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Stress markers and physiochemical responses of the Mediterranean shrub *Phillyrea angustifolia* under current and future drought and ozone scenarios

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ABSTRACT

Mediterranean plants are particularly threatened by the exacerbation of prolonged periods of summer drought and increasing concentrations of ground-level ozone (O_3). The aims of the present study were to (i) test if selected markers (i.e., reactive oxygen species, ROS; malondialdehyde, MDA; photosynthetic pigments) are able to discriminate the oxidative pressure due to single and combined stress conditions, and (ii) elucidate the physiochemical adjustments adopted by *Phillyrea angustifolia* (evergreen woody species representative of the maquis, also known as narrow-leaved mock privet) to perceive and counter to drought and/or O_3. Plants were grown from May to October under the combination of two levels of water irrigation [i.e., well-watered (WW) and water-stressed (WS)] and three levels of O_3 [i.e., 1.0, 1.5 and 2.0 times the ambient air concentrations, i.e. AA (current O_3 scenario), 1.5 × AA and 2.0 × AA (future O_3 scenarios), respectively], using a new-generation O_3 Free Air Controlled Exposure (FACE) system. Overall, this species appeared relatively sensitive to drought (e.g., net CO_2 assimilation rate and stomatal conductance significantly decreased, as well as total chlorophyll and carotenoid contents), and tolerant to O_3 (e.g., as confirmed by the absence of visible foliar injury, the unchanged values of total carotenoids, and the detrimental effects on stomatal conductance, total chlorophylls and terpene emission only under elevated O_3 concentrations). The combination of both stressors led to harsher oxidative stress. Only when evaluated together (i.e., combining the information provided by the analysis of each stress marker), ROS, MDA and photosynthetic pigments, were suitable stress markers to discriminate the differential oxidative stress induced by drought and increasing O_3 concentrations applied singly or in combination: (i) all these stress markers were affected under drought *per se*; (ii) hydrogen peroxide (H_2O_2) and MDA increased under O_3 *per se*, following the gradient of O_3 concentrations (H_2O_2: about 2- and 4-fold higher; MDA: +22 and + 91%; in 1.5 × AA_WW and 2.0 × AA_WW, respectively); (iii) joining together the ROS it was possible to report harsher effects under 2.0 × AA_WS and 1.5 × AA_WS (both anion superoxide and H_2O_2 increased) than under 2.0 × AA_WW (only H_2O_2 increased); and (iv) MDA showed harsher effects under 2.0 × AA_WS than under 1.5 × AA_WS (increased by 49 and 18%, respectively). Plants activated physiological and biochemical adjustments in order to partially avoid (e.g., stomatal closure) and tolerate (e.g., increased terpene emission) the effects of drought when combined with increasing O_3 concentrations, suggesting that the water use strategy (isohydric) and the sclerophyllous habit can further increase the plant tolerance to environmental constraints in the Mediterranean area.

1. Introduction

Mediterranean plants are particularly threatened by the exacerbation of several natural and anthropogenic abiotic factors related to climate change, which include prolonged periods of summer drought and increasing concentrations of ground-level ozone (O_3) (Mèdail, 2017; Doblas-Miranda et al., 2017; IPCC, 2018). These abiotic factors have the potential to induce oxidative stress in plants (Foyer and Noctor, 2005), so resulting in many physiological and bioabiotic factors have the potential to inducechemical impairments such as reduction in

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photosynthesis, imbalance between the production of reactive oxygen species (ROS) and the availability of antioxidant defences, cell dehydration, early senescence, and/or leaf necrosis (Bohler et al., 2015; Pellegrini et al., 2016). Although these effects due to drought and O_3 *per se* have been largely investigated and sufficiently understood in Mediterranean plants (Munnè-Bosch and Peñuelas, 2003; Nali et al., 2004; Ogaya and Peñuelas, 2006), controversial outcomes and interpretations have been reported on their interactive effects. The combination of drought and O_3 can induce responses considerably different from those observed when each stressor is applied independently (Matyssek et al., 2006; Bohler et al., 2015; Cotrozzi et al., 2017; Landi et al., 2019; Otu-Larbi et al., 2020). The most common assumption for which drought-induced stomatal closure would limit O_3 entering the leaf and the consequent O_3-induced pressure abiotic factors have the potential to induceseems not to be always acceptable, since in some cases drought further exacerbates the effects of O_3 on plants (Alonso et al., 2014). Further research is thus needed to predict future responses of Mediterranean plants to co-occurring climate change-related abiotic stressors.

In order to estimate the oxidative stress in plant tissue, several stress markers have been identified. Advantages/limitations of using these stress markers have been recently evaluated in terms of stress detection accuracy, as well as of difficulty, time consumption, sample destructiveness, and costs related to their measurements (Pintó-Marijuan and Munné-Bosch, 2014). Among these stress clues, a wide use has been made of ROS as indicators of oxidative challenge (Demidchik, 2015), reactive carbonyl species, e.g., malondialdehyde (MDA) (Morales and Munné-Bosch, 2019) as indicators of oxidative damage in general, and photosynthetic pigments as indicators of oxidative pressure on photosynthetic apparatus (Pellegrini et al., 2019; Sharma et al., 2020). Overall, multiple indicators should be used to properly assess oxidative stress in plant tissues in relation to their suitability and accuracy. However, the suitability and accuracy in stress detection of these stress markers should be further validated in various plant species and experimental conditions. Specifically, in the case of abiotic stress combination, the capability of these markers to discriminate whether the cause of stress is a single stressor or the combination of stressors should be elucidated.

