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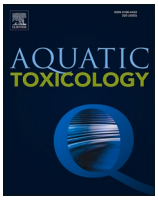

Ocean acidification impact on the uptake of trace elements by mussels and their biochemical effects

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ABSTRACT

This study delves into the intricate interplay between ocean acidification (OA), metal bioaccumulation, and cellular responses using mussels (*Mytilus galloprovincialis*) as bioindicators. For this purpose, environmentally realistic concentrations of isotopically labelled metals (Cd, Cu, Ag, Ce) were added to investigate whether the OA increase would modify metal bioaccumulation and induce adverse effects at the cellular level. The study reveals that while certain elements like Cd and Ag might remain unaffected by OA, the bioavailability of Cu and Ce could potentially escalate, leading to amplified accumulation in marine organisms. The present findings highlight a significant rise in Ce concentrations within different mussel organs under elevated pCO_2 conditions, accompanied by an increased isotopic fractionation of Ce ($^{140/142}$Ce), suggesting a heightened potential for metal accumulation under OA. The results suggested that OA influenced metal accumulation in the gills of mussels. Conversely, metal accumulation in the digestive gland was unaffected by OA. The exposure to both trace metals and OA affects the biochemical responses of *M. galloprovincialis*, leading to increased metabolic capacity, changes in energy reserves, and alterations in oxidative stress markers, but the specific effects on other biomarkers (e.g., lipid peroxidation, some enzymatic responses or acetylcholinesterase activity) were not uniform, suggesting complex interactions between the stressors and the biochemical pathways in the mussels.

1. Introduction

In marine ecosystems, global change is expected to be associated with a decrease in global mean sea surface pH from preindustrial conditions up to 0.29 units by the year 2081–2100 (SSP5–8.5, IPCC, 2022). This predicted ocean acidification (OA) has been identified as one of the main stressors in marine environments (Prada et al., 2017), impacting different biochemical and physiological processes in living organisms, such as growth or biological interactions, among others (Clements and Hunt, 2015). Therefore, advancing the understanding of the structure and functioning of the ocean and coastal ecosystems under OA conditions will be essential to forecast the responses of the marine fauna to different processes that are affected by project scenarios. Under elevated CO_2 levels, the determination of physiological and biochemical indices in living organisms appear to be correlated with the capacity for acid-base tolerance, including survival, growth, development, and metabolism of marine organisms (Feely et al., 2004; De Marchi et al., 2019).

Within oceanic environments, the predicted environmental changes, such as the increase in CO_2 (and the subsequent decrease in pH), may play a key role not only in the performance of organisms but also in the potential toxicity of contaminants. Several trace metals act as oligo-elements (e.g. Cu, Co, Zn) and are, therefore, essential for organisms; however, they can display adverse effects at elevated concentrations (Perošević et al., 2018). The decrease in pH may alter the interaction of metals with marine organisms due to changes in their speciation and therefore their bioavailability, rate of uptake, and toxicity (Millero et al., 2009). Although many studies have described the impacts caused by metals on marine species, scarce information is available regarding the relationship between seawater CO_2 increase and metal bioavailability (Passarelli et al., 2018). Nevertheless, in the last years, studies concerning how climate change-related factors may affect metal

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bioavailability and thus toxicity to organisms are gaining more interest (Stahl et al., 2013; Romero Freire et al., 2020; Kibria et al., 2021). There is evidence that high CO_2 levels, and the corresponding low pH, may impact the solubility and speciation of several metals in seawater (Nardi et al., 2017), mainly by forming strong metal complexes with carbonate ions, whilst higher uptake due to acidification has been described in several marine invertebrates, including bivalves (Nardi et al., 2018).

Amongst the different bivalve species, the Mediterranean mussel *Mytilus galloprovincialis* (Lamarck, 1819), is widely used as a sentinel and biological indicator (Andrade et al., 2019) for monitoring the marine environment (Neira del Río et al., 2015). Mussels present a wide distribution and abundance, and their sessile nature gives them the capacity to adapt to environmental fluctuations (Belivermiş et al., 2016; Azizi et al., 2018). Therefore, they pose a high tolerance to a wide range of environmental conditions and the capacity to accumulate and reflect the concentrations of contaminants in the water column (Banni et al., 2014; Faggio et al., 2016; Oliveira et al., 2017; Andrade et al., 2019). However, the OA and the potential corresponding metal bioavailability will alter the mussel environment and may induce modifications in their behavioral and biological responses, such as changes in growth, condition index, or byssus secretion (Babarro and Carrington, 2013), modifications in valve opening and closures (Clements et al., 2018) and their feeding rates, or the accumulation of metals into their soft tissues, predominately during feeding (Stewart et al., 2021). Mytilid bivalves respond dynamically to elevated pCO_2, displaying an enhanced capacity for acid-base regulation. This physiological adjustment likely involves a strategic allocation of resources toward maintaining somatic tissue equilibrium (Range et al., 2012). While, under intensified CO_2 conditions, growth reduction was reported, their adaptability ensures a higher survival rate (Hendriks et al., 2010; Range et al., 2012), indicating a remarkable ability to confront acidification stressors. This adaptive response, witnessed across various experiments and field observations (Zhao et al., 2020; Ross et al., 2016; Range et al., 2012; Hendriks et al., 2010), hints at a potential mechanism for biological equilibrium in the face of impending ocean acidification challenges. Thus, measurements of the different physiological rates of a bivalve (clearance, ingestion, absorption, respiration, excretion) have been widely determined to reflect spatio-temporal fluctuations in environmental conditions (Albentosa et al., 2012; Irisarri et al., 2014). Another common tool used in marine pollution monitoring is the determination of cellular machinery alterations. The most studied biochemical markers to assess the health status of bivalves are related to oxidative stress, metabolic capacity, and neurotoxicity (Freitas et al., 2017; Coppola et al., 2017; De Marchi et al., 2019; Freitas et al., 2020). When bivalves are exposed to stressful conditions, including the presence of metals and climate change-related factors, the intracellular formation of reactive oxygen species (ROS) may greatly enhance, leading to alterations in the antioxidant mechanisms, cellular damage, and loss of redox homeostasis (Regoli and Giuliani, 2014; Freitas et al., 2020). Bivalves may also alter their metabolic capacity, which can be assessed by the activity of the electron transport system, and energy reserves content, to fight against threats such as pollutants and climate change-related factors (Anestis et al., 2007; Sokolova, 2013; Freitas et al., 2017). Also, injuries produced by exposure to pollutants in aquatic ecosystems are commonly assessed by measuring the inhibition of the acetylcholinesterase enzyme, an indication of neurotoxicity (De Marchi et al., 2018a, 2018b; Lee et al., 2019; Kim et al., 2020; Freitas et al., 2020). Therefore, several studies indicate that elevated CO_2 levels associated with ocean acidification can affect trace element uptake, altering physiological processes in bivalves, and potentially impacting shell formation, metabolic functions, and overall resilience in these organisms. However, a comprehensive understanding of the specific mechanisms and the extent of these interactions in bivalve species warrants further investigation.

