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EFFECTS OF OZONE FUMIGATION ON EPIPHYTIC MACROLICHENS: ULTRASTRUCTURE, CO_2 GAS EXCHANGE AND CHLOROPHYLL FLUORESCENCE

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Abstract

The lichen species Anaptychia ciliaris, Collema nigrescens, Evernia prunastri, Hypogymnia bitteri, Lobaria pulmonaria, Pseudevernia furfuracea *and* Usnea rigida *s.l. were fumigated with site-relevant concentrations (for Central Europe) of ozone over 80 days (180 μg m^{-3} during daytime, 80 μg m^{-3} during the night). Chlorophyll fluorescence measurements revealed a significant reduction of F_v/F_m after ozone fumigation in five of the species investigated, indicating severe stress on photosystem II due to ozone. The physiological impairment paralleled our fine structural investigations, revealing a significantly higher percentage of collapsed photobiont cells. This indicates that the effects of ambient ozone concentrations under experimental conditions included biophysical and physiological, as well as structural impairment in the lichens studied.*

Keywords: Lichenized ascomycetes, ozone fumigation, low-temperature scanning electron microscopy, chlorophyll fluorescence, CO_2 gas exchange.

Abbreviations: DR—dark respiration; EF—exoplasmic fracture face; F_m—maximal fluorescence yield when all PS II centres are closed; F_o—initial fluorescence yield when all PS II centres are open; F_v—variable fluorescence ($F_m - F_o$); LTSEM—low-temperature scanning electron microscopy; NP—net photosynthesis; OD—optical density at a given wavelength; PAR—photosynthetically-active radiation; PF—plasmic fracture face; PS II—photosystem II.

INTRODUCTION

Ozone is known to be a major gaseous pollutant in rural regions of industrialized countries and its phytotoxicity has been widely accepted since the late 1950s (Heggestad & Middleton, 1959). Since then, numerous plant taxa with a high sensitivity to site-relevant concentrations of ozone have been identified, including both annual and perennial species (Arndt *et al.*, 1987). It has also been established that current ozone concentrations considerably reduce the yield of crop plants, e.g. spring wheat (Fuhrer *et al.*, 1989), or the biomass production of trees such as *Betula pendula* (Reich, 1987; Pye, 1988; Matyssek *et al.*, 1992) under experimental conditions.

Ultrastructural changes of the chloroplasts (Fink, 1988), collapsed mesophyll cells and unetchable droplet-like exudates (Scheidegger *et al.*, 1991; Günthardt-Goerg *et al.*, 1993) have been reported in various higher plant species. Physiological responses include reduction of CO_2 gas exchange (Matyssek *et al.*, 1991), loss of intracellular potassium (Chimiklis & Heath, 1975), shifts in carbohydrate composition — such as cyclitols (Landolt *et al.*, 1989), and stress effects on the electron transport chain in PS II, as measured by chlorophyll fluorescence (Schreiber *et al.*, 1978; Shimazaki, 1988).

Lichens are well known and widely used bio-indicators and bio-accumulators of various air pollutants (Le Blanc & De Sloover, 1970; Hawksworth & Rose, 1976; Nieboer & Richardson, 1981; Nash III & Wirth, 1988; Herzig & Urech, 1991). Numerous experiments and field observations have revealed a high sensitivity of many foliose and fruticose lichens to SO_2 (Holopainen & Kärenlampi, 1984; Richardson, 1988; Sanz *et al.*, 1992). In regions with low SO_2 levels, however, other gaseous pollutants such as NO, NO_2, O_3 (Ross & Nash III, 1983; Eversman & Sigal, 1987) and peroxyacetyl nitrate (PAN) (Sigal & Taylor, 1979) become equally important (Nash III & Gries, 1991). Particulate pollutants including dusts or lead deposition are also reported to reduce the number of epiphytic lichen species and their frequencies (Herzig *et al.*, 1989).

Previous lichenological experiments with ozone have involved short-term fumigations, some of them working with very high concentrations (Brown & Smirnoff, 1978; Nash III & Sigal, 1979) which may be irrelevant to environmental studies.

In Central Europe, so far, no experiments have been conducted in which site-relevant ozone concentrations were applied over an extended period during the growing season.

Additionally, many laboratories still generate the ozone from air and not from pure oxygen, as recommended by Brown and Roberts (1988). Unless direct

measurements give evidence that no nitrogen deposition occurred during the experiment, possible fumigation artifacts should be taken into consideration in these reports (Taylor *et al.*, 1993).

