

1 Effects of acute NH₃ air pollution on N-sensitive and N-tolerant lichen species

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11 12 Abstract

13
14 Lichens are sensitive to the presence of ammonia (NH₃) in the environment. However, in order to
15 use them as reliable indicators in biomonitoring studies, it is necessary to establish unequivocally
16 the occurrence of certain symptoms following the exposure to NH₃ in the environment. In this paper,
17 we simulated an episode of acute air pollution due to the release of NH₃. The biological effects of
18 acute air pollution by atmospheric NH₃ have been investigated using N-sensitive (*Flavoparmelia*
19 *caperata*) and N-tolerant (*Xanthoria parietina*) species. Lichen samples were exposed to
20 ecologically relevant NH₃ concentrations for 8 weeks, simulating three areas of impact: a control
21 area (2 µg/m³), an area of intermediate impact (2–35 µg/m³) and an area of high impact (10–315
22 µg/m³), with a peak of pollution reached between the fourth and fifth week. Ammonia affected both
23 the photobiont and the mycobiont in *F. caperata*, while in *X. parietina* only the photosynthetic
24 performance of the photobiont was altered after exposure to the highest concentration. In the
25 photobiont of *F. caperata* we recorded chlorophyll degradation as indicated by OD_{435/415} ratio,
26 decrease of the photosynthetic performance (as reflected by the maximum quantum yield of primary
27 photochemistry F_v/F_M and the performance index PI_{ABS}); in the mycobiont, ergosterol reduction,
28 membrane lipid peroxidation (as reflected by the increase of thiobarbituric acid reactive
29 substances), alteration (decrease) of the secondary metabolite usnic acid. No effects were detected
30 on caperatic acid and dehydrogenase activity. In *X. parietina*, the only signal determined by NH₃
31 was the alteration of F_v/F_M and the performance index PI_{ABS}. The results suggest that physiological
32 parameters in N-sensitive lichens well reflect the effects of NH₃ exposure and can be applied as
33 early indicators in monitoring studies.

34
35 **Keywords:** chlorophyll fluorescence; dehydrogenase activity; ergosterol; industrial composting;
36 lichens; TBARS

37 38 1. Introduction

39
40 Air pollution by ammonia (NH₃) is a notable environmental concern since NH₃ contributes to the
41 deposition of eutrophating substances which exceeds the critical loads for many ecosystems
42 (Asman et al., 1998) causing impacts ranging from decreased biodiversity, changes in species
43 composition and dominance, and toxicity effects (Fangmeier et al., 1994). Agricultural activities are
44 responsible for over 90% of NH₃ emissions (Galloway et al., 2004), which occur primarily from
45 animal husbandry, manure storage and spreading and application of fertilizers. However, another
46 notable NH₃ source, which is increasingly expanding in many countries, is from decomposition
47 (composting) of organic waste, since the mineralization of organic N-containing amino acids and
48 urea releases considerable amounts of NH₃, the main cause of N pollution during composting of
49 organic waste (Zeng et al., 2012). In fact, N loss from industrial composting is mainly due to NH₃
50 emissions, which account for 24–33% and 47–77% of the initial N content of household waste and
51 manure respectively (Beck-Friis et al., 2001; Martins and Dewes, 1992).

52 Ammonia emission (and hence pollution) is not a uniform and continuous phenomenon, but rather
53 goes through acute episodes. In fact, during aerobic treatment of organic waste, according to the
54 activity of different groups of microorganisms, mineralization of organic N results in two NH₃

