Is lichen $\delta^{15}N$ an indicator of nitrogen pollution and a surrogate of nitrogen atmospheric composition? Evidence from manipulative experiments

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Abstract
Due to the relevance of nitrogen (N) as a pollutant, setting up an effective method to determine spatial distribution of N sources would help to develop management and mitigation strategies. Although promising, the use of isotopic signature of lichens to map atmospheric N deposition is still difficult due to the synergism between climatic and anthropogenic factors and the superimposition of multiple N sources. To understand how lichen's isotopic signature is affected by N, thalli of the sensitive *Evernia prunastri* and of the tolerant *Xanthoria parietina* were exposed for ten weeks to different forms and doses of N in a manipulative experiment, and physiological parameters, total N, $\delta^{15}N$ and chlorophyll *a* fluorescence were measured. In parallel, thalli of *Cladonia portentosa* exposed to the same treatments for 11 years or 6 months were analyzed to investigate the role of time of exposure. Our results showed that lichen N content and $\delta^{15}N$ were correlated with the N dose of the N treatments and that lichen $\delta^{15}N$ tended to become similar to the source's signature. However, nitrophytic and acidophytic species showed different $\delta^{15}N$ in response to the same treatments, probably due to their cation exchange capacity. Finally, the correlation between N content and $\delta^{15}N$ was higher in case of long-term exposure. Nitrogen isotopic signature in lichens can potentially be used as indicator to determine spatial distribution of N sources in the field. However, further investigation is needed to confirm these results in the field, and species functional traits must be taken in particular consideration.

Key Words
biomonitoring, lichen functional diversity, reactive nitrogen, isotopic signature, agricultural practices, Whim bog experimental site

Introduction

Human activities, mainly fossil fuels combustion and industrialized forms of agriculture, have reached levels detrimental to the environment around the world. Eventually, all the reactive N introduced into the ecosystem ends up in the environment, slowly eroding the resilience of important Earth subsystems (Steffen et al. 2015). Therefore, monitoring N deposition and effects on the ecosystem is pivotal for the establishment of suitable management and mitigation strategies.
Lichens, symbiotic associations between algae (green algae or cyanobacteria) and fungi, are well-known biomonitors responding to increased N availability (Munzi et al. 2014a; Pinho et al. 2008), since they depend on the atmospheric compartment (wet and dry atmospheric depositions) for their nutrient requirements. Several approaches and methods have been established in the last decades to allow the use of lichens as biomonitoring tools. The most traditional one is the community composition response to environmental changes, with a shift from acidophytic to nitrophytic species increasing N availability. Functional diversity showed to be an effective tool also to disentangle different drivers of lichen ecological response (Munzi et al. 2014b). More recently, a physiological approach has been developed that provides further parameters (chlorophyll *a* fluorescence, membrane integrity, etc.) sensitive to N stress (Munzi et al. 2010, 2012).
It is well known that epiphytic lichens reflect N deposition, especially from agriculture-derived N-containing compounds (e.g. Ruoss 1999; Frati et al. 2007). The lack of roots, cuticle and stomata in lichens also implies that thallus N content and isotopic signature reflect directly land use (N sources) and atmospheric transport of N pollutants in the surroundings.
However, few studies dealt with interrelations between N concentrations and $\delta^{15}N$ in lichens and different N depositions so far (e.g. Skinner et al. 2006; Boltersdorf and Werner 2013). Although promising, the use of isotopic signature of lichens to map atmospheric N deposition is still difficult due to the synergism between climatic and anthropogenic factors and the superimposition of multiple N sources (Boltersdorf and Werner 2013).
To understand how lichen's isotopic signature is affected by N, thalli of the sensitive *Evernia prunastri* and of the tolerant *Xanthoria parietina* were exposed for ten weeks to different forms (dry-NH_3, wet-NH_4^+, wet-NO_3^-)

and doses (16; 32 and 64 kg N/ha/yr) of N in a manipulative experiment, and physiological parameters (Fv/Fm), total N and $\delta^{15}N$ were measured. In parallel, thalli of *Cladonia portentosa* (moderately sensitive) exposed to the same treatments for 11 years or 6 months were analyzed to investigate the role of time of exposure. Setting up an effective method to determine spatial distribution of N sources would help to develop management and mitigation strategies.

Methods

Lichen material

Thalli of *C. portentosa* were collected from the soil immediately outside the manipulated area and placed in the treated plots, two thalli per plot, receiving both oxidized and reduced N forms. Where available, *in situ* thalli were also collected for comparison between short- (6 months) and long-term (11 years) N treatments.
Thalli of *X. parietina* and *E. prunastri* 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).
Branches of *Sambucus nigra* and of *Quercus robur*, carrying respectively *X. parietina* and *E. prunastri* were transplanted in the different plots. All the transplanted branches supported by plastic sticks were inserted facing the N source at the same height in the open. Transplants were collected after 1, 5 and 10 weeks.

Nitrogen treatments

At Whim bog experimental site (see Sheppard et al. 2004, 2011 for details), reactive N treatments (wet) have been applied since June 2002. Four control plots receive only natural rainfall with a background total inorganic N deposition of 8 kg/ha/yr. The other experimental plots receive additional N applied as a spray at rates of 8, 24 and 56 kg N/ha/yr, each treatment is replicated in 4 plots. An NH_3 gradient is established by NH_3 release from a 10 m pipe at 1 m height when wind direction is 180–215° and speed is 2.5 m/s. NH_3 concentrations were measured at the transplant locations, located 12, 30 and 60 m from the NH_3 source, using passive ALPHA samplers 0.1 m above the vegetation (Tang et al. 2001). Isotopic signature of chemicals used for the treatments were -0.1‰ for NH_4Cl, 3.8‰ for $NaNO_3$ and 6.2‰ for NH_3.

N isotopes

$^{15}N/^{14}N$ ratio in the samples were 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 analyzer for online sample preparation by Dumas-combustion. The standards used were Sorghum Flour Standard OAS (Elemental Microanalysis, UK) for N isotope ratio; $\delta^{15}N$ results were referred to Air. Precision of the isotope ratio analysis was ≤ 0.2‰.

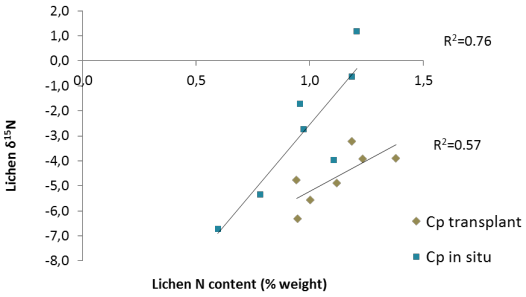
Chlorophyll a *fluorescence*

Measurements of the Fv/Fm ratio of the transplanted lichens were taken as a stress indicator (Munzi et al. 2014a). Samples were 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).

Results

Figure 1 shows the relationship between lichen N content and $\delta^{15}N$ in samples of *C. portentosa* treated for 6 months or 11 years (wet deposition, including both NH_4Cl and $NaNO_3$). Long-term treated samples showed a better correlation than short-term ones. The value of $\delta^{15}N$ was more negative in control samples (with the lowest N content) and tended to become more positive increasing the amount of N taken up, as expected considering the $\delta^{15}N$ of the chemicals.
This suggests that this species tends to reach the $\delta^{15}N$ of the N source along time.
Figures 2 shows the correlation between $\delta^{15}N$ and N content in *X. parietina* and *E. prunastri* treated with NO_3^- (a) and NH_4^+ (b) (wet deposition).
While both species didn't respond to nitrate treatment, in the case of ammonium N, $\delta^{15}N$ of *E. prunastri* tended to get closer to the value of the N provided giving a better response than *X. parietina*.
This suggests that N form is important and that in case of multiple N sources ammonium can have a larger impact on isotopic signature of lichens than nitrate. It is also clear that the same environmental conditions (N availability) influence differently the isotopic composition of species belonging to different functional groups (acidophytic or nitrophytic). Similarly, the results of dry treatment (Figure 3) show the best correlation between N content and $\delta^{15}N$ in *X. parietina* when compared with *C. portentosa* and *E. prunastri*.
This is not surprising since gaseous NH_3 is more toxic to lichen than wet deposition and, on the other hand, ammonium is more toxic than nitrate.

Our results can be explained if we look at the vitality index of the two species (acidophytic and nitrophytic) along the experiment with NH_3 (Figure 4a): the Fv/Fm parameter showed a decrease in *E. prunastri*



$\delta^{15}N$ of NH_4Cl = -0.1‰
$\delta^{15}N$ of $NaNO_3$ = 3.8‰
$\delta^{15}N$ of NH_3 = 6.2‰

Figure 1. Correlation between lichen N content and $\delta^{15}N$ in samples of *C. portentosa* treated for 6 months (Cp transplant) or 11 years (Cp in situ) with wet deposition (including both NH_4Cl and $NaNO_3$).

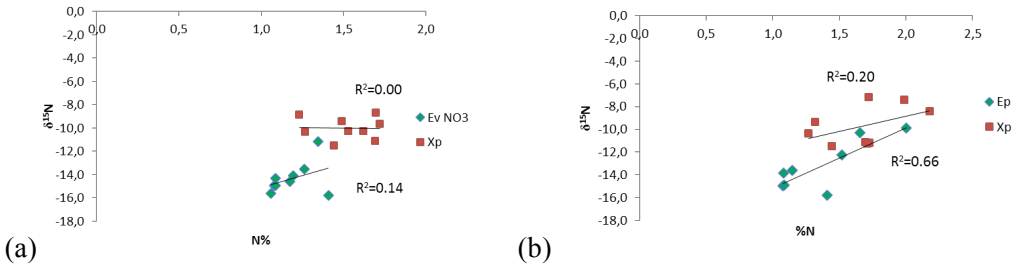


Figure 2. Correlation between $\delta^{15}N$ and N content in *X. parietina* (Xp) and *E. prunastri* (Ep) treated with $NaNO_3$ (a) and NH_4Cl (b).

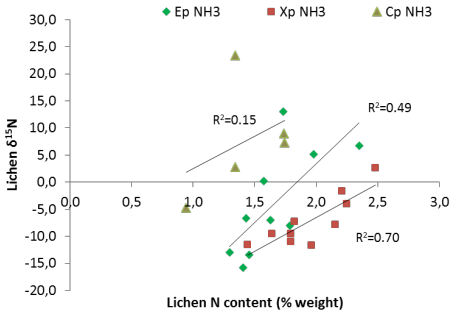


Figure 3. Correlation between $\delta^{15}N$ and N content in *X. parietina* (Xp), *E. prunastri* (Ep) and *C. portentosa* (Cp) treated with NH_3 (dry treatment).

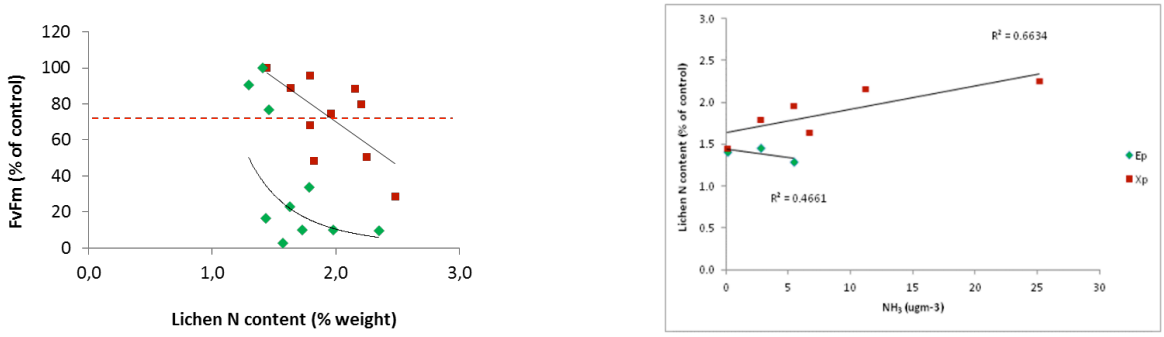


Figure 4. (a) Fv/Fm parameter used as a vitality index in *E. prunastri* and *X. parietina* in relation to thalli's N content. Above the red line, samples considered viable; below the red line, samples considered not viable. (b) Correlation between lichen N content and amount of treatment (NH_3) provided in viable thalli of *X. parietina* (Xp), *E. prunastri* (Ep).

increasing the amount of N in sample thalli, likely due to an impairment of physiological functioning in this species when subjected to N stress. Consequently, if we consider only viable samples (Figure 4b), it is evident

that in sensitive species above a certain threshold there is no correlation between the environmental N and the N amount in the thalli.

Conclusion

Nitrogen isotopic signature in lichens can potentially be used as indicator to determine spatial distribution of N sources in the field. In particular, *X. parietina* reflected isotopic signature of the dry treatment, while *E. prunastri* was a better indicator in case of ammonium treatment. *Cladonia portentosa* showed that this method can be suitable for long-term monitoring. However, further investigation is needed to confirm these results in the field, where multiple sources and long range transportation make the interpretation of $\delta^{15}N$ difficult, and species functional traits must be taken in particular consideration.

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