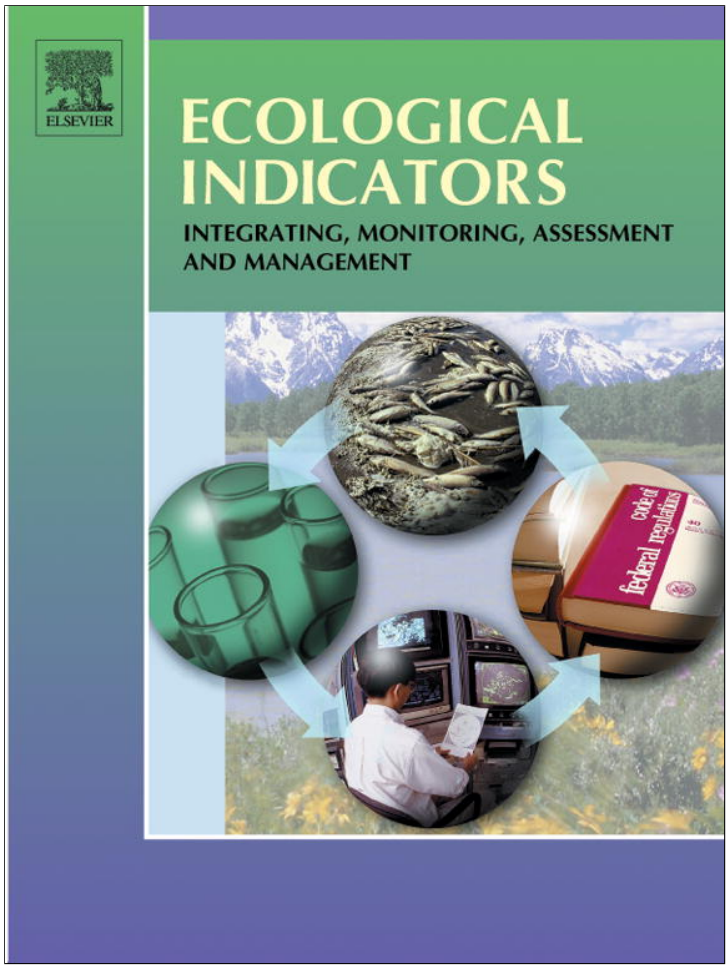

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Original article

Why lichens are bad biomonitors of ozone pollution?

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ABSTRACT

The combined effects of water availability and ozone (O_3) on chlorophyll *a* fluorescence (Chl_aF) were studied in three epiphytic lichens selected for their different ecology. The samples were exposed in open top chambers (OTCs) under different watering regimes with O_3 AOT40 in the range 0–50,000 ppb. Further samples were exposed in a nearby wood, as controls. Chl_aF measurements were taken before exposure, after 3- and 6-week exposure and after a subsequent 2-day recovery period to verify the long-term effects of O_3 exposure. All species tolerated the pollutant well. However, there was a strong influence associated with the mode of exposure: the Chl_aF emission remained steady over time in the controls, whereas it varied significantly in chamber-exposed samples, with a strong decrease of F_v/F_m in non-watered and morning-watered samples, and a small decrease in evening-watered samples. Chl_aF emission characteristics were also influenced by the weather conditions of the day preceding measurements, with some species-specific differences possibly related to species ecology. The ozone-tolerance of lichens is thoroughly discussed on the basis of the cellular mechanisms that allow these organisms to overcome the oxidative burst associated with the cycles of dehydration–rehydration typical of poikilohydrous organisms.

1. Introduction

Tropospheric ozone (O_3) is an important component of global change that is mostly determined by human activities, such as the incomplete combustion of fossil fuels (gasoline, diesel, etc.) (Logan, 1985; Wu et al., 2008). O_3 has direct, deleterious consequences on plants and animals, exerted through the fast formation of reactive oxygen species (ROS) that are particularly damaging to cell membranes, enzymes and DNA (Pell et al., 1997). In vascular plants ROS are readily formed as soon as O_3 enters the leaf through stomata by diffusion (Laisk et al., 1989), and therefore a large number of vascular plants are very sensitive to O_3. Lichens, composite organisms consisting of a symbiotic association of a fungus (the mycobiont) and one or more photosynthetic partners (the photobionts), have been studied only sporadically in relation to ozone toxicity, notwithstanding their frequent use as biomonitors of air pollution (Bargagli and Nimis, 2002). The few field studies available so far show that high concentrations of O_3 do not cause an appreciable impoverishment of the lichen flora (Lorenzini et al., 2003; Ruoss and Vonaburg, 1995), at least if peroxyacetylnitrates or other organic pollutants are not co-occurring (Egger et al., 1994; Sigal and Nash, 1983; Zambrano and Nash, 2000). Physiological studies gave more conflicting results. No significant consequence to O_3 exposure were detected by Calatayud et al. (2000), Riddell et al. (2010; 2012) and Rosentreter and Ahmadjian (1977), while limited effects were identified by Nash and Sigal (1979), Ross and Nash (1983), and Tarhanen et al. (1997). Severe damage to the integrity of photosystems, the collapse of a high percentage of photobiont cells and, occasionally, blanching of the thallus surface were instead reported by Scheidegger and Schroeter (1995). The latter study, frequently cited in the literature as a proof of lichen sensitivity to O_3 pollution, was based on lichens fumigated with O_3 in open top chambers (OTCs). Riddell et al. (2012), however, identified a clear chamber-effect associated with lichen exposure in OTCs and, therefore, it might be argued that the conclusions of Scheidegger and Schroeter (1995) were influenced as much by the exposure conditions than by the pollutant itself. A critical point is certainly the artificial rehydration process to which samples are typically subjected in this type of exposure. In contrast to vascular plants, lichens are poikilohydrous organisms, and therefore their water status varies passively according to the surrounding environmental conditions (Nash, 2008). In the OTCs lichens must be artificially watered. This process might affect lichen response to O_3 as a consequence of small differences in timing, frequency and/or intensity of sample watering. In fact, a recent field study with lichen transplants suggests that water availability is the key factor moderating O_3 resistance, because daily