55 emissions peaks (Zeng et al., 2012) and levels of atmospheric NH₃ up to 700 mg/m³ have been
56 reported around waste water sludge composting facilities (Haug, 1993). Ammonia is severely
57 irritating to the nose, throat and lungs, and human exposure to excess NH₃ has been shown to be a
58 relevant concern for the health and safety of exposed workers (Rahman et al., 2007).
59 Once released to the environment, NH₃ is readily converted to NH₄⁺ or subject to dry deposition
60 (Fangmeier et al., 1994). Toxicity by NH₄⁺/NH₃ has been extensively studied in higher plants
61 (Fangmeier et al., 1994; Britto and Kronzucker, 2002) and detrimental effects can be early detected
62 at physiological level, involving alteration of secondary metabolism and changes due to increased
63 uptake and assimilation of N (Fangmeier et al., 1994). Higher plants, bryophytes, lichens, soil
64 organisms and invertebrates can be profitably used as bioindicators of the effects of N pollution in
65 the environment, integrating non biological methods of analysis (Sutton et al., 2004).
66 In particular, lichens are very sensitive to atmospheric reactive N, especially NH₃ (Sutton et al.,
67 2004). Being symbiotic organisms made up by an alga and a fungus, excess N is detrimental to the
68 equilibrium between the two symbiotic partners and hence to the whole lichen, especially if one of
69 the two partners is more able than the other to cope with high N levels (Gries, 1996). The results of
70 previous studies suggested that the photosynthetic apparatus of lichens exposed to ecologically
71 relevant NH₄⁺/NH₃ concentration is directly susceptible to these pollutants in the vapour/gas phase
72 (Paoli et al., 2010a; Munzi et al., 2012). In addition, relevant NH₄⁺/NH₃ levels may affect membrane
73 lipids and hence alter cell membrane permeability (Fangmeier et al., 1994) and there is evidence
74 that physiological parameters connected to membrane permeability are suitable tools for monitoring
75 biological effects of acute N pollution (Munzi et al., 2009).
76 In a previous work we investigated whether NH₃ emissions released during composting of organic
77 waste influenced during short-term exposures the lichens in the surrounding environment (Paoli et
78 al., 2014a). It was shown that exposing lichens around a composting plant allowed detecting early
79 physiological indications of potential biological changes before these consequences were apparent
80 at the community level. In particular, N-tolerant species were not affected by the proximity to the
81 facility and some parameters even suggested a better performance, while N-sensitive species
82 showed reduced performances approaching the source. In addition, it was hypothesized that the
83 concentrations of NH₃ were highly fluctuating, with peaks during outdoor handling and maturation
84 of the compost, suggesting that acute episodes of pollution could be the reason for the observed
85 effects (Paoli et al., 2014a). It was concluded that lichens can provide useful data for decision-
86 makers to establish correct science-based environmentally sustainable waste management policies.
87 However, since the interpretation of the results of field studies is often complicated by the
88 interactions among many environmental factors, experiments under controlled conditions are
89 necessary to separate the effects of specific environmental variables. The present experiment was
90 thus carried out to investigate the biological effects of a simulated acute air pollution by
91 atmospheric NH₃ on N-sensitive (*Flavoparmelia caperata*) and N-tolerant (*Xanthoria parietina*)
92 lichens.

93 94 **2. Materials and methods**

95 96 **2.1 Lichen species**

97
98 Samples of the lichens *Flavoparmelia caperata* (L.) Hale and *Xanthoria parietina* (L.) Th.Fr. were
99 collected at the beginning of May 2013 from a remote area of central Italy far from pollution
100 sources (Murlo, Tuscany 43°11'60" N, 11°21'33" E, 310 m a.s.l.) and transferred to the Botanical
101 Garden of the University of Siena.

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

107 *caperata* is a mesophytic species chiefly growing in sites with diffuse light but scarce direct solar
108 irradiation, up to sun-exposed sites, but avoiding extreme solar irradiation (Nimis and Martellos
109 2008), while *X. parietina* is rather xerophytic and can tolerate extreme radiations. Both species are
110 widely spread in lichen communities of the eu-mediterranean belt (i.e., in areas with a humid-warm
111 climate, such as Tyrrhenian Italy): *F. caperata* is one of the most common species in *Quercus* stands
112 and *X. parietina* is diffused in open stands, also in dry environments.

113

114 **2.2 Experimental design and sample treatment**

115

116 Samples of *F. caperata* and *X. parietina* were divided in 3 batches and placed inside 3 experimental
117 fumigation chambers of 60×40×25 cm³, located within one of the greenhouses of the Botanical
118 Garden of the University of Siena. Based on previous field studies (Paoli et al., 2014a), each
119 experimental chamber simulated a different situation of impact according to a gradient of NH₃
120 pollution: no impact (control), intermediate impact, high impact. In order to work with a similar
121 lichen biomass, each experimental chamber contained about 50 thalli of *F. caperata* and about 100
122 thalli of *X. parietina* (whose thalli are generally more little than those of *F. caperata*).