Phillyrea angustifolia also known as narrow-leaved mock privet (belonging to the Oleaceae family) is one of the most diffused vascular plant species in the Mediterranean maquis. A sclerophyllous evergreen broadleaved woody species, is distributed as shrub or small tree in the south-western Mediterranean region, growing in very dry and hot habit below 500 m a.s.l. (Pignatti, 1982). *Phillyrea* species have generally shown a good degree of tolerance to various environmental stresses including drought (Munné-Bosch and Peñuelas, 2003; Peñuelas et al., 2004; Álvarez et al., 2019), O_3 (Nali et al., 2004; Paoletti et al., 2004; Hoshika et al., 2020), salinity (Tattini et al., 2002; Tattini and Traversi, 2008) and high solar radiation (Tattini et al., 2005). Specifically, *P. angustifolia* has been shown to be able to activate several mechanisms of photo- and antioxidant protection, as well as water regulation, to withstand drought stress (Peñuelas et al., 2004; Álvarez et al., 2019); for this reason, it has been suggested as an interesting ornamental species for gardening and landscaping purposes (Álvarez et al., 2019). Conversely, *P. angustifolia* sensitivity to O_3, applied singularly or in combination with drought, has been investigated only by Hoshika et al. (2020), who focused exclusively on physiological responses. They showed that under high-O_3 and well-watered conditions, *P. angustifolia* limited O_3 fluxes by stomatal closure; whereas the carbon assimilation was impaired under drought, regardless of O_3 concentration. Thus, there are still many open questions about whether and how the water use strategy adopted by *P. angustifolia* under drought may affect its potential capability to avoid O_3 stress by stomatal closure and/or tolerate O_3-induced effects (e.g., by activating antioxidant processes).

The aim of the present study was to characterize morphological and physiochemical mechanisms adopted by *P. angustifolia* to cope with drought combined with three levels of O_3 exposure in an O_3 Free Air Controlled Exposure (O_3-FACE) facility. Specifically, we asked the following questions: (i) Are ROS, MDA, and photosynthetic pigments suitable markers to discriminate whether oxidative stress is due to drought and increasing O_3 concentrations applied singularly or a combination of them? (ii) What physiological and biochemical adjustments are adopted by *P. angustifolia* to perceive and counter to drought and/or O_3? We postulated that (i) the selected markers are able to discriminate the oxidative pressure due to singular and combined stress conditions, and (ii) drought can alter the photo- and antioxidative mechanisms which regulate the strategy of coping with O_3-induced oxidative stress.

2. Materials and methods

2.1. Plant material and experimental design

In December 2017, three-year-old seedlings of *P. angustifolia* were purchased from a commercial nursery and transferred to the O_3-FACE facility at Sesto Fiorentino, Florence, Italy (43°48′59″ N, 11°12′01″ E, 55 m a.s.l.), where the experimental activities were conducted. A detailed description of the O_3 exposure methodology is available in Paoletti et al. (2017). Here, plants were directly transplanted into 25-L plastic pots filled with a sand:peat:soil mixture (1:1:1 in volume) and maintained well-irrigated under field conditions until the beginning of the treatments. On May 1, 2018, uniform-sized plants were selected and grown under the combination of two levels of water irrigation [100 and 40% of field capacity on average, denoted as well-watered (WW) and water-stressed (WS), respectively] and three levels of O_3 [1.0, 1.5 and 2.0 times the ambient air concentrations, denoted as AA (current O_3 scenario), 1.5 × AA and 2.0 × AA (future O_3 scenarios, Young et al., 2013), respectively], until October 31, 2018. Throughout the experimental period, the hourly mean O_3 concentrations were 35, 53 and 65 ppb in AA, 1.5 × AA and 2.0 × AA, respectively; while the Accumulated exposure Over Threshold of 40 ppb (AOT40, *sensu* Kärenlampi and Skärby, 1996) values were 22.8, 60.2 and 92.6 ppm h in AA, 1.5 × AA and 2.0 × AA, respectively. The amount of irrigation was related to the soil field capacity (FC, volumetric soil water content = approximately 29.5%, Paoletti et al., 2017), i.e. the maximum volume of water that was retained into the soil of the pots [the volumetric soil water content was measured in the root layer (5-cm depth) by EC-5 soil moisture sensors equipped with an EM5b data logger (Decagon Devices, Pullman, WA, USA); Hoshika et al. (2018)].

A well-replicated split-plot design comprised of three replicated plots (5 × 5 × 2 m, n = 3 plots) was used with the whole-plot factor consisting of the O_3 levels, whereas the two watering regimes [WW (100% FC) and WS (40% FC)] were randomly assigned to six pots in each plot (three pots per each watering regime), distributed among the three plots of each O_3 levels (in total 54 pots). At the end of the experiment, 6 plants per each watering regime × O_3 level condition were randomly selected and dedicated to the assessment of plant biomass. Furthermore, fully expanded and sun-exposed leaves (5th to 8th position from the tip of the shoot) of the remaining plants were investigated for the selected stress markers (i.e., ROS, MDA, photosynthetic pigments), as well as for other morphological (i.e., leaf mass per area), hydric (i.e., water status), photosynthetic (i.e., gas exchange), and biochemical parameters (i.e., volatile organic compounds). For the assessment of selected stress markers, leaves were collected, divided into aliquots (obtained from each combination of watering regime and O_3 level), immediately frozen in liquid nitrogen and stored at −80 °C until analyses. Measurements and sampling were performed from 11:00 a.m. to 1:00 p.m.

2.2. Plant biomass, leaf mass per area and water status

Plants dedicated to the assessment of plant biomass were harvested and separated into leaves, stems, branches, and roots. Roots were washed very carefully to remove soil particles. Leaves were dried at 80 °C, and the other organs were dried at 103 °C (standard temperature

for drying woody parts such as stems, branches and roots, Williamson and Wiemann, 2010) until a constant weight was reached. To determine the leaf mass per area (LMA), three leaf discs (Ø: 12 mm, avoiding the main vein) per plant were collected and dried at 80 °C until a constant weight was reached, and LMA was calculated as leaf dry weight (DW) divided by leaf area (Watanabe et al., 2018). The water status was evaluated by determining the leaf water content (LWC), calculated as (FW – DW)/FW × 100, where FW and DW are fresh and dry weight, respectively.

2.3. Reactive oxygen species content and oxidative damage

Anion superoxide ($^{\bullet}O_2^-$) content was measured according to Tonelli et al. (2015), after extraction with potassium/phosphate (K/P) buffer (50 mM, pH 7.8) with a fluorescence/absorbance microplate reader (Victor3 1420 Multilabel Counter, PerkinElmer, Waltham, MA, USA) at 470 nm, after subtracting the background absorbance due to the buffer solution and to the assay reagents. This assay is based on the reduction of a tetrazolium dye sodium, 3'-(1-[phenylamino carbonyl]-3, 4-tetrazolium)-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate (XTT) by O_2 to a soluble XTT formazan (Able et al., 1998). Hydrogen peroxide (H_2O_2) content was measured according to Shin et al. (2005), after extraction with K/P buffer (20 mM, pH 6.5) by using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Life Technologies Corp. Carlsbad, CA, USA). The determination was performed with the same fluorescence/absorbance microplate reader reported above at 530 and 590 nm (excitation and emission of resofurin fluorescence, respectively). Oxidative damage was estimated by determining the MDA by-product accumulation, according to the method of Hodges et al. (1999) with minor modifications, as reported by Guidi et al. (2017), after extraction with trichloroacetic acid (0.1%, w/v). The determination was performed with a spectrophotometer (UV-1900 UV–vis, Shimadzu, Kyoto, Japan) at 532 and 600 nm.

