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Interactions between *Laminaria ochroleuca* and Mytilus sp. under copper exposure and salinity variability in IMTA systems

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ABSTRACT

Copper-based antifouling paints are widely used to reduce biofouling on marine and estuarine structures. Yet, copper (Cu) can be toxic to marine organisms in the surrounding environment and aquaculture systems. Such toxicity may be enhanced by salinity reduction caused by extreme weather precipitation events that are becoming more frequent under climate change-induced alterations. The present study aims to analyze the physiological effects of the salinity (34 and 25 PSU) and copper (environmentally relevant levels, 20 μg/L) interaction in an macroalgae-mussel integrated multitrophic aquaculture (IMTA) aquaculture of *Laminaria ochroleuca* and *Mytilus* sp. Growth, photosynthetic pigments, fluorescence and bioactivity were used as indicators for *L. ochroleuca*, while oxidative stress biomarkers and stress response biomarkers were assessed in both species. The tested Cu concentrations did not affect macroalgae growth nor *Mytilus* sp. condition index. The presence of *Mytilus* sp. enabled a reduction in Cu accumulation in macroalgae under IMTA. Salinity reduction triggered higher Cu accumulation in *Mytilus* sp. rather than in *L. ochroleuca*. Mussels exposed to Cu exhibited similar effects of whether specimens were kept under IMTA or monoculture. Salinity had no effect on macroalgae, while macroalgae exposed to Cu under monoculture conditions suffered a reduction in chl *a*. The presence of *Mytilus* sp. enabled increased *L. ochroleuca* photosynthetic performance with higher Fv/Fm ratios and chl *a* concentrations, but reduced carotene concentrations, total phenolic content and antioxidant activity. Therefore, IMTA proved to be an effective solution against Cu contamination though with a cost in macroalgae bioactivity.

1. Introduction

Biofouling – the fixation of organisms on anthropogenic structures as cables and aquaculture structures – is one of the main issues for marine, coastal and estuarine infrastructures [1]. Copper (Cu) is a ubiquitous contaminant in coastal areas, originating primarily from vessel discharges and its use as a biocide in antifouling paints employed in aquatic infrastructures [2,3], including aquaculture systems [4,5]. Cu-based antifoulings have become the main alternative to the highly toxic, now banned organotin compound tributyltin [3]. Although some less toxic alternatives have been developed in recent years, Cu-based antifoulings are currently the primary tool used to control the biofouling that affects ships and coastal structures [6,7]. However, these antifouling coatings release Cu into the water, potentially contaminating marine life in nearby areas [8,9]. Limits for waterborne Cu in aquaculture areas vary throughout the world, ranging between 1 and 2 mg/L [10]. Limited information is available on dissolved copper in estuarine and marine aquacultures, with most studies focusing on the copper absorbed by organisms (e.g. [11]) or on sediments (e.g. [12]). From the few available data, it is possible to observe that usual values for dissolved copper range from 0 to 5 μg/L [13]. However, in certain areas values reach as high as 16.3 μg/L as in the coastal waters of Bohai Bay area [13] while values around 50 μg/L can be found in estuarine environments [14].

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Integrated Multi-Trophic Aquaculture (IMTA) is a sustainable practice in which the organic matter and nutrients excreted by one farmed species are utilized by other organisms such as mussels, shrimps, or macroalgae [15]. This process reduces organic particulate matter and nutrient loads in the surrounding environment, while increasing profitability through the simultaneous production of multiple commercially valuable organisms [15]. Macroalgae play an important role in marine IMTA systems, as they perform a unique ecological function unmatched by other commercially valuable organisms — the removal of organic and inorganic forms of nitrogen and phosphorus, thereby preventing eutrophication in aquaculture areas [16]. Moreover, the commercial value of macroalgae has steadily increased due to the growing interest in seaweeds across Western markets for applications in food, feed, nutraceuticals, cosmetics and biofuels [17]. Macroalgae also possess the ability to remove various contaminants from the water, and some studies suggest that they can even contribute to the decontamination of sewage waters [18]. Therefore, their inclusion in IMTA systems not only enhances ecological sustainability but also helps protect co-cultured organisms from environmental contaminants such as waterborne Cu.

The ability of macroalgae to accumulate various metal elements is largely due to their richness in chelating agents, such as polysaccharides alginates, fucoidan and ulvans [19,20], tolerating both dissolved and nanoparticle forms [21]. This capacity is particularly high in algae belonging to the phylum Phaeophyta (brown algae), which contain elevated concentrations of both alginates and fucoidan, as well as phenolic compounds such as phlorotannins [22,23]. In addition, photosynthetic pigments like fucoxanthin exhibit strong antioxidant activity, further enhancing the value of macroalgae as a source of bioactive compounds [24]. Consequently, brown algae are among the most extensively studied groups in terms of bioactivity.

Mussels are widely used as extractive organisms in IMTA systems [25,26]. Their main contribution lies in reducing organic loads released by fish, as they feed on fecal material and suspended particles [25,26]. However, due to their benthic filter-feeding nature, mussels are more prone to metal accumulation than fish [27,28]. Therefore, these bivalves are extensively used to assess the risk of contaminant exposure in aquaculture environments. Both mussels and macroalgae are considered extractive organisms and, in IMTA structures, these organisms are commonly cultivated together, reducing the particulate matter and nutrients that the aquaculture releases to the surrounding environment [29,30]. However, information regarding these organisms interaction in IMTA systems in terms of resistance to waterborne contaminants is scarce, except for shellfish toxin accumulation [31].

Besides biological characteristics, environmental (physicochemical) parameters such as temperature, salinity, and pH may also influence metal accumulation in organisms [32,33]. Among these factors, the interaction between salinity and metal accumulation is one of the most studied, with a well-documented negative relationship between salinity and metal uptake in macroalgae and aquatic plants [34–36]. Therefore, in estuarine or shallow coastal areas, macroalgae are expected to exhibit increased susceptibility to metal accumulation during heavy rainfall events, when salinity levels decrease. Extreme events such as storms, which greatly decrease estuarine and coastal salinity [37,38], are expected to become stronger and more frequent under climate change scenarios [39], increasing the importance of understanding both the direct salinity effects on cultured species and the synergistic effects of salinity with other stressors such as waterborne contaminants.

Hence, in order to fulfill the gap of information on the effects of copper on macroalgae-bivalves IMTA systems when exposed to salinity shifts, the objective of the present study is to evaluate how Cu-based antifouling affects the macroalgae-mussel interaction in aquaculture systems, as well as how such interactions are influenced by salinity reduction. This main objective is set to respond to the following scientific questions: i) How do salinity variations affect mussel and macroalgae copper absorption? ii) How do environmental copper concentrations affect macroalgae and mussels physiology?; iii) Does an IMTA system lead to a more resilient aquaculture to waterborne copper contamination? In this context, the brown macroalgae *Laminaria ochroleuca* and the mussel *Mytilus* sp. were exposed, individually and together, to waterborne Cu contamination, salinity reduction, and the combination of both. Although the current publication represents a controlled conditions experiment it is an important starting point to understand the true variations observed in the IMTA aquacultures in which greater natural variability could hide the sought effects.