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rehydrated thalli can repair O_3 damage effectively and replenish their reservoir of antioxidants (Tretiach et al., 2012b).

In this work the effects of O_3 on three epiphytic lichens with different ecologies were investigated by exposing portions of thalli subjected to different hydration regimes in OTCs with O_3 AOT40 in the range 0–50,000 ppb. To estimate the response of lichens to the different treatments, chlorophyll *a* fluorescence (Chl_aF) emission was measured. This is a non-destructive technique commonly used in plant physiology and nowadays frequently applied also to lichens (e.g. Calatayud et al., 2000; Scheidegger and Schroeter, 1995; Tretiach et al., 2007). Derived Chl_aF parameters can provide reliable information about the state of photosystems and relationships with a wide range of environmental factors, including specific pollutants (Baker and Rosenqvist, 2004; Bertuzzi and Tretiach, 2013; Nash, 2008).

2. Materials and methods

2.1. Lichen sampling and pre-treatment of samples

Three lichens, characterized by relatively well known biologies, were chosen: *Xanthoria parietina* (L.) Th. Fr., *Flavoparmelia caperata* (L.) Hale and *Parmotrema perlatum* (Huds.) M. Choisy. The species have different ecological requirements and resistance to pollutants, as shown in Table 1 and references cited therein; their photobionts are green coccoid algae of the genus T*rebouxia* de Puymaly (Ahmadjian, 1993, 2001). The samples were collected from deciduous trees in sites of NE Italy far from known air pollution sources (Table 1). Several healthy-looking thalli were detached from the bark using a sharp blade, put in open petri dishes and immediately transported to the laboratory, where the material was left to dry out at room temperature, in darkness for two days. Randomly selected thalli were then closed in petri dishes, sealed in vacuum bags and sent to the OPAL project laboratories at Imperial College's Silwood Park campus in Ascot, GB, for the following exposure experiments. Here the thalli were carefully cleaned of debris and bryophytes; 52 (*X. parietina*) and 78 (*F. caperata* and *P. perlatum*) samples were randomly cut from different thalli, numbered and photographed. Each sample (60 ± 5 mg dry weight) was tied with nylon threads to a small cork strut ($2.5 \times 4.0 \times 0.3$ cm) to facilitate moving and exposure, and kept dry under diffuse light (<10 μmol photons $m^{-2}\,s^{-1}$) until use, within a maximum of 21 days from sampling. Prior to exposure, the samples were subjected to a conditioning process for two days: they were immersed in distilled water for 3 min every 12 h and, in the remaining time, placed on rigid plastic nets within plastic boxes containing water at the bottom; the boxes were covered (but not sealed) with transparent plastic wrap (>95% RH) and placed in an incubator at $20 \pm 1\,^{\circ}C$, with a light/dark regime of 12/12 h and a light intensity corresponding to one sixth of the species-specific photosynthetic photon flux at which the quantum yield of CO_2 assimilation is the highest (see Table 1).

2.2. Sample exposure

The lichen samples were exposed for 6 weeks from July to September 2010, with a delay of one week between two successive species, starting with *P. perlatum* and finishing with *X. parietina*, in eight open top chambers (OTCs) ventilated with charcoal filtered air. In six chambers, O_3 generated using an O_3 generator (model GEN02-03, Bio-Fresh Ltd. UK), was added to filtered air to expose samples to different pollutant concentrations. Concentration measurements were continuously recorded with an O_3 monitor (model 202, 2B Technologies Inc., Boulder, Colorado, USA). The accumulated ozone exposure (AOT40) was calculated above a threshold concentration of 40 ppb as $\Sigma(c - c_0)$ for every $(c - c_0) > 40$ ppb. The maximum concentration of O_3 in the chambers were reached during the central hour of the day, but since the control of the O_3 generator did not allow, for short periods of time, to achieve concentrations below 40 ppb during the night, the AOT40 was calculated over the 24 h and not only in the interval 8:00 am–8:00 pm as indicated by the Directive 2002/3/EC (Nali et al., 2009).

