Effects of acute NH_3 air pollution on N-sensitive and N-tolerant lichen species

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Abstract

Lichens are sensitive to the presence of ammonia (NH_3) in the environment. However, in order to use them as reliable indicators in biomonitoring studies, it is necessary to establish unequivocally the occurrence of certain symptoms following the exposure to NH_3 in the environment. In this paper, we simulated an episode of acute air pollution due to the release of NH_3. The biological effects of acute air pollution by atmospheric NH_3 have been investigated using N-sensitive (*Flavoparmelia caperata*) and N-tolerant (*Xanthoria parietina*) species. Lichen samples were exposed to ecologically relevant NH_3 concentrations for 8 weeks, simulating three areas of impact: a control area (2 $\mu g/m^3$), an area of intermediate impact (2–35 $\mu g/m^3$) and an area of high impact (10–315 $\mu g/m^3$), with a peak of pollution reached between the fourth and fifth week. Ammonia affected both the photobiont and the mycobiont in *F. caperata*, while in *X. parietina* only the photosynthetic performance of the photobiont was altered after exposure to the highest concentration. In the photobiont of *F. caperata* we recorded chlorophyll degradation as indicated by $OD_{435/415}$ ratio, decrease of the photosynthetic performance (as reflected by the maximum quantum yield of primary photochemistry F_V/F_M and the performance index PI_{ABS}); in the mycobiont, ergosterol reduction, membrane lipid peroxidation (as reflected by the increase of thiobarbituric acid reactive substances), alteration (decrease) of the secondary metabolite usnic acid. No effects were detected on caperatic acid and dehydrogenase activity. In *X. parietina*, the only signal determined by NH_3 was the alteration of F_V/F_M and the performance index PI_{ABS}. The results suggest that physiological parameters in N-sensitive lichens well reflect the effects of NH_3 exposure and can be applied as early indicators in monitoring studies.

Keywords: chlorophyll fluorescence; dehydrogenase activity; ergosterol; industrial composting; lichens; TBARS

1. Introduction

Air pollution by ammonia (NH_3) is a notable environmental concern since NH_3 contributes to the deposition of eutrophicating substances which exceeds the critical loads for many ecosystems (Asman et al., 1998) causing impacts ranging from decreased biodiversity, changes in species composition and dominance, and toxicity effects (Fangmeier et al., 1994). Agricultural activities are responsible for over 90% of NH_3 emissions (Galloway et al., 2004), which occur primarily from animal husbandry, manure storage and spreading and application of fertilizers. However, another notable NH_3 source, which is increasingly expanding in many countries, is from decomposition (composting) of organic waste, since the mineralization of organic N-containing amino acids and urea releases considerable amounts of NH_3, the main cause of N pollution during composting of organic waste (Zeng et al., 2012). In fact, N loss from industrial composting is mainly due to NH_3 emissions, which account for 24–33% and 47–77% of the initial N content of household waste and manure respectively (Beck-Friis et al., 2001; Martins and Dewes, 1992).
Ammonia emission (and hence pollution) is not a uniform and continuous phenomenon, but rather goes through acute episodes. In fact, during aerobic treatment of organic waste, according to the activity of different groups of microorganisms, mineralization of organic N results in two NH_3

emissions peaks (Zeng et al., 2012) and levels of atmospheric NH_3 up to 700 mg/m^3 have been reported around waste water sludge composting facilities (Haug, 1993). Ammonia is severely irritating to the nose, throat and lungs, and human exposure to excess NH_3 has been shown to be a relevant concern for the health and safety of exposed workers (Rahman et al., 2007).

Once released to the environment, NH_3 is readily converted to NH_4^+ or subject to dry deposition (Fangmeier et al., 1994). Toxicity by NH_4^+/NH_3 has been extensively studied in higher plants (Fangmeier et al., 1994; Britto and Kronzucker, 2002) and detrimental effects can be early detected at physiological level, involving alteration of secondary metabolism and changes due to increased uptake and assimilation of N (Fangmeier et al., 1994). Higher plants, bryophytes, lichens, soil organisms and invertebrates can be profitably used as bioindicators of the effects of N pollution in the environment, integrating non biological methods of analysis (Sutton et al., 2004).

