows (Devin et al., 2014): Ai = Si * Si + 1 * sin(2π/k) / 2, thus yielding IBR=$\sum_{i=1}^{k} Ai$. Higher IBR values indicate a stronger response in polychaetes. In the computational analysis, the biological effects obtained, whether inhibition or activation, were determined for each biomarker while considering the control condition.

3. Results

3.1. Chemical analysis

The quantification of copper in the experimental water confirmed

Table 2A
Levels of copper in experimental water spiked with Cu at 50 μg/L. Semi-static experiment with weekly renewal of water plus contaminant. Temperature conditions were 20 °C and marine heat waves (MHWs). Results are expressed as mean ± SD of three independent samples.

	T0 CTRL	T0 Cu	T7 CTRL	T7 Cu	T7 MHWs	T7 MHWs+Cu
mean ± SD μg/L	3.11 ± 0.14	48.84 ± 0.23	5.02 ± 0.57	12.07 ± 0.73	4.99 ± 0.68	10.76 ± 0.40

the nominal concentration (Table 2A). At the end of experiments, copper-exposed tubeworms (T28 Cu and T28 MHWs+Cu) revealed a significant, even if slight, bioaccumulation in both conditions respect to control (T0) (Table 2B).

3.2. Biochemical parameters

3.2.1. Cell membrane damage

A statistically significant increase of 2-fold was observed in PC values when exposed to a combination of MHWs and Cu contamination compared to the control (Fig. 2), while no significant variations in LPO levels were observed among conditions tested (Fig. 2). LPO control levels were 4.22 ± 0.31 nmol MDA/g F.W.

3.2.2. Enzymatic antioxidant defences

No statistically significant differences were found in the values of both SOD and GPx (Fig. 2). Control levels of SOD activity were 2.28 ± 0.28 U/mL; control levels of GPx activity were 4.56 ± 0.42 nmol/min/mg protein.

3.2.3. Metabolic activity

F. enigmaticus samples exposed to MHWs alone and in combination with Cu showed levels of GST activity significantly higher in comparison to control (26 % and 30 % increase, respectively) and Cu exposure conditions (Fig. 2). Control levels of GST activity were 12.78 ± 1.02 nmol/min/mg protein.

In terms of CE activity, significantly lower values were observed in the MHWs and Cu combined condition in comparison to all the other conditions (Fig. 2). A decrease of 20 % was observed, compared to the control. Control levels of CE activity were 14.55 ± 1.14 nmol/min/mg protein.

3.2.4. Energy reserves

The PROT content showed a significantly lower amount (around 50 % lower) when exposed to MHWs alone or combined with Cu compared to control and to Cu contamination condition alone (Fig. 2). Measured control levels of PROT total content were 10.03 ± 0.14 mg/g F.W.

Exposure at MHWs combined with Cu produced a significant effect with a decrease in CARB content in comparison with the control and the increased Cu condition (−39 % and − 98 %, respectively), while MHWs alone showed a significant decrease (−45 %) compared to the Cu contamination condition (Fig. 2h). Measured control levels of CARB total content were 61.71 ± 5.34 mg/g F.W.

3.2.5. Neurotoxicity

AChE levels did not show any significant differences for the conditions assessed (Fig. 2). Control levels of AChE activity were 3.89 ± 0.67 nmol/min/mg protein.

3.2.6. DNA integrity

F. enigmaticus DNA integrity was not affected by any of the tested treatments (Fig. 2).

Table 2B
Levels of copper in tissues of *F. enigmaticus* at T0 and T28 (end of experiment) in different tested conditions. Results are expressed as mean ± SD of three independent samples. Units are mg/Kg of dry weight (D.W.). Data in bold represent significant differences ($p < 0.01$) respect to T0 (one-way Anova, Dunnet's test).

	T0	T28 MHWs	T28 Cu	T28 MHWs+Cu
mean ± SD mg/Kg D.W.	20.26 ± 0.80	22.63 ± 0.47	**29.75 ± 0.44**	**28.80 ± 0.83**

3.3. Multivariate analysis

The results from PCO analyses, based on biochemical responses in all tested conditions, were reported in Fig. 3.

In detail, the first principal component (PCO1), which accounted for 54.7 % of the total variability, clearly separated MHWs and MHWs+Cu conditions (on the positive side), from CTRL and Cu conditions (on the negative side). The variables PC, GPx, AChE, SOD and GST were found to be the most influential in explaining the positive side of PCO1. The most relevant correlation was found between MHW + Cu and PC, while other biomarkers explaining the positive side of PCO1 are weakly correlated with assessed conditions. Regarding the negative side of PCO1, the main correlation can be found between CARB and CTRL. A peculiar condition is represented by MHW which seems to be correlated with both SOD (positive side) and LPO (negative side). On the other hand, PCO2 explained 37.3 % of the total variance among conditions, distinguishing Cu and MHWs+Cu conditions and DNAssb, AChE and PC variables on the positive side, while the negative side consisted of CTRL and MHWs conditions and CARB, CE, LPO, SOD GST and GPX variables.

3.4. Integrated biomarker response (IBR)

The IBR radar diagram is presented in Fig. 4 together with the global scores of the measured parameters (bar graphs). Bar graphs showed that MHWs+Cu condition scored the highest IBR index respect to the other conditions (4.03, about 2-fold higher than Cu condition). The radar diagram clearly depicted scores how, in particular, PC, CE and GST contributed to the overall score of MHWs+Cu condition. Moreover, GST appear to similarly contribute to all three assessed conditions, while CE contributes specifically to MHWs+Cu and PC shows a decreasing in contribution with the highest to MHWs+Cu, followed by MHWs and, lastly, by Cu.

4. Discussion

One of the most widespread threats to reef-forming ecosystems is the increase in temperature (Halpern et al., 2008), which has caused several mass mortality events also in the Mediterranean. In addition to the effects of climate change, sessile organisms are also impacted by local threats, such as pollutants. Ocean warming has a significant impact on several marine organisms at various levels of biological organization *e.g.* cellular, physiological, ecological, and evolutionary (Kong et al., 2019). A lesser number of studies has focused on the effects of extreme weather events such as the MHWs (Minuti et al., 2021; Xu et al., 2022). The potential of MHWs to disrupt the homeostasis of organisms, the balanced equilibrium of marine ecosystems and socio-economic conditions (Smale et al., 2019), have revealed it as a serious threat. Moreover, since it is expected that MHWs will occur more frequently in the short term, it is interesting to understand how marine organisms can counteract these extreme events after experiencing them. In this study, we examined the biochemical responses of the reef-forming serpulid *F. enigmaticus* after exposure to copper and MHWs, alone or in combination.