Considering the above-mentioned, the present study is focused on a systematic approach, including metal bioaccumulation in different organs of mussels and the biochemical response to metals in a scenario of ocean acidification. Thus, the present study aims to assess the impact of oceanic acidification on the bioavailability of several trace metals (Cd, Cu, Ag, Ce), added isotopically labelled at environmentally realistic concentrations, to understand if these environmental changes will increase organism metal bioaccumulation and adverse effects (at the cellular level) in aquatic environments, using mussels (*M. galloprovincialis*) as bioindicators. The selection of Cd, Cu, Ag, and Ce for studying the impact of ocean acidification on mussel behaviour is driven by their recognized toxicity to marine life, distinct uptake in bivalves, and prevalent environmental presence. Cadmium, Cu, and Ag have been extensively studied as pollutants, while Ce, with a current increase in its use, has been less studied. Therefore, this study contributes to revealing fundamental shifts in the bioavailability of multiple metals, improving the comprehension of their dynamics in marine systems, and offering insights into potential increased metal bioaccumulation and cellular-level adverse effects in the scenario of global change.

2. Materials and methods

2.1. Mussel collection

Specimens of the mussel species *M. galloprovincialis* of similar sizes (64±2 mm) were collected in winter in an area of intense commercial culture from a raft in the subtidal zone of the mouth of the Arousa Ria (Galicia, NW Iberian Peninsula, Atlantic Ocean) (water average pH at raft location: 8.02, Figure SI.1, lat:42.482, lon:−8.963). Once in the Marine Institute Research Laboratory (IIM, CSIC, Vigo) mussels epibionts were removed from the shells and byssal threads were carefully cut from the ventral margin to prevent damaging the foot organ. For acclimation, the mussels were maintained, before starting the experiment, for two weeks in an open-through flow of a natural filtered seawater system (20 μm) at 16±1 °C and with a 12:12 h (light:dark) photoperiod cycle, resembling natural conditions. The system supplied approximately 1.5 mg/L of seston as a mixture of two phytoplankton cultures of *Isochrysis galbana* clone T-Iso and *Rhodomonas* lens (1:1).

2.2. Experimental design

The experimentation was performed in a microcosm system composed of eighteen experimental tanks (9-L polyethylene tanks, 34×23×19 cm; LxWxH), supplied with seawater from three header 200-L polyethylene tanks via peristaltic pumps (ISMATEC) by a continuous flow rate of 9 mL min^{-1}. The flow rate applied enabled the removal of ammonium and other waste products due to mussel metabolism or bacteria (Lassoued et al., 2019). To inject the CO_2 into the experimental tanks atmospheric air and pure CO_2 gas were previously mixed in separate glass flasks before being constantly bubbled through the experimental tanks by probes, which also helped to homogenize the water.

The header tanks were filled with natural 20 μm-filtered seawater supplemented with an optimal diet (1:1, *T-Iso:Rhodomonas*). In two of the three header tanks the metal treatments were spiked directly by a mixture of isotopically-labelled metals at realistic concentrations (Rodríguez-Verlarte et al. 2022): ^{111}Cd (80 ng/L), ^{65}Cu (2000 ng/L), ^{109}Ag (20 ng/L) and ^{142}Ce (20 ng/L) previously prepared in 1 L of seawater, whereas the remaining header tank was used for supplying the control treatments (without metal addition). Seawater in the three header tanks was renewed three times per week by adding fresh nutrients and the specific metals under study.

The atmospheric scenarios were generated directly in the experimental tanks, by bubbling atmospheric air with two different CO_2 concentrations: 400 ppm (roughly 2015 value) and 1200 ppm (three times scenario, slightly above SSP5–8.5 for 2100 according to IPCC, 2022). To achieve these CO_2 flow concentrations, a combination of atmospheric air, a CO_2 trap and pure CO_2 gas were previously mixed in a custom

system controlled with two parallel infrared gas analyzers (Li-COR 850). In addition, control treatments for both CO_2 contents, but without metal addition were also prepared. Therefore, the final treatments were (n = 4): LM (low CO_2 level −400 ppm- with metals added) and its corresponding control (L0, low CO_2 without trace metal addition); and HM (high CO_2 level −1200 ppm- with metals added) and its corresponding control (H0, high CO_2 without trace metal addition). In each experimental 9 L tank, five mussels of similar size were introduced (20 mussels per treatment). Besides, a blank for each CO_2 treatment, tanks without mussels but where physicochemical properties -including CO_2 concentration- were measuredcontinuously during the experiment, were monitored. In controls and treatments, mussels were exposed for twenty-one days under control conditions 16 °C and, 12:12 photoperiod, with a constant flow from the header tanks (9 ml/min).

2.3. Chemical and metal determinations in seawater and mussel tissues

Gas concentrations were logged continuously using LI-COR 850 CO_2 gas analyzers in custom systems that adjust the gas mixture to the required values through software-controlled mass-flow controllers. Several physical-chemical seawater parameters (pH, salinity, total alkalinity) were determined during the length of the experiment. The recorded CO_2 and pH in studied waters during the long of the experiment can be consulted in Figure SI.2. The methodologies used to determine each specific parameter are described in detail by Lassoued et al. (2019) and Romero Freire et al. (2020). Briefly, in experimental tanks, twice a week (n = 24), the pH on Total Scale at 25 °C was determined spectrophotometrically by using m-cresol purple indicator following Clayton and Byrne (1993), the salinity was measured using a salinometer (8410 Portasal, Guildline), and total alkalinity was calculated by potentiometry following Pérez and Fraga (1987) and Pérez et al. (2000), ensuring accuracy with certified reference material for CO_2 in seawater (CRM, batch #163, provided by A. Dickson, Scripps Institution of Oceanography, University of California, San Diego, CA, USA).

Water aliquots for the determination of metals (Cd, Cu, Ag, and Ce) in experimental tanks and controls were collected twice a week (n = 24) by an acid-wash plastic syringe and then syringe-filtered through 0.45 μm filter capsules (polyethersulfone, VWR). Samples were then acidified (pH 1.7, HCl) pending determination. The determination of dissolved metals was attained by the use of a sea*FAST* (Elemental Scientific) preconcentration module coupled to an ICP-MS (7900 ICP-MS, Agilent Technologies Inc.). Before analysis, samples were transferred to 10-mL FEP-capped tubes and UV-irradiated for 16 h to destroy organic matter (see Rodríguez-Velarte et al., 2022 for further details). The reference materials used for water determination were CASS-6 (Coastal Sea Water, NRC Canada) and SLEW-3 (Estuarine Water, NRC Canada). Both were subjected to the same preparation steps. The certified reference waters exhibited a recovery percentage ranging between 106 and 97 % for the four selected elements (n = 8).

