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Short Communication

$\delta^{15}N$ of lichens reflects the isotopic signature of ammonia source

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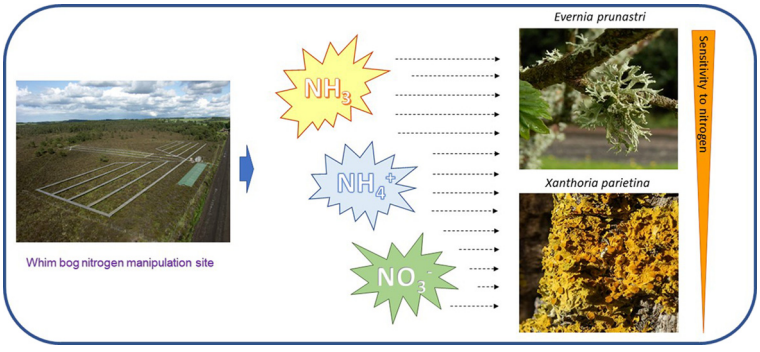
HIGHLIGHTS

- Lichen $\delta^{15}N$ is a suitable tool to interpret the spatial distribution of NH_3 sources.
- *X. parietina* kept its potential photosynthetic activity at higher NH_3 doses than *E. prunastri*.
- Tolerant species are preferred to sensitive ones in areas with high N pollution.

GRAPHICAL ABSTRACT



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ABSTRACT

Although it is generally accepted that $\delta^{15}N$ in lichen reflects predominating N isotope sources in the environment, confirmation of the direct correlation between lichen $\delta^{15}N$ and atmospheric $\delta^{15}N$ is still missing, especially under field conditions with most confounding factors controlled. To fill this gap and investigate the response of lichens with different tolerance to atmospheric N deposition, thalli of the sensitive *Evernia prunastri* and the tolerant *Xanthoria parietina* were exposed for ten weeks to different forms and doses of N in a field manipulation experiment where confounding factors were minimized. During this period, several parameters, namely total N, $\delta^{15}N$ and chlorophyll *a* fluorescence, were measured. Under the experimental conditions, $\delta^{15}N$ in lichens quantitatively responded to the $\delta^{15}N$ of released gaseous ammonia (NH_3). Although a high correlation between the isotopic signatures in lichen tissue and supplied N was found both in tolerant and sensitive species, chlorophyll *a* fluorescence indicated that the sensitive species very soon lost its photosynthetic functionality with increasing N availability. The most damaging response to the different N chemical forms was observed with dry deposition of NH_3, although wet deposition of ammonium ions had a significant observable physiological impact. Conversely, there was no significant effect of nitrate ions on chlorophyll *a* fluorescence, implying differential sensitivity to dry deposition versus wet deposition and to ammonium versus nitrate in wet deposition. *Evernia prunastri* was most sensitive to NH_3, then NH_4^+, with lowest sensitivity to NO_3^-. Moreover, these results confirm that lichen $\delta^{15}N$ can be used to indicate the $\delta^{15}N$ of atmospheric ammonia, providing a suitable tool for the interpretation of the spatial distribution of NH_3 sources in relation to their $\delta^{15}N$ signal.

1. Introduction

Nitrogen (N) is considered one of the main drivers of environmental change and one of the major pollutants of anthropogenic origin

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(Kanakidou et al., 2016; Payne et al., 2017; Stevens et al., 2018). Nitrogenous gases and in particular ammonia (NH_3) represent the most relevant part of total N deposition, originating mainly from production of fertilizers, munitions and other products, biological fixation of N_2 and decomposition of organic compounds (Sutton et al., 2013).

The excess of reactive N introduced into the ecosystems has been increasingly recognized as a threat to biodiversity and to ecosystem function and resilience, affecting processes occurring in the atmosphere, oceans and terrestrial habitats (Bobbink et al., 2010; Steffen et al., 2015). Gaseous NH_3 is also produced by catalytic convertors of vehicles with internal combustion engine, thus representing a main threat to human health in urban environments (Suarez-Bertoa et al., 2014). Therefore, monitoring N sources and deposition as well as assessing the effects on ecosystems is pivotal for the establishment of suitable management and mitigation strategies.

Deposition measurement stations are expensive and have restricted spatial distribution, thus providing only a partial and uncertain picture of N deposition rates in limited areas (Sutton et al., 2003). Moreover, currently available measurement methods present technical uncertainties related to emissions, dispersion, chemistry, and data collecting and handling, making the assessment of N deposition still an open issue (Fowler et al., 2009). Equally, apart from specific landscape scale assessments (e.g. Dragosits et al., 2002), local conditions can hardly be represented by regional models existing for N deposition at larger scales.

Biomonitoring has been proposed and used as an effective tool to quantify the exposure to and evaluate the effects of atmospheric N on sensitive components of the ecosystems (e.g. Izquieta-Rojano et al., 2016; Munzi et al., 2012; Pinho et al., 2011, 2012, 2014; Varela et al., 2016; Wolseley et al., 2006). In particular, N isotopic signature ($\delta^{15}N$) in different organisms and tissues, such as lichens, mosses and bark, provides an informative tool for monitoring sources and pathways of atmospheric N (Boltersdorf et al., 2014 and references therein).