In particular, lichens are very sensitive to atmospheric reactive N, especially NH_3 (Sutton et al., 2004). Being symbiotic organisms made up by an alga and a fungus, excess N is detrimental to the equilibrium between the two symbiotic partners and hence to the whole lichen, especially if one of the two partners is more able than the other to cope with high N levels (Gries, 1996). The results of previous studies suggested that the photosynthetic apparatus of lichens exposed to ecologically relevant NH_4^+/NH_3 concentration is directly susceptible to these pollutants in the vapour/gas phase (Paoli et al., 2010a; Munzi et al., 2012). In addition, relevant NH_4^+/NH_3 levels may affect membrane lipids and hence alter cell membrane permeability (Fangmeier et al., 1994) and there is evidence that physiological parameters connected to membrane permeability are suitable tools for monitoring biological effects of acute N pollution (Munzi et al., 2009).

In a previous work we investigated whether NH_3 emissions released during composting of organic waste influenced during short-term exposures the lichens in the surrounding environment (Paoli et al., 2014a). It was shown that exposing lichens around a composting plant allowed detecting early physiological indications of potential biological changes before these consequences were apparent at the community level. In particular, N-tolerant species were not affected by the proximity to the facility and some parameters even suggested a better performance, while N-sensitive species showed reduced performances approaching the source. In addition, it was hypothesized that the concentrations of NH_3 were highly fluctuating, with peaks during outdoor handling and maturation of the compost, suggesting that acute episodes of pollution could be the reason for the observed effects (Paoli et al., 2014a). It was concluded that lichens can provide useful data for decision-makers to establish correct science-based environmentally sustainable waste management policies. However, since the interpretation of the results of field studies is often complicated by the interactions among many environmental factors, experiments under controlled conditions are necessary to separate the effects of specific environmental variables. The present experiment was thus carried out to investigate the biological effects of a simulated acute air pollution by atmospheric NH_3 on N-sensitive (*Flavoparmelia caperata*) and N-tolerant (*Xanthoria parietina*) lichens.

2. Materials and methods

2.1 Lichen species

Samples of the lichens *Flavoparmelia caperata* (L.) Hale and *Xanthoria parietina* (L.) Th.Fr. were collected at the beginning of May 2013 from a remote area of central Italy far from pollution sources (Murlo, Tuscany 43°11'60" N, 11°21'33" E, 310 m a.s.l.) and transferred to the Botanical Garden of the University of Siena.

Both lichen species have a similar foliose habitus and a green-algal photobiont (*Trebouxia*). They are however characterized by a different sensitivity to the presence of N compounds in the environment (Nimis and Martellos, 2008): *F. caperata* grows in sites with no or weak eutrophication (non-nitrophilous) and is sensitive to excess N in the environment, whereas *X. parietina* is a nitrophilous lichen, which may grow in sites with high eutrophication. In addition, *F.*

caperata is a mesophytic species chiefly growing in sites with diffuse light but scarce direct solar irradiation, up to sun-exposed sites, but avoiding extreme solar irradiation (Nimis and Martellos 2008), while *X. parietina* is rather xerophytic and can tolerate extreme radiations. Both species are widely spread in lichen communities of the eu-mediterranean belt (i.e., in areas with a humid-warm climate, such as Tyrrhenian Italy): *F. caperata* is one of the most common species in *Quercus* stands and *X. parietina* is diffused in open stands, also in dry environments.