The samples were positioned 2 m above ground on the north-facing portion of the internal wall of each OTC. Small black cardboard panels were placed perpendicular to the wall between three contiguous samples to avoid direct light. The samples within each OTC were divided into three groups (A–C) of three samples each, that were subjected to different hydration regimes: watered with a spray of distilled water at 9:00 am ("morning watered", group A); watered at 6:00 pm ("evening watered", group B); non-watered (group C). During exposure, therefore, group C could benefit only from the humidity of the air, whereas the two others were certainly active for some hours during the O_3 treatment (group A) or immediately after it (group B).

One further group of samples (group D; namely, 4 samples of *X. parientina* and 6 samples of *F. caperata* and *P. perlatum*) was exposed in a wood on the trunks of some oaks only 100 m away from the OTCs. These samples were exposed to normal rainfall and dew events, and served as control to check the effects of microclimatic conditions within the OTCs.

2.3. Chl_aF measurements

Chl_aF measurements were taken before and after 3 and 6 weeks of exposure, and after a further two days of recovery in the incubator under the conditions described above, to verify the long-term

Table 1
Investigated lichens, with respective photobionts, sampling sites, altitude (Alt., m above sea level), collection dates, substrata, species-specific photosynthetic photon flux at which the quantum yield of CO_2 assimilation is the highest ($PPFD_{lk}$, μmol photons $m^{-2}\,s^{-1}$) (Piccotto and Tretiach, 2010) and categories of acidophytism (pH), hygrophytism (H), eutrophication (N), poleophobism (Pol.) according to Nimis and Martellos (2008).

Species	Photobionts	Sampling site	Alt.	Date(s)	Substratum	$PPFD_{lk}$	pH	H	N	Pol.
Xanthoria parietina (L.) Th.Fr.	*T. irregularis* Hildreth & Ahmadjian *T. arboricola* de Puymaly	Italy, Friulan-Venetian Plain, Udine, prov., Latisana	5	29-June-2010	Northerly exposed bark of *Juglans* sp.	131	2–4	3–5	3–4	3–1
Parmotrema perlatum (Huds.) M.Choisy	*T. crenulata* Archibald *T. decolorans* Ahmadjian	Italy, Classic Karst Plateau, Trieste prov., Borgo Grotta Gigante	250	23-June-2010	Northerly exposed bark of *Quercus petraea* (Matt.) Liebl.	108	2	2–3	1–2	3–2
Flavoparmelia caperata (L.) Hale	*Trebouxia crenulata* Archibald *T. gelatinosa* Archibald	Italy, Classic Karst Plateau, Trieste prov., Borgo Grotta Gigante	260	23-June-2010	Northerly exposed bark of *Fraxinus ornus* L.	112	2–3	3	1–3	3–2

Table 2
O_3 AOT40 (ppm h) measured during the 3- (a) and 6-week (b) exposure of the three target lichens in the 8 open top chambers.

		AOT 40 (ppm h)							
		1	2	3	4	5	6	7	8
Xanthoria parietina	a	0.000	0.000	2.674	3.460	6.368	5.716	8.537	26.153
	b	0.000	0.021	3.670	7.318	11.687	14.232	19.754	46.893
Flavoparmelia caperata	a	0.000	0.000	3.128	4.113	4.600	6.514	10.311	16.649
	b	0.000	0.021	3.271	8.292	11.670	15.393	21.300	43.376
Parmotrema perlatum	a	0.000	0.000	4.256	7.718	4.523	6.020	13.251	13.115
	b	0.000	0.000	4.822	10.608	11.546	10.503	17.863	36.088

effects of O_3 exposure. Chl$_a$F measurements were carried out with a pulse–amplitude–modulated fluorimeter Mini-PAM (Walz, Effeltrich, D), positioning the measuring fibre optic (length: 100 cm; active diameter: 5.5 mm) at 60° on the upper surface of terminal parts of lobe margins, because these portions have considerably higher Chl$_a$F emission than the central ones (Tretiach et al., 2007). The modulated light was turned on to obtain F_0 (minimal Chl$_a$F level). A saturating light pulse of ca. 8000 μmol photons m^{-2} s^{-1} for 0.8 s was emitted to obtain F_m (transient maximum Chl$_a$F level) and thus to calculate F_v (variable Chl$_a$F level, i.e. $F_m - F_0$) and F_v/F_m (maximum quantum efficiency of PSII photochemistry) (Genty et al., 1989). An external actinic light provided by a light unit FL-460 (Walz, Effeltrich, D) with halogen lamp was turned on to record the Kautsky effect at an intensity consistent with the specific $PPFD_{lk}$ value of each species (Piccotto and Tretiach, 2010; see Table 1). Once the peak F_p was achieved, saturating light pulses were applied at 60 s intervals during actinic illumination to determine the photochemical (qP) and non photochemical (qN and NPQ) quenching (see, e.g. Baker, 2008; Roháček, 2002).