2. Material and methods

To access the effect of IMTA on the copper accumulation by macroalgae and mussel cultivated separately or in IMTA and how such accumulation varies with salinity, in this work, the macroalgae *L. ochroleuca* and the mussels *Mytilus* sp. were kept separately (monoculture) or together (IMTA) in plastic cups submitted to control conditions, copper exposure, reduced salinity and copper exposure with reduced salinity. This allows to analyze the physiological effects of copper-based antifoulings accumulation in bivalves and macroalgae when cultured in monocultures and in IMTA allowing to access a possible bioremediation effect of the IMTA cultures. The physiological parameters analyzed include mortality, growth/condition index (form macroalgae and mussels, respectively), macroalgae photosynthetic efficiency, macroalgae photosynthetic pigment composition, macroalgae bioactive compound composition and both organisms oxidative stress, providing an overview of the physiological state of the organisms.

2.1. Organism collection

A total of 450 mussels (*Mytilus* sp.) were collected in Algés, Lisbon Bay, Portugal (38.694687 N, 9.227873 W) in January 2025, and transported in refrigerated plastic containers to IPMA's Live Marine Organisms Bioterium (LABVIVOS) in Algés. The collection area presented a mean water temperature of 15 °C, salinity of 33.5 and pH of 7.95 ([101] geoportal.coastnet.pt). The collected mussels had approximately 6.40 ± 0.74 cm length, 3.57 ± 0.36 cm width, 26.80 ± 7.04 g mean total weight, and 5.87 ± 2.10 g mean edible weight (measured in a Kern EMS 300–3 scale (Kern & Sohn, Balingen, Germany) and a caliper). All collected *Mytilus* sp. were cleaned from biofouling by scrapping and were randomly and equally divided into two stock aquarium systems in which they were slowly acclimated to 18 °C for one week. These systems were composed by two aquariums with a capacity of approximately 500 L each. Mussels were then fed with the microalgae mix concentration shellbreed® from Necton, Olhão, Portugal (8% dry weight (DW) - 8% of industrially produced microalgae biomass in DW).

Approximately 2 kg of *L. ochroleuca* were collected in NW Portuguese coast, at Praia do Norte (Viana do Castelo; 41.697337 N, 8.852327 W) in January 2025 and transported in polystyrene boxes in the dark, humid and cold conditions to IPMA's LABVIVOS bioterium in Algés. The collection area of Praia do Norte presented a mean water temperature of 13 °C, salinity of 34.0 and pH of 8.10 measured with an Hanna HI98194 multiparameter probe (Hanna Instruments, Póvoa de Varzim). The macroalgae stripes or holdfasts were removed and only the leaves were used in the experiments to normalize the tissues biomass used. The leaves biomass were randomly and equally divided into four aquarium systems with 500 L in total. While in stock aquaria (3 days), *L. ochroleuca* was supplemented with the following nutrients: 0.075 g/L sodium nitrate ($NaNO_3$) and 0.005 g/L sodium phosphate (NaH_2PO_4 H_2O), following the same approach used by [102]. Illumination was kept on a 14:10 Light:Dark cycle with an Aquabar T-series single Blue led lamp with a mean of 24 ± 4 μmol/m^2 at the water level.

While in stock aquaria, all organisms were maintained in recirculation systems. Each system was composed by two 150 L aquaria and a sump with a mechanical filtration with 100 μm pore size, skimmer, biological filter (bioballs), UV sterilization and temperature controlled by a profillux Outdoor 3 from GHL (Rheinland-Pfalz, Germany) through

a heat pump and a water cooler. Systems physicochemical conditions were kept as specified in Table 1. Saltwater was obtained in Cabo Raso, Cascais, Portugal and kept in polypropylene reservoirs. To better maintain the water temperature, the water for the experiment was transferred, at least 24 h before usage, to polypropylene reservoirs stored in the same room in which the experiment was being performed (from now on referred to as inner reservoirs).

2.2. Exposure treatments

The exposure experiment is schematized in Fig. 1. Environmental relevant copper levels were defined as 20 μg/L considering [13,14] (see introduction). Treatments included: control (CTR), low salinity - 25 PSU (LS), Cu exposure at 20 μg/L with 34 PSU (Cu), and Cu exposure at 20 μg/L with 25 PSU (Cu-LS). Mussels and macroalgae were divided into 5 L plastic cups, so there were, for each treatment: 5 cups with 10 mussels (Mussels cups), 5 cups with 30 g of *L. ochroleuca* (Algae cups) and 5 cups with 10 mussels +30 g of *L. ochroleuca* (IMTA cups). The experimental period lasted 14 days. Sampling was conducted at day 0, 7 and 14 for Cu accumulation, macroalgae fluorescence and both organisms' morphometric. At day 14 additional analyses were performed for photosynthetic pigments and bioactivity parameters in algae, while oxidative stress biomarkers in both organisms, was conducted only at day 14. This experimental period was defined in accordance with previous experiments using Cu contamination performed by our team [8], as it was possible to see several alterations in biomarker indicators in both organisms, while still being longer than the exposure durations commonly used in *Mytilus* sp. experiments [40–42]. Cu exposure was achieved by pipetting 5 mL of the Cu antifouling Aquanet LG360 dissolved at 1:1000 in each of the Cu exposure cups. For salinity reduction, dechlorinated freshwater was added to one of the inner reservoirs to lower the seawater to 25 PSU at least 24 h before usage. Mussels were fed daily with microalgae mix shellbreed® from Necton (Olhão, Portugal), at a concentration of 112 mg/L of DW algae equivalent to 0.7 mL per mussel per day. Mussels were let to feed for 5 h and then all water was removed to avoid the increase of ammonia levels, which were kept under 0.5 μmol/L throughout the experimental time. After the water removal, the cups were refiled with clean water at either 34 or 25 PSU, and Cu concentrations were readjusted to 20 μg/L in the exposure treatments. Cu contaminated water was disposed by maintaining it for 24 h in an 800 L container with around 5 kg of activated carbon. From this point, the treatments will be referred to as follows: control with mussels in monoculture at 34 PSU (CTR-M), algae in monoculture at 34 PSU (CTR-A), and both organisms together simulating IMTA culture (CTR-I); low salinity (25 PSU) treatments without copper (LS-M, LS-A and LS-I, for mussels, algae and IMTA, respectively); Cu exposure treatments with control salinity (34 PSU) (Cu-M, Cu-A and Cu—I, for mussels, algae and IMTA, respectively) and Cu exposure with low salinity (Cu-LS-M, Cu-LS-A and Cu-LS-I, for mussels, algae and IMTA, respectively).

2.3. Sampling

Macroalgae growth was determined by weighing algae mass at day 0, 7 and 14 using a Kern EMS 300–3 scale (Kern & Sohn, Balingen, Germany), while the algae fluorescence was assessed using a Li-Cor FMS300-B (Li-Cor, Siemensstrasse, Germany). For fluorescence, macroalgae were left in the dark for 30 min and afterwards the maximum quantum yield was measured using a white light pulse with an intensity of 5000 μmol/m^2/s with a pulse width of 1 s, which is within the normal light range previously used [43,44]. The ratio Fv/Fm was used as a proxy of photosystem efficiency [43].