At the end of the exposure period, two mussels from each experimental tank were sacrificed (n = 8) and the digestive gland, gill, foot, byssus, and remaining soft tissues were separately dissected. The biological material for metal content determination was carefully rinsed in Milli-Q water and freeze-dried. Dry weights of the different mussel dissections were recorded and then digested using HNO_3 (69 % Hiperpure, Panreac, Spain) in the hot plate (195 °C) for 4 h. Additionally, fish protein CRM certified for trace metals (DORM-2) and mussel tissue for Ce (BCR-668) were selected as certified reference materials. Metal content in body parts was also determined by ICP-MS, and % of recoveries for the certified reference materials were 100±17 % (Cd), 84±5 % (Cu), 79±13 % (Ag) and 94±5 % (Ce) (n = 8). Detection limits expressed as 3 times the standard deviation of the blanks, were 0.2 and 0.01 μg g^{-1} for Cu and Cd, and 1 ng g^{-1} for Ag and Ce.

2.4. Physiological responses

The condition index was monitored, before (n = 16) and at the end of the experimentation, to ensure the proper energetic status of mussels during the experiment. The dry weight of the soft tissues in relation to shell tissues was weighed according to Freeman (1974). During the experimental period, clearance rates in all tanks and treatments were monitored once a week (n = 12) by PAMAS laser particle counter by monitoring particle decline with time and retention efficiency of distinct particle sizes following Cranford et al. (2016). At the end of the experimental period, in two mussels of each tank (n = 8), the condition index and the byssus strength were measured for the same specimens. Byssus strength was determined by connecting one individual from the cluster to a dynamometer (Digital Force Gauge DN431 with peak hold indication, resolution of 0.01 N) according to Babarro and Comeau (2014).

2.5. Biochemical responses

At the end of the experimental period (21 d), one mussel from each tank was sacrificed for biomarker determination (n = 4). Shells were removed and the soft tissues of the whole organism were stored frozen at −80 ºC after deep-freezing in liquid nitrogen. Before biomarker analyses, frozen soft tissues were pulverized using a mortar and pestle with liquid nitrogen. The homogenized tissue of each organism was then distributed in 0.5 g fresh weight (FW) aliquots for further extractions with specific buffers. The biochemical parameters determined were:

i) Metabolic capacity and energy reserves content: electron transport system activity (ETS), was measured at 490 nm over 10 min intervals, and the extinction coefficient (Ɛ) of 15,900 M^{-1} cm^{-1} was used to calculate the amount of formazan formed; glycogen content (GLY), was quantified following the sulfuric acid method, evaluating absorbance at 492 nm after incubation at room temperature for 30 min; protein content (PROT), was measured using the Biuret method, assessing absorbance at 540 nm and using bovine serum albumin standards (0–40 mg mL^{-1}).
ii) Indicators of oxidative stress (enzymatic markers): superoxide dismutase (SOD) activity was determined using SOD standards and measuring the absorbance at 560 nm after 20 min incubation at room temperature; catalase (CAT) activity was quantified using formaldehyde standards, measuring absorbance at 540 nm; glutathione peroxidase (GPx) activity was assessed through absorbance measurements at 340 nm for 5 min using (Ɛ) of 6.22 mM^{-1} cm^{-1}; glutathione S-transferase (GSTs) activity was quantified by measuring absorbance at 340 nm during 5 min and using (Ɛ) of 9.6 mM^{-1} cm^{-1};
iii) Indicators of oxidative damage and redox balance (no enzymatic markers): lipid peroxidation levels (LPO) were determined by quantifying malondialdehyde (MDA) through absorbance at 535 nm using Ɛ of 156 mM^{-1} cm^{-1}; protein carbonylation (PC) levels were assessed following the 2,4-dinitrophenylhydrazide alkaline method, measuring the absorbance at 450 nm and using the Ɛ of 22.308 mM^{-1} cm^{-1}; reduce to oxidized glutathione content ratio (GSH/GSSG) was determined using GSH and GSSG standards measured at an absorbance of 412 nm;
iv) Neurotoxicity: acetylcholinesterase (AChE) activity was measured at 412 nm using acetylthiocholine iodide (ATChI) substrates.

Two analytical replicates *per* sample were used for the assessment of each biochemical parameter, and the mean values were used. The methodologies used to perform each specific biomarker were described in detail by De Marchi et al. (2018a,b), Andrade et al. (2019), and Henriques et al. (2019).

2.6. Data analysis

Descriptive statistics of the metal content in water and mussels' body parts and the mussel responses were calculated to check their normality (individual histogram, mean, median, minimum, maximum, and quartiles), and Levene's test to check the homogeneity of variances. All data were log-transformed when necessary, and if heterogeneity persisted, the rank transformation was used (Conover, 2012). To examine significant differences, a two-way ANOVA was conducted with fixed factors being metal and pCO_2, evaluating their impact on mussel biological responses (detailed in Table SI.1). Subsequently, a one-way ANOVA followed by multiple comparison analyses using the Tukey Honestly Significant Difference (HSD) test (at $p < 0.05$) and pairwise comparisons employing the *t*-Student test (at $p < 0.05$) were performed. To study the influence of metal bioaccumulation on the biological and biochemical mussel responses principal component analysis (PCA) after varimax rotation was applied to discriminate different groups of variables according to statistical similarities of the normalized dataset. All the analyses were performed with a confidence level of 95 % by using the SPSS v.20.0 software package (SPSS Inc. Chicago, USA) and STATISTICA 7.0 software (TIBCO Software Inc., Palo Alto, CA, United States). All data are reported as mean±SD.

3. Results

3.1. Water chemistry

Table 1 presents the average values of the primary chemical parameters observed in the experimental tanks throughout the conducted tests. During the length of the experiment (21 d) the measured seawater CO_2 concentration, which was continuously logged remotely, closely matched the selected nominal values. For the treatments where nominal values of 400 ppm of CO_2 were applied, pH was maintained at 8.0 (Table 1). Conversely, in scenarios simulating ocean acidification with nominal CO_2 values of 1200 ppm, the pH exhibited a decrease of 0.4 units, reaching an average value of 7.6. Both salinity and alkalinity were consistently maintained across all applied treatments, with an average salinity level of 34.7 and an average alkalinity of 2272 μmol kg^{-1}.

The initial average concentrations of the studied elements in seawater hovered at approximately 8.5 ng L^{-1} for Cd, 0.6 ng L^{-1} for Cu, 2.7 ng L^{-1} for Ag, and 6 ng L^{-1} for Ce. Following the introduction of these elements into the experimental setup, the average metal content throughout the experiment altered notably, increasing around 80 ng Cd L^{-1}, 2000 ng Cu L^{-1}, 20 ng Ag L^{-1}, and 20 ng Ce L^{-1}, respectively, after the spiking process (Table 1).

Table 1
Average physicochemical parameters and dissolved metal concentrations in the experimental tanks during the test ($n = 24$).