Fumigations of lichens with ozone have been reviewed by Nash and Gries (1991). Most of the experiments report a low sensitivity of lichen species to ozone, but these results are not entirely consistent with field observations (Nash III & Gries, 1991).

Ozone concentrations in Central Europe are enhanced during summer months, when hourly mean values often exceed 200 $\mu g\ m^{-3}$. However, during such periods where the ozone concentrations are high, the air humidity is low and only low amounts of precipitation occur. Both of these factors lead to longer periods with low rates of CO_2 gas exchange in lichens (Farrar, 1976; Kershaw, 1985; Nash III *et al.*, 1990). It may therefore be suggested that, during dry periods, ozone fumigation leads to insignificant injuries due to limited photosynthetic and respiratory activity.

In our study, we investigated effects on biophysical, physiological and morphological parameters in lichens after ozone fumigations with site-relevant concentrations in a long-term experiment (80 days). The exposure was carried out under relatively dry conditions, as is typical for the season, with enhanced ozone concentrations.

MATERIALS AND METHODS

Plants and treatments

Specimens were collected in natural forests in the northern Alps of Switzerland. *Anaptychia ciliaris* (Ach.) Koerber, and *Evernia prunastri* (L.) Ach. were collected on *Acer pseudoplatanus* L. in the canton of Freiburg, commune of Montbovon; Bonodon d' en Bas, at 1174 m above sea-level. *Lobaria pulmonaria* (L.) Hoffm. was collected in the same area, but at 1300 m above sea-level, and *Collema nigrescens* (Hudson) DC. was collected in a near valley of the same commune (Preise au Maidzo), 1220 m above sea-level. *Usnea rigida* (Ach.) Mot. s.l. and *Pseudevernia furfuracea* (L.) Zopf were collected on *Abies alba* Mill. in the canton of Saint Gall, commune of Pfäfers; Taminatal, Bläserberg, at 1400 m above sea-level. *Hypogymnia bitteri* (Lynge) Ahti was from the canton of Berne, commune of Zweisimmen; Grubenwald at 1600 m above sea-level, collected on *Picea abies* (L.) H. Karsten.

Species were selected according to their different tolerances to pollution (Herzig & Urech, 1991; Wirth, 1991), ecological requirements (Wirth, 1980), growth forms and photobiont types (Tschermak-Woess, 1988). *A. ciliaris* is a fruticose lichen with green algae as photobiont. It is very sensitive to air pollution (Herzig & Urech, 1991). *C. nigrescens* is a rather rare foliose lichen, restricted to sub-oceanic areas. Its tolerance of pollutants is unknown. The cyanobacterial photobiont belongs to the genus *Nostoc*. *E. prunastri* is a widespread, fruticose green algal lichen. It is moderately sensitive to air pollution (Herzig & Urech, 1991). *H. bitteri* is a foliose green algal lichen restricted to upper montane and subalpine forests. Its tolerance to pollutants is unknown. *L. pulmonaria* is a rare, foliose green algal lichen with internal cephalodia. It is very sensitive to air pollution. The green algal photobiont is *Dictyochloropsis reticulata* (Tsch.-Woess) Tsch.-Woess (Tschermak-Woess, 1988). *P. furfuracea* is a fruticose green algal lichen very widespread in lowland up to subalpine forests. It is rather tolerant of pollution (Herzig *et al.*, 1985). The photobiont belongs to *Trebouxia jamesii* (Hildr. & Ahmadjian) Gärtner (Friedl, 1989). *U. rigida* is a fruticose green algal lichen restricted to sub-oceanic upper-montane forests. Its tolerance to pollutants is not reported, but the species is probably very sensitive.

From 14 June to 23 August 1991 (80 days), samples of the selected lichens were mounted with velcro (Fillafix, Senn, CH) on wood panels at 150 cm above ground. The panels were transferred into the field fumigation chambers in Birmensdorf (10 panels/treatment, 1 panel/chamber) (Keller, 1976). The lichens were pointed north in order to avoid direct sunlight. Because under field conditions ozone concentrations are high mainly during dry climatic conditions, the watering of lichens in the chambers was minimal during the experiment, and the lichens were shielded from direct rain. The lichens were sprayed to full-saturation with distilled water in the early mornings of Monday, Wednesday and Friday, every week. Desiccation of the sprayed thalli occurred within 1–3 h. The O_3 concentrations were $< 5\ \mu g\ m^{-3}$ (charcoal-filtered air; control) and 180 $\mu g\ m^{-3}$ (ozone fumigation) during daytime (0700–2100), and $< 5\ \mu g\ m^{-3}$ (control) and 80 $\mu g\ m^{-3}$ (ozone fumigation) during the night (2100–0700), and were monitored by a 8810 instrument (Monitor Labs, USA). Ozone was generated from pure oxygen by a model 502 (Fischer, Germany) and continuously added to charcoal-filtered air (Landolt *et al.*, 1989).

