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Herbaria century record of increasing eutrophication in Spanish terrestrial ecosystems

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Abstract

Additional biological evidence is presented for the alteration of biogeochemical cycles by human activities. The leaf $\delta^{15}\text{N}$ and the concentrations of nutrients in herbarium specimens of 24 species of vascular plants and 3 species of bryophytes collected in northern and eastern regions of Spain have substantially changed throughout the XX century. In the second half of the century, when anthropogenic nitrogen fixation and mobilization started to increase rapidly, leaf $\delta^{15}\text{N}$ values started to decrease strongly, indicating that additional anthropogenic nitrogen is being retained in Spanish terrestrial ecosystems. The concentration of nutrients in vascular plants did not present any clear pattern, but there were increasing concentrations of N and other nutrients (P, K, and S) in the last decades in bryophytes, which are usually better biomonitors of airborne chemicals than vascular plants. Important consequences for ecosystem structure and functioning such as enhancement of the carbon sink or changes in community biodiversity and species distribution may be expected from this increase in eutrophication.

Keywords: bryophytes, $\delta^{15}\text{N}$, nitrogen, nutrients, vascular plants

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Introduction

Global biogeochemical cycles are being altered strongly by anthropic activities. The global N cycle has now reached the point where more N is fixed annually by human-driven processes (fertilisers, combustion of fossil fuels, legume crops) than by natural ones (Vitousek *et al.* 1997). In addition to fixing nitrogen, human activity mobilizes nitrogen from long-term storage pools through biomass burning, land clearing and conversion, and drainage of wetlands (Crutzen & Andreae 1990; Vitousek *et al.* 1997). Both newly fixed and mobilized nitrogen can be transported in solution or in the atmosphere and thus alterations to the N cycle can spread regionally and globally. Such an enormous increase in the eutrophication by biologically available forms of this often-limiting nutrient will affect species composition and ecosystem functioning (Melillo *et al.* 1993; Vitousek *et al.* 1997). Similar reasoning can be applied to the concentrations of other nutrients such as phosphorus emitted from industrial activities, detergent production and agricul-

ture, or sulphur emitted from coal and residual fuel oil combustion. Their emissions have increased in Europe throughout the century up to the 1980s (European Environment Agency 1998).

Because of their capacity to act as efficient interceptors and accumulators of chemicals, plants are used widely as passive biomonitors in urban and rural environments (Jones *et al.* 1992; Markert 1993; Monaci & Bargagli 1997). Among plants, bryophytes are being used increasingly as biomonitors of airborne chemicals (Rühling 1994). Because they lack a root system, they are dependent on the aerial environment to which they are exposed for the uptake of chemical substances. Moreover, they lack a cuticle and epidermis and an active mechanism to mobilize chemicals. These traits facilitate chemical penetration and the integration of temporal fluctuations of chemicals in the environment.

With the aim of studying possible plant chemistry changes in response to the huge input of anthropogenic nutrients into the biosphere, herbarium specimens of 24 species of vascular plants and 3 species of bryophytes collected throughout the XX century in Spain were

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analysed. In order to determine whether there was a huge input of anthropogenic nitrogen, and if so, whether the additional anthropogenic nitrogen was retained in terrestrial ecosystems or lost by denitrification to N_2 , volatilization of nitrogen oxides and ammonia, or lixiviation to aquatic systems, leaf $\