123 Samples were treated for 8 weeks as shown in Table 1: during the first 3 weeks samples were
124 acclimated to low atmospheric NH₃; then an episode of acute pollution from atmospheric NH₃ was
125 simulated for 2 weeks and during the last 3 weeks a moderate impact was simulated. Control
126 samples were constantly treated at the concentration of 2 µg/m³, roughly corresponding to
127 background values in Tuscany (Frati et al., 2007). Intermediate samples were treated at
128 concentrations of 2 µg/m³ during the first 3 weeks, at a peak of 100 µg/m³ during the 4th and 5th
129 weeks of exposure and at 10 µg/m³ during last 3 weeks. High impact samples were treated at
130 concentrations of 10 µg/m³ during the first 3 weeks, at peaks of 300 µg/m³ during the episode of
131 acute pollution (4th and 5th week) and at 100 µg/m³ during last three weeks (Table 1).

132 Ammonia was applied as follows: water solutions containing liquid NH₃ were prepared and placed
133 into open Petri dishes within the experimental chamber, then let evaporate within each chamber,
134 which remained closed. In the control chamber only water was applied. Relative humidity increased
135 during water evaporation: every two days, after water evaporated, a further solution containing NH₃
136 (or only water in controls) was applied opening the chamber only for the time necessary and closing
137 it after the treatment. Therefore, each chamber represented a sort of closed environment. The level
138 of atmospheric NH₃ (Table 1) was measured with passive air samplers (Radiello® diffusion tubes,
139 Aquaria). For each treatment two samplers were placed in each chamber for 7 days during the 3rd,
140 5th and 8th week. Samplers contained a filter impregnated with phosphoric acid that adsorbs gas-
141 phase NH₃ as NH₄⁺, which can be measured spectrophotometrically by the indophenol blue method
142 (Allen 1989). The detection limit was 0.7 µg/m³, uncertainty was 6.5%.

143 The experiment was run between May–June 2013. Microclimatic parameters under the
144 experimental conditions were regularly recorded between 12:00 and 1:00 p.m. and values were in
145 the following range: solar radiation (1000–1550 µmol s⁻¹ m⁻²), temperature (23–30°C), relative
146 humidity (45–65%). All chambers were characterized by the same microclimatic conditions during
147 the experiment, which followed their normal daily fluctuation between day and night, so that the
148 main difference among the experimental chambers was the average level of NH₃. Since there are no
149 known examples of dying lichens releasing volatile chemicals that could affect the physiological
150 responses in neighbouring thalli (independently of NH₃ impacts) lichen thalli within the same
151 chamber have been considered as independent samples.

152

153 **2.3 Physiological parameters investigated**

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

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

166

167 2.3.1 Chlorophyll degradation

168 Photosynthetic pigments were extracted using dimethylsulfoxide (DMSO), adding
169 polyvinylpyrrolidone (PVP) and filtering the solution before use (Barnes et al., 1992).
170 *Flavoparmelia caperata* contains lichen substances, which could degrade chlorophyll during
171 extraction causing phaeophytinization (Brown and Hooker 1977). In order to remove these
172 substances, before pigment extraction, lichen samples (20 mg) were subjected to six 5-min
173 washings in 3 mL 100% acetone buffered with CaCO₃ (Pisani et al., 2007). Two extraction cycles,
174 45 min each, were run in a warm bath (65°C), using 5 mL of DMSO. Absorbance of the extracts
175 was measured using a UV-visible spectrophotometer (Agilent 8453). Chlorophyll degradation was
176 expressed by the ratio between the absorbance at 435 and 415 nm (OD₄₃₅/OD₄₁₅), as suggested by
177 Ronen and Galun (1984). Five replicates were measured for each treatment.

178

179 2.3.2 Photosynthetic efficiency

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

191

192 2.3.3 Membrane lipid peroxidation

193 Membrane lipid peroxidation was estimated using the thiobarbituric acid reactive substances
194 (TBARS) assay. About 50 mg of lichen material was rinsed in distilled water and then homogenized
195 in a mortar using 2 mL of 0.1% (w/v) trichloroacetic acid (TCA) with the addition of sand. 1.5 mL of
196 the homogenate was put in eppendorf tubes and centrifuged at 12000 g for 20 min. 0.5 mL of the
197 supernatant were collected and added to 1.5 mL of 0.6% thiobarbituric acid in 10% TCA and put in
198 glass tubes. Tubes were put in the oven at 95°C for 30 min, cooled in an ice bath and then solutions
199 were centrifuged again at 12000 g for 10 min. The absorbance of the supernatant was measured at
200 532 nm and corrected for non-specific absorption at 600 nm. Concentration of TBARS was
201 calculated using the extinction coefficient for the TBA-MDA complex (155 mM⁻¹cm⁻¹) and the
202 results expressed as μmol/g (dw). Five replicates were measured for each treatment.

