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Wildfire ash undermines the physiology of the Mediterranean coral *Cladocora caespitosa*

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ABSTRACT

Wildfires represent a natural disturbance phenomenon whose frequency and intensity are expected to increase due to climate change-driven heatwaves, droughts, and anthropogenic pressure. This is particularly concerning in fire-susceptible areas, such as the Mediterranean basin, raising concerns about ecosystem functioning and biodiversity. While wildfire impacts have been extensively investigated in terrestrial environments, little is known about their effects on marine organisms. Here, nubbins of the temperate coral *Cladocora caespitosa*, an endemic key ecological species in the Mediterranean Sea, were subjected to a 93-h exposure to four environmentally relevant wildfire ash concentrations (0.05, 0.5, 2.5, and 5 g L^{-1}) under controlled laboratory conditions. Coral responses were assessed through oxidative stress biomarkers (activity of antioxidant enzymes SOD, CAT, GR, and LPO levels) and bleaching parameters (chlorophyll *a and c2* concentration and *Symbiodiniaceae density*). A fluctuating response of SOD, a significant decrease in CAT activity, and an increase in GR activity were observed, suggesting oxidative stress overwhelming primary defenses. This interpretation was supported by a significant increase in LPO, indicating oxidative damage to cellular structures, possibly exacerbated by elevated metal concentrations in the ash. *Symbiodiniaceae density* decreased significantly across all tested ash concentrations, while chlorophyll content per cell increased, potentially reflecting photoacclimation to reduced light penetration caused by water turbidity. Overall, our results could indicate that *C. caespitosa* is vulnerable to wildfire ash exposure. These findings highlight wildfire ash as an emerging marine stressor, thereby broadening our understanding of wildfire impacts on benthic ecosystems.

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1. Introduction

Wildfires are typically considered a natural landscape disturbance phenomenon worldwide, capable of altering the functioning of several ecosystems and the composition of biotic and abiotic compartments (Wardle et al., 2003; Burkle et al., 2015; Agbeshie et al., 2022). Nevertheless, during the last decades, factors such as land-use changes, rural area abandonment, and poor forest management have increased the frequency and intensity of wildland fires, especially in areas susceptible to fires, such as the Mediterranean basin (Pausas et al., 2008; Martínez et al., 2009; Caon et al., 2014; Nolè et al., 2022; Marfella et al., 2024). Additionally, climate change-driven heatwaves and droughts are expected to promote wildfire events further (Turco et al., 2019; Ruffault et al., 2020). As such, wildfires are now considered a critical, emerging threat to public and ecosystem health (Salis et al., 2016), and there is an impelling need to better understand their potential effects on ecological key organisms that could therefore influence a large fraction of biodiversity (Kelly et al., 2020; Campos and Abrantes, 2021).

While wildfire impacts have mainly been investigated for terrestrial environments (Chia et al., 2016; Bowd et al., 2019; Marfella et al., 2023; Niccoli et al., 2025) fewer studies have been carried out on aquatic, freshwater, and marine ecosystems (Earl and Blinn, 2003; Bixby et al., 2015; Gomez Isaza et al., 2022; Riera and Pausas, 2024). In particular, fire byproducts, such as ashes and sediments, can enter the seawater column through riverine and terrestrial run-off or oceanic deposition via aerosols (Certini, 2005; Kelly et al., 2021; Herbert-Read et al., 2022; Riera and Pausas, 2024). These compounds may contain various concentrations of nutrients, metals, polycyclic aromatic hydrocarbons, and other elements (Santín et al., 2013; Sánchez-García et al., 2025), with the potential to alter the physical and chemical properties of water, or can be absorbed by organisms, with possible toxic effects on their metabolism, development, or behavior (Pradhan et al., 2020; Jesus et al., 2023). For example, iron- and/or nitrogen-rich wildfire aerosols enhanced extensive phytoplanktonic blooms in different oceans (Tang et al., 2021; Liu et al., 2022). Conversely, a large coral mortality event was linked to a giant red tide and low oxygen levels following Indonesian wildfires in 1997 (Abram et al., 2003). Concerning scleractinian corals, to date, very few studies have investigated their response to exposure to wildfire ash/products, and they all involved tropical coral species. For instance, wildfire fine particulate matter determined a reduction in photosynthetic activity of the symbiotic algae associated with the scleractinian tropical species *Porites lutea,* which was supported by an increased coral heterotrophic strategy (Qin et al., 2024), while *Acropora formosa* exhibited oxidative damage and disrupted metabolic functions (Liu et al., 2025). Nevertheless, the effects of wildfire ashes have not yet been investigated on temperate scleractinians.

The colonial, zooxanthellate scleractinian coral *Cladocora caespitosa* is an endemic species of the Mediterranean Sea (Morri et al., 1994). It is the only shallow-water hard coral of this basin that can be considered as a habitat-forming species since, although it typically grows as relatively small spherical and subspherical colonies, *C. caespitosa* is also capable of forming large reef-like bioconstructions (Kružić and Benković, 2008; Kersting and Linares, 2012). Its actual populations are declining throughout the Mediterranean Sea due to global and local threats (Kružić et al., 2025), such as seawater warming and marine heatwaves (Rodolfo-Metalpa et al., 2005; Jiménez-González et al., 2016; Vergotti et al., 2025), competition with invasive algae (Kružić et al., 2008; Kersting et al., 2014), anthropogenic activities (Kružić and Požar-Domac, 2007; Kersting et al., 2023), and pollution (El Kateb et al., 2016; Reuning et al., 2025). Given the important ecological role and the observed mortality events, several studies have investigated the response of *C. caespitosa* to environmental stressors under controlled experimental conditions, including for example thermal stress (Rodolfo-Metalpa et al., 2006a, 2006b; Ankon et al., 2025), acidification (Rodolfo-Metalpa et al., 2010; Trotter et al., 2011; Movilla et al., 2012), irradiance (Rodolfo-Metalpa et al., 2008; Hoogenboom et al., 2010; Tremblay et al., 2012), and eutrophication (Hadjioannou et al., 2019). To our knowledge, the response of *C. caespitosa* to trace metals and/or potential emerging contaminants has remained relatively scarcely studied, except for the role of iron, which at high levels has been demonstrated to be detrimental for the symbiosis between *C. caespitosa* and its associated photosynthetic dinoflagellate, leading to bleaching and necrosis (Dellisanti et al., 2024). On the contrary, *C. caespitosa* colonies subjected to nutrient-rich (nitrogen and phosphorus) waters were less susceptible to bleaching (Hadjioannou et al., 2019), probably due to the high heterotrophic capacity of this coral species (Ferrier-Pagés et al., 2011).

Marine sessile benthic invertebrates, such as *C. caespitosa*, are unable to migrate in order to escape external stressors. Therefore, they are forced to cope with all the environmental stressors, relying entirely on their cellular defense mechanisms as their first line of defense against stress for acclimatization and adaptation (Seveso et al., 2014, 2024; Drury, 2020). Different environmental stressors can cause physiological and cellular stress in coral holobionts, leading to oxidative stress and metabolic impairments that trigger coral bleaching (Montalbetti et al., 2023; Helgoe et al., 2024; Isa et al., 2024, 2025). Therefore, oxidative stress, resulting from an overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS), is a common indicator of environmental stress in corals (Flores-Ramírez and Liñán-Cabello, 2007). In response to the increased levels of these radicals, corals typically enhance their antioxidant defense mechanisms, which include antioxidant enzymes and stress proteins (Traylor-Knowles et al., 2022; Tisthammer et al., 2024). Consequently, antioxidant assays and oxidative damage biomarkers have been widely used as indicators of stress in corals (Downs et al., 2000; Lesser, 2024).