Morphological studies

Lichen thalli were photographed in the chamber in wet conditions before and after the experiment. Alterations in colour, such as bleaching, were noted and the percentage of any necrotic area was estimated.

At the end of the 80 days exposure period, non-necrotic parts of lichen thalli were excised (diameter 5 mm) for ultra-structural investigations and put into distilled water for 10 min for complete water-saturation. The specimens were mounted perpendicularly on an aluminum stub, embedded in a small water-drop and quick-frozen in liquid nitrogen (LN_2) immediately thereafter. Specimens were stored under LN_2 until further preparation. For LTSEM the specimens were transferred to the cold stage of the preparation chamber of a SCU 020 (Bal-tec, FL) (Müller *et al.*, 1991; Scheidegger *et al.*, 1991; Scheidegger & Brunner, 1993). The specimens were fractured with a microtome at –90°C and partially freeze-dried in a high vacuum ($P < 2 \times 10^{-4}$ Pa) for 1 min. Platinum sputter-coating was then undertaken after raising the pressure to 2·2 Pa. The coating thickness was 15 nm, measured by a quartz thin-film monitor. After coating, the specimens

were transferred with a manipulator through the sliding vacuum valve onto the SEM cold stage in a SEM 515 (Philips, NL). The temperature on the cold stage in the microscope was kept below −120°C.

Physiological experiments

Measurements of chlorophyll fluorescence were carried out by a PAM-101 (Walz, Germany) fluorescence measuring system controlled by an Olivetti M 290 PC with a DA-100 data acquisition system (Walz, Germany). A detailed description of the measuring system and method can be found in Schreiber (1986), Schreiber *et al.* (1986) and Bolhar-Nordenkampf *et al.* (1989). Fluorescence measurements were undertaken at the end of the 80 days exposure period. Lichen samples were removed from the fumigation chambers and transferred into the laboratory immediately before measuring the chlorophyll fluorescence. The thalli were fully hydrated by spraying with distilled water and, after 30 min, the samples were blotted with filter paper to remove adhering surface water. After a period of dark adaptation of 10 min, the F_o-level of chlorophyll fluorescence was measured by a weak, red measuring beam, followed by a saturation light-pulse to determine the F_m-level. The variable fluorescence yield F_v ($F_v = F_m - F_o$) and the ratio F_v/F_m were calculated. The ratio F_v/F_m describes the photochemical efficiency of PS II (Kitajima & Butler, 1975) and can be used as an indicator of stress effects on PS II.

Gas exchange of CO_2 was monitored in an open flow system (Walz, Germany) using a differential IRGA (Binos, Rosemount, Germany) and temperature-controlled measuring cuvettes (300 ml volume) with a flow rate of 500 ml min^{-1} (Sancho & Kappen, 1989; Schroeter, 1991). To investigate long-term effects of the ozone fumigation, CO_2 gas exchange was measured 4–6 weeks after the treatment in both the fumigated and the control samples. Between ozone treatment and photosynthetic measurements, the lichens were kept at 10°C in a climatised chamber under ambient air with a 14 h light/10 h dark-treatment to allow recovery of reversible damage caused by the ozone fumigation. Samples were sprayed irregularly during this time. Immediately before the CO_2 exchange measurements, the samples were fully hydrated by spraying with water and blotted with filter paper in order to remove adhering surface water. All gas exchange measurements were carried out at 15°C. Each sample was measured individually. After placing the samples in the measuring cuvettes, DR was monitored until a steady-state was reached. During several hours of treatment under constant light conditions (400 μmol m^{-2} s^{-1} PAR), thalli dehydrated slowly and the CO_2 exchange was monitored until a marked decrease of gas exchange rates were recorded. The maximum value of NP and the steady-state value of DR were selected and based either on the dry weight (105°C, 24 h) or the chlorophyll content using a CO_2 gas exchange calculation programme PHOTOPLOT (Schroeter, 1991).

Chlorophyll content was measured after the CO_2 gas exchange measurements according to Schroeter (1991). Additionally, the phaeophytination index (OD 435/OD 415 nm) was used to detect the potential degradation of chlorophyll (Ronen & Galun, 1984).

Data were computed with Statgraphics (1986). Mann–Whitney U-test was used to calculate significance levels.