2.4. Microclimatic and sample water content measurements

During exposure, air temperature, humidity, and solar irradiation were continuously collected, outside and inside the chambers. The following instruments were used: air temperature and humidity data logger iButton (Maxim Integrated, San Jose, CA, USA); contact thermometer MK 5310 (Mitek, Reggio Calabria, I); quantum radiometer HD 2302.0 (Delta Ohm, Padua, I). In order to know the thallus relative water content immediately before watering, and to estimate the time of dehydration of sprayed samples, measurements were taken with a Protimeter mini-3 (Parametrics, Shannon, Ireland) on samples used as references. The electronic circuit of this instrument measures the resistance of the material to the passage of a low voltage current between two needles driven in at a distance of 1 cm. The reading values are given as ordinal numbers, from 6 (low water content) to 25 (high water content). The thallus relative water content (%RWC) was then estimated on the basis of a calibration curve built up in the laboratory at 20 °C for each species.

2.5. Statistics

All calculations were performed with Microsoft Office Excel 2003 SP3 (Microsoft corporation, WA, U.S.A.), STATISTICA 6.0 (StatSoft Inc., Tulsa, OK, U.S.A.) and R version 2.15.1 (R Foundation for Statistical Computing). F_v/F_m values are presented with non-parametric descriptors (median, first and third quartile). Statistical analyses were conducted applying the non-parametric Mann–Whitney *U* test, also known as Wilcoxon non-paired test, as suggested by Baruffo and Tretiach (2007) and Lazár and Nauš (1998).

3. Results

The ambient O_3 AOT40 registered outside the OTCs during the 6-week period was less than 400 ppb, comparable with the concentration of a non-polluted site, whereas the O_3 AOT40 in the OTCs ranged from 0 (control) to 46.9 ppm h (Table 2). After 6 weeks, the critical levels (CLe_c) for agricultural crops and semi-natural vegetation of 3 ppm h (Ferretti et al., 2007; UN/ECE, 2004) was reached in all the chambers except the control ones, whereas the critical levels for forests (5 ppm h) and for horticultural crops (6 ppm h) were reached in the chambers nr. 4–8 of Table 2.

Pre-exposure Chl$_a$F levels significantly differed among the three lichen species (Table 3). However, all F_v/F_m values were higher than 0.700, confirming that the samples were healthy before exposure.

The watering regimes applied to the sample groups A–C in the OTCs were responsible for a large variation in degree and time of thallus hydration, and purportedly of metabolic activity. Significant differences were observed among the three species, with *X. parietina* typically remaining wet for longer times (>1 h) than the two parmelioid lichens (Fig. 1a and b). Depending on ambient weather conditions, morning-watered samples remained wet for no more than 2 h on warm and dry days, although they were wet for longer periods on cold and rainy days (Fig. 1). Evening-watered samples, on the other hand, remained wet for most of the night, and sometimes even until late morning on rainy days (Fig. 1f). By contrast, non-watered samples rarely reached RWC above 30% (*F. caperata*, *P. perlatum*) or 50% (*X. parietina*), and then only on rainy days or when ambient humidity was higher than 90%. Non-watered samples were therefore arguably metabolically inactive for most of the exposure period.

The post-exposure Chl$_a$F emission values of the wood-exposed controls did not significantly change with respect to pre-exposure levels, whereas that of the OTC-exposed samples varied greatly (Figs. 2 and 3; see also Online Resource 1 and 2). Interestingly, no significant differences were observed between O_3-free and

Table 3
Pre-exposure values of minimum (F_0) and transient maximum (F_m) emission yield of chlorophyll a fluorescence, maximum efficiency of PSII (F_v/F_m, where $F_v = F_m - F_0$), and non photochemical quenching (NPQ) measured in the three target lichens before the exposure experiment. Measurements are given as mean ± one standard deviation (F_0 and F_m) and 1st quartile, median and 3rd quartile (Q1, Q2, Q3) (F_v/F_m and NPQ).

		F_0	F_m	F_v/F_m			NPQ		
	n	mean ± SD	mean ± SD	Q1	Q2	Q3	Q1	Q2	Q3
Xanthoria parietina	52	273 ± 28	1086 ± 121	0.740	0.751	0.758	0.379	0.450	0.574
Flavoparmelia caperata	78	318 ± 32	1238 ± 126	0.733	0.746	0.756	0.488	0.566	0.710
Parmotrema perlatum	78	344 ± 42	1254 ± 158	0.716	0.725	0.738	0.406	0.528	0.679