The Mediterranean coral *Cladocora caespitosa* typically inhabits depths between 5 and 20 m (Morri et al., 1994) and is therefore likely to be exposed to contaminants introduced into coastal waters through riverine run-off. Based on this, we hypothesized that short-term exposure to wildfire ash would impair both the coral antioxidant defense system and the stability of its symbiotic relationship with Symbiodiniaceae. To test this hypothesis, we exposed *C. caespitosa* to environmentally relevant concentrations of aqueous wildfire ash extracts collected on a natural wildfire ground under controlled laboratory conditions for 93 h. Specifically, we evaluated the coral antioxidant defense system by measuring the activity of three key enzymes involved in ROS detoxification (superoxide dismutase SOD, catalase CAT, and glutathione reductase GR) and the level of lipid peroxidation (LPO) as an indicator of oxidative damage. In parallel, we assessed the status of the coral symbiosis by quantifying *Symbiodiniaceae density* and the cellular concentrations of chlorophyll *a and c2*. These endpoints were integrated with the chemical characterization of wildfire ash, including nutrients (C, N, P) and trace metals (Cd, Cu, Fe, Mn, Ni, Pb, and Zn), as well as measurements of water turbidity across the experimental conditions. This study aimed to provide new insights into the physiological responses of *C. caespitosa* to wildfire ash exposure and to identify potential mechanisms of stress and acclimation in this threatened Mediterranean coral species.

2. Materials and methods

2.1. Coral sample preparation

A total of three donor colonies (about 10 cm diameter) of the Mediterranean scleractinian coral *Cladocora caespitosa* were collected with hammer and chisel at about 9–13 m depth at Punta Mesco, Cinque Terre Marine Protected Area, Ligurian Sea (44°08′46.9″N, 9°37′02.6″E) (Fig. 1A), in October 2023 by scuba diving. To avoid the potential presence of clones, colonies were collected at least 10 m apart (Casado-Amezúa et al., 2014; López-Márquez et al., 2019). After the sampling, colonies were kept in a tank with aerated seawater and reduced-light conditions and immediately transported to the facilities of

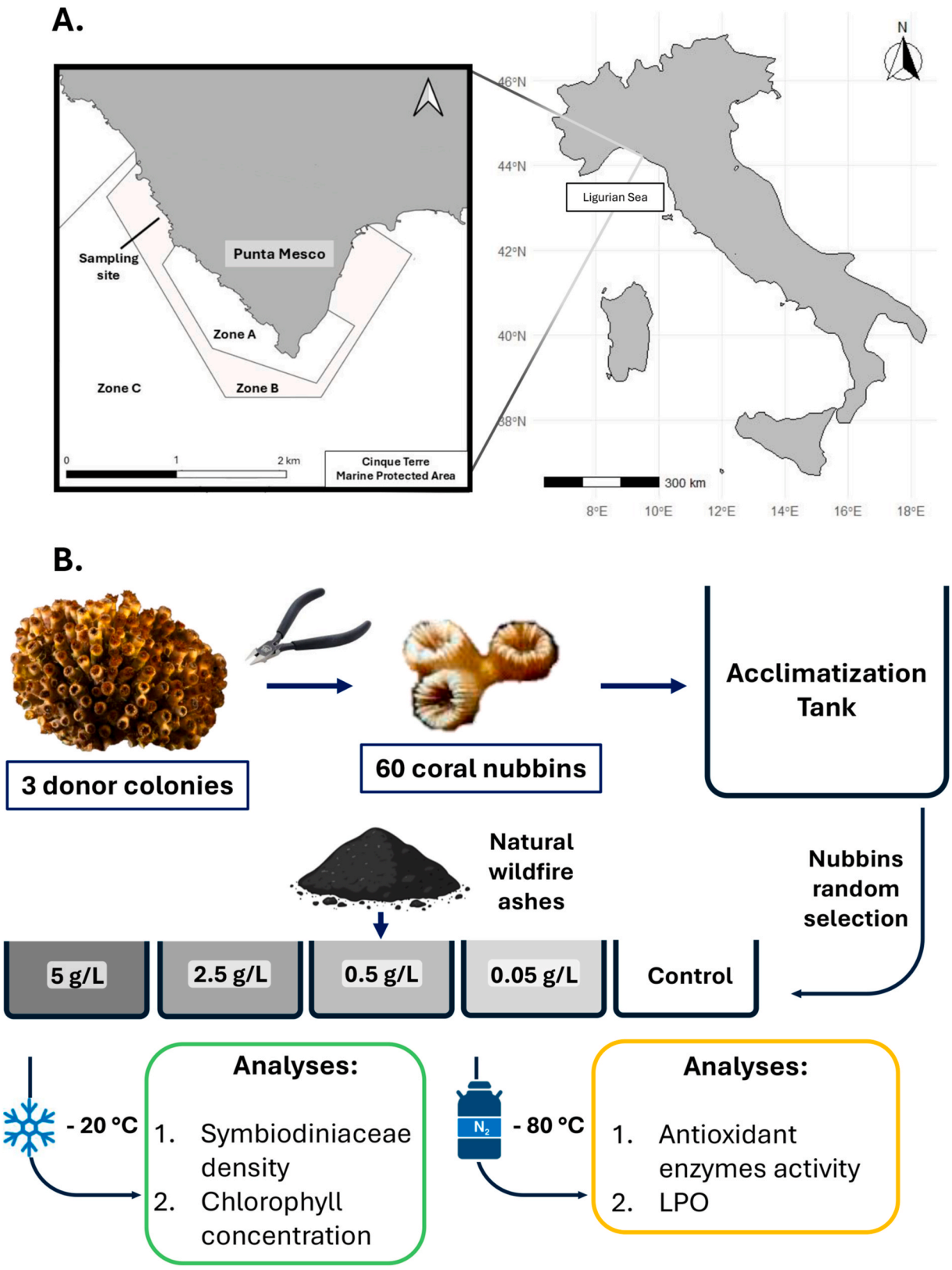

Fig. 1. – (A) Detail of the sampling area, Punta Mesco (Italy, Mediterranean Sea). Three zones of increasing protection (Zone A, maximum protection) of the Cinque Terre Marine Protected Area are indicated. The sampling site is also indicated. **(B)** Diagram showing the experimental design of the study. Further details are present in paragraphs 2.1 and 2.3.

Acquario di Genova (Genoa, Italy), where they were maintained in a 200 L acclimatization tank for 4 weeks. During this period, corals were fed daily with *Artemia salina* nauplii, using a syringe to feed each single coral nubbin with about 1.5 mL of a seawater solution at a concentration of about 200 individuals of *A. salina* mL^{-1}. Moreover, corals were reared under controlled conditions, including temperature (20 °C), salinity (37.1), pH (8.1), irradiance 135 ± 10 μmol photons m^{-2} s^{-1}, and photoperiod (12h:12h light:dark). The Life Support System (LSS) of this thank was composed by a pump (Astralpool Victoria Plus) with a 24-h flow rate to ensure complete water change every 30 min, a filtration system consisting of a sand filter (Astralpool Artic, filtering particles 0.4–2 mm), a UV filter (Panaque 750 s AB equipped with four 40 W lamps), and an heat exchanger controlled by electrovalves. After the 4-week acclimatization, 60 nubbins of *C. caespitosa* (2–4 polyps each) were prepared from donor colonies using sharp iron pliers and promptly fixed to plastic supports with epoxy resin (Fig. 1B). Subsequently, nubbins of *C. caespitosa* were kept in the acclimatization tank for an additional 2 weeks under the same conditions, to visually assess whether any polyps were affected by the physical separation and for possible signs of bleaching or necrosis.

