Title: The influence of nitrate pollution on elemental and isotopic composition of aquatic and semi-aquatic bryophytes

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Abstract

Bryophytes can play an important role in key ecosystem processes and represent potential candidates as bioindicators for environmental monitoring programmes. Nitrate (NO_3^-) pollution poses a growing threat to both aquatic and terrestrial ecosystems, potentially leading to imbalances in nutrient levels and altering the chemical composition of organisms, thereby impacting ecosystem function. However, the specific effects of NO_3^- pollution on the elemental and isotopic composition of aquatic and semi-aquatic bryophytes remain uncertain. In this study, we examined the influence of NO_3^- pollution from spring water on the elemental composition of aquatic and semi-aquatic (hygrophytic) bryophyte species and their respective water sources. Our investigation encompassed diverse land use, lithology, and climate conditions to identify suitable bryophyte species as bioindicators of NO_3^- pollution. We observed higher NO_3^- concentrations in spring water from intensively farmed and urban areas compared to natural and extensively farmed areas (e.g., pastures). These higher concentrations were positively correlated with the nitrogen (N) content and $\delta^{15}N$ isotope ratio in bryophytes. However, spring water NO_3^- concentrations did not significantly affect the overall chemical composition of the water sources, except for N-related elements such as Na, Ca, and Mg. Our findings highlight *Apopellia endiviifolia* and *Oxyrrhynchium speciosum* as promising candidate species for bioindication of aquatic NO_3^- pollution, due to their $\delta^{15}N$ sensitivity to increasing NO_3^-, i.e., they respond to variations in the ratio of $\delta^{15}N$ isotopes in their environment. The identification of these species will assist land managers in effectively monitoring NO_3^- pollution in freshwater systems, thereby addressing public health concerns and supporting wildlife conservation priorities.

Keywords: Nitrogen, nitrogen pollution, spring water, farmed areas, urban areas, bioindicators, elementome

1. Introduction

Bryophytes include moss, liverwort and hornwort species that provide microhabitats for a diversity of organisms (Lindo and Gonzalez, 2010). Additionally, because some bryophyte taxa contain N_2 fixing symbionts, they can be important in the N cycle in some ecosystems, for example boreal forest (Turetsky, 2003; Lindo and Whiteley, 2011; Cleveland et al., 2022), and they also play a key role in the global carbon (C) balance and in carbon dioxide exchange (Lindo et al., 2013; Porada et al., 2013; Spitale et al., 2020). However, losses in bryophyte diversity are expected with ongoing climate warming (He et al., 2016) and under global environmental change, such as increased levels of nitrogen (N) availability (Fernández-Martínez et al., 2020, 2021) that may lead to subsequent impacts on ecosystem function, including nutrient cycling and C balance.

Nitrogen pollution is a growing problem in aquatic and terrestrial ecosystems (Vitousek et al., 1997; Camargo and Alonso, 2006) due to atmospheric deposition of N from the burning of fossil fuels and groundwater inputs of inorganic forms of N (ammonia-NH_3, ammonium-NH_4, nitrite-NO_2, nitrate-NO_3) from intensive crop and livestock management practices (Ju et al., 2006; Huebsch et al., 2013), as well as runoff from agricultural areas (Skaggs et al., 1994; Collao Barrios, 2008; Huang et al., 2011). Increases in N availability reduce levels of plant and animal biodiversity (Porter et al., 2012) and lead to shifts in nutrient stoichiometry (Sardans and Peñuelas, 2012); for example, contrasting responses of bryophyte species to environmental pollutants (Pyari et al., 2010; Lindo et al., 2013; Stanković et al., 2018) have led to shifts in bryophyte community composition and decreases in species diversity under NO_3^- pollution, due to greater abundance of N-tolerant species (nitrophilous) and reductions in abundance of N-sensitive

species (nitrophobous) (Fernández-Martínez et al., 2020). Increases in stoichiometric ratios of N to phosphorus (P) (N:P) may alter the nutritional status of organisms, with implications for growth and performance (Elser et al., 2010; Penuelas et al., 2013, 2020; Sardans et al., 2016). Increasing plant N:P ratios is a common observation under N pollution, but the effect of increasing N on other elements beyond P has been seldom explored (but see Penuelas et al., 2020). Although there is evidence for toxic effects of excessive N on bryophytes, including damage to cell membranes, leading to solute leakage, reduced growth and shoot death (Potter et al., 1995; Carroll et al., 2000; Pearce et al., 2003; van der Wal et al., 2005), there is limited understanding of the effects of NO_3^- pollution and associated shifts in nutrient stoichiometry on aquatic and semi-aquatic bryophyte species physiology and community structure and function.

Bryophytes are reliable environmental indicators of global warming (L. Dennis Gignac, 2001), having been used to explain previous climate changes (Désamoré et al., 2012), to validate climate models (Spitale et al., 2020), and are ideal candidates for pollution bioindicators (Pyari et al., 2010). This is because of their structural simplicity (lack of true roots and thick cuticles), rapid rates of growth and reproduction, diversity of host habitats, and sensitivity to environmental conditions (He et al., 2016; Fernández-Martínez et al., 2021). For example, bryophytes are characterised by a high capacity to accumulate toxic elements, such as heavy metals (Salemaa et al., 2004), that affect their distribution and functional traits (Fernández-Martínez et al., 2019). In addition, there is a need to improve our understanding of the impacts of N on bryophytes, and the confirmation of the sources of N pollution is required to improve environmental management. For example, analysis of $\delta^{15}N$ may indicate that N comes from

103 animal origin (high values) (Vanderklift and Ponsard, 2003) or N derived from
104 inorganic fertilizer (values close to zero, similar to atmospheric nitrogen-N_2) (Choi
105 et al., 2007; Diebel and Zanden, 2009). However, there is a need to quantify the
106 isotopic N composition of bryophytes for use as a reliable indicator of NO_3^-
107 pollution in aquatic and semi-aquatic ecosystems.
108 The study of springs, draining groundwater, is important because these aquatic
109 systems are unique and different from other water bodies such as lakes or rivers
110 (Foster et al., 2000; Choi et al., 2007; Fleckenstein et al., 2010). Groundwater
111 tends to have higher susceptibility to pollution than rivers (Kačaroğlu, 1999) and
112 has a lower dilution capacity due to its size and water flow (Sophocleous, 2002).
113 In addition, it may be surrounded by sensitive terrestrial ecosystems, such as
114 spring ecosystems, which may be affected by groundwater quality (Danielopol et
115 al., 2003; Kløve et al., 2011; Chiloane et al., 2022). Many springs are the main
116 source of water supply in arid and semi-arid regions for economic activities and
117 to meet the needs of the population. In addition, it has been observed that some
118 bryophytes associated with springs may be different from those found in flowing
119 waters in general, suggesting that these habitats play an important role in
120 biodiversity conservation (Kuglerová et al., 2016).
121 The aims of this study were to investigate the effects of NO_3^- water pollution on
122 overall spring water chemistry, on the elemental and isotopic bryophyte
123 composition in aquatic and semi-aquatic environments (springs) and to identify
124 the most appropriate bioindicator species of N pollution for environmental
125 monitoring protocols. We here hypothesized that nitrate pollution would alter the
126 elemental composition of bryophytes, particularly that of N, $\delta^{15}N$ and C:N and N:P
127 stoichiometric ratios. Thus, we analysed bryophyte tissue concentrations of C,

$\delta^{13}C$, N, $\delta^{15}N$, and XX other elements (including P, K, Ca, Na…) for 43 bryophyte species from 178 springs distributed across a climate-hydrogeochemical gradient (Fernández-Martínez et al., 2019). In our study we consider climate, altitude and water availability as they have an effect on bryophyte communities. We test for effects of land use and NO_3^- pollution on water chemistry, quantify NO_3^- pollution effects on bryophyte elemental concentration and stoichiometry, and identify N-sensitive species to increased NO_3^- pollution in terms of changing their elemental composition, and $\delta^{15}N$ in particular.