2.4. Gas exchange and biogenic volatile organic compounds assessment

Biogenic Volatile Organic Compounds (BVOC) sampling with concurrent measurement of leaf gas exchange was made according to the methodology suggested by Loreto et al. (2001). Measurements were performed using a portable infrared gas analyser (Li-Cor 6400, Li-Cor, Lincoln, USA) with 6-cm^2 leaf cuvette operating at 400 ppm (ambient) CO_2 concentration and 1000 μmol m^{-2} s^{-1} photosynthetic active radiation (standard PAR for the BVOC sampling, Loreto et al., 2001) during 1st to August 4, 2018. The temperature and relative humidity inside the leaf cuvette were 30 °C and 50%, respectively. All gas exchange measurements were carried out in the morning (9:00 a.m. to 12:00 a.m.) to avoid the midday depression of photosynthesis. First, net CO_2 assimilation rate (A), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were determined. After that, when a steady-state A was reached, the air existing inside a leaf cuvette was collected by using stainless steel cartridges packed with 200 mg of Tenax TA and Carbograph 1TD (Markes International, Ltd., Llantrisant, United Kingdom) directly connected to the Li-Cor 6400 cuvette with a Teflon tube. The flow rate inside the Li-Cor cuvette was set to 500 μmol s^{-1}, and a subsample air was taken at a flow rate of 200 mL min^{-1} through the cartridge with an external pump (VSS-1, AP Buck, Orlando, FL, USA) for 15 min (total volume: 3-l of air). Background samples (without leaf inside a cuvette) were also collected at the beginning and end of sampling in each day. All traps were sealed and then stored at −20 °C until the analysis. Isoprene and monoterpene emissions were analysed as described in Baraldi et al. (2019). In detail, sampled traps were analysed using a thermal-desorber (Markes International, Series 2 Unity), and a 7890A gas chromatograph connected with a 5975C mass detector (GC-MS, Agilent Technologies, Wilmington, DE, USA). Thermal desorption of the sampling tubes was carried out for 15 min at 280 °C with a helium flow rate of 50 ml min^{-1}, then rapid heating of the cold trap from 30 °C to 280 °C and fast injection of analytes onto the capillary column (HP-1, 60 m × 0.25 mm I.D. × 0.25 μm film of polymethylsiloxane; J&W Scientific, Agilent Technologies, Palo Alto, CA, USA) via a transfer line heated at 200 °C. The separation of BVOC was achieved according to Rapparini et al. (2004), and identified by software comparison of retention times and fragmentation pattern with the Wiley 275 database of mass spectra and external reference compounds. The identified BVOC were quantified using external standard calibration procedure.

2.5. Photosynthetic pigments

Total chlorophylls and carotenoids were measured according to Podda et al. (2019), after extraction with 100% HPLC-grade methanol by using a Dionex UltiMate 3000 system (Thermo Scientific, Waltham, MA, USA) equipped with an Acclaim 120 Dionex column (C18, 4.6 mm internal diameter × 150 mm length, 5 μm particle size; Thermo Scientific). Pigments were eluted at a flow rate of 1 ml min^{-1} using 100% solvent A (acetonitrile/methanol, 75/25, v/v) for the first 14 min followed by a 1.5 min linear gradient to 100% solvent B (methanol/ethylacetate, 68/32, v/v), 15 min with 100% solvent B, followed by 2 min linear gradient to 100% solvent A. Pigments were detected at 445 nm with a Dionex UVD 170 U UV–Vis detector (Thermo Scientific). Total chlorophyll content was calculated as the sum of chlorophyll *a* and *b*, while total carotenoid content was calculated as the sum of neoxanthin, violaxanthin, anteraxanthin, lutein, zeaxanthin and β-carotene.

2.6. Statistical analysis

Data were collected for one to three plants per each combination of O_3 and water treatments per each plot in a FACE and plot means were used as statistical unit, i.e., n = 3 plots. All data were firstly tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene test), and then analysed by a full-factorial two-way analysis of variance (ANOVA) with O_3 and watering regime as fixed factors. For this ANOVA test, BVOC emissions were log-transformed prior to the analyses. Differences among means were tested by the Tukey's honestly significant difference (HSD) *post-hoc* test. Statistically significant effects were considered for $P \leq 0.05$. Statistical analyses were performed in JMP 11.0 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Plant biomass, leaf mass per area and water status

At the end of the exposure, plants did not show visible foliar injury. For biometric parameters, the two-way ANOVA showed a significant effect only for the unifactorial watering regime (Table 1): WS induced a marked reduction of both shoot and root biomass [−42 and −21%, respectively, compared with WW conditions (throughout the whole text, percentages of WS and/or increased O_3 effects are calculated in comparison with AA_WW conditions); Fig. S1].

The two-way ANOVA of LMA and LWC showed that the interactive effect between watering regime and O_3 was significant (Table 1). A slight increase of LMA was found as a consequence of moderate O_3 concentrations under WW conditions (i.e., 1.5 × AA_WW; +16%; Fig. 1A). Conversely, only WS induced a significant reduction of LWC, regardless of O_3 level (−17, −13 and −24% in AA_WS, 1.5 × AA_WS and 2.0 × AA_WS, respectively; Fig. 1B).

3.2. Reactive oxygen species and oxidative damage

The two-way ANOVA of $^{\bullet}O_2^-$, H_2O_2 and MDA by-product contents showed that the effects of watering regime, O_3, and their interaction were significant (Table 1). A significant production of $^{\bullet}O_2^-$ was found as a consequence of WS, which was amplified by the co-occurrence of

Table 1
P levels (***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$, ns $P > 0.05$) of two-way ANOVA for the effects of ozone (O_3), watering regime and their interaction on shoot biomass, root biomass, leaf mass per area (LMA), leaf water content (LWC), anion superoxide ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), assimilation rate (A), stomatal conductance (g_s), internal CO_2 concentration (C_i), terpenes (α-thujene, α-pinene, camphene, sabinene, β-pinene, myrcene, α-phellandrene, δ-3-carene, p-cymene, limonene, cis-ocimene, trans-ocimene, γ-terpinene, terpinolene and linalol), other biogenic volatile compounds (BVOC, heptaldehyde, cis-3-hexenyl acetate, acetophenone, nonanal and decanal), total chlorophylls and total carotenoids in *Phillyrea angustifolia* leaves subjected to different watering regimes and exposed to increasing O_3 concentrations. d.f. represents the degrees of freedom.