Table 1. F_v/F_m of fumigated (n = 10) and control (n = 10) lichens. Values of two measurements of each specimen are averaged. Standard error and percentage of reduction (Red.) are indicated. ns — difference not significant; [a] — $P < 0.001$ = significance level for the comparison with the control; [b] — $P < 0.01$

Species	Fumigated	Control	Red.
A. ciliaris	0·619 ± 0·014 [a]	0·686 ± 0·009	9·7
C. nigrescens	0·271 ± 0·012 [a]	0·330 ± 0·011	17·6
E. prunastri	0·453 ± 0·023 [a]	0·566 ± 0·012	20·0
H. bitteri	0·535 ± 0·014 [b]	0·583 ± 0·007	8·2
L. pulmonaria	0·325 ± 0·045 [a]	0·579 ± 0·016	43·8
P. furfuracea	0·526 ± 0·029 ns	0·579 ± 0·024	9·0
U. rigida	0·542 ± 0·012 ns	0·565 ± 0·016	4·2

RESULTS

Macroscopic symptoms

Visible symptoms were found only in *L. pulmonaria* and in *U. rigida*. Three out of 10 fumigated thalli of *L. pulmonaria* showed marked bleached areas in the centre of the thalli, covering up to 30% of the total surface. One thallus of the control had a similar symptom, covering 5% of the surface. Three thalli of fumigated *U. rigida* had a few bleached branches, covering up to 10% of the thallus. The other species investigated did not show any discolourations or necroses.

Chlorophyll fluorescence

F_v/F_m-values decreased in all fumigated specimens (Table 1). The reduction was highest in *L. pulmonaria*, with fluorescence lowered by 43·8%, but *E. prunastri* and *C. nigrescens* also showed depressions of more than 15%. In *A. ciliaris* and *H. bitteri*, the reduction was less than 10%. In these five species, the reduction of F_v/F_m measured on 10 replicates was statistically significant ($P < 0.01$). *P. furfuracea* and *U. rigida* presented a slight reduction of F_v/F_m (<10%), but these differences were statistically insignificant.

Gas exchange

Four–six weeks after fumigation the NP (related to dry weight and to chlorophyll content) was lower in six of the species investigated, but higher by about 65% (related to chlorophyll content, difference insignificant) in *H. bitteri* (Table 2). Significant reductions were observed in *A. ciliaris* (36%) when related to chlorophyll content, and in *U. rigida* (59%) when related to dry weight. In *E. prunastri*, *L. pulmonaria*, *C. nigrescens* and *P. furfuracea*, the reduction of photosynthesis was not significant.

DR was reduced in *Evernia prunastri* (−22%; statistically significant), *H. bitteri* (−34%) and *L. pulmonaria*

Table 2. Net photosynthesis (NP) and dark respiration (DR) of fumigated (Fum.) and control (Contr.) lichens. Mean values, standard error and the percentage of reduction (Red.) are given. *n*—sample size. *—Significance level for the comparison with the control ($P < 0.05$)

	NP (mg CO_2 kg dry wt^{-1} h^{-1})					NP (mg CO_2 mg Chl^{-1} h^{-1})			DR (mg CO_2 kg dry wt^{-1} h^{-1})				
Species	Fum.	*n*	Contr.	*n*	Red.	Fum.	Contr.	Red.	Fum.	*n*	Contr.	*n*	Red.
A. ciliaris	6·7 ± 2·1	5	10·4 ± 2·1	5	−36	0·91 ± 0·12*	1·43 ± 0·12	−36	−4·0 ± 0·7	5	−3·4 ± 0·3	5	+17
C. nigrescens	2·0 ± 1·5	4	2·2 ± 2·1	4	− 6	0·25 ± 0·19	0·37 ± 0·37	−30	−4·9 ± 0·9	4	−4·5 ± 1·1	4	+14
E. prunastri	5·9 ± 0·8	5	9·0 ± 1·4	5	−34	1·47 ± 0·13	1·56 ± 0·21	− 6	−4·0 ± 0·1*	5	−5·2 ± 0·5	5	−22
H. bitteri	4·3 ± 0·6	5	3·0 ± 1·2	4	+44	1·24 ± 0·12	0·75 ± 0·29	+65	−2·2 ± 0·4	4	−2·9 ± 1·1	4	−34
L. pulmonaria	7·6 ± 1·0	10	9·1 ± 0·8	9	−16	0·79 ± 0·11	0·92 ± 0·07	−15	−4·6 ± 0·4	10	−5·4 ± 0·8	9	−17
P. furfuracea	3·7 ± 1·6	5	4·8 ± 0·8	5	−22	0·59 ± 0·20	0·73 ± 0·18	−18	−4·1 ± 0·3	5	−4·0 ± 0·8	5	+ 2
U. rigida	3·8 ± 0·7*	6	9·5 ± 1·7	6	−59	0·95 ± 0·24	1·64 ± 0·31	−41	−6·9 ± 2·4	6	−6·1 ± 0·8	6	+12

(−17%), but increased (statistically insignificant) in *A. ciliaris* (17%), *Collema nigrescens* (14%), *P. furfuracea* (2%) and *U. rigida* (12%).

