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Exposure of the seagrass Cymodocea nodosa to seawater contaminated by the pharmaceutical Ibuprofen: an analysis of the potential impact at multiple plant levels

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Abstract:	Pharmaceuticals such as ibuprofen entering marine environments are of great concern due to their increasing consumption and impact on aquatic organisms. Very little is still known about the toxicity of ibuprofen to marine photosynthetic organisms, and no information is available for foundation species like seagrasses, which are declining globally to anthropogenic factors. Here, the effects of short-term exposure (12 days) of the seagrass Cymodocea nodosa to environmentally realistic IBU concentrations (0.25-2.5-25 μg L−1) at multiple levels (plant growth, oxidative status, photosynthetic efficiency, and secondary metabolites content) were assessed in mesocosm. Chemical analyses to detect the presence of ibuprofen and its products in seawater medium and plants were also performed. Ibuprofen was undetected in plants and did not affect their growth but caused oxidative damage and altered antioxidant enzyme activity. At the highest concentration, ibuprofen also increased photosynthetic pigment content and damaged the photosynthetic machinery, particularly PSII and its donor side. Moreover, it halved phenolic acid and flavonoid content while increased rutin, gallic acid, and coumaric acid. These findings suggest that C. nodosa could tolerate short-term ibuprofen exposure through stress mitigation strategies, but they raise concern about the potential risk of a prolonged exposure on plant resilience and on associated ecosystems.
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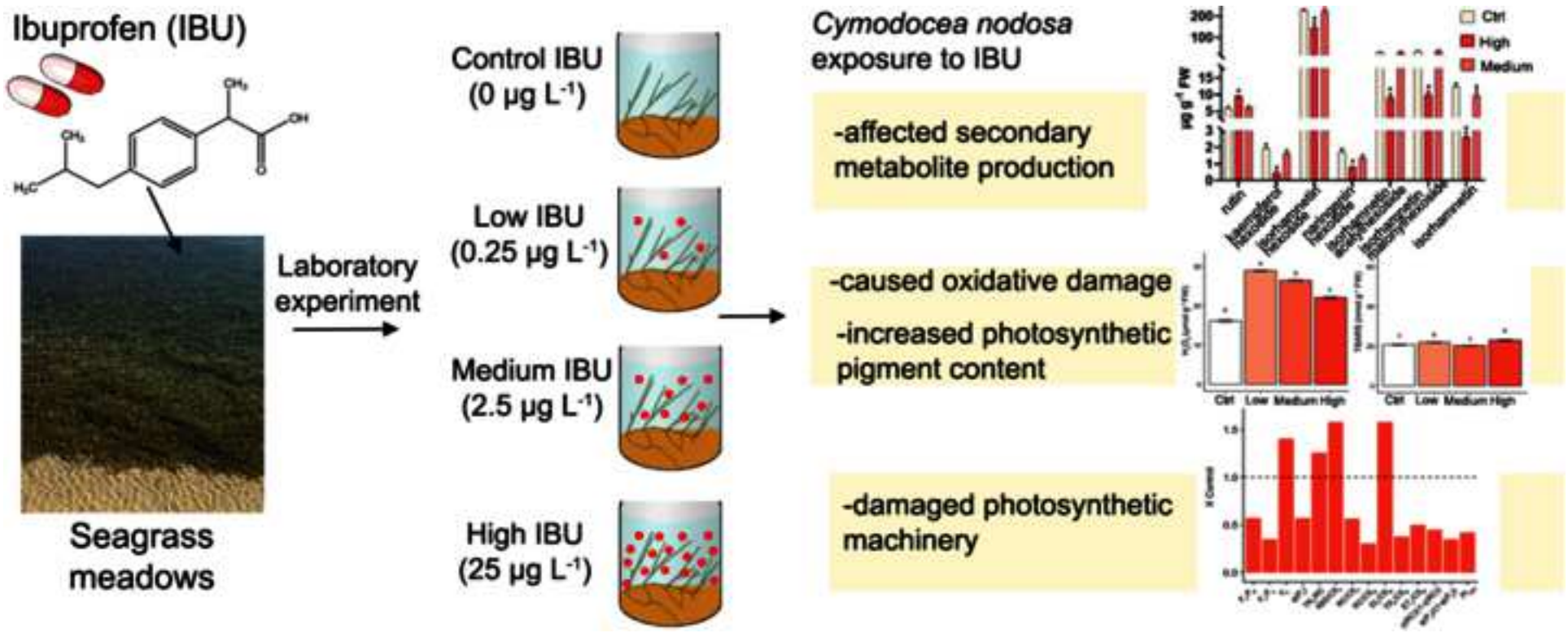
Environmental Implication (maximum limit:100 words)

Pharmaceuticals, such as ibuprofen, are considered as emerging contaminants due to their potential adverse effects on marine wildlife. This study is the first documenting the effects of environmentally relevant concentrations of ibuprofen detected in the Mediterranean Sea on marine plants (seagrasses), which play fundamental ecological roles, at multiple levels. It shows that short-term exposure of *Cymodocea nodosa* plants to ibuprofen, especially to the highest concentration, caused oxidative stress and photosynthetic machinery damage. These findings provide valuable insights for assessing the potential risk posed by a prolonged exposure to this pollutant to seagrasses and their resilience against environmental stressors.

Graphical Abstract



- Effects of seawater contamination by IBU on *Cymodocea nodosa* plants was assessed.
- IBU was detected in the growth medium but not inside plant tissues.
- IBU altered secondary metabolite production and caused oxidative stress in plants.
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- Prolonged exposure to high IBU amounts may reduce seagrass resilience.

Exposure of the seagrass *Cymodocea nodosa* to seawater contaminated by the pharmaceutical Ibuprofen: an analysis of the potential impact at multiple plant levels

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Abstract

Pharmaceuticals such as ibuprofen entering marine environments are of great concern due to their increasing consumption and impact on aquatic organisms. Very little is still known about the toxicity of ibuprofen to marine photosynthetic organisms, and no information is available for foundation species like seagrasses, which are declining globally to anthropogenic factors. Here, the effects of short-term exposure (12 days) of the seagrass *Cymodocea nodosa* to environmentally realistic IBU concentrations (0.25-2.5-25 μg L^{-1}) at multiple levels (plant growth, oxidative status, photosynthetic efficiency, and secondary metabolites content) were assessed in mesocosm. Chemical analyses to detect the presence of ibuprofen and its products in seawater medium and plants were also performed. Ibuprofen was undetected in plants and did not affect their growth but caused oxidative damage and altered antioxidant enzyme activity. At the highest concentration, ibuprofen also increased photosynthetic pigment content and damaged the photosynthetic machinery, particularly PSII and its donor side. Moreover, it halved phenolic acid and flavonoid content while increased rutin, gallic acid, and coumaric acid. These findings suggest that *C. nodosa* could tolerate short-term ibuprofen exposure through stress mitigation strategies, but they raise concern about the potential risk of a prolonged exposure on plant resilience and on associated ecosystems.

Environmental implication statement

Pharmaceuticals, such as ibuprofen, are considered as emerging contaminants due to their potential adverse effects on marine wildlife. This study is the first documenting the effects of environmentally relevant concentrations of ibuprofen detected in the Mediterranean Sea on marine plants (seagrasses), which play fundamental ecological roles, at multiple levels. It shows that short-term exposure of *Cymodocea nodosa* plants to ibuprofen, especially to the highest concentration, caused oxidative stress and photosynthetic machinery damage. These findings provide valuable

insights for assessing the potential risk posed by a prolonged exposure to this pollutant to seagrasses and their resilience against environmental stressors.

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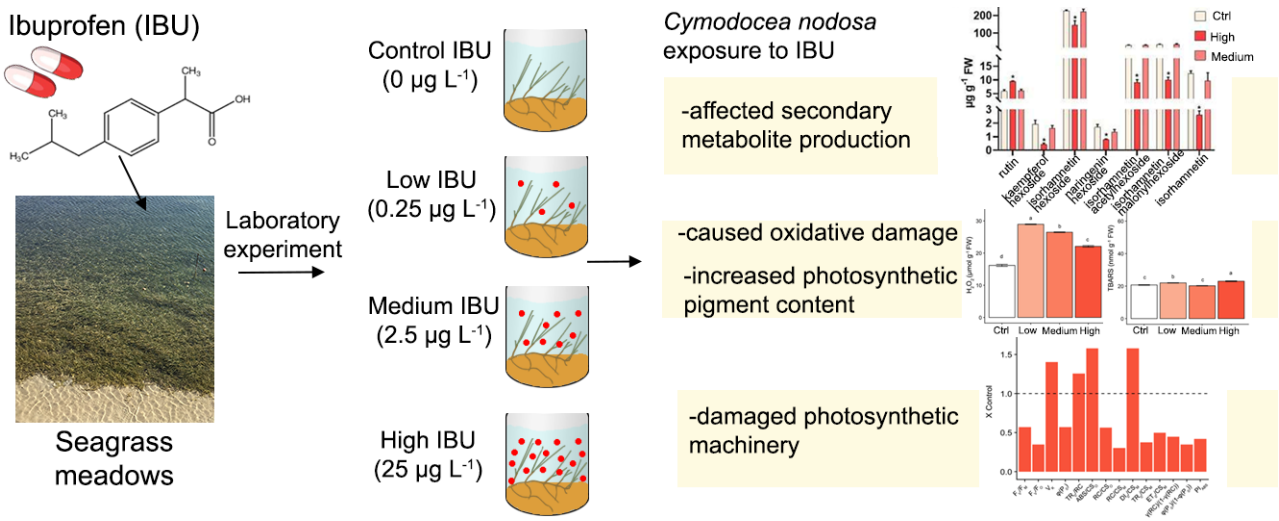

Highlights

- Effects of seawater contamination by IBU on *Cymodocea nodosa* plants was assessed.
- IBU was detected in the growth medium but not inside plant tissues.
- IBU altered secondary metabolite production and caused oxidative stress in plants.
- IBU damaged photosynthetic machinery, especially PSII, at high concentration.
- Prolonged exposure to high IBU amounts may reduce seagrass resilience.

1 Introduction

The presence of active pharmaceutical ingredients (APIs) as well as of their metabolites and degradation products in marine environments is an issue of increasing global concern due to their potential adverse effects, both in isolation and in combination with other global-change-related stressors, on aquatic organisms (Ankley et al., 2007; Branchet et al., 2021; Ibanez et al., 2021; Blasco and Trombini, 2023; Kock et al., 2023). These chemicals enter the marine environment continuously through various point and nonpoint sources (e.g., urban and hospital wastewater treatment plant effluents, water bodies, animal husbandry and aquacultures) where they are present at concentrations in the range of ng L^{-1}- µg L^{-1} due to their systemic use and lack of effective removal technologies (Madikizela et al., 2020; Alfonso-Muniozguren et al., 2021; Blasco and Trombini, 2023). Up to 600 pharmaceutical substances have been detected in seawater and marine sediments (Adeleye et al., 2022; Blasco and Trombini, 2023), and some of them have been included in the European Water Framework Directive "Watch-list" as potentially harmful (EU, 2013) even if a strict and comprehensive regulation is still lacking. Some pharmaceuticals have also been found in marine animals, like mollusks, crustaceans, and fish (Álvarez-Muñoz et al., 2015; Maranho et al., 2015; Świacka et al., 2019), as well as in macrophytes, such as macroalgae and seagrasses (Álvarez-Muñoz et al., 2015; Ali et al., 2018; Long et al., 2023). However, current knowledge on the toxicity of these chemicals to marine organisms is limited and mostly referred to few specific animal taxa and target species (Gonzales-Rey and Bebianno, 2011; Matozzo et al., 2012; de Orte et al., 2013; Mezzelani et al., 2018; Almeida et al., 2020; Silva et al., 2020; Mezzelani and Regoli, 2022; Świacka et al., 2022).