		TREATMENTS			
		L0	LM	H0	HM
pCO_2	*ppm*	441±47	416±24	1327±1	1335±20
pH		8.01±0.04	8.03±0.03	7.57±0.01	7.57±0.02
Salinity		34.7 ± 0.01	34.7 ± 0.03	34.7 ±<0.01	34.7 ± 0.02
Alkalinity	*μmol kg^{-1}*	2277±6.4	2273±8.6	2271±4	2266±9.3
Cd	*ng L^{-1}*	9 ± 1	89±1	8 ± 1	88±1
$^{110/111}$Cd		0.97±0.03	0.019 ±0.002	0.96±0.03	0.018 ±0.001
Cu	*μg L^{-1}*	0.57±0.08	2.57±0.08	0.61±0.06	2.61±0.06
$^{63/65}$Cu		2.25±0.02	0.19±0.02	2.22±0.03	0.21±0.02
Ag	*ng L^{-1}*	2.40±0.3	22.4 ± 0.3	3.0 ± 0.4	23.0 ± 0.4
$^{107/109}$Ag		1.08±0.02	0.062 ±0.008	1.07±0.02	0.070 ±0.007
Ce	*ng L^{-1}*	6 ± 1	26±1	6 ± 1	26±1
$^{140/142}$Ce		8.01±0.04	0.34±0.02	7.96±0.03	0.34±0.03

3.2. Metal bioaccumulation in mussels

3.2.1. Basal concentrations and organotropism

The basal metal concentrations between the control groups L0 and H0 did not exhibit statistically significant differences. Consequently, the average concentrations for both treatments were calculated (refer to Fig. 1). In the control soft tissue, the metal concentrations ranged from 2.3 ± 0.7 μg g^{-1} for Cd, 5.2 ± 0.4 μg g^{-1} for Cu, 10 ± 1 ng g^{-1} for Ag, and 39 ±2 ng g^{-1} for Ce (mean ± SD). Across the study, the gills demonstrated higher concentrations of Ag in controls compared to the soft tissue. However, no significant differences were observed for Cd and Ce. Notably, for Cu, even lower concentrations were observed in the control gills. Regarding the digestive gland, Cu, Ag, and Ce showed notably higher concentrations compared to the soft tissue, except Cd. The metal concentrations in the foot were similarly low compared to the soft tissue for Ag and as low as in the gills for Cu. Cadmium also showed low content in the foot, but the lowest concentration appeared in the byssus. Conversely, the foot exhibited the lowest content in the case of Ce. In comparison, significantly higher concentrations were observed in the byssus than in the soft tissue for Cu, Ag, and Ce, while Cd concentrations showed the lowest concentration in the byssus. The average byssus to soft tissue ratios (BST) obtained was 0.3 for Cd, 3.8 for Cu, 2.9 for Ag, and 7.0 for Ce.

3.2.2. Impact of ocean acidification on metal bioaccumulation

Comparisons between treatments on metal concentration in each organ revealed consistent findings for cadmium (Cd). We observed elevated Cd concentrations under higher CO_2 conditions (HM), suggesting increased internalization, as indicated by the $^{110/111}$Cd ratios. However, these ratios did not exhibit differences compared to those obtained under current pH conditions (LM) (Fig. 1). In contrast, we did not observe distinct concentration trends for copper (Cu) between the LM and HM treatments. Similarly, the degree of metal internalization did not exhibit a clear pattern when comparing both treatments. However, an interesting finding emerged from the 63/65Cu ratio analysis, indicating an increased uptake and internalization of the spiked Cu specifically within the soft tissue compared to other body parts. In the case of silver (Ag), our findings showed lower bioaccumulation across the digestive gland, soft tissue, gills, and foot under elevated pCO_2 compared to current conditions. Notably, significant differences were observed in the latter three organs (Fig. 1). However, the internalization of silver, indicated by the $^{107/109}$Ag ratios, did not differ between treatments (LM, HM) and among mussel body parts. Regarding cerium (Ce), concentrations in various organs under spiked treatments at elevated pCO_2 (HM) were higher than those under current conditions (LM). However, statistically significant differences were only observed in the soft tissue (Fig. 1). This general trend suggested a greater change in the isotopic fractionation of Ce ($^{140/142}$Ce), indicating increased uptake and internalization of the spiked metal under low pH conditions (LM).

3.3. Biological and biochemical responses based on metal bioaccumulation and OA

3.3.1. Biological responses

The condition index (CI) was measured before starting the experimentation for a representative number of mussels. In the present study, the CI did not show any differences between treatments at the beginning of the experiment (CI = 16.1 ± 1.7 %) and for the studied treatments (CI = 16.7 ± 0.7 % -LM- and CI = 16.4 ± 1.0 % -HM-) (data not shown).

Mean values for the clearance rate (L h^{-1}) and byssus strength – expressed as the mussel attachment force (in N) – for the different studied treatments are shown in Fig. 3. Results showed significant differences for mussels exposed to treatments with the highest CO_2 level, with a decrease greater than 20 % in clearance rates at 1200 ppm CO_2 compared to 400 ppm CO_2. In the case of byssus strength of attachment for mussels in this study did not show significant differences between