203

204 2.3.4 Dehydrogenase activity

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

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

213

214 2.3.5 Ergosterol content

215 Ergosterol content in lichens is sensitive to the exposure to heavy metals, which likely reduces the
216 integrity of cell membranes of the mycobiont. Three replicates were measured at each site. Samples
217 of 100 mg of lichen material were homogenized for 10 min in 99% ethanol. Extracts were
218 transferred to 1.5 mL Eppendorf tubes and shaken in the dark at 25 °C for 30 min, then vortexed and
219 centrifuged at 10000 g for 20 min. The resulting supernatant was immediately analysed by HPLC in
220 a Kromasil 100 C18 column (150 x 4.6 mm, particle size 7 µm) as separator, with flow rate 0.8 mL
221 min⁻¹ and isocratic elution with methanol as mobile phase (Dahlman et al., 2002). Total analysis
222 time was 15 min. Ergosterol absorption at 280 nm was measured with a UV detector (Ecom LCD
223 2084). A standard curve was prepared ranging 1-200 µg ergosterol (Sigma–Aldrich, USA) dissolved
224 in 1mL of ethanol. As ergosterol is sensitive to light, all steps were conducted almost in the dark.
225 Three replicates were measured for each treatment.

226

227 2.3.6 Secondary metabolites

228 Secondary metabolites were measured as indicated by Bačkor et al. (2011). Usnic and caperatic acid
229 were measured in *F. caperata* and parietin was analysed in *X. parietina*. Cleaned samples (15 mg
230 dw) were extracted in 1 mL cool acetone till acetone evaporation. Acetone extracts were collected
231 and the residues were dissolved with fresh 1 mL of acetone, during 40 s were materials blended on a
232 whirl mixer and filtered extracts were analysed by gradient HPLC under the following conditions:
233 column Tessek SGX C₁₈, flow rate: 0.7 mL min⁻¹, mobile phase: A= H₂O: acetonitrile: H₃PO₄
234 (80:19:1) and B= 95% acetonitrile. Gradient program: 0 min 25% B, 5 min 50% B, 20 min 100% B,
235 25 min 25% B. The detection wavelength was 245 nm (detector Ecom LCD 2084). Usnic acid
236 (Aldrich) was used as standard. Standard of caperatic acid was prepared from crystallized acetone
237 extracts from *F. caperata* (purity 100%). Standard of parietin was prepared from crystallized
238 acetone extracts from *X. parietina* (purity 98%). Three replicates were measured for each
239 treatment.

240

241 2.4 Statistical analysis

242

243 After checking the normality of data distribution (Shapiro-Wilk, 95% confidence interval), one-way
244 analysis of variance and Tukey's pairwise comparison ($P < 0.05$) were run to investigate the effects
245 of NH₃ concentrations on the investigated physiological parameters.

246

247 3. Results

248

249 The maximum quantum yield of primary photochemistry (F_v/F_M) was used as a non-destructive tool
250 for a rapid screening of the vitality of the samples during the experiment (Figure 1). Signs of
251 alteration following NH₃ treatments emerged after 5 weeks in *F. caperata* (N-sensitive) both in the
252 cases with high and intermediate impact, while *X. parietina* (N-tolerant) was affected only in the
253 case with the highest impact. A comparison accounting the time of exposure revealed a weak
254 decrease of F_v/F_M also in the samples of the control case (respect to values pre-treatment). The
255 experimental conditions were thus partially selective on this parameter after five weeks in *F.*
256 *caperata* and eight weeks in *X. parietina*.

257 The results of ANOVA indicated that at the end of the treatments NH₃ affected both the photobiont
258 and the mycobiont in *F. caperata*, while in *X. parietina* only the photosynthetic performance of the
259 photobiont was altered at the highest concentration (Tables 2 and 3).