The few available information on the effects of pharmaceuticals on marine macrophytes as well as on their uptake mechanisms concerns macroalgae (Wiklund et al., 2011; Oskarsson et al., 2012; Ali et al., 2018). No study has dealt so far with the effects of these substances on marine angiosperms (seagrasses) to our knowledge. These plants are exposed to a wide range of chemicals, including sunscreen UV filters contained in personal care products and pharmaceuticals, since they

grow in shallow coastal areas even in proximity to urban and industrial effluents (Lewis and Devereux, 2009; García-Marquez et al., 2023; Li et al., 2023; Long et al., 2023). Some species like *Zostera marina* L., *Cymodocea nodosa* (Ucria) Ascherson, and *Posidonia oceanica* L. Delile are recommended as biological indicators for marine environmental quality assessment due to their capacity to bioaccumulate chemicals present in sediments and water column (Montefalcone, 2009; Marbà et al., 2013). However, seagrass populations are declining globally due to climate change and anthropogenic disturbances resulting in the loss of important ecological functions and multiple services they provide to humans (Boudouresque et al., 2009; Barbier et al., 2011; McMahon et al., 2022). Thus, assessing whether the occurrence of pollutants of emerging concern like pharmaceuticals in marine habitats may pose a further threat to these plants is crucial and will help in developing more effective seagrass conservation strategies and management interventions.

One of the most frequently detected pharmaceuticals in coastal surface seawaters is Ibuprofen (hereafter IBU). This compound, belonging to the therapeutic group of non-steroidal anti-inflammatory drugs (NSAIDs), has an estimated annual global consumption of over 10,000 metric tons and has attracted much attention in the last years because of its use during the COVID-19 pandemic (Wilton and Brant, 2013; Almeida et al., 2020; Wang et al., 2021; Ferreira et al., 2023). IBU can be introduced in aquatic environments both in its original form (i.e., the parent compound 2-(4-Isobutylphenyl) propionic acid) and in its metabolites generated after consumption by humans or animals (Rainsford, 2009). IBU has been found in European and Mediterranean coastal seawater at average concentrations of 1.5-2.5 μg L^{-1} (Togola and Budzinski, 2008; Loos et al., 2013; Mezzelani et al., 2018; Madikizela et al., 2020), and it is considered as a pseudo-persistent pollutant that can undergo degradation (half-life in freshwater is of 12 days; Ding et al., 2017) and phototransformation resulting in the formation of many intermediates (Vione et al., 2011). Lower concentrations (range of ng L^{-1}) have been observed in marine sediments probably due to the low sorption capacity of sediments for IBU at pH around 8 (Blasco and Trombini, 2023).

Studies on the effects of the seawater contamination by IBU on marine microalgae have shown that the exposure of the target species, the diatom *Pheodactylum tricornutum* Bohlin, to concentrations (100-300 µg L^{-1}) higher than those detected in natural marine environments resulted in a reduction of the growth rate, oxidative stress, and changes in the photosynthetic pathway functioning (Silva et al., 2020). Instead, no effect was found in the macroalga *Fucus vesiculosus* L., following the exposure to much higher IBU concentrations (up to 10,000 µg L^{-1}; Wiklund et al., 2011; Oskarsson et al., 2012). Studies on the impact of IBU on macrophytes inhabiting other aquatic environments, like saltmarshes, rivers, and lakes (Brain et al., 2004; Pomati et al., 2004; Iori et al., 2013; Pietrini et al., 2015; Li et al., 2016; Di Baccio et al., 2017; He et al., 2017) have shown that some species, including *Typha angustifolia* L., *Phragmites australis* (Cav.) Trin. ex Steud. and *Salix alba* L., can metabolize and degrade this compound via detoxifying reactions involving specific enzymes (e.g., cytochrome P450 monooxygenase, glycosyltransferase, and glutathione-S transferase) resulting in no adverse effects on plants (Li et al., 2016; He et al., 2017). However, a growth inhibition in *Lemna minor* L. and a growth stimulation in *Lemma gibba* L. was detected upon the exposure to a 1000 µg L^{-1} IBU concentration (Pomati et al., 2004; Pietrini et al., 2015).

The aim of this study was to evaluate the potential impact of seawater contamination by IBU on the performance of seagrasses using *C. nodosa* as a model. This species was selected because of its occurrence in shallow coastal areas often in proximity to freshwater inputs, fast growth rate and responsiveness to environmental changes and contaminants (e.g., metals and plastics; Cancemi et al., 2002; Agostini et al., 2003; Borum et al., 2004; Malea et al., 2018; Menicagli et al., 2021, 2022). Specifically, through a multidisciplinary approach involving plant morphological traits and growth measurements, qualitative and quantitative secondary metabolites analyses, oxidative stress evaluation (i.e., oxidative stress markers, antioxidant enzyme activity, and histochemical assay), and photosynthetic efficiency assessment, the effects of a short-term exposure (12 days) of plants to environmentally realistic IBU concentrations for the Mediterranean basin were explored.

2 Materials and Methods

2.1 Chemicals

Standard IBU (CAS number 15687-27-1) was purchased from Sigma Aldrich, Germany (purity ≥ 98%). Physico-chemical properties of IBU are reported in Table S1. A stock solution of IBU (10 mg L^{-1} in ethanol) was prepared and stored at room temperature in laboratory. Ultra High-Performance Liquid Chromatography (UHPLC) grade methanol, water, formic acid, and the standards rutin (≥ 98% purity) and chicoric acid (≥ 95% purity) were purchased from Merck KGaA (Darmstadt, Germany). Catechin standard was previously isolated and characterized by 1D- and 2D-NMR, and HR-MS techniques in authors laboratory from other plant extracts. All the analytical grade solvents were acquired from VWR (Milano, Italy).

2.2 Plant material and experimental set-up

In May 2023, *C. nodosa* plagiotropic rhizome fragments were harvested in a shallow site (0.5 m) located in the Ligurian Sea (Italy, 43°22′55.66″N, 10°26′7.05″E). Fragments were transported to the laboratory and cut into experimental plant units of homogenous size (7.05 ± 0.08 cm, mean ± SE, n=60, rhizome length; 4.18 ± 0.09 shoot number; 0.71 ± 0.43 root number). Each plant unit was gently placed into a mesocosm consisting of a glass culture vessel (0.5 L) containing natural seawater (NSW, pH = 8.13 ± 0.03, practical salinity unit = 38.03 ± 0.03) and a layer (3 cm) of silica sand (grain size 0.5–1 mm, density 1.6 g mL^{-1}, and less than 0.01% of organic matter content), previously washed and sterilized. NSW was provided by INVE Aquaculture Research Center of Rosignano Solvay and filtered as described in Menicagli et al. (2022) before its use. A commercial NPK fertilizer (Cifo, 20-10-10, 0.44 g L^{-1}) was added in each mesocosm to sustain plant growth. All mesocosms were placed in a culture chamber at environmental conditions like those experienced by plants in their natural habitat at the time of collection (20 °C, 12/12h light/dark regime, photosynthetic photon flux density 110-150 μmol m^{-2} s^{-1}) and left undisturbed for 7 days for plant acclimatization. At the end of the acclimatization, the NSW in each mesocosm was renewed, and

each mesocosm was assigned to one of the four nominal IBU seawater concentrations (0 μg L^{-1} or Control, 0.25 μg L^{-1} or Low, 2.5 μg L^{-1} or Medium, 25 μg L^{-1} or High) mimicking those detected in European coastal seawaters (average concentration of 1.5-2.5 μg L^{-1}; Togola and Budzinski, 2008; Loos et al., 2013; Mezzelani et al., 2018; Madikizela et al., 2020). Such concentrations were achieved by dissolving appropriate aliquots of the IBU stock solution in the NSW added to mesocosms. There were 15 replicates for each treatment (60 plants in total). Before assigning plants to the IBU treatments, their morphological variables (number of shoots, average number of leaves per shoot, length of the longest leaf on the apical shoot, and rhizome length) were recorded. Additional mesocosms (12) containing sand, NSW and IBU at the same four concentrations as described above but without the plant unit inside were also prepared (3 replicates per each treatment) and used as blank. Plants were maintained under the same culture conditions as those experienced during the acclimatization period. The NSW was renewed after 7 days from the experiment beginning and spiked with aliquots of the IBU stock solution according to the nominal concentrations. Plants were daily inspected, and the mesocosms were reallocated spatially to avoid position effects. At the end of the experiment (after 12 days of exposure to IBU), ten plants per each treatment were collected and used for morphological trait and growth measurements (section 2.3), detection of IBU and qualitative and quantitative analyses of specialized plant metabolites (sections 2.4 and 2.5), and oxidative stress evaluation (i.e., oxidative stress markers, antioxidant enzyme activity, and histochemical assay; from section 2.6 to 2.8). The remaining five plants per each treatment were used for measurements of the effect of IBU exposure on the photosynthetic efficiency (section 2.9). NSW samples were also collected from mesocosms and analyzed to detect IBU and its main metabolites (section 2.4 and 2.5).

2.3 Plant morphological traits and growth measurements

At the end of the experiment, the number of survived plants in each treatment was determined and their morphological variables were measured. The net change in shoot number for each plant was

calculated as the difference between the number of newly produced shoots and that of dead shoots within the experimental exposure period. The net change in leaf number was calculated as the difference between the number of newly produced leaves and that of detached leaves averaged across all the standing shoots present on each plant within the experimental exposure period. Leaf (or rhizome) elongation was calculated as the difference between the final length and the initial length of the longest leaf on the apical shoot (or the rhizome) over the initial length and expressed as a percentage.

2.4 Preparation of NSW and plant extract samples for chemical analyses

Before assigning plants to IBU treatments, NSW samples (50 mL) were collected from mesocosms containing only NSW (negative control) or IBU at high, medium, and low concentration. IBU-supplemented NSW samples were also collected at the end of the experiment from mesocosms containing *C. nodosa* plants as well as from blank mesocosms (without *C. nodosa* plants). All NSW samples were evaporated under vacuum (Buchi Rotavapor®, Milano, Italy) and the residues were partitioned with *n*-BuOH/H_2O (1:2 v/v) to remove salts and centrifuged for 5 min at 2710 × g. The *n*-butanol fractions were subjected to vacuum drying and finally dissolved in 100 µL of methanol for liquid chromatography (LC) coupled to mass spectrometry (MS) analyses. The whole *C. nodosa* plants (1 g), grown in mesocosms without IBU (i.e., control) and containing the different IBU concentrations, were subjected to extraction with 5 mL of methanol for 15 min by ultrasonic bath LBS2 (Falc Instruments s.r.l., Treviglio, Italy) and finally centrifuged for 5 min at 2710 × g. The supernatants were transferred into vials to be injected into the LC-MS system.