Fine structure

Fully water-saturated samples were freeze-fractured and investigated by LTSEM.

More than 40% of the cyanobacterial photobionts of *C. nigrescens* (*Nostoc* sp.) were collapsed in the fumigated specimens (Table 3, Fig. 1(d)), whereas in the control only about 2% of the *Nostoc* cells were collapsed. Living *Nostoc* cells were globular, with a smooth outer membrane (Fig. 1(b)). In *C. nigrescens*, the filamentous colonies of the photobiont *Nostoc* sp. were irregularly arranged in the homoeomerous thallus, and were embedded in a dense extracellular matrix with no air-spaces in the thallus (Figs 1(a) and (b)). Due to the relatively slow freezing process of the bulk specimen in LN_2, the high amount of water in the matrix crystallized during freezing and led to ice crystal damage in the matrix. After etching of the fracture, the minute dark spots which were evenly distributed in the matrix probably represent ice crystal artifacts (Fig. 1(b)). Thin-walled hyphae of the mycobiont were visible in the matrix, where colonies of *Nostoc* sp. were also embedded (Fig. 1(a)). Between the gelatinous capsule and the outer membrane, a gap smaller than 1 μm was regularly present (Fig. 1(b)). In parts of fumigated thalli however, this gap was often much wider, measuring up to 2·5 μm, while in this early stage of morphological impairment the photobiont cells were still globular (Fig. 1(c)). Ongoing damage then led to collapsed photobiont cells with a wrinkled outer membrane, and to a 2-μm broad spongeous layer of the gelatinous capsule around the broad gap (Fig. 1(d)).

Table 3. Percentage of collapsed photobiont cells in fumigated and control lichens (mean value and standard error). Number of samples per treatment is five (two for fumigated *E. prunastri*). [a] — $P < 0.05$; [b] — $P < 0.001$ — significance level for the comparison with the control. ns—Difference statistically not significant

Species	Total obs.	Fumigated	Control
A. ciliaris	(382; 336)	7·5 ± 1·3 [b]	1·5 ± 0·7
C. nigrescens	(713; 992)	43·9 ± 1·8 [b]	2·2 ± 0·5
E. prunastri	(161; 96)	4·3 ± 1·6 [a]	0·0 ± 0·0
H. bitteri	(118; 201)	8·4 ± 2·6 ns	9·5 ± 2·1
L. pulmonaria	(726; 651)	10·6 ± 1·1 [b]	1·2 ± 0·4
P. furfuracea	(143; 311)	11·1 ± 2·6 [a]	4·8 ± 0·1
U. rigida	(89; 206)	8·9 ± 3·0 ns	2·2 ± 2·0

In *L. pulmonaria*, the unicellular green algal photobionts *Dictyochloropsis reticulata* were arranged below the cortex in the algal layer of the stratified thallus. In contrast to the dense upper cortex, the algal and medullary layers were loosely packed plectenchyma with large air-spaces in which no free, liquid water was present even under water-saturated conditions. A similar thallus organization was found in the other species investigated with green algal photobionts. In LTSEM of freeze-fractured control specimens (Fig. 2(a)), thick-walled, turgescent hyphae of the mycobiont formed a paraplectenchymatous upper cortex and many plasmic and exoplasmic fractures of the plasmalemma and transversal fractures through the photobiont and fungal cells were visible.

The *Dictyochloropsis reticulata* photobiont of *L. pulmonaria* (Figs 2(c) and (d)) and also the *Trebouxia jamesii* photobiont of *Pseudevernia furfuracea*, had more than 10% of collapsed cells in the fumigated thalli. In *A. ciliaris* and *E. prunastri* less than 10% of collapsed green algal photobiont cells were found, however, differences were still statistically significant. Only in *H. bitteri* and *U. rigida* were the differences statistically insignificant (Table 3).

Collapsed cells, especially in *C. nigrescens* and in *L. pulmonaria*, were usually clustered. This is shown for fumigated thalli of *L. pulmonaria*, where some specimens had no, or very few collapsed photobiont cells (Fig. 2(b)), while in others almost all algal cells, and even the cortical and medullary hyphae, were collapsed (Figs 2(c) and (d)). The observation of *E. prunastri* was very difficult because of excessive charging in the microscope, probably due to the high amount of needle-like crystalline lichen products which incrustated the fungal cells.