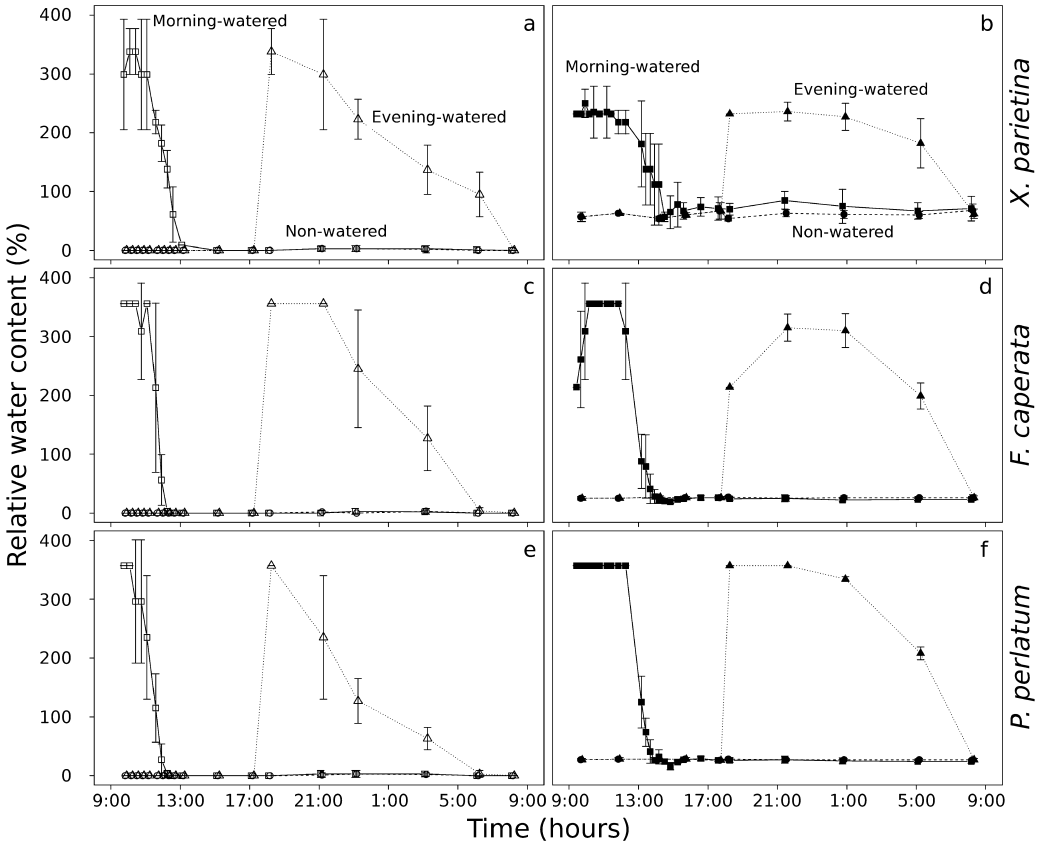

Fig. 1. Example of the variation in relative water content (RWC%) observed in samples of the lichens *Xanthoria parietina* (a) and (b), *Flavoparmelia caperata* (c) and (d), and *Parmotrema perlatum* (e) and (f) exposed in the OTCs during a sunny, warm day (a), (c) and (e) and a rainy, cool day (b), (d) and (f).

O_3-exposed samples in the OTCs, and no significant relationship was observed between O_3 AOT40 and F_v/F_m (Fig. 4) or any other measured Chl$_a$F parameter (e.g. NPQ, see Online Resource 3). The observed F_v/F_m decrease illustrated in Fig. 2 reflected, on one hand, the gradient of xerophytism among species (Table 1) – low in *X. parietina*, intermediate in *F. caperata* and more pronounced (up to 60%) in *P. perlatum* – and, on the other hand, the watering regime applied to the sample groups. In fact, the evening-watered samples maintained F_v/F_m values similar to (*X. parietina* and *F. caperata*,), or slightly lower (*P. perlatum*) than the outside controls, whereas the morning-watered and non-watered samples suffered a pronounced F_v/F_m decrease, from 15% (*X. parietina*) to 80% (*P. perlatum*). In *F. caperata* and *X. parietina* watering treatments modified NPQ in two different ways (Fig. 3). Morning watering was associated with an increase in NPQ (from +20% to +100%), whereas evening watering was associated with a decrease of between 20% and 50%. In *P. perlatum* the two watering treatments caused a significant decrease in NPQ (ca. −50%) (Fig. 3).

The decrease in F_v/F_m was similar between the two 3-week exposure periods in *F. caperata*. In *X. parietina* the F_v/F_m decrease was more pronounced after the second 3-week exposure period, whereas the opposite was true for *P. perlatum*. This suggests that the duration of exposure was not a key factor. By contrast, the weather conditions of the last days of exposure, and particularly the very last day, seem to be more influential, with a negative impact of high light (Table 4).

Interestingly, the conditioning process of two days applied to all samples at the end of the exposure allowed the total or partial recovery of the original Chl$_a$F emission (Figs. 2 and 3 and Online Resource 1 and 2, right hand column). *X. parietina* recovered fully. In contrast, morning-watered samples of *F. caperata* and *P. perlatum*, and non-watered samples of *P. perlatum*, did not reach pre-exposure values of F_v/F_m, a sign that the environmental conditions within the OTCs were not completely suitable for these two lichens, irrespective of ozone treatments.

4. Discussion

In this study, the species-specific tolerance of three foliose epiphytic lichens to O_3 has been tested by exposing portions of healthy thalli in OTCs under different watering regimes. After a fumigation of 6 weeks, no significant effects were observed that could be traced back to the action of the pollutant. This suggests that the functionality of photosystems is not impaired by ozone, supporting recent findings by Calatayud et al. (2000) and Riddell et al. (2010, 2012). However, our three lichens clearly suffered a chamber effect, likely associated with small increases in air temperature and light, and reduced air humidity. Watering regime did, however, have a strong effect on the efficiency of lichen photosystems, because non-watered and morning-watered samples showed a stronger F_v/F_m decrease than the evening-watered ones. The decrease in non-watered samples is not surprising since Hájek et al. (2001, 2006) demonstrated that F_v/F_m is sensitive to desiccation. Indeed, a recent study by Tretiach et al. (2012b) showed that the prolonged lack of rain, high temperatures and high light can decrease the maximum quantum yield of PSII photochemistry, as seen in unwatered samples of all three species. The low chlorophyll *a* fluorescence typical of thalli desiccated for long periods is likely to be associated with a long-wavelength quencher embedded in the antenna complex of photosystem II that captures absorbed energy faster than the reaction centres, dissipating it as heat (Heber et al., 2006). This specific protective mechanism would be progressively activated and de-activated according to thallus hydration (Veerman et al., 2007).