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

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

268

269 4. Discussion

270

271 The experiment simulated the exposure of lichens into three different situations around a point
272 source concerned by an episode of acute air pollution from atmospheric NH_3 . Our NH_3
273 concentrations, spanning from a peak of $300 \mu g/m^3$ in the simulated high impact, down to $2 \mu g/m^3$ in
274 the simulated control area, are in line with those documented by Frati et al. (2007) around a pig
275 farm, since they reported a peak of $267 \mu g/m^3 NH_3$ and a 98% reduction of NH_3 achieved already in
276 the first 200 m from the source. Atmospheric NH_3 generally decreases exponentially with distance
277 from the polluting source (Pinho et al., 2012; Fowler et al., 1998; Jones et al., 2013). Paoli et al.
278 (2010a) assessed the effects of NH_3 pollution on lichen photosynthesis and measured 62.4 ± 4.3
279 $\mu g/m^3$ at a sheep farm in Greece, which decreased to $15 \mu g/m^3$ at 60 m from the farm down to 2
280 $\mu g/m^3$ in a remote area 5 km away, in parallel with the improvement of the photosynthetic
281 performance of the lichens.

282 During the aerobic composting of biowaste, concentrations of NH_3 in the waste gas up to 227
283 mg/m^3 were reported (Smet et al., 1999), while during animal housing and transportation, values up
284 to $22.5 mg/m^3$ have been documented (Costa et al., 2003), corresponding to levels potentially
285 harmful for the animals (Gustin et al., 1994). Paoli et al. (2014a) assessed the biological effects of
286 NH_3 released during three months of activity from a composting plant of organic wastes using
287 lichen transplants. Respect to unexposed samples, NH_3 around the facility (peak of 48.7 ± 18.9
288 $\mu g/m^3$) affected the overall vitality (in particular the photosynthetic performance) of non-
289 nitrophilous lichens (*E. prunastri*), but did not affect negatively that of nitrophilous species (*X.*
290 *parietina*). In our study, NH_3 led to oxidative stresses, which affected both the photobiont and the
291 mycobiont in *F. caperata*, while in *X. parietina* only the photosynthetic performance of the
292 photobiont was altered.

293 Concerning the photobiont, besides altering the photosynthetic performance, NH_3 led to a
294 significant chlorophyll degradation in *F. caperata*. Consistently with the observations of Munzi et
295 al. (2009), chlorophyll degradation was not detected in *X. parietina*. Frati et al. (2007) reported
296 signs of injury in samples of *F. caperata* exposed in the centre of a pig farm, while these symptoms
297 were not visible in *X. parietina*, confirming that the former is a more sensitive species to NH_3
298 pollution and the latter is a resistant one. However, our prolonged exposure to peak concentrations
299 (for two weeks) reduced the vitality of the photobiont also in *X. parietina*. Relevant NH_4^+/NH_3
300 levels may be toxic to the photosynthetic apparatus as they function as electron acceptors,
301 uncoupling electron transport (Losada and Arnon, 1963) and the photosynthetic performance of the
302 lichen photobiont can be considered a suitable indicator of the effects of NH_4^+/NH_3 in the
303 environment (Munzi et al. 2010; Paoli et al. 2010a). Similarly, treatments of N-sensitive lichens (*E.*
304 *prunastri*) with ecologically relevant NH_4^+ concentrations ($50\text{--}500 \mu M NH_4Cl$, simulating
305 prolonged exposure) reduced the photosynthetic performance of the photobiont (Munzi et al.,
306 2012). The fact that the photosynthetic performance partially decreased also in our (simulated)
307 control case (after five weeks in *F. caperata* and later, after eight weeks in *X. parietina*) is perfectly
308 in line with the autoecology of these species: *F. caperata* is mesophytic and potentially sensitive to
309 extreme radiations under stressing microclimatic conditions, such as during a Mediterranean
310 summer, while *X. parietina* is rather xerophytic and can better tolerate extreme conditions (Paoli et
311 al., 2010b).