2. Methods

2.1 *Study area*

The 50-km wide study area extended along a 100-km longitudinal gradient in the north-eastern Iberian Peninsula, along the Serralada Litoral and across the regions of Montseny-Guilleries, Lluçanès, Moianès and Bages, Eastern Pyrenees and La Garrotxa that are characterised by contrasting climate and lithological conditions (Fig. S1). The Pyrenean climate is represented by cold winters and wet, cool summers, while lithology comprises plutonic and sedimentary rocks; Garrotxa climate conditions are continental and wet, while lithology is comprising calcareous and volcanic rocks; and climate of the Serralada Litoral Central is maritime Mediterranean and lithology is largely dominated by granite, with metamorphic rocks in the northeast (Sabater et al., 2015; Fernández-Martínez et al., 2016)The climate of Montseny-Guilleries and Lluçanès is sub-humid and lithology of Montseny is composed of granitic, metamorphic and calcareous rocks, while granite and other plutonic rocks dominate in Guilleries; lithology of Lluçanès-Moianès-Bages is entirely calcareous. These variations in lithology

affect the dissolved ion and chemical composition of spring water and associated levels of conductivity and pH (Sabater et al., 2015).

2.2 *Study sites*

We selected springs across the Serralada Litoral (N=32), and regions of Montseny-Guilleries (N=77), Lluçanès, Moianès and Bages (N=42), Eastern Pyrenees (N=6) and La Garrotxa (N=21). Study sites comprised point-sources of groundwater drained from aquifers or natural springs to an outflow and vegetation of the surrounding areas. The construction of the water point-sources were similar, comprising a rock wall, drainage channel and retaining sink fitted with an outflow spout. The area surrounding the water point-sources (c. 200–1000 m) was defined as the area of potential aquifer recharge and land use was classified in the field and using aerial imagery (http://www.icc.cat/vissir3/) as natural (forest, shrubland, non-grazed grassland), extensively farmed (livestock), intensively agriculture (cropland) or urbanized. Study site lithology was extracted from http://www.icgc.cat/ and classified according to Fernández-Martínez et al. (2019).

Using GPS geolocated coordinates of the water point-sources, study site monthly precipitation and temperature data were extracted from the digital Climatic Atlas of Catalonia (http://www.opengis.uab.cat/acdc/index.htm) and seasonal values were calculated as the average across March–May (spring), June–August (summer), September–November (autumn) and December–February (winter); given high levels of collinearity between climate variables, we used spring and summer values as determinants of spring water runoff, and we calculated seasonality of precipitation and temperature as the coefficient of variation of monthly values. Study site altitude was extracted using an elevation digital model with 30-m resolution, water availability was calculated as reference

177 evapotranspiration minus monthly precipitation (Hargreaves, 1994) and a binary
178 measure of shade was used as a proxy for insolation.

179 2.3 *Spring water sampling and analysis*

180 Electrical conductivity and pH of the spring water were measured in situ at the
181 study sites using a combined conductivity and pH meter (Hanna Instruments
182 model HI 98129, Woonsocket, Rhode Island, USA). We collected water samples
183 (Fig. S2) that were filtered at 0.45 µm and stored at -20°C prior to analysis.

184 Anion content (NO_3^-, sulphate-SO_4^{2-} and chloride-Cl^-) was measured using ionic
185 chromatography (STD_50ppm-CH2) and content of cations (potassium-K^+,
186 calcium-Ca^{2+}, sodium-Na^+ and magnesium-Mg^{2+}) was measured ion exchange
187 chromatography; we recorded PO_4^{3-} content using the colorimetric method
188 (Murphy and Riley, 1962). Concentrations of the metals arsenic (As), chromium
189 (Cr), nickel (Ni), cobalt (Co), iron (Fe), zinc (Zn), copper (Cu), manganese (Mn),
190 aluminium (Al), cadmium (Cd), mercury (Hg) and lead (Pb) were quantified using
191 inductively coupled plasma mass spectrometry (ICP-MS). Further details of water
192 chemical composition analysis are reported by Fernández-Martínez et al. (2019).

193 2.4 *Bryophyte sampling and analysis*

194 Across the study sites, we collected 253 bryophyte plants from 2013 to 2019
195 during spring, summer and autumn, representing 35 hygrophytic species (six
196 liverworts and 29 mosses) that were either in direct contact with the spring water
197 or within the splash zone; continuous contact with the water was interrupted only
198 in some springs, because of intense drought in the preceding summer, or due to
199 frozen water during winter. The bryophytes were identified to species according
200 to Casas et al. (2001) and Smith (2004), following nomenclature established by
201 Hill et al. (2006).

Following identification, samples were stored dry prior to chemical composition analyses, when they were submerged in a solution of acetic acid (pH 2.7) to remove incrustations of calcium carbonate ($CaCO_3$) and then rinsed using distilled water; samples were subsequently dried at 60°C for 48 h and ground to a powder in liquid N using a mortar and pestle. Elemental concentrations of C and N and the isotopic ratios of $\delta^{13}C$ and $\delta^{15}N$ were quantified using isotope ratio MS (IRMS) using a Flash EA1112 and TC/EA coupled to a Delta C stable-isotope mass spectrometer through a ConFlo III interface (Thermo Finnigan, Thermo Electron Corporation, Bremen, Germany). Analyses of $\delta^{13}C$ and $\delta^{15}N$ isotope ratios in bryophyte samples allows the influence of nitrogen pollution on nutrient cycling to be assessed. These measurements can provide information on the source of carbon and nitrogen they are using and how these nutrients are being transformed and mobilised in the ecosystem. In addition, $\delta^{13}C$ and $\delta^{15}N$ isotope ratios in bryophytes can also be used as a tool to assess the impact of nitrogen pollution on biodiversity and ecosystem health. Concentrations of Na, K, Mg, Ca, Cl, As, Cr, Ni, Co, Fe, Zn, Cu, Mn, Al, Cd, Hg and Pb were determined using inductively coupled plasma optical emission/mass (ICP-OES/MS) spectrophotometry (ELAN 600 and Optima 8300, Perkin Elmer, Waltham, Massachusetts, USA) following pre-digestion overnight at 90 °C in a 2:1 nitric acid:hydrogen peroxide solution.

During the autumn of 2020, we collected 105 bryophytes from 51 springs to test for elemental composition of leachates; samples comprised 16 species of moss and 9 species of liverwort. This campaign belonged to another project aiming to study bryophyte leachates and desiccation resistance. These bryophytes were maintained at room temperature for 24 h and then rinsed at low-intensity agitation

for 10 min using distilled water before they were dried at 50 °C for 48 h. Leachates were extracted by rehydrating each bryophyte in 100 mL of distilled water for 1 h, with mild agitation; following extraction, 18 samples that were incrusted with $CaCO_3$ were soaked in acetic acid (pH 2.7) for 1 h for its removal. Concentration of elemental cations and metals in the leachate solution was quantified using ICP-MS, as described above for spring water, including sulphur and phosphorus that had previously been analysed as SO_4^{2-} and PO_4^{3-}. Leachate content of total non-particulate organic carbon (NPOC) and total N (TN) was determined by (non-purgeable organic C, using the Analytik Jena-Analyser multi N/C 3100, Analytik Jena, Jena, Germany). Following leachate extraction, the bryophytes were dried at 60 °C for 48h to a constant weight and then ground to a powder prior to determination of elemental composition using IRM-MS and ICP-MS, as described above for the previous set of bryophyte samples.

We calculated total elemental composition of the bryophytes by summing together element masses extracted from each sample of bryophyte (concentration × sample dry weight) and the corresponding leachate (concentration × 100 mL) and then dividing by the total dry weight of the sample.