2.2. Aqueous ash extracts preparation

Ashes were collected from a burned Mediterranean shrubland vegetation (Rossetti et al., 2022) in the western sector of the Montiferru massif (40° 09′ 39,03″ N, 8° 36′ 39,21" E), Sardinia, Italy, one week after a localized wildfire that occurred in 2022. The area had previously been heavily affected by the major 2021 megafire, which burned ~13,000 ha of forest and shrubland (Rossetti et al., 2022). The 2022 fire affected a much smaller portion of the same landscape, burning only a few tens of hectares of Mediterranean shrubland from which the sampled ashes derived. Wildfire ashes were sampled in situ by selecting multiple points randomly distributed within the 2022 burned area. At each point, surface ash was gently scooped from the soil using sterile tools, transferred to dark plastic bags, and transported to the University of Cagliari laboratory, where it was sieved through a 2 mm mesh and dried at 30 °C for 24 h. After this, three independent samples were taken from the main reservoir for the ashes chemical characterization described in paragraph 2.4. The aqueous ash extracts (AEAs) were prepared following the protocol by (Silva et al., 2015), further adapted in Jesus et al. (2023), with modifications described hereafter, by mixing ashes and seawater to a final ash concentration of 5 g L^{-1}. This value was selected following laboratory experimental conditions tested in previous studies and based on environmental conditions found in post-fire runoff waters (5–10 g L^{-1}) (Jesus et al., 2023; Muñiz González et al., 2023; Santos et al., 2023). Briefly, ashes were mixed with seawater and placed in an orbital shaker at 200 rpm for 2 h, and allowed to sit overnight for 16 h. The aqueous fraction was then collected by siphoning and sieved through a 50 μm filter to remove solid particles further suspended in the water column that could potentially obstruct coral polyps' mouths during the treatments.

2.3. Aquaria experimental setup

At the end of the total 6-week acclimatization period, the 60 coral nubbins were transferred and immersed in 10 experimental tanks (dimensions 30 x 18 × 18 cm) to have 6 replicate nubbins of *C. caespitosa* in each tank. A total of 5 different conditions were tested, each of which was performed in two duplicate experimental tanks to take into account any eventual tank effects: a controlled condition without wildfire ashes and the following four ash concentrations, 5 g L^{-1} (100 %), 2.5 g L^{-1} (50 %), 0.5 g L^{-1} (10 %), and 0.05 g L^{-1} (1 %), applying proper dilutions of the mother solution (Fig. 1B). Each tank was filled with 8 L of experimental seawater solution and equipped with an air pump to allow water motion and oxygenation, maintained at 36.5 salinity, 8.1 pH, 12h:12h light:dark photoperiod with an irradiance of about 135 μmol photons m^{-2} s^{-1}. The 10 experimental tanks were allocated in a water bath (composed of three 400 L tanks) to maintain the temperature stable at 20 °C. Coral nubbins were exposed to the experimental conditions for 93 h, without being fed for the entire treatment period. Afterward, nubbins were cut with tongs to detach them from the epoxy resin and placed in 15 mL tubes. Half of them (N = 30) were immediately frozen in liquid nitrogen and subsequently stored at −80 °C for coral host biochemical analyses, while the remaining nubbins (N = 30) were immediately stored at −20 °C for symbiotic algae content analyses (Fig. 1B).

2.4. Ashes chemical characterization

The pH and conductivity measurements of wildfire ashes were conducted on an aqueous suspension of air-dried ashes using a 1:10 ash-to-water ratio, as suggested by Šulc et al. (2022) for determinations on ash samples. For each sample, 5 g of ashes were mixed with 50 mL of distilled water (Šulc et al., 2022). The mixture was homogenized for 2 h and then filtered through Whatman filter paper No. 42 (Úbeda et al., 2009). The pH value of the extracts was measured using a calibrated pH meter (pH50 VioLab), while the electrical conductivity was assessed with a COND 51+ instrument.

Total C, N, and S content was determined using an NCS elemental analyzer (NA 1110 Nitrogen/Carbon/Sulfur Analyzer, Carlo Erba Instruments). Measurements were conducted on aliquots of 0.5 mg of pulverized and dried ash samples placed in silver capsules. The same procedure was used to measure total organic C in ash samples previously treated with hydrochloric acid (HCl). Specifically, 40 μL of a 10 % HCl solution diluted 1:10 was added to each sample. Samples were allowed to stand overnight, after which an additional 40 μL of HCl solution was added. These samples were then left to sit for 4 h. Subsequently, the capsules were placed on a heating plate, heated to 65 °C, and dried for 3 h. After cooling, the capsules were sealed and inserted into the sampler of the elemental analyzer. The calcium carbonate content of the ashes was estimated based on the difference between total and organic carbon (Harris et al., 2001).

We additionally quantified seven metals (Cd, Cu, Fe, Mn, Ni, Pb, and Zn) since various concentrations of these elements have been demonstrated to have beneficial or detrimental effects on the physiology of corals and their symbiotic algae (*e.g.*, Ferrier-Pagés et al., 2011; Montalbetti et al., 2021; Reich et al., 2023). As described in paragraph 2.2, three independent samples of ashes taken from the main reservoir, also used for the aquaria experiments (approximately 100 mg each), were hot digested in Teflon vials with 8 mL of nitric acid (65 % HNO_3) and 2 mL of hydrogen peroxide (40 % H_2O_2) in specifically designed mineralizer Anton Paar Multiwave 5000 microwave ovens. After digestion, samples were transferred to 50 mL plastic tubes and diluted in deionized water. Analyses of the trace elements Cd, Cu, Fe, Mn, Ni, and Zn were carried out using the Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES Optima 7000 DV PerkinElmer), following the protocol described by Pancaldi et al. (2019). For Pb quantification, the Atomic Absorption Spectrophotometry with Graphite Furnace (GFAAS) PerkinElmer Analyst 600 was used.

2.5. Coral host oxidative stress analyses

Coral fragments stored at −80 °C were used to conduct biochemical analyses on biomarkers of oxidative stress and oxidative damage. Samples were crushed using a pre-cooled mortar and pestle, then transferred to tubes and homogenized in a lysis buffer consisting of 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 10 % glycerol, 1 % NP40 detergent, and 5 mM EDTA, along with a protease inhibitor cocktail, and PMSF (phenylmethylsulfonyl fluoride), as previously described (Montalbetti et al., 2022, 2023). After centrifugation at 14000 rpm at 4 °C for 15 min to remove any skeletal debris, the supernatant was divided into aliquots for protein quantification and biomarker analyses,

respectively. Aliquots not immediately processed were stored at −80 °C for subsequent analyses.

Total protein content was determined using Bradford's colorimetric detection and spectrophotometric quantification method (Jasco V-730 UV–Vis Spectrophotometer), employing bovine serum albumin (BSA) as a calibration standard (Bradford, 1976). The activities of superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) were analyzed as previously described (Isa et al., 2025).

To measure SOD activity, which competes with ferricytochrome c for reactions with oxygen radicals, the capacity of SOD to inhibit the reduction of ferricytochrome c by O_2^- (generated in the xanthine/xanthine oxidase system) was evaluated. The reaction mixture contained ferricytochrome c (0.01 mM), EDTA (0.1 mM), xanthine (0.01 mM), and xanthine oxidase (0.0061 U) in a final volume of 1 mL, with all reagents sourced from Sigma-Aldrich. The reduction rate of ferricytochrome c was monitored spectrophotometrically at 550 nm (Jasco V-730 UV–Vis Spectrophotometer). Under these conditions, one unit of SOD activity was defined as the amount of enzyme that inhibits 50 % of the reduction of ferricytochrome c. Results are reported as enzyme units (U) per mg of protein.

To analyze CAT activity, which acts on hydrogen peroxide (H_2O_2), a reaction solution containing 50 mM sodium phosphate buffer (pH 7.5) and 12 mM H_2O_2 was added to a 1 mL cuvette with varying sample volumes. The decrease in H_2O_2 concentration was measured spectrophotometrically at 240 nm using a Varian Cary 50 Scan spectrophotometer (Agilent Technologies). Results are expressed as units (U) of enzyme per mg of protein, where U refers to k, the first-order kinetic constant (min^{-1}), as described by Aebi (1984).