Effects	d. f.	Shoot biomass	Root biomass	LMA	LWC	$^{\bullet}O_2^-$	H_2O_2	MDA	A	g_s	C_i	Terpenes	BVOC	Total chlorophylls	Total carotenoids
Watering regime	1	***	***	ns	***	***	***	***	***	***	***	ns	ns	***	***
O_3	2	ns	ns	*	ns	***	***	***	**	***	*	*	ns	***	*
Watering regime × O_3	1	ns	ns	*	*	*	***	*	*	***	**	ns	*	*	*

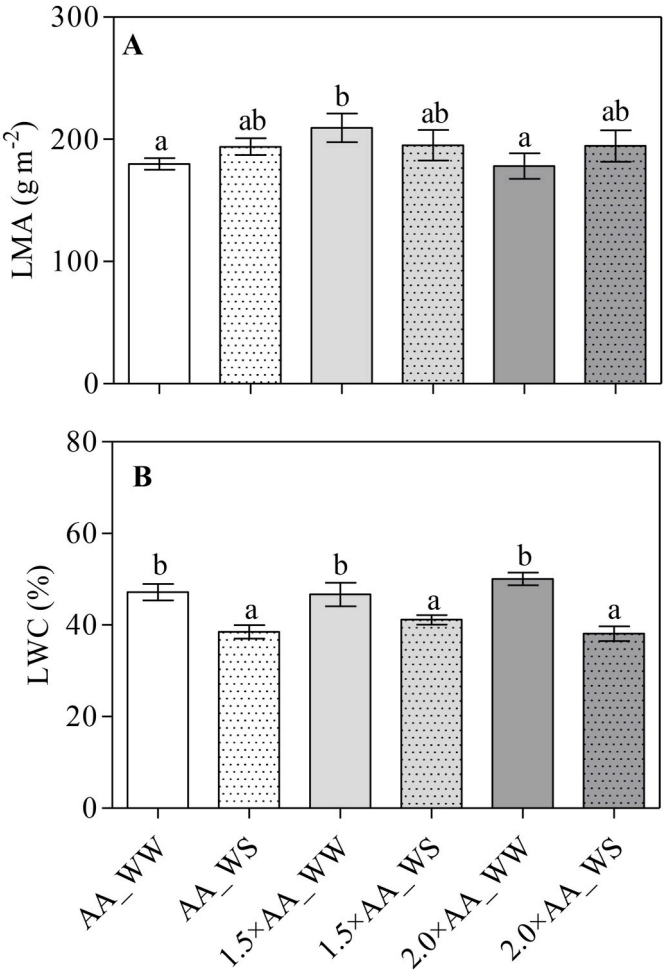

Fig. 1. Leaf mass per area (LMA; **A**) and leaf water content (LWC; **B**) in *Phillyrea angustifolia* plants subjected to different watering regimes [well-watered (WW, *solid fill*) and water-stressed (WS, *pattern fill*)] and exposed to increasing O_3 concentrations [applied for 4.5 months: ambient air (AA; *white fill*), 1.5 × and 2.0 × ambient O_3 (1.5 × AA and 2.0 × AA; *grey* and *dark grey fill*, respectively)]. Data are shown as mean ± standard error (n = 3 plots). Since two-way ANOVA reveals a significant watering regime and O_3 interaction on LMA and LWC, according to Tukey's post-hoc test, different letters indicate significant differences among means in each graph ($P \leq 0.05$).

increased O_3 concentrations (+26, +41 and + 34% in AA_WS, 1.5 × AA_WS and 2.0 × AA_WS, respectively; Fig. 2A). Differently, H_2O_2 and MDA by-products increased due to WS *per se* (i.e., in AA_WS; about 2- and 3-fold higher, respectively), as well as to increased O_3 *per se*, following the O_3 concentration gradient (H_2O_2: about 2- and 4-fold higher; MDA: +22 and + 91%; in 1.5 × AA_WW and 2.0 × AA_WW, respectively). The co-occurrence of 1.5 × AA and WS increased H_2O_2 and MDA to 2.0 × AA_WW levels, while no further increase due to WS was observed under 2.0 × AA. Actually, only MDA by-products increased more under 2.0 × AA_WS than under 1.5 × AA_WS (+49 and + 18%, respectively; Fig. 2B and C).

3.3. Gas exchange and biogenic volatile organic compounds

The two-way ANOVA of A, g_s and C_i showed that the effects of watering regime, O_3, and their interaction were significant (Table 1). A significant reduction of A, g_s and C_i was found as a consequence of WS applied singly or in combination with increased O_3 (−46, −64 and −14%, respectively; as average among O_3 concentrations; Fig. 3). Differently, comparing WW samples, although lower A values were reported under 2.0 × AA_WW than 1.5 × AA_WW, no significant differences were found with AA_WW; while only the highest O_3 concentration decreased g_s and C_i (−45 and −9%, respectively; Fig. 3B and C).

The two-way ANOVA showed that the interaction between watering regime and O_3 was not significant for the emission of terpenes while it was significant for the emission of other BVOC (Table 1). The effects of single factors (except "watering regime" for BVOC) were significant for the emission of terpenes and other BVOC. Twenty BVOC spontaneously emitted in the headspace by foliar samples were identified, belonging to the terpene (α- and β-pinene, sabinene, myrcene, Δ-3- carene, para-cymene, limonene, cis and trans β -o-cymene and linalool), aldehyde (heptaldehyde, nonanal and decanal), ester (cis-3-hexenyl acetate) and ketone (1-phenyl-ethanone) classes (S1). Among monoterpene hydrocarbons, α-pinene was the most abundant in all plants grown under WW conditions. Other monoterpenes detected in all samples and exhibiting noteworthy relative abundances (in particular under WS conditions) were sabinene, para-cymene and trans-ocymene. Only elevated O_3 concentrations *per se* raised the content of terpenes, independently of watering regime (up to 4-fold higher; Fig. 4A). Differently, the emission of terpenes slightly decreased due to WS *per se*, except for the highest O_3 concentration (about +40%). Among other BVOC, nonanal, acetophenone and cis-3-hexenyl acetate were the most abundant in almost all samples. However, a significant reduction of other BVOC emission was observed under 1.5 × AA_WW and 2.0 × AA_WS conditions (about −60 and −69%, respectively; Fig. 4B).