2.5 *Statistical analyses*

In this study, the objective of the PCA was to assess the effect of land use and NO_3^- pollution on groundwater chemical composition (beyond that of nitrate), as well as to identify the whether the concentration of other variables that were associated with nitrate pollution (Fig. 1A,B). We denoted the three PCA performed with water chemistry as PCWQ1, PCWQ2, and PCWQ3. PCWQ1 was associated with high values of K^+, Cl^-, SO_4^2, Na^+ and Mg^{2+}, while PCWQ2 was negatively related to metals, such as Fe, Co, Mn and Al, and PCWQ3 was

252 negatively related to Ni and Cu; PCs 1, 2 and 3 accounted for 27, 15, 10% of the
253 variation in spring water chemistry. To test for main effects of NO_3^- pollution on
254 water chemistry and elemental composition of spring water, we used linear
255 models in which the response variable comprised the first three axes of the PCA
256 for each element and the explanatory variables were NO_3^- concentration, mean
257 annual precipitation (MAP), lithology (a factor of N levels) and land use; MAP was
258 included, because it is negatively related to spring water solute content
259 (Fernández-Martínez et al., 2019). To test for main effects of land use on NO_3^-
260 pollution, we built a linear model in which NO_3^- was the response variable and
261 MAP, lithology and land use were explanatory variables. Variance explained by
262 the explanatory variables (R^2) was calculated using in the *relaimpo* R package
263 using the "*calc.relimp*" function, with the metric "*lmg*" (Ulrike Grömping, 2006).

264 We performed another PCA in order to reduce the dimensionality of the climate
265 dataset and take into account the influence of the climate on the elemental
266 composition of bryophytes while investigating the role of nitrate. The first climate
267 axis (Fig. 1C) was positively related to mean annual precipitation, mean annual
268 water availability, altitude, and seasonality in temperature (explaining 61%). The
269 second axis was positively related to MAT mean annual temperature, MAP and
270 WA mean annual water availability (explaining 16%). We additionally performed
271 a PCA to reduce the dimensionality of the bryophyte elemental composition
272 where the first two axes explained, respectively, 21% and 14% of the variability
273 in the data, (Fig. 1D). The first axis was positively related to C and K and
274 negatively related to Al, Fe, Co, Cr, Mn, Ni, As and Cu. The second axis was
275 positively related to Mg, P, S, Na and K and very weakly negatively related to Cu
276 and As.

To analyse the effect of nitrate pollution on bryophyte chemistry we performed mixed models for each element and the two first axes extracted for bryophyte chemistry where species, the field seasons (2013-2019, and 2020 field seasons) and the spring (site) were selected as random factors and land-use, shade, and the selected PCA axes of water chemistry (the first, second and third axis as water chemistry data as explanatory variables) and climate as explanatory variables (the first and second axes of the PCA performed with climate data as explanatory variables).

We used linear mixed models to test for sensitivity to N for species with sample N>10 (2 liverworts and 9 mosses), with response variables comprising bryophyte content of $\delta^{15}N$, C, N, P, and ratios of C:P, C:N and N:P, and PCBC1–2 for bryophyte chemistry, and explanatory variables comprising spring water NO_3^- content, PCWQ1 for water chemistry and PCC1 for climate effects on bryophyte chemistry, with campaign as a random factor. We used linear models to estimate sensitivity to N for species represented by data for a one single campaign.

Data for elemental concentrations were log-transformed prior to analysis, as were data for NO_3^- concentrations prior to analysis using linear models, to account for potential non-linear effects derived from saturated pollution; visual inspection of model residuals confirmed that all model assumptions were met. We applied the false discovery rate correction (Benjamini and Hochberg, 1995) to *p*-values to avoid alpha-inflation when fitting models with the same predictors for different response variables ("p.adjust" function in "stats" R package). All statistical analyses were performed using R (version 4.2.0; R Core Team, 2021).

3. Results

3.1 *Drivers of spring water chemistry*

Land use influenced spring water NO_3^- concentrations, particularly in agricultural and urban areas, while levels of NO_3^- were similar in extensively farmed and natural areas (Fig. 2A). The concentration of other elements in spring water, such as Na^+, Co, Mn and Pb, were also significantly affected by land use (Table 1). Because springs drain groundwater, the nitrate concentration in the spring water is equivalent to that of the groundwater feeding the spring. The effect of nitrogen-derived byproducts (e.g., manure), which not only contain NO_3^-, was low on water chemistry. However, NO_3^- can be an indicator that ions such as Cl^-, Cr, and Zn are positively associated, and Fe and Mn are negatively associated with groundwater contamination (Table 1). In contrast, lithology was a key driver of spring water chemical composition for all recorded elements, with the exception of Co, Zn and Al (Table 1). Springs subjected to larger amounts of precipitation generally presented lower concentrations of elements (including NO_3^-; Fig. 2B), except for PO_4^3 and Cr, for which the relationship was positive and As, Zn, Cu, Cd and Pb for which there was no significant relationship (Table 1).

3.2 *Drivers of bryophyte elemental composition*

The results of the mixed models showed that the effect of nitrate on bryophyte chemistry was relatively low (Table 2). Land use showed a significant effect on $\delta^{15}N$, presenting higher values in springs near crops and urban areas compared to those from natural or extensive farming areas. Bryophyte N concentration was higher in springs near urban areas compared to those in natural areas, and bryophyte Pb concentration was higher in springs near urban areas compared to those near crops or natural areas. Shaded springs presented lower values of $\delta^{15}N$ and P, and higher concentrations of C in the bryophytes compared to those living in springs in direct sunlight.

Nitrate pollution significantly increased bryophyte N and $\delta^{15}N$ concentration (Fig. 3), albeit the positive relationship between nitrate and bryophyte N concentration disappeared when considering other environmental factors (Table 2). Actually, the concentration of most elements in bryophytes did not show any significant relationship with the water chemistry of the springs, included in the models as the first three axes of a PCA (Table 2). PCWQ1, related to hard water with high K, Cl^-, SO_4^{2-}, Na and Mg concentration, was positively related to higher concentrations of Na, Ca and Cu in bryophytes. On the other hand, PCWQ2, which was negatively associated with heavy metals in spring water (Fe, Co, Mn and Al, see Figure 1A), was also negatively related to bryophyte concentrations of $\delta^{13}C$, Fe, Cd and Co, where a strong relationship was observed with Cd and Co (Table 2). Co, again, had a strong negative relationship in PCWQ3 (mainly driven by Cu and Ni but also Co), where a negative effect was also observed for Cd, C, P, Ni, Zn and $\delta^{15}N$ and the reaction was positive only for Mn (Table 2).

Climate, overall, presented a very weak relationship with bryophyte elemental composition. Our models indicated that only N and P presented positive and significant relationships with PCC2 (indicating rainy climate and harsh summer) and a negative and significant relationship for As. With PCC2 only Cu presented a positive relationship, indicating wet and cold weather.

3.3 Elemental composition responses of species to nitrate pollution

Although overall elemental composition or C:N:P ratios of the tested bryophytes did not strongly respond to spring water NO_3^- concentration, there were positive relationships between bryophyte $\delta^{15}N$ concentration and spring water

concentrations of NO_3^- for more than 50% of the species (Table 3; see Figure 4 for *Apopellia endiviifolia*). We also found a positive relationship with bryophyte N concentration in *Apopellia endiviifolia* and *Thamnobryum alopecurum* and a negative relation in *Oxyrrhynxium speciosum* (Table 3). Sensitivity refers to the responsiveness of bryophytes to nitrate concentrations in water, affecting their elemental composition and C:N:P ratios. *Apopellia endiviifolia* showed greater sensitivity to NO_3^- concentration in spring water, as there were positive relationships with $\delta^{15}N$, C and N concentrations, and a negative relationship with C:N ratios. *Plagiomnium undulatum* and *Brachythecium rivulare* showed a lack of sensitivity to groundwater NO_3^- content (Table 3).