4.1. Biomarker responses

Although *F. enigmaticus* has been described as a model organism (Oliva et al., 2018), there is a scarce availability of information about the biochemical responses of this species *versus* stressors related to climate change. Furthermore, scarce information is available on how these stressors can affect the uptake and bioaccumulation of pollutants in organisms (De Marchi et al., 2017e; Moleiro et al., 2022). Some authors demonstrated synergistic effects of temperature and copper in the biochemical responses of the Atlantic coral *Mussismilia harttii* (Fonseca et al., 2017, 2019, 2021a, 2021b). Fonseca et al. (2021a) showed that temperature increase had a more significant impact on the trophic

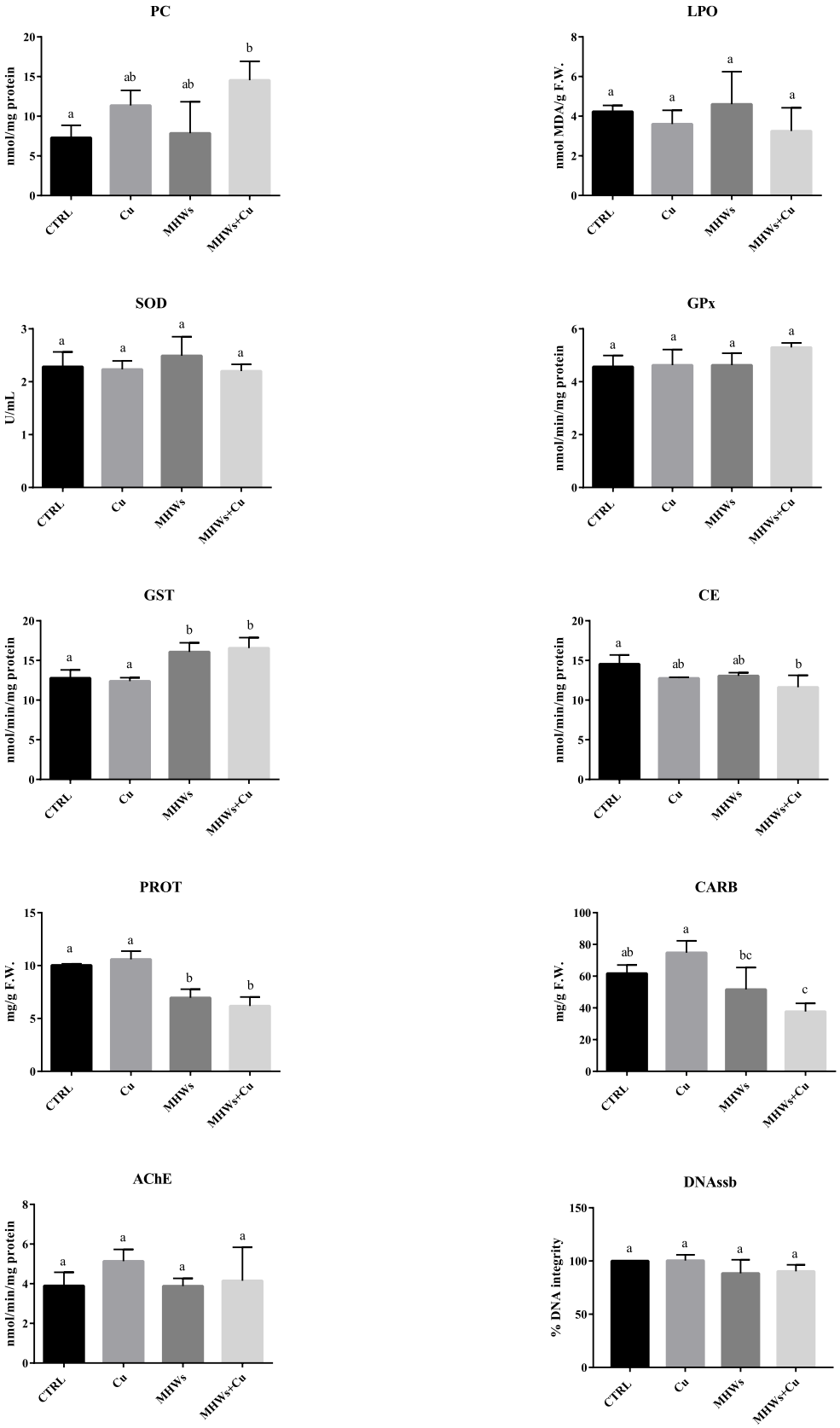

Fig. 2. Biomarkes responses. Measured biomarkers in CTRLs and Cu-, MHWs-, MHWs+Cu-exposed *F. enigmaticus* in 28 days experiment. Values (mean ± SD of three independent experiments, 3 pools/~70 individuals) of SOD, GPx, GST, AChE and CE were expressed as nmol/min/mg protein.; PC as nmol/mg protein; LPO as nmol of MDA/g F.W.; PROT and CARB as mg/g F.W.; DNAssb as % (where <100 % represents strand breaks). $p = 0.05$ was set as the significance threshold level. Statistically significant differences respect to controls and among conditions were expressed with different letters in the figures. (one-way ANOVA, Tukeys's test). MDA: malondialdehyde; F.W.: fresh weight.