2.5 Ibuprofen-targeted and quali-quantitative liquid chromatography-high-resolution mass spectrometry (LC-HR-MS) analyses of plant specialized metabolites

The solutions (5 µL) obtained from NSW and plant samples were injected into an UHPLC coupled with a diode array detector (DAD) and a high resolution (HR) Q Exactive Plus Orbitrap MS, equipped

with an electrospray ionization (ESI) source and a hybrid quadrupole analyzer (Thermo Fischer Scientific Inc., Bremem, Germany). The chromatographic runs were performed by using a Kinetex® Biphenyl C-18 column (2.1 x 100 mm, 2.6 µm) equipped with a Security Guard™ Ultra Cartridge (Phenomenex, Bologna, Italy) at a flow rate of 0.5 mL min^{-1}. The autosampler and the column oven were maintained at a temperature of 4 °C and 35 °C, respectively. As a mobile phase, a mixture of HCOOH/H_2O 0.1% v/v (solvent A) and HCOOH/MeOH 0.1% v/v (solvent B) was chosen and a linear gradient was used, increasing from 5 to 80% B in 20 min. The ESI-HR mass spectra were recorded in negative and positive ion modes, operating in Parallel Reaction Monitoring (PRM) for IBU detection. Standard solutions of IBU were used as reference standards, with IBU detected both in negative ($[M-H]^-$ at *m/z* 205.1228; $[M+H]^+$ at *m/z* 207.1376, Figure S1). For the analysis of specialized metabolites, a scan range of *m/z* 135-2000 was applied, recording MS both in full (70000 resolution, 220 ms maximum injection time) and data dependent-MS/MS scan (17500 resolution, 60 ms maximum injection time). UV data were recorded in a range of 200–600 nm, using 254, 280 and 325 nm as preferential channels. Nebulization voltage of 3500 V, capillary temperature of 300 °C, sheath gas (N_2) 20 arbitrary units, auxiliary gas (N_2) 3 arbitrary units, HCD (Higher-energy C-trap dissociation) of 18 eV were applied as ionization settings (Cioni et al., 2024). IBU and its main metabolites (hydroxyibuprofen, dihydroxyibuprofen, ibuprofen glucoside, and carboxyibuprofen), possibly produced by phase I and II metabolism of plants (He et al., 2017), were compared among samples by considering the areas of the extracted ion peaks from the chromatograms obtained by LC-MS analyses. The specialized metabolites in the plant extracts were quantified by using three pure external standards such as chicoric acid, rutin and catechin. Triplicate solutions of chicoric acid and rutin were prepared in a concentration range of 1.95-62.5 µg mL^{-1}, obtaining calibration curves with a good linearity over the entire range and a correlation coefficient (R^2) equal to 0.999 and 0.998, respectively. Catechin calibration curve was prepared in a concentration range of 12.5-100 µg mL^{-1}, showing R^2 = 0.992. Xcalibur 4.1 software (Thermo Fisher Scientific Inc., Bremen, Germany) was

used to process acquired chromatographic profiles and MS^n (n = 1, 2 levels) spectra. Results were expressed as μg g^{-1} of FW ± standard deviation (SD).

2.6 Oxidative stress markers: hydrogen peroxide, thiobarbituric acid reactive substances and histochemical assay

The determination of hydrogen peroxide (H_2O_2) and thiobarbituric acid reactive substances (TBARS) in plant samples was performed according to Jana and Choudhuri (1982) and Spanò et al. (2017), respectively. To measure H_2O_2 concentrations, leaves and rhizomes were homogenized in 50 mM phosphate buffer (pH 6.5), centrifuged, and the supernatant was mixed with 0.1% titanium chloride in 20% (v/v) H_2SO_4, and the absorbance was read at 410 nm. The concentration of H_2O_2 was determined by employing a standard curve and then expressed in micromoles per gram of fresh weight (μmol g^{-1} FW). TBARS concentration was determined by measuring absorbance at 532 nm after subtracting non-specific absorbance at 600 nm, and quantified as nmol g^{-1} FW, following homogenization and extraction of plant material.

Leaves of similar size and length from five randomly chosen plants per treatment were isolated and divided into tip and mid-leaf segments. H_2O_2 was detected histochemically by dipping leaf portions in a staining solution containing 1 mg mL^{-1} DAB at pH 3.8, followed by vacuum infiltration for 20 min (Daudi and O'Brien, 2012). After that, the samples were left overnight in the same solution, then treated with 96% ethanol for 60 min at 65 °C, and finally examined under a light microscope to assess the presence of brown precipitates. In situ determination of lipid peroxidation was conducted using Schiff's reagent (Yamamoto et al., 2001) (VWR Chemicals BDH), which binds to free aldehyde groups, serving as a qualitative indicator of lipid peroxidation. Leaf segments were incubated with the dye for 60 min at room temperature, followed by bleaching in 96% ethanol for 60 min at 65 °C. The samples were then examined under a light microscope to evaluate the development of a purple coloration. Histochemical analyses were performed using a Leitz Diaplan microscope, with images captured using a Leica DFC 420 camera.

Cross sections of rhizomes from the same five plants were prepared using a hand microtome. Detection of H_2O_2 within the rhizome slices was conducted following the method outlined by Giorgetti et al. (2019), utilizing Amplex UltraRed Reagent (Life Technologies, USA). Following staining, the slices were mounted in glycerol and examined using a fluorescence microscope with excitation/emission wavelengths of 568ex/681em nm. Lipid peroxidation levels were assessed using a fluorescence microscope by observing the change in fluorescence emission peak from red to green after staining with the BODIPY 581/591 C11 probe (Life Technologies, USA), as described by Spanò et al. (2020). Microscope evaluation involved acquiring both green (485ex/510em nm) and red fluorescence (581ex/591em nm) signals simultaneously and merging the two images. Fluorescence microscope analysis was carried out using a Leica DMLB microscope equipped with the appropriate excitation/emission filters, along with a Leica DFC7000 T camera.

2.7 Extraction and determination of the activity of antioxidant enzymes in plants

Antioxidant enzymes were extracted from plant samples in 100 mM potassium phosphate buffer (pH 7.5), following the procedure outlined in Spanò et al. (2013). The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was measured according to Nakano and Asada (1981), monitoring the decrease in absorbance at 290 nm (extinction coefficient of 2.8 mM^{-1} cm^{-1}) as ascorbate was oxidized. A correction for the non-enzymatic oxidation of ascorbate by hydrogen peroxide (blank) was applied. Catalase (CAT, EC 1.11.1.6) activity was determined following the method described by Aebi (1984) and calculated using the extinction coefficient of 39.4 mM^{-1} cm^{-1}. A blank containing only the enzymatic solution was prepared. Guaiacol peroxidase (POX, EC 1.11.1.7) activity was determined according to the method described by Arezki et al. (2001), using 1% guaiacol as substrate and measuring guaiacol oxidation by H_2O_2 at 470 nm (extinction coefficient of 26.6 mM^{-1} cm^{-1}), with one unit oxidizing 1.0 µmol guaiacol per minute. Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined as per Beyer and Fridovich (1987), with slight modifications as detailed in Spanò et al. (2016). One SOD unit was defined as the amount required

to inhibit by 50% the photoreduction of nitroblue tetrazolium, measured spectrophotometrically at 550 nm. All enzymatic activities were assessed at 25°C and expressed as units per milligram of protein (U mg^{-1} protein). Protein quantification was conducted following the method of Bradford (1976), utilizing bovine serum albumin (BSA) as a standard.

2.8 Extraction and determination of plant photosynthetic pigments

Leaf chlorophylls (a, b, and total) and carotenoids were extracted following the method outlined by Hassanzadeh et al. (2009). In summary, leaves were homogenized in 80% acetone, and the extracts were then centrifuged for 10 min at 6000 g at 4°C. After collecting the supernatants, the pellets were resuspended and re-extracted with 80% acetone until they became colourless. The combined supernatants were analyzed using a spectrophotometer at wavelengths of 645 nm, 663 nm, and 470 nm. Pigment contents were calculated using the formula developed by Lichtenthaler (1987) and expressed as milligrams per gram of fresh weight (mg g^{-1} FW).

2.9 Chlorophyll a Fluorescence Transient Kinetics

The analysis of PSII fluorescence was carried out to determine the effects of IBU on the total functional efficiency of plants. A Handy PEA fluorometer (Hansatech Instruments Ltd., Pentney, King's Lynn, UK) was used to record fluorescence at four times: just before the treatment with IBU (T0), and one, five and twelve days following the treatment (T1, T5 and T12, respectively). Plants were acclimated to darkness for 30 min, then they were withdrawn from water and quickly blotted on paper to eliminate the excess of water. Two leaf clips were applied to each plant (one clip for each shoot) and the two values recorded on each plant were averaged to yield a single replicate: consequently, five replicates were available for each treatment. After having recorded chlorophyll fluorescence, plants were put back in the water. All operations were carried out in darkness, with the aid of a dim, diffuse light which was used only during the preparation of the plants. To avoid interferences on fluorescence kinetic data resulting from exposure to such light source,

measurements were taken after the leaves had been with the clips applied for 4 min: based on preliminary tests, this time lapse had proven to be sufficient for the purpose. Chlorophyll a fluorescence (ChlF) was measured after the darkened areas were exposed for 1 s to 3500 µmol photons m^{-2} s^{-1} (peak wavelength of 650 nm). Data were processed by PEA Plus software (Hansatech Instruments Ltd.), which carried out the analysis of the fast fluorescence kinetics, or JIP test (Stirbet et al., 2018). Each treatment's records were averaged to produce a single value, which was subsequently handled as a separate replicate. The JIP test parameters were computed using F_O, F_J, F_I, and F_M in addition to the ChlF values that were obtained at 50 µs, 100 µs, and 300 µs (Paunov et al., 2018). Table S2 contains a list of the parameters.

2.10 Statistical analyses

The effects of the exposure of *C. nodosa* plants to IBU contaminated seawater on morphological traits and growth measurements, specialized metabolites, oxidative stress markers, antioxidant enzyme activity, photosynthetic pigments, and photosynthetic efficiency were assessed through a one-way analysis of variance (ANOVA, "GAD" package, Sandrini-Neto and Camargo, 2014). Before performing ANOVAs, the normality and homoscedasticity of data were assessed by Shapiro−Wilk test and Cochran C test, respectively. Since some data did not meet ANOVA assumptions, they were analyzed by a Kruskal-Wallis test. In case of a significant effect, a Dunnett's test was conducted to compare the amount of detected specialized metabolite between IBU treated plants and control ones while Tukey HSD post-hoc tests (or Dunn test for APX in leaves) were carried out on other response variables. The statistical analyses were performed in R environment (version 3.5.2; R Core Team, 2018) and by JMP® Pro 16.0.0 (SAS Institute Inc., Cary, NC, USA) software.

3 Results and Discussion

3.1 Plant morphological traits and growth measurements

No difference in shoot number, average number of leaves per shoot, length of the longest leaf, and rhizome length of plants attributed to different IBU concentrations was detected at the beginning of the experiment (i.e., before the exposure to IBU; Table S3, Figure S2). At the end of the experiment, statistical analyses did not detect any significant effects of IBU on plant morphological and growth traits (Table S4, Figure 1, S3). These results agree with previous studies reporting no effect on the growth of the marine macroalga *F. vesiculosus* (Wiklund et al., 2011; Oskarsson et al., 2012), the marine diatom *P. tricornutum* (Silva et al., 2020) as well as on freshwater plants like *L. gibba*, *L. minor,* and *S. alba* (Pomati et al., 2004; Iori et al., 2013; Di Baccio et al., 2017) after their exposure to IBU concentrations within the range of those used in the present study.

3.2 Profiling Ibuprofen and plant specialized metabolites

LC-MS analyses revealed the presence of linearly decreasing concentrations of IBU in the NSW medium supplemented with high, medium, and low concentrations of IBU (positive controls) and the absence of IBU in the NSW samples (negative controls)(data not shown). The IBU-supplemented NSW collected at the end of the experiment from mesocosms containing or not *C. nodosa* plants showed IBU levels in agreement with the positive controls at all tested concentrations. The presence of IBU or its metabolites in the *C. nodosa* extracts was not detected, suggesting that this compound was not internalized by plants or alternatively that it was under the detection limit of the analytical method.