4. Discussion

4.1 Environmental drivers of spring water chemistry

Intensive agriculture and urbanization increase N inputs to water bodies and contribute to the eutrophication of surface and coastal waters (Pierobon et al., 2013). Indeed, our results indicated that groundwater NO_3^- concentrations were higher in urbanised areas, and particularly high in intensively farmed areas, compared with natural and extensively farmed areas, likely due to leaching of N from inputs of slurry, wastewater and organic and inorganic fertilisers (Pierobon et al., 2013). In the study region, fine free-draining soils and karst aquifers coincide in agricultural areas, increasing the vulnerability of groundwater to NO_3^- pollution (Huebsch et al., 2014). Previous research has shown that NO_3^- pollution can interact with climate, as regions with larger precipitation tend to present lower concentrations of NO_3^- due to the diluting effect of precipitation (Fernández-Martínez et al., 2019). It should also be noted that areas with higher levels of

377 precipitation may experience greater leaching of N due to the increased amount
378 of water available to dissolve nitrogen compounds and transport them to
379 underground aquifers. Our results indicate that persistent NO_3^- pollution of
380 groundwater is the result of intensive anthropogenic land use (Egmond et al.,
381 2002).
382
383 In contrast to N, P did not appear to increase in spring water close to agricultural
384 lands (Table 1) even though P leaches into water courses due to fertiliser use
385 and erosion of soil in agricultural systems and through drainage of water in
386 urbanized areas. Our results clearly showed lower spring water N concentrations
387 in natural and extensively farmed areas (Figure 2) indicating lower inputs of N
388 and full ecosystem uptake of N. Our finding that bryophyte $\delta^{15}N$ concentration
389 was positively associated with levels of groundwater NO_3^- pollution (Table 2,
390 Figure 3 and 4) indicates that this is from animal sources of N, most likely as
391 manure fertiliser.
392
393 We found few changes in spring water chemical composition in response to NO_3^-
394 pollution, even though some sources of NO_3^- pollution, such as manure, could
395 leach other ions into groundwater. For example, levels of groundwater NO_3^-
396 pollution were positively related to content of Cl^-, Cr and Zn and negatively related
397 to concentrations of Fe and Mn (Table 1). Increases in Cl^- concentration with
398 levels of NO_3^- pollution may indicate waste water leakages, particularly in
399 urbanized and agricultural areas where chlorinated water is used, while increases
400 in Cr and Zn are likely to be related to fertiliser use and household activities (Khan
401 et al., 1981). However, the reason why Fe, Mn and other heavy metals decrease

in springs presenting nitrate pollution could be related to the fact that, in our dataset, nitrate pollution is mainly located in regions with carbonated lithology, which typically presents lower concentrations of heavy metals compared to granitic aquifers (Fernández-Martínez et al., 2019). Hence, these results should be interpreted with caution.

Nonetheless, the spring lithology played a very important role determining spring water chemistry. This result was expected given that some of the most significant elements come directly from the dissolution of the minerals within the aquifer. Variations in water chemistry show a clear correlation with regional lithological distribution and topography (Sabater et al., 2015; Thivya et al., 2013) i.e. the chemical quality of groundwater depends on the characteristics of the exposed soil and rock masses present along the path of the groundwater saturation zone (Foster et al., 2000; Thivya et al., 2013). Mean annual precipitation was also an important factor controlling spring water chemistry, mostly reducing the concentration of elements except for a few cases (e.g., phosphate). Our results fully support the dilution effect produced by the mixing of rain water with ground water which is one of the most important process controlling groundwater chemistry (Fernández-Martínez et al., 2019).

4.2 Impacts of spring water nitrate on bryophyte chemistry

Our analyses showed that nitrate pollution did not systematically alter the elemental composition of bryophytes beyond $\delta^{15}N$, and potentially N (Figure 4), when analysed all together (Table 2), even though several species presented strong responses to nitrate pollution (Table 3, Figure 5). Thus, our results

indicated that overall water chemistry and, to a lesser extent, land use may elicit stronger impacts on bryophyte chemistry than water concentrations of NO_3^-. While water chemistry is recognized as an important driver of bryophyte chemistry in the study area (Fernández-Martínez et al., 2021), the impacts of land use on aquatic and semi-aquatic bryophyte chemistry were unknown.

The nitrogen cycle is a complex process that involves multiple steps and transformations (Imsande and Touraine, 1994; Canfield et al., 2010). During these processes, nitrogen can be fractionated, meaning its heavy isotope (15N) can be preferentially absorbed or released depending on environmental conditions (Vitousek and Hobbie, 2000; Casciotti and McIlvin, 2007). The analysis of $\delta^{15}N$ content of bryophytes allows the identification of anthropogenic sources of N emissions (Solga, n.d.; Delgado et al., 2013; Varela et al., 2013; Izquieta-Rojano et al., 2016), with organic sources being indicated by values >0 (when N passes through animals, the lighter ^{14}N is absorbed preferentially thus releasing proportionally the heavier ^{15}N), whereas inorganic sources (fertilisers) are indicated by values ≈0 (levels of atmospheric N_2) (Robinson, 2001; Casciotti and McIlvin, 2007). We showed that increases in bryophyte $\delta^{15}N$ concentrations with levels of NO_3^- indicate that the N sources are most likely derived from farming or manure applications to agricultural fields.

4.3 Bryophytes as bioindicators of nitrate pollution

In a context of pollution, we refer to "elemental sensitivity" to the ability of certain plant or animal species to accumulate or respond to a specific element (Gaillardet et al., 2003; Gumienna-Kontecka et al., 2018), such as in this case, nitrogen in the form of NO_3^- (Frost et al., 2005; Hall et al., 2005). Of the eleven bryophyte

452 species, we found evidence for elemental sensitivity to NO_3^- pollution in five
453 mosses (*Rhynchostegium riparioides, Eucladium verticillatum*, *Didymodon*
454 *tophaceus, Cratoneuron filicinum, Oxyrrhynchium speciosum*) and a liverwort
455 (*Apopellia endiviifolia*), where sensitivity was greatest in *A. endiviifolia*. Previous
456 research has shown that *A. endiviifolia* is the most widespread and frequently
457 occurring species in the study region (Fernández-Martínez et al., 2020) and,
458 combined with its sensitivity to groundwater NO_3^- (Figure 4), we suggest this
459 species is an appropriate candidate as an indicator in NO_3^- biomonitoring
460 programmes in aquatic and semi-aquatic environments. The presence of *A.*
461 *endiviifolia* in an area can be an indirect indicator of the presence of nitrate in
462 groundwater or soil, as this species has been found in sites with high nitrate
463 concentrations. However, it is also true that tissue concentrations of *A. endiviifolia*
464 reflect environmental nitrate concentrations in groundwater, making it a direct
465 indicator of the presence of nitrate in water over long periods (Sossey Alaoui and
466 Rosillon, 2013; Puczko et al., 2018; Mohamed, 2020).
467
468 Our finding that $\delta^{15}N$ content in *O. speciosum, D. tophaceous, C. filicinum, E.*
469 *verticillatum* and *R. riparioides* was sensitive to groundwater levels of NO_3^- may
470 contribute to their consideration as bioindicator candidates, given their common
471 occurrence in aquatic and semi-aquatic habitats (Loo et al., 2008; Fernández-
472 Martínez et al., 2020; Rimac et al., 2022a, 2022b) and known ecology. For
473 example, *R. riparioides* is a widely used bioindicator of heavy metals (Carballeira
474 and López, 1997; Samecka-Cymerman et al., 2002), including Cu (Claveri and
475 Mouvet, 1995), eutrophic waters (Vanderpoorten et al., 2013), where it is
476 positively associated with P, K and N (García-Álvaro et al., 2000), and some rare

earth elements (Pratas et al., 2017), while *C. filicinum* and *E. verticillatum* are associated with oligotrophic waters (Ceschin et al., 2012). We found that just two species showed sensitivity in tissue N concentration to NO_3^- pollution (*T. alopecurum* and *A. endiviifolia*); *T. alopecurum* has been used to biomonitor metal pollution (Pratas et al., 2017) and atmospheric depositions of Zn and Fe (Cojocareanu et al., 2010).