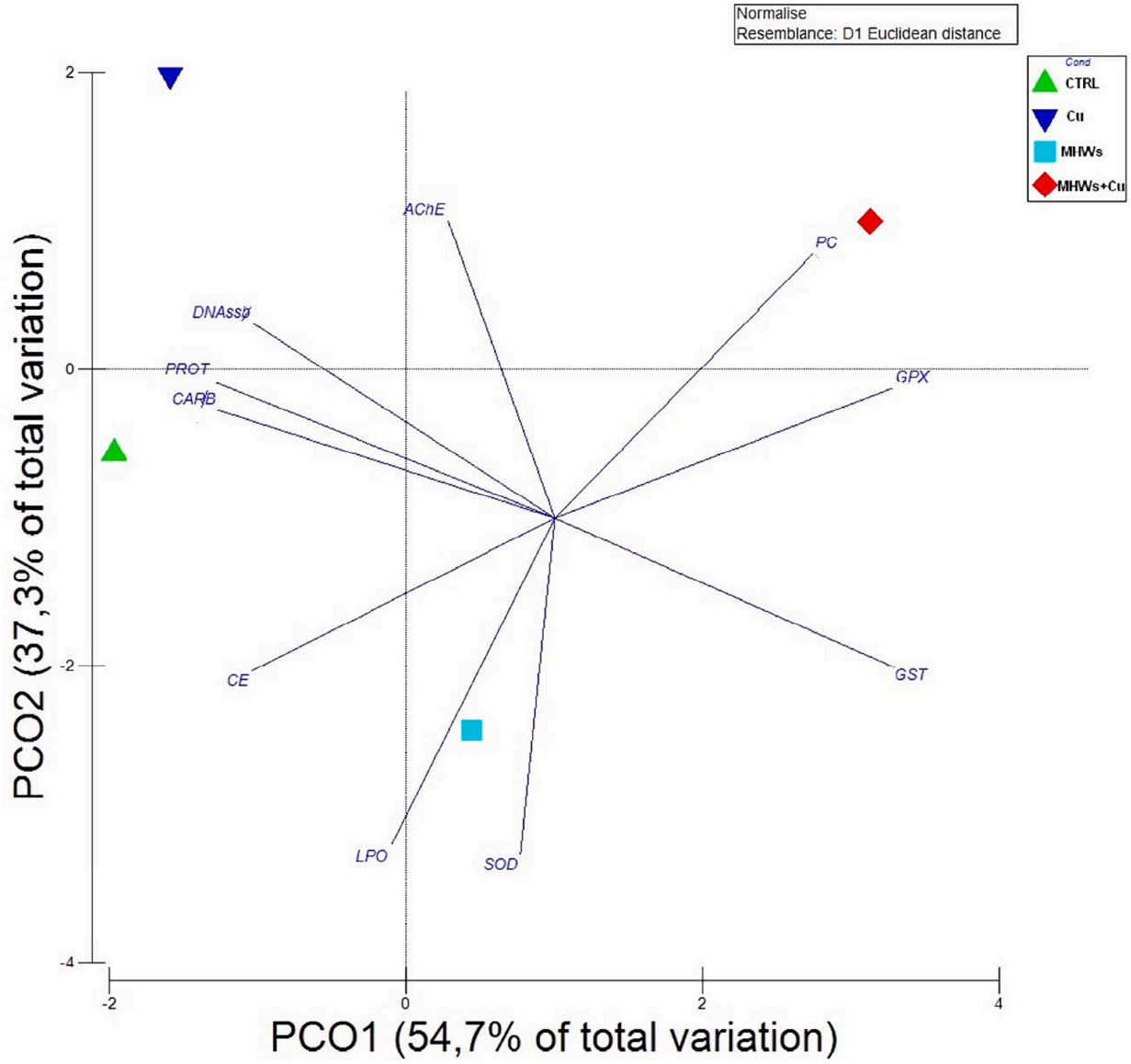

Fig. 3. PCO analysis. Centroids ordination diagram (PCO) measured in CTRL and Cu-, MHWs-, MHWs+Cu-exposed *F. enigmaticus*. Pearson correlation vectors are superimposed as supplementary variables, namely biochemical data ($r > 0.75$): LPO; PC; SOD; GPx; GST; CE; PROT; CARB; AChE, DNAssb.

behavior of this species compared to copper exposure. These findings agree with our results, in which the temperature as MHWs played a more substantial role. The combined exposure to temperature and copper produced a dysregulation of 5 analyzed biomarkers (PC, GST, CE, PROT and CARB). Moreover, the IBR was found to be strongly related to this combined condition, which is the treatment that shows a higher response regarding IBR scores (Fig. 4). In contrast, exposure to copper alone did not produce significant effects in our study. It is important to observe that, for the experimental purposes of this research, we intentionally selected a copper concentration of 50 μg/L. This concentration, although relevant from an environmental point of view, is considered relatively high (being a concentration that has been recorded close to the emission sources). This choice was motivated by our specific goal of evaluating the potential influence of temperature on copper toxicity. It should be noted that the dose of copper selected is less than half the emission limit values for surface water and sewage discharges established by Italian legislation, which is defined as 100 μg/L (D. Lgs. 152/06, Part Three, Annex 5, Table 3).

PC is an indicator of ROS-mediated protein oxidation and thus this biomarker can be considered indicative of proteins modification that can be responsible for loss of function (Carney Almroth et al., 2005). Increased levels of PC were detected in several heat-stressed marine invertebrates as the Atlantic sea urchin *Arbacia punctulata* (Lamarck, 1816) (Johnstone et al., 2019), the hydroid *Hydractinia echinata* (Fleming, 1828) (Eder et al., 2018) and the polychaete *Hediste diversicolor* (O.F. Müller, 1776) (Valente et al., 2022). The findings from this study emphasized that neither heat-stress alone nor copper exposure alone were sufficient to induce PC. Differently, the increase of PC was detected when the heat-stress was combined with copper exposure. In this scenario, heat-stress could act as a triggering factor that enhances metabolism, leading to intensified responses when combined with copper exposure.

Relatively to biotransformation biomarker, GST, the increase of its activity coupled with the decrease of PROT in *F. enigmaticus*-MHWs exposed could be interpreted as early signals of increased metabolism. On one side, the rise in GST levels correlated with increased metabolic and biotransformation activity. Conversely, the decline in PROT indicated an expenditure of energy to cope the increased metabolism triggered by stress, as also stated by Knochel (2017). This expenditure becomes more pronounced when tubeworms were exposed to the

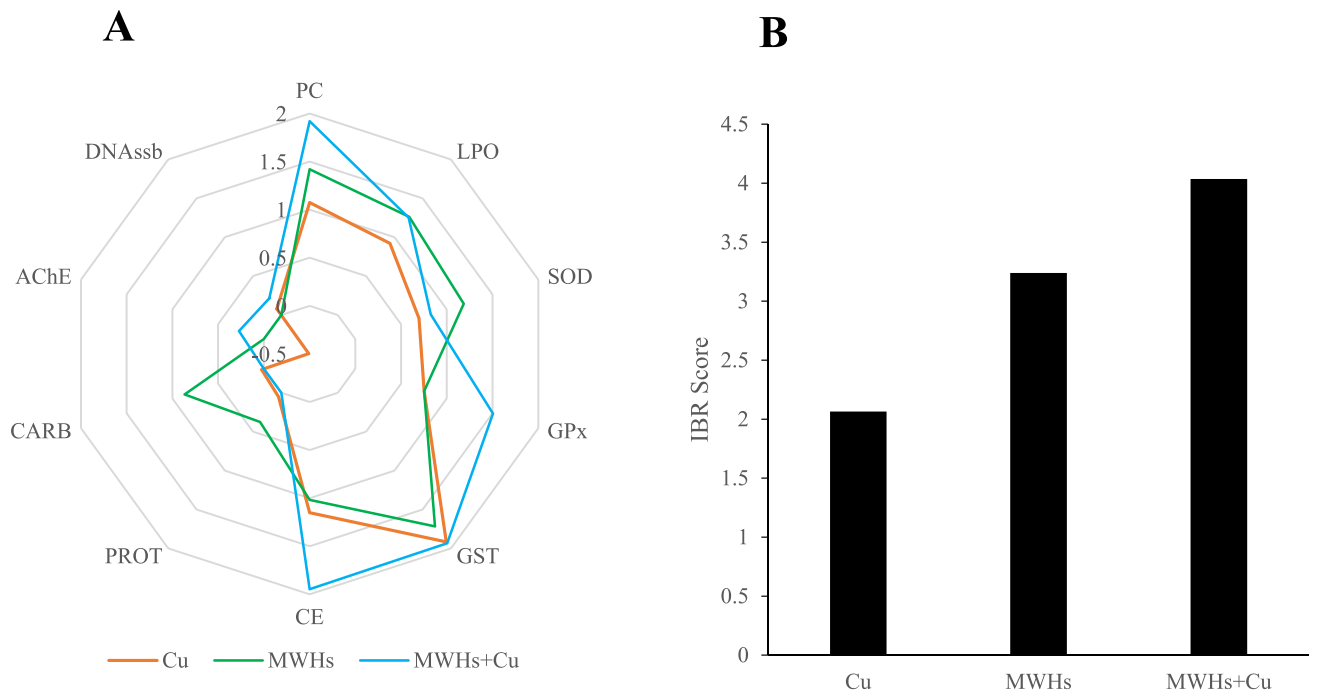

Fig. 4. Integrated Biomarker Response (IBR) assessment. Integrated Biomarker Response (IBR) radar diagram (panel A) and IBR score (panel B) for all assessed biomarkers (ten) in *F. enigmaticus* after exposure to Cu, MHWs and MHWs+Cu conditions.