Other characteristics of photobiont cells, such as shape and number of plasmalemma invaginations, did not differ between control and O_3-fumigated thalli (Fig. 3) in any species. However, the shape and length of plasmalemma invaginations differed between photobionts of different species. The photobiont of *L. pulmonaria* had long, curved, and ramified plasmalemma

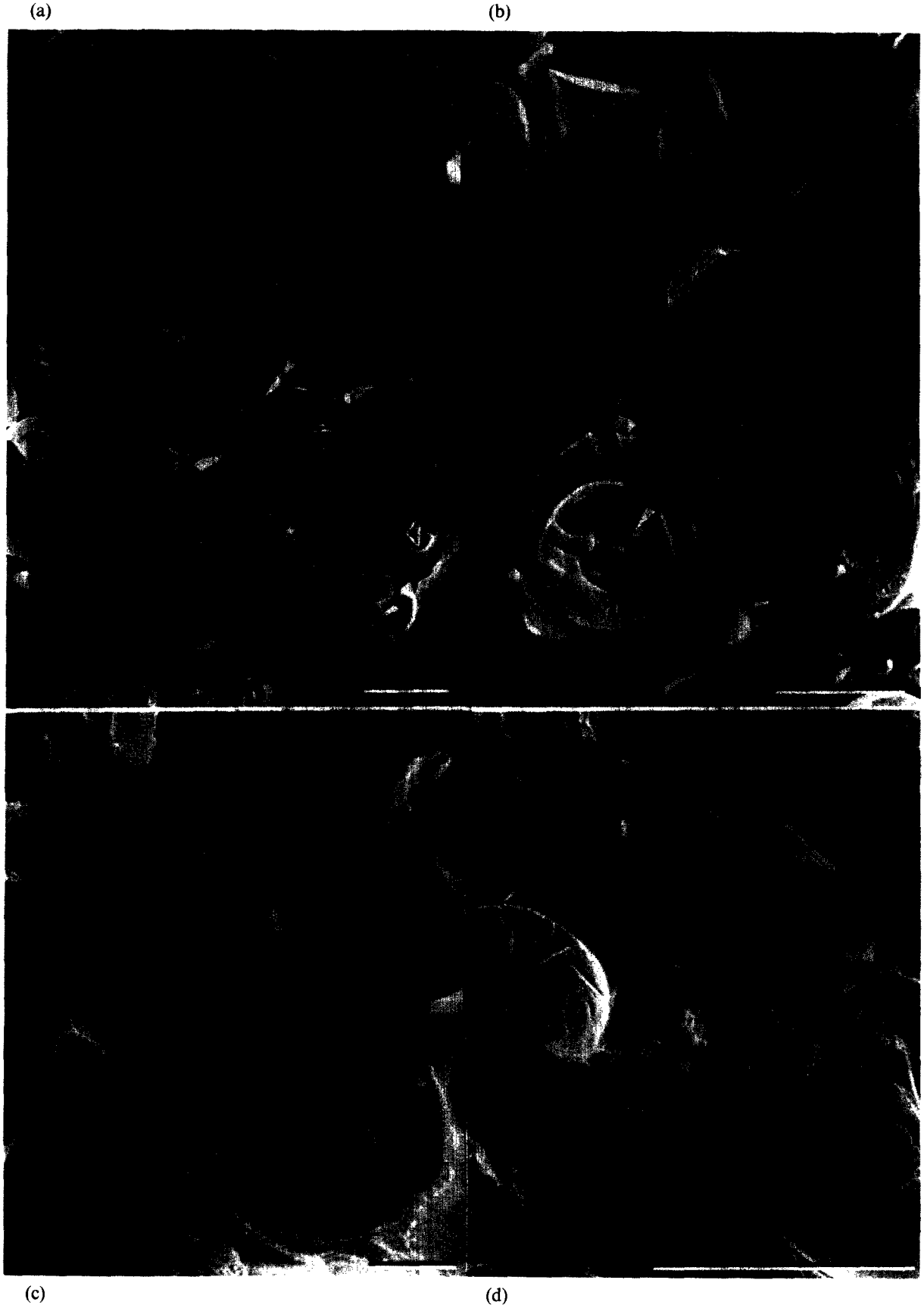

Fig. 1. *Collema nigrescens*, LTSEM of fully water-saturated freeze-fractured thalli. (a) Control: homoeomerous thallus with colonies of cyanobacterial photobiont *Nostoc* sp. (arrowheads) and thin-walled hyphae of the mycobiont (double arrowhead). Note the dense gelatinous capsule; no air spaces are present. (b) Control: fractured colonies of *Nostoc* with small gaps between photobiont cells and gelatinous capsule. Note the plastoglobules (arrowhead) in transversely fractured *Nostoc* cells and the plasmic fractures of the outer membrane (double arrowhead). (c) O_3-fumigated thallus: early stage of morphological impairment of *Nostoc*. Cells are turgescent but a broad gap between the cells and the gelatinous capsule is visible (asterisk). Fibrous structures are present in the gap (arrowhead). (d) O_3-fumigated thallus: collapsed *Nostoc* cells with wrinkled outer membrane, broad gaps between cells and gelatinous capsule and a marked zone of porous gelatinous capsule (arrowhead).

Fig. 2. *Lobaria pulmonaria*: LTSEM of fully water-saturated freeze-fractured thalli. (a) Control: stratified thallus with upper cortex with turgescent cortical hyphae. Algal layer with large air-spaces and fully turgescent *Dictyochloropsis reticulata* photobiont cells (double arrowhead). Plasmic (PF) and exoplasmic (EF) fracture faces of the fungal and algal plasmalemma as well as transverse fractures of the fungal and algal (arrowhead) cells are visible. (b) O_3-fumigated thallus without visible symptoms: hyphae of cortical and algal layer are turgescent. Most algal cells are turgescent but a few are collapsed (arrowhead). (c)–(d) O_3-fumigated thallus where parts of the thallus are necrotic. Non-necrotic part of the thallus: fungal cells of the cortex and the algal layer are collapsed (arrowheads). All algal cells are collapsed (double arrowheads). Fractures through collapsed cells are transverse; no fractures through the fungal or algal plasmalemma can be seen.