Table 4
Mean values (± one standard deviation) of photosynthetic photon flux density (PPFD), air temperature and relative humidity within the OTCs, and period of time (h) during which the relative water content of the exposed thalli of the three target lichens was above 60% (RWC > 60%); measurements taken the day before the Chl_aF measurements of Fig. 1 (boxplot), at the end of the 3- (a) and 6-week (b) exposure.

		PPFD (μmol photons m^{-2} s^{-1})	Air temp. (°C)	RH (%)	RWC > 60% (h)
Xanthoria parietina	a	204 ± 24	18.5 ± 1.7	84.5 ± 4.4	9
	b	160 ± 74	16.7 ± 0.6	75.0 ± 5.1	3
Flavoparmelia caperata	a	244 ± 48	16.7 ± 1.0	88.5 ± 2.4	9
	b	518 ± 116	17.0 ± 1.0	62.8 ± 2.5	2
Parmotrema perlatum	a	452 ± 138	18.5 ± 1.0	62.5 ± 3.7	2
	b	320 ± 25	20.8 ± 0.6	79.3 ± 2.9	9

The pronounced difference between morning- and evening-watered samples is particularly intriguing. The samples were exposed in the same chambers, so this excludes a single chamber effect. An explanation must therefore take into account the environmental conditions to which the two sample groups were exposed when metabolically active. Evening-watering generally occurred when temperatures were low, and there was no light or just dim light, evidently the best conditions to avoid negative effects on the photosystems; indeed these samples registered the highest F_v/F_m, apart from chamber-free (wood-exposed) controls.

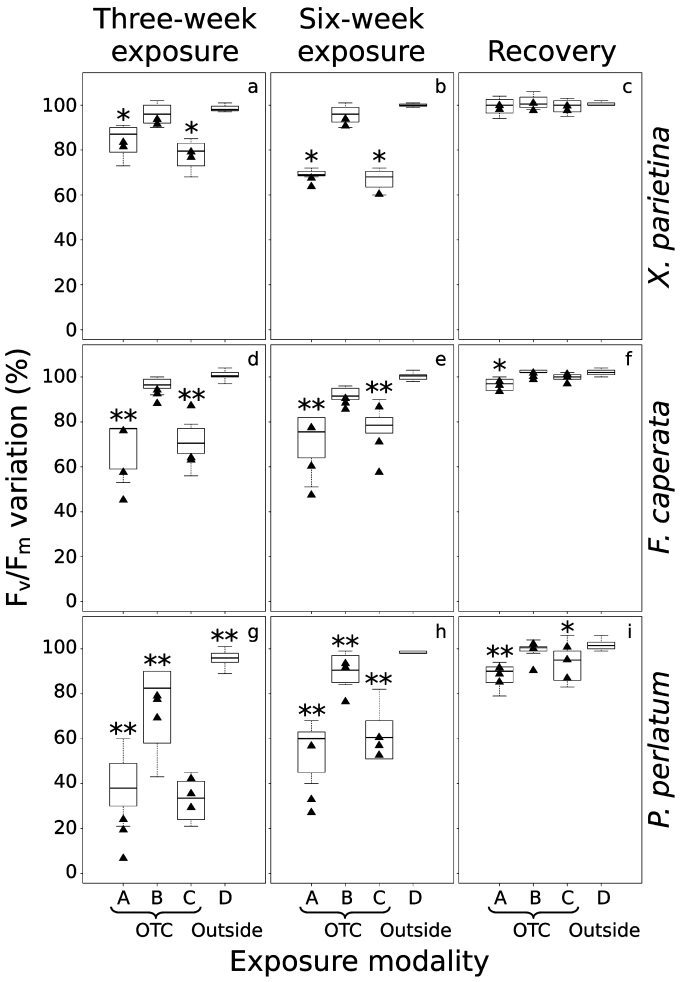

Fig. 2. Decrease of F_v/F_m after 3-week and 6-week exposure and after recovery with respect to the pre-exposure values (Table 3) in samples of *X. parietina* (a–c), *F. caperata* (d–f), *P. perlatum* (g–i) exposed in the wood and in OTC without O_3 (boxplot) and with the highest O_3 AOT40 (solid triangles). For each boxplot median, 25°–75° percentiles and non-outlier minimum and maximum are reported; $n = 4$ in a–c, $n = 6$ in d–i. (A) morning-watered samples; (B) evening-watered samples; (C) non-watered samples; (D) control samples exposed in the wood; statistically significant differences are marked (**$p \leq 0.01$; *$0.01 < p \leq 0.05$; Mann–Whitney *U* test).