2.2 Experimental design and sample treatment

Samples of *F. caperata* and *X. parietina* were divided in 3 batches and placed inside 3 experimental fumigation chambers of 60×40×25 cm^3, located within one of the greenhouses of the Botanical Garden of the University of Siena. Based on previous field studies (Paoli et al., 2014a), each experimental chamber simulated a different situation of impact according to a gradient of NH_3 pollution: no impact (control), intermediate impact, high impact. In order to work with a similar lichen biomass, each experimental chamber contained about 50 thalli of *F. caperata* and about 100 thalli of *X. parietina* (whose thalli are generally more little than those of *F. caperata*).
Samples were treated for 8 weeks as shown in Table 1: during the first 3 weeks samples were acclimated to low atmospheric NH_3; then an episode of acute pollution from atmospheric NH_3 was simulated for 2 weeks and during the last 3 weeks a moderate impact was simulated. Control samples were constantly treated at the concentration of 2 $\mu g/m^3$, roughly corresponding to background values in Tuscany (Frati et al., 2007). Intermediate samples were treated at concentrations of 2 $\mu g/m^3$ during the first 3 weeks, at a peak of 100 $\mu g/m^3$ during the 4^{th} and 5^{th} weeks of exposure and at 10 $\mu g/m^3$ during last 3 weeks. High impact samples were treated at concentrations of 10 $\mu g/m^3$ during the first 3 weeks, at peaks of 300 $\mu g/m^3$ during the episode of acute pollution (4^{th} and 5^{th} week) and at 100 $\mu g/m^3$ during last three weeks (Table 1).
Ammonia was applied as follows: water solutions containing liquid NH_3 were prepared and placed into open Petri dishes within the experimental chamber, then let evaporate within each chamber, which remained closed. In the control chamber only water was applied. Relative humidity increased during water evaporation: every two days, after water evaporated, a further solution containing NH_3 (or only water in controls) was applied opening the chamber only for the time necessary and closing it after the treatment. Therefore, each chamber represented a sort of closed environment. The level of atmospheric NH_3 (Table 1) was measured with passive air samplers (Radiello® diffusion tubes, Aquaria). For each treatment two samplers were placed in each chamber for 7 days during the 3^{rd}, 5^{th} and 8^{th} week. Samplers contained a filter impregnated with phosphoric acid that adsorbs gas-phase NH_3 as NH_4^+, which can be measured spectrophotometrically by the indophenol blue method (Allen 1989). The detection limit was 0.7 $\mu g/m^3$, uncertainty was 6.5%.
The experiment was run between May–June 2013. Microclimatic parameters under the experimental conditions were regularly recorded between 12:00 and 1:00 p.m. and values were in the following range: solar radiation (1000–1550 $\mu mol\ s^{-1}\ m^{-2}$), temperature (23–30°C), relative humidity (45–65%). All chambers were characterized by the same microclimatic conditions during the experiment, which followed their normal daily fluctuation between day and night, so that the main difference among the experimental chambers was the average level of NH_3. Since there are no known examples of dying lichens releasing volatile chemicals that could affect the physiological responses in neighbouring thalli (independently of NH_3 impacts) lichen thalli within the same chamber have been considered as independent samples.

2.3 Physiological parameters investigated

The following parameters were used to assess the physiological conditions of the samples: in the photobiont chlorophyll degradation and photosynthetic efficiency; in the mycobiont, membrane lipid peroxidation, dehydrogenase activity, ergosterol content and secondary metabolites. These latter parameters are chiefly or exclusively referred to the mycobiont, since it constitutes about 90% of the lichen biomass. Chlorophyll *a* fluorescence emission (indicator of the photosynthetic

efficiency) was used as a non-destructive tool for a rapid screening of the vitality of the samples during the experiment. In this case, after 3 and 5 weeks of exposure, the limited amount of material necessary for the measurements was carefully cut from the marginal parts of the thalli and the thalli were placed again within the experimental chambers. After 8 weeks all the thalli were removed and used for the analyses foreseen at the end of the treatments. In order to reduce any source of variability, the lichen material was randomly selected cutting the marginal parts (up to 1 cm) of the thalli and mixed. Then the fraction necessary for each test was selected.

2.3.1 Chlorophyll degradation

Photosynthetic pigments were extracted using dimethylsulfoxide (DMSO), adding polyvinylpyrrolidone (PVP) and filtering the solution before use (Barnes et al., 1992). *Flavoparmelia caperata* contains lichen substances, which could degrade chlorophyll during extraction causing phaeophytinization (Brown and Hooker 1977). In order to remove these substances, before pigment extraction, lichen samples (20 mg) were subjected to six 5-min washings in 3 mL 100% acetone buffered with $CaCO_3$ (Pisani et al., 2007). Two extraction cycles, 45 min each, were run in a warm bath (65°C), using 5 mL of DMSO. Absorbance of the extracts was measured using a UV–visible spectrophotometer (Agilent 8453). Chlorophyll degradation was expressed by the ratio between the absorbance at 435 and 415 nm (OD_{435}/OD_{415}), as suggested by Ronen and Galun (1984). Five replicates were measured for each treatment.

2.3.2 Photosynthetic efficiency

The "vitality" of the lichen photobiont was checked by the maximum quantum yield of primary photochemistry as inferred from chlorophyll *a* fluorescence emission: $F_V/F_M = (F_M - F_0)/F_M$, where F_0 and F_M are minimum and maximum chlorophyll *a* fluorescence and $F_V = (F_M - F_0)$ is the variable fluorescence. Measurements were carried out with a Plant Efficiency Analyser (Handy PEA, Hansatech Ltd, Norfolk, UK). In addition, the performance index (PI_{ABS}), a global indicator of the photosynthetic performance was calculated to express the overall vitality of the samples (Strasser et al. 2000). The parameter PI_{ABS} combines in a single expression the three functional steps of the photosynthetic activity (light absorption, excitation energy trapping, and conversion of excitation energy to electron transport), resulting in a very sensitive indicator of stress suitable to be applied for physiological and environmental screenings. Up to ten replicates were measured for each treatment and time.