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

315 Ammonia pollution enhanced TBARS production in *F. caperata*, but not in *X. parietina*. However,
316 TBARS production has been reported as a consequence of the assimilation of high concentrations of
317 chemical elements (e.g. Ce, Sb) also in the lichen *X. parietina*, when all physiological parameters
318 pinpoint the overall status of alteration of the thalli (Paoli et al., 2013; 2014b).
319 Under the experimental conditions, ergosterol content decreased in the mycobiont of *F. caperata*,
320 according to NH₃ concentration, but was unaffected in that of *X. parietina*. Ergosterol is the
321 principal sterol of the plasma membrane of fungi and may thus reflect the amount of metabolically
322 active cells in the mycobiont (Sundberg et al., 1999). Fungal membranes are a suitable target to
323 detect the effects of acute N pollution (Munzi et al., 2009) and ergosterol content has been reported
324 as a parameter negatively effected by air pollution, e.g., by heavy metals (Bačkor et al., 2006).
325 Ammonia did not significantly affect dehydrogenase activity (TTC reduction to triphenylformazan),
326 both in *F. caperata* and *X. parietina*, however, the results of a previous study suggested that NH₃
327 may induce changes in dark respiration in the N-sensitive lichen *E. prunastri* (Paoli et al., 2014a).
328 Usnic acid, a yellow cortical pigment with antibiotic effects, is deposited in the form of crystals on
329 the surface of lichen mycobionts as well as photobionts. Produced by the mycobiont, it can regulate
330 photobiont cell division in the thalli, screen from excessive sunlight, detoxify from metal pollution
331 and prevent oxidative stresses (Bačkor et al., 2010; Caviglia et al., 2001; Cocchietto et al., 2002).
332 Exposure to NH₃ in *F. caperata* altered the content of usnic acid, but did not affected that of
333 caperatic acid, a medullary compound with a protective role. Interestingly, the ratio between
334 caperatic and usnic acid raised from the control (3.6), intermediate (7.1), up to the high impact case
335 (9.5).
336 Our results suggest that the mycobiont of *F. caperata* was not able to produce usnic acid as a
337 consequence of NH₃ exposure and a series of oxidative stresses occurred, as witnessed by ergosterol
338 decrease and malondialdehyde production. It seems that the mycobiont was affected by high NH₃
339 concentrations (as usnic acid and ergosterol content decrease support) but probably it was still
340 metabolic active, as justified by stable dehydrogenase activity and caperatic acid content. Caperatic
341 acid probably plays a more important role in tolerance to N pollution than usnic acid, which is
342 important in detoxification from metal pollution. On the other hand, in *X. parietina* ergosterol and
343 malondialdehyde content remained stable and the exposure to NH₃ likely induced parietin
344 production, evident comparing exposed (irrespective of the concentration) vs control thalli (at $P =$
345 0.10). Our results would suggest that parietin, a photoprotective metabolite of the lichen *X.*
346 *parietina*, can presumably act as an antioxidant preventing cell membranes from oxidative stresses.
347 In fact, parietin production was reported as a likely defensive mechanism upon exposure of *X.*
348 *parietina* to air pollutants in the environment (Silberstein et al., 1996).
349 The reason of the tolerance of *X. parietina* to excess N can be, at least partially, explained by the
350 low cation exchange capacity, which allows avoiding excess N uptake and the ability to provide
351 carbon (C) skeletons for N assimilation (Gaio-Oliveira et al., 2005). It was estimated that *X.*
352 *parietina* can tolerate an NH₄⁺ deposition load of 1,000 kg ha⁻¹ yr⁻¹ (Gaio-Oliveira et al., 2004).
353 At ecological level, it was shown the diffusion of *X. parietina* in areas with higher NH₃ in the
354 environment, despite a decreased photosynthetic efficiency (F_v/F_M) was observed in sites above 50
355 µg/m³, suggesting that the ecological success of *X. parietina* at NH₃-rich sites might be related to
356 indirect effects of increased N availability (Munzi et al., 2014). The photosynthetic efficiency
357 already decreased for N-sensitive species (*E. prunastri*) already above the level of 3 µg/m³ (Munzi
358 et al., 2014). Similarly, in the field we observed the diffusion of nitrophilous species in the presence
359 of NH₃ released from a composting plant and a shift from lichen communities composed chiefly by
360 meso-acidophilous species (at ca. 3 µg/m³) to more nitrophilous communities approaching the
361 source (ca. 49 µg/m³) (Paoli et al., 2014a).