In contrast, the high RWC of morning-watered samples suggested that they were metabolically active from 9.00 am to c. 12.00 am, even on the warmest days, when evening-watered samples were completely dry. The combination of high hydration and high light (typical of the middle part of the day and worsened by the increased transmittance through the hydrated cortical layer) is obviously rather unnatural for an epiphytic lichen: lichens generally dry out as soon as the conditions become sunny and warm, and a fully hydrated thallus experiences high light only exceptionally, e.g. immediately after a thunderstorm (Gauslaa et al., 2012; Green et al., 1995; Jonsson et al., 2008). Furthermore, lichens, thermo-tolerant when dry, are thermo-sensitive when they are metabolically active (Kappen, 1973; Macfarlane and Kershaw, 1978; Tretiach et al., 2012a). Therefore, the F_v/F_m decrease of morning-watered samples can be explained as the consequence of the exposure of wet

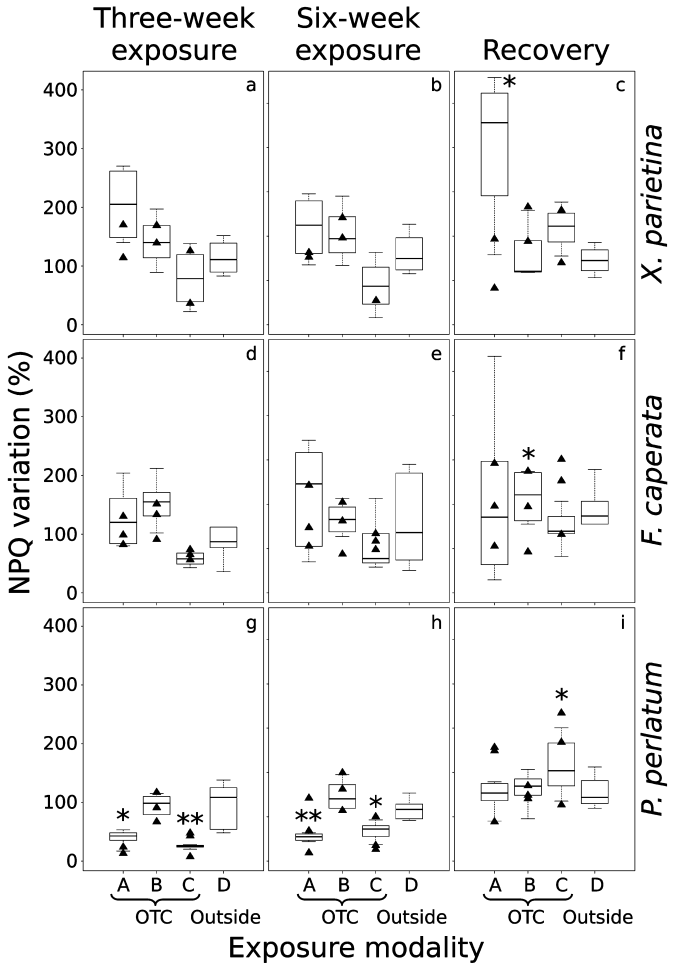

Fig. 3. Decrease of NPQ after 3-week and 6-week exposure and after recovery with respect to the pre-exposure values (Table 3) in samples of *X. parietina* (a–c), *F. caperata* (d–f), *P. perlatum* (g–i) exposed in the wood and in OTC without O_3 (boxplot) and with the highest O_3 AOT40 (solid triangles). Symbols as in Fig. 2.