The enzymatic activity of glutathione reductase (GR) was evaluated as described by Wang et al. (2001). GR activity was determined by spectrophotometrically measuring the absorbance at 340 nm (Jasco V-730 UV–Vis Spectrophotometer) of NADPH oxidation to NADP+, which occurs alongside glutathione reduction. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.6), 0.16 mM NADPH, 1 mg mL^{-1} BSA, and 4.6 mM oxidized glutathione, to which varying volumes of the sample were added. GR activity was calculated from the difference in absorbance values. One unit of GR activity was defined as the oxidation of 1 nmol NADPH min^{-1} at 25 °C. Results are expressed as units (U) of enzyme per mg of protein.

Lipid peroxidation (LPO) was assessed through malondialdehyde (MDA) quantification using an MDA assay kit (Bioxytech LPO-586, Oxis International), based on the reaction of N-methyl-2-phenylindole with MDA. Gastrodermis samples were pulverized and homogenized in 1 mL of 20 mM phosphate buffer (pH 7.4), with 0.5 M butylated hydroxytoluene added in acetonitrile to prevent oxidation. Following centrifugation at 3000×*g* at 4 °C for 10 min, the assay was performed according to the manufacturer's instructions. The resulting blue product was quantified by measuring absorbance at 586 nm (Jasco V-730 UV–Vis Spectrophotometer) (Erdelmeier et al., 1998). Results are presented in μmol MDA per μg of protein.

2.6. Chlorophyll *a* and *c2* content and Symbiodiniaceae density assessment

The response of coral-associated algae Symbiodiniaceae was assessed by quantifying the concentrations of chlorophyll *a* and *c*2 per algal cell and the algal cell density per polyp. To determine chlorophyll concentration, each sample was ground in 5 mL of ice-cold phosphate-buffered saline (Voolstra et al., 2020), centrifuged (3600×*g* for 4 min), and further homogenized using a syringe and needle. Aliquots (1 mL) were stored at −20 °C and fixed with 4 % formalin for Symbiodiniaceae counts (Ladrière et al., 2014; Louis et al., 2016). The remaining pellet was extracted in 100 % acetone for 24 h at 4 °C in the dark. After centrifugation, the supernatant was analyzed for chlorophyll *a and c2* by measuring absorbance (Jasco V-730 UV–Vis Spectrophotometer) at 630, 663, and 750 nm, and concentrations were calculated using dinoflagellate-specific equations (Jeffrey and Humphrey, 1975). Due to the phaceloid structure of *C. caespitosa*, chlorophyll values were normalized per polyp (Meron et al., 2012).

To evaluate algal cell density, Symbiodiniaceae cell counts were performed in six independent counts using Improved Neubauer hemocytometers under an optical microscope (Leica). Cell densities were standardized per polyp following (Meron et al., 2012).

2.7. Statistical analyses

To evaluate significant differences in antioxidant enzyme activities (SOD, CAT, GR), lipid peroxidation (LPO), symbiont density, chlorophyll contents (Chl *a* and Chl *c2*), and chlorophyll concentration per symbiont cell among the four ash concentrations, separate one-way ANOVAs followed by Tukey's HSD post hoc tests were used when assumptions of normality and homogeneity of variance were met. Data normality and homogeneity of variances were verified using the Shapiro–Wilk test, and when assumptions were violated, appropriate transformations were applied. When transformation did not satisfy assumptions, the non-parametric Kruskal–Wallis test followed by Dunn's post hoc comparisons with Bonferroni correction was applied. All univariate statistical analyses were performed using SPSS version 29 (IBM), with statistical significance set at $p < 0.05$. Data are presented as arithmetic means ± SE (n = 6 for each biomarker), unless stated otherwise.

Additionally, a multivariate analysis was performed using PRIMER-E v.7 (Clarke KR and Gorley RN, 2015) with the PERMANOVA + add-on (Anderson et al., 2008) to assess the combined modulation of all biomarkers in response to increasing ash concentrations. Biomarker data were normalized and square-root transformed to calculate a Bray–Curtis similarity matrix. A non-parametric permutational multivariate analysis of variance (PERMANOVA) was performed with ash concentration as a fixed factor, using 999 permutations. Pairwise PERMANOVA tests were conducted to identify significant differences between individual ash treatments, and due to the limited number of unique permutations, *p*-values were also obtained from Monte Carlo samplings (Anderson and Robinson, 2003).

3. Results

3.1. Ash chemical characterization

The chemical characterization of wildfire ash revealed a strongly alkaline material (pH 9.9 ± 0.1) with high electrical conductivity (8029.5 ± 32.9 μS cm^{-1}) (Table 1A). Ash samples showed elevated carbon content (40.3 ± 0.01 %), of which 18.7 ± 0.17 % was organic, and 21.6 ± 0.18 % was present as $CaCO_3$ equivalents. Nitrogen and sulfur contents were comparatively low, with mean values of 0.41 ± 0.01 % and 0.012 ± 0.001 %, respectively.

Metal content analysis of wildfire ash revealed high concentrations of Fe (29,439.5 ± 944.5 ppm; 3013.3 ± 109.1 μg g^{-1}) and Mn (987.4 ± 66.6 ppm; 101.0 ± 6.5 μg g^{-1}), while Zn (385.2 ± 56.4 ppm; 57.5 ± 7.4 μg g^{-1}), Ni (76.2 ± 2.5 ppm; 7.8 ± 0.3 μg g^{-1}), and Cu (15.6 ± 0.1 ppm; 1.6 μg g^{-1}) were detected at comparatively lower levels (Table 1B). No traces of Cd and Pb were found in the ash samples through our analyses.

3.2. Antioxidant enzyme activity

To evaluate oxidative stress levels in corals exposed to varying concentrations of wildfire ash, the activity of three antioxidant enzymes commonly involved in ROS detoxification was measured. The antioxidant activity of SOD, CAT and GR showed statistically significant differences across the various treatments (One-way ANOVA, $F(4,12) = 5.481$, $p = 0.01$ for SOD; Kruskal-Wallis test, $H(4, 12) = 9.673$, $p = 0.046$ for CAT; One-way ANOVA, $F(4,12) = 5.728$, $p = 0.008$ for GR), (Fig. 2).

In particular, corals exposed to ash concentrations of 0.5 and 2.5 g

Table 1
(A) Chemical characterization of wildfire ashes used in this study: pH, electric conductivity (EC), total carbon (C_{tot}), organic carbon (C_{org}), calcium carbonate equivalent ($CaCO_3$ eq), nitrogen (N), sulfur (S). Data are expressed as mean ± SE (n = 3). **(B)** Metal content analysis of wildfire ashes used in this study: Copper (Cu), Iron (Fe), Manganese (Mn), Nickel (Ni), Zinc (Zn), Cadmium (Cd), and Lead (Pb). Ppm and μg of metal per g of ashes are indicated as mean ± SE (n = 3). n.d. not detected. These data describe the overall composition of the ash material used to prepare the aqueous extracts and are intended to provide indicative information on potential chemical constituents, rather than representing the concentrations present in the dissolved experimental extracts.

A.

pH	EC (μS cm^{-1})	C_{tot} (%)	C_{org} (%)	$CaCO_3$ eq (%)	N (%)	S (%)
9.9 (±0.1)	8029.5 (±32.9)	40.3 (±0.01)	18.7 (±0.17)	21.6 (±0.18)	0.41 (±0.01)	0.012 (±0.001)

B.

	ppm	μg/g
Cu	15.64 ± 0.13	1.6
Fe	29,439.51 ± 944.5	3013.33 ± 109.14
Mn	987.38 ± 66.56	101 ± 6.49
Ni	76.19 ± 2.45	7.8 ± 0.31
Zn	385.23 ± 56.44	57.48 ± 7.39
Cd	n.d.	n.d.
Pb	n.d.	n.d.