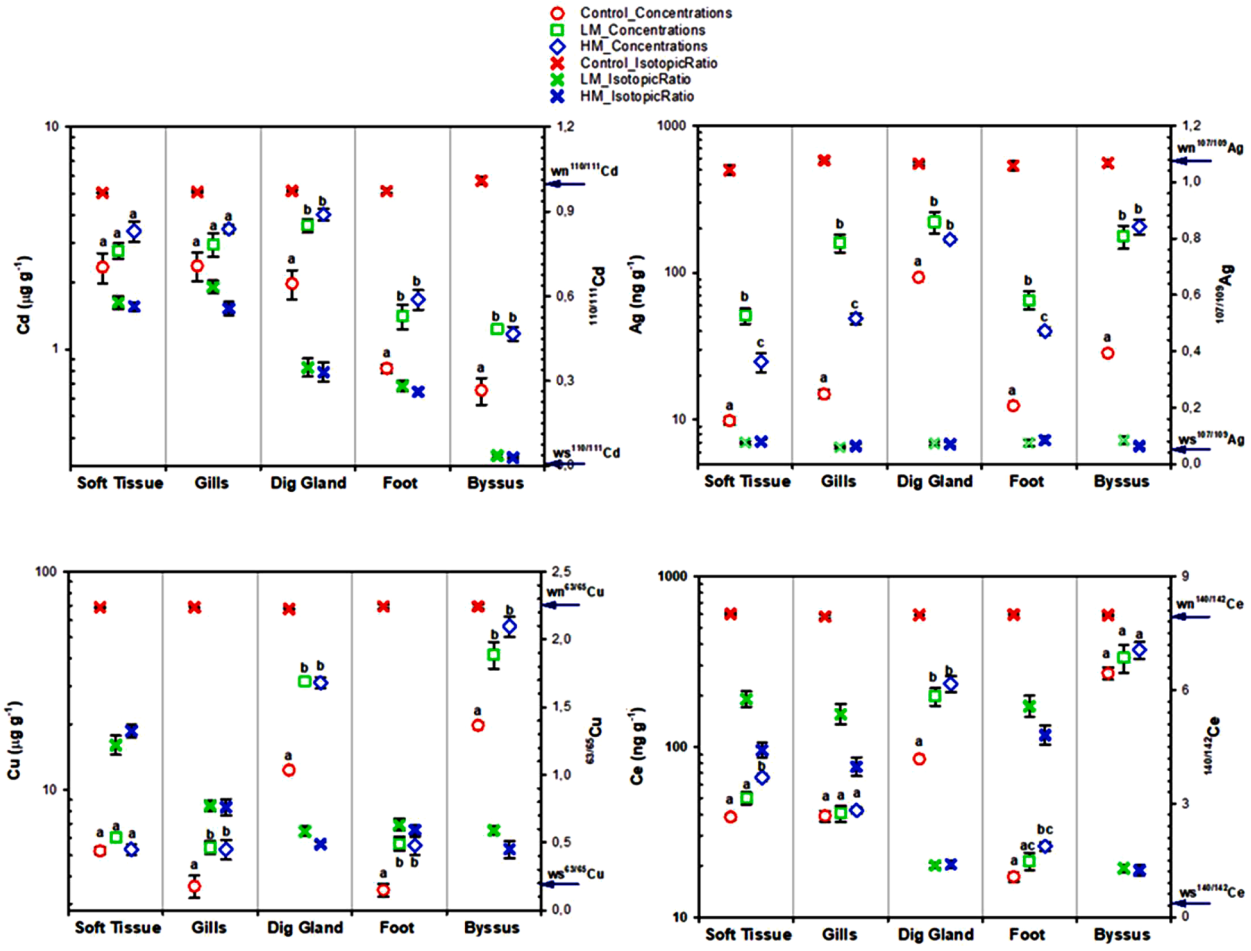

Fig. 1. Cadmium, Cu, Ag, and Ce concentrations and isotopic ratios in the controls ($n = 16$) and in both metal-spiked treatments at current (LM; 400 ppm) and elevated (HM, 1200 ppm) CO_2 levels ($n = 8$). Arrows in the right-hand axis indicate natural isotopic ratios of metals in the water of controls (wn) and the spiked treatments (ws). Letters indicate significant differences between internal metal concentrations from the ANOVA test (Tukey $p < 0.05$).

treatments.

Fig. 2.

3.3.2. *Biochemical parameters*

The biochemical responses in terms of metabolic capacity and energy reserves, oxidative stress (from enzymatic and non-enzymatic markers) and neuro status of the mussel *M. galloprovincialis* exposed to different stress conditions (acidifications and metal spike) are compiled in Table 2. The Electron Transport System (ETS), a marker of metabolic capacity, displayed heightened activity in organisms facing stress from

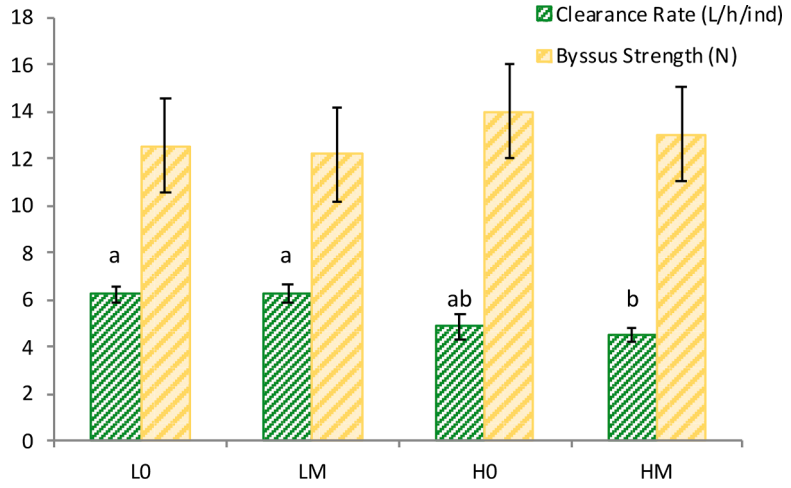

Fig. 2. Average mussel clearance rate (L h^{-1} $individual^{-1}$) ($n = 12$) and mussel byssus strength (N) ($n = 8$) with standard deviation (SD) for the 4 studied treatments. See Table 1 for the CO_2 and metal concentrations for each of the treatments. Letters indicate significant differences between treatments from the ANOVA test (Tukey $p < 0.05$).

Table 2
Effect on metabolic capacity markers (ETS, GLY, PROT), enzymatic markers (SOD, CAT, GPX, GSTs, PC), no-enzymatic markers (LPO, GSH/GSSG), and neurotoxicity (AChE) in *Mytillus galloprovincialis* exposed to different conditions during 21 days ($n = 4$). See Table 1 for the CO_2 and metal concentrations for each of the treatments. Letters represent significant differences from ANOVA test (Tukey $p < 0.05$) among treatments for each studied biomarker.

	Biomarker	*Unit*	TREATMENTS			
			L0-control	LM	H0	HM
			400 ppm CO_2	*400 ppm CO_2+ TM*	*1200 ppm CO_2*	*1200 ppm CO_2+ TM*
Metabolic capacity and energy reserves	ETS	*nmol*/min/*g FW*	113±18a	284±55b	127±29ab	238±74ab
	GLY	*mg/ g FW*	1.9 ± 0.8ab	4.4 ± 1.1c	1.7 ± 0.4a	3.7 ± 0.3bc
	PROT	*mg/g FW*	140±16a	112±15ab	71±18c	85±6bc
Enzymatic Markers	SOD	*U/ g FW*	0.08±0.02ab	0.06±0.01a	0.09±0.01b	0.07±0.01ab
	CAT	*U/ g FW*	7 ± 2a	20±3b	12±2ab	17±5ab
	GPx	*U/ g FW*	0.13±0.01b	0.09±0.01a	0.09±0.02a	0.08±0.02a
	GSTs	*U/ g FW*	0.03±0.02a	0.12±0.04b	0.11±0.05ab	0.09±0.07ab
	PC	*lumol*/min/*g FW*	1.2 ± 0.1a	1.7 ± 0.1b	2.1 ± 0.1c	1.8 ± 0.1bc
No enzymatic markers	LPO	*nmol MDA/ g FW*	47±5	55±9	47±10	50±9
	GSH/GSSG	-	4.2 ± 0.3a	3.1 ± 0.3ab	4.2 ± 0.2a	3.0 ± 0.6b
Neurotoxicity	AChE	*nmol*/min/*g FW*	0.5 ± 0.1a	0.6 ± 0.1a	0.9 ± 0.2b	0.6 ± 0.1ab

both trace elements and water acidification. This activity peaked when both stressors were combined, indicating an amplified metabolic response under simultaneous stress. Mussels exposed to treatments with trace elements exhibited a notable increase in glycogen reserves (GLY) compared to control organisms. On the contrary, a contrasting trend was observed in protein content (PROT), which significantly decreased across all stress scenarios, especially in treatments with elevated CO_2 levels.

Enzymatic responses revealed noteworthy alterations. Superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities declined significantly in mussels exposed to trace metals, while catalase (CAT) activity exhibited an increase. Glutathione s-transferases (GSTs), displayed increased activity when mussels were exposed to trace metals and/or elevated CO_2 levels.