Overall, our results indicate that the analysis of $\delta^{15}N$ and N concentration of bryophytes by IRMS, rather than a comprehensive elemental analysis using ICP-MS, represents a cost-effective and effective approach to NO_3^- pollution monitoring in aquatic and semi-aquatic habitats. Biomonitoring of bioindicator species such as *A. endiviifolia* is a useful and effective way to assess water quality and detect the presence of pollutants (Parzych et al., 2018), as these species can reflect environmental conditions over time and space (Elser et al., 2010; Gecheva and Yurukova, 2013, 2014; Vieira et al., 2018), and are able to accumulate and magnify contaminants in their tissues (Shacklette and Erdman, 1982; Basile et al., 2012; Favas et al., 2018)**.**

CRediT authorship contribution statement

AM, JP, FS and MFM planned and designed the study. AM, JC, OC, CP, FS and MFM, conducted fieldwork and laboratory analyses. AM analysed data. All authors contributed to writing the manuscript.

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Figure captions

Figure 1: Biplots showing the results of the PCAs performed with water chemistry from 178 spring (panels A and B), climate (panel C) and chemistry of 358 bryophyte samples (panel D). Dots show PCA scores for springs in panels A, B and C (coloured by land use for A and B) and bryophyte sample scores in panel D (bottom and left axes). Arrow length and orientation from the origin indicate variable loading and direction of effect, respectively. MAP: mean annual precipitation; WA: mean annual water availability; WAS: seasonality of water availability; MAT: mean annual temperature; TS: temperature seasonality; and PS: precipitation seasonality.

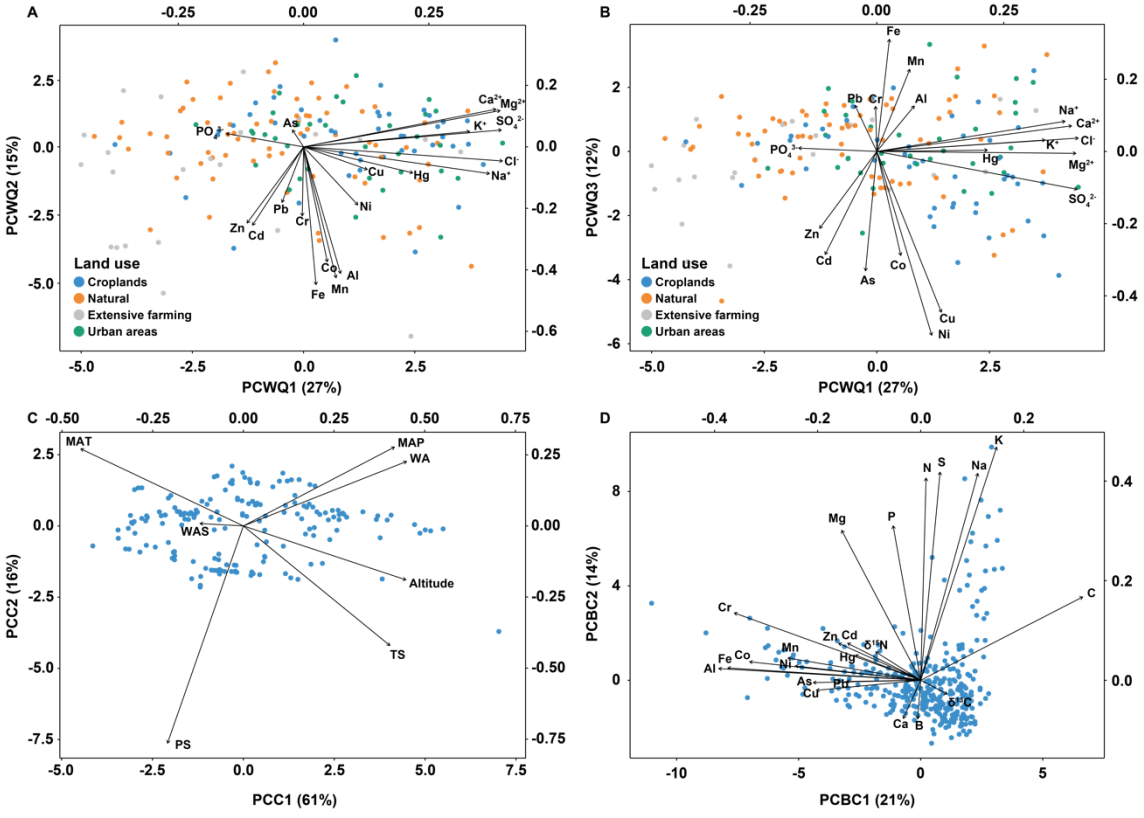


Figure 2. Estimated effects of land use (A) and mean annual precipitation (B) on 178 spring water nitrate concentrations. *β* indicates standardised parameter estimates (±SEM).