362

363 **Conclusions**

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365 The simulated episode of acute air pollution by atmospheric NH₃ induced alterations on both the
366 photobiont and the mycobiont in the N-sensitive lichen *F. caperata*: we reported chlorophyll

367 degradation and decrease of the photosynthetic performance in the photobiont; ergosterol reduction,
368 membrane lipid peroxidation, decrease of the content of usnic acid in the mycobiont. In the N-
369 tolerant *X. parietina* only the photosynthetic performance of the photobiont was altered after the
370 exposure to the highest NH₃ concentration. The resistance of the mycobiont of *X. parietina* can
371 explain the ability of this species to tolerate NH₃ pollution and hence its nitrophilous behaviour. On
372 the whole, the results indicated that physiological parameters in N-sensitive lichens well reflect the
373 effects of NH₃ exposure and can be applied as early indicators in monitoring studies in order to
374 detect early signs of potential biological changes.

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527 Table 1. Atmospheric NH₃ concentrations (µg/m³) measured with passive air samplers.
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weeks	Ammonia concentrations (µg/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

529

530

531 Table 2. Physiological parameters in *Flavoparmelia caperata* after 8 weeks of exposure to NH₃. *F*
 532 and *P* values of ANOVA. Values in each line followed by a different letter differ according to the
 533 impact of NH₃ (Tukey's pairwise comparison, *P* < 0.05).
 534

Physiological parameters	Environmental conditions			
	Control	Intermediate impact	High impact	
OD_{435/415}	1.03 ± 0.01a	0.81 ± 0.04b	0.67 ± 0.03c	<i>F</i> = 115.3 <i>P</i> = 0.000
F_v/F_M	0.551 ± 0.078a	0.321 ± 0.098b	0.155 ± 0.141c	<i>F</i> = 30.03 <i>P</i> = 0.000
PI_{ABS}	0.133 ± 0.060a	0.009 ± 0.010b	0.004 ± 0.010b	<i>F</i> = 37.45 <i>P</i> = 0.000
Dehydrogenase (A₄₉₂/g)	2.45 ± 0.46	3.40 ± 0.86	3.02 ± 0.69	<i>F</i> = 1.440 <i>P</i> = 0.308
TBARS (µmol/g)	7.3 ± 1.4c	19.4 ± 7.9b	29.7 ± 7.7a	<i>F</i> = 4.218 <i>P</i> = 0.084
Ergosterol (mg/g)	0.58 ± 0.05a	0.36 ± 0.04b	0.29 ± 0.03c	<i>F</i> = 39.73 <i>P</i> = 0.000
Caperatic acid (% dw)	8.5 ± 1.0	8.0 ± 2.3	9.5 ± 1.7	<i>F</i> = 1.035 <i>P</i> = 0.379
Usnic acid (% dw)	1.48 ± 0.22a	0.78 ± 0.27b	0.67 ± 0.16b	<i>F</i> = 23.10 <i>P</i> = 0.000

535

536 Table 3. Physiological parameters in *Xanthoria parietina* after 8 weeks of exposure to NH₃. *F* and *P*
 537 values of ANOVA. Values in each line followed by a different letter differ according to the impact
 538 of NH₃ (Tukey's pairwise comparison, *P* < 0.05).
 539

Physiological parameters	Experimental conditions			
	Control	Intermediate impact	High impact	
OD_{435/415}	1.42 ± 0.01	1.39 ± 0.02	1.39 ± 0.03	<i>F</i> = 0.894 <i>P</i> = 0.457
F_v/F_M	0.581 ± 0.064a	0.525 ± 0.097a	0.166 ± 0.212b	<i>F</i> = 18.66 <i>P</i> = 0.000
PI_{ABS}	0.112 ± 0.070a	0.080 ± 0.060a	0.016 ± 0.030b	<i>F</i> = 6.909 <i>P</i> = 0.006
Dehydrogenase (A₄₉₂/g)	11.11 ± 1.71	10.13 ± 1.87	8.43 ± 1.40	<i>F</i> = 1.966 <i>P</i> = 0.220
TBARS (µmol/g)	32.8 ± 7.3	32.1 ± 1.3	30.3 ± 2.3	<i>F</i> = 4.146 <i>P</i> = 0.106
Ergosterol (mg/g)	0.76 ± 0.09	0.76 ± 0.08	0.75 ± 0.05	<i>F</i> = 0.013 <i>P</i> = 0.987
Parietin (% dw)	0.88 ± 0.56	1.36 ± 0.90	1.37 ± 0.63	<i>F</i> = 0.910 <i>P</i> = 0.424