The levels of lipid peroxidation (LPO) were not significantly different, despite a tendency to increase when metals were added, regardless of the acidification process. The protein carbonylation (PC) showed significantly higher values in organisms exposed to stress conditions both with trace element addition and water acidification, with the highest values under acidification conditions. The ratio that represents the transformation of reduced glutathione (GSSG) to oxidized glutathione (GSH) (GSH/GSSG), showed a decrease with the increase of trace elements in the experimental tanks, regardless of the CO_2 content in seawater.

The acetylcholinesterase activity (AChE) exhibited significant activation in treatments with high CO_2 levels, while no discernible differences were detected due to the presence of added trace elements.

3.3.3. *Multivariate analysis based on metal bioaccumulation and OA and mussel responses*

Principal Component analysis (PCA) was applied to help explain the effects of metal bioaccumulation and OA on biological and biomarkers responses (Fig. 3). The PCA included pCO_2, metal bioaccumulation in the soft tissue, selected for its higher mass in the total mussel weight, and the biological and biochemical (biomarkers) responses. Results showed that 55.7 % of the variance was explained by two components. In SI (figure SI.2) PCAs for all individual metals (Cd, Cu, Ag and Ce) in the dissected studied mussels' parts (digestive gland, gill, foot, byssus, and remaining soft tissues) with all biological and biochemical parameters can be consulted, with trends similar to the observed in Fig. 3.

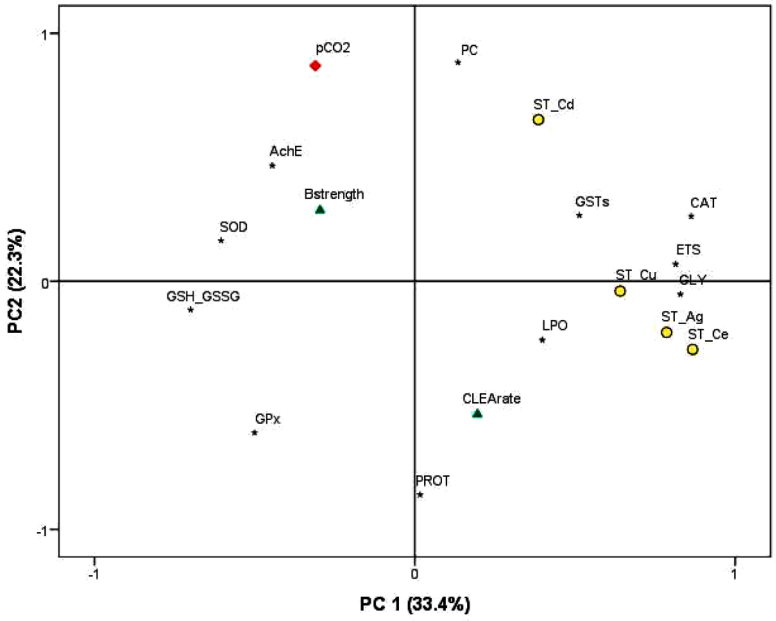

Fig. 3. Principal component analysis (PCA) including pCO_2 in waters -♦-, the studied biological parameters (clearance rate and byssus strength) -▲-, the different biomarkers studied (ETS, GLY, PRO, SOD, CAT, GPx, GSTs, LPO, PC, GSH/GSSG and AchE) -*-, and the metal bioaccumulation in the soft tissue (ST) of the 4 studied elements (Cd, Cu, Ag and Ce) -●-. Accumulate variance explained for component 1 = 33.4% and for component 2 = 22.3%.

Component 1 grouped on the positive side the metals accumulation (mainly Cu, Ag and Ce), together with the metabolic capacity (ETS), the energy reserve (GLY) and the enzymatic marker (CAT). While on the negative side appear the antioxidant enzyme (SOD) and the GSH/GSSG ratio. The physiological response clearance rate (CLEArate) is grouped in component 2 with an indirect relation with pCO_2 in the study water treatments. According to the biomarkers, in the same PC 2, protein content (PRO) is present on the positive side, whilst protein carbonylation (PC) is found on the negative side.

Overall, results observed from the PCA discriminate that ocean acidification (OA) is related to the effects on protein content (PC, PRO) in mussels. Whereas metal accumulation seems not influenced by OA, but was the main external factor explaining ETS, GLY, CAT, SOD and GSH/GSSG biomarkers.

4. Discussion

4.1. Experimental water chemistry analysis and basal metal distribution in mussels

The obtained decrease in water pH values is in line with the predicted ocean acidification (IPCC, 2022). Whereas, salinity and alkalinity values resembled real conditions (Padin et al., 2020). The sea water concentration for the studied elements was similar to those reported by other authors in the same region (Santos-Echeandía et al., 2009). During the length of the experiment the average metal content for these elements, once spiked, was within typical concentrations for nearshore water bodies plus the seawater basal content (Rodríguez-Velarte et al., 2022).

Metal content obtained in the mussel soft tissue is generally within those observed in the Galician rias (e.g. Rodríguez-Velarte et al., 2022 and references therein). The concentrations of metals in mussels generally vary within different organs, with higher concentrations usually observed in the digestive gland and gills compared to the rest of the soft tissue (e.g. Chandurvelan et al., 2015; Rodríguez-Velarte et al., 2022). Here, however, the concentrations in gills were only higher than in the soft tissue for Ag. Significantly higher concentrations in the byssus than in the soft tissue were obtained only for Cu, Ag, and Ce. Accordingly, the average byssus to soft tissue ratios (BST) obtained are similar to those previously reported in the literature (*Mytilus edulis* from the Baltic Sea; Nicholson and Szefer 2003). Byssal threads are well-known for their capacity to bind metals, which has been explained by the presence of metal-binding ligands in histidine proteins (Zhang et al., 2019). In particular, these histidine-rich peptides from byssal threads can produce mechanically stable and reversibly breakable intermolecular protein-metal linkages, which are behind their stiffness, high hysteresis, and self-healing properties (Schmidt et al., 2014). However, it has been argued that metal concentrations in byssal threads do not respond to these physiological requirements alone; accordingly, it has been suggested that metal concentrations in byssus also respond to ambient water concentrations through direct adsorption and/or acting as an elimination – detoxification – pathway from the soft tissues of the mussel (Szefer et al., 2004).

4.2. Impact of ocean acidification on metal bioaccumulation in mussels

In the present study, metals in mussel body parts showed different trends. Summarizing, under higher pCO_2 levels, concentrations of Cd rose, Cu remained similar, Ag decreased in different organs, and Ce increased, especially in soft tissue.