MHWs+Cu condition, as evidenced by the observed decrease in CARB levels. It is known that stress factors can generally lead to changes in energy distribution in invertebrates (Bednarska et al., 2013; De Coen and Janssen, 2003). It has been observed, for *F. enigmaticus*, other polychaetes and bivalves, a reduction of energy reserves in terms of proteins (PROT) and glycogen content, when exposed to pollutants such as carbon nanomaterials, organic and inorganic UV filters (De Marchi et al., 2017a, 2017b, 2017c, 2017d, 2017e, 2018, 2019; Cuccaro et al., 2022). Rosic et al. (2014) described an increased GST activity in *Acropora aspera* (Dana, 1846) coral adults after a short thermal stress exposure at increased temperature (6–7 °C above ambient temperature) in order to underline the relationship between heat stress and metabolism. An increase in this biomarker response was also detected in nine Indo-Pacific scleractinian coral species after exposure at elevated temperature (30 °C) and low salinity (Dias et al., 2019b). Prolonged exposure (12 days) at 26.6 °C also produced an increase in GST in the Atlantic coral *M. harttii* (Fonseca et al., 2021b).

CE resulted significantly inhibited in MHWs+Cu condition only. CEs are a superfamily of serine esterase with several function ranging from the metabolism of xenobiotics to the involvement of metabolism and homeostasis of lipids (Solé and Sanchez-Hernandez, 2018). The inhibitory effect of several metals, including copper, was observed both in bivalves and fish (Morais et al., 2023; de Lima et al., 2013). The obtained results could reflect a situation of generalized dysregulation of metabolic activity following the heat stress leading to an inhibition of CE activity.

Such dysregulation was not observed for the oxidative status of cell membranes (LPO) and for the antioxidant defences (SOD and GPx). The absence of increased LPO levels and antioxidant enzymatic defences could be explained by the increased activity of GST (biotransformation enzyme) that was enough to counterbalance the deleterious effects of ROS on lipids, as suggested by Dias et al. (2019a, 2019b).

Results related to DNA integrity indicated that adult organisms exposed to all tested conditions did not exhibit any alteration. In contrast, Cuccaro et al. (2021) observed a loss of DNA integrity on *F. enigmaticus* sperm at this concentration. This aligns with the general biological assumption that early life stages are typically more sensitive than adults (Mohammed, 2013). In contrast to our results, Marangoni et al. (2017) detected significant DNA lesions at apurinic/apyrimidinic (AP) sites in adult coral *M. harttii* after 12 days of exposure to 4.8 and 6.7 μg/L copper, with a dose-dependent increase. In certain invertebrates, temperature has been identified as a factor influencing DNA integrity. For example, in *M. galloprovincialis*, higher damages were observed during summer (Almeida et al., 2011). Similar observations were made in *Mytilus californianus* (Conrad, 1837) and oysters (Yao and Somero, 2012; Rahman et al., 2023). However, the effects of high temperatures on DNA damage, such as strand breaks, in temperate reef-forming organisms are not extensively documented. In Indo-Pacific corals, thermal stress has been shown to affect this biomarker (Fitt et al., 2009). Utilizing a combination of different genotoxicity assays, such as the Fast Micromethod, comet assay, and micronucleus test, may enable the assessment of early and cumulative pollutant effects (Monserrat et al., 2007).

The neurotoxicity biomarker (AChE activity) appeared unaffected in *F. enigmaticus* by exposure to copper, MHWs, or their combination (MHWs+Cu). This observation is consistent with the findings in the earthworm *Eisenia fetida* (Savigny, 1826) (Bednarska et al., 2017), where AChE activity was not affected by similar exposures. However, other authors reported an increase in AChE activity in embryo and larval stages of *M. galloprovincialis* when subjected to combined stress from temperature and metals (Boukadida et al., 2022). These data primarily pertain to early stages of development, necessitating further analyses to understand how this biomarker responds in reef-forming organisms to the combined stress of temperature and metals.

4.2. Multi-biomarker approach implications

The application of a multi-biomarker approach, integrated with the IBR index, enabled a more comprehensive view of the effect of MHWs and copper contamination on the reef-forming tubeworm *F. enigmaticus*. The temperature was found to be the factor with the highest impact on the biochemical level of this bio-builder, while copper had no significant effect, although bioaccumulation was noted. Regarding the interaction between copper and MHWs, this exposure condition showed the highest

IBR index, with high CE, GST, PC and GPx relative area in the star plot, indicating an enhanced effect if compared to both stressors (copper and MHWs). This observation indicates how toxicity of legacy contaminants, such as copper, should be re-evaluated in light of global change scenarios, in order to identify additive and synergistics effects of these different sources of stress (Dinh et al., 2022).

5. Conclusion

Results presented and discussed in this research work pointed to an enhanced toxic effect exerted by copper, intended as legacy contaminant, when under specific global change events, namely heat waves. While the selected copper concentration, as single stressor, exerted negligible effects on *F. enigmaticus* adult biochemical parameters, the exposure of animals to MHWs alone or in combination with the same copper concentration, showed clear effects on several biochemical parameters. However, despite MHWs, as extreme events related to temperature increase, being one of the major problems for intertidal sessile benthic organisms, information on their effects on reef-formers, either individually or in combination with additional stressors, is still scarce. Furthermore, the individuation of biochemical and physiological implication of these events, which constantly increase their frequency and magnitude, on different calcifying organisms, may represent important knowledge for future preservation of more sensible species, such as hard corals. In fact, integrated biochemical/physiological approaches, as suggested by the Marine Strategy Framework Directive (MSFD 2008/56/EC) at the European level, are key-tools for early individuation of hazard affecting endangered ecosystems.

CRediT authorship contribution statement

Verdiana Vellani: Formal analysis, Investigation, Writing – original draft. **Alessia Cuccaro:** Data curation, Formal analysis, Investigation, Methodology. **Matteo Oliva:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Carlo Pretti:** Conceptualization, Data curation, Funding acquisition, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. **Monia Renzi:** Conceptualization, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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References

Almeida, C., Pereira, C., Gomes, T., Bebianno, M.J., Cravo, A., 2011. DNA damage as a biomarker of genotoxic contamination in *Mytilus galloprovincialis* from the south coast of Portugal. J. Environ. Monit. 13 (9), 2559–2567. https://doi.org/10.1039/C1EM10190K.