3.4. Photosynthetic pigments

The two-way ANOVA of total chlorophylls and total carotenoids revealed that the effects of watering regime, O_3, and their interaction were significant (Table 1). Water shortage significantly decreased total chlorophylls, especially under increased O_3 concentrations (−16, −22 and −26% in AA_WS, 1.5 × AA_WS and 2.0 × AA_WS, respectively, compared with WW conditions; Fig. 5A). Under WW conditions, only the highest O_3 concentration decreased total chlorophylls (−18% compared with AA). A slight reduction of total carotenoids was also found as a

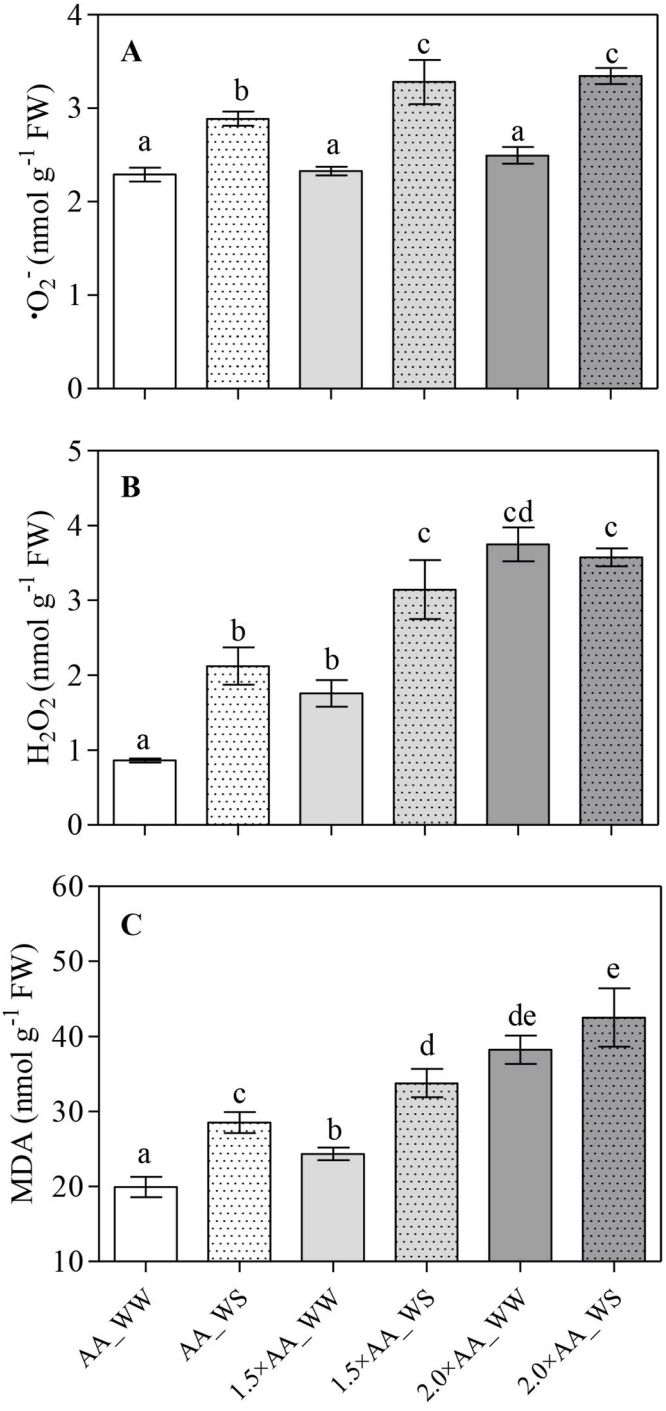

Fig. 2. Anion superoxide ($\bullet O_2^-$; **A**), hydrogen peroxide (H_2O_2; **B**) and malondialdehyde (MDA; **C**) by-products content in *Phillyrea angustifolia* plants subjected to different watering regimes [well-watered (WW, *solid fill*) and water-stressed (WS, *pattern fill*)] and exposed to increasing O_3 concentrations [applied for 4.5 months: ambient air (AA; *white fill*), 1.5 × and 2.0 × ambient O_3 (1.5 × AA and 2.0 × AA; *grey* and *dark grey fill*, respectively)]. Data are shown as mean ± standard error (n = 3 plots), on a fresh weight (FW) basis. Since two-way ANOVA reveals a significant watering regime and O_3 interaction on $\bullet O_2^-$, H_2O_2 and MDA, according to Tukey's *post-hoc* test, different letters indicate significant differences among means in each graph ($P \leq 0.05$).