2.3.3 Membrane lipid peroxidation

Membrane lipid peroxidation was estimated using the thiobarbituric acid reactive substances (TBARS) assay. About 50 mg of lichen material was rinsed in distilled water and then homogenized in a mortar using 2 mL of 0.1% (w/v) trichloracetic acid (TCA) with the addition of sand. 1.5 mL of the homogenate was put in eppendorf tubes and centrifuged at 12000 g for 20 min. 0.5 mL of the supernatant were collected and added to 1.5 mL of 0.6% thiobarbituric acid in 10% TCA and put in glass tubes. Tubes were put in the oven at 95°C for 30 min, cooled in an ice bath and then solutions were centrifuged again at 12000 g for 10 min. The absorbance of the supernatant was measured at 532 nm and corrected for non-specific absorption at 600 nm. Concentration of TBARS was calculated using the extinction coefficient for the TBA-MDA complex (155 $mM^{-1} \cdot cm^{-1}$) and the results expressed as µmol/g (dw). Five replicates were measured for each treatment.

2.3.4 Dehydrogenase activity

Triphenyltetrazolium chloride (TTC) reduction to triphenylformazan (TPF) is a good indicator of dehydrogenase activity (dark respiration) and was used to assess sample viability (Bačkor and Fahselt, 2005). Ca. 15 mg of lichen material was incubated in the dark for 20 hours in 2 mL of 0.6% TTC and 0.005% Triton X 100 solution in 50 mM phosphate buffer. Solutions were then removed and samples rinsed in distilled water until bubbles of Triton X were produced. Water-insoluble formazan was extracted with 6 mL of ethanol at 65°C for 1 h. Tubes were then centrifuged at 4000

g for 10 min and absorbance read at 492 nm. Results were expressed as absorbance units/g (dw). Five replicates were measured for each treatment.

2.3.5 Ergosterol content

Ergosterol content in lichens is sensitive to the exposure to heavy metals, which likely reduces the integrity of cell membranes of the mycobiont. Three replicates were measured at each site. Samples of 100 mg of lichen material were homogenized for 10 min in 99% ethanol. Extracts were transferred to 1.5 mL Eppendorf tubes and shaken in the dark at 25 ºC for 30 min, then vortexed and centrifuged at 10000 g for 20 min. The resulting supernatant was immediately analysed by HPLC in a Kromasil 100 C18 column (150 x 4.6 mm, particle size 7 µm) as separator, with flow rate 0.8 mL min^{-1} and isocratic elution with methanol as mobile phase (Dahlman et al., 2002). Total analysis time was 15 min. Ergosterol absorption at 280 nm was measured with a UV detector (Ecom LCD 2084). A standard curve was prepared ranging 1-200 µg ergosterol (Sigma–Aldrich, USA) dissolved in 1mL of ethanol. As ergosterol is sensitive to light, all steps were conducted almost in the dark. Three replicates were measured for each treatment.

2.3.6 Secondary metabolites

Secondary metabolites were measured as indicated by Bačkor et al. (2011). Usnic and caperatic acid were measured in *F. caperata* and parietin was analysed in *X. parietina*. Cleaned samples (15 mg dw) were extracted in 1 mL cool acetone till acetone evaporation. Acetone extracts were collected and the residues were dissolved with fresh 1 mL of acetone, during 40 s were materials blended on a whirl mixer and filtered extracts were analysed by gradient HPLC under the following conditions: column Tessek SGX C_{18}, flow rate: 0.7 mL min^{-1}, mobile phase: A= H_2O: acetonitrile: H_3PO_4 (80:19:1) and B= 95% acetonitrile. Gradient program: 0 min 25% B, 5 min 50% B, 20 min 100% B, 25 min 25% B. The detection wavelength was 245 nm (detector Ecom LCD 2084). Usnic acid (Aldrich) was used as standard. Standard of caperatic acid was prepared from crystallized acetone extracts from *F. caperata* (purity 100%). Standard of parietin was prepared from crystallized acetone extracts from *X. partietina* (purity 98%). Three replicates were measured for each treatment.

2.4 Statistical analysis

After checking the normality of data distribution (Shapiro-Wilk, 95% confidence interval), one-way analysis of variance and Tukey´s pairwise comparison ($P < 0.05$) were run to investigate the effects of NH_3 concentrations on the investigated physiological parameters.