Anderson, M., Gorley, R., Clarke, K., 2008. PERMANOVA+ for primer: guide to software and statistical methods. PRIMER-E Plymouth (UK).

Badary, O.A., Abdel-Maksoud, S., Ahmed, W.A., Owieda, G.H., 2005. Naringenin attenuates cisplatin nephrotoxicity in rats. Life Sci. 76 (18), 2125–2135. https://doi.org/10.1016/j.lfs.2004.11.005.

Bednarska, A.J., Stachowicz, I., Kuriańska, L., 2013. Energy reserves and accumulation of metals in the ground beetle *Pterostichus oblongopunctatus* from two metal-polluted gradients. Environ. Sci. Pollut. Res. Int. 20 (1), 390–398. https://doi.org/10.1007/s11356-012-0993-y.

Bednarska, A.J., Choczyński, M., Laskowski, R., Walczak, M., 2017. Combined effects of chlorpyriphos, copper and temperature on acetylcholinesterase activity and toxicokinetics of the chemicals in the earthworm *Eisenia fetida*. Environmental Pollution (Barking, Essex: 1987) 220 (Pt A), 567–576. https://doi.org/10.1016/j.envpol.2016.10.004.

Beliaeff, B., Burgeot, T., 2002. Integrated biomarker response: A useful tool for ecological risk assessment. Environ. Toxicol. Chem. 21 (6), 1316–1322. https://doi.org/10.1002/etc.5620210629.

Bezuidenhout, M., 2021. The implications of climate change for the invasive tube worm Ficopomatus enigmaticus [thesis, Stellenbosch: Stellenbosch University]. https://scholar.sun.ac.za:443/handle/10019.1/110254.

Boukadida, K., Banni, M., Romero-Ramirez, A., Clerandeau, C., Gourves, P.-Y., Cachot, J., 2022. Metal contamination and heat stress impair swimming behavior and acetylcholinesterase activity in embryo-larval stages of the Mediterranean mussel, *Mytilus galloprovincialis*. Marine Environmental Research 179, 105677. https://doi.org/10.1016/j.marenvres.2022.105677.

Brown, R.J., Galloway, T.S., Lowe, D., Browne, M.A., Dissanayake, A., Jones, M.B., Depledge, M.H., 2004. Differential sensitivity of three marine invertebrates to copper assessed using multiple biomarkers. Aquatic Toxicology (Amsterdam, Netherlands) 66 (3), 267–278. https://doi.org/10.1016/j.aquatox.2003.10.001.

Carney Almroth, B., Sturve, J., Berglund, A., Förlin, L., 2005. Oxidative damage in eelpout (*Zoarces viviparus*), measured as protein carbonyls and TBARS, as biomarkers. Aquatic Toxicology (Amsterdam, Netherlands) 73 (2), 171–180. https://doi.org/10.1016/j.aquatox.2005.03.007.

Cuccaro, A., De Marchi, L., Oliva, M., Sanches, M.V., Freitas, R., Casu, V., Monni, G., Miragliotta, V., Pretti, C., 2021. Sperm quality assessment in *Ficopomatus enigmaticus* (Fauvel, 1923): effects of selected organic and inorganic chemicals across salinity levels. Ecotoxicol. Environ. Saf. 207, 111219 https://doi.org/10.1016/j.ecoenv.2020.111219.

Cuccaro, A., Freitas, R., De Marchi, L., Oliva, M., Pretti, C., 2022. UV-filters in marine environments: A review of research trends, meta-analysis, and ecotoxicological impacts of 4-methylbenzylidene-camphor and benzophenone-3 on marine invertebrate communities. Environ. Sci. Pollut. Res. Int. 29 (43), 64370–64391. https://doi.org/10.1007/s11356-022-21913-4.

van Dam, J.W., Negri, A.P., Uthicke, S., Mueller, J.F., 2011. Chemical pollution on coral reefs: exposure and ecological effects. In: Sánchez-Bayo, Francisco, van den Brink, Paul J., Mann, Reinier M. (Eds.), Ecological Impacts of Toxic Chemicals: Vol. Chapter 9, pp. 187–211. https://doi.org/10.2174/978160805121210187.

De Coen, W.M., Janssen, C.R., 2003. The missing biomarker link: relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. Environ Toxicol Chem 22 (7), 1632–1641. https://doi.org/10.1002/etc.5620220727.

De Marchi, L., Neto, V., Pretti, C., Figueira, E., Brambilla, L., Rodriguez-Douton, M.J., Rossella, F., Tommasini, M., Furtado, C., Soares, A.M.V.M., Freitas, R., 2017a. Physiological and biochemical impacts of graphene oxide in polychaetes: the case of *Diopatra neapolitana*. Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP 193, 50–60. https://doi.org/10.1016/j.cbpc.2017.01.005.

De Marchi, L., Neto, V., Pretti, C., Figueira, E., Chiellini, F., Soares, A.M.V.M., Freitas, R., 2017b. Physiological and biochemical responses of two keystone polychaete species: *Diopatra neapolitana* and *Hediste diversicolor* to multi-walled carbon nanotubes. Environ. Res. 154, 126–138. https://doi.org/10.1016/j.envres.2016.12.018.

De Marchi, L., Neto, V., Pretti, C., Figueira, E., Chiellini, F., Morelli, A., Soares, A.M.V.M., Freitas, R., 2017c. Toxic effects of multi-walled carbon nanotubes on bivalves: comparison between functionalized and nonfunctionalized nanoparticles. Sci. Total Environ. 622–623, 1532–1542. https://doi.org/10.1016/j.scitotenv.2017.10.031.

De Marchi, L., Neto, V., Pretti, C., Figueira, E., Federica, C., Soares, A., Freitas, R., 2017d. The impacts of emergent pollutants on *Ruditapes philippinarum*: biochemical responses to carbon nanoparticles exposure. Aquat. Toxicol. 187 https://doi.org/10.1016/j.aquatox.2017.03.010.

De Marchi, L., Neto, V., Pretti, C., Figueira, E., Chiellini, F., Morelli, A., Soares, A.M.V.M., Freitas, R., 2017e. The impacts of seawater acidification on *Ruditapes philippinarum* sensitivity to carbon nanoparticles. Environ. Sci. Nano 4 (8), 1692–1704. https://doi.org/10.1039/C7EN00335H.

De Marchi, L., Neto, V., Pretti, C., Figueira, E., Chiellini, F., Morelli, A., Soares, A.M.V.M., Freitas, R., 2018. Effects of multi-walled carbon nanotube materials on *Ruditapes philippinarum* under climate change: the case of salinity shifts. Aquatic Toxicology (Amsterdam, Netherlands) 199, 199–211. https://doi.org/10.1016/j.aquatox.2018.04.001.