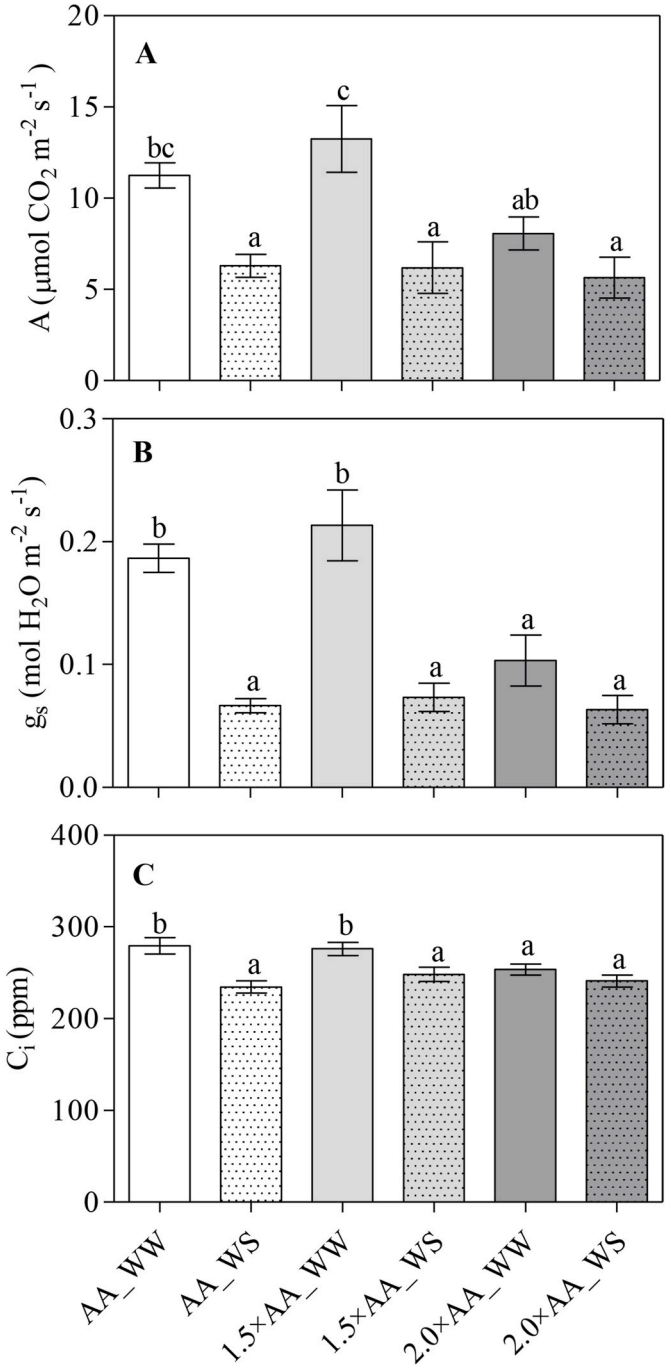

Fig. 3. Assimilation rate (A; **A**), stomatal conductance (g_s; **B**) and internal CO_2 concentration (C_i; **C**) in *Phillyrea angustifolia* plants subjected to different watering regimes [well-watered (WW, *solid fill*) and water-stressed (WS, *pattern fill*)] and exposed to increasing O_3 concentrations [applied for 4.5 months: ambient air (AA; *white fill*), 1.5 × and 2.0 × ambient O_3 (1.5 × AA and 2.0 × AA; *grey* and *dark grey fill*, respectively)]. Data are shown as mean ± standard error (n = 3 plots). Since two-way ANOVA reveals a significant watering regime and O_3 interaction on A, g_s and C_i, according to Tukey's *post-hoc* test, different letters indicate significant differences among means in each graph ($P \leq 0.05$).

consequence of WS, regardless of O_3 level (−15, −13 and −22% in AA_WS, 1.5 × AA_WS and 2.0 × AA_WS, respectively, compared with WW conditions; Fig. 5B). Under WW conditions, increased O_3 did not change total carotenoids.

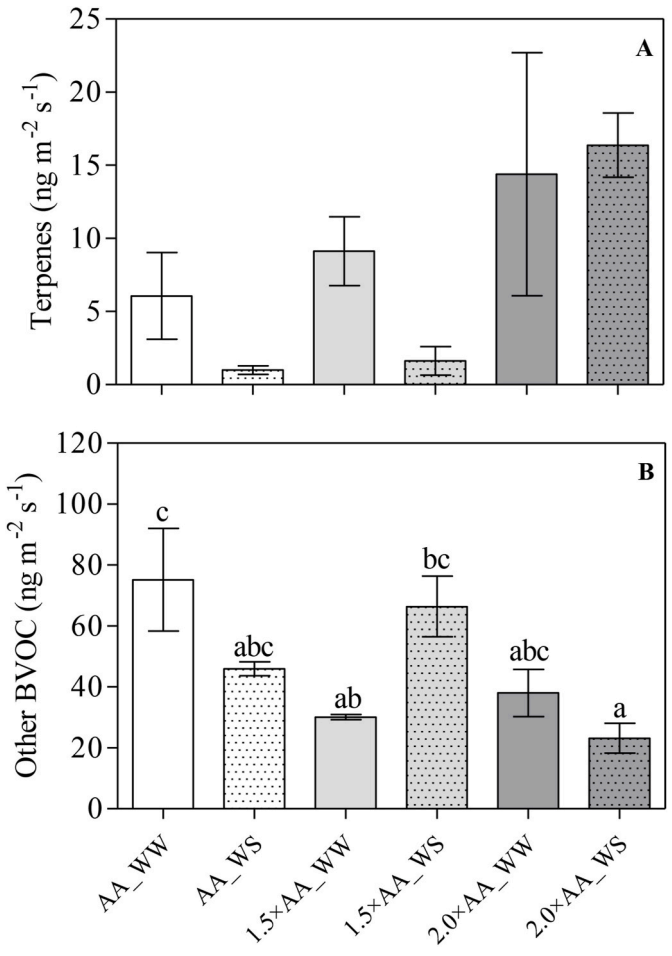

Fig. 4. Terpenes (α-thujene, α-pinene, camphene, sabinene, β-pinene, myrcene, α-phellandrene, δ-3-carene, para-cymene, limonene, cis-ocymene, trans-ocymene, γ-terpinene, terpinolene and linalool; **A**) and other volatile hydrocarbons belonging to different classes compounds (BVOC, heptaldehyde, cis-3-hexenyl acetate, acetophenone, nonanal and decanal; **B**) in *Phillyrea angustifolia* plants subjected to different watering regimes [well-watered (WW, *solid fill*) and water-stressed (WS, *pattern fill*)] and exposed to increasing O_3 concentrations [applied for 4.5 months: ambient air (AA; *white fill*), 1.5 × and 2.0 × ambient O_3 (1.5 × AA and 2.0 × AA; *grey* and *dark grey fill*, respectively)]. Data are shown as mean ± standard error (n = 3 plots). Since two-way ANOVA reveals a significant watering regime and O_3 interaction on other volatile hydrocarbons belonging to different classes compounds, according to Tukey's *post-hoc* test, different letters indicate significant differences among means ($P \leq 0.05$).