Furthermore, to investigate if the presence of IBU influenced the production of specialized metabolites, a quali-quantitative chemical analysis of specialized metabolites in extracts prepared from *C. nodosa* plants exposed to medium and high IBU concentrations was carried out by LC-MS techniques. This work stands out as a crucial contribution to the elucidation of the chemical composition of *C. nodosa* plant, as there are few other investigations in the literature (Grignon-Dubois and Rezzonico, 2013; Milović et al., 2019). The chromatographic profiles of control plants compared to treated ones are shown in Figure 2. A total of 39 compounds (Table S5) were

tentatively identified by comparing their retention times (t_R), HR full mass spectra, and fragmentation patterns with data reported in the literature, considering an accepted mass error <5 ppm on the experimental molecular formula. A tricarboxylic acid, 23 phenolic acids and derivatives, 13 flavonoids, and 2 dihydrochalcones were found. Citric acid (**1**) displayed the loss of CO_2 and two water molecules (base ion peak at *m/z* 111.00) (Fernández-Fernández et al., 2010). Among phenolic acids, gallic acid (**2**), hydroxybenzoic acid (**3**, $[M - H]^-$ at *m/z* 137.0239), coumaric acid (**17**, $[M - H]^-$ at *m/z* 163.0396), dihydrocoumaric acid (**18**, $[M - H]^-$ at *m/z* 165.0553) and ferulic acid (**23**, $[M - H]^-$ at *m/z* 193.0503) exhibited all the loss of a CO_2 molecule (-44 u) (Astudillo-Pascual et al., 2021). Compound **5** was identified as dihydroxybenzoic acid hexoside, showing the loss of a monosaccharide portion (-162 u). Compounds **4**, **6** and **11** were not completely annotated, but the presence of base ion peaks at *m/z* 137.02, 151.04, 165.06, respectively, suggested that these molecules are hydroxybenzoic, hydroxymethylbenzoic, dihydrocoumaric acid derivatives (Ostrowski et al., 2014). Coumaroyl hexoside isomers (**12** and **13**) showed a diagnostic product ion at *m/z* 163.04 (-162 u). Feruloyltartaric acid (**14**), feruloylhexoside (**16**) and feruloylmalic acid (**24**) displayed ferulic acid as a product ion at *m/z* 193.05. Caftaric acid isomers were attributed to peaks **7** and **21** by considering the product ion at *m/z* 149.01 (tartaroyl residue). Similarly, coutaric acid (**10**) and dicoumaroyltartaric acid (**32**) exhibited a tartaroyl product ion (Carazzone et al., 2013; Milović et al., 2019). Coumaroylmalic acid (**19**) showed a base ion peak at *m/z* 163.04 due to the cleavage of an ester bond with malic acid. Chicoric acid (**20**), a valuable compound previously found in *C. nodosa* plant (Grignon-Dubois and Rezzonico, 2013), showed the consecutive loss of two caffeoyl residues (product ions at *m/z* 311.04 and 149.01) and tartaric acid as a product ion (peak at *m/z* 179.03). Compounds **26** and **31** differed from caftaric acid only for the presence of an additional coumaroyl and feruloyl moieties, respectively. Similarly, compound **38** showed an additional feruloyl unit, compared to feruloyltartaric acid (**14**). Since roots were included in the extraction of the *C. nodosa* plants, also catechins and derivatives, typically found in barks, were detected in the extracts. Catechin and epicatechin (**8** and **15**) showed the

product ion at *m/z* 245.08 (Navarro et al., 2018). Procyanidin B-type dimer (**9**) displayed diagnostic ions at *m/z* 451.10, 425.09 and 287.05 as previously reported by Cioni et al. (2024). Among flavonoids, rutin (**22**), quercetin hexoside (**25**), quercetin malonylhexoside (**27**) exhibited the presence in the mass spectra of the same base ion peak at *m/z* 300.03 (aglycon portion) (López-Fernández et al., 2020; Navarro-Hoyos et al., 2021). Furthermore, MS^2 analysis revealed the presence of kaempferol hexoside (**28**) and naringenin hexoside (**33**) both displaying the loss of a hexose unit (Sánchez-Rabaneda et al., 2003). Isorhamnetin rutinoside (**29**), isorhamnetin hexoside (**30**), isorhamnetin acetylhexoside (**35**), and isorhamnetin malonylhexoside (**36**) all exhibited a base ion peak at *m/z* 315.04 in the MS/MS, corresponding to the aglycon portion isorhamnetin, also detected in the extracts (**39**). Finally, the metabolomic analysis revealed the presence of two dihydrochalcones, phloretin (**37**) and its glucoside form phlorizin (**34**), exhibiting ESI-MS^2 peaks in agreement with data reported in the literature (Lijia et al., 2014).

The quantitative analysis showed a significant change in the production of some specialized metabolites among both phenolic acids and flavonoids, especially in the extracts from plants exposed to high concentrations of IBU compared to the control ones. In general, total phenolic acids and flavonoids were almost halved in the plants treated with high IBU concentrations (480 ± 61 μg g^{-1} FW ± SD), compared to the control ones (821 ± 52 μg g^{-1} FW ± SD) (Table S6). The specialized metabolites reported in Figure 3 significantly decreased in their content in the plants treated with high/medium concentrations of IBU, except for rutin, gallic acid, coumaric acid, and dihydrocoumaric acid derivative which, instead, showed an increase. Finally, catechin, epicatechin and procyanidin B-type dimer showed a not significantly change under IBU treatment. These results, showing that high IBU concentrations affect the production of specialized metabolites, are consistent with previous studies (Ismail et al., 2015; Gorni et al., 2022; Zhang et al., 2022), where abiotic stress conditions induce plants to modulate the expression of genes involved in the biosynthetic pathways of some specialized metabolites, and in particular, to overproduce the ones

known for the antioxidant properties capable of increasing the plant stress tolerance, such as rutin, gallic acid and coumaric acid.

3.3 Oxidative stress evaluation, antioxidant enzyme activity and photosynthetic pigments

Data in the literature support that IBU, like other pharmaceutical pollutants, can induce an oxidative burst in the terrestrial crop *Vigna unguiculata* (L.) Walp. (Wijaya et al., 2020), with the overproduction of ROS, among which H_2O_2, that plays a key role in oxidative stress, and can cause damage to cell structure and macromolecules. In the present study, no significant difference in H_2O_2 rhizome concentration among control and treatments was recorded, except at the medium concentration in which the lowest value was detected (Table S7; Figure 4a). In our study, the lack of an increase in H_2O_2 levels in *C. nodosa* aligns with the results observed in *V. unguiculata* (Wijaya et al., 2020). In this latter, indeed, no significant increases in the concentration of this signaling molecule was found at a concentration of 400 ppm.

Applying the histochemical approach, which allows direct visualization of H_2O_2 in the rhizome, the staining linked to the fluorescent probe used (Amplex Ultrared) showed specific localization patterns within the organ tissues (Figure 5). The red signal was uniformly distributed in the control, slightly more intensely colouring the epidermis and cortical vascular bundles (Figure 5a). In plants exposed to the low IBU concentration, the pattern extended to part of the compact outer cortical tissue (Figure 5c), which in plants attributed to the medium and high concentration became overall intensely positive to the probe (Figure 5e,g). Furthermore, in plants exposed to the medium IBU concentration, but especially in those at the highest one, even the inner cortical tissue with large air spaces was positive for the dye, including the central stele (Figure 5e,g). These histochemical findings indicate that IBU, while not changing the overall H_2O_2 levels, can influence tissue-specific reactions depending on the concentration.

Biochemical results obtained in leaves were quite different, as all the plants exposed to IBU had levels of H_2O_2 significantly higher than control ones, the highest content being recorded at the low

concentration, with values that progressively decreased at medium and high concentration (Table S7; Figure 4b). The present findings highlight the importance of using narrow concentration ranges as the response can change even with small changes in concentration, which may not be detected when these ranges are very broad (Wijaya et al., 2020). These trends also underline that the response to environmental stimuli depends on the organ, being the leaves more sensitive than the rhizome. Indeed, histochemical results also confirm an intense H_2O_2-dependent colorimetric response in the leaf, which was obtained with the DAB dye (Figure 6), whose signal was detectable by light microscopy, to avoid interferences with the red autofluorescence of chlorophyll. All the leaves of IBU exposed plants were more intensely dark-brown stained in respect to the control, both in the tips and in the mid-leaf segments, with no specific staining pattern, demonstrating a general disturbance, slightly more prominent in the middle portion of the leaf of plants exposed to the medium and high IBU concentration (Figure 6f,h). These findings in histochemistry support the previous biochemical evidence, confirming that the leaf is more sensitive to IBU than the rhizome. This higher sensitivity may be related to the direct contact of leaves with the drug within water column.

Oxidative damage estimated as TBARS, indirect measurement of membrane damage, had in rhizome similar values in all treatments (Table S7; Figure 4c), while its histochemical determination (Figure 5) has shown specific differences as for H_2O_2: in control plants, only the epidermis was positive for Bodipy staining, while in plants exposed to low and medium IBU concentration, the staining was also present in the vascular cortical bundles (Figure 5d, f) and extended to the internal cortical tissues in those at the high concentration (Figure 5h). For the leaves, the quantitatively determined oxidative damage was higher at the maximum IBU concentration, the lowest values characterized controls and plants under the medium concentration of this pharmaceutical, while those exposed at the low concentration had intermediate contents (Table S7; Figure 4d) in accordance with results obtained histochemically with the Schiff reagent (Figure 7). Overall, both biochemical and histochemical data sustain that IBU can induce oxidative

damage even when administered at concentrations environmentally relevant. These results are consistent with previous data on the diatom *P. tricornutum*, in which an overall increment in lipid peroxidation, estimated as TBARS, in an IBU concentration-dependent manner was recorded (Silva et al., 2020).

To counteract oxidative injury, plants have evolved a complex antioxidant machinery, including enzymes such as POX, CAT, APX and SOD. With the only exception of SOD, differences in the activity of antioxidant enzymes were recorded, with increase and/or decrease in the two organs (Table 1a, S7). Present results differ from those obtained under comparable IBU concentrations in *P. tricornutum* (Silva et al., 2020), in which significant differences were detected for SOD activity and not for APX and CAT activities. In fact, in rhizome APX activity had the highest value at the low and medium IBU concentration and it had a lower value at the high concentration and in control samples (Table 1a, S7). POX and CAT activities progressively decreased and increased respectively in plants exposed to the low and high IBU concentration with intermediate values at medium concentration (Table 1a, S7). Slight increase in activity of APX (for plants at the low and medium concentration) and CAT (for plants at the high concentration) seemed able to limit oxidative stress. In leaves, while CAT and SOD activities were similar in all treatments, APX and POX activities progressively decreased from the low to the high concentration (Table 1a, S7). In plants treated with the high IBU concentration, the highest oxidative damage in terms of TBARS coincided with the lowest activities of POX and APX.

Among pigments (Table 1b), significant differences were recorded in the concentration of chlorophyll b, that was significantly higher in plants exposed to the high IBU concentration (Table S7). This was reflected in the content of total chlorophyll that reached the highest value just in this treatment, with intermediate values at medium concentration. The increase in chlorophyll b is of particular interest, and it could be an attempt of plants to increase the ability to harvest light in the restrictive conditions induced by stress, and that nevertheless could increase the probability of photoinhibition (Bascuñán-Godoy et al., 2012). Considering this, the significant increase in

carotenoids found in plants exposed to the highest concentration, might be an attempt to protect the photosynthetic apparatus (Figure S4). In contrast with the present results, under 1 mg L^{-1} IBU, Pietrini et al. (2015), showed no difference in pigment concentrations between the IBU-treated materials and the controls in *L. gibba.*

3.4 Chlorophyll a Fluorescence Transient Kinetics

Some pollutants have been found to negatively affect photosynthetic parameters that may be used as markers of phytotoxicity (Iori et al., 2013). In the present work, the analysis of the fast fluorescence kinetics was carried out to determine the effects of IBU on the photosynthetic efficiency of *C. nodosa*. Data interpretation is based primarily on Krüger et al. (2014), Paunov et al. (2018), Tsimilli-Michael (2020) and Zagorchev et al. (2021).

The parameters of the JIP test at T0 and T1 did not demonstrate any significant difference among control and treated plants, therefore these data are not shown. Similarly, exposure to the low and medium IBU concentrations did not induce significant changes at any time. Conversely, the effects of IBU at the highest concentration were evident after 5 days and 12 days of exposure (at T5 and T12). At T5, plants exposed to the highest concentration displayed numerous differences from the controls (Figure 8), with some parameters that demonstrated the onset of a state of stress, whereas others apparently suggested that IBU also produced some positive effect. These parameters are reported in the following graphs as xControl values, that is, as ratios between treatment and control data, clustered according to their magnitudes, to facilitate comparisons. The dashed lines indicate the value 1, i.e., the Control, to which all the values of treated plants are referred (Figure 8).