Additionally, samplings were performed at day 0 and 7 for Cu concentrations in both organisms and on day 14 for Cu concentration, stress biomarkers and mussels' morphologic parameters (total weight and edible weight; equipment described in Section 2.1). Additionally, macroalgae's photosynthetic pigments and bioactivity were also assessed at day 14. From macroalgae, 5 g were taken for the Cu quantification, 1 g for stress biomarker analysis, 0.1 g for photosynthetic pigments and 5 g for bioactivity analysis, all in 5 replicates. For mussels, the whole specimen was used for Cu quantification, while gills and digestive glands were separated for stress biomarkers, using 5 replicates per treatment. All samples were stored at −80 °C.

Table 1
Aquarium system parameters during the experimental procedures.

Parameter	Condition
Temperature	18 ± 1 °C
Salinity	34 ± 1 PSU and 25 PSU (treatment dependent)
Dissolved oxygen (%)	> 85%.
Light condition	24 ± 4 μmol/m^2

2.4. Analytical procedures

Analytical procedure included the quantification of Cu by ICP-OES, the oxidative stress biomarkers superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), Ascorbate peroxidase (APx) and lipid peroxidation (LPO, by the malondialdehyde (MDA), ubiquitins and HSP70 concentrations were determined according to Madeira et al. [45], Cereja et al. [8,46]. SOD, CAT, GST and LPO were determined for both *Mytilus* sp. and *L. ochroleuca*, but GST did not present any activity with the macroalgae concentrations used. Alternatively, APx were determined for *L. ochroleuca.* According to antibody affinity, ubiquitin was determined for mussels while HSP70 was determined for *L. ochroleuca.* All biomarkers were normalized by total protein [47,48]. Additionally, bioactivity, namely, Cu and iron chelating capacities, total phenolic compounds (TPC) and total antioxidant activity by 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-difenil-1-picrilhidrazil (DPPH) methods optimized to 96-well plate as described by Cereja et al. [8], and photosynthetic pigments were determined according to Mendes et al. [49] For the assessment as biomarkers, the pheophorbide *a* and carotene concentrations were divided by the chlorophyll *a* (chl *a*) concentrations leading to phaeo/chl and car/chl ratios, respectively. Both ratios, together with chl *a* absolute concentrations, are known to be proxies of primary producers' physiology [50,51].

For further information on analytical procedures please see supplementary material – methodology.

2.5. Statistical analysis

Cu concentrations were compared between the different treatments and organisms through a univariate PERMANOVA with a pair-wise comparison as a post-hoc. This analysis was first nested in the sampling time and then in the organism. The remaining parameters were included in multivariate PERMANOVAs according to the nature of the data: photosynthetic biomarkers (fluorescence, Chl*a* concentration, phaeopigments:Chl*a* ratio, carotene:Chl*a* ratio), carotene relative concentration (Fucoxanthin, Violaxanthin, Zeaxanthin, Diadinoxanthin, β-carotene relative concentrations), macroalgae bioactivity (ABTS, DPPH, Cu chelating activity, iron chelating activity and TPC), macroalgae stress biomarkers (APx, SOD and CAT activities and LPO concentrations) and mussels stress biomarkers (SOD, CAT, GST and LPO) in a total of 6 analysis. All PERMANOVA ran with 9999 permutations under type III sum of squares and using unrestricted permutations of raw data. Final *p*-values were corrected using Bonferroni correction for the 6 analyses. For the multivariate PERMANOVAs post-hoc analysis was performed through PCA analysis.

3. Results

Both macroalgae and mussels showed significant differences in most of the assessed parameters (Table SM1). However, mussel condition

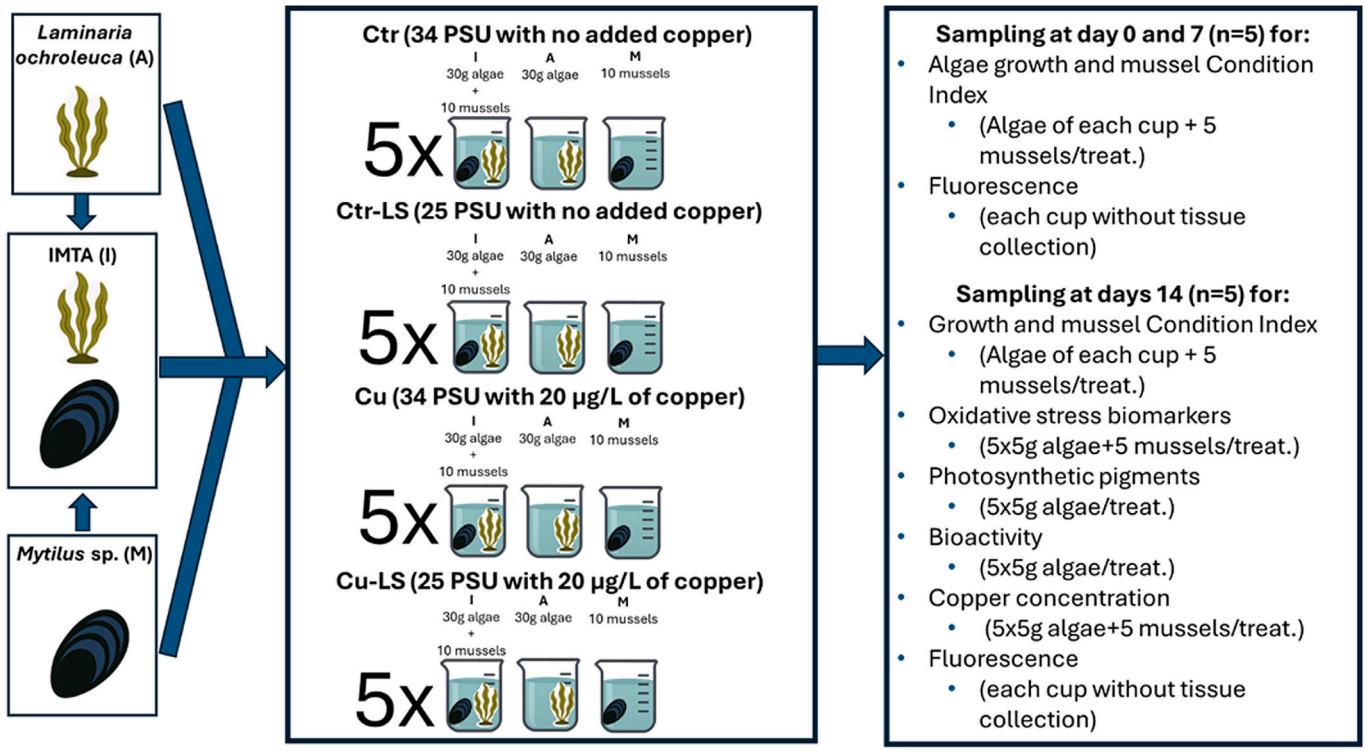

Fig. 1. Experimental design for the Cu exposure experiments. Treatments include control (Ctr), low salinity (LS), copper exposure (Cu), and copper exposure with low salinity (Cu-LS). Within each treatment, there were 5 cups with algae (A), 5 cups with mussels (M), and 5 cups with both, simulating an IMTA (I), totaling 60 cups in the entire experiment.

index did not exhibit relevant or consistent significant differences; therefore, information on this parameter is provided in Fig. SM1. The statistics and results for the remaining parameters are presented below, and in Table SM1 and Fig. SM2 for PERMANOVA statistics and univariate data for all analyses, respectively.