L^{-1} exhibited reduced SOD activity compared to controls. At the highest concentration (5 g L^{-1}), SOD activity increased relative to the 0.5 and 2.5 g L^{-1} treatments, yet remained comparable to the control and 0.05 g L^{-1} groups (Fig. 2). CAT activity peaked in the control treatment, after which a progressive decline was observed. Significantly lower CAT activity was recorded at ash concentrations of 0.5 and 5 g L^{-1}, while exposures to 0.05 and 2.5 g L^{-1} induced a non-significant reduction relative to the control (Fig. 2). GR activity reached its maximum at the highest ash concentration tested (5 g L^{-1}), showing a significant increase compared to the control. Interestingly, exposure at 0.5 g L^{-1} did not differ significantly from the control, whereas the 0.05 and 2.5 g L^{-1} treatments produced a non-significant increase (Fig. 2).

3.3. Lipid peroxidation

To assess oxidative damage in intracellular lipid structures, lipid peroxidation was quantified through malondialdehyde (MDA) levels. Malondialdehyde concentrations showed statistically significant difference across treatments (One-way ANOVA, $F(4,15) = 10.599$, $p < 0.001$) (Fig. 3), increasing with ash exposure, with significantly higher values detected at 2.5 and 5 g L^{-1}. In contrast, no significant changes were observed at the lower concentrations of 0.05 and 0.5 g L^{-1} (Fig. 3).

3.4. Symbiont density and chlorophyll quantification

To evaluate the effect of exposure to different ash concentrations on the density of algal symbionts within coral tissues, Symbiodiniaceae cell content was quantified relative to single coral polyps (n = 6). *Symbiodiniaceae density* differed significantly among treatments (Kruskal-Wallis test, $H(4,18) = 13.409$, $p = 0.009$) (Fig. 4A). The control treatment exhibited the highest *Symbiodiniaceae density*. A significant reduction was already observed at 0.05 g L^{-1}, with a slight, though non-significant, recovery at 0.5 g L^{-1}. Algal symbiont densities remained significantly low in treatments relative to the controls at 2.5 and 5 g L^{-1} (Fig. 4A).

To assess chlorophyll concentrations in the algal symbionts, Chl *a* and Chl *c2* contents were measured relative to single coral polyps (n = 6). Both pigments showed significant differences across treatments (Chl *a*: One-way ANOVA, $F(4,12) = 11.602$, $p < 0.001$; Chl *c2*: One-way ANOVA, $F(4,12) = 12.439$, $p < 0.001$), (Fig. 4A). Chlorophyll *a* concentrations increased with ash concentration and reached significantly higher levels than the control at 2.5 and 5 g L^{-1}. Similarly, Chl *c2* concentrations increased with ash exposure, with significantly higher values observed at 5 g L^{-1} compared to the control (Fig. 4A).

To evaluate the adaptive capacity and photoacclimation of symbionts under experimental conditions, Chl *a* and Chl *c2* concentrations were normalized to symbiont cell density, obtaining chlorophyll concentration per Symbiodiniaceae cell (Cunning and Baker, 2014). Significant differences were detected on a per-cell basis for both Chl *a* (Kruskal–Wallis test, $H(4,15) = 10.775$, $p = 0.029$; Fig. 4B) and Chl *c2* (Kruskal–Wallis test, $H(4,15) = 12.140$, $p = 0.016$; Fig. 4C). Specifically, Chl *a* content per symbiont cell was significantly higher than the control at the two higher ash concentrations (2.5 and 5 g L^{-1}), (Fig. 4B). Chlorophyll *c2* content per symbiont cell followed a similar trend, with values significantly higher than both the control and the 0.5 g L^{-1} treatment at the maximum ash concentration of 5 g L^{-1}, (Fig. 4C).

3.5. Multivariate analysis

PERMANOVA revealed a significant effect of ash concentration on the overall physiological profile of *C. caespitosa* (Pseudo-$F = 13.077$, $p = 0.001$, 999 permutations) (Table 2). Pairwise tests showed significant differences between the control and all ash treatments ($p = 0.001$–0.042), as well as between several intermediate and high concentrations, including 0.05 vs 0.5 g L^{-1} ($p = 0.005$) and 0.5 vs 5 g L^{-1} ($p = 0.021$), (Table 3). Some comparisons among higher concentrations, such as 0.5 vs 2.5 g L^{-1} ($p = 0.222$) and 2.5 vs 5 g L^{-1} ($p = 0.139$), were not significant. Average Euclidean distances indicated the greatest divergence between the control and the highest ash concentration (5 g L^{-1}).

4. Discussion

Wildfires in coastal areas of the Mediterranean Sea represent an emerging threat not only to terrestrial ecosystems but also to adjacent marine environments, due to the substantial production and subsequent deposition of combustion-derived solid residues into the sea (Ruffault et al., 2020; Jesus et al., 2025). Several extreme wildfire events that have occurred in recent years across Mediterranean countries have reached the scale of so-called *megafires*, and their recurrence appears to be closely linked to the increasing frequency of heatwaves observed over the past two decades (Tedim et al., 2018; Turco et al., 2019; Rossetti et al., 2022; Costa-Saura et al., 2025). This connection with climate change–related conditions suggested that, in the coming years, wildfires will pose an increasingly serious threat to both terrestrial and marine organisms, with escalating impacts on natural resources and biodiversity (Ruffault et al., 2020).

In this study, we focused on the potential effects of wildfire-derived ash on an important bioconstructor of Mediterranean benthic habitats, the scleractinian coral *Cladocora caespitosa*, an endemic species already threatened by multiple stressors (Jiménez et al., 2016; Kersting et al., 2013, 2023). In particular, we assessed the impact of aqueous ash