Lichens are well-known as biomonitors, responding to increased N availability (Munzi et al., 2014; Pinho et al., 2012, 2014) since they depend on the atmospheric compartment (wet and dry atmospheric depositions) for their nutrient requirements. Several works demonstrated that epiphytic lichens reflect N deposition, especially from agriculture-derived N-containing compounds (e.g. Gombert et al., 2003; Ruoss, 1999).

It is generally accepted that thallus N content and isotopic signature ($\delta^{15}N$) in lichens reflect land use (N sources) and atmospheric transport of N pollutants in the surroundings, and several studies have dealt with relations between N concentration and isotopic signature in lichen organic material. Tests on epiphytic lichens collected near deposition measurement field stations in Germany found that N content and $\delta^{15}N$ values in lichens, when compared with measured and modelled N deposition data, reflected the local N deposition originating from agriculture activities (Boltersdorf et al., 2014; Boltersdorf and Werner, 2013, 2014). Manninen (2018) found that $\delta^{15}N$ in *Hypogymnia physodes* supported the uptake of oxidized N mainly originating from road traffic in Helsinki metropolitan area. For Antarctic environments, Cipro et al. (2011) and Crittenden et al. (2015) investigated organic pollutants and stable isotopes in vegetation finding that N isotopic signature in lichens and mosses indicate the influence of animal-derived N. Hogan et al. (2017) used N and sulphur isotopic composition of the species *Cladia retipora* to pinpoint anthropogenic influences on atmospheric background deposition in Tasmania. Similarly, Pinho et al. (2017) ranked land-cover types according to the amount and form of emitted atmospheric reactive N in a complex landscape with multiple N sources correlating N concentration and isotopic composition in lichen thalli to land-cover data.

Atmospheric $\delta^{15}N$ has not been measured and compared directly with $\delta^{15}N$ in lichen tissue; previous results merely report correlations with $\delta^{15}N$ in other organisms living in the area or with models. The main difficulties are due to the synergism between climatic and anthropogenic factors and the superimposition of multiple N sources, the spatial variability of NH_3, the reactivity of some N forms, and the scarce number of measuring devices in the field (Boltersdorf and Werner, 2013). Moreover, the range of variability of N-NH_3 isotopic signature from different sources is huge, as shown by Ti et al. (2018). The authors found that in China δ^{15} of N-NH_3 ranged from −30.8 to −3.3‰ for volatilized fertilizer, from −35.1 to −10.5‰ for emissions from a pig farm, and from −24.7 to −11.3‰ for emissions from a dairy farm.

Although Skinner et al. (2006) found a significant correlation between experimental supply of dry N deposition (ammonia concentration ranging between 0 and 125 μg m^{-3}) and N content and $\delta^{15}N$ in the terricolous lichen *Cladonia portentosa*, a confirmation of the direct correlation between the source and epiphytic lichen $\delta^{15}N$ was missing.

The present communication investigates how N deposition affects lichen's isotopic signature in a manipulative ecosystem experiment (Whim Bog, Scotland, Leith et al., 2004). The aim is to evaluate the potential for $\delta^{15}N$ as a natural tracer for N pollution, especially for atmospheric N sources. Moreover, since the Whim Bog site is in an environment with low background N deposition (Leith et al., 2004), the use of physiological tests allows investigation of the specific effects of different forms of N (dry vs. wet; ammonium vs. nitrate), thereby minimizing other confounding elements.

Based on the working hypotheses that lichens will take up the N supplied, and that $\delta^{15}N$ in epiphytic lichens exposed to atmospheric N deposition will tend toward the $\delta^{15}N$ of the N deposition source, we aimed to compare: i) $\delta^{15}N$ of lichen thalli and the atmospheric N source; ii) the performance of and the fractionation of $\delta^{15}N$ of lichen organic material of N-tolerant and N-sensitive species; iii) the effects of nitrate vs. ammonium on lichens exposed to conditions resembling natural ones; and iv) the effects of wet deposited nitrogen vs. dry deposition as gaseous NH_3.

2. Methods

2.1. Nitrogen treatments

Reactive N deposition treatments have been applied at the Whim Bog experimental site, a peatland ecosystem 26 km from the sea (Sheppard et al., 2004, 2011), including manipulation of both wet and dry deposition. For wet deposition, four control plots receive only natural rainfall with a background total inorganic N deposition of 8 kg ha^{-1} yr^{-1} (wet plus dry). The other wet deposition experimental plots receive different additional N doses (as NH_4Cl or $NaNO_3$) applied through a sprinkler irrigation system, to simulate natural rainfall, at rates of 8, 24 and 56 kg ha^{-1} yr^{-1}. Each treatment is replicated in 4 plots in each of four blocks, which take account of site geography. Plots, circular areas of 12.5 m^2, are 3 m apart to minimise cross-contamination.