The inorganic speciation of dissolved Cd is dominated by the formation of chloride complexes, and therefore no significant effect is expected due to ocean acidification (Millero et al., 2009). However, organic chelates are the dominant forms of Cd in coastal systems (e.g. Kozelka and Bruland 1998), but these appear to be mainly in labile – and therefore potentially bioavailable – forms (Cindric et al., 2020). Therefore, a decrease in pH is not expected to have a major influence on the speciation and bioavailability of Cd. Contrasting findings regarding the impact of pH on Cd bioaccumulation in marine bivalves have emerged from various studies. Nardi et al. (2018) noted no notable rise in Cd concentrations at reduced pH (7.4) in *M. galloprovincialis* exposed to 20 μg L^{-1}, attributing increased Cd uptake instead to other facets of global change, such as elevated temperature. Sezer et al. (2020), utilizing a radiotracer method, observed a pH-associated increase in Cd uptake among juvenile *M. galloprovincialis* but not in adults. Conversely, in other bivalves, such as the oyster *Crassostrea gigas*, Cao et al. (2018) documented augmented Cd accumulation at lower pH levels (7.8 and 7.6) when specimens were subjected to 10 μg L^{-1} compared to control pH. Moreover, Shi et al. (2016) reported heightened Cd bioaccumulation in multiple bivalve species (*M. edulis, Tegillarca granosa, Meretrix meretrix*) under elevated pCO_2 conditions (pH 7.8 and 7.4). The authors posited several potential physiological explanations for this increased uptake under acidified conditions, including a speculated higher Cd^{2+}/Ca^{2+} ratio due to decreased Ca^{2+} levels at lower pH. This alteration might facilitate Cd^{2+} entry via Ca^{2+} channels. Additionally, they also suggested a potential downregulation of genes crucial for intracellular Cd exclusion or constraints on energy availability for Cd exclusion under stress conditions.

The inorganic speciation of dissolved Cu is dominated by the formation of carbonate complexes and, therefore, a significant effect is expected due to acidification (Millero et al., 2009), implying a reduction of the carbonate fraction from ~85 % at current pH down to ~72 % at pH 7.6, and a concomitant increase in the free Cu^{2+} from ~8 to ~22 % (Millero et al., 2009). As for Cd, however, organic chelates are the dominant forms of Cu in seawater (e.g. Cobelo-García and Prego 2004), of which a small fraction is present as a labile form (Cindric et al., 2020). For such elements strongly bound to organic matter, a pH decrease is expected to increase the bioavailable free Cu^{2+} (Millero et al., 2009) but the extent of the increase can be considered modest (Stockdale et al., 2016). In the present experiment, no clear trends in the concentrations were observed between the LM and HM treatments. Previous studies on the effect of elevated CO_2 concentrations on marine bivalves have led to contradictory results. For example, in the case of oysters (*Crassostrea virginica* and *C. gigas*) Götze et al. (2014) and Cao et al. (2019) observed an increase in the Cu accumulation in soft tissues under ocean acidification conditions, but the reverse was observed by Ivanina et al. (2015). In this context, it has been shown a decrease in the Cu uptake rate with a decrease in pH in toxicokinetic experiments with zebra mussels, implying an interaction between H^+ and Cu at uptake sites (Le et al., 2021).

In the case of Ag, for which its inorganic speciation is dominated by the formation of chlorides and with no evidence of organic complexation (Miller and Bruland 1995), no effect on their species is expected due to a decrease in pH (Millero et al., 2009). That is, the major form of Ag in seawater is the dichloride complex $AgCl_2^-$ irrespective of the predicted ocean acidification, and this would suggest a low to null effect, from a chemical standpoint, on its bioaccumulation as the pH decreases (Lacoue-Labarthe et al., 2009). However, our results indicate lower bioaccumulation in the digestive gland, gills, and soft tissue – the two latter significantly different – at elevated pCO_2 compared to current conditions. Rather than a change in the chemical speciation of Ag, these results suggest that the different Ag uptake concerning pH may result from physiological alterations. In contrast with our results, Sezer et al. (2020) did not observe any effect of elevated pCO_2 on the uptake, depuration, and tissue distribution in juvenile and adult specimens of *M. galloprovincialis*. However, the effect of pCO_2/pH on the accumulation of Ag in marine organisms has been reported for the eggshell of the cuttlefish *Sepia officinalis*, which shows a lower Ag retention at elevated pCO_2 (pH 7.85 and 7.60) compared to ambient values (Lacoue-Labarthe et al., 2009), suggesting that pH affects the permeability properties of the eggshell.

The decrease in the pH of seawater will result in the reduction in the

concentrations of hydroxide and carbonate ions and therefore a change in the speciation of metals strongly bound to these inorganic complexes is expected. Cerium – together with the rest of rare earth elements – are present in the dissolved phase in seawater mainly in the form of carbonate complexes ($CeCO_3^+$ and $Ce(CO_3)_2^-$), with the free uncomplexed metal (Ce^{3+}) – which has been proved to be the bioavailable fraction (Strady et al., 2015) – representing only a minor fraction (roughly 13 % at pH 8.1; Millero et al., 2009). This free, bioavailable fraction is however expected to increase as the seawater pH decreases, reaching values of ~31 % for Ce^{3+} at pH 7.6 (Millero et al., 2009). Accordingly, in our experiments, the Ce concentrations in the different organs in the spiked treatments at elevated pCO_2 (HM) were higher than current conditions (LM) with a higher degree of change in the isotopic fractionation of Ce ($^{140/142}Ce$) at low pH conditions, indicating a higher uptake and internalization of the spiked metal.

These findings emphasize the intricate links between pH shifts and metal accumulation in marine bivalves, showcasing varied responses across metals. While Cu yielded inconclusive results, Ag exhibited reduced bioaccumulation under higher CO_2, contrasting the potential rise in Cd and Ce bioaccumulation in mussels due to increased CO_2 levels.

4.3. Physiological responses

The condition index (CI), measured before starting the experimentation for a representative number of mussels, is useful as an indicator of the general physiological status of the organisms (Andral et al., 2004) and it is strongly linked to the availability of food resources and the reproductive cycle (Mourgaud et al., 2002). The absence of notable differences among treatments at the experiment's onset indicates the favourable condition of the mussels and suggests sustained adequacy in food resources throughout the study period.

Studies concerning the impact of ocean acidification have demonstrated a high variability for mussel biological responses. Accordingly, Fernández-Reiriz et al. (2012) did not find differences in the acidification in clearance rates for *M. galloprovincialis*, whereas Romero Freire et al. (2020), under optimal feeding conditions, observed an increase in clearance rate when pCO_2 increased at the same CO_2 level (1200 pCO_2) than in the present experiment, suggesting that the same mussel species may remain resistant to acidification by increasing their feeding rates (Lassoued et al., 2019), provided they have an optimal diet to respond accordingly. However, our results showed the inverse trend, with feeding activity decreasing with the acidification. This opposite trend observed in studies performed with similar conditions (Lassoued et al., 2019; Romero Freire et al., 2020), could imply the (sub-lethal) effect of trace metal availability for mussels by the acidification, although they were added in concentrations near realistic values. But lower clearance rates were also reported at high pCO_2 concentration (1000 pCO_2) for mussels (*M. chilensis*) and other species of bivalves in other studies (Navarro et al., 2016).

Although several studies have revealed that the byssus filaments may be negatively affected by ocean acidification (Babarro et al., 2018; Lassoued et al., 2019), in the present study it was demonstrated that byssus strength of attachment did not show significant differences between treatments. Some authors noted that the effect of byssal attachment could be directly related to other factors such as food regime (Lassoued et al., 2019) or condition index (Clements et al., 2018). Optimally-fed mussels, as was the case for this experiment, may be capable of maintaining attachment strength under acidified conditions (see also Lassoued et al., 2019).