3. Results

The maximum quantum yield of primary photochemistry (F_V/F_M) was used as a non-destructive tool for a rapid screening of the vitality of the samples during the experiment (Figure 1). Signs of alteration following NH_3 treatments emerged after 5 weeks in *F. caperata* (N-sensitive) both in the cases with high and intermediate impact, while *X. parietina* (N-tolerant) was affected only in the case with the highest impact. A comparison accounting the time of exposure revealed a weak decrease of F_V/F_M also in the samples of the control case (respect to values pre-treatment). The experimental conditions were thus partially selective on this parameter after five weeks in *F. caperata* and eight weeks in *X. parietina*.

The results of ANOVA indicated that at the end of the treatments NH_3 affected both the photobiont and the mycobiont in *F. caperata*, while in *X. parietina* only the photosynthetic performance of the photobiont was altered at the highest concentration (Tables 2 and 3).

In detail, in *F. caperata* (N-sensitive) we recorded chlorophyll degradation, impairment of the photosynthetic performance, (F_V/F_M and PI_{ABS}) ergosterol reduction, membrane lipid peroxidation, reduction of the secondary metabolite usnic acid at both intermediate and high concentrations. No

effects were detected on caperatic acid and dehydrogenase activity. In *X. parietina* (N-tolerant), we only recorded a decrease of F_V/F_M and PI_{ABS} at the highest concentration. The exposure to NH_3 under the experimental conditions did not alter chlorophyll integrity, dehydrogenase activity, TBARS production, ergosterol concentration. The content of secondary metabolites (parietin) showed a tendency for the production of parietin in samples exposed to NH_3.

4. Discussion

The experiment simulated the exposure of lichens into three different situations around a point source concerned by an episode of acute air pollution from atmospheric NH_3. Our NH_3 concentrations, spanning from a peak of 300 $\mu g/m^3$ in the simulated high impact, down to 2 $\mu g/m^3$ in the simulated control area, are in line with those documented by Frati et al. (2007) around a pig farm, since they reported a peak of 267 $\mu g/m^3$ NH_3 and a 98% reduction of NH_3 achieved already in the first 200 m from the source. Atmospheric NH_3 generally decreases exponentially with distance from the polluting source (Pinho et al., 2012; Fowler et al., 1998; Jones et al., 2013). Paoli et al. (2010a) assessed the effects of NH_3 pollution on lichen photosynthesis and measured 62.4±4.3 $\mu g/m^3$ at a sheep farm in Greece, which decreased to 15 $\mu g/m^3$ at 60 m from the farm down to 2 $\mu g/m^3$ in a remote area 5 km away, in parallel with the improvement of the photosynthetic performance of the lichens.

During the aerobic composting of biowaste, concentrations of NH_3 in the waste gas up to 227 mg/m^3 were reported (Smet et al., 1999), while during animal housing and transportation, values up to 22.5 mg/m^3 have been documented (Costa et al., 2003), corresponding to levels potentially harmful for the animals (Gustin et al., 1994). Paoli et al. (2014a) assessed the biological effects of NH_3 released during three months of activity from a composting plant of organic wastes using lichen transplants. Respect to unexposed samples, NH_3 around the facility (peak of 48.7±18.9 $\mu g/m^3$) affected the overall vitality (in particular the photosynthetic performance) of non-nitrophilous lichens (*E. prunastri*), but did not affect negatively that of nitrophilous species (*X. parietina*). In our study, NH_3 led to oxidative stresses, which affected both the photobiont and the mycobiont in *F. caperata*, while in *X. parietina* only the photosynthetic performance of the photobiont was altered.

Concerning the photobiont, besides altering the photosynthetic performance, NH_3 led to a significant chlorophyll degradation in *F. caperata*. Consistently with the observations of Munzi et al. (2009), chlorophyll degradation was not detected in *X. parietina*. Frati et al. (2007) reported signs of injury in samples of *F. caperata* exposed in the centre of a pig farm, while these symptoms were not visible in *X. parietina*, confirming that the former is a more sensitive species to NH_3 pollution and the latter is a resistant one. However, our prolonged exposure to peak concentrations (for two weeks) reduced the vitality of the photobiont also in *X. parietina*. Relevant NH_4^+/NH_3 levels may be toxic to the photosynthetic apparatus as they function as electron acceptors, uncoupling electron transport (Losada and Arnon, 1963) and the photosynthetic performance of the lichen photobiont can be considered a suitable indicator of the effects of NH_4^+/NH_3 in the environment (Munzi et al. 2010; Paoli et al. 2010a). Similarly, treatments of N-sensitive lichens (*E. prunastri*) with ecologically relevant NH_4^+ concentrations (50–500 μM NH_4Cl, simulating prolonged exposure) reduced the photosynthetic performance of the photobiont (Munzi et al., 2012). The fact that the photosynthetic performance partially decreased also in our (simulated) control case (after five weeks in *F. caperata* and later, after eight weeks in *X. parietina*) is perfectly in line with the autoecology of these species: *F. caperata* is mesophytic and potentially sensitive to extreme radiations under stressing microclimatic conditions, such as during a Mediterranean summer, while *X. parietina* is rather xerophytic and can better tolerate extreme conditions (Paoli et al., 2010b).