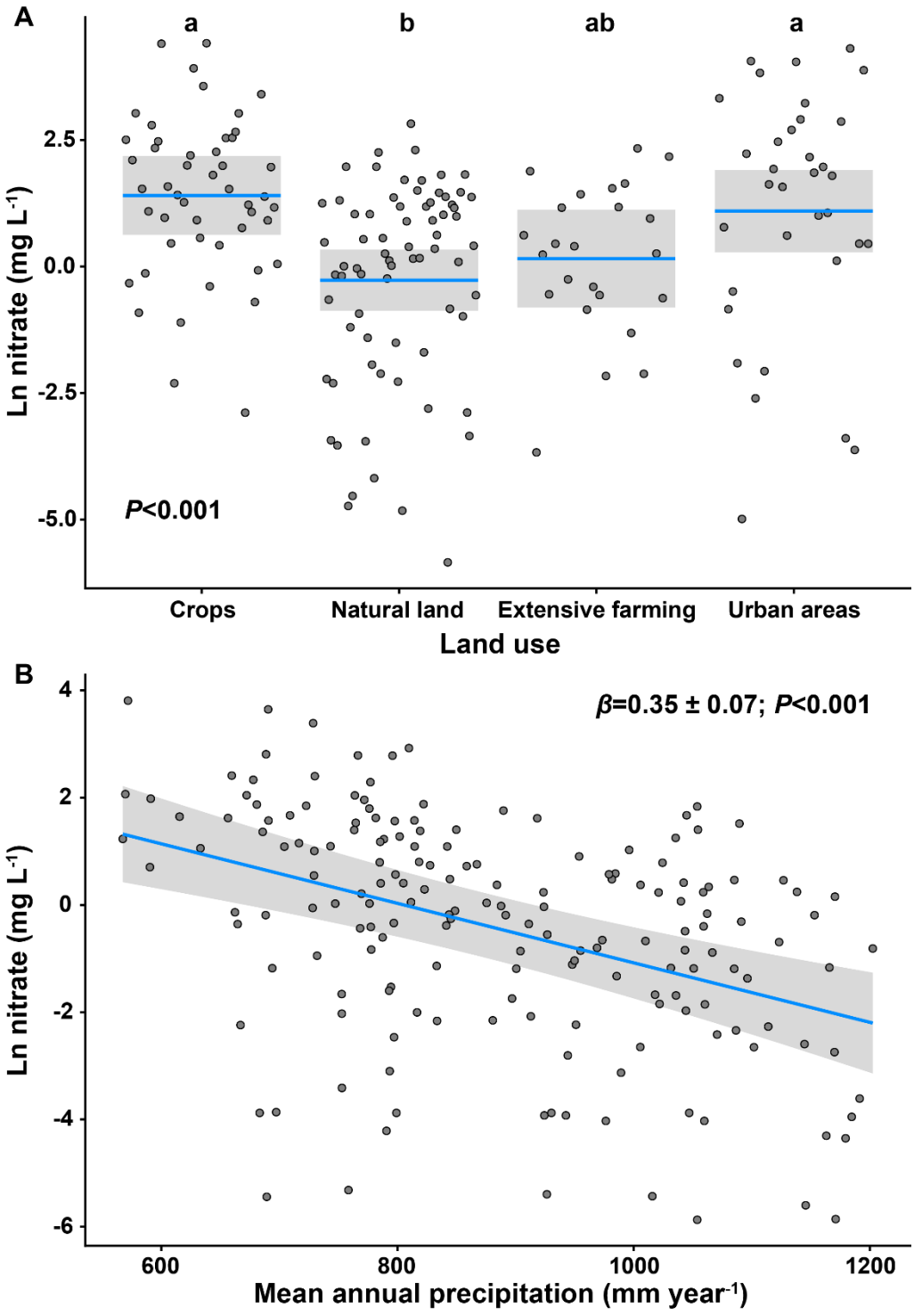


Figure 3. Partial residual plots of univariate mixed model estimates of N (panel A) and $\delta^{15}N$ (panel B) concentrations of the 358 bryophyte samples as a function of spring water nitrate concentrations. Data for N and $\delta^{15}N$ were log and log(*x*+8) transformed, respectively, to avoid negative values. Marginal (nitrate effect only) and conditional (including random factors) R^2 were, respectively, 0.02 and 0.60 for bryophyte N concentration and 0.11 and 0.80 for $\delta^{15}N$.

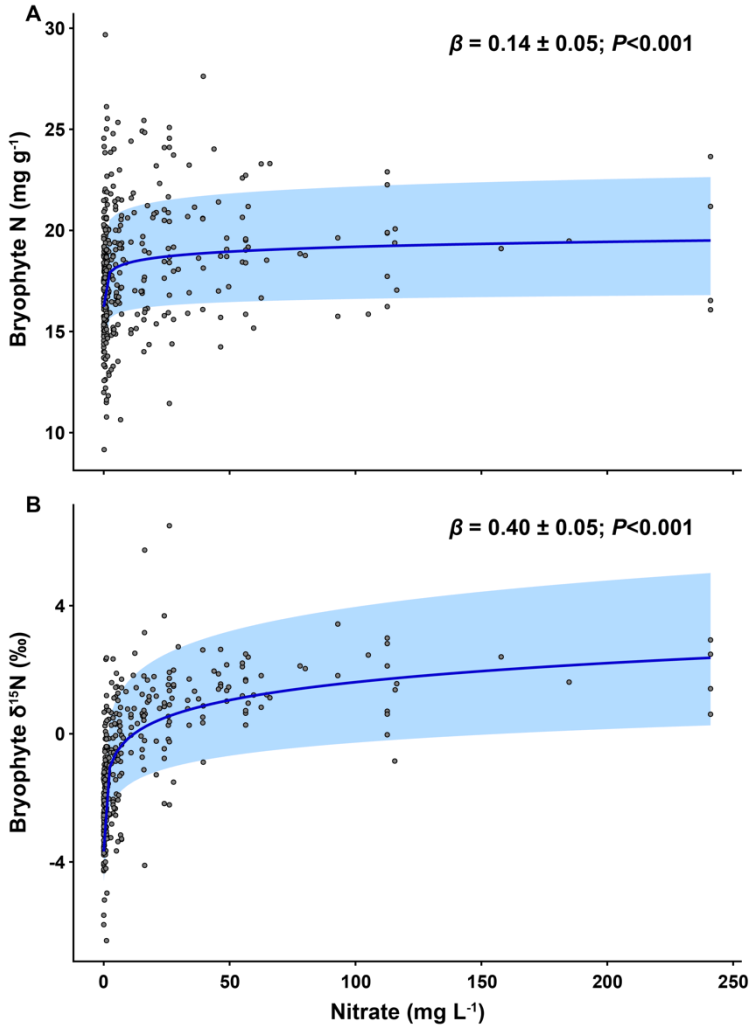
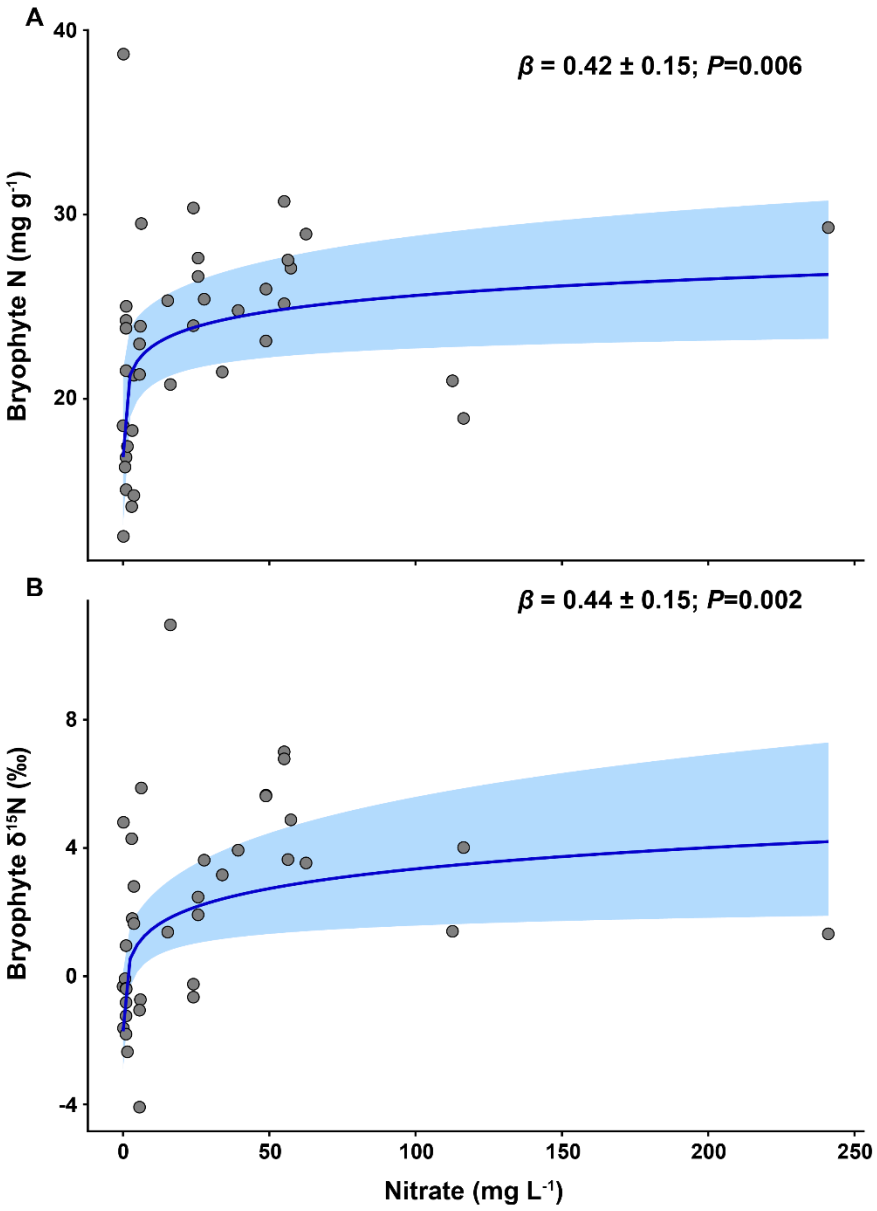


Figure 4. Partial residual plots of univariate mixed model estimates for N (panel A) and $\delta^{15}N$ (panel B) concentrations of 38 *Apopellia endiviifolia* samples as a function of groundwater nitrate concentrations. Data for N and $\delta^{15}N$ were log and log(*x*+5) transformed, respectively, to avoid negative values. Marginal (nitrate effect only) and conditional (including random factors) R^2 were, respectively, 0.17 and 0.24 for bryophyte N concentration and 0.20 and 0.20 for $\delta^{15}N$.