For dry deposition, an NH_3 gradient is established by NH_3 release from a 10 m long pipe line source at 1 m height (tangential to the main wind direction), when the wind direction is 180–215° and speed is at least 2.5 m s^{-1}. NH_3 concentrations and loads were measured at the transplant locations, located 12, 30 and 60 m downwind (north east) from the NH_3 source, using passive ALPHA samplers (Tang et al., 2001) set 0.1 m above the vegetation, and estimates calculated following Cape et al. (2008).

2.2. Lichen material

Thalli of the tolerant foliose species *X. parietina*, which is considered well suited for monitoring using N isotopic signature (e.g. Boltersdorf and Werner, 2013), and the sensitive fruticose species *E. prunastri*, commonly used in biomonitoring surveys (e.g. Paoli et al., 2015b), were collected at sites with an NH_3 concentration of 1.6 μg m^{-3} for *X. parietina* (Penicuik, Midlothian Scotland) and 0.6 μg m^{-3} for *E. prunastri* (Peebles,

Tweeddale, Scotland). NH_3 concentrations were extracted from the APIS – Air Pollution Information System website (http://www.apis.ac.uk), which reports a modelled 3-year average concentration for the 5 km × 5 km grid square that the specified location is in. Four branches of *Sambucus nigra* and of *Quercus robur*, carrying respectively 5–10 thalli of different size of *X. parietina* and *E. prunastri* were transplanted together into each of the different wet plots and along the dry transect at 12, 30 and 60 m from the NH_3 source (where ALPHA samplers were located) at Whim Bog. All the transplanted branches were supported by plastic sticks and inserted facing the N source at the same height and distance in the open (Supplementary material, Fig. S1). Transplants, exposed from the end of June to the beginning of September 2012, were collected after 10 weeks from wet deposition plots (around 20 thalli per species) and in 3 different times (10–12 thalli per species each time), after 3, 6 and 10 weeks respectively, in the case of dry deposition. During those periods, lichens were exposed to different N loads depending on meteorological conditions. Meteorological conditions were the same for dry and wet sampling plots, and meteorological parameters were measured on site by an automatic weather station. All parameters were calculated considering not the exposure time or the distance from the source, but the cumulative N loads to which lichen thalli were subjected in the different periods (see Supplementary material, Table S1 for N loads of single periods).

Measurements were taken in collected material pre-transplantation, which were considered as control values. Additionally, some samples were subjected to irrigation with rain water without N supply (control plots). These two plots were used to check if the transplant process affected lichen health at two different times (in the middle and at the end of the experiment).

2.3. Stable isotopes and total N

Stable isotope ratio analysis was performed at the Centro de Recursos em Isótopos Estáveis - Stable Isotopes and Instrumental Analysis Facility, at the Faculdade de Ciências, Universidade de Lisboa - Portugal. $\delta^{15}N$ in the samples was determined by continuous flow isotope mass spectrometry (CF-IRMS) (Preston and Owens, 1983), on a Sercon Hydra 20–22 (Sercon, UK) stable isotope ratio mass spectrometer, coupled to a EuroEA (EuroVector, Italy) elemental analyser for online sample preparation by Dumas-combustion. Delta Calculation was performed according to $\delta = [(R_{sample} - R_{standard}) / R_{standard}] * 1000$, where R is the ratio between the heavier isotope and the lighter one. $\delta^{15}N_{Air}$ values are referred to air. The reference materials used were USGS-25, USGS-35, BCR-657 and IAEA-CH7 (Coleman and Meier-Augenstein, 2014); the laboratory standard used was Protein Standard OAS/Isotope (Elemental Microanalysis, UK). The major mass signal of N was used to calculate total N abundance, using Wheat Flour Standard OAS (Elemental Microanalysis, UK, with 1.36%N) as elemental composition reference materials. Uncertainty of the isotope ratio and N content analysis, calculated using values from 6 to 9 replicates of laboratory standard, interspersed among samples in every batch analysis, was ≤0.1‰. Elemental and isotopic composition of reference material can be found in Supplementary material Table S2. Three replicates for each treatment and species were analyzed, with replicates plot-averaged and treated as independent sampling units.

Isotopic signature of chemicals used for the treatments were −0.1‰ for NH_4Cl, 3.8‰ for $NaNO_3$ used in the wet deposition treatments and 6.2‰ for NH_3 released in the dry deposition treatment.

2.4. Chlorophyll a fluorescence

Measurements of the chlorophyll *a* fluorescence parameter Fv/Fm of the transplanted lichens were taken as a stress indicator (Munzi et al., 2014). Samples were hydrated and dark-adapted at room temperature for 15 min before measuring fluorescence. The Fv/Fm ratio was measured with the Plant Efficiency Analyzer Handy PEA (Hansatech LTD, UK).