De Marchi, L., Oliva, M., Freitas, R., Neto, V., Figueira, E., Chiellini, F., Morelli, A., Soares, A.M.V.M., Pretti, C., 2019. Toxicity evaluation of carboxylated carbon

nanotubes to the reef-forming tubeworm *Ficopomatus enigmaticus* (Fauvel, 1923). Mar. Environ. Res. 143, 1–9. https://doi.org/10.1016/j.marenvres.2018.10.015.
Devin, S., Burgeot, T., Giambérini, L., Minguez, L., Pain-Devin, S., 2014. The integrated biomarker response revisited: optimization to avoid misuse. Environ. Sci. Pollut. Res. 21 (4), 2448–2454. https://doi.org/10.1007/s11356-013-2169-9.
Dias, M., Ferreira, A., Gouveia, R., Madeira, C., Jogee, N., Cabral, H., Diniz, M., Vinagre, C., 2019a. Long-term exposure to increasing temperatures on scleractinian coral fragments reveals oxidative stress. Mar. Environ. Res. 150, 104758 https://doi.org/10.1016/j.marenvres.2019.104758.
Dias, M., Madeira, C., Jogee, N., Ferreira, A., Gouveia, R., Cabral, H., Diniz, M., Vinagre, C., 2019b. Oxidative stress on scleractinian coral fragments following exposure to high temperature and low salinity. Ecol. Indic. 107, 105586 https://doi.org/10.1016/j.ecolind.2019.105586.
Dinh, K.V., Konestabo, H.S., Borgå, K., Hylland, K., Macaulay, S.J., Jackson, M.C., Verheyen, J., Stoks, R., 2022. Interactive effects of warming and pollutants on marine and freshwater invertebrates. Curr. Pollut. Rep. 8 (4), 341–359. https://doi.org/10.1007/s40726-022-00245-4.
Dondero, F., Piacentini, L., Banni, M., Rebelo, M., Burlando, B., Viarengo, A., 2005. Quantitative PCR analysis of two molluscan metallothionein genes unveils differential expression and regulation. Gene 345 (2), 259–270. https://doi.org/10.1016/j.gene.2004.11.031.
DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, Fred, 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28 (3), 350–356. https://doi.org/10.1021/ac60111a017.
Eakin, C.M., Morgan, J.A., Heron, S.F., Smith, T.B., Liu, G., Alvarez-Filip, L., Baca, B., Bartels, E., Bastidas, C., Bouchon, C., Brandt, M., Bruckner, A.W., Bunkley-Williams, L., Cameron, A., Causey, B.D., Chiappone, M., Christensen, T.R., Crabbe, M.J., Day, O., de la Guardia, E., Díaz-Pulido, G., DiResta, D., Gil-Agudelo, D. L., Gilliam, D.S., Ginsburg, R.N., Gore, S., Guzmán, H.M., Hendee, J.C., Hernández-Delgado, E.A., Husain, E., Jeffrey, C.F., Jones, R.J., Jordán-Dahlgren, E., Kaufman, L. S., Kline, D.I., Kramer, P.A., Lang, J.C., Lirman, D., Mallela, J., Manfrino, C., Maréchal, J.P., Marks, K., Mihaly, J., Miller, W.J., Mueller, E.M., Muller, E.M., Orozco Toro, C.A., Oxenford, H.A., Ponce-Taylor, D., Quinn, N., Ritchie, K.B., Rodríguez, S., Ramírez, A.R., Romano, S., Samhouri, J.F., Sánchez, J.A., Schmahl, G. P., Shank, B.V., Skirving, W.J., Steiner, S.C., Villamizar, E., Walsh, S.M., Walter, C., Weil, E., Williams, E.H., Roberson, K.W., Yusuf, Y., 2010. Caribbean corals in crisis: record thermal stress, bleaching, and mortality in 2005. PloS One 5, e13969. https://doi.org/10.1371/journal.pone.0013969.
Eder, Y., Tschink, D., Gerlach, G., Strahl, J., 2018. Physiology of juvenile hydroids-high food availability mitigates stress responses of *Hydractinia echinata* to increasing seawater temperatures. J. Exp. Mar. Biol. Ecol. 508, 64–72.
Ellman, G.L., Courtney, K.D., Andres, V., Feather-Stone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95. https://doi.org/10.1016/0006-2952(61)90145-9.
Fitt, W.K., Gates, R.D., Hoegh-Guldberg, O., Bythell, J.C., Jatkar, A., Grottoli, A.G., Gomez, M., Fisher, P., Lajuenesse, T.C., Pantos, O., Iglesias-Prieto, R., Franklin, D.J., Rodrigues, L.J., Torregiani, J.M., van Woesik, R., Lesser, M.P., 2009. Response of two species of Indo-Pacific corals, *Porites cylindrica* and *Stylophora pistillata*, to short-term thermal stress: the host does matter in determining the tolerance of corals to bleaching. J. Exp. Mar. Biol. Ecol. 373 (2), 102–110. https://doi.org/10.1016/j.jembe.2009.03.011.
Fonseca, J. da S., Marangoni, L.F. de B., Marques, J.A., Bianchini, A., 2017. Effects of increasing temperature alone and combined with copper exposure on biochemical and physiological parameters in the zooxanthellate scleractinian coral *Mussismilia harttii*. Aquatic Toxicology (Amsterdam, Netherlands) 190, 121–132. https://doi.org/10.1016/j.aquatox.2017.07.002.
Fonseca, J. da S., Marangoni, L.F. de B., Marques, J.A., Bianchini, A., 2019. Carbonic anhydrase activity as a potential biomarker for acute exposure to copper in corals. Chemosphere 227, 598–605. https://doi.org/10.1016/j.chemosphere.2019.04.089.
Fonseca, J. Da S., de Barros Marangoni, L.F., Marques, J.A., Bianchini, A., 2021a. Elevated temperature and exposure to copper leads to changes in the antioxidant defense system of the reef-building coral *Mussismilia harttii*. Front. Physiol. 12, 804678 https://doi.org/10.3389/fphys.2021.804678.
Fonseca, J. da S., Mies, M., Paranhos, A., Taniguchi, S., Güth, A.Z., Bícego, M.C., Marques, J.A., Fernandes de Barros Marangoni, L., Bianchini, A., 2021b. Isolated and combined effects of thermal stress and copper exposure on the trophic behavior and oxidative status of the reef-building coral *Mussismilia harttii*. Environ. Pollut. 268, 115892 https://doi.org/10.1016/j.envpol.2020.115892.
Frölicher, T.L., Laufkötter, C., 2018. Emerging risks from marine heat waves. Nat. Commun. 9, 650. https://doi.org/10.1038/s41467-018-03163-6.
Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249 (22), 7130–7139. https://doi.org/10.1016/S0021-9258(19)42083-8.
Halpern, B.S., Walbridge, S., Selkoe, K.A., Kappel, C.V., Micheli, F., D'Agrosa, C., Bruno, J.F., Casey, K.S., Ebert, C., Fox, H.E., Fujita, R., Heinemann, D., Lenihan, H.S., Madin, E.M.P., Perry, M.T., Selig, E.R., Spalding, M., Steneck, R., Watson, R., 2008. A global map of human impact on marine ecosystems. Science (New York, N.Y.) 319 (5865), 948–952. https://doi.org/10.1126/science.1149345.
Halpern, B.S., Frazier, M., Potapenko, J., Casey, K.S., Koenig, K., Longo, C., Lowndes, J. S., Rockwood, R.C., Selig, E.R., Selkoe, K.A., Walbridge, S., 2015. Spatial and temporal changes in cumulative human impacts on the world's ocean. Nature Communications 6 (1), 1. https://doi.org/10.1038/ncomms8615.