Fig. 3. Plasmic and exoplasmic fracture faces of the algal plasmalemma: LTSEM of fully water-saturated, freeze-fractured thalli. (a)–(b) *Anaptychia ciliaris*: green algal photobiont with plasmic fracture of plasmalemma (PF) with irregularly arranged, rod-shaped plasmalemma invaginations (arrowhead). There are no differences between the fumigated thalli (a) and the control (b). (c)–(d) *Pseudevernia furfuracea*: green algal photobiont with exoplasmic fracture of the plasmalemma (EF) with irregularly arranged, rod-shaped plasmalemma invaginations (arrowhead). No differences exist between the fumigated thalli (c) and the control (d). Note PF (double arrowhead) and cross fracture of fungal appressoria (asterisk).

invaginations (figure not shown). *A. ciliaris* (Figs 3(a) and (b)), *H. bitteri* and *P. furfuracea* (Figs 3(c) and (d)) had straight and ca 0·5 μm long plasmalemma invaginations. In *E. prunastri* the plasmalemma invaginations were also straight but slightly longer (0·8 μm; figs not shown). In *Nostoc* sp., the cyanobiont of *C. nigrescens*, no structural details were found on the fracture faces of the outer or inner membranes (Fig. 1(b)).

DISCUSSION

Long-term fumigation with low concentrations of ozone caused a significant reduction of the F_v/F_m values in *A. ciliaris*, *C. nigrescens*, *E. prunastri*, *H. bitteri* and *L. pulmonaria*. Because dead algal cells with degraded chlorophyll are not taken into consideration by the fluorescence parameter, the results can be interpreted in terms of an effect on the living photobiont cells. A reduction of F_v/F_m shows a reduced maximum capacity of the primary reaction of the photosynthesis, and therefore indicates severe stress to PS II as a result of ozone fumigation.

Possible effects of gaseous pollutants on lichens have not been monitored so far by chlorophyll fluorescence methods. However, Gries (personal communication) reported an application of chlorophyll fluorescence to the detection of stress effects of ozone on PS II. *In vivo* fluorescence methods were applied in lichens to describe the influence of Diuron (DCMU) and lead on PS II by Lühmann (1987).

The results presented here reveal that fluorescence measurements with a pulse-amplitude modulated system is a valuable method for detection of an early impairment of PS II (Schreiber & Bilger, 1987). The method can be applied in the field and it is non-destructive (Schroeter, 1991; Schroeter *et al.*, 1991*a*, 1992), a major advantage in environmental studies where measurements should be repeated regularly.