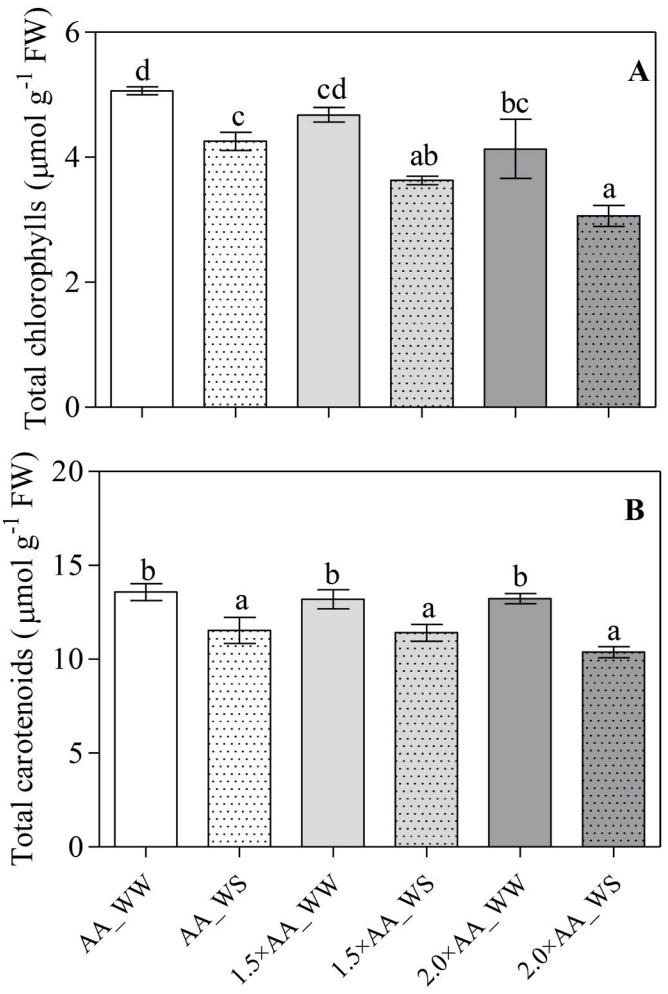

Fig. 5. Total chlorophylls (**A**) and total carotenoids (**B**) in *Phillyrea angustifolia* plants subjected to different watering regimes [well-watered (WW, *solid fill*) and water-stressed (WS, *pattern fill*)] and exposed to increasing O_3 concentrations [applied for 4.5 months: ambient air (AA; *white fill*), 1.5 × and 2.0 × ambient O_3 (1.5 × AA and 2.0 × AA; *grey* and *dark grey fill*, respectively)]. Data are shown as mean ± standard error (n = 3 plots), on a fresh weight (FW) basis. Since two-way ANOVA reveals a significant watering regime and O_3 interaction on total chlorophylls and carotenoids, according to Tukey's *post-hoc* test, different letters indicate significant differences among means in each graph ($P \leq 0.05$). FW, fresh weight.

4. Discussion

4.1. Are ROS, MDA, and photosynthetic pigments suitable markers to discriminate whether oxidative stress is due to drought and increasing O_3 concentrations applied singularly or a combination of them?

Abiotic stresses such as drought and O_3 may lead to an imbalance between the amount of ROS and the antioxidant defences (Hasanuzzaman et al., 2020). Although ROS are required for intra- and intercellular signalling, their accumulation may induce oxidative stress (Foyer and Noctor, 2005). Hydrogen peroxide, which is formed in the PSI from $^{\bullet}O_2^-$ owing to superoxide dismutase activity, seems to be one of the major ROS involved in oxidative stress-induced cell death (Triantaphylidès et al., 2008). In the present experiment, a stress-specific behaviour of ROS was reported: $^{\bullet}O_2^-$ levels were not affected by increasing O_3 *per se*, while their accumulation due to drought was exacerbated by the co-occurrence of increasing O_3 concentrations; H_2O_2 accumulation induced by both drought and increasing O_3 *per se* (importantly, O_3-induced accumulation was positively related to the increased O_3 concentrations) was exacerbated when stressors occurred concomitantly, but no significant differences were observed between 2.0 × AA_WW and 2.0 × AA_WS conditions (likely because the highest O_3 concentration already induced the maximum H_2O_2 accumulation). The observed ROS generation and the spontaneous reaction of O_3 with unsaturated lipids of the plasma membrane led to peroxidative processes (as confirmed by the increased MDA by-product values), confirming the oxidative pressure caused by both stressors (Foyer et al., 1997; Cotrozzi et al., 2017). Specifically, oxidative damage (i.e., MDA; one of the most widely investigated reactive carbonyl species correlated with oxidative stress; Pintó-Marijuan and Munné-Bosch, 2014) showed a similar behaviour to H_2O_2, but with significant differences between 1.5 × AA_WS and 2.0 × AA_WS (i.e., harsher environmental conditions). Thus, $^{\bullet}O_2^-$, H_2O_2 and MDA, when observed individually, were not suitable markers to discriminate the differential oxidative stress induced by the various environmental conditions, as opposed to when they were evaluated together (i.e., combining the information provided by the analysis of each stress marker): (i) all these stress markers increased under

drought *per se*; (ii) H_2O_2 and MDA increased under O_3 *per se*, following the gradient of O_3 concentrations; (iii) joining together the ROS it was possible to report harsher effects under 2.0 × AA_WS and 1.5 × AA_WS (both $^{\bullet}O_2^-$ and H_2O_2 increased) than under 2.0 × AA_WW (only H_2O_2 increased); and (iv) MDA showed harsher effects under 2.0 × AA_WS than under 1.5 × AA_WS.