Samples were considered affected but viable when Fv/Fm > 0.32 based on Munzi et al. (2013). In that study, samples of *X. parietina* with similar value of Fv/Fm after N treatment were able to recover almost to control value showing that the algal partner, belonging to the genus *Trebouxia*, and also present in *E. prunastri*, was still viable.

2.5. Statistics

Significance of differences ($P < 0.05$) in chlorophyll *a* fluorescence values between treatments and controls was checked by one-way analysis of variance (ANOVA), using the Dunnett test for post-hoc comparisons in Statistica (Stasoft Inc.). Linear regression analysis was used to model the relationships between $\delta^{15}N$, N content and N dry and wet deposition and between Fv/Fm and $\delta^{15}N$ using SPSS software (IBM Corp. Released 2016. IBM SPSS Statistics Version 24.0).

3. Results and discussion

In the experimental conditions, $\delta^{15}N$ of lichens responded to the $\delta^{15}N$ of the released atmospheric ammonia (Fig. 1A). Although a close correlation between the isotopic signatures of lichen tissue and supplied N was found both in the tolerant and sensitive species, the sensitive species lost its functionality with increasing N availability (Fig. 2).

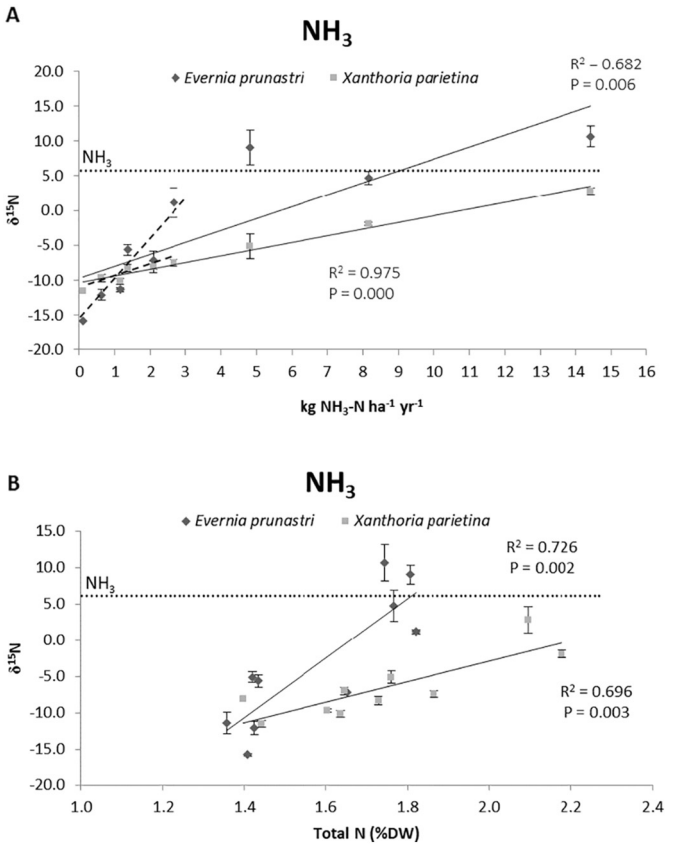

Fig. 1. Results of linear regression analysis between $\delta^{15}N$ (average ± SE, n = 3) of lichen thallus and NH_3 deposition rate (besides the total background value of 8 kg ha^{-1} yr^{-1}) (A) and between $\delta^{15}N$ (average ± SE, n = 3) and thallus N concentration expressed as percentage dry weight (DW) (B), in *X. parietina* (squares) and *E. prunastri* (diamonds). The dotted line in (A) and (B) represents the value of $\delta^{15}N$ in the released NH_3 of the dry treatment. The dashed lines in part (A) represent the uptake rate of NH_3 in the two lichen species for depositions of up to 3 kg N ha^{-1} yr^{-1}. 95% confidence intervals of the slopes are from 2.707 to 9.170 for *E. prunastri* and from 0.605 to 2.363 for *X. parietina* in (A) up to 3 kg N ha^{-1} yr^{-1}; from 21.545 to 65.172 for *E. prunastri* and from 5.801 to 22.128 for *X. parietina* in (B). R^2 is the coefficient of determination and P is the statistical significance.