3.1. Cu concentrations

The macroalga *L. ochroleuca* showed no significant differences in Cu concentrations in comparison to *Mytilus* sp., with only Cu treatment presenting some differences among organisms and thus not being detected in main effects of PERMANOVA. For both organisms, Cu exposure treatments resulted in Cu concentrations ranging between 18 and 22 µg/g DW in mussels and between 17 and 48 µg/g DW in algae, compared with values below 5 µg/g DW in both control treatments. In *Mytilus* sp., the Cu levels in CTR, Cu—I, and Cu-LS-I treatments did not differ significantly, whereas low salinity led to an increase from 18 ± 6 µg/g in Cu to 33 ± 12 µg/g in Cu-LS; both treatments showed no significant differences from Cu-LS-I. In *L. ochroleuca*, low salinity treatments had no significant effect, with Cu and Cu-LS showing similar Cu concentrations (47 ± 7 and 35 ± 9 µg/g, respectively), as did Cu—I and Cu-LS-I (17 ± 3 and 18 ± 3 µg/g, respectively). Conversely, the IMTA treatments significantly reduced Cu accumulation in *L. ochroleuca*, with both Cu—I and Cu-LS-I exhibiting markedly lower Cu concentrations than their corresponding non-IMTA treatments (17 and 18 ± 3 µg/g, respectively, compared with 47 ± 7 and 35 ± 9 µg/g in Cu and Cu-LS) (Fig. 2).

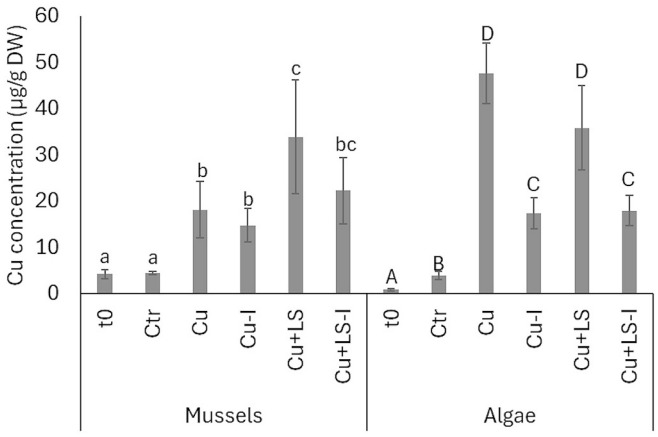

Fig. 2. Cu concentration (µg/g DW) in mussels Mytilus sp. and macroalgae *L. ochroleuca* exposed to the treatments t0 (wild), and at day 14 no Cu exposure (Ctr), Cu exposure at 20 µg/L with isolated species (Cu), Cu exposure with both species (Cu—I), Cu exposure under low salinity with isolated species (Cu-LS) and with both species (Cu-LS-I). Ctr and LS treatments had no significant differences between each other in either single species or IMTA treatments. Lower case letters represent significant differences between Mytilus sp. treatments while upper case letters represent significant differences between *L. ochroleuca* treatments.

3.2. Photosynthetic biomarkers and growth

In the photosynthetic biomarkers, fluorescence Fv/FM presented significant differences only between contaminant treatments ($p = 0.017$) and monoculture VS IMTA treatments ($p = 0.003$), with no significant differences in factor interaction. IMTA treatments presented significantly higher Fv/Fm in comparison to monoculture treatments both at day 7 and 14 (Fig. 3A), while Cu treatments presented lower Fv/Fm ratio only at day 14 (Fig. 3B).

Chl *a* was significantly higher in IMTA treatments, in special in Cu-LS-I (Fig. 3C), while monocultured organisms presented higher photoprotective pigments (car:CHL ratio) (Fig. 3C). None of the treatment groups presented variations in terms of photosystem degradation (Pheo:

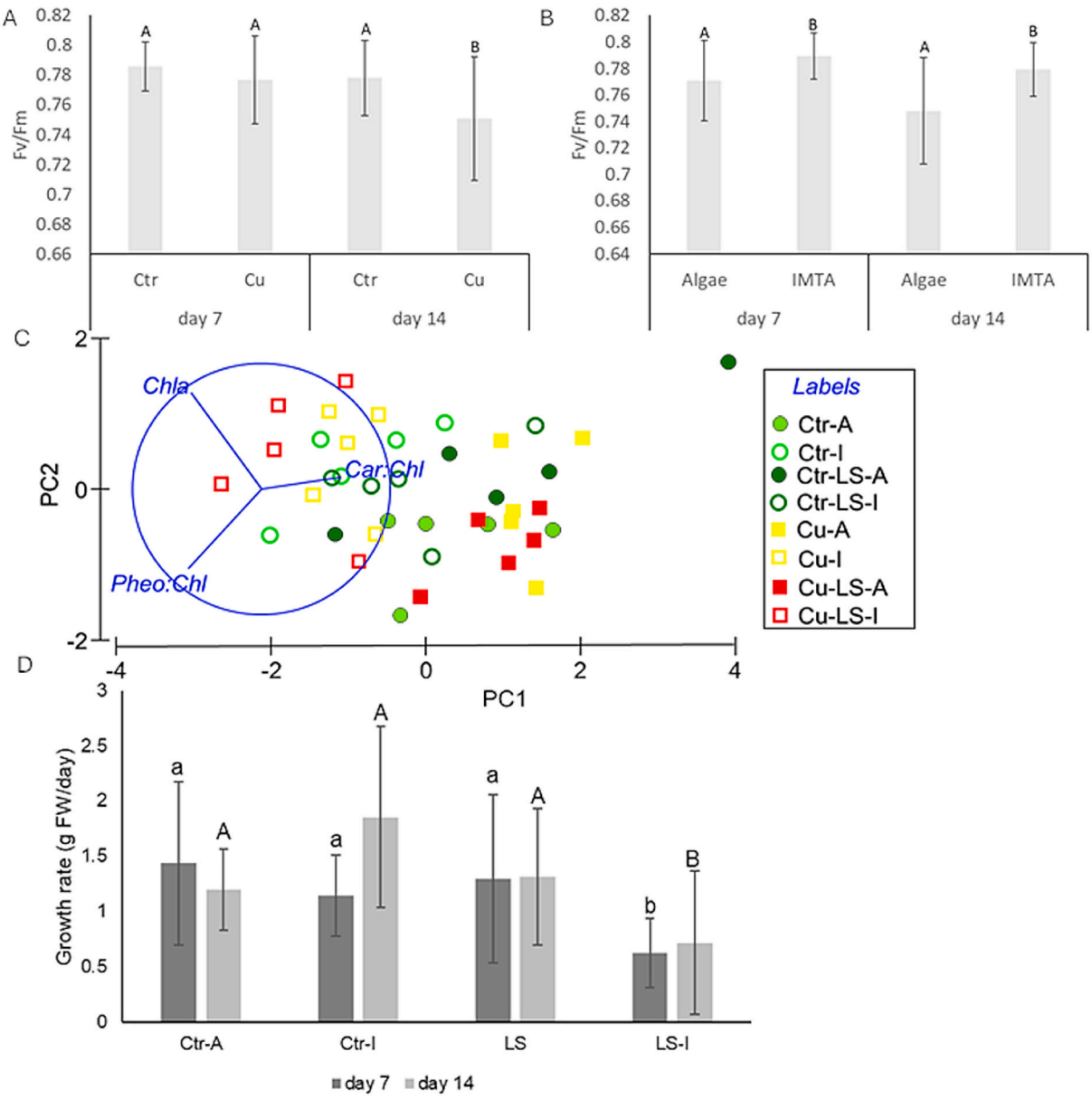

Fig. 3. Photosynthetic biomarkers: Fv/Fm ratio (fluorescence) for day 7 and 14 of the experiment organized by (A) contaminant treatment (control-"Ctr", and copper-"Cu") and by (B) culture method (monoculture-"A", or IMTA-"I"), while the PCA (C) present Chla, carotene:chlorophyll *a* ratio (Car:Chl), pheophytin: chlorophyll *a* ration (Pheo:Chl) for macroalgae in control (circles) and Cu exposure treatments (Cu, squares), low salinity treatments ("LS", dark green and red), isolated (monocultures "A", filled circles and filled squares) or together with mussels (IMTA, "I", empty circles and empty squares) under 34 PSU (light-green and yellow) and 25 PSU (dark-green and red). PCA statistics are presented in table SM2. The macroalgae growth (D) is presented by the factor groups with significant differences, namely 34 PSU treatment, 34 PSU in IMTA (34-I), 25 PSU treatment and 25 PSU in IMTA (25-I) for day 7 (dark bars) and 14 (light bars). Different letters on the bar plots mark significant differences. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Chl ratio) (Fig. 3C).

Algae presented significantly lower growth at reduced salinity ($p = 0.038$) and with the interaction of salinity and IMTA treatment ($p = 0.024$).

3.3. Pigment relative concentrations

Comparing each pigment:chl *a* ratio individually, both Cu exposure ($p = 0.003$) and IMTA ($p = 0.001$) presented significant differences and factor interactions, except salinity-IMTA interaction (statistic significances in table SM1).

Monocultured macroalgae presented significantly higher carotene concentrations in relation to chl *a* concentrations (Car:Chl *a* ratio). However, such trend altered depending on Cu exposure, with CTR-LS-A treatment presenting higher fucoxanthin, diadinoxanthin and chl *c* (*c1* and *c2*), while monocultured organisms exposed to Cu presented higher antheraxanthin, violaxanthin and β-carotene (Fig. 4). All IMTA treatments presented low relative concentrations of all pigments, specially in Cu exposed treatments.

3.4. Macroalgae bioactivity

In relation to bioactivity parameters, significant differences were detected for Cu exposure ($p = 0.0067$), IMTA treatments ($p < 0.001$), Cu-LS exposure ($p = 0.030$) and the three factors interaction ($p = 0.007$) (statistical significance in table SM1).

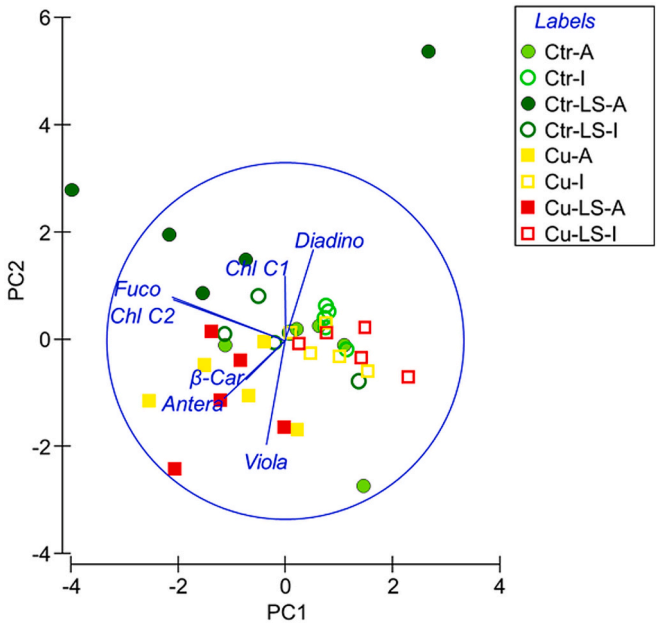

Fig. 4. Pigment ratio by chlorophyll *a* for the accessory and photoprotective pigments including chlorophyll *c* (1 and 2), Fucoxanthin (Fuco), Diatoxanthin (Diato), Violaxanthin (Viola), Antheraxanthin (Anthera) and β-carotene (β-car) for macroalgae in control (circles) and Cu exposure treatments (Cu, squares), low salinity treatments ("LS", dark green and red), isolated (monocultures "A", filled circles and filled squares) or together with mussels (IMTA, "I", empty circles and empty squares) under 34 PSU (light-green and yellow) and 25 PSU (dark-green and red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Monocultured macroalgae presented higher antioxidant capacity (either for DPPH and ABTS) and TPC in comparison to IMTA treatments, which in turn presented higher chelating capacity (Fig. 5). Cu-LS-I was the treatment presenting the highest chelating activity to Cu and Fe, followed by CTR-LS-I. Cu-LS-I was also the one presenting lowest ABTS

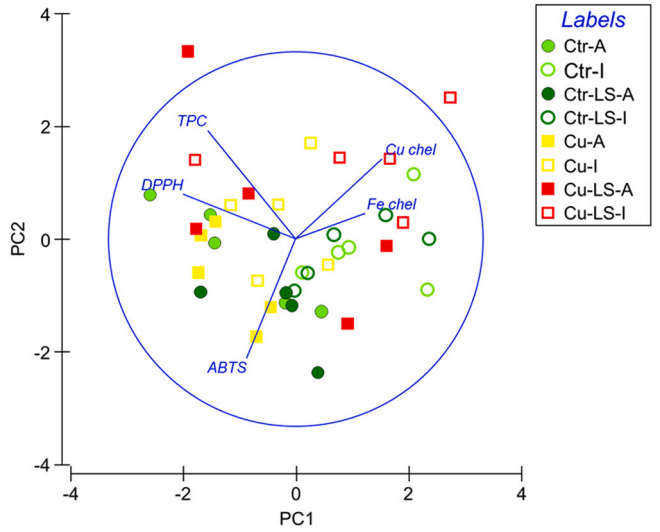

Fig. 5. Antioxidant capacity (ABTS and DPPH), total phenolic compounds (TPC), Cu and iron chelating capacity (Cu chel and Fe chel, respectively) for macroalgae in control (circles) and Cu exposure treatments (Cu, squares), low salinity treatments ("LS", dark green and red), isolated (monocultures "A", filled circles and filled squares) or together with mussels (IMTA, "I", empty circles and empty squares) under 34 PSU (light-green and yellow) and 25 PSU (dark-green and red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

antioxidant capacity and, together with CTR-LS-I, the one presenting lowest DPPH (Fig. 5). All 25 PSU, Cu exposure treatments presented low ABTS, while control treatments presented the highest antioxidant capacity (CTR-A – highest DPPH, while CTR-LS-A – highest ABTS) (Fig. 5).