875 **Table 1:** Linear model standardised coefficients of variation (±SEM) in water
876 chemistry. Explained predictor variability (R^2) is in parentheses; *P*-values from
877 mean annual precipitation (MAP), lithology and land use were adjusted for 23
878 models using the false discovery rate correction, while *P*-values for nitrate were
879 adjusted for 22 models. Ranked land use differences at $P < 0.05$ are shown (C :
880 crops; N: natural areas; E: extensive farming; and, U: to urbanized areas); ***$P <$
881 0.001, **$P < 0.01$ and *$P < 0.05$.

	MAP	Nitrate	Lithology	Land use
PCWQ1	**-0.61 ± 0.05, (0.29)*** **	0.03 ± 0.06 (0.08).	**(0.22) *** **	(0.07)
PCWQ2	**0.26 ± 0.09, (0.03)** **	0.05 ± 0.09 (0.00)	**(0.13)** **	**(0.06)**, N>E**
PCWQ3	-0.0 1± 0.07, (0.06)	**-0.25 ± 0.07 (0.06)** **	**(0.3)*** **	**(0.04)*, U>E**
NO_3^-	**-0.35 ± 0.07 (0.12) *** **	-	**(0.15)*** **	**(0.14)***, C>N; U>N**
Na^+	**-0.58 ± 0.06, (0.26)*** **	-0.008 ±0.06, (0.06)	**(0.16)*** **	**(0.12)***, U>N; U>E**
K^+	**-0.23 ± 0.07 (0.06) *** **	0.12 ± 0.07 (0.08)	**(0.29)*** **	(0.06)
Mg_2^+	**-0.49 ± 0.06 (0.2)*** **	-0.03 ± 0.06, (0.05)	**(0.26)*** **	(0.07)
Ca_2^+	**-0.41 ± 0.07 (0.14) *** **	-0.06 ± 0.07, (0.04)	**(0.29) *** **	(0.06)
Cl-	**-0.58 ± 0.05 (0.29)*** **	**0.12 ± 0.06 (0.11)***	**(0.16)*** **	(0.09)
SO_4^{2-}	**-0.51 ± 0.06 (0.22) *** **	0.09 ± 0.06 (0.09)	**(0.22)*** **	(0.05)
PO_4^3	**0.22 ± 0.09 (0.04)***	0.04 ± 0.09 (0.00)	**(0.13)** **	(0.01)
As	0.08 ± 0.08 (0.00)	0.16 ± 0.08, (0.02)	**(0.32)*** **	(0.00)
Cr	**0.26 ± 0.09, 0.03** **	**0.30 ± 0.09 (0.03)** **	**(0.09)** **	(0.00)
Ni	**-0.25 ± 0.08, (0.04)** **	0.11 ± 0.09. (0.03)	**(0.15)*** **	(0.02)
Co	**-0.21 ± 0.09 (0.01)***	0.02 ± 0.09 (0.00)	(0.01)	**(0.13)***, E>C; E>N; E>U**
Fe	**-0.31 ± 0.08 (0.05)*** **	**-0.21 ± 0.09 (0.04)***	**(0.16)** **	(0.02)
Cu	-0.12 ± 0.08, (0.01)	0.08 ± 0.08, (0.04)	**(0.24)*** **	(0.04)
Mn	**-0.24 ± 0.08, (0.02)** **	**-0.23 ± 0.09, (0.03)***	**(0.12)** **	**(0.04)*, E>N**
Al	**-0.39 ± 0.09, (0.07)*** **	-0.11 ± 0.09, (0.00)	(0.06)	(0.01)
Cd	-0.04 ± 0.09, (0.00)	0.03 ± 0.10, (0.00)	**(0.11)***	(0.01)
Hg	**-0.59 ± 0.07, (0.29)*** **	0.06 ± 0.07, (0.03)	**(0.09)***	(0.02)
Pb	0.01 ± 0.08, (0.00)	-0.08 ± 0.09, (0.00)	**(0.18)*** **	**(0.06)**, U>N, U>E**
Zn	0.13 ± 0.09 (0.01)	**0.23 ± 0.09 (0.02)***	(0.08)	(0.01)

882

Table 2: Standardised model coefficients (± standard error of the mean) of linear mixed models explaining the effect of nitrate on bryophyte chemistry. PCWQ1, PCWQ2, PQWQ3, PCC1, PC2 indicate values for PCA where WQ is water chemistry and C is climate. Ranked land use differences (C: crops: N: natural areas; E: extensive farming; and, U: urbanized areas) and differences between shaded (Y) and non-shaded (N) springs at $P < 0.05$ are shown. *P*-values were adjusted for 23 models using the false discovery rate correction. ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$.

	Nitrate	Land use	Shade	PCWQ1	PCWQ2	PCWQ3	PCC1	PCC2
PCBC1	0.02±0.07	-	-	-0.06±0.10	**0.18±0.06****	0.06±0.06	-0.10±0.10	-0.04±0.06
PCBC2	-0.04±0.05	-	-	0.07±0.07	-0.05±0.04	**-0.10±0.05***	-0.09±0.07	0.02±0.05
$\delta^{15}N$	**0.18 ± 0.06****	***, C>N, U>N, U>E	**, N>Y	**0.23 ±0.09 ***	0.03± 0.05	**-0.13 ± 0.06***	0.00 ± 0.09	-0.06 ± 0.06
$\delta^{13}C$	-0.08 ± 0.06	-	-	**-0.21±0.09***	**-0.14±0.05***	0.06 ± 0.06	0.05 ± 0.09	-0.09 ± 0.06
C	0.01 ± 0.06	-	**, Y>N	-0.04 ± 0.08	0.06 ± 0.05	**-0.11 ± 0.05***	-0.00±0.08	0.02 ± 0.05
N	0.07 ± 0.06	***, U>N	-	-0.13 ± 0.09	-0.08 ± 0.05	-0.08 ± 0.05	-0.10±0.09	**0.12 ± 0.05***
P	0.01 ± 0.06	-	*, N>Y	-0.07 ± 0.09	-0.10 ± 0.05	**-0.17 ± 0.06****	-0.02±0.09	**0.21±40.06*****
Na	-0.10 ± 0.05	-	-	**0.16 ±0.07****	-0.05 ± 0.04	0.06 ± 0.05	0.00 ± 0.07	-0.08 ± 0.05
K	-0.09 ± 0.05	-	-	0.06 ± 0.07	0.04 ± 0.04	-0.05 ± 0.04	-0.07±0.07	-0.03 ± 0.04
Mg	0.05 ± 0.07	-	-	0.08 ± 0.10	0.04 ± 0.06	0.00 ± 0.06	-0.04±0.10	-0.03 ± 0.06
Ca	-0.07 ± 0.08	-	-	**0.37 ± 0.11****	0.07 ± 0.06	-0.06 ± 0.07	-0.03±0.11	-0.04 ± 0.07
S	-0.11 ± 0.06	-	-	**0.30 ± .08*****	0.02 ± 0.04	-0.05±0.05	-0.10±0.08	-0.04 ± 0.05.
As	0.04 ± 0.12	-	-	-0.15 ± 0.17	0.12 ± 0.10	0.00± 0.11	0.09±0.17	**-0.25 ± 0.11***
Cr	-0.03 ± 0.08	-	-	0.05 ± 0.12	-0.02 ± 0.07	-0.09 ± 0.08	0.07 ± 0.12	0.09 ± 0.08
Ni	-0.09 ± 0.08	*, none	-	0.07 ± 0.11	**-0.18 ± 0.06****	**-0.32 ± 0.07*****	0.03 ± 0.12	0.09 ± 0.07
Co	-0.10 ± 0.07	-	-	0.02 ± 0.10	**-0.33 ± 0.06*****	**-0.24 ± 0.06*****	0.14 ± 0.10	0.10 ± 0.06
Fe	-0.003 ± 0.08	-	-	0.03 ± 0.12	**-0.18 ± 0.07***	0.14 ± 0.08	0.07 ± 0.13	0.03 ± 0.08
Cu	**-0.18 ± 0.08***	-	-	**0.48 ± 0.11*****	0.01 ± 0.06	-0.07 ± 0.07	**0.38 ± 0.11*****	0.09 ± 0.07
Mn	-0.01 ± 0.08	-	-	0.23 ± 0.12	**-0.26 ± 0.07*****	**0.17 ±0.08***	0.13 ± 0.12	0.03 ± 0.07
Al	0.02 ± 0.08	-	-	-0.03 ± 0.11	-0.11 ± 0.06	0.07 ± 0.07	-0.00±0.12	0.04 ± 0.07
B	-0.08 ± 0.10	-	-	0.23 ± 0.15	-0.11 ± 0.09	0.08 ± 0.10	-0.14±0.16	-0.11 ± 0.10.
Cd	-0.02 ± 0.06	-	-	**-0.23 ± 0.09****	**-0.17 ± 0.05*****	**-0.35 ± 0.05****	-0.04±0.09	0.07 ± 0.05
Hg	0.04 ± 0.07	-	-	0.02 ± 0.09	-0.07 ± 0.05	0.00 ± 0.06	0.09 ± 0.09	-0.04 ± 0.06
Pb	0.02 ± 0.07	*, U>C, U>N	-	0.01 ± 0.10	-0.07 ± 0.05	-0.07 ± 0.06	0.11 ± 0.10	-0.09 ± 0.06