Concerning the mycobiont, the investigated parameters suggested that oxidative stresses occurred in *F. caperata* and have been prevented in *X. parietina*. Reactive oxygen species degrade polyunsaturated lipids, forming malondialdehyde, which is the main constituent of TBARS.

Ammonia pollution enhanced TBARS production in *F. caperata*, but not in *X. parietina*. However, TBARS production has been reported as a consequence of the assimilation of high concentrations of chemical elements (e.g. Ce, Sb) also in the lichen *X. parietina*, when all physiological parameters pinpoint the overall status of alteration of the thalli (Paoli et al., 2013; 2014b).

Under the experimental conditions, ergosterol content decreased in the mycobiont of *F. caperata*, according to NH_3 concentration, but was unaffected in that of *X. parietina*. Ergosterol is the principal sterol of the plasma membrane of fungi and may thus reflect the amount of metabolically active cells in the mycobiont (Sundberg et al., 1999). Fungal membranes are a suitable target to detect the effects of acute N pollution (Munzi et al., 2009) and ergosterol content has been reported as a parameter negatively effected by air pollution, e.g., by heavy metals (Bačkor et al., 2006).

Ammonia did not significantly affect dehydrogenase activity (TTC reduction to triphenylformazan), both in *F. caperata* and *X. parietina*, however, the results of a previous study suggested that NH_3 may induce changes in dark respiration in the N-sensitive lichen *E. prunastri* (Paoli et al., 2014a).

Usnic acid, a yellow cortical pigment with antibiotic effects, is deposited in the form of crystals on the surface of lichen mycobionts as well as photobionts. Produced by the mycobiont, it can regulate photobiont cell division in the thalli, screen from excessive sunlight, detoxify from metal pollution and prevent oxidative stresses (Bačkor et al., 2010; Caviglia et al., 2001; Cocchietto et al., 2002). Exposure to NH_3 in *F. caperata* altered the content of usnic acid, but did not affected that of caperatic acid, a medullary compound with a protective role. Interestingly, the ratio between caperatic and usnic acid raised from the control (3.6), intermediate (7.1), up to the high impact case (9.5).

Our results suggest that the mycobiont of *F. caperata* was not able to produce usnic acid as a consequence of NH_3 exposure and a series of oxidative stresses occurred, as witnessed by ergosterol decrease and malondialdehyde production. It seems that the mycobiont was affected by high NH_3 concentrations (as usnic acid and ergosterol content decrease support) but probably it was still metabolic active, as justified by stable dehydrogenase activity and caperatic acid content. Caperatic acid probably plays a more important role in tolerance to N pollution than usnic acid, which is important in detoxification from metal pollution. On the other hand, in *X. parietina* ergosterol and malondialdehyde content remained stable and the exposure to NH_3 likely induced parietin production, evident comparing exposed (irrespective of the concentration) *vs* control thalli (at $P = 0.10$). Our results would suggest that parietin, a photoprotective metabolite of the lichen *X. parietina*, can presumably act as an antioxidant preventing cell membranes from oxidative stresses. In fact, parietin production was reported as a likely defensive mechanism upon exposure of *X. parietina* to air pollutants in the environment (Silberstein et al., 1996).

The reason of the tolerance of *X. parietina* to excess N can be, at least partially, explained by the low cation exchange capacity, which allows avoiding excess N uptake and the ability to provide carbon (C) skeletons for N assimilation (Gaio-Oliveira et al., 2005). It was estimated that *X. parietina* can tolerate an NH_4^+ deposition load of 1,000 kg ha^{-1} yr^{-1} (Gaio-Oliveira et al., 2004).

At ecological level, it was shown the diffusion of *X. parietina* in areas with higher NH_3 in the environment, despite a decreased photosynthetic efficiency (F_V/F_M) was observed in sites above 50 $\mu g/m^3$, suggesting that the ecological success of *X. parietina* at NH_3-rich sites might be related to indirect effects of increased N availability (Munzi et al., 2014). The photosynthetic efficiency already decreased for N-sensitive species (*E. prunastri*) already above the level of 3 $\mu g/m^3$ (Munzi et al., 2014). Similarly, in the field we observed the diffusion of nitrophilous species in the presence of NH_3 released from a composting plant and a shift from lichen communities composed chiefly by meso-acidophilous species (at ca. 3 $\mu g/m^3$) to more nitrophilous communities approaching the source (ca. 49 $\mu g/m^3$) (Paoli et al., 2014a).