3.5. Stress biomarkers

Macroalgae stress biomarkers showed significant differences for the Cu exposure ($p = 0.005$), reduced salinity ($p = 0.006$) and the interaction between reduced salinity and IMTA ($p < 0.001$). Except for CTR-LS-I, all CTR treatments presented lower oxidative stress, i.e. lower MDA, SOD and APx (Fig. 6). Cu exposure in general led to higher SOD and APx activity, but no increment in MDA or CAT levels. Cu-LS-A and Cu—I presented the highest SOD and APx activity, and Cu—I also revealed the highest CAT activity (Fig. 6). The treatment presenting the highest MDA was CTR-LS-I, followed by all Cu exposure samples (Fig. 6).

Mussel biomarkers presented few significant differences, i.e. only in main effect Cu-exposure for the digestive gland ($p = 0.014$) and in the mono vs IMTA comparison in the gills ($p < 0.001$) (statistical differences in table SM1). Despite multiple overlaps between groups, it was found that gills of Cu-exposed mussels presented lower SOD and higher GST activities (Fig. 7A) (further details in Fig. SM2). In the digestive gland, IMTA samples presented higher LPO, GST and SOD levels (Fig. 7B).

4. Discussion

The present study investigated the effects of Cu exposure and low salinity on mussels and macroalgae cultured in IMTA and monoculture systems, namely the physiological status, bioactivity, and susceptibility to Cu. Overall, IMTA systems were found to influence both Cu accumulation and macroalgae bioactivity. Also, IMTA had a greater effect over the tested parameters for macroalgae than either Cu or salinity.

4.1. Cu accumulation and salinity driven effects

Although wild *L. ochroleuca* showed lower Cu concentrations compared to mussels, this macroalga also exhibited a much higher susceptibility to variations in environmental Cu, as even the experimental control resulted in a significant increase in Cu concentrations. This high susceptibility to waterborne Cu is likely related to the elevated levels of metal-chelating compounds characteristic of the genus *Laminaria* [52,53], which often shows higher metal concentrations compared with other macroalgal species [54]. In general, all Phaeophyta exhibit strong metal-chelating capacity due to their high content of polysaccharides such as laminarin (used as a storage carbohydrate) and structural components, including alginate, fucoidan, and cellulose in their cell walls [24]. Moreover, brown algae demonstrate high tolerance to metal accumulation and frequently reveal high levels in metal-contaminated environments [55]. This combination of metal accumulation and resistance makes them promising candidates for domestic and wastewater decontamination applications [56,57]. However, in the present study, the high susceptibility of *L. ochroleuca* to Cu accumulation appears to have been influenced by the presence of *Mytilus* sp., even though this species generally does not accumulate Cu to the same extent as *L. ochroleuca* or macroalgae in general [8]. Macroalgae can accumulate metals both within their cell walls and in internal tissues [58], whereas in *Mytilus* sp., metal uptake from water occurs primarily in the gills and digestive gland, with substantial absorption occurring within a few hours [59,60]. Although insufficient information is currently available to directly compare the Cu uptake rates between *Mytilus* sp. and *L. ochroleuca*, bivalves frequently exhibit higher metal concentrations than macroalgae. For example, Alkan et al. [61], analyzing *Mytilus* sp., *Ulva intestinalis*, and *Ceramium rubrum* from metal-contaminated sites, found higher accumulations of certain elements (e.g., As, Cu, Mo, and Zn) in *Mytilus* sp. than in the macroalgae. Therefore, future studies comparing the short-term dynamics of metal accumulation in

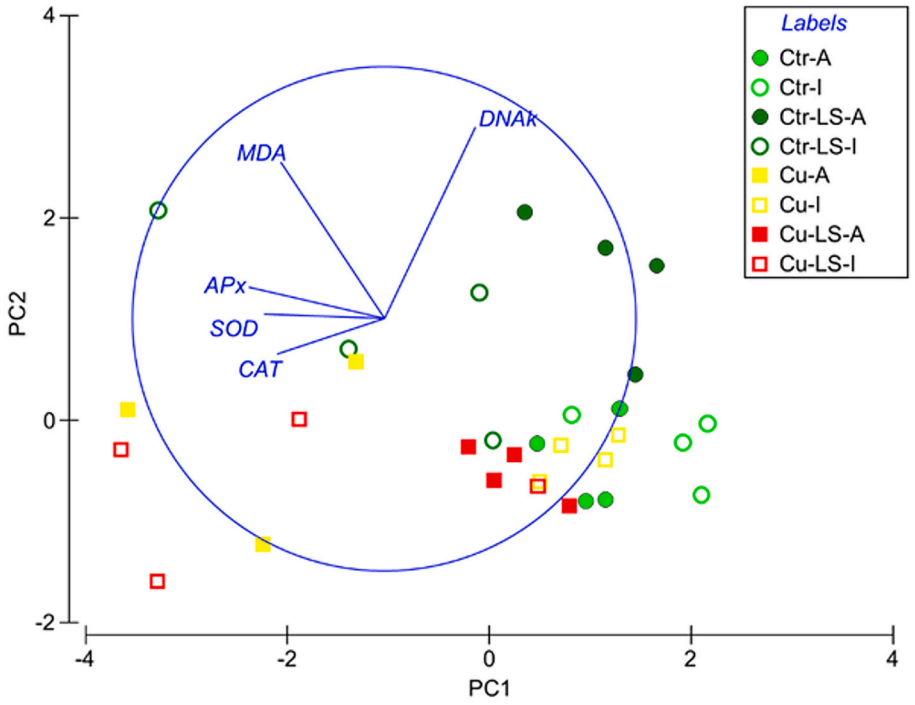

Fig. 6. Stress biomarkers superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APx), malondialdehyde (MDA, proxy of lipid peroxidation) and heat shock proteins 70 kDa (HSP70) for macroalgae in control (circles) and Cu exposure treatments (Cu, squares), low salinity treatments ("LS", dark green and red), isolated (monocultures "A", filled circles and filled squares) or together with mussels (IMTA, "I", empty circles and empty squares) under 34 PSU (light-green and yellow) and 25 PSU (dark-green and red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

macroalgae and bivalve species could help clarify the nature of this interaction.