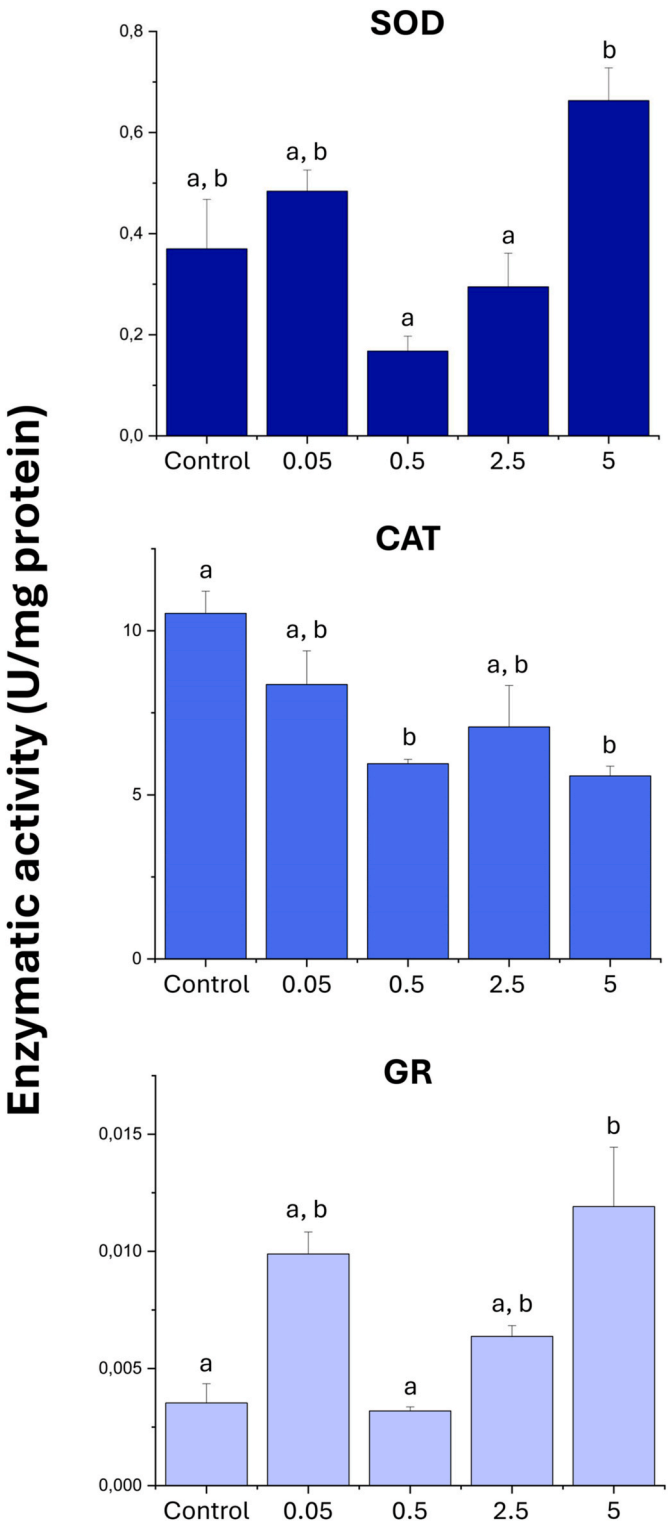

Fig. 2. Enzymatic activity of SOD, CAT, and GR detected in samples of *C. caespitosa* exposed to different ash concentrations (Control; 0.05; 0.5; 2.5; 5 [g L^{-1}]). Data are expressed as enzymatic units (U) per mg of protein and as mean ± SE ($n = 6$). Lower-case letters indicate significant differences between corals maintained under different conditions ($p < 0.05$).

extract at environmentally relevant concentrations on the cellular and physiological profile of the coral by using oxidative stress biomarkers and bleaching parameters as indicators/proxies of coral health status (Jesus et al., 2023; Muñiz González et al., 2023; Santos et al., 2023).

To assess the level of oxidative stress induced by the presence of ash

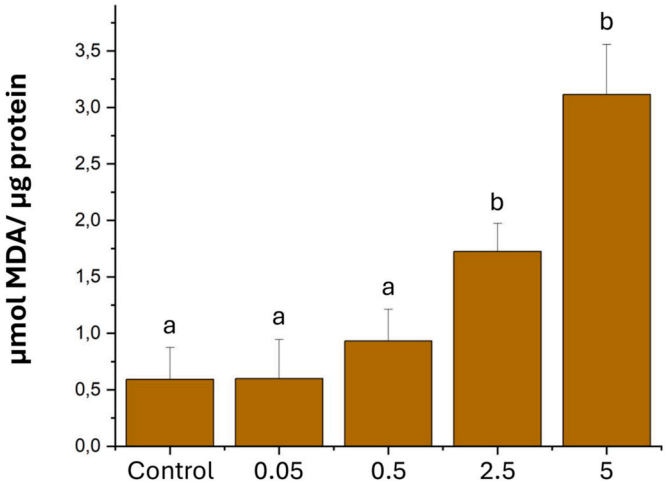

Fig. 3. Levels of lipid peroxidation (LPO) detected in samples of *C. caespitosa* exposed to different ash concentrations (Control; 0.05; 0.5; 2.5; 5 [g L^{-1}]). Data are expressed as µmol of MDA per µg of proteins and as mean ± SE ($n = 6$). Lower-case letters indicate significant differences between corals maintained under different conditions ($p < 0.05$).

in seawater on *C. caespitosa*, we examined the activity of three key antioxidant enzymes: SOD, CAT, and GR. SOD activity displayed fluctuating levels, with low activity at lower concentrations and increased activity at the highest concentration, although none of these conditions were significantly different from the control. SOD represents a primary defense mechanism against the oxidative action of the superoxide anion (O_2^-), an extremely reactive molecule produced physiologically during metabolic processes and in response to environmental stressors in corals (Yakovleva et al., 2004; Montalbetti et al., 2021). This enzyme is able to respond rapidly to changes in the intracellular redox state and is considered the first line of defense against oxidative stress (Nielsen et al., 2018). The fluctuating pattern of SOD activity may be attributable to several factors. First, our analysis focused on the total enzymatic activity converting superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2), without distinguishing between the responses of the different isoforms physiologically present at the cellular level. Previous studies have identified the occurrence of distinct isoforms, including Cu/Zn-SOD, Mn-SOD, and Fe-SOD, which vary in prevalence across compartments of the coral holobiont and the symbiotic zooxanthellae, both at the protein and transcriptomic levels (Richier et al., 2003; Krueger et al., 2015). In this context, the reduction in zooxanthellae density observed even at the lowest ash concentrations implied a concomitant loss of symbiont-associated SOD isoforms. Thus, the complex and non-linear SOD response may reflect the differential activity of its isoforms, combined with the cellular adjustments required to cope with prolonged stress, altered redox homeostasis, and the downstream physiological consequences of symbiont loss (Dixon et al., 2002). Secondly, SOD is known to exhibit a hormetic response, whereby enzymatic activity increases under moderate stress but does not necessarily rise further with intensifying stress, due to the onset of compensatory mechanisms or metabolic limitations (Lesser, 2006; Calabrese and Mattson, 2017). Indeed, SOD generally exhibits relatively narrow fluctuations compared to other antioxidant enzymes, reflecting its role as a frontline, yet highly conserved, defense mechanism (Downs et al., 2002; Ainsworth et al., 2008). Furthermore, the accumulation of H_2O_2, resulting from the enhanced dismutation of superoxide, may generate feedback inhibition if downstream detoxification enzymes, such as CAT, are unable to effectively neutralize it, thereby limiting further increases in SOD activity (Lesser, 2006). Indeed, our results for CAT showed a general decreasing trend with increasing ash concentration. A decrease in CAT under conditions of elevated oxidative stress has previously been reported in corals, attributed to both direct damage to the enzyme and