The oxidation chain can affect several cellular structures such as membranes, lipids and proteins involved in photosynthetic reactions (e.g., electron transport, photochemical processes; Huang et al., 2019). The quantification of photosynthetic pigments involved in light absorption is a clue to the actual functional capacity of photosystems (Pintó-Marijuan and Munné-Bosch, 2014). In the present experiment, a stress-specific degradation of photosynthetic pigments was also reported, in particular the concomitant reduction of total chlorophylls and carotenoids mostly observed under combined stress conditions (except total carotenoids under 2.0 × AA_WS conditions). Actually, O_3 *per se* did not reduce photosynthetic pigments (except for 2.0 × AA on total chlorophylls), indicating a drought-dependent involvement of these molecules in the oxidative processes in *P. angustifolia* (Munné-Bosch and Peñuelas, 2003). The reduction of photosynthetic pigments was associated with a lower photosynthetic performance of plants grown under WS than those under WW, regardless of O_3 concentration (as confirmed by the gas exchange analysis) hinting that a failure of adaptation to oxidative conditions occurred (as confirmed by the reduced total biomass; Calatayud and Barreno, 2004). Some authors suggested that the decline of photosynthetic pigments may be considered a secondary effect of oxidative stress related to accelerated aging and nutrient remobilization (Köllner and Krause, 2000; Pellegrini et al., 2015). Conversely, the chlorophyll loss due to elevated O_3 concentrations *per se* (i.e., 2.0 × AA_WW) likely played a regulatory role to protect chloroplasts still retaining substantial photosynthetic activity (as confirmed by the unchanged A values compared with AA_WW conditions; Kyparissis et al., 1995). Chlorophyll degradation alone does not necessarily indicate oxidative processes in all cases, as these pigments can also be degraded enzymatically (Munné-Bosch and Peñuelas, 2003). Consequently, the potential decline in light absorption due to the decrease of total chlorophylls likely reduced the potential damaging heating effect due to high solar radiation in O_3-stressed plants grown under WW conditions, whose stomata were closed (as confirmed by the decrease of g_s levels). The absence of permanent damage induced by O_3 (no visible injury and unchanged LMA values were observed independently of watering regime) indicated a low sensitivity of *P. angustifolia* to O_3. These results suggest that photosynthetic pigments (total chlorophylls + carotenoids) were suitable markers only to monitor oxidative stress due to drought, and only total chlorophylls were able to discriminate the harsher effects due to the co-occurrence of drought and increased O_3 concentrations. In accordance with the above, these outcomes suggest to use photosynthetic pigments in combination with other stress markers (e.g., ROS and MDA).

4.2. What physiological and biochemical adjustments are adopted by *P. angustifolia* to perceive and counter to drought and/or O_3?

According to Levitt (1980), avoidance is the first strategy for a plant to resist a chemical insult. In particular, the capacity of stomata to control water loss and O_3 flux (i.e., avoidance) can be considered as one of the most important physiological mechanisms involved in plant acclimation/adaptation (Cotrozzi et al., 2016). Our results show that drought (singularly or in combination with increasing O_3 concentrations) impaired the photosynthetic process. In particular, the drop in photosynthesis was mostly attributable to WS-induced stomatal closure confirming that stomata posed the predominant limitation to CO_2 assimilation (dependent on watering regime) and represented the main avoidance strategy against O_3 (corroborating the isohydric behaviour of *P. angustifolia*, Hoshika et al., 2020). Ozone *per se* reduced g_s values only at the highest concentrations (2.0 × AA) suggesting that stomatal closure may be just a reaction against O_3 (not a physiological adjustment), thus protecting plants (Hoshika et al., 2020). Plant resistance to oxidative stress also depends on leaf biochemical traits which are crucial for avoiding and preventing oxidative stress (Sharma et al., 2012). A plethora of studies have described how antioxidant concentrations and antioxidant enzymatic activities are modified under abiotic stresses, also in *P. angustifolia* plants subjected to individual and combined stresses (e.g., drought, O_3, high temperature; Munné-Bosch and Peñuelas, 2003; Peñuelas et al., 2004; Ciccarelli et al., 2019). Carotenoids not only absorb blue light used for photosynthesis (as previously discussed), but they are also involved in energy dissipation by heat through the xanthophyll cycle, quenching $^1O_2$ formed during photo-oxidation, abscisic acid biosynthesis, and regulation of thylakoid membrane fluidity (Havaux et al., 2005). For this reason, they can transiently complement the action of the primary antioxidants (i.e., ascorbic acid and enzymatic antioxidant compounds; Brunetti et al., 2015). Our results show that drought *per se* and in combination with increasing O_3 induced a reduction of total carotenoids suggesting that these antioxidants could be consumed by the cell to counteract the possible ROS generation due to increased oxidative metabolism, so reducing the risk of oxidative stress (Niinemets et al., 2003). The utilization of these compounds likely improved the stress tolerance of *P. angustifolia* suggesting that this species was able to partially avoid increasing O_3 concentrations (as previously reported) and tolerate O_3-induced effects under WS conditions. Ozone *per se* did not give rise to the same effects in terms of decreased total carotenoids, but it significantly elicited terpene emission, which may be considered as a factor to protect plants from oxidative damage due to O_3 ("hormesis perspective"; Agathokleous et al., 2018). In particular, the stimulation of isoprenoid metabolites may contribute to the reduction of ROS excessively generated under elevated O_3 concentrations (as confirmed by the unchanged values of $\bullet O_2^-$) by priming antioxidant responses and systemic acquired resistance (Riedlmeier et al., 2017). Therefore, the activation of this protective mechanism and the fact that the effects of O_3 on photosynthesis and biomass were not significant confirm a low sensitivity of *P. angustifolia* to O_3. To date, too few monoterpene-emitting species have been investigated concerning their response to stressors (Feng et al., 2019) and further investigations are required to elucidate the redox chemistry of isoprenoid metabolites under oxidative stress.

5. Conclusions

In conclusion, our study shows that *P. angustifolia* was relatively sensitive to drought, and moderately tolerant to current and future O_3 concentrations. The combination of both stressors led to harsher oxidative stress. The selected stress markers (i.e., ROS, MDA and photosynthetic pigments), when observed individually, were not suitable to discriminate the differential oxidative stress induced by drought and increasing O_3 concentrations applied singularly or a combination of them, as opposed to when these stress markers were evaluated together (i.e., combining the information provided by the analysis of each stress marker). Plants activated some physiological and biochemical adjustments in order to partially avoid (e.g., stomatal closure) and tolerate (e.g., increased terpene emission) the effects of drought when combined with increasing O_3 concentrations suggesting that the water use strategy (isohydric) and the sclerophyllous habit can further increase the plant tolerance to environmental constraints in the Mediterranean area.

Credit author statement

Elisa Pellegrini: Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. Lorenzo Cotrozzi: Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. Luisa Neri: Formal analysis, Investigation, Data curation, Writing – review & editing. Rita Baraldi: Formal analysis, Investigation, Data curation, Writing – review & editing. Elisa Carrari: Formal analysis,

Investigation, Data curation, Writing – review & editing. Cristina Nali: Conceptualization, Writing – review & editing. Giacomo Lorenzini: Conceptualization, Writing – review & editing. Elena Paoletti: Methodology, Conceptualization, Writing – review & editing, Supervision, Funding acquisition. Yasutomo Hoshika: Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing, Supervision.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2021.111615.

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