In terms of commercial usage of these macroalgae, EFSA determines the upper limit as 0.07 mg/ kg of body weight, being around 4.9 mg for a 70 kg adult (EFSA [62]). Therefore, the macroalgae exposed to Cu still present values within the safety values defined by EFSA as 100 g of fresh algae from either Cu-A and Cu-LS-A treatments (around 45 μg/g of dry weight macroalgae) represents around 16% of the copper upper limit of a 70 kg adult, (considering around 85% moisture determined during processing). Still the decrease induced by the bivalves decreased such dosages to just 7% of the copper upper limit (considering the 85% moisture and an adult with 70 kg) thus contributing to the safety of the macroalgae being sold for human consumption.

Salinity variations are another factor directly associated with Cu accumulation, with organisms subjected to low-salinity conditions generally being more susceptible to Cu uptake than those in marine environments (e.g., [63]). However, this pattern is not consistent across all organisms, as some macroalgae have been shown to reduce metal accumulation under decreased salinity [64]. It is important to note that both organisms are estuarine and coastal species, naturally exposed to salinity variations to some extent; however, these variations may affect their metal accumulation. In the present study, the effect of salinity was only evident in *Mytilus* sp., which showed an increase in Cu concentrations (from 18 μg/g in the Cu treatment to 33 μg/g in the Cu-LS treatment), which may be related to the species' faster rate of metal accumulation when compared with *L. ochroleuca*. Therefore, based on the results obtained, it appears that mussels may have provided a protective effect over *L. ochroleuca*, either by reducing its exposure to Cu contamination or by buffering the impacts of salinity variations.

4.2. Alterations to photosynthetic activity

Two main factors influenced photosynthetic activity in the present study, with the effects of IMTA treatments being the most pronounced. The co-cultivation of *Mytilus* sp. with *L. ochroleuca* enhanced photosystem efficiency, as indicated by fluorescence ratios, and resulted in higher chl *a* concentrations along with a reduced carotene:chl *a* ratio. This effect is likely due to the increased nutrient availability in IMTA treatments, stemming from bivalve excretions. Although productivity is generally dependent on available nutrients [65], the effects of nutrient enrichment on photosynthetic parameters such as fluorescence and pigment concentrations or ratios are not always straightforward. Several studies have reported no detectable effects of specific nutrient variations on these parameters [66] or even on growth [67]. Such variability is likely due to photosynthetic response to multiple interacting factors, including light limitation, nutrient limitations (including carbon; [68]), and physical or chemical stressors [69,70]. Nonetheless, numerous studies have reported increases in chl *a* and Fv/Fm under favorable nutrient sufficiency or enrichment [68,71,72]. Additionally, plants and algae grown under nutrient limitations are known to be more susceptible to photoinhibition [73,74]. Therefore, in long-term experiments, macroalgae exposed to IMTA treatments are expected to exhibit higher growth rates as a result of increased productivity, even if photoprotective pigments are relatively reduced.

The second factor affecting photosynthetic activity was Cu exposure. Algae exposed to Cu exhibited a slight reduction in photosystem health (fluorescence ratio) by day 14 of the experiment, accompanied by a decrease in chl *a*, but only in the single-organism treatments. Cu-exposed algae are expected to show a reduction in chl *a* concentrations in response to the exposure [8,75], and the fact that this effect occurred only in the monoculture treatments demonstrates the effectiveness of IMTA in protecting the macroalgae from Cu-induced stress. Interestingly, Cu-exposed algae in IMTA treatments displayed higher chl *a* concentrations compared with the controls. Regarding pigment composition, Cu-exposed algae exhibited higher relative concentrations of violaxanthin, antheraxanthin, β-carotene, and fucoxanthin. Carotenoids are known to respond to stress by dissipating excess thermal energy or scavenging reactive oxygen species, increasing in abundance under stressful conditions [76]. In the present study, the monocultures exposed to Cu treatments showed the highest relative concentrations of these

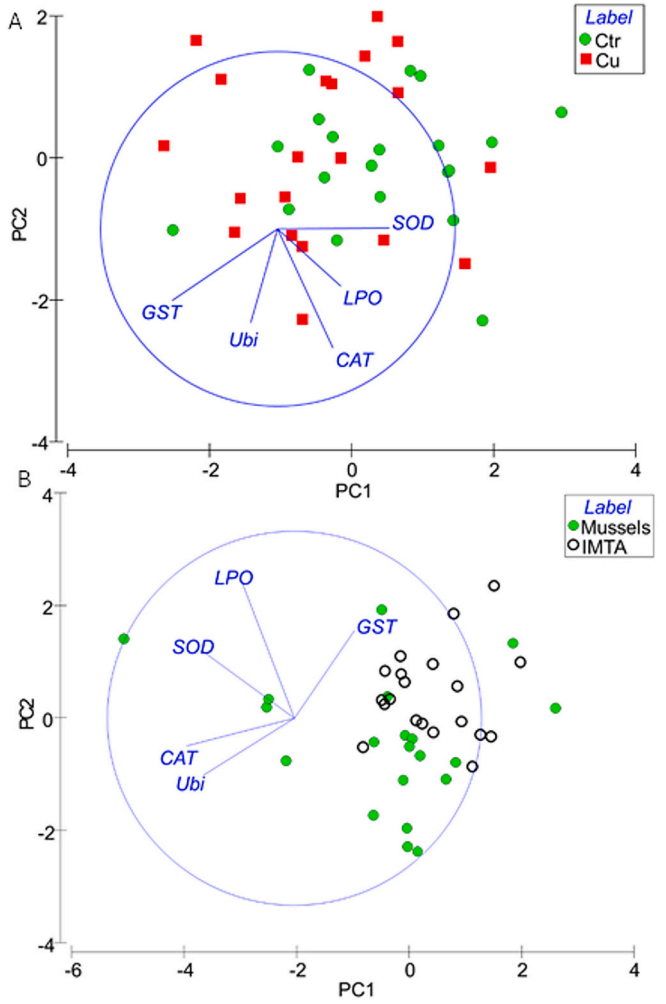

Fig. 7. Stress biomarkers superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), malondialdehyde (MDA, proxy of lipid peroxidation) and Ubiquitin (Ubi) in mussel digestive gland (A) and gills (B). The PCA are organized by the factor that presented significant differences (Cu exposure in the digestive gland and IMTA in the gills) with green dots representing control/monoculture (A/B), red squares represent Cu exposure (in A) and black empty circles represent IMTA treatment (in B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pigments, suggesting that IMTA treatments were able to mitigate stress and protect the macroalgae.