4.4. Biochemical responses

The observed rise in mussels' metabolic capacity (ETS activity) upon exposure to higher metal concentrations, as demonstrated in this experiment, aligns with previous findings (De Marchi et al., 2019). Such an increase typically corresponds with the depletion of energy reserves. It's plausible that this heightened metabolic activity might contribute to the observed increase in certain metals within mussel tissues. Voets et al. (2006) observed that zebra mussels exposed to metals presented lower GLY content and no changes in PROT content compared to specimens collected in a no-polluted area. However, this was not the case for our study since, despite PROT content decrease, the GLY content increased parallel to the ETS. In contrast to lipids and GLY, proteins are typically utilized as a final source of energy in situations of heightened energy demands or during periods of starvation, when alternative energy reserves have been exhausted (Shang et al., 2023). Freitas et al. (2020) observed that mussels did not expend GLY and PROT content when exposed to low-stress factors, while the stress increased, the PROT content decreased, which can be in parallel with our results that used realistic metal concentrations and, expected, higher metal internalization in mussels under OA conditions.

In this experiment, mussels exposed to metals increased their metabolism most probably associated with the activation of antioxidant defences, in particular CAT enzyme. However, SOD and GPx enzyme activities decreased in organisms exposed to stressful scenarios which might indicate a high-stress level and, consequently, inhibition of these enzymes. A similar response was already described by e.g. Martins et al. (2017), who observed that the mussel *Bathymodiolus azoricus,* under high concentrations of Cu, decreased in enzyme activity. Mussels also showed a significant increasing trend in GSTs activity when trace metals were added and/or water CO_2 content was increased. Other studies have shown that adverse effects produced by exogenous compounds, including metals (Moreira et al., 2006), can be modulated by GSTs induction (Hampel et al., 2016) even under low pH conditions. Similar to our results, Cao et al. (2018) observed that both seawater acidification and metal (in their case Cd) induced GSTs activities in the Pacific oyster. This heightened GSTs activity could indicate a detoxification mechanism to protect the organism from harmful effects (Srikanth et al., 2013) and might also compensate for the reduced activities of other antioxidant enzymes (Cui et al., 2020), such as the observed for GPx in our results, which could hypothetically account for the lower concentrations of certain tested metals.

The levels of LPO showed a tendency to increase when metals were added, regardless of the acidification process. Other researchers have shown that exposing mussels to minimal metal levels could induce lipid peroxidation, but no clear link was found between long-term metal (Cu) effects (Jorge et al., 2013). Recent studies indicated that the response of the same species to various metals (Hg, Co, and Ni) showed an antagonistic trend (Morais et al., 2023). Additionally, for *M. galloprovincialis* gonads, exposure to a mixture of contaminants for more than 3 days demonstrated an antagonistic effect (Gonçalves and Bebianno, 2023). Therefore, it is possible that in our experiment, due to the combination of several metals and the duration of exposure, the absence of LPO response might have resulted from a blend of stressful conditions. In addition, the protein carbonylation (PC) showed significantly higher values in organisms exposed to stress conditions both with trace element addition and water acidification. The absence of LPO and the increase of PC were previously reported on the same species exposed to other stress conditions (heating conditions and/or presence of contaminants). These researchers suggested that the fact that no LPO occurred, while the proteins were carbonylated, could indicate faster oxidation of proteins than lipids (Pirone et al., 2019; Moleiro et al., 2022; Paciello et al., 2023). On the other hand, the ratio of GSH/GSSG decreased with the increase of trace elements. This ratio is considered an indicator of the redox state of cells (Mocan et al., 2010), therefore such a decrease would indicate the presence of oxidative damage and, therefore, that organisms are under stressful conditions due to the presence of trace metals. Although the evidence of damage to the lipids of the membrane could not be demonstrated, oxidative stress was identified in mussels exposed to metals through alterations in antioxidant enzyme activities and a decrease in such ratio. Other studies with *M. galloprovincialis* also

demonstrated a decrease in the GSH/GSSG ratio or an increase in GSSG content after exposure to global warming context (Hg and Temperature increase) (Coppola et al., 2018).

Despite the typical expectation that the AChE enzyme is usually inhibited under stress, the present findings showed a significant activation in mussels subjected to high CO_2 levels and the absence of metals which could be explained by an unforeseen compensatory response triggered by the specific combination of high CO_2 levels and the absence of metals, potentially indicating an alternative regulatory pathway remain unclear. In the literature contrasting results are reported. Brown et al. (2004) observed varying responses of AChE activity in three marine invertebrate species exposed to Cu, with notable AChE increase in *P. vulgata*, significant inhibition in *C. maenas*, and no impact in *M. edulis*. Additionally, AChE activity in bivalves has been described to be influenced by several abiotic factors (Pfeifer et al., 2005; Attig et al., 2014). In their research, Boukadida et al. (2022) found that the combined effect of temperature and metals significantly amplified AChE activity in mussel larvae. Similarly, Fulton and Key (2009) reported AChE activity in estuarine fishes exposed to low levels of toxicants. Moreover, Qu et al. (2022) observed that the response of the AChE enzyme in *M. galloprovincialis*, when exposed to OA and Cu, depended on the body part (gill or digestive gland). Their findings indicated that OA alone, Cu alone, and combined OA-Cu decreased AChE activity in gills, while a high concentration of Cu only marginally increased AChE activity in the digestive gland. Based on our results, we can conclude that the use of realistic metal concentrations is unlikely to induce neurotoxic effects in mussels, even with a decrease in seawater pH. However, further studies are warranted, including the determination of AChE activity in different parts of the mussel's body.

5. Conclusions

This study highlights the potential implications of ocean acidification on metal bioaccumulation patterns and cellular responses in mussels. Results showed that ocean acidification may not greatly impact the availability of Cd and Ag, as they are primarily present in chloride complexes. However, it could potentially elevate the availability of metals dominated by hydroxide and carbonate ions in seawater systems (e.g., Cu or Ce), leading to their increased accumulation in marine organisms. We observed that exposure to both trace metals and ocean acidification affects the biochemical responses of *M. galloprovincialis*, leading to an increase in metabolic capacity, changes in energy reserves, and alterations in oxidative stress markers. The results highlight that metals present in an ocean acidification scenario at realistic concentrations can cause biochemical alterations in *M. galloprovincialis*, inducing oxidative stress. The findings contribute to a better understanding of the impacts of changing environmental conditions on marine ecosystems and emphasize the importance of considering metal pollution in conjunction with OA effects when assessing ecosystem health.

CRediT authorship contribution statement

A. Romero-Freire: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **L. De Marchi:** Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **R. Freitas:** Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Supervision. **A. Velo:** Formal analysis, Investigation. **J.M.F. Babarro:** Conceptualization, Methodology, Validation, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **A. Cobelo-García:** Conceptualization, Methodology, Validation, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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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