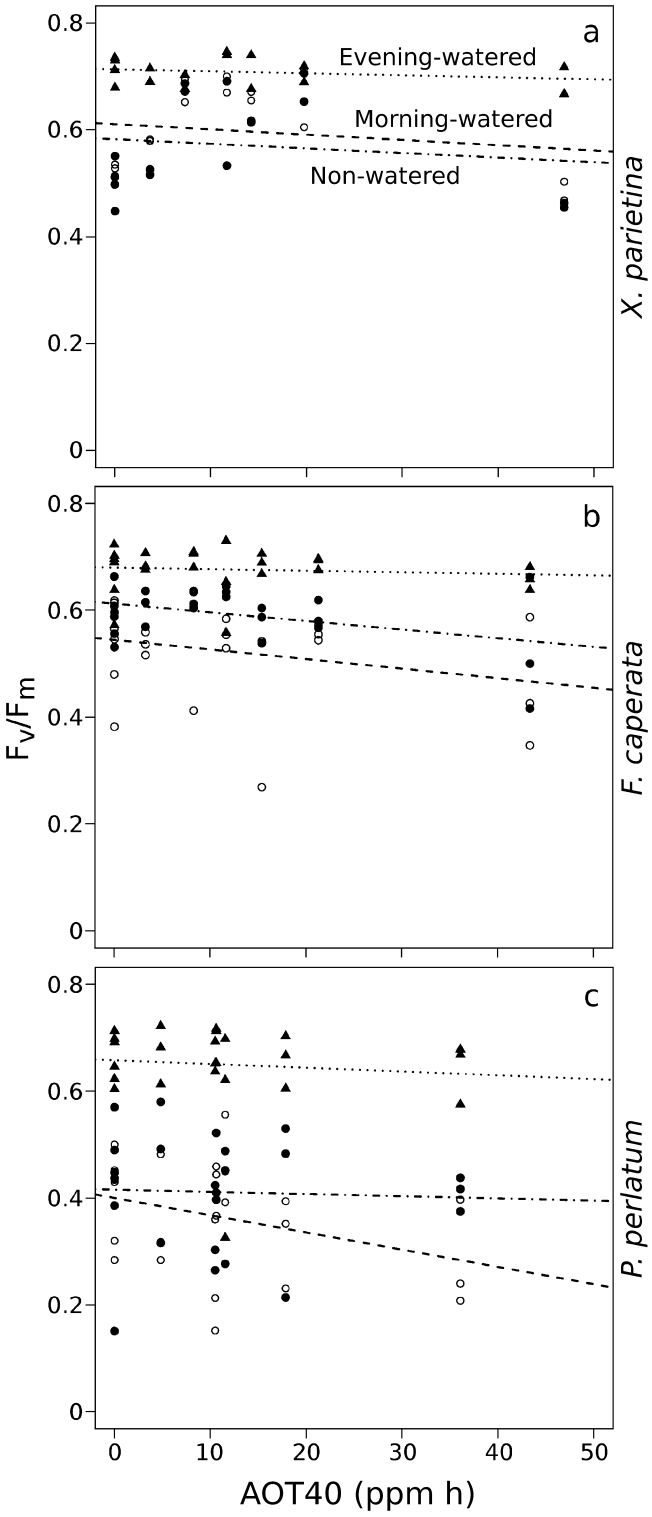

Fig. 4. Relationship between O_3 AOT 40 (ppm h) in the OTCs after 6-week exposure and the respective F_v/F_m values measured in *X. parietina* (a) *F. caperata* (b) and *P. perlatum* (c). (a) Morning-watered, $y = -0.00099x + 0.61$, $r^2 = 0.031$, $p = 0.51$; evening-watered, $y = -0.00037x + 0.71$, $r^2 = 0.045$, $p = 0.42$; non-watered, $y = -0.00086x + 0.58$, $r^2 = 0.02$, $p = 0.60$. (b) Morning-watered, $y = -0.0018x + 0.54$, $r^2 = 0.073$, $p = 0.20$; evening-watered, $y = -0.00029x + 0.67$, $r^2 = 0.0094$, $p = 0.65$; non-watered, $y = -0.0016x + 0.61$, $r^2 = 0.16$, $p = 0.051$. (c) Morning-watered, $y = -0.0032x + 0.40$, $r^2 = 0.12$, $p = 0.10$; evening-watered, $y = -0.00071x + 0.66$, $r^2 = 0.0097$, $p = 0.65$; non-watered, $y = -0.00041x + 0.42$, $r^2 = 0.0017$, $p = 0.84$.

thalli to relatively high light and relatively high temperatures, as supported by NPQ (Fig. 3) and qP (not shown) values.

It is worth noting that in many vascular plants O_3 is highly phytotoxic, due to the effects of production of reactive oxygen species (ROS) on cell membrane permeability, via lipid peroxidation, and oxidation of sulphydril groups in protein and enzymes (Lorenzini and Nali, 2005; Sharma and Davis, 1997). Our experiment demonstrated that whatever the water regime applied, O_3 was not harmful for the three lichens studied. Given the moderately high ozone exposures in this experiment, the question is how lichens can escape this damage. An important source of ROS production is water stress (França et al., 2007). As poikilohydrous organisms, lichens gain and lose water very rapidly. The change in water content from the extreme desiccation state to full turgor (and vice versa) causes dramatic changes, such as the progressive shrinkage of the cytoplasm, rearrangement of the membranes, suspension of enzymatic activities, and the partial or total deactivation of photosystems (Honegger et al., 1996). These effects are accompanied by alterations in the ionic strength and pH of the cytoplasm and by an intense oxidative burden (Kranner and Birtić, 2005), against which lichens have evolved a set of antioxidant defences, from non enzymatic mechanisms (e.g. ascorbic acid, glutathione, carotenoids, tocopherols), to scavenging enzymes (superoxide dismutases, catalases and ascorbate peroxidases) (Gechev et al., 2006). Lichens are therefore well equipped to face an internal source of ROS that takes origin from the peculiarity of their life style, i.e. the strong fluctuation of their water content (Valencia-Islas et al., 2007). The stress derived from an exposure to ozone, like the one applied in this study, would be just a small input to a larger oxidative stress that is faced almost daily.

In conclusion, this study suggests that lichens are actually O_3-tolerant organisms; this is not because they act as O_3-avoiders, but it is probably due to the high level of natural defences against different forms of oxidative stress, including that caused by O_3. Furthermore, we demonstrated that the use of lichen transplants in OTCs is a good approach to test the effects of pollutants in these organisms if precautions are taken to limit chamber effects in relation to air temperature and humidity. Since an essential prerequisite to OTC studies is the artificial watering of lichen samples, it is necessary to mimic the natural daily cycle of rehydration and dehydration, and we therefore suggest that pre-dawn- or evening-watering is recommended in such protocols.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecolind.2013.05.023.

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