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Eco-physiological response of *Hypnum cupressiforme* Hedw. to increased atmospheric ammonia concentrations in a forest agrosystem

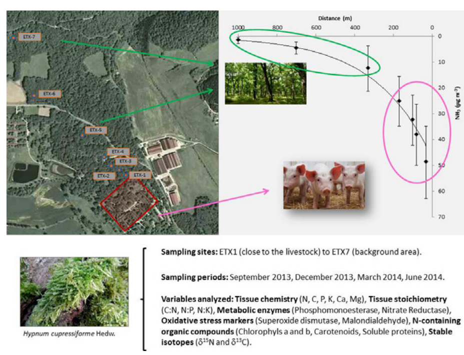
S. Izquieta-Rojano [a], M. López-Aizpún [a], J.J. Irigoyen [b], J.M. Santamaría [a,*], C. Santamaría [a], E. Lasheras [a], R. Ochoa-Hueso [c], D. Elustondo [a]

[a] Universidad de Navarra, Facultad de Ciencias, Departamento de Química, Laboratorio Integrado de Calidad Ambiental (LICA), Campus Universitario, Irunlarrea 1, 31008 Pamplona, Spain
[b] Universidad de Navarra, Facultad de Ciencias, Departamento de Biología Ambiental, Campus Universitario, Irunlarrea 1, 31008 Pamplona, Spain
[c] Universidad Autónoma de Madrid, Departmento de Ecología, Darwin 2, 28049 Madrid, Spain

HIGHLIGHTS

- Atmospheric NH_3 significantly affected the physiological response of mosses.
- The greatest effects were detected at sites close to the livestock farm.
- Seasonal dynamics are of primary importance in the study of NH_3-related impacts.
- Responsive but highly scattered variables are not suitable to set critical levels.

GRAPHICAL ABSTRACT



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ABSTRACT

Ammonia (NH_3) emissions are linked to eutrophication, plant toxicity and ecosystem shifts from N to P limitation. Bryophytes are key components of terrestrial ecosystems, yet highly sensitive to N deposition. Hence, physiological responses of mosses may be indicative of NH_3-related impacts, and thus useful to foresee future ecosystem damages and establish atmospheric Critical Levels (CLEs). In this work, samples of *Hypnum cupressiforme* Hedw. were seasonally collected along a well-defined NH_3 concentration gradient in an oak woodland during a one-year period. We performed a comprehensive evaluation of tissue chemistry, stoichiometry, metabolic enzymes, antioxidant response, membrane damages, photosynthetic pigments, soluble protein content and N and C isotopic fractionation. Our results showed that all the physiological parameters studied (except P, K, Ca and C) responded to the NH_3 gradient in predictable ways, although the magnitude and significance of the response were dependent on the sampling season, especially for enzymatic activities and pigments content. Nutritional imbalances, membrane damages and disturbance of cellular C and N metabolism were found as a consequence to NH_3 exposure, being more affected the mosses more exposed to the barn atmosphere. These findings suggested significant implications of intensive farming for the correct functioning of oak woodlands and highlighted the importance of seasonal dynamics in the study of key physiological processes related to photosynthesis, mosses nutrition and responses to oxidative stress. Finally, tissue N showed the greatest potential for the identification of NH_3-related ecological end points (estimated CLE = 3.5 μg m^{-3}), whereas highly scattered physiological responses, although highly sensitive, were not suitable to that end.

* Corresponding author.
E-mail address: chusmi@unav.es (J.M. Santamaría).

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1. Introduction

Human disturbance of the nitrogen (N) cycle in Europe is primarily driven by agricultural activities (Jensen et al., 2011; Hertel et al., 2012) with ammonia (NH_3) being one of the emitted compounds of major concern (Sutton et al., 2008; Van Damme et al., 2014). According to the European Environment Agency (EEA), 4324 Gg of NH_3 were emitted in 2012 in Europe (EEA-33 country group), of which 93.5% was released in agricultural activities (EEA-European Environment Agency, 2015). Animal production and volatilization from livestock excreta accounts for the major share, which was estimated for 2011 in three quarters of agricultural NH_3 emissions, whereas agricultural soils accounted for the rest (Eurostat, 2013). Inventories from China (Zhou et al., 2015) and North America (Bittman and Mikkelsen, 2009) also found that livestock was the dominant source of NH_3 emissions, showing that this is a global-scale problem.

Once in the environment, NH_3 can cause important damage, including ecosystem eutrophication, changes in pH, nutritional imbalances (particularly N in relation to C and P), loss of biodiversity and direct toxic effects on vegetation and human health (Krupa, 2003; Erisman et al., 2013; Bittsánszky et al., 2015; Shibata et al., 2015). To reduce these negative consequences, many international treaties and policies have been developed. The Gothenburg protocol and the National Emission Ceilings Directive (2001/81/EC) are addressing NH_3 emissions, setting upper limits for each Member State to be met by 2020 according to recent amendments. The main long-term objective is to avoid the exceedance of critical levels (CLEs) and loads, and achieve an effective protection of people and ecosystems against the recognized health risks from air pollution (NEC Directive 2001/81/EC). The critical level refers to the gaseous concentration of a pollutant in the air, whereas the critical load relates to the quantity of pollutant deposited from air to the ground. Thus, CLEs are not habitat specific as in critical loads, but has been set to cover broad vegetation types (CLRTAP, 2014). The Critical Level for NH_3 is described as 'the concentration in the atmosphere above which direct effects of this gas on receptors such as human beings, plants, ecosystems or materials may occur according to present knowledge' (Cape et al., 2009). Following this definition, NH_3 CLEs are revised periodically and updated according to the progress of scientific knowledge. The last review recommended a long-term NH_3 CLE for lichens and bryophytes of 1 $\mu g\ m^{-3}$ (reduction from 8 $\mu g\ m^{-3}$), including those ecosystems where lichens and bryophytes are key part of the ecosystem integrity (Sutton et al., 2009; CLRTAP, 2014). The observed changes in species composition (ecological endpoint) in response to measured air concentrations of NH_3 were the basis to update this CLE (Sutton et al., 2009).