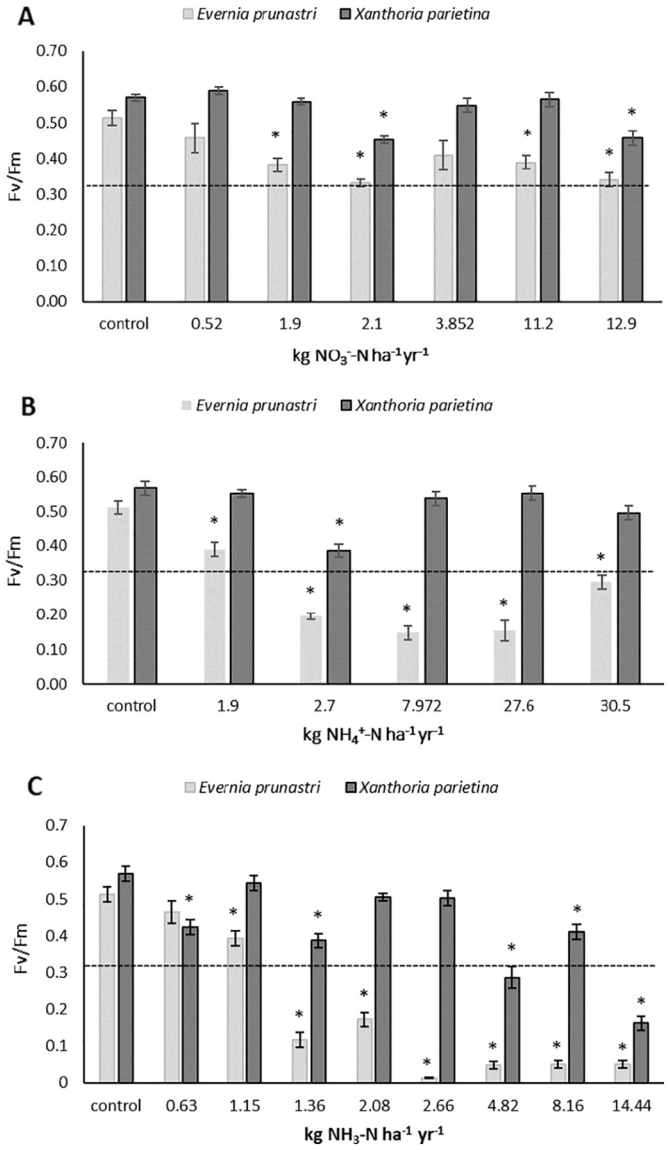

Fig. 2. Average values of Fv/Fm (±SE; n = 20 for each species for wet deposition and n = 10 for each species for dry deposition) in transplanted samples of *X. parietina* and *E. prunastri* after treatments with different doses of NO_3^- (A), NH_4^+ (B) and NH_3 (C) (N kg besides the background value of 8 kg ha^{-1} yr^{-1}). * = significantly different from the control ($P < 0.05$); dashed line represents the estimated lichen viability threshold for Fv/Fm.

Differential physiological responses due to different N forms were observed, with the strongest response observed in case of dry deposition; wet deposition was moderately effective in reducing Fv/Fm in case of NH_4^+, but much less effective in case of NO_3^- (Fig. 2).

3.1. Chlorophyll a fluorescence

The maximum quantum yield, based on the Fv/Fm parameter, was used to evaluate the metabolic and physiological status of the lichen thalli (Munzi et al., 2014). Measurements taken on the control samples, at three times in the experiment (t_0 = 1 week, t_1 = 5 weeks and t_2 = 10 weeks), revealed that 10 weeks after the transplantation the process did not affect *X. parietina* and slightly affected the health status of *E. prunastri*, though without compromising its viability (Supplementary material, Table S3). A decrease in photosynthetic performance was observed by Paoli et al. (2015a) in thalli of the sensitive species *Flavoparmelia caperata* transplanted in a climatic chamber for 5 weeks, whereas *X. parietina* tolerated the same conditions for a longer time.

Chlorophyll *a* fluorescence (Fig. 2A, B; Supplementary material, Table S4) showed only modest effect of wet deposition on *X. parietina*, with occasional decreases in few treatments. The Fv/Fm parameter in *E. prunastri* decreased in transplants already at low doses of ammonium and nitrate, even though samples remained viable in the case of NO_3^-, with Fv/Fm values larger than the estimated critical value of 0.32. In the case of *X. parietina* the minor overall response was similar for impacts of NH_4^+ and NO_3^-, whereas for *E. prunastri*, the adverse effect was much larger from NH_4^+ than from NO_3^-, with all except the lowest treatments (1.9 kg NH_4^+-N ha^{-1}) having Fv/Fm values less than the critical value of 0.32.

Exposure to mean concentrations of gaseous NH_3 equivalent to a deposition of >1.15 kg N ha^{-1} yr^{-1} strongly affected photosystem II of *E. prunastri*, with values of Fv/Fm near to zero at the highest doses (Fig. 2C; Supplementary material, Table S5). *Xanthoria parietina* exhibited decreased fluorescence values only at the highest depositions of ammonia (>4.8 kg N ha^{-1} yr^{-1}) (Fig. 2C; Supplementary material, Table S5).

These findings are in agreement with previous observations about the effects of N compounds on lichens. In several studies, *X. parietina* appeared tolerant to wet deposited NO_3^- and NH_4^+ (Munzi et al., 2009a, 2010; Pirintsos et al., 2009), but more sensitive to NH_3 (Munzi et al., 2014; Paoli et al., 2015a). This is consistent with the reported deleterious effects of dry deposition on peatland vegetation and more specifically for *E. prunastri*, as compared with wet deposition (Munzi et al., 2009a, 2010; Pinho et al., 2012; Sheppard et al., 2011).