Zn	-0.01 ± 0.06	-	-	-0.10 ± 0.09	**-0.12 ± 0.05***	**-0.17 ± 0.05****	-0.02±0.09	0.08 ± 0.05

892 **Table 3:** Linear model standardised coefficients of variation (±SEM) in effects of
893 host site spring water chemistry on bryophyte species chemical composition.
894 PCBC1 and 2 are principal components the PCA of bryophyte elemental
895 composition. *P*-values were adjusted for 9 models using the false discovery rate
896 correction. ****P* < 0.001, ***P* < 0.01, **P* < 0.05.
897

	PCBC1	**PCBC2**	**$\delta^{15}N$**	**C**	**P**	**N**	**C:N**	**N:P**	**C:P**
Apopellia endiviifolia	0.22± 0.11	0.07 ± 0.07	**0.40 ± 0.13****	**0.26 ± 0.10***	-0.15 ± 0.16	**0.35 ± 0.15***	**-0.48 ± 0.14*****	0.12 ± 0.16	-0.14 ± 0.16
Conocephalum conicum	-0.04 ± 0.14	0.13 ± 0.13	0.16 ± 0.14	0.06 ± 0.16	0.09 ± 0.15	0.26 ± 0.15	**-0.41 ± 0.16****	-0.14 ± 0.18	-0.25 ± 0.17
Oxyrrhynchium speciosum	-0.11 ± 0.17	-0.004 ± 0.17	**0.57± 0.13*****	-0.23 ± 0.17	-0.04 ±0.17	**-0.15 ± 0.13*****	**0.03 ± 0.13*****	0.02 ± 0.18	0.06 ± 0.17
Plagiomnium undulatum	-0.04 ± 0.18	0.29 ± 0.17	0.24 ± 0.17	0.15 ± 0.16	0.07 ± 0.19	0.30 ± 0.15	-0.28 ± 0.16	-0.17 ± 0.19	-0.32 ± 0.19
Didymodon tophaceus	0.22 ± 0.18	-0.13 ± 0.14	**0.45 ± 0.16****	-0.12 ± 0.18	-0.12 ± 0.18	0.01 ± 0.15	-0.02 ± 0.18	0.04 ± 0.19	-0.05 ± 0.18
Cratoneuron filicinum	-0.17 ± 0.24	0.18 ± 0.24	**0.55 ± 0.23***	0.09 ± 0.25	0.09 ± 0.25	-0.22 ± 0.25	0.18 ± 0.26	-0.08 ± 0.26	0.04 ± 0.26
Eucladium verticillatum	0.32 ± 0.20	0.05 ± 0.21	**0.48 ± 0.19***	-0.18 ± 0.21	-0.18 ± 0.21	0.25 ± 0.21	-0.13 ± 0.22	0.31 ± 0.21	0.30 ± 0.21
Rhynchostegium riparioides	0.24 ± 0.33	-0.09 ± 0.36	**0.59 ± 0.21***	0.23 ± 0.36	0.23 ± 0.36	-0.30 ± 0.36	0.17 ± 0.36	**-0.69 ± 0.32***	-0.59 ± 0.33
Palustriella commutata	0.09 ± 0.31	0.56 ± 0.28	0.53 ± 0.28	0.61 ± 0.28n	**0.61 ± 0.28***	0.55 ± 0.28	-0.37 ± 0.30	-0.40 ± 0.30	-0.44 ± 0.29
Brachythecium rivulare	-0.08 ± 0.32	0.03 ± 0.27	0.18 ± 0.26.	-0.004 ± 0.32	0.11 ± 0.27	-0.00 ± 0.27	0.07 ± 0.28	0.03 ± 0.31	0.07 ± 0.29
Thamnobryum alopecurum	-0.38 ± 0.43	0.59 ± 0.43	-0.32 ± 0.35	0.45 ±0.41n.s	0.75 ± 0.41	**0.99 ± 0.45***	**-0.92 ± 0.46***	0.15 ± 0.35.	-0.30 ± 0.42

898

Supplementary material

Figure S1: Location of the study sites in Catalonia.

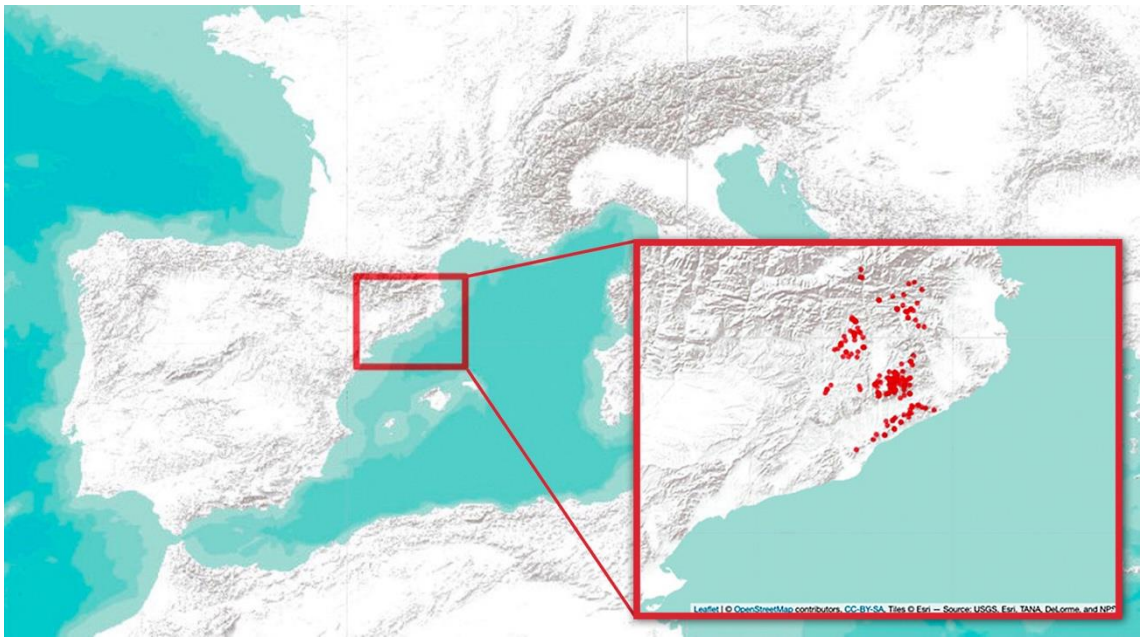


Figure S2: Image of a typical study site and spring water outflow. The white line delimits the area of influence of the water, within which bryophytes were collected.