At T5 (Figure 8a), in treated *C. nodosa* plants the stress was detectable in the greater dissipation of chlorophyll excitation energy (higher F_O/F_M and DI_0/RC) and lower efficiency of the Hill reaction (F_V/F_O), perhaps a consequence of damage to the oxygen evolving complex, OEC (Gupta, 2020); also the parameter $\varphi(P_0)/(1-\varphi(P_0))$, functionally analogous to F_V/F_O, was lower in plants exposed to the highest IBU concentration. Perhaps the most obvious symptom of the onset of the

stress was the decrease in the number of active reaction centers per cross section of excited PSII (RC/CS_M). In addition, each of the RCs that were still active at the time of fluorescence recording experienced a decrease in the maximum quantum yield of the primary photochemistry of PSII (F_V/F_M and $\varphi(P_0)$). Apparent positive effects of IBU could be inferred from the following parameters, that were higher in treated plants: ET_0/RC (electron flux from Q_A^- to Q_B per active RC), $\psi(E_0)$ (efficiency with which an electron trapped by PSII is transferred from Q_A^- to Q_B, $\psi(R_0)$ (efficiency with which an electron trapped by PSII is transferred to PSI end acceptors), $\delta(R_0)$ (efficiency with which an electron is transferred from PQH_2 to PSI end acceptors), RE_0/RC (electron flux transferred from PQH_2 to PSI end acceptors per active RC), $\psi(E_0)/(1-\psi(E_0))$ (contribution of intersystem electron transport to the overall performance of photosynthesis light reactions), and $\delta(R_0)/(1-\delta(R_0))$ (contribution of electron transport from Q_B to PSI end acceptors to the overall performance of photosynthesis light reactions). Altogether, these parameters demonstrate that the energy flux in the intersystem and on the acceptor side of PSI, as well as the efficiencies with which the electrons were transferred through these sections of the transport chain, were higher following the exposure to IBU. This may be the consequence of increased photosynthetic cyclic and pseudocyclic electron flow around PSI (CEF and PEF, respectively), which are known to help generating a ΔpH across the thylakoid membranes that enhances non-radiative dissipation of light energy (Makino et al., 2002): the high dissipation of excitation energy (F_O/F_M and DI_0/RC) recorded in treated individuals substantiates this interpretation. A further beneficial effect arising from an enhanced PEF may be the increase of antioxidant enzymes activity and of antioxidant molecules concentration (Cheng et al., 2021). Nevertheless, these responses were not sufficient to counteract the advancement of the stress.

After 12 days of exposure (i.e., T12), the JIP test highlighted a worsening of the photochemical efficiency, with several parameters that were significantly different between control and treated *C. nodosa* (Figure 8b, c). As occurred at T5, the IBU treatment induced a greater dissipation of chlorophyll excitation energy: however, at T12 this was demonstrated not only by higher values of

F_O/F_M and DI_0/RC (Figure 8c), but also by higher DI_0/CS_O (Figure 8c) and DI_0/CS_M (Figure 8b). In particular, DI_0/RC shows how RCs were struggling with the management of absorbed light energy, which was dissipated at high rates. It is reasonable to assume that during the few days (twelve) elapsed from the start of the treatment, the plants could not regulate light energy absorption through the adjustment of the size of their antennas. This is suggested by the values of ABS/CS_O (Figure 8b) and ABS/CS_M: the former parameter was about 1.5 xControl, while the latter did not differ from the Control. This agrees with the concentration of chlorophyll b, which was higher in IBU treated plants than in control ones at T12. Consequently, photon absorption under IBU treatment went on without changes, while operation of RCs was severely hindered. The value of ABS/RC, in fact (Figure 8c), was primarily the consequence of the inactivation of a large number of RCs. This process is confirmed by RC/CS_O and RC/CS_M (Figure 8b), that in treated *C. nodosa* were half, or even less, compared to the control. In addition, $\gamma(RC)/(1-\gamma(RC))$ (Figure 8b) was lower in treated leaves, showing that IBU also caused the decrease of the number of active RCs per chlorophyll molecule of PSII antenna. The residual active RCs showed a lower maximum quantum yield of the primary photochemistry of PSII (F_V/F_M and $\varphi(P_0)$; Figure 8b). The OECs appeared to be damaged more severely than at T5, because in addition to lower F_V/F_O and $\varphi(P_0)/(1-\varphi(P_0))$, the JIP test yielded also a higher V_K (Figure 8b). The decrease in the number of active RCs explains why some parameters (S_M, N, $S_M/t(F_M)$ and TR_0/RC) seemed to indicate that IBU also had some positive effect. S_M was higher in plants exposed to the highest IBU concentration (Figure 8c), which meant more electron transporters per chain: however, S_M represents the number of transporters that were reduced between O and P per active RC, so if the number of active RCs declined, the value of S_M increased. Also, N was higher in treated plants (Figure 8c), probably for the same reason: with fewer active RCs, the number of times they were reduced between O and P increased. Similarly, the higher $S_M/t(F_M)$ (higher average excitation energy of the open RCs between O and P) of IBU–treated individuals (Figure 8c) can be explained by the low number of RCs remained active, which were therefore burdened with more energy. Another data that is only apparently positive is the

higher TR_0/RC of the plants exposed to the highest IBU concentration (Figure 8b): this too can be attributed to the low number of RCs remained active, each of which trapped more excitons because they were subjected to a strong flux of photon energy from the antennas, whose dimensions had not changed from the start of the experiment. In fact, both the trapping of excitons (TR_0/CS_M) and the electron flux from Q_A^- to Q_B per cross section of excited PSII (ET_0/CS_M) (Figure 8b) were lower in treated *C. nodosa*, because of the decreased energy input into the photosynthetic transport chain consequent to the inactivation of many RCs. All the negative effects described so far can also explain the lower PI_{ABS} (performance index of energy conservation of photons absorbed until Q_B reduction) of the treated individuals (Figure 8b). Another set of parameters demonstrates that the energy flux in the final part of the photosynthetic electron transport chain was greater in IBU–treated plants. This cannot be attributed, as it was in the case of the previously described parameters, to the decline of the number of active RCs. Although the higher RE_0/RC (Figure 8c) of treated plants might indeed depend on the lower number of active RCs, some parameters clearly suggest another explanation. Among these, RE_0/CS_O and φR_0 (Figure 8c), i.e., the electron flux from PQH_2 to PSI end acceptors per cross section of excited PSII and the quantum yield of electron transport to PSI end acceptors, respectively, were higher in treated *C. nodosa*, demonstrating that a greater energy flux actually took place in the last section of the electron transport chain. In agreement with these data and with this interpretation, also $\psi(E_0)/(1-\psi(E_0))$ and $\delta(R_0)/(1-\delta(R_0))$ (Figure 8c), respectively the contribution of intersystem electron transport and of electron transport from Q_B to PSI end acceptors to the overall performance of the light reactions of photosynthesis, were higher in treated plants. As already pointed out for T5, this greater energy flux in the final section of the transport chain may be the consequence of increased photosynthetic CEF and PEF around PSI.

Overall, exposure to 25 μg L^{-1} IBU caused severe damage to the photosynthetic machinery of *C. nodosa*, in a relatively short time. The first symptoms of physiological stress were detected by the JIP test already at T5, but they could have been occurred even earlier. The main targets of the toxic

action of IBU were PSII and its donor side, with damages to RCs and to OECs, while antenna complexes did not seem to be affected. An attempt of exposed plants to protect PSII from the impact of IBU might be the increase of carotenoids concentration, which was detected at T12. In treated plants, the flux of energy through the intersystem, PSI and its acceptor side was enhanced, probably because of the greater activity of CEF and PEF, a response with which the plant aimed at accelerating energy dissipation (Figure S4). Apparently, PEF did not strengthen antioxidant defenses because the related enzymes showed only a decline of APX and POX activity in plants exposed to the highest IBU concentration, that also underwent oxidative damage, as revealed by the levels of their TBARS.

Less severe impacts have been observed on hydroponically grown *S. alba* plants, treated with 3 or 30 mg L^{-1} IBU for two weeks (Iori et al., 2013). Plants showed a decline of ΦPSII, F_V/F_M, qP and increased NPQ, that were attributed to the impairment of PSII RCs and the overexcitation of the photochemical system, which might had led to the generation of reactive radicals, responsible for the damage to PSII components. The aquatic plant *L. gibba* exhibited a notable resistance to IBU. Plants treated for 8 days with 20, 200 or 1000 μg L^{-1} IBU did not show any changes in the values of F_V/F_M, ΦPSII, qP and ETR and the lowest concentration even increased ΦPSII (Di Baccio et al., 2017). It is worth noting that, in most cases, both *S. alba* and *L. gibba* had been exposed to IBU concentrations that were higher than those applied in the present work on *C. nodosa*, whose sensitivity and vulnerability to IBU and, probably, to its metabolites are far greater than in the above-mentioned species. The disturbance of the functioning of photosynthetic machinery could be considered as an early warning signal of the exposure to IBU. Thus, the analysis of the fast fluorescence kinetics may be effective in monitoring the impact of this pollutant on *C. nodosa*.

4 Conclusions

The presence of pharmaceuticals in marine macrophytes has been reported, but their effects on seagrasses have not been assessed so far. This information may help in developing more effective

seagrass conservation strategies and management interventions. Our study demonstrates that a short-term exposure of the seagrass *C. nodosa* to IBU concentrations detected in coastal seawaters cannot elicit detrimental growth effects. However, it shows that this chemical causes oxidative stress and damages the photosynthetic machinery, especially at the highest concentration. The observed increased production of specific secondary metabolites and antioxidant compounds, including acid gallic acid and rutin, as well as the enhancement of the excitation energy dissipation of PSII and the acceleration of electron transport in the intersystem, could be an attempt of plants to mitigate the effects of this pollutant. But the potential risks posed by a prolonged exposure to IBU for seagrasses need to be assessed. Further studies should also monitor the behavior of IBU in seagrass meadows and evaluate whether it makes them less resilient to other global environmental stressors including climate changes.

CRediT authorship contribution statement

Virginia Menicagli: Methodology, Formal analysis, Investigation (plant harvesting, plant cultivation, morphological trait and growth measurements), Visualization, Writing - Review & Editing. **Monica Ruffini Castiglione:** Methodology, Formal analysis, Investigation (plants histochemical assay), Visualization, Writing- Review & Editing, Funding acquisition. **Emily Cioni**: Methodology, Formal analysis, Investigation (detection of IBU in plants and NSW, specialized plant secondary metabolites), Visualization, Writing- Review & Editing. **Carmelina Spanò**: Methodology, Formal analysis, Investigation (oxidative stress evaluation, photosynthetic pigments measurement, antioxidant enzyme activity), Visualization, Writing - Review & Editing, Funding acquisition. **Elena Balestri:** Conceptualization, Methodology, Formal analysis, Investigation (plant harvesting, plant cultivation, morphological trait and growth measurements), Visualization, Supervision, Writing - Review & Editing, Funding acquisition. **Marinella De Leo**: Methodology, Formal analysis, Investigation (detection of IBU in plants and NSW, specialized plant secondary metabolites), Visualization, Writing- Review & Editing, Funding acquisition. **Stefania Bottega**:

Methodology, Formal analysis, Investigation (oxidative stress evaluation, photosynthetic pigments measurement, antioxidant enzyme activity), Visualization, Writing - Review & Editing. **Carlo Sorce**: Methodology, Formal analysis, Investigation (photosynthetic efficiency measurements), Visualization, Writing - Review & Editing, Funding acquisition. **Claudio Lardicci**: Conceptualization, Methodology, Formal analysis, Investigation (plant harvesting, plant cultivation, morphological trait and growth measurements), Writing – Review & Editing, Supervision, Funding acquisition.