Hobday, A.J., Alexander, L.V., Perkins, S.E., Smale, D.A., Straub, S.C., Oliver, E.C.J., Benthuysen, J.A., Burrows, M.T., Donat, M.G., Feng, M., Holbrook, N.J., Moore, P.J., Scannell, H.A., Sen Gupta, A., Wernberg, T., 2016. A hierarchical approach to defining marine heatwaves. Prog. Oceanogr. 141, 227–238. https://doi.org/10.1016/j.pocean.2015.12.014.
Hosokawa, M., Satoh, T., 2001. Measurement of carboxylesterase (CES) activities. Current Protocols in Toxicology 10 (1). https://doi.org/10.1002/0471140856.tx0407s10, 4.7.1-4.7.14.
Johnstone, J., Nash, S., Hernandez, E., Rahman, M.S., 2019. Effects of elevated temperature on gonadal functions, cellular apoptosis, and oxidative stress in Atlantic sea urchin *Arbacia punculata*. Mar. Environ. Res. 149, 40–49. https://doi.org/10.1016/j.marenvres.2019.05.017.
Knochel, A., 2017. The effects of thermal stress on fluorescent protein expression in an indo-Pacific scleractinian coral species. In: Acropora tenuis. SIT Graduate Institute/SIT Study Abroad. SIT Digital Collections Independent Study Project (ISP) Collection, 2637, p. 36.
Kong, Q., Zheng, J., Fowler, H.J., Ge, Q., Xi, J., 2019. Climate change and summer thermal comfort in China. Theor. Appl. Climatol. 137 (1), 1077–1088. https://doi.org/10.1007/s00704-018-2648-5.
Kružić, P., 2014. Bioconstructions in the Mediterranean: Present and future. In: Goffredo, S., Dubinsky, Z. (Eds.), *The Mediterranean Sea: Its History and Present Challenges* (Pp. 435–447). https://doi.org/10.1007/978-94-007-6704-1_25. Springer Netherlands.
Kuglitsch, F.G., Toreti, A., Xoplaki, E., Della-Marta, P., Zerefos, C.S., Türkeş, M., Luterbacher, J., 2010. Heat wave changes in the eastern Mediterranean since 1960. Geophys. Res. Lett. 37 https://doi.org/10.1029/2009GL041841.
Laufkötter, C., Zscheischler, J., Frölicher, T.L., 2020. High-impact marine heatwaves attributable to human-induced global warming. Science 369 (6511), 1621–1625. https://doi.org/10.1126/science.aba0690.
de Lima, D., Roque, G.M., de Almeida, E.A., 2013. In vitro and in vivo inhibition of acetylcholinesterase and carboxylesterase by metals in zebrafish (*Danio rerio*). Mar. Environ. Res. 91, 45–51.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193 (1), 265–275.
Magnani, L., Gaydou, E., Hubaud, J., 2000. Spectrophotometric measurement of antioxidant properties of flavones and flavonols against superoxide anion. Anal. Chim. Acta 411, 209–216. https://doi.org/10.1016/S0003-2670(00)00717-0.
Marangoni, L.F. de B., Marques, J.A., Duarte, G.A.S., Pereira, C.M., Calderon, E.N., Castro, C.B.E., Bianchini, A., 2017. Copper effects on biomarkers associated with photosynthesis, oxidative status and calcification in the Brazilian coral *Mussismilia harttii* (Scleractinia, Mussidae). Mar. Environ. Res. 130, 248–257. https://doi.org/10.1016/j.marenvres.2017.08.002.
Maria, V.L., Bebianno, M.J., 2011. Antioxidant and lipid peroxidation responses in *Mytilus galloprovincialis* exposed to mixtures of benzo(a)pyrene and copper. Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP 154 (1), 56–63. https://doi.org/10.1016/j.cbpc.2011.02.004.
Mesquita, C.S., Oliveira, R., Bento, F., Geraldo, D., Rodrigues, J.V., Marcos, J.C., 2014. Simplified 2,4-dinitrophenylhydrazine spectrophotometric assay for quantification of carbonyls in oxidized proteins. Anal. Biochem. 458, 69–71. https://doi.org/10.1016/j.ab.2014.04.034.
Minuti, J.J., Byrne, M., Hemraj, D.A., Russell, B.D., 2021. Capacity of an ecologically key urchin to recover from extreme events: physiological impacts of heatwaves and the road to recovery. Sci. Total Environ. 785, 147281 https://doi.org/10.1016/j.scitotenv.2021.147281.
Mohammed, A., 2013. Why Are Early Life Stages of Aquatic Organisms more Sensitive to Toxicants than Adults?. In: New Insights into Toxicity and Drug Testing. IntechOpen. https://doi.org/10.5772/55187.
Moleiro, P., Morais, T., Leite, C., Coppola, F., Henriques, B., Pinto, J., Soares, A., Pereira, E., Freitas, R., 2022. The effect of ocean warming on accumulation and cellular responsiveness to cobalt in *Mytilus galloprovincialis*. Mar. Pollut. Bull. 182, 113944 https://doi.org/10.1016/j.marpolbul.2022.113944.
Monserrat, J.M., Martínez, P.E., Geracitano, L.A., Lund Amado, L., Martinez Gaspar Martins, C., Lopes Leães Pinho, G., Soares Chaves, I., Ferreira-Cravo, M., Ventura-Lima, J., Bianchini, A., 2007. Pollution biomarkers in estuarine animals: critical review and new perspectives. Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP 146 (1–2), 221–234. https://doi.org/10.1016/j.cbpc.2006.08.012.
Morais, T., Moleiro, P., Leite, C., Coppola, F., Pinto, J., Henriques, B., Soares, A.M.V.M., Pereira, E., Freitas, R., 2023. Ecotoxicological impacts of metals in single and co-exposure on mussels: comparison of observable and predicted results. Sci. Total Environ. 881, 163165.
Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95 (2), 351–358. https://doi.org/10.1016/0003-2697(79)90738-3.
Oliva, M., Mennillo, E., Barbaglia, M., Monni, G., Tardelli, F., Casu, V., Pretti, C., 2018. The serpulid *Ficopomatus enigmaticus* (Fauvel, 1923) as candidate organisms for ecotoxicological assays in brackish and marine waters. Ecotoxicol. Environ. Saf. 148 https://doi.org/10.1016/j.ecoenv.2015.10.006.
Oliva, M., Manzini, C., Bontà Pittaluga, G., Kozinkova, L., De Marchi, L., Freitas, R., Fabi, G., Pretti, C., 2019. *Ficopomatus enigmaticus* larval development assay: an application for toxicity assessment of marine sediments. Mar. Pollut. Bull. 139, 189–196. https://doi.org/10.1016/j.marpolbul.2018.12.033.
Rahman, M.F., Billah, M.M., Kline, R.J., Rahman, M.S., 2023. Effects of elevated temperature on 8-OHdG expression in the American oyster (*Crassostrea virginica*): induction of oxidative stress biomarkers, cellular apoptosis, DNA damage and γH2AX signaling pathways. Fish and Shellfish Immunology Reports 4, 100079. https://doi.org/10.1016/j.fsirep.2022.100079.