Although on average a decrease of 30% in agricultural NH_3 emissions across the EU-27 was found between 1990 and 2010, countries like Spain have increased their emissions in that period (Eurostat, 2012; EEA, 2014). Regarding total emissions, no significant reduction has been found in the last years in the EU-28 countries (2004–2014, Eurostat, 2017); even a slight increase (5%) has been recorded if EU-33 countries are considered (2000–2014, EEA, 2016). Therefore, despite the efforts of policy makers and the commitments made by different countries, NH_3 still is a relevant atmospheric pollutant worldwide, whose impacts are expected to persist and be magnified in response to the increasing Nr losses projected for the next decades (Reis et al., 2009; van Vuuren et al., 2011; Fowler et al., 2015).

Bryophytes are among the most vulnerable organisms to N pollution (Cape et al., 2009; Bobbink et al., 2010). The absence of a well-developed cuticle and the lack of a true root system to acquire N from substratum are the main features that make them specially sensitivity to atmospheric pollution, allowing their use as biomonitors of N deposition and also as indicators of direct effects of ammonia in the gas phase (Cape et al., 2009; Branquinho et al., 2010; Pinho et al., 2012). In fact, the CLE for NH_3 has been established after experimental work carried out with mosses and lichens (Cape et al., 2009; Pinho et al., 2012, 2014; CLRTAP, 2014). Although the N deposition-induced effects on moss-dominated communities from central and northern Europe are well-known (Pitcairn et al., 1998; Sheppard et al., 2011; Verhoeven et al., 2011), we lack a complete and integrated understanding of the physiological response to NH_3, particularly in the seasonally-dry woodlands of southern Europe. Several studies have attempted to couple N-induced changes in species richness, cover, growth or biomass production, with variations in the photosynthesis performance, the appearance of nutritional constraints, shifts in the response of metabolic enzymes or damages on the cell membrane (Pearce et al., 2003; Pitcairn et al., 2003; Granath et al., 2009, 2012; Du et al., 2014; Munzi et al., 2014; Paoli et al., 2014; Wang et al., 2016). However, so far no study has evaluated the impacts of gaseous ammonia on mosses from a multivariate, comprehensive and temporal perspective in real conditions, even though its effects are known to be significantly more deleterious than those caused by wet deposited nitrogen species (Leith et al., 2002; Sheppard et al., 2011). On the other hand, the study of some physiological responses has also been proposed as a potential tool for anticipating likely N-related impacts at the ecosystem level (Arróniz-Crespo et al., 2008). Contrary to higher plants, mosses react quicker to increased N loads, acting as early warning indicators of risk. Equally, very responsive physiological variables can be used to evaluate ecosystem recovery after N pollution declines (Arróniz-Crespo et al., 2008).

Hypnum cupressiforme Hedw. is one of the four species recommended by the International Cooperative Programme on Effects of Air Pollution on Natural Vegetation and Crops (ICP Vegetation), 2015 for biomonitoring purposes. This species, commonly distributed in the seasonality-dry oak woodlands from northern Spain, has been widely used in N monitoring surveys across Europe (Harmens et al., 2011; Schröder et al., 2014), where it has proved to be specially tolerant to high levels of atmospheric pollution (González-Miqueo et al., 2010).

The main objective of the present work was to analyze the impacts of NH_3 on the moss functioning and to determine its effects on vital processes. We hypothesize that ammonia is inducing alterations on the nutritional status and requirements of the moss, causing imbalances of C:N, N:P and N:K ratios because of the higher N accumulation in those samples exposed to higher NH_3 concentrations and up-regulation and down-regulation of phosphomonoesterase and nitrate reductase enzyme activities, respectively. We also hypothesize that ammonia has a toxic effect on this sensitive organism. As a consequence, anti-stress and detoxification strategies would have been activated: increasing activity of superoxide dismutase enzyme activity in samples collected close to the livestock building and greater synthesis of N-containing compounds like proteins or pigments at those sites. When these mechanisms are surpassed, we forecast NH_3-induced impacts on the photosynthesis performance (content and relationships between pigments; C fixation and isotopic fractionation) and damages on the cell membranes (increasing levels of lipid peroxidation). The study of multiple variables related to different physiological processes will allow us to define which of the considered variables are the most responsive, and therefore, which ones are the most promising for the use of *H. cupressiforme* in ecosystem surveys as early warning indicators of NH_3 toxicity. A second objective of this project was to make an approximation to study the intra-annual variability on the response of the studied variables, which is of primary importance for developing sampling protocols and conducting biomonitoring surveys based on the analysis of physiological parameters. Finally, we aimed at testing the suitability of physiological responses at species level to establish critical ecological thresholds in oak woodlands from northern Spain.

Our study is particularly well-suited to tackle these questions because, unlike N-addition fertilization studies, which are not frequently performed with enough time and reasonable N doses, it was developed in a real, highly-polluted farming area, which constitutes a more realistic approach.