Conclusions

The simulated episode of acute air pollution by atmospheric NH_3 induced alterations on both the photobiont and the mycobiont in the N-sensitive lichen *F. caperata*: we reported chlorophyll

degradation and decrease of the photosynthetic performance in the photobiont; ergosterol reduction, membrane lipid peroxidation, decrease of the content of usnic acid in the mycobiont. In the N-tolerant *X. parietina* only the photosynthetic performance of the photobiont was altered after the exposure to the highest NH_3 concentration. The resistance of the mycobiont of *X. parietina* can explain the ability of this species to tolerate NH_3 pollution and hence its nitrophilous behaviour. On the whole, the results indicated that physiological parameters in N-sensitive lichens well reflect the effects of NH_3 exposure and can be applied as early indicators in monitoring studies in order to detect early signs of potential biological changes.

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Table 1. Atmospheric NH_3 concentrations ($\mu g/m^3$) measured with passive air samplers.

weeks	Ammonia concentrations ($\mu g/m^3$)		
	Control	**Intermediate impact**	**High impact**
1 – 3 acclimation	2.1 ± 0.8	1.9 ± 0.7	10.3 ± 0.6
4 – 5 acute pollution	2.2 ± 0.8	35 ± 4	315 ± 4
6 – 8 pollution	1.9 ± 0.5	10 ± 1	101 ± 2

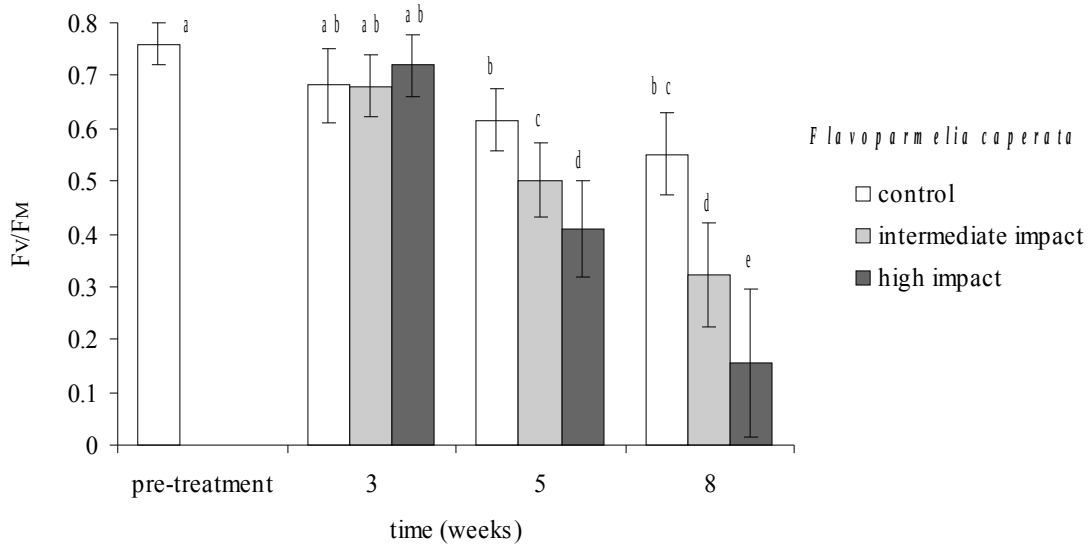
Table 2. Physiological parameters in *Flavoparmelia caperata* after 8 weeks of exposure to NH_3. *F* and *P* values of ANOVA. Values in each line followed by a different letter differ according to the impact of NH_3 (Tukey´s pairwise comparison, $P < 0.05$).

Physiological parameters	Environmental conditions			
	Control	**Intermediate impact**	**High impact**	
$OD_{435/415}$	1.03 ± 0.01a	0.81 ± 0.04b	0.67 ± 0.03c	***F* = 115.3 *P* = 0.000**
F_V/F_M	0.551 ± 0.078a	0.321 ± 0.098b	0.155 ± 0.141c	***F* = 30.03 *P* = 0.000**
PI_{ABS}	0.133 ± 0.060a	0.009 ± 0.010b	0.004 ± 0.010b	***F* = 37.45 *P* = 0.000**
Dehydrogenase (A_{492}/g)	2.45 ± 0.46	3.40 ± 0.86	3.02 ± 0.69	*F* = 1.440 *P* = 0.308
TBARS (µmol/g)	7.3 ± 1.4c	19.4 ± 7.9b	29.7 ± 7.7a	*F* = 4.218 *P* = 0.084
Ergosterol (mg/g)	0.58 ± 0.05a	0.36 ± 0.04b	0.29 ± 0.03c	***F* = 39.73 *P* = 0.000**
Caperatic acid (% dw)	8.5 ± 1.0	8.0 ± 2.3	9.5 ± 1.7	*F* = 1.035 *P* = 0.379
Usnic acid (% dw)	1.48 ± 0.22a	0.78 ± 0.27b	0.67 ± 0.16b	***F* = 23.10 *P* = 0.000**