After a recovery period of more than four weeks, NP was increased in *H. bitteri*, but reduced in all other species, although no significant changes of chlorophyll content, chlorophyll A/B ratio or phaeophytination were found (results not shown). DR was significantly reduced in *E. prunastri*, whereas no significant differences were found in the other species. These gas exchange results indicate an ozone impact leading to statistically significant long-term impairment of NP in *A. ciliaris* and *U. rigida*, and to non-significant reductions in *C. nigrescens*, *E. prunastri*, *L. pulmonaria* and *P. furfuracea*. Only in *H. bitteri* was a stimulating effect of ozone found, which parallels the lower percentage of collapsed photobiont cells after fumigation but conflicts with the fluorescence values. In *U. rigida* neither significant changes in fluorescence nor an increased percentage of collapsed photobiont cells were found, suggesting that photosynthesis would probably be unchanged. However, the increased DR indicates an increased fungal respiration, which can be interpreted as a stress reaction of the mycobiont. A reduction of NP rates and/or an increased DR indicates irreversible damage of the photosynthetic apparatus (Kappen & Smith, 1980; Schroeter *et al.*, 1991*b*).

Ross and Nash (1983) found no significant decline of gross photosynthesis in *Ramalina menziesii* at comparable and extremely high concentrations of O_3 in short-term experiments. However, *Parmelia caperata* showed a significant decline in the rate of $^{14}CO_2$ assimilation, even at an O_3 concentration of 200 μg m^{-3}. In sensitive annual (Fuhrer *et al.*, 1989) and perennial (Matyssek *et al.*, 1991) phanerogams, the photosynthetic capacity is also considerably reduced after O_3 fumigation.

LTSEM allows the observation of turgescent structures of hydrated lichens such as photobiont cells (Brown *et al.*, 1987; Scheidegger, 1993) and a wide range of other biological specimens (Echlin, 1992). Because liquid water uptake is a rapid process in desiccated lichens, turgescence of the photobiont cells is obtained within 100 s after spraying (Scheidegger, unpubl.). After 10 min of hydration, collapsed photobiont cells can therefore be interpreted to be seriously damaged or dead because they were unable to reach turgescence. Our investigations generally revealed significantly higher percentages of collapsed photobiont cells after ozone fumigation. Collapsed photobiont cells of lichens at high water-content give evidence of severe structural damages including the plasmalemma because turgescence cannot be established after rehydration. Collapsed cells were clustered and not homogeneously distributed, indicating spatial inhomogeneities of morphological damage. Such spots with a high percentage of seriously damaged or dead photobiont cells were not visible externally but could later lead to necroses. This patchiness may possibly influence the fluorescence measurements and explain the observed higher standard errors in the fumigated specimens of *A. ciliaris*, *E. prunastri*, *H. bitteri* and *L. pulmonaria*.

Although the clearly broader gap between the outer membrane of *Nostoc* cells from the surrounding gelatinous capsule may be interpreted as an early morphological alteration of the photobiont cells in *C. nigrescens* (which later results in collapsed *Nostoc* cells), the absence of early symptoms in the plasmalemma and cell wall of the green algal photobionts contrasts with ultrastructural observations in phanerogams. Ojanperä *et al.* (1992) observed a bubbling of plasmalemma in TEM, and Scheidegger *et al.* (1991), Matyssek *et al.* (1991) and Günthardt-Goerg *et al.* (1993) described unetchable droplet-like exudates on the outer part of mesophyll cell walls in ozone fumigated birch (*Betula pendula*) leaves (which later resulted in collapsed cells). Ultrastructural alterations in lichens have so far only been found in TEM investigations. Deformation of the mitochondria and chloroplasts have been reported after SO_2 fumigations (Holopainen & Kärenlampi, 1984), and increased accumulation of starch in the chloroplasts, together with increased lipids in the cytoplasm, were found by Eversman and Sigal (1987) after O_3 fumigation of *Parmelia caperata*.

Our experiment was carried out under relatively dry (but close to natural) climatic conditions, and optimal

NP was only possible during a few hours a week (because of limited water supply). The significant impairment after ozone fumigation indicates that this pollutant may injure sensitive lichen photobionts also during dry periods, when photosynthetic and respiratory activities are highly reduced. Because under water saturated condition gas diffusion is considerably reduced by the upper cortex (Snelgar *et al.*, 1981; Matthes-Sears *et al.*, 1987) in many lichens, the influx of ozone may be higher during dry periods.

Our results indicate significant stress on PS II, directly after O_3 fumigation, for five of the species studied. This stress is still expressed four–six weeks after the exposure in NP of all species, except *H. bitteri*, although the differences in NP were often not statistically significant. The physiological impairments were paralleled in our fine structural investigations which revealed a significantly higher percentage of collapsed photobiont cells. This indicates that the effects of ambient ozone-concentrations under experimental conditions included biophysical, physiological and structural impairment in the lichens studied.

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