4.3. Alterations to macroalgae bioactivity

As discussed in the previous section, IMTA systems led to a decrease in the carotene:chl *a* ratio. Analysis of individual pigment:chl *a* ratios revealed that IMTA-reared algae exhibited lower relative concentrations of all accessory and photoprotective pigments, including chl *c* (1 and 2), fucoxanthin, β-carotene, and other pigments. Many pigments, particularly fucoxanthin (a major photoprotective pigment in Phaeophyceae), are potent antioxidants [77], and their reduction may indicate a decrease in the potential benefits of consuming this species. This decrease may also be linked to the observed reductions in total phenolic content (TPC) and DPPH antioxidant activity. In Phaeophyceae, phenolic compounds are primarily phlorotannins, which can constitute up to 12% of dry weight [22]. Phlorotannin production is often associated with photosynthetic activity, as both processes are linked to carbon metabolism, acting as photoprotective agents [22,78,79]. Additionally, increased nutrient availability, such as that provided by IMTA systems, has been shown to reduce phlorotannin concentrations due to shifts in carbon allocation [78]. Therefore, the observed decrease in photoprotective pigments, alongside an increase in chl *a*, likely reflects physiological changes that also contributed to reduce total phenolic compounds. Since many photosynthetic pigments and phenolic compounds are lipophilic, their reduction may underlie the observed decrease in DPPH antioxidant activity in IMTA treatments. Conversely, the single-organism treatments showed higher carotenoid concentrations, particularly in Cu-exposed algae, probably due the applied Cu concentrations falling inside *L. ochroleuca* physiological requirements. The increase in major carotenoids, including violaxanthin and fucoxanthin, likely enhances the antioxidant capacity of the algae, thereby increasing its nutritional value [72,76]. This establishes a balance between the protective effects provided by IMTA—observed in previous sections—and the enhanced nutritional potential of algae in the single organism treatments.

4.4. Stress biomarkers alterations by Cu and salinity

Cu can alter macroalgae physiology, impairing both productivity and respiration [75]. In the present work, Cu led to an increase in SOD, CAT and APX activities in the macroalgae, independently of salinity, representing an intensification of superoxide and hydrogen peroxide production. However, no alterations were registered in MDA levels, with also no detectable effects on growth or tissue degeneration, which is likely due to the low Cu concentration applied here in comparison to other studies (e.g. [8,75]). It is important to emphasize that the Cu concentrations applied in the present study fall within the range of those found in contaminated environments (e.g. [80–82]), with higher values found only at the vicinity of active mines [83] or wastewater outfalls [14], where aquaculture production is not expected to occur.

In relation to the salinity effects, in the present study macroalgae suffered only slight salinity-driven alterations, with no increase in MDA. Short to mid-terms (hours to few days) exposure to low salinity are known to have low effects over intertidal macroalgae in terms of lipid peroxidation (MDA) and antioxidant enzyme activities [84,85]. Even decreases of up to 10 PSU for 6 days had only slight effects in *Ulva* sp. [85], as well as 15 PSU for 4 days with *Gracillaria corticata* [84]. Kumar et al. [84] also revealed that 25 PSU treatments (like the one applied in the present study) had no or just a slight effect in MDA levels, growth rate and reactive oxygen species (ROS) production, consistent with the results observed in the present study. Nonetheless, an increase in the initial levels of antioxidant response, such as SOD, CAT and APX, are common to most studies [84,85]. Although such increase in antioxidant enzymes was not observed in the present study, an increase in DNAk was registered in the CTR-LS and CTR-LS-I treatments, which may reflect metabolic alterations performed by the macroalgae to adapt to such low salinity [86]. The increase in the levels of such enzymes may be associated with a rerouting of energy from the photosynthesis and primary metabolism to specific stress responses [87], with the absence of response in growth and photosynthetic markers being a consequence of the small salinity decrease (−10 PSU).

In mussels, Cu exposure decreased SOD and increased GST activities in the digestive glands, while showing no effects in the gills. Cu concentrations exceeding physiological requirements can promote the generation of ROS via Fenton reactions [88], triggering antioxidant systems to prevent cellular and tissue damage. In cases of waterborne Cu contamination, both gills and digestive glands are continuously exposed to stress and can suffer extensive damage at high Cu concentrations [8]. However, the stress observed in the present study was minimal, likely due to the relatively low Cu concentrations tested. While some studies reported significant alterations in mussels exposed to as little as 3–5 μg/L [42], others observe changes only at concentrations above ~30 μg/L [40]. Such variability may also result from differences in exposure duration, with studies finding response in antioxidant defenses to

similar Cu levels in one to four days of exposure [41,89], suggesting that adaptation to Cu may have occurred during the current study (14 days) exposure. GST catalyzes the glutathione conjugation with xenobiotics present in the cell cytosol, enabling the detoxification of metal ions [90–92]. Thus, the observed trend may be a Cu-mediated Fenton reaction and the resulting increase in cell hydroxyl ROS [88] inhibiting the SOD activity [93], while increasing GST for detoxification of either ROS or Cu. Other important detoxification pathways that may be promoted by Cu induced stress include metallothionein, which are an important detoxification pathway in bivalves [94].

Another verified effect in the present study was the higher oxidative stress registered on the gills of *Mytilus* sp. produced in IMTA systems. Such higher stress suggests that macroalgae might be producing waterborne metabolites that affect *Mytilus* sp. gills, as this is the most exposed organ to waterborne contaminants [95,96]. Although not limited information exists regarding the negative effects of waterborne metabolites on bivalves, it is known that many secondary metabolites are used as grazing defense [97], with some being released to the water to avoid fouling [98]. Thus, the stress registered in the gills may be due to the direct action of these molecules on mussels' gills. In aquaculture production systems, the high-water renovation may attenuate this effect.

Being adapted to estuarine environments, *Mytilus* sp. are usually resistant to salinity variations to the extent of the one tested in the present study (−10 PSU), which justifies the limited changes in the biomarkers observed in the present study[99,100]. Although reduced salinity facilitating Cu accumulation in *Mytilus* sp., synergistic effects were not observed in this study. This pattern may have resulted from the minor differences detected between Cu accumulation in the normal salinity treatment and low salinity treatments (around 20 μg/g vs 30 μg/g, respectively).

5. Conclusions

The present study reveals that IMTA production systems may benefit the cultured organisms not only by enhancing their physiological condition, but also by reducing the adverse effects associated with exposure to waterborne contaminants. Both organisms were not highly susceptible to the tested salinity reduction. Mussels under low salinity presented higher copper accumulation, but did not suffer additional physiologic stress from such increase. *L. ochroleuca* produced in IMTA conditions is overall likely to present higher growth rates but reduced bioactivity, specifically in terms of antioxidant capacity, associated with the decrease in photosynthetic pigments with strong antioxidant activity (carotenoids) and in total phenolic compounds. While IMTA contributes positively to the overall performance of macroalgae, these benefits may be offset by reductions in macroalgal bioactivity and in the associated potential benefits to human consumers. Also, IMTA did not have a clear benefic effect on mussel condition or physiology, neither in protecting it from metal accumulation. Nevertheless, it is important to validate these findings under commercial operating conditions, where additional factors, such as solar irradiance, may induce carotene production and counteract the reduction in bioactivity observed in macroalgae.

CRediT authorship contribution statement

Rui Cereja: Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tomás Chainho:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Alícia Pereira:** Writing – review & editing, Investigation, Data curation. **Rita V.C. Gomes:** Writing – review & editing, Investigation, Data curation. **Daniel Bolotas:** Writing – review & editing, Investigation, Data curation. **Ana C. Brito:** Writing – review & editing, Validation, Resources, Methodology. **Mário S. Diniz:** Writing – review & editing, Validation, Resources, Methodology. **Pedro R. Costa:** Writing – review & editing, Resources, Funding acquisition. **António Marques:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2026.104913.

Data availability

Data will be made available on request.

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