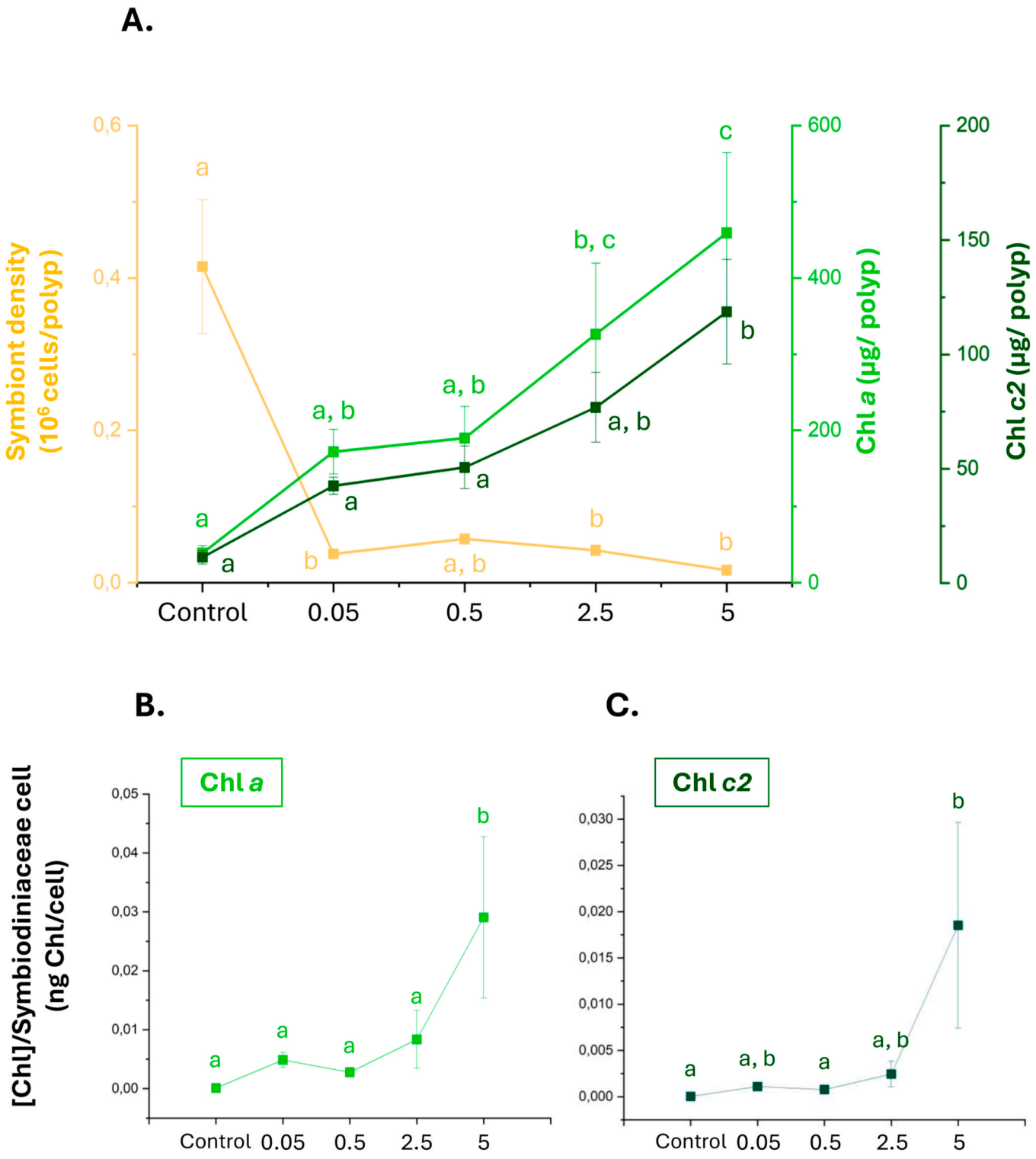

Fig. 4. – **(A)** *Symbiodiniaceae density* (dark yellow), and concentration of chlorophyll *a* (light green) and *c2* (dark green) in *C. caespitosa* following exposure to different ash concentrations (Control; 0.05; 0.5; 2.5; 5 [g L^{-1}]). **(B)** Concentration of Chl *a* and **(C)** Chl *c2* per Symbiodiniaceae cell. Data are expressed as mean ± SE ($n = 6$). Lower-case letters indicate significant differences between corals maintained under different conditions ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

metabolic limitations that compromise its proper functioning (Lesser, 2006). Since CAT primarily catalyzes the dismutation of H_2O_2 into H_2O and O_2, inhibition of CAT can lead to H_2O_2 accumulation, which must then be managed by other antioxidant systems, such as the glutathione pathway (Downs et al., 2002; Galasso et al., 2021). This compensatory mechanism was reflected in our GR activity results, where a significant increase was observed at the highest ash concentration. GR acts to maintain reduced glutathione (GSH), a tripeptide that is oxidized by ROS in place of biological macromolecules, providing essential cellular protection (Couto et al., 2016; Montalbetti et al., 2021). The observed dose-dependent increase in GR activity could be interpreted as an attempt to compensate for deficits in primary antioxidant defenses, such

Table 2
Results of the multivariate PERMANOVA analysis testing the effects of the different ash concentrations (Control; 0.05; 0.5; 2.5; 5 [g L^{-1}]) on SOD, CAT, GR, LPO, *Symbiodiniaceae density*, and concentration of Chl *a* and Chl *c2* obtained by permutations (perm) for each group. Significant *p*-values, P (perms), are in bold.

Source	df	SS	MS	Pseudo-F	P (perm)	Unique perms	P (MC)
Ash Concentrations	5	2698.3	674.56	13.077	**0.001**	998	**0.002**
Res	21	1083.3	51.585				
Total	26	3781.5					

Table 3
Results of the PERMANOVA pairwise comparisons between different ash concentrations obtained by permutations (perm) for each group. Significant *p*-values, both P (perms) and Monte Carlo P (MC), are in bold.

Groups	t	P (perm)	perms	P (MC)
0 vs. 0.05	3.7436	**0.015**	313	**0.029**
0 vs. 0.5	3.3177	**0.029**	309	**0.0423**
0 vs. 2.5	4.3959	**0.005**	566	**0.0137**
0 vs. 5	5.4916	**0.001**	732	**0.0061**
0.05 vs. 0.5	2.8861	**0.043**	35	**0.005**
0.05 vs. 2.5	1.999	0.05	126	0.0714
0.05 vs. 5	2.8293	**0.007**	210	**0.017**
0.5 vs. 2.5	1.4343	0.251	126	0.2221
0.5 vs. 5	2.9472	**0.009**	210	**0.021**
2.5 vs. 5	1.6565	0.173	417	0.1393

as SOD and CAT. Transcriptomic studies have also shown that GR was upregulated in corals downstream of primary defense systems when these were inhibited or overwhelmed (Krueger et al., 2015).

Although we did not directly measure the bioaccumulation of metals such as iron (Fe), manganese (Mn), cadmium (Cd), nickel (Ni), copper (Cu), or zinc (Zn) in coral tissues in this study, it is important to consider their presence in ash, since these metals may further influence the observed antioxidant responses in *C. caespitosa*. Indeed, transition metals like Fe and Cu can participate in Fenton and Haber–Weiss reactions, generating highly reactive hydroxyl radicals (•OH) from H_2O_2, thereby amplifying oxidative stress (Valko et al., 2005; Lesser, 2006). Such metal-mediated ROS production can overload primary antioxidant defenses, contributing to the observed decrease in CAT activity at higher ash concentrations. In contrast, metals such as Mn and Zn serve as essential cofactors for specific SOD isoforms (Mn-SOD and Cu/Zn-SOD, respectively), potentially altering the balance between isoform activities under stress (Krueger et al., 2015). Excessive accumulation of non-essential metals, such as Ni, may directly or indirectly inhibit enzyme activity by displacing essential metal cofactors or inducing protein oxidation (Rainbow and Luoma, 2011; Sharma et al., 2012). To better understand how metal concentrations in wildfire ash affect the antioxidant response of corals, future studies should directly investigate bioaccumulation in coral tissues and broaden the range of enzymatic biomarkers examined. Indeed, the effects of additional antioxidant defenses, such as glutathione peroxidase (GPx) and glutathione S-transferase (GST), could be investigated. This would help to more accurately assess the impact of this pollution source on these biological pathways.