Declaration of competing interest

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

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Figure Legends

Figure 1 *Cymodocea nodosa* plants exposed to different levels of seawater contamination by IBU (0 μg L^{-1} or Control-Ctrl, 0.25 μg L^{-1} or Low, 2.5 μg L^{-1} or Medium, 25 μg L^{-1} or High) at the beginning and at the end of the experiment.

2-colums fitting image, color in the online version only

Figure 2 Comparison of qualitative profiles of *Cymodocea nodosa* extracts from plants exposed to high (H) concentrations of IBU and control plants (C) grown without IBU. LC-MS/MS analyses were recorded in negative ion mode. Each number corresponds to those listed in Table S5.

2-colums fitting image, color in the online version only

Figure 3 Phenolic acids (a) and flavonoids (b) found in the extracts of plants treated with high (H) and medium (M) IBU concentrations differing significantly (* = $p < 0.05$; ** = $p < 0.005$) in amount (μg g^{-1} FW) from the control group (C). GA = gallic acid; HBAd = hydroxybenzoic acid derivative; DHBAH = dihydroxybenzoic acid hexoside; HMBAd = hydroxymethylbenzoic acid derivative; DHCAd = dihydrocoumaric acid derivative; CH(I) and CH(II) = coumaroyl hexoside isomers; FH = feruloylhexoside; CA = coumaric acid; CMA= coumaroylmalic acid; FMA = feruloylmalic acid; R = rutin; KH = kaempferol hexoside; IH = isorhamnetin hexoside; NH = naringenin hexoside; IAH = isorhamnetin acetylhexoside; IMH = isorhamnetin malonylhexoside; I = isorhamnetin.

Single column fitting image, color in the online version only

Figure 4 Concentration of (a,b) hydrogen peroxide (H_2O_2) and (c,d) thiobarbituric acid reactive substances (TBARS) in rhizome (left column) and leaves (right column) of *Cymodocea nodosa* plants exposed to different levels of seawater contamination by IBU (0 μg L^{-1} or control-Ctrl, 0.25 μg L^{-1} or Low, 2.5 μg L^{-1} or Medium, 25 μg L^{-1} or High). Different letters denote significant differences ($p < 0.05$) among treatments. Mean ± SE, n = 4.

1.5-colum fitting image, color in the online version only

Figure 5 Representative images of rhizome cross sections of *Cymodocea nodosa* plants processed to histochemical detection of oxidative stress markers. Amplex probe (H_2O_2 indicator, images left) and Bodipy probe (lipid peroxidation indicator, images right). a, b = control; c, d = low IBU concentration; e, f = medium IBU concentration; g, h = high IBU concentration. Scale bar = 100 µm.

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Figure 6 Representative images of leaf portions of *Cymodocea nodosa* plants processed to histochemical detection of H_2O_2 by DAB staining. Tip-leaf segments, images left; mid-leaf segments, images right. a, b = control; c, d = low IBU concentration; e, f = medium IBU concentration; g, h = high IBU concentration. Scale bar = 1 mm.

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Figure 7. Representative images of leaf portions of *Cymodocea nodosa* plants processed to histochemical detection of lipid peroxidation by Schiff' reagent staining. Tip-leaf segments, images left; mid-leaf segments, images right. a, b = control; c, d = low IBU concentration; e, f = medium IBU concentration; g, h = high IBU concentration. Scale bar = 1 mm.

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Figure 8 Effects of the exposure to 25 µg L^{-1} IBU for (a) 5 days and (b, c) 12 days in dark-adapted *Cymodocea nodosa* leaves. The bar plots report the parameters of JIP test (described in Table S2), normalized to the values of the control, which were set as one. Dashed lines = control (value = 1). Only those parameters that differed significantly from the control ($p < 0.05$) are shown. All values are the mean of five replicates.

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Table 1 (a) Activity of ascorbate peroxidase (APX), guaiacol peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD) in rhizomes and leaves of *Cymodocea nodosa* plants exposed to different levels of seawater contamination by IBU (0 μg L^{-1} or Ctrl, 0.25 μg L^{-1} or Low, 2.5 μg L^{-1} or Medium, 25 μg L^{-1} or High), and (b) concentration of chlorophyll a (Chla), chlorophyll b (Chlb), total chlorophyll (Total Chl) and carotenoids in leaves of *Cymodocea nodosa* plants exposed to different levels of seawater contamination by IBU. Different letters denote significant differences ($p < 0.05$) among treatments.

(a)	**APX**		**POX**		**CAT**		**SOD**	
	Rhizomes	Leaves	Rhizomes	Leaves	Rhizomes	Leaves	Rhizomes	Leaves
Ctrl	0.66±0.01 b	0.77±0.02 bc	5.50±0.30 a	5.38±0.29 b	2.44±0.24 ab	4.41±0.28 a	39.66±1.45 a	40.23±0.76 a
Low	0.90±0.08 ab	1.09±0.08 a	5.92±0.36 a	10.23±0.62 a	2.00±0.21 b	5.12±0.32 a	37.26±2.46 a	29.06±7.15 a
Medium	0.94±0.02 a	0.84±0.02 ab	4.98±0.53 ab	5.52±0.34 b	2.44±0.24 ab	4.08±0.34 a	33.96±4.66 a	40.80±1.42 a
High	0.66±0.01 b	0.67±0.03 c	3.37±0.06 b	3.26±0.32 c	3.02±0.14a	4.32±0.26 a	40.80±0.93 a	36.12±4.74 a

(b)	**Chla**	**Chlb**	**Total Chl**	**Carotenoids**
Ctrl	0.42±0.09a	0.27±0.05b	0.69±0.17b	0.10±0.01b
Low	0.38±0.04a	0.29±0.02b	0.67±0.07b	0.09±0.02b
Medium	0.50±0.07a	0.31±0.02b	0.81±0.11ab	0.15±0.02b
High	0.77±0.08a	0.50±0.02a	1.27±0.08a	0.22±0.00a

Figure 1

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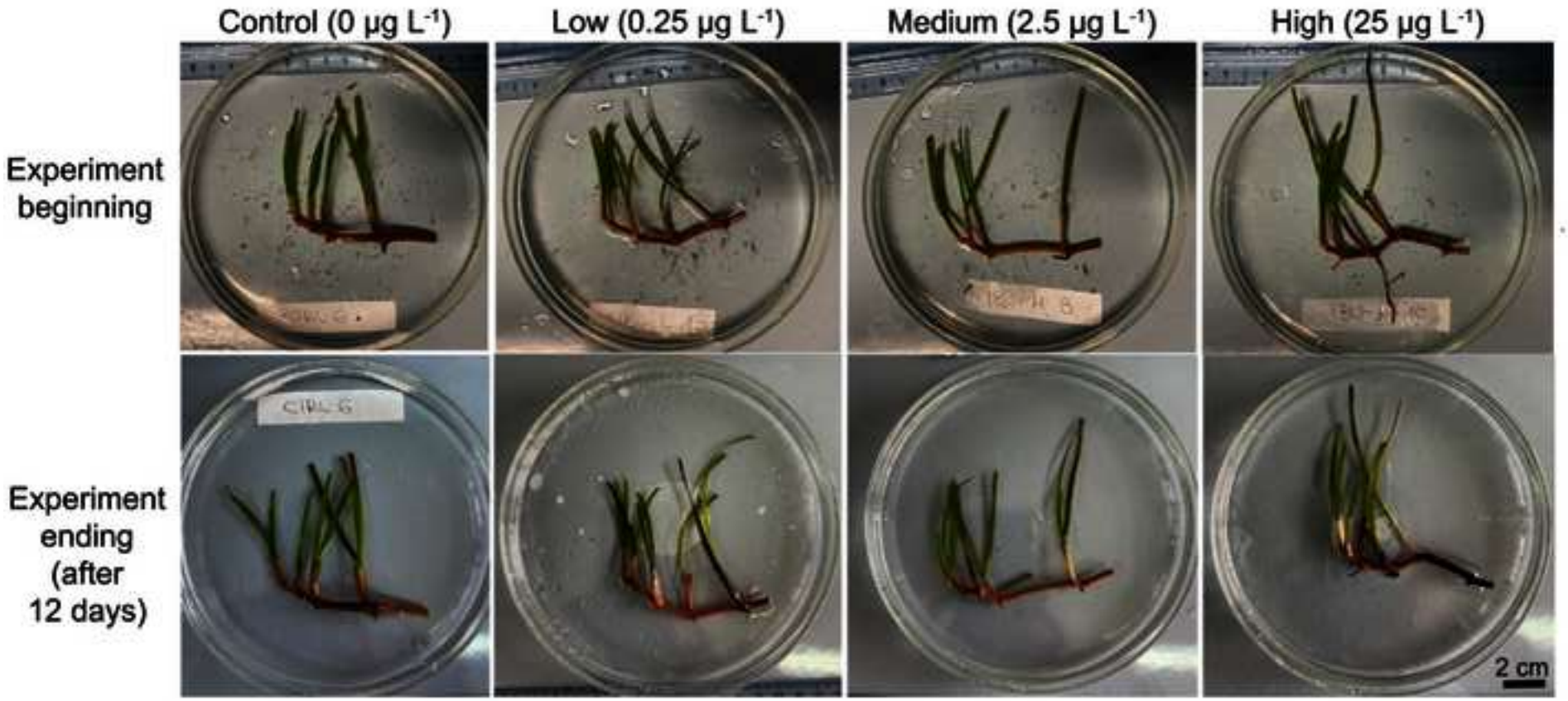

Figure 2

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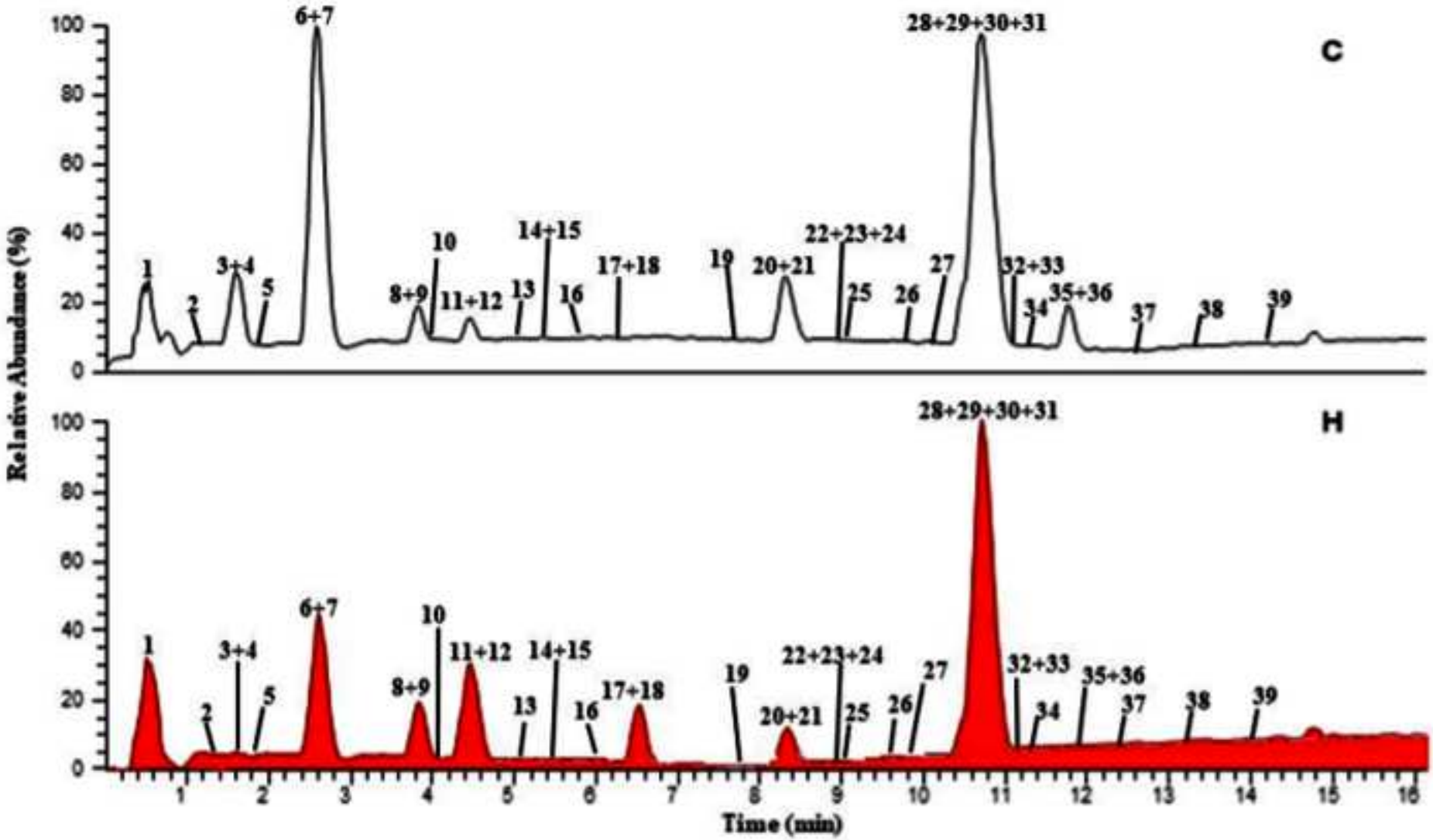