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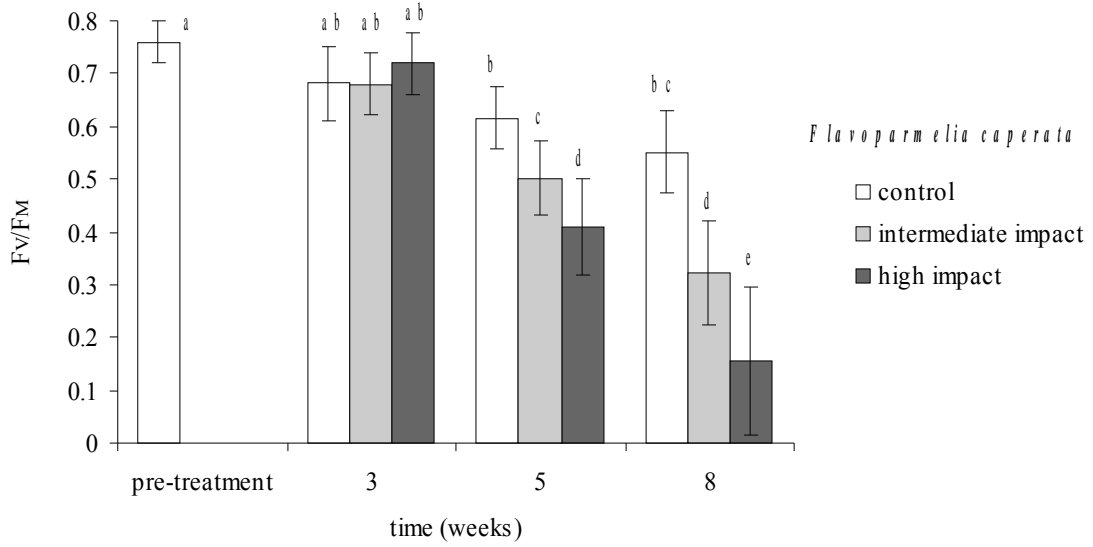
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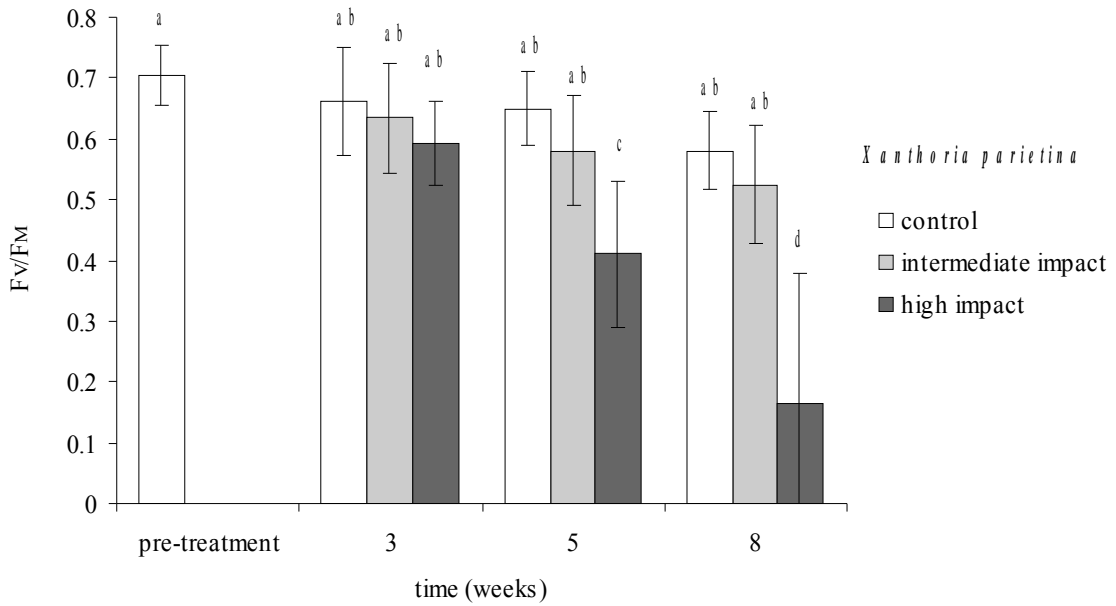
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546 Figure 1. The maximum quantum yield of primary photochemistry ($F_v/F_M \pm SD$) in *Flavoparmelia*
 547 *caperata* and *Xanthoria parietina* during the experiment. Histograms marked by a different letter
 548 are statistically different ($P <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



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