3.2. Dry deposition

The results showed an increase in thalli N concentration when NH_3 was provided to both species (Fig. 3). For *X. parietina*, the data were fitted to a logarithmic increase ($y = 0.1415\ln(x) + 1.6549$). For *E. prunastri*, the N content remained stable at the lowest values of NH_3 deposition, increased linearly between 1.15 and 2.66 kg N ha^{-1} yr^{-1} to reach, after that, a limit value which appears to be reflective of a breakdown of this species at high NH_3 concentrations.

In a laboratory experiment, Miller and Brown (1999) found that, when exposed to NH_3, most of the ammonia vapour was recovered in the lichen *Peltigera membranacea* as ammonium ions, both in the intercellular spaces and bound to exchange sites. In the field, several authors have reported that lichen N content reflects spatial variations in the amount of deposited N, particularly ammonia, even when species present clear difference in growth form, morphology and other general functional traits (Branquinho et al., 2010; Gaio-Oliveira et al., 2001; Nielsen et al., 2014).

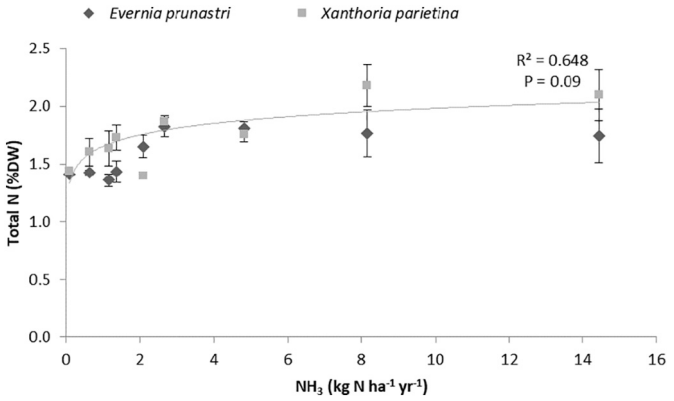

Fig. 3. Total N content (% of dry weight, DW, average ± SE, n = 3) in response to atmospheric NH_3 depositions provided to samples (kg N ha^{-1} yr^{-1} besides the total background value of 8 kg ha^{-1} yr^{-1}) in thalli of *X. parietina* (squares) and *E. prunastri* (diamonds). For *X. parietina*, the data were fitted to a logarithmic increase ($y = 0.1415\ln(x) + 1.6549$). R^2 is the coefficient of determination and P is the statistical probability.

In the cases of *X. parietina* an initial increase of total N concentration is seen in Fig. 3 followed by attenuation to a steady state value suggesting a saturation pattern once NH_3 depositions exceed 4 kg N ha^{-1} yr^{-1}. In *E. prunastri* there was no correlation between NH_3 and N lichen content above 2.66 kg N ha^{-1} yr^{-1} (Fig. 3), which is consistent with a breakdown of the lichen functioning at high N availability.

This observation is in agreement with findings by Olsen et al. (2010) who found a maximum tissue N concentration within one month of exposure in *X. parietina* transplanted into a heavily NH_3 polluted area. Their maximum thallus N concentration was 2.3%, which was similar to the values found in our study (2.5% in *X. parietina* and 2.2% in *E. prunastri*).

The different response of the two species can be explained looking at their viability (Fig. 2). Already at a deposition of 1.2 kg N ha^{-1} yr^{-1} of NH_3, *E. prunastri* showed an impairment of its vitality. That, together with other possible damages attributable to N (Munzi et al., 2009b), likely prevented a correct metabolic functioning and a clear response to the treatment during the experiment. The increase in thallus N concentration for an NH_3 deposition of >1.2 kg N ha^{-1} yr^{-1} in *E. prunastri* is not surprising since previous observations suggested that the uptake mechanism is passive and physical rather than metabolic and showed that NH_3 uptake occurred not only in living but also to some extent in dead lichen material (Miller and Brown, 1999).

A decrease of Fv/Fm below the vitality threshold was also observed in *X. parietina* around 4.8 kg N ha^{-1} yr^{-1} of NH_3 (Fig. 2C). The consequent decreased functionality of the thalli can thus explain the saturation pattern.

This difference between tolerant and sensitive species was found also by Boltersdorf and Werner (2013) in a field survey including various species of epiphytic lichens, where only data obtained from nitrophytic species were correlated with data obtained from N monitoring networks based on physicochemical measurements.

Results displayed in Fig. 1 provide further support for our hypothesis that N isotopic signature observed in lichens tends to resemble the value of the N source (6.2‰ for added NH_3), since increasing the concentration of available NH_3 (Fig. 1A) and increasing lichen N concentration (Fig. 1B), lichen $\delta^{15}N$ is driven toward more positive values.

Dashed lines in Fig. 1A show the initial linear stage of uptake before saturation pattern or total impairment were reached in *X. parietina* and *E. prunastri*. The slopes of the lines indicate a 3-fold greater increase of $\delta^{15}N$ due to increasing NH_3 supply in *E. prunastri* than in *X. parietina*. This value is compatible with the higher cation exchange capacity of non-nitrophilous than of nitrophilous species (Branquinho, 2001). When Gaio-Oliveira et al. (2001) compared cation exchange capacity in *E. prunastri* and *X. parietina*, they found a 5-fold higher capacity for the former than for the latter. In a humid environment such as Whim Bog, it is expected that NH_3 will react to form NH_4^+ which binds to exchange sites. Support for the occurrence of such chemical reactions comes from the observation that surface pH of thalli of the lichen *C. portentosa* exposed to NH_3 in the Whim Bog was increased to 136% of the control value, changing from 4.41 to 6.02 (Munzi et al., *in preparation*). Such a drastic change in pH, which influences the cellular environment and functioning, can contribute to explain the high toxicity to lichens of NH_3 dry deposition.

While carbon fractionation has been considered in previous papers (e.g. Máguas et al., 1995), N fractionation in lichens has been almost completely neglected. Although several metabolic processes.

could potentially alter $\delta^{15}N$ of lichens (Beck and Mayr, 2012), our findings showed that fractionation, if any, did not cause a relevant divergence between lichen and source $\delta^{15}N$. A possible exception to this is the $\delta^{15}N$ values of +10 and + 9 for *E. prunastri* at high NH_3 deposition levels above the impairment threshold of 1.2 kg ha^{-1} yr^{-1}, which exceeded the source value. It is reasonable to think that *E. prunastri* had already lost the capacity to maintain cellular integrity and physiological processes regulated at these levels, including uptake processes, which might lead to a fractionating effect with a preferential uptake of the heaviest isotope. However, further studies are needed to clarify this aspect and a new experiment has been designed at purpose.

Finally, Fig. 4 shows that the maximum potential quantum efficiency of photosystem II of lichens (Fv/Fm) is significantly correlated with $\delta^{15}N$. Again, this can be explained since $\delta^{15}N$ depends on the amount of N taken up from the N deposition source that can be toxic at high concentrations. In agreement with previous results (Munzi et al., 2014), the Fv/Fm parameter decreased in both *E. prunastri* and *X. parietina*. However, due to a different N tolerance between these two species, *E. prunastri*, showed lower fluorescence values than *X. parietina*.

Taken together these results suggest an oligotrophic species responded more efficiently than a nitrophytic species to NH_3 treatment in the short term, but when subjected to increased N availability for a prolonged time, they lose their functionality and, consequently, the ability to respond to changing environmental conditions. By contrast, the nitrophytic species which can tolerate high N supplies, can act as a reliable indicator of NH_3 deposition even in areas with high N pollution. This is true not only for transplantation experiments, but also when in situ lichens are used. For example, a study conducted near a cattle barn in Portugal showed *E. prunastri* disappearing and *X. parietina* increasing in frequency along a gradient of NH_3 concentrations (Munzi et al., 2014; Pinho et al., 2011, 2012).

3.3. Wet deposition

Significant correlations were found between lichen total amount of NH_4^+ provided in wet deposition and both total N content of lichen thalli and lichen $\delta^{15}N$ signal (Fig. 5). Conversely, no significant correlations were found between N content and $\delta^{15}N$ in thalli of both species when treated with NH_4^+ (Supplementary material, Fig. S2). For the same parameters in case of NO_3^-, the only significant correlation was found between $\delta^{15}N$ and NO_3^- provided in thalli of *E. prunastri* (Supplementary material, Fig. S3).

Lichens can take up several N forms from wet deposition like NH_4^+, NO_3^-, and organic N (Hauck, 2010). Although in nature lichens take up NH_4^+ and NO_3^- from rainfall with similar efficiency (Crittenden, 1998), when exposed to solutions of high N concentration, it has been found that lichens take up NH_4^+ preferentially to NO_3^- (Dahlman et al., 2004).

This finding is confirmed by our experiment, where measurements of total N content in thalli of both species showed a slight but significant increase when thalli were treated with NH_4^+ (Fig. 5A) and indicated no significant uptake of the NO_3^- supplied (Supplementary material, Fig. S3A). Accordingly, the isotopic signature of samples treated with NH_4^+ suggests the uptake of N which was then found in lichen tissue (Fig. 5B), while in case of NO_3^- only *E. prunastri* at the highest concentration showed a change in the isotopic signature (Supplementary material, Fig. S3B).

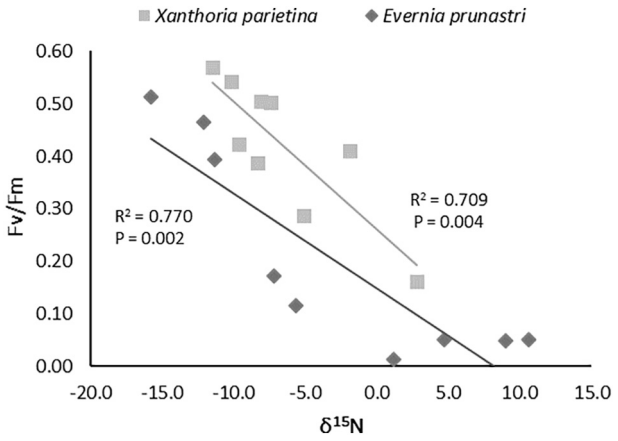

Fig. 4. Relation between lichen $\delta^{15}N$ and Fv/Fm in thalli of *X. parietina* and *E. prunastri* treated with NH_3. R^2 is the coefficient of determination and P is the statistical significance.

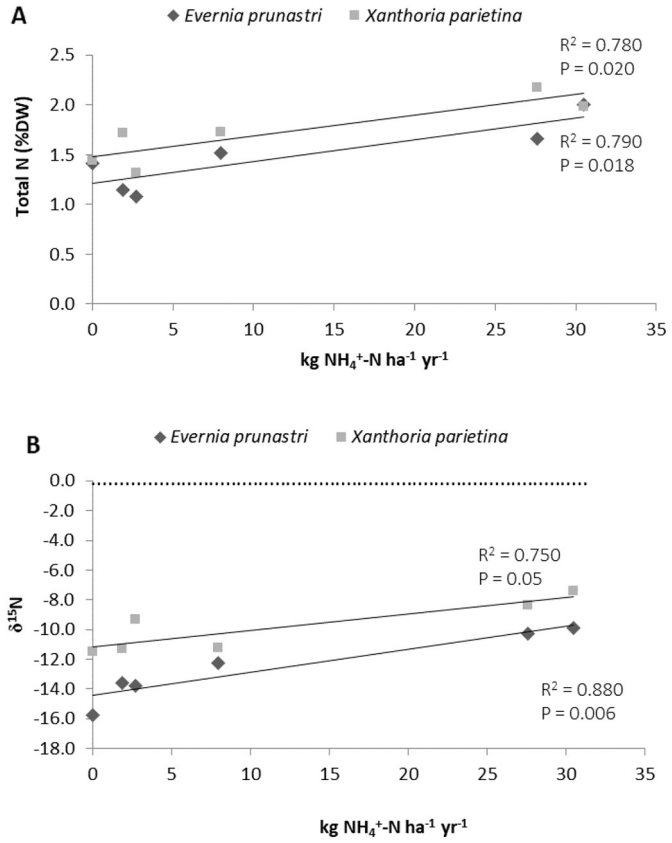

Fig. 5. Linear regression between N content and doses of NH_4^+ provided (besides the total background value of 8 kg ha^{-1} yr^{-1}) (A) and between $\delta^{15}N$ and doses of NH_4^+ provided (B) in thalli of *X. parietina* and *E. prunastri*. The dotted line in part (B) represents the value of $\delta^{15}N$ in the released NH_4^+ of the wet treatment. R^2 is the coefficient of determination and P is the statistical significance.

A different uptake rate can also explain the physiological results (Fig. 2). In fact, the less efficient uptake of NO_3^- and the consequent lower amount of N in the cells in comparison with NH_4^+ can justify the less harmful effects observed in the experiment indicated by the Fv/Fm ratio. In this way, it appears that one reason for the lower impact of NO_3^- than NH_4^+ is the lower rate of uptake of NO_3^-. This is explored in Supplementary material, Fig. S4 which shows the extent of reduction in Fv/Fm (by comparison to the control) normalized by the degree to which the $\delta^{15}N$ content approaches the $\delta^{15}N$ value of the deposited N, and Fig. S5 which shows the same per unit of tissue N.

4. Conclusions

Our findings confirmed that lichen $\delta^{15}N$ varies accordingly to the isotopic signature of the N source (6.2‰ for NH_3, −0.1‰ for NH_4Cl and 3.8‰ for $NaNO_3$). By contrast, the results do not point to any significant fractionation in N deposition to lichens. An exception to this may be two values of $\delta^{15}N$ at 10 and 9‰ for high NH_3 treatments in *E. prunastri*, potentially linked to thallus breakdown, though in the absence of other data this requires further investigation.

We found that the nitrophytic *X. parietina* was able to survive in the tested conditions and maintain its functionality at higher NH_3 concentrations than *E. prunastri*. From a practical point of view, this means that $\delta^{15}N$ of lichen tissue reflects the $\delta^{15}N$ of atmospheric ammonia, providing a suitable tool for the interpretation of the spatial distribution of NH_3 sources even after few weeks of exposure. The use of tolerant species is preferred to sensitive ones to obtain a reliable indication in areas with high N pollution.

In conditions resembling natural ones, wet deposition of NO_3^- did not affect lichen N concentrations through the duration of the experiment and caused less impact on chlorophyll *a* than NH_4^+ as indicated by the Fv/Fm ratio. Dry deposition caused a much greater impact, which is consistent with different and more efficient uptake mechanisms. As this study focused on exposure over a 10-week period, further experiments are needed to clarify the response to NH_4^+ and NO_3^- over the long term.

These results clarify the response of sensitive components of the environment to N pollutants in the field, where multiple N sources often make the interpretation of biomonitoring surveys difficult.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.11.010.

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