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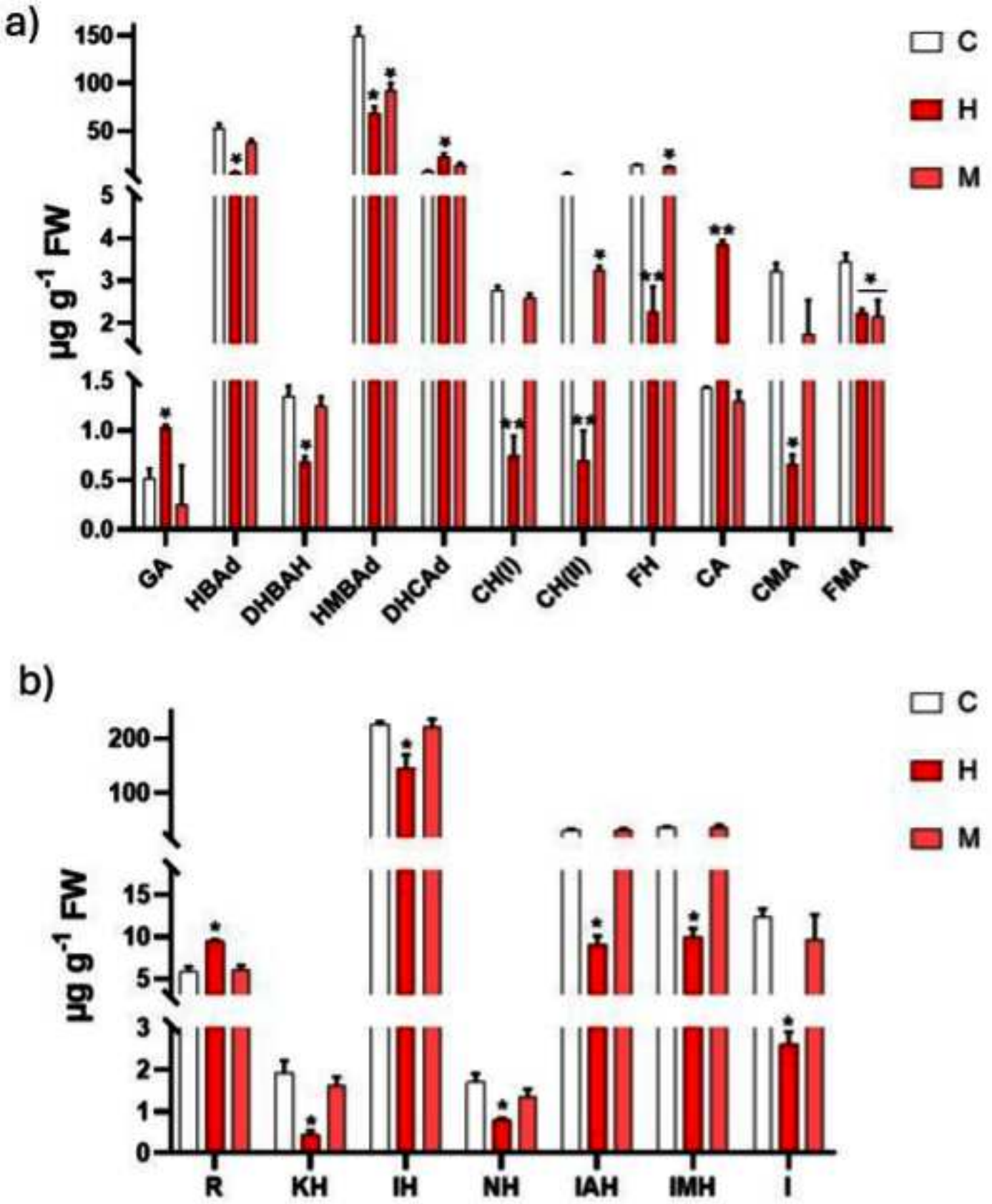

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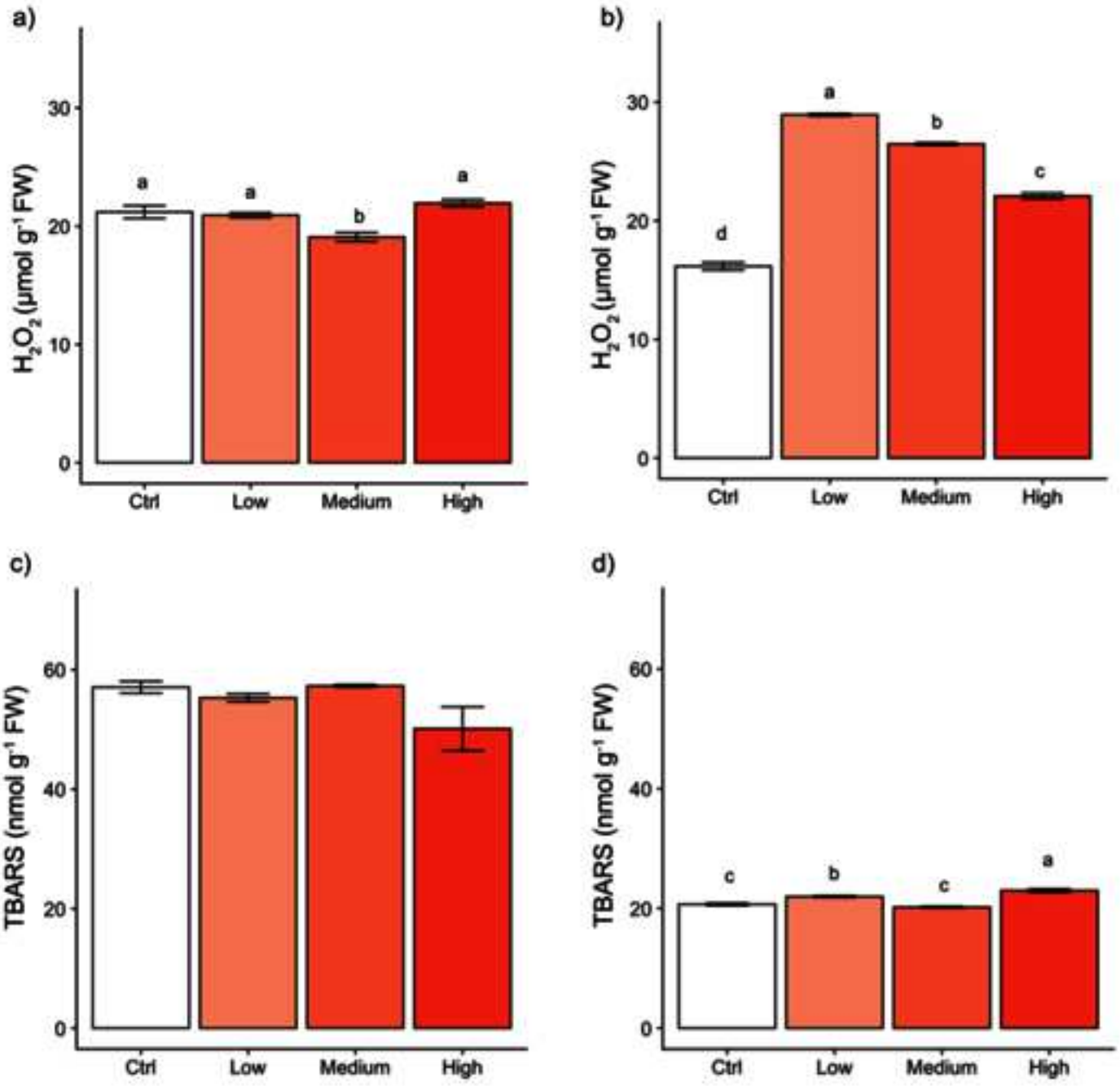

Figure 5

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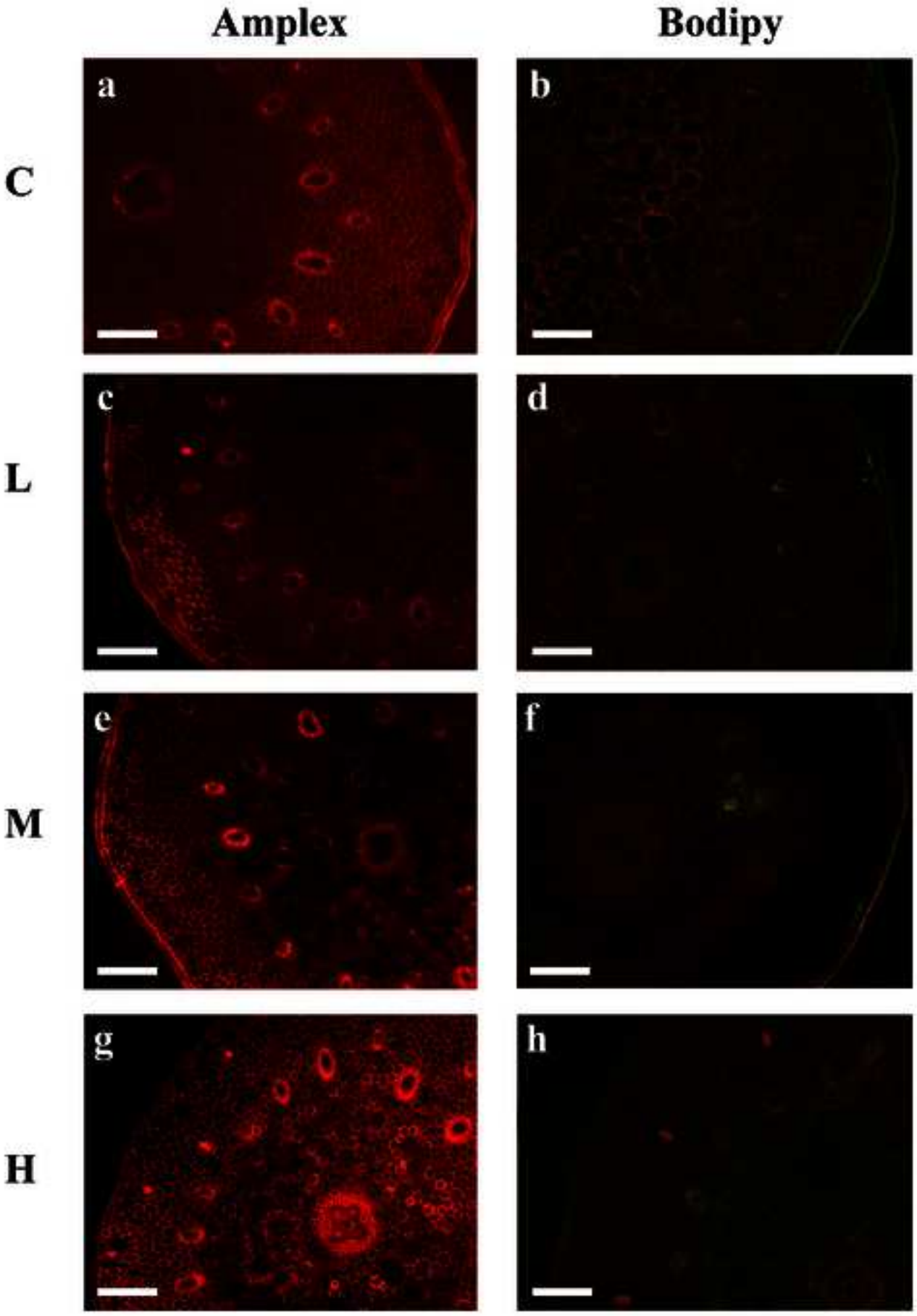