Table 3. Physiological parameters in *Xanthoria parietina* after 8 weeks of exposure to NH_3. *F* and *P* values of ANOVA. Values in each line followed by a different letter differ according to the impact of NH_3 (Tukey´s pairwise comparison, $P < 0.05$).

Physiological parameters	Experimental conditions			
	Control	**Intermediate impact**	**High impact**	
$OD_{435/415}$	1.42 ± 0.01	1.39 ± 0.02	1.39 ± 0.03	*F* = 0.894 *P* = 0.457
F_V/F_M	0.581 ± 0.064a	0.525 ± 0.097a	0.166 ± 0.212b	***F* = 18.66 *P* = 0.000**
PI_{ABS}	0.112 ± 0.070a	0.080 ± 0.060a	0.016 ± 0.030b	***F* = 6.909 *P* = 0.006**
Dehydrogenase (A_{492}/g)	11.11 ± 1.71	10.13 ± 1.87	8.43 ± 1.40	*F* = 1.966 *P* = 0.220
TBARS (µmol/g)	32.8 ± 7.3	32.1 ± 1.3	30.3 ± 2.3	*F* = 4.146 *P* = 0.106
Ergosterol (mg/g)	0.76 ± 0.09	0.76 ± 0.08	0.75 ± 0.05	*F* = 0.013 *P* = 0.987
Parietin (% dw)	0.88 ± 0.56	1.36 ± 0.90	1.37 ± 0.63	*F* = 0.910 *P* = 0.424

546 Figure 1. The maximum quantum yield of primary photochemistry ($F_V/F_M \pm$ SD) in *Flavoparmelia*
547 *caperata* and *Xanthoria parietina* during the experiment. Histograms marked by a different letter
548 are statistically different ($P < 0.05$).



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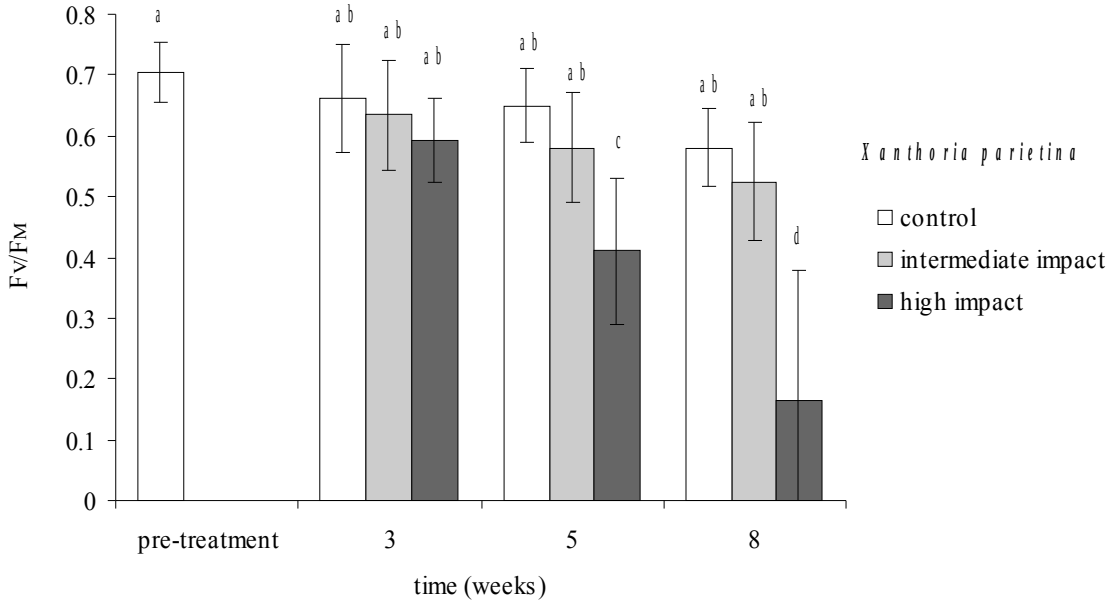

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