Rainbow, P.S., 2002. Trace metal concentrations in aquatic invertebrates: why and so what? Environ. Pollut. 120 (3), 497–507. https://doi.org/10.1016/S0269-7491(02)00238-5.

Rivetti, I., Fraschetti, S., Lionello, P., Zambianchi, E., Boero, F., 2014. Global warming and mass mortalities of benthic invertebrates in the Mediterranean Sea. PloS One 9 (12), e115655. https://doi.org/10.1371/journal.pone.0115655.

Rosic, N., Kaniewska, P., Chan, C.-K.K., Ling, E.Y.S., Edwards, D., Dove, S., Hoegh-Guldberg, O., 2014. Early transcriptional changes in the reef-building coral *Acropora aspera* in response to thermal and nutrient stress. BMC Genomics 15 (1), 1052. https://doi.org/10.1186/1471-2164-15-1052.

Schröder, H.C., Batel, R., Schwertner, H., Boreiko, O., Müller, W.E.G., 2006. Fast micromethod DNA single-strand-break assay. Methods in Molecular Biology (Clifton, N.J.) 314, 287–305. https://doi.org/10.1385/1-59259-973-7:287.

Schwarz, J.A., Mitchelmore, C.L., Jones, R., O'Dea, A., Seymour, S., 2013. Exposure to copper induces oxidative and stress responses and DNA damage in the coral *Montastraea franksi*. Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP 157 (3), 272–279. https://doi.org/10.1016/j.cbpc.2012.12.003.

Sen Gupta, A., Thomsen, M., Benthuysen, J.A., Hobday, A.J., Oliver, E., Alexander, L.V., Burrows, M.T., Donat, M.G., Feng, M., Holbrook, N.J., Perkins-Kirkpatrick, S., Moore, P.J., Rodrigues, R.R., Scannell, H.A., Taschetto, A.S., Ummenhofer, C.C., Wernberg, T., Smale, D.A., 2020. Drivers and impacts of the most extreme marine heatwave events. Sci. Rep. 10 (1), 1 https://doi.org/10.1038/s41598-020-75445-3.

Smale, D.A., Wernberg, T., Oliver, E.C.J., Thomsen, M., Harvey, B.P., Straub, S.C., Burrows, M.T., Alexander, L.V., Benthuysen, J.A., Donat, M.G., Feng, M., Hobday, A.J., Holbrook, N.J., Perkins-Kirkpatrick, S.E., Scannell, H.A., Sen Gupta, A., Payne, B.L., Moore, P.J., 2019. Marine heatwaves threaten global biodiversity and the provision of ecosystem services. Nat. Clim. Chang. 9 (4), 306–312. https://doi.org/10.1038/s41558-019-0412-1.

Sokolova, I., Lannig, G., 2008. Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: implications of global climate change. Climate Res. 37, 181–201. https://doi.org/10.3354/cr00764.

Solé, M., Sanchez-Hernandez, J.C., 2018. Elucidating the importance of mussel carboxylesterase activity as exposure biomarker of environmental contaminants of current concern: an in vitro study. Ecol. Indic. 85, 432–439.

UNEP, 1996. Assessment of the State of Pollution of the Mediterranean Sea by Zinc, Copper and Their Compounds. (No. 105). United Nations Environment Programme. https://www.academia.edu/24112499/Assessment_of_the_State_of_Pollution_of_the_Mediterranean_Sea_by_Zinc_Copper_and_Their_Compounds_Evaluation_De_LEtat_De_La_Pollution_De_La_Mer_Mediterranee_Par_Le_Zinc_Le_Cuivre_et_Leurs_Composes.

Valente, P., Cardoso, P., Giménez, V., Silva, M.S.S., Sá, C., Figueira, E., Pires, A., 2022. Biochemical and behavioural alterations induced by arsenic and temperature in *Hediste diversicolor* of different growth stages. Int. J. Environ. Res. Public Health 19 (23), 15426.

Viarengo, A., Burlando, B., Bolognesi, C., 2002. Cellular responses to copper in aquatic organisms. In: DiDonato, M. (Ed.), Handbook of Copper Toxicology. Edward J. Massaro. (ed. Totowa, Vol. 48, p. 624 pp.). https://doi.org/10.1093/clinchem/48.12.2300a.

Vieira Sanches, M., Oliva, M., Pires, A., De Marchi, L., Cuccaro, A., Freitas, R., Baratti, M., Pretti, C., 2020. Relationship between wild-caught organisms for bioassays and sampling areas: widespread serpulid early-development comparison between two distinct populations after trace element exposure. Ecotoxicol. Environ. Saf. 205, 111094 https://doi.org/10.1016/j.ecoenv.2020.111094.

Xu, Y., Wang, Z., Zhang, Y., Liang, J., He, G., Liu, X., Zheng, Z., Deng, Y., Zhao, L., 2022. Transcriptome analysis reveals acclimation responses of pearl oysters to marine heatwaves. Sci. Total Environ. 810, 151189 https://doi.org/10.1016/j.scitotenv.2021.151189.

Yao, C.-L., Somero, G.N., 2012. The impact of acute temperature stress on hemocytes of invasive and native mussels (*Mytilus galloprovincialis* and *Mytilus californianus*): DNA damage, membrane integrity, apoptosis and signaling pathways. J. Exp. Biol. 215 (Pt 24), 4267–4277. https://doi.org/10.1242/jeb.073577.

Zorita, I., Bilbao, E., Schad, A., Cancio, I., Soto, M., Cajaraville, M.P., 2007. Tissue- and cell-specific expression of metallothionein genes in cadmium- and copper-exposed mussels analyzed by in situ hybridization and RT-PCR. Toxicol. Appl. Pharmacol. 220 (2), 186–196. https://doi.org/10.1016/j.taap.2007.01.003.