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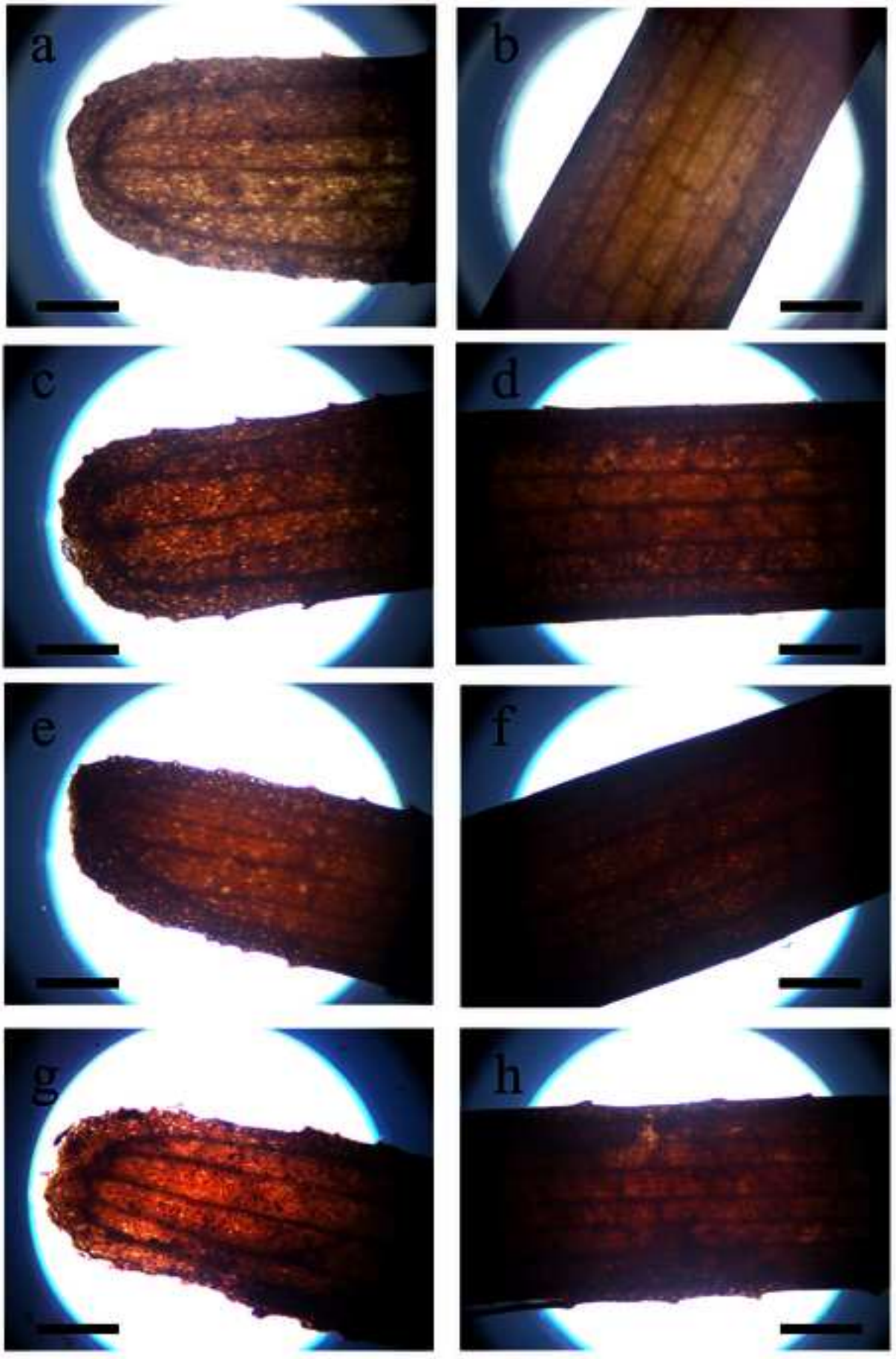

Figure 7

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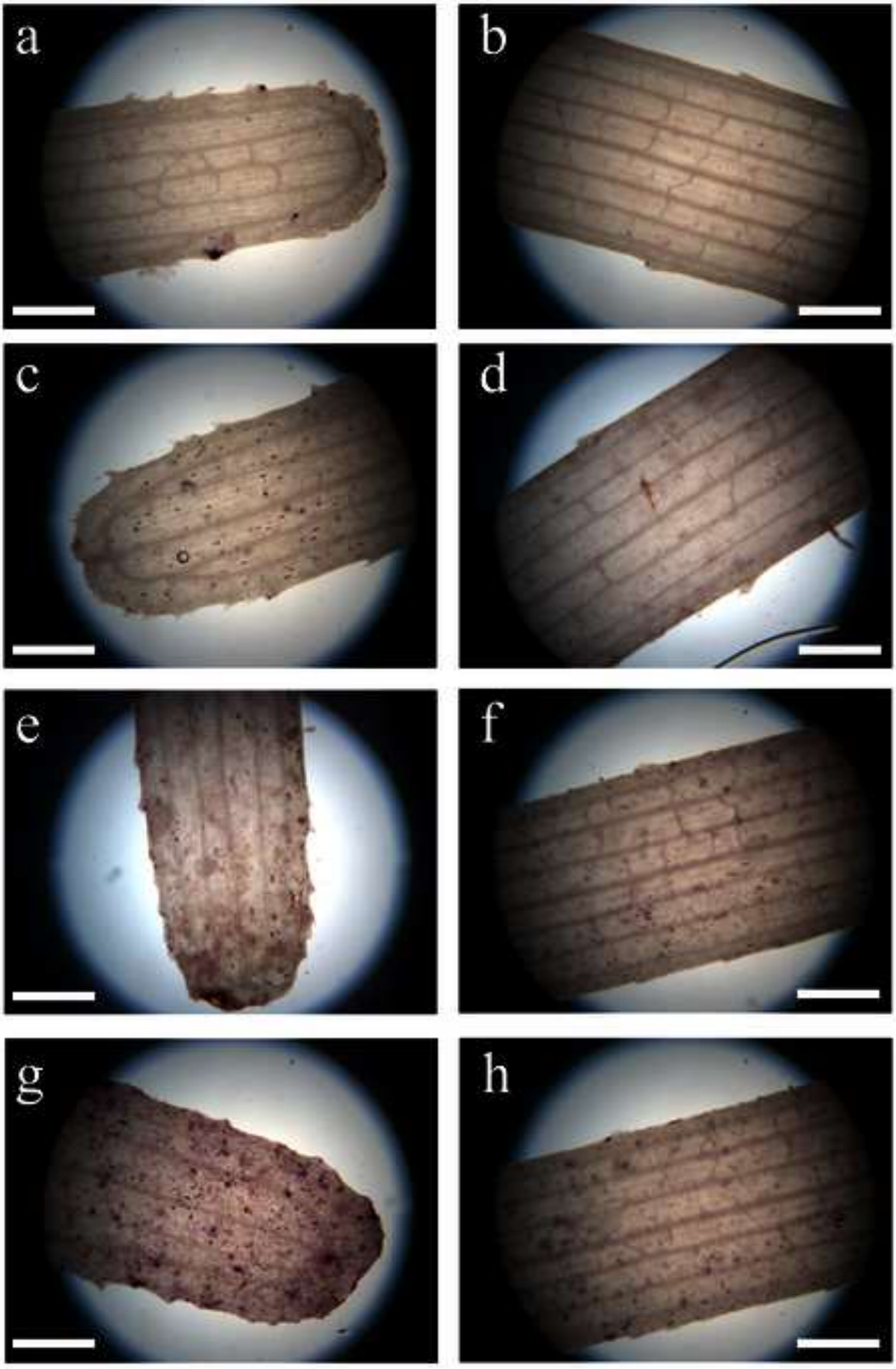

Figure 8

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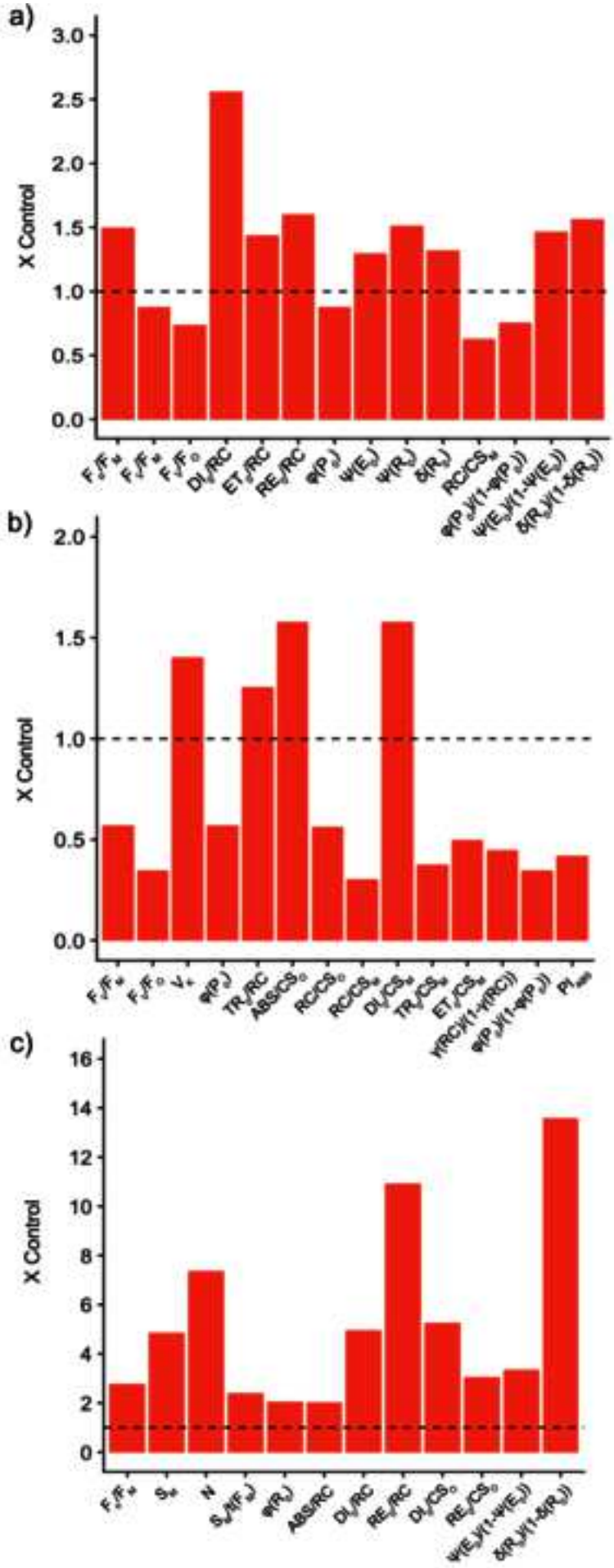

Declaration of interests

☒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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: