More tolerant than expected: Taking into account the ability of Cladonia portentosa to cope with increased nitrogen availability in environmental policy

More tolerant than expected: Taking into account the ability of *Cladonia portentosa* to cope with increased nitrogen availability in environmental policy

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ABSTRAC T

The lichen *Cladonia portentosa* is generally considered to be sensitive to increased environmental nitrogen (N) deposition. However, the presence of this lichen in impacted environments suggests that it can cope with prolonged exposure to high N availability. To test the tolerance of this species to N, photosynthetic parameters, carbon and N concentrations and isotopic signature, chitin concentration, surface pH and extracellular enzy-matic activity were measured in samples exposed for 11 years to different N doses and forms at the Whim bog N manipulation experimental site (United Kingdom). The results showed that *C. portentosa* is tolerant to long-term exposure to wet N deposition, maintaining its functionality with almost unaltered physiological parameters. The comparison of the proteome of short- and long-term exposed samples showed similar changes in protein expression suggesting that mechanisms to cope with N are not dependent on the exposure time even after more than a decade. Since empirical N Critical Loads are based on the response of sensitive components of the eco-system, like *C. portentosa*, its capacity to cope with short- and long-term exposure to N needs to be recognized and taken into account when setting them, likewise, the significance of the form of N.

Capsule: Updated knowledge on tolerance of sensitive species to nitrogen must be taken into account to establish environmental policy.

1. Introduction

Lichen functional groups, defined as groups of species with a similar response to specific environmental factors (Lavorel et al., 2002), are widely used as a monitoring tool (e.g. Benítez et al., 2018; Paoli et al., 2015). The attribution of a species to a certain functional group usually reflects an expert assessment based on data available in the literature (for ex. LIAS, 2020; Nimis and Martellos, 2017; United States Forest Service, 2020). This knowledge of how different functional groups re spond to particular pollutants can provide support to environmental policy, as shown by Pinho et al. (2009) in the assessment of Critical Levels of atmospheric ammonia. It is therefore of pivotal importance to keep knowledge about species used in environmental surveys as de tailed and updated as possible.

Critical Loads for nitrogen (N) deposition are established through empirical observation of the effects of known or estimated deposition on sensitive components of a certain ecosystem (https://www.icpmapping.org/Definitions and abbreviations). For example, lichen communities, particularly rich in species of the genus *Cladonia*, have been used to establish the current critical loads for raised and blanket bogs, that are set to a range between 5 and 10 kg N ha^{-1} yr^{-1} (APIS, 2019). Knowledge of lichen physiological response and a comprehen sive understanding of the effects of long term exposure to N is therefore required for the science based establishment of environmental policies.

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Parameters such as dose, chemical form and time of exposure were found to be crucial in determining type and extent of biological effects of N on lichens and some studies showed a different response of the symbiotic partners. For example, in the sensitive species *Evernia pru nastri*, the mycobiont responded to very high N concentrations supplied for a short time (Munzi et al., 2009), while the photobiont responded to lower concentrations (Munzi et al., 2010, 2012). When Munzi et al. (2013a,b) exposed *E. prunastri* and the tolerant *Xanthoria parietina* for 4 weeks to a range of N concentrations simulating conditions occurring in different areas worldwide, they found that only the photobiont was affected.

Since livestock, agricultural practices like the use of fertilizers, and fossil fuel combustion (Bobbink et al., 2010; Sutton et al., 2003) are the main sources of reactive N, episodes of chronic N stress are more common than acute stress and lichens can be exposed to atmospheric N for years. Sheppard et al. (2009) highlighted the importance of the length of the exposure since they found that NH_3 threshold for damage in an ombrotrophic bog depends on the duration of the treatment and decreases significantly over periods longer than 1 year.

Although the importance of understanding the effects of prolonged exposures to N on the ecosystem and the extensive literature existing on the N lichen relationship, most of these studies merely scratch the surface by describing symptoms of N excess. A deeper insight has been provided in a recent work investigating changes in the proteome of *C. portentosa* exposed to oxidized and reduced forms of N for 6 months (Munzi et al., 2017b). Results showed that different N forms affected different metabolic pathways in the lichen with the most relevant changes in protein expression observed in the fungal partner while in the photobiont only energy production was sensitive.

In this work, we investigated the changes occurring in the lichen *Cladonia portentosa* after a long term (11 years) exposure to various forms and doses of N: focusing on the different response of the two partners. Although the species is generally considered sensitive to pollution and N (Nimis, 2016), the presence of this terricolous lichen in N polluted environments (Gheza, 2015, 2018) indicates its ability to develop tolerance mechanisms to cope with N deposition exceeding the natural background. Based on this we hypothesized that *C. portentosa* is able to maintain its physiological parameters unaltered, ensuring its functionality, even after long term exposure to increased N availability. To test the hypothesis, we collected samples of *C. portentosa* in the Whim bog experimental site, where long term N manipulation experi ments have been running for over a decade. Parameters of both sym bionts, namely photosynthetic parameters and ultrastructure of the photobiont, surface pH, chitin content, N and carbon (C) concentra tions, N and C stable isotope signatures and extracellular enzymatic activity in the mycobiont, were measured in control samples and samples exposed for 11 years to wet N deposition. Finally, the proteome of thalli exposed both for 6 months and 11 years were compared to check for differences due to long term vs. short term exposure.

The results contribute to the knowledge required in order to use lichens as indicators for environmental protection.

2. Materials and methods

2.1. Nitrogen treatments

Whim bog (282 m a.s.L, 3° 16′ W, 55° 46′ N, Grid Ref: NT203532) represents a transition between lowland raised bog and blanket bog, on 3 6 m of deep peat. Atmospheric deposition (wet + dry) consists of 4 kg S ha^{-1} yr^{-1} and ca. 8 kg N ha^{-1} yr^{-1}, with air concentrations (at 1.5 m) of 0.7 lg NH_3 m^{-3}. The peat is very acid, with pH ca. 3.4 (3.27 3.91 in water) (Sheppard et al., 2011).

At Whim, N treatments have been applied since June 2002 to cir cular plots, each of which are 12.5 m^2 and separated by a distance of 3 m. Four control plots receive only natural rainfall with a background total inorganic N deposition of 8 kg ha^{-1} yr^{-1}. The other experimental plots receive additional N applied as a spray at rates of 8, 24 and 56 kg N ha^{-1} yr^{-1}, each treatment replicated in 4 plots. Including ambient N deposition, treatment plots therefore receive a total of 16, 32 or 64 kg N ha^{-1} yr^{-1} in either oxidized ($NaNO_3$) or reduced (NH_4Cl) forms (Sheppard et al., 2004). Treatments are provided in rainfall col lected at the site and are only applied during rainfall and when air temperature exceeds 0 °C and wind speed is < 5 m s^{-1} over a 5 min averaged period.

Nitrogen isotopic signature of chemicals used for the treatments were -0.1‰ for NH_4Cl and 3.8‰ for $NaNO_3$.

2.2. Lichen sampling

Thalli of *C. portentosa* were collected in May 2013 in control and treated plots receiving both oxidized and reduced N forms. Samples transplanted for 6 months in the treatment plots were also collected. Lichens were transported to the laboratory, carefully cleaned of im purities, and parts of other bog species such as *Calluna* and *Erica*. Collected thalli were photographed. Since some treatments caused an algal proliferation on the lichen such material was excluded from the samples. To avoid protein degradation, material for proteomic analysis was cleaned, rinsed in liquid nitrogen and immediately stored at -80 °C.

Before performing any measurements, samples were completely rehydrated. Lichens were stored at room temperature and analyzed in the first week after collection.

In some plots, because the lichen was originally absent or had been denuded by earlier sampling, it was not possible to collect enough material for the analyses. These cases are indicated as NA (not avail able) in the results.

A set of parameters referring either to the photobiont or the my cobiont was measured to compare the physiological status of control samples, short exposed and long exposed samples.

2.3. Chlorophyll a fluorescence

Measurements of the Fv/Fm ratio and the Performance Index (PI) were taken after each N treatment as a stress indicator (Strasser et al., 2000, 2004). Lichen samples were gently blotted in blotting paper and then dark adapted for 15 min before fluorescence measurements were taken, to maximize oxidation of the primary quinone electron acceptor of PSII. The Fv/Fm ratio and PI were measured at room temperature, with the Plant Efficiency Analyzer Handy PEA (Hansatech Instruments LTD, UK). For each treatment, 6 replicates were used.

2.4. Rate of photosynthesis

Uptake of CO_2 was used as a measure of rate of photosynthesis. Measurements were taken by infrared gas analyzer Licor 6400 (LI COR, Lincoln, NE, USA) on completely hydrated samples illuminated at 600 mmol m^{-2} s^{-1} at room temperature. Values were recorded when stable.

Depending on availability, 3 6 replicates were used for each treat ment.

2.5. Chlorophyll content

Spectral reflectance indices can be a non destructive tool to assess photosynthetic pigment levels and their ratios in leaves (Li et al., 2010). In our samples, total chlorophyll was determined using the chlorophyll content index (ratio between reflectance at a wavelength of 750 and at 700 nm) measured with a portable reflectometer UniSpec SC Spectral Analysis System (PP Systems). For each treatment, 15 replicates were used.

2.6. Photobiont ultrastructure

In the present study, we preferentially analyzed the algal compo nent of lichens as some microscopic parameters of algae could be more easily analyzed and compared. The protocol is detailed in Behr et al.(2019). For transmission electron microscopy, samples were fixed in 3% glutaraldehyde in cacodilate buffer 0.066 M pH 7.2, for 1 h at room temperature. After fixation, samples were rinsed with cacodilate buffer and post fixated with osmium tetroxide 1% in cacodilate buffer for 1 h. Then, samples were rinsed with water and dehydrated gradually in increasing concentrations of ethanol (from 10% to 100%). Samples were embedded in Spurr's resin (Spurr, 1969), polymerized for 8 h at 70 °C, and then cut into 600 Å sections using an LKB Nova ultra microtome provided with diamond knife. Sections were stained with uranyl acetate and lead citrate for 10 min, respectively, and finally observed with a Philips Morgagni 268D transmission electron micro scope operating at 80KV and equipped with a MegaView II CCd camera (Philips electronics).

2.7. pH measurements

Although pH measurements refer to upper cortex composed only by fungal cells, hyphae completely enclosing the algal cells thus re presenting their living environment. In this sense, lichen pH is the en vironmental pH for the algae, contributing to regulate their exchanges with the outside.

Thallus pH was measured by moistening the lichen surface with distilled water and using a portable contact pH electrode (Crison PH25 DL). Values were recorded when stable. For each treatment, 10 re plicates were measured.

2.8. Chitin quantification

Chitin was quantified following Munzi et al. (2017a). Prior to ex traction, samples of 10 25 mg pulverized, freeze dried lichens were suspended in 1 ml 0.2 M NaOH then incubated on a rotary agitator at room temperature, for 6 h; after centrifugation at maximum speed for 15 min, another 1 ml 0.2 M NaOH was added to the pellet before in cubation overnight at 100 °C to remove amino acids and proteins. After centrifugation (max. speed, 15 min.), the pellet was suspended in 1 ml 6 M HCl and incubated at 100 °C for 5 h to hydrolyse chitin; after cooling to room temperature and centrifuging (max. speed, 15 min.), the supernatant, containing any glucosamine, was collected in a new Eppendorf vial, then evaporated under a vacuum. 200 µl water (Milli Q), 250 µl FMOC Cl (15 mM in acetone) derivatisation reagent and 50 µl borate buffer (1 M, pH 6.3) were added to the extract. After mild agitation and incubation for 10 min, excess FMOC Cl was removed by two phase partitioning with 1 ml heptane, repeated twice, and samples were injected into the HPLC within 10 min.

HPLC UV analysis was performed with a Shimadzu (Japan) LC 6A pump and a Shimadzu (Japan) SPD 6AV UV Vis detector; data were recorded and analyzed using generic signal recorder software (Azur, Datalys, France); separation was achieved with a Merck LiChroCART 250 mm 4.6 mm Puroshper STAR reversed phase 18e (5 µm) column kept at 25 °C. The mobile phase was 100% methanol (SIGMA) at a flow rate of 1.4 ml min^{-1}, and detection was performed at the wavelength of maximum absorbance (242 nm), determined by spectrophotometric essays on standard solutions or glucosamine (SIGMA). The injection volume was 20 µl. Samples were analyzed in triplicate.

Chitin was quantified through external calibration, using standard solutions of Glucosamine (SIGMA) in water, which was derivatised as the sample (6 levels, encompassing sample chromatographic response range, $R^2 = 0.9935$). The mean relative standard deviation of the quantification was 7.68%.

2.9. Stable isotopes and total C and 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. $\delta^{13}C$ and $\delta^{15}N$ 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 spec trometer, coupled to a EuroEA (EuroVector, Italy) elemental analyser for online sample preparation by Dumas combustion. Delta Calculation was performed according to $\delta = [(Rsample - Rstandard)/Rstan dard] * 1000$, where R is the ratio between the heavier isotope and the lighter one. $\delta^{15}N$Air values are referred to air and $\delta^{13}C$VPDB values are referred to PDB (Pee Dee Belemnite). The reference materials used were USGS 25, USGS 35, BCR 657 and IAEA CH7 (Coleman and Meier Augenstein, 2014); the laboratory standard used was Wheat Flour Standard OAS/ Isotope (Elemental Microanalysis, UK). Uncertainty of the isotope ratio analysis, calculated using values from 6 to 9 replicates of laboratory standard interspersed among samples in every batch analysis, was $\leq 0.1‰$. The major mass signals of N and C were used to calculate total N and C abundances, using Wheat Flour Standard OAS (Elemental Microanalysis, UK, with 1.47%N, 39.53%C) as elemental composition reference materials.

Isotopic signature of chemicals used for the treatments were $-0.1‰$ for NH_4Cl and 3.8‰ for $NaNO_3$. One bulk sample per treat ment was analyzed.

2.10. Enzymatic activity

Activity of 7 extracellular enzymes, namely xylosidase, glucur onidase, cellobiohydrolase, N acetyl glucosaminidase, β glucosidase, phosphatase and leucine aminopeptidase, was measured following the protocol described by Pritsch et al. (2011). Fragment of lichens were placed into 96 well filter plates where they stayed for the whole assay procedure. Fluorogenic substrates were freshly prepared as 5 mM stock solutions in 2 methoxyethanol and then further diluted to obtain de scribed incubation concentrations. Samples were incubated with 100 µl incubation buffer and fluorogenic substrates on a microplate shaker at room temperature for 70 min for Leucine AMC, 15 min for N Acetyl glucosaminidase and β Glucosidase and 30 min for the others. The concentration of the product of each reaction was subsequently mea sured at 364 nm excitation and 450 nm emission in a fluorescence microplate reader (BIOTEK FLx800, BioTek Instruments, Winooski, USA). The values obtained were calibrated against a standard curve of 100 µl calibration solutions (0, 1, 2, 3, 4, or 5 µM) and related to the total dry weight taken by drying the lichen samples at 60 °C after the procedure. For each treatment, 3 replicates were measured.

2.11. Proteomic analyses

Lichen proteins were extracted and identified according to Munzi et al. (2017b). Due to the scarcity of material, the comparison between short and long term treatments was possible only for the intermediate concentration of the two N forms. Briefly, a so called Master (virtual) gels were created as described in Crossett et al. (2008), which re presents all spots detected in all samples. Master gel from 2DE analysis of samples exposed to 32 NO_3^- and 32 NH_4^+ for 11 years was com pared with the Master gel of samples treated with 32 NO_3^- and 32 NH_4^+ for 6 months, in order to detect differences in spot presence and intensity. Spot analysis was performed using a PDQuest software (Bio Rad). Only spots exhibiting at least 2 fold intensity differences were analyzed in order to remove random selection errors.

2.12. Statistics

Significance of differences ($P < 0.05$) between treatments and

Fig. 1. Thalli of *C. portentosa* collected in control plots (A-B), 16 NO_3^- plots (C-D), 16 NH_4^+ plots (E-F), 32 NO_3^- plots (G-H), 32 NH_4^+ plots (I-J), 64 NO_3^- plots (K-L), and 64 NH_4^+ plots (M N).

controls was checked by one way analysis of variance (ANOVA), using the Dunnett test for post hoc comparisons. Prior to analysis, data not matching a normal distribution (Shapiro Wilk W test at the 95% con fidence interval) were log transformed to correct for skewed distribu tions (Zar, 1996).

Pearson correlation coefficient calculations were performed using the SPSS (IBMSPSS Statistics Version 26.0). Significance was taken at $P < 0.05$.

3. Results

In general, thalli didn't show necrosis or other morphological da mage. However, some of the treatments caused the proliferation of free algae on the surface of *C. portentosa* (Fig. 1).

3.1. Physiological response

Table 1 synthesizes the results of physiological analyses carried out on samples of *C. portentosa* collected in control and treatment plots (11 year exposure).

Considering the parameters related to the algal partner, all the samples were viable, and no impairment was caused by the long term supply of N. The rate of photosynthesis showed a significant decrease in thalli treated with NO_3^-, while tended to increase slightly when treated with NH_4^+. The Fv/Fm parameter only lowered significantly with the lowest concentrations of NO_3^-. Coincidentally, the PI didn't show any significant variation. A linear and significant increase with treatment dose was observed in the content of chlorophyll, both in case of NH_4^+ and NO_3^-.

Due to chemical reactions occurring in the N compounds on lichen surfaces in a humid environment, the pH showed a significant linear increase in case of NO_3^- in relation to the N content of the thalli. On

Table 1
Mean values (± SD) of rate of photosynthesis (RoP, N = 3–6, CO_2 efflux rate μmol m^{-2} s^{-1}), fluorescence parameters (Fv/Fm and PI, N = 6), chlorophyll concentration (CHL, N = 15, μg cm^{-2}), surface pH (N = 10), chitin concentration (mg g^{-1}, N = 3), total N and C (% DW, N = 1), N and C isotopic signature (N = 1) in *C. portentosa* from control plots and plots supplied for 11 years with nitrate (NO_3^-) or ammonium (NH_4^+) at different N doses (ie concentrations) (16, 32 and 64 kg N ha^{-1} yr^{-1}). Values in bold are significantly different from the control (one-way ANOVA, Dunnett test, $P < 0.05$); NA = not available.

	control	NH_4^+-16	NH_4^+–32	NH_4^+-64	NO_3 -16	NO_3 –32	NO_3 -64
Parameters related to the photobiont							
RoP	7.22 ± 0.99	7.42 ± 2.14	8.48 ± 1.74	NA	**5.03 ± 0.89**	5.57 ± 1.31	**5.13 ± 0.94**
Fv/Fm	0.606 ± 0.065	0.581 ± 0.068	0.574 ± 0.079	0.623 ± 0.034	**0.511 ± 0.086**	**0.528 ± 0.097**	0.565 ± 0.040
PI CHL	0.175 ± 0.126	0.151 ± 0.085	0.171 ± 0.106	0.248 ± 0.102	0.148 ± 0.107	0.175 ± 0.132	0.137 ± 0.086
	1.17 ± 0.06	**1.31 ± 0.08**	**1.40 ± 0.08**	**1.56 ± 0.09**	**1.26 ± 0.07**	**1.29 ± 0.07**	**1.31 ± 0.06**
Parameters mainly related to the mycobiont							
pH	3.74 ± 0.13	3.87 ± 0.18	3.69 ± 0.19	3.67 ± 0.11	**4.33 ± 0.22**	**4.45 ± 0.25**	**5.09 ± 0.19**
Chitin	1.01 ± 0.26	1.14 ± 0.26	**1.49 ± 0.3**	1.4 ± 0.28	1.35 ± 0.37	1.27 ± 0.25	**1.53 ± 0.44**
Total N	0.6	1.11	0.97	NA	0.78	0.96	1.21
Total C	43.2	43.83	44.37	NA	42.93	43.52	43.43
$\delta^{15}N$	6.73	3.96	2.72	NA	5.35	1.73	1.19
$\delta^{13}C$	25.25	25.2	24.92	NA	25.21	23.91	23.74

the other hand, the pH values remained quite stable when NH_4^+ was provided.

As expected, the higher N availability led to an increase in N con centration and chitin content in lichens tissue in all treatments. Accordingly, the N isotopic signature of the samples changed ap proaching the N isotopic signature of the provided chemicals, −0.1‰ for NH_4Cl and 3.8‰ for $NaNO_3$. Total C concentration remained quite stable with differences under 1%. $\delta^{13}C$ became less negative with in creasing treatment dose/concentration both with NO_3^- and NH_4^+.

The measured potential extracellular enzyme activity (Fig. 2) showed a clear pattern where activity of all tested enzymes was lower in samples treated with the lowest dose of NH_4^+ and the highest dose of NO_3^-. The only exception was the leucine aminopeptidase which was significantly lower also at the intermediate dose of NH_4^+ and the lowest dose of NO_3^-.

Coefficients of Pearson correlation among the N concentration in tissue of *C. portentosa* and activity of the tested enzymes are shown in Table 2. All the enzymes decreased their activity with increasing N in lichen thalli.

3.2. Electron microscopy analysis of algal component

Algal cells in control samples presented typical characteristics, such as an extended chloroplast that occupies most of the cell volume (C in Fig. 3a), an abundant amount of pyrenoglobuli in the middle of the chloroplast (P), a group of peripheral, abundant and unsaturated lipid bodies (L) and a substantial cell wall that delimits the alga (CW). Other cellular organelles were difficult to observe due to the limited cyto plasm that remains between the chloroplast and the plasma membrane; nevertheless, sometimes mitochondria (M in Fig. 3b) were visible. The number of pyrenoglobuli was very high and they exhibited different levels of electron density. Lipid bodies were plentiful and generally arranged in contact with the cell membrane. The cell wall appeared generally homogeneous and of fibrillar nature (Fig. 3b).

In samples treated with 16 NO_3^-, the ultrastructure of the alga appeared compromised or at least affected. Apart from the cell wall and the extensive lipid bodies that were more or less similar to the control, the most marked difference was the absence of pyrenoglobuli at the center of the alga (asterisk in Fig. 3c) in all the different sections ana lyzed. The treated samples presented a different level of organization of the chloroplast membranes from the control: membranes were less extended, fragmented and disorganized (arrows in Fig. 3c). Moreover, membranes were packed together on top of one another in a way not found in control (arrow in Fig. 3d). The space inside the chloroplast was also characterized by the presence of electron dense deposits of various shape and size (arrowheads), the nature of which is unknown.

Surprisingly, the treatment with 64 NO_3^- did not cause the same effects on the ultrastructure of the alga, whose cytology seemed much less compromised with little damage. The cell wall appeared as regular both in size and in structure. Lipid bodies were abundant and com parable to control ones (Fig. 3e). Being relatively more extended, the membranes of the chloroplast looked like much more those of control when compared to the 16 NO_3^- treated sample; nevertheless, mem branes appeared again to maintain a certain degree of compactness and were not always clearly defined as in the control (arrow in Fig. 3f). The center of the chloroplast was characterized by the presence of many pyrenoglobuli, whose difference from the control consisted of a greater variability of electron density (asterisk in Fig. 3e f).

In the sample treated with 16 NH_4^+, the situation appeared com promised and the algal system was profoundly altered. In several cases, the cell wall appeared to be characterized by pronounced local thick enings, defined as invaginations (asterisk in Fig. 4a). These thickenings seemed characterized by different levels of electron density, as if the additional cell wall contains different carbohydrate components. Lipid bodies were present but not well defined; particularly, their dark electron density suggested an enrichment in saturated lipids (arrow in Fig. 4a). The predominant damage was observed at the level of the chloroplast membrane system that appeared profoundly altered so that individual membranes were not readily observable (arrow in Fig. 4b). The inside of chloroplasts showed no trace of pyrenoglobuli.

As in the case of samples treated with NO_3^-, those treated with 64 NH_4^+ also showed a condition analogous to that of control; compared to the 16 NH_4^+ treated sample. We can affirm that the 64 NH_4^+ treated sample recovered under standard conditions (Fig. 4c). First of all, the cell wall appeared again as normal, no invagination or thickening were visible. Secondly, the membrane system was relatively extensive, much more like the control and definitely not comparable to the sample treated with 16 NH_4^+. The center of the alga was again occupied by an extensive system of pyrenoglobuli. The only difference compared to the control were in peripheral lipid bodies probably saturated (as suggested by dark staining). Another important difference was the presence of large numbers of irregular dark aggregates inside the chloroplast, the nature of which is unknown (Fig. 4d, arrows).

3.3. Long vs. short term the proteome

Samples exposed to 32 NO_3^- and 32 NH_4^+ for 11 years were compared with samples treated with 32 NO_3^- and 32 NH_4^+ for 6 months. Master gels obtained by comparing 2DE analyses of samples are presented in Figs. S1 and S2 in the supplementary material. Proteins identified by mass spectrometry are reported in Table 3, with protein names, accession numbers with correspondent data base, matched peptides, organisms and theoretical isoelectric point and mass weight. Two spots were identified from comparison of NO_3^- treatments

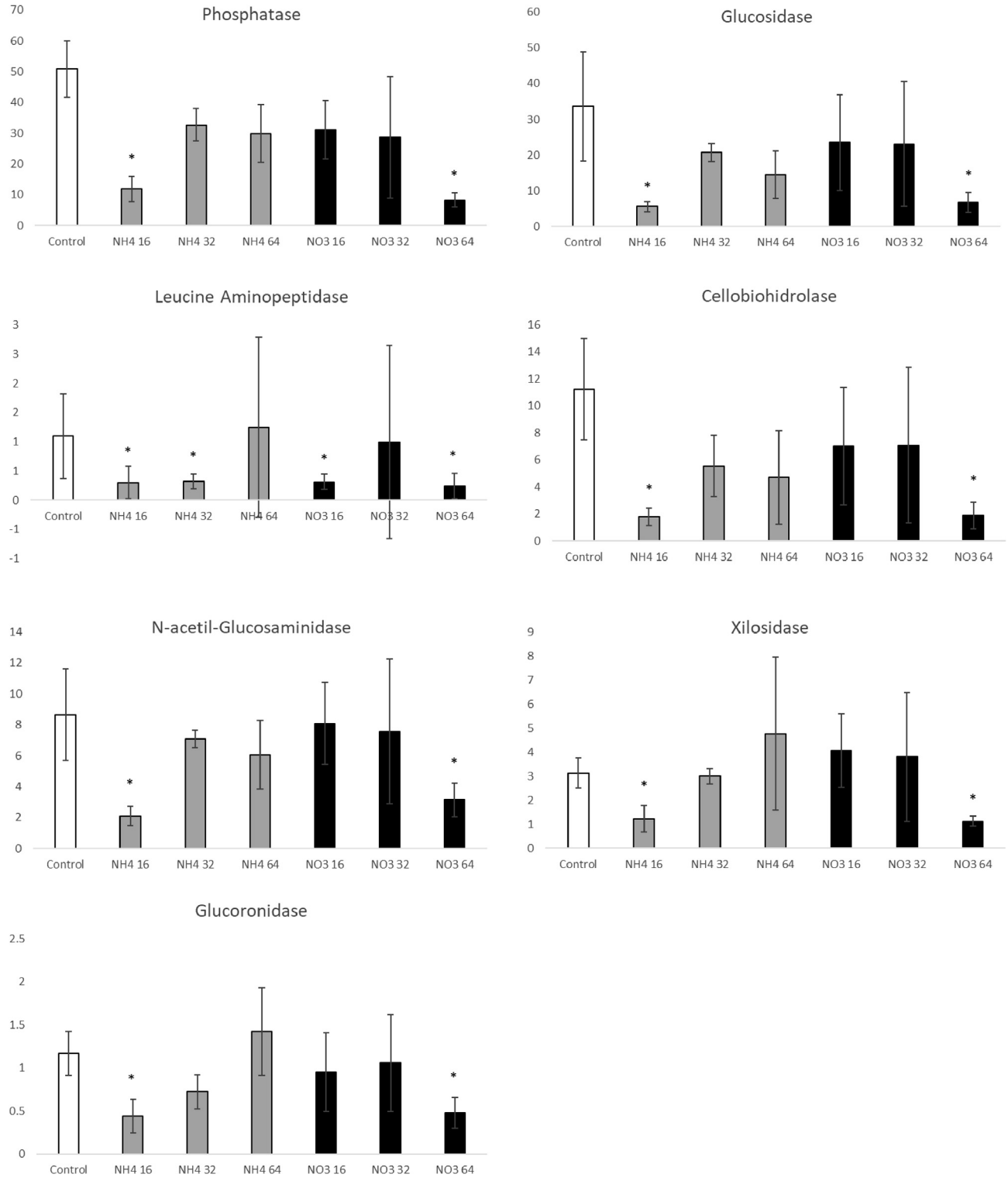

Fig. 2. Mean values of the potential extracellular enzymatic activity (nmol g lichen^{-1} h^{-1}, N = 3) in *C. portentosa* from control plots and plots supplied for 11 years with nitrate (NO_3^-) or ammonium (NH_4^+) at different dose/(concentration) (16, 32 and 64 kg N ha^{-1} yr^{-1}). Vertical bars represent standard deviation. * = significantly different from the control (one-way ANOVA, Dunnett test, $P < 0.05$).

Table 2
Pearson correlation coefficient among enzymatic activities and lichen tissue N (significance *$P < 0.05$; **$P < 0.01$).

	Cellobio hidrolase	Phosphatase	Glucosidase	Glucoronidase	N-acetil-Glucosaminidase	Xilosidase	Leucine-aminopeptidase
Tot N	-0.965**	-0.961**	-0.950**	-0.896**	-0.862*	0.673	0.727

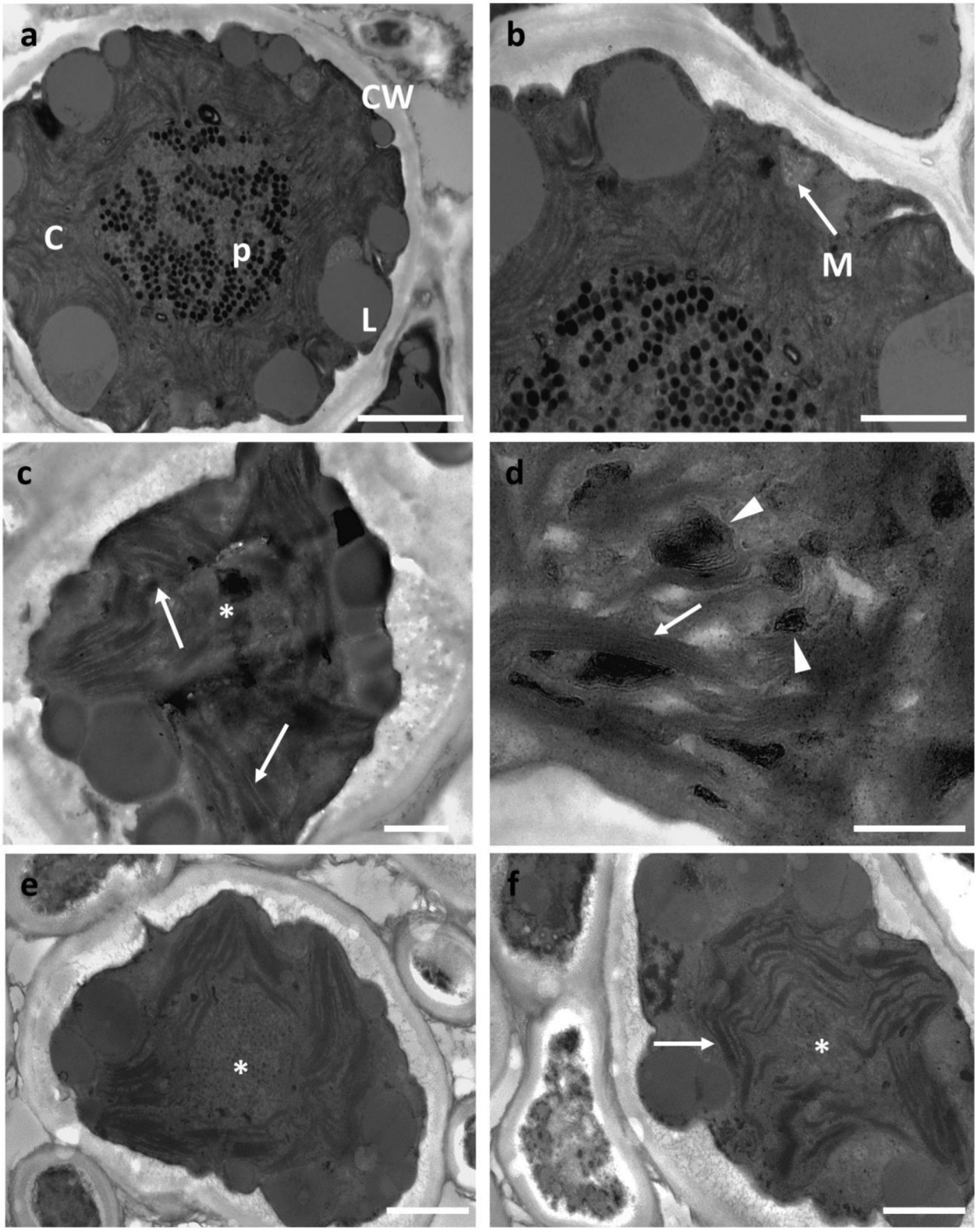

Fig. 3. Algal cells in control and treated samples. (a) In controls, an extended chloroplast (C), abundant pyrenoglobuli (P) in the chloroplast, large and unsaturated lipid bodies (L), the cell wall (CW) and mitochondria (M) are visible. Bar 2000 nm. (b) The cell wall is homogeneous and fibrillar. Bar 1000 nm. (c) In 16 NO_3^- samples, the ultrastructure of algae is compromised, the most striking difference being the absence of pyrenoglobuli in the center of algae (asterisk). The chloroplast membranes are less extended, fragmented, and disorganized (arrows). Bar 1000 nm. (d) Sometimes the chloroplast membranes are packed (arrow) and the space within the chloroplast is characterized by electron dense deposits (arrowheads). Bar 500 nm. (e) 64 NO_3^- treatments did not have the same effects. The cell wall is regular, lipid bodies are abundant as in control. Bar 1000 nm. (f) Chloroplast membranes maintain some degree of compactness (arrowhead). The center of the chloroplast is characterized by many pyrenoglobuli that vary in electron density (asterisk in e-f). Bar 1000 nm.

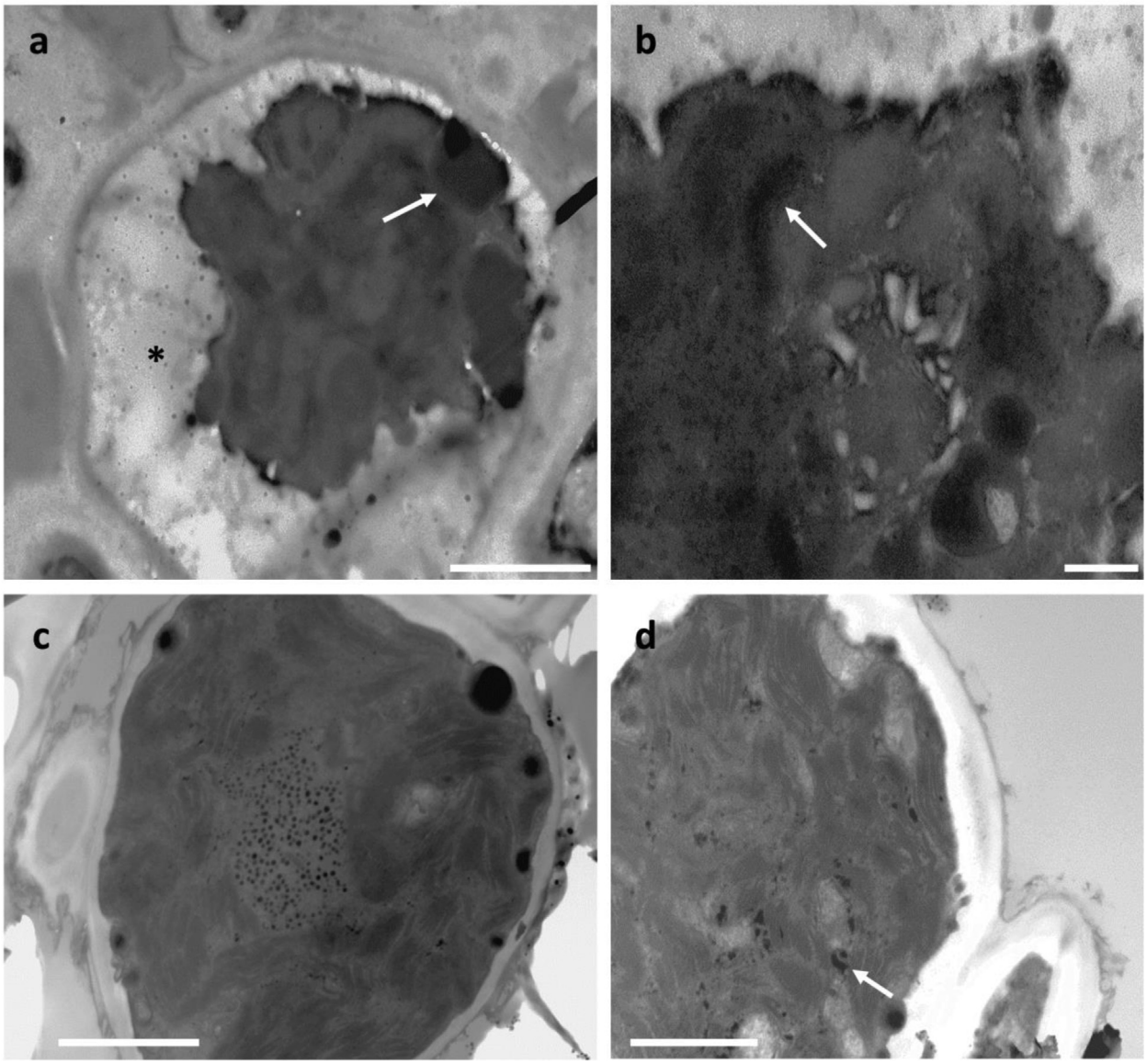

Fig. 4. Samples treated with NH_4^+. (a) In 16 NH_4^+ samples, the situation is compromised. The cell wall is characterized by strong thickening (asterisk). Lipid bodies are present but enriched in saturated lipids (arrow). Bar 2000 nm. (b) The most severe damage is at the level of the chloroplast membranes (arrow). The interior of chloroplasts shows no pyrenoglobuli. Bar 500 nm. (c) 64 NH_4^+ samples show a condition like the control. The cell wall is regular, without thickening and the membrane system is as extensive as in the control. The center of algae is filled with many pyrenoglobuli. Bar 2000 nm. (d) Important differences are the presence of saturated lipid bodies and irregular dark aggregates within the chloroplast (arrows). Bar 2000 nm.

(spots 1 and 2 in Table 3) and 2 spots from NH_4^+ treatments (spots 3 and 4 in Table 3), whose abundance differed at least two fold between treatments with overexpression after the 11 years exposure.

For spot 1 (NO_3^-) two different correspondences were found in the database: a mitochondrial ATP synthase subunit beta 1 from *Arabidopsis thaliana* and a F1 ATPase from *Pisum sativum*. Similarly, spot 3 (NH_4^+) corresponds to mitochondrial ATP synthase subunit alpha, from *Kluyveromyces lactis*.

In NO_3^- treatment, spot 2 has a correspondence to a "probable proteasome subunit alpha" from *Neurospora crassa*, while spot 4 cor responds to a hypothetical protein whose expression level increased in long term exposure to NH_4^+.

Table 3
Proteins identified by MALDI-TOF and/or MALDI-TOF/TOF mass spectrometry (pI/MW = isoelectric point/molecular weight).

Spot n°	Protein Name	Accession number	Matched peptides	Organism	pI/MW
1	ATP synthase subunit beta-1, mitochondrial F1 ATPase	P83483[a]	K.VVDLLAPYQR.G	*Arabidopsis thaliana*	6.18/59.7
		gi|2116558[b]	R.DAEGQDVLLFVDNIFR.F	*Pisum sativum*	6.63/60.2
2	Probable proteasome subunit alpha type-2	Q8X077[c]	R.IYNEYPPTR.I	*Neurospora crassa*	5.72/27
3	ATP synthase subunit alpha, mitochondrial	P49375[c]	R.EAYPGDVFYLHSR.L	*Kluyveromyces lactis*	9.37/59.1
4	Hypothetical protein MYCGRDRAFT 105661	gi|398391699[d]	R.YDGFEGEPR.S	*Zymoseptoria tritici* IPO323	6.26/28.9

a – Swiss-Prot (Viridiplantae), b – NCBInr (Viridiplantae), c – Swiss-Prot (fungi), d – NCBInr (fungi).

4. Discussion

Although some changes occurred in the physiological parameters and in the algal ultrastructure, *C. portentosa* showed it was able to cope with long term N supply, maintaining stable metabolic function with all the doses and forms of N supplied (16 64 kg ha^{-1} y^{-1}).

4.1. Photobiont response

Since the photobiont is responsible for energy production, its functioning is crucial for the survival of the lichen association. In our experiment, NO_3^- affected the photobiont more than NH_4^-. This is probably due to associated increases in pH values, that represents a drastic change in the chemical environment where the algal cells exist. Nevertheless, vitality indexes either remained stable, like the PI, or slightly changed, like the Fv/Fm parameter, staying within the range at which healthy lichens can operate (Munzi et al., 2013a,b). Modifica tions of algal ultrastructure were also not severe enough to incapacitate photosynthetic activity that was still present in all samples, as showed by the RoP that varied between 70 and 117% of the control one. That ensured the production of energy needed for the survival of the lichen even in case of costly mechanisms of N tolerance. Changes observed in the algal ultrastructure, namely loss of organization in the thylakoid system, appearance of stromal electron dense droplets and heavy plasmolysis, are comparable to modifications observed in samples ex posed to other types of stressor, such as heavy metals or trace elements (Paoli et al., 2013) and environmental pollution (Sorbo et al., 2011). This suggests an unspecific response to stress rather than specific N related damage to the cells.

At the highest dose (concentrations) of both N forms, algal orga nelles showed characteristics more similar to the control samples than at the lowest concentrations. This agrees with results obtained from proteomic analysis (Munzi et al., 2017b). In that work, authors found a bell shaped pattern for the expression of several proteins, suggesting the existence of a threshold of N concentration/dose beyond which the homeostasis mechanisms change, provoking the down regulation of proteins upregulated at lower N concentrations. Similarly, ultra structural changes can reflect a temporary stress before tolerance me chanisms reestablish the correct functioning of the cells even at the highest N concentrations.

A fertilizing effect of N supply was observed, with higher content of chlorophyll after all treatments. This is in agreement with previous observation of increased chlorophyll content in thalli exposed to moderate pollution (Sujetoviene and Sliumpaite, 2013). A general fer tilizing effect has been also observed in other experiments: under the threshold of toxicity, N works as a nutrient, in particular for lichen species of oligotrophic environments as *Cladonia* (Carreras et al., 1998; Munzi et al., 2013a,b).

4.2. Mycobiont response

Thalli appeared to be healthy, with unaltered morphological char acteristics after all treatments. The proliferation of free alga on the surface can be explained by the increased availability of nutrients, namely N, for these microalgae, as happens in cases of eutrophication. Another possibility is that lichens spend more energy and resources to respond to N excess, reducing other tasks like the production of alle lopathic secondary compounds in the mycobiont that can function as regulators of other organisms' proliferation (Goga et al., 2017; Lokajová et al., 2014). However, measurements of secondary compounds would be needed to test this hypothesis.

It is well known that the N concentration in lichen tissue reflects environmental N availability and that the N isotopic signature in li chens depends on the signature of the N source (e.g. Boltersdorf and Werner, 2013; Munzi et al., 2019 and references therein). This kind of response is typically attributed to the mycobiont, that represents around 90% of lichen biomass (Honegger, 1993). In our samples, the higher N concentration in tissue of treated lichens with respect to control indicates an effective uptake of N by transplanted thalli of both chemical forms. Values of isotopic signature confirm this, being highly correlated to N concentration and with $\delta^{15}N$ approaching the value of the chemical used for the treatments in a dose dependent way. Simi larly, chitin content tends to increase with all N treatments. That is expectable since N represents 6.3% of chitin weight so a higher avail ability of N allows the production of more chitin. Moreover, it has been suggested that chitin production can work as a detoxification me chanism for N excess (Munzi et al., 2017b). It is therefore reasonable to think that *C. portentosa* stores N as harmless molecules of chitin.

Carbon concentration and $\delta^{13}C$ showed a much more restrained, dose independent change than N concentration. In lichens, $\delta^{13}C$ values result from a combination of resistance to inward CO_2 fluxes, CO_2 source signature and the photobiont CO_2 fixation mechanism (Máguas et al., 2020). Since these parameters are not affected by N availability and since photosynthetic activity was not heavily compromised, the steadiness of $\delta^{13}C$ and C concentration is not surprising.

4.3. Changes in pH

Ammonium and nitrate are known to cause deregulation of pH homeostasis in plants (Marino and Moran, 2019) respectively by de creasing and increasing its value (Subbarao et al., 2015; Wang et al., 2016). Our data confirm the effect of nitrate on the lichen surface, however, no changes in pH were caused by exposure to ammonium. In plants, ammonium can collapse the pH gradient of tonoplast vesicles, high levels of NH_4^+ in the cytosol may be toxic and consequently, cellular levels of this ion tend to remain stable by a passive mechanism of NH_4^+ efflux or other mechanisms (Ludewig et al., 2002). We can expect a similar effect on tonoplast vesicles in algal cells.

Hutchinson et al. (1986) reported that prolonged exposure of spe cies of the genus *Cladina* to a pH of 3.5 caused a substantial decline in both podetial height and podetial dry weight. Similarly, Fritz Sheridan (1985) observed a total inhibition of nitrogenase in cyano lichens at pH 3. The harmful consequences of acidification justify a greater invest ment of resources by *C. portentosa* in maintaining the pH stable when exposed to NH_4^+ than to NO_3^-. This protection mechanism could be supported by the enhanced energetic metabolism observed in pro teomic analysis in samples of *Cladonia* exposed to N (Munzi et al., 2017b).

4.4. Enzymatic activity

Extracellular enzymes are secreted by fungi in nature to break down complex molecules into small, more easily assimilable molecules, thus fulfilling their nutrient requirements (Bell et al., 2013). Although li chens mainly rely on the algal partner for C assimilation, alternative nutrition modes requiring the digestion of organic molecules are also known (Gassmann and Ott, 2000). It is therefore reasonable to hy pothesize that extracellular enzymes can be involved in N and C turn over in lichens and that an increase in N availability can influence their activities as it happens, for example, in soil microorganisms (Jian et al., 2016).

The influence of N fertilization on enzymatic response in our sam ples is shown by a significant negative correlation between the N concentration found in lichen tissue and the activity of 5 out of 7 en zymes analyzed (Table 2). For enzymes using N compounds as a sub strate, a reduced activity in the presence of increased N availability is expected. This is the case, for example, of N acetyl glucosaminidase, which targets chitin: a reduction of this enzyme's activity is in agree ment with the enhanced chitin content found in all treated samples.

After11 years of N enrichment, hydrolases were significantly en hanced, while oxidases declined (Fig. 2). The lack of data on cellulases (glucosidase, xylosidase, and cellobiosidase) in lichens prevents a

detailed interpretation of the results, however, our data suggest a co regulation of the enzymes involved in C and N turn over. This is rea sonable if we think that a regulated C/N economy seems to exist in lichens (Munzi et al., 2013a,b; Palmqvist et al., 2002).

Fungi mineralize organic phosphorus (P) through the release of phosphatase enzymes that catalyze the hydrolysis of organic P, turning organic into inorganic P, which can be taken up by plants and other organisms. The production and activity of phosphatase enzymes de pends on many factors including the fungal species, local pH and N availability (Olander and Vitousek, 2000; Sinsabaugh et al., 2008). Hogan et al. (2010) showed that becoming N demand saturated, an upregulation of phosphomonoesterase activity occurred due to the in creased demand for P in *C. portentosa*.

Hence, increased N deposition (in contrast to that of P) is expected to increase phosphatase activity (Olander and Vitousek, 2000; Sinsabaugh et al., 2008). Optimal pH for phosphomonoesterase activity was determined as being around 2.2 for *C. portentosa* and *Lobaria pul monaria* (Lane and Puckett, 1979). Our results show that for the same N source (nitrate or ammonium) the inhibitory effect of surface pH in crement associated with increased N deposition was stronger than the putative stimulant effect of the increased N availability on the potential acid phosphatase activity. Although the pH suppressed the N con centration effect, and ammonium deposition lead to lichen surfaces with lower pH than those subject to nitrate deposition, the lichens re ceiving nitrate N presented higher potential for acid phosphatase ac tivity. Phosphatase is mainly located at the surface (internal and ex ternal) of young hyphae, and it has been described for several organisms that ammonium deposition is associated with early senes cence (Esteban et al., 2016), which may explain the lower levels of phosphatase activity observed for ammonium treated lichens.

Notably, the response curve described by enzymatic activity is in agreement with the results of proteomic analysis in samples of *C. por tentosa* (Munzi et al., 2017b), where most of the proteins increased with the intermediate doses of N and decreased with the highest doses.

4.5. Proteome response

Munzi et al. (2017b) highlighted that already after 6 months of exposure to N, some changes in the protein expression could be ob served in the lichen *C. portentosa*. These changes mainly occurred in the fungal partner, with NO_3^- mostly affecting the energetic metabolism and NH_4^+ affecting transport and regulation of proteins and the en ergetic metabolism more intensely than NO_3^- did. The few differences in the proteomes analyzed after 6 months and 11 years of exposure suggest that the molecular mechanisms necessary to cope with in creased N availability are put in action shortly after the beginning of the stress and remain quite stable over time.

Changes in protein expression indicate a specific role of ATP syn thase of *C. portentosa* thalli in relation to both form of N treatments. Mitochondrial ATP synthase produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain. F type ATPase consist of two structural domains, F1 containing the extramembranous catalytic core, and F0 containing the membrane proton channel, linked to gether by a central stalk and a peripheral stalk. During catalysis, ATP synthesis in the catalytic domain of F1 is coupled via a rotary me chanism of the central stalk subunits to proton translocation. Subunits alpha and beta form the catalytic core in F1.

Although only probable, spot 3 corresponds to a proteasome, i.e. a multicatalytic proteinase complex which cleaves peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH. The proteasome has an ATP dependent proteolytic activity, so it can be linked to the increase in spot 1. The last spot analyzed corresponds to a hypothetical protein.

Even if different proteins are involved, they are always related to the energetic metabolism and protein regulation, in agreement with the results of proteomic analyses after short term exposure.

5. Conclusions

The establishment of environmental policies, like pollutants' critical loads and levels, tends to be based on the response of the most sensitive components of an ecosystem, to ensure the protection of all the com ponents. The selection of these sensitive and responsive species is therefore crucial for the success of any protection measure.

Given that lichens have already been adopted to follow environ mental changes through empirical observations (Bobbink and Hettelingh, 2011), using actual moderately tolerant species, which has previously been considered to be highly sensitive, could lead to an underestimation of potential damage and to a mismatch between the aims and the effects of a protection measure. The more the monitoring species sensitivity is underestimated, the larger the number of sensitive species that are left unprotected.

Cladonia portentosa, although considered a nitrogen sensitive species (e.g. Nimis and Martellos, 2017), was shown to be able to cope with long term N depositions, both in the field (Gheza, 2015, 2018) and in the Whim bog experimental site.

While this work does not provide a quantitative assessment of *C. portentosa* functional traits, it nevertheless highlights the need to con sider updated knowledge in expert assessment of functional response to be used in biomonitoring survey and environmental policies establish ment.

CRediT authorship contribution statement

S. Munzi: Conceptualization, Investigation, Data curation, Formal Analysis, Visualization, Writing original draft. **C. Cruz:** Conceptualization, Data curation, Writing review & editing. **C. Branquinho:** Conceptualization. **G. Cai:** Data curation, Formal ana lysis, Methodology, Writing original draft. **C. Faleri:** Formal analysis, Data Curation. **L. Parrotta:** Formal analysis, Methodology, Data cura tion, Writing original draft. **L. Bini:** Formal analysis, Data curation, Methodology. **A. Gagliardi:** Formal analysis, Data curation. **I.D. Leith:** Investigation, Resources, Writing review & editing. **L.J. Sheppard:** Conceptualization, Investigation, Resources, Writing review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influ ence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https:// doi.org/10.1016/j.ecolind.2020.106817.

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