Overall, antioxidant enzyme activity results suggest that increasing wildfire ash concentrations may induce elevated oxidative stress that the cellular antioxidant mechanisms of *C. caespitosa* cannot effectively manage. Overwhelming of antioxidant defense mechanisms has often been associated with oxidative damage to cellular lipids and proteins, consistent with our findings showing an increase in lipid peroxidation levels as ash concentration rises (Luz et al., 2018; Dias et al., 2019). The increase in LPO levels in response to ash exposure could therefore reinforce the hypothesis that exposure to high ash concentrations may induce oxidative stress in *C. caespitosa*. It is well known that contaminants in seawater can increase LPO levels in many tropical coral species, and these effects can be exacerbated by additional stressors (Bednarz et al., 2022; Isa et al., 2024). The presence of metals, in particular, appeared to have a significant effect on LPO status both in the host and the symbiont, as demonstrated for several tropical coral species, and this effect may vary depending on the metal and the coral species investigated (Kuzminov et al., 2013; Dal Pizzol et al., 2022). Chemical analyses of our wildfire ash revealed the presence of metals such as Fe, Zn, Cu, Ni, and Mn. The presence of Fe has been shown to increase LPO in tropical corals such as *Mussimilia hartii*, *Millepora alcicornis*, *Siderastrea* sp., and *Stylophora pistillata*, even at environmentally relevant concentrations (Montalbetti et al., 2021; Fukushima et al., 2025). Moreover, metal accumulation has been shown to alter the activity of enzymes involved in the antioxidant response (Dal Pizzol et al., 2022). In this context, the LPO response observed in our study may be partly linked to the increasing presence of metals released from ash in seawater. This hypothesis is particularly relevant considering the ability of *C. caespitosa* to compensate for reduced photosynthetic input from its symbionts by enhancing its heterotrophic metabolism and feeding on particulate matter suspended in the water column (Hoogenboom et al., 2010; Tremblay et al., 2011). Future studies should therefore investigate the actual capacity of this coral to accumulate metals through heterotrophic intake, in order to determine whether increasing ash concentrations correspond to elevated metal levels in the tissues of the holobiont. In addition, recent evidence supports the interpretation that wildfire ash introduces a chemically complex mixture of pollutants into coastal waters, extending beyond inorganic metals to include polycyclic aromatic hydrocarbons (PAHs) and additional combustion-derived organic compounds (Downs et al., 2024). Notably, PAHs are known to induce mitochondrial dysfunction, ROS overproduction, and lipid peroxidation in marine invertebrates, acting synergistically with metals to exacerbate oxidative stress (Xiang et al., 2018). It remains to be determined whether additional contaminants not specifically investigated in this study could provide a plausible mechanistic explanation for the substantial LPO increase and the increased antioxidant system activity we observed in *C. caespitosa*.

The presence of filtered ash extracts in seawater, even at the lowest concentration, also caused a significant and very rapid loss of algal symbionts in the investigated coral. *Cladocora caespitosa* is a temperate coral adapted to thermal and light regimes that can vary dramatically between summer and winter (Rodolfo-Metalpa et al., 2006a; Ferrier-Pagés et al., 2011). Several physiological processes in *C. caespitosa*, such as photosynthesis and calcification, have been shown to be more efficiently regulated in response to temperature fluctuations than to alterations in seawater chemistry, such as changes in pH, highlighting a strong adaptation to the environmental conditions of a temperate sea, such as the Mediterranean (Rodolfo-Metalpa et al., 2010). This suggests that chemical alterations of seawater, such as those reproduced in our experimental system, can rapidly generate a stress condition at the level of the holobiont. Moreover, *C. caespitosa* has been reported to exhibit a high degree of heterotrophic plasticity, providing an efficient energy source when autotrophic input from photosynthesis is compromised due to symbiont loss or limited due to reduced light conditions during cold seasons (Hoogenboom et al., 2010; Tremblay et al., 2011). Further studies should investigate whether the early loss of symbionts under stress conditions, as observed in this study and in previous reports, could represent not only a physiological limit, but an adaptation strategy of *C. caespitosa* to eliminate an additional source of stress arising from alterations in the symbiotic relationship with zooxanthellae (Rodolfo-Metalpa et al., 2008; Lesser et al., 2013).

Our results also suggested that corals may activate photoacclimation

mechanisms in response to turbidity caused by ash in seawater and the resulting reduced light availability for photosynthesis. In fact, our data showed that the significant reduction in symbiont density was accompanied by a marked increase in photosynthetic pigment concentration per symbiont cell at the highest ash concentration. This type of photoacclimation is well documented in tropical corals, as well as in *C. caespitosa*, whereby Symbiodiniaceae increase their pigment content to maintain photosynthetic efficiency under reduced light conditions (Falkowski and Dubinsky, 1981; Iglesias-Prieto and Trench, 1994; Rodolfo-Metalpa et al., 2008; Hoogenboom et al., 2012; Toniolo et al., 2025). However, an increase in pigment content may also exacerbate redox imbalance and promote the overproduction of reactive oxygen species (ROS), ultimately leading to oxidative damage (Warner et al., 1999; Lesser, 2006). This was particularly relevant in our study, considering that the increase in chlorophyll per cell occurred in parallel with a significant decrease in symbiont density. The overall outcome suggests that enhanced pigmentation may represent a compensatory mechanism to counterbalance the reduced symbiont population, but it may be insufficient to maintain the energetic balance of the coral holobiont, given the decrease in symbiont density relative to the control. Further investigations are needed to clarify whether the increase in pigment concentration observed here represents a functional physiological strategy of the holobiont, or rather a stress response of the symbionts with downstream consequences for the coral host.

Finally, PERMANOVA confirmed our hypothesis that wildfire ash concentration could exert a strong overall effect on the cellular homeostasis of *C. caespitosa*. The analysis indicated that the treatment effect accounted for the majority of variability among samples, underscoring that the observed changes in SOD, CAT, GR, LPO, and algal symbiont parameters were strongly dependent on ash presence and concentration across the holobiont. In addition, significant differences were observed between the control and all ash treatments, indicating that even the lowest concentration of 0.05 g L^{-1} induced measurable changes across multiple physiological parameters. The significant dilution that occurs when wildfire ash enters the marine environment necessitates investigating whether these physiological changes persist at concentrations below 0.05 g L^{-1}. Differences were also detected among intermediate and high concentrations (e.g., 0.05 vs 0.5 g L^{-1}, 0.5 vs 5 g L^{-1}), suggesting a progressive intensification of physiological stress with increasing wildfire ash concentrations. Collectively, these multivariate analyses confirmed that ash exposure can disrupt antioxidant defenses, enhance oxidative damage, and alter the homeostasis of the symbiosis.

In conclusion, our findings suggest that exposure to wildfire ash induced marked physiological stress in the Mediterranean coral *Cladocora caespitosa*. Taken together, the presented results provide novel insights into the vulnerability of *C. caespitosa* to ash inputs, identifying oxidative stress and symbiont destabilization as critical pathways of impact. Given the increasing frequency and intensity of wildfires in the Mediterranean region under climate change, our findings raise concern about an emerging and poorly understood threat to temperate coral populations. Future research should investigate the potential bioaccumulation of metals from ash-derived particles, the role of heterotrophic plasticity in mitigating the loss of autotrophic input, and the long-term capacity of *C. caespitosa* to recover from ash exposure, while monitoring metal concentrations across the different experimental phases. Furthermore, our study was limited to 93 h, simulating a short-term acute exposure. Therefore, it did not consider the long-term effects of ash exposure or the recovery capacity of corals, which remain to be fully understood. Addressing these aspects will be essential for understanding the resilience of this endangered coral species and for developing conservation strategies in the face of multiple, interacting anthropogenic stressors.

CRediT authorship contribution statement

Enrico Montalbetti: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Davide Seveso:** Writing – original draft, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Simone Farina:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Simone Bava:** Writing – review & editing, Resources, Investigation. **Elisa Carta:** Writing – review & editing, Investigation, Data curation. **Laura Castellano:** Writing – review & editing, Resources, Investigation. **Paolo Galli:** Writing – review & editing, Resources, Funding acquisition. **Valerio Isa:** Writing – review & editing, Methodology, Investigation. **Yohan Didier Louis:** Writing – review & editing, Methodology, Investigation. **Rossana Marzaioli:** Writing – review & editing, Investigation. **Davide Moccia:** Writing – review & editing, Resources. **Stefania Papa:** Writing – review & editing, Investigation. **Flora Angela Rutigliano:** Writing – review & editing, Resources, Investigation. **Laura Tamburello:** Writing – review & editing, Resources. **Roberto Arrigoni:** Writing – original draft, Resources, Methodology, Investigation, 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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Data availability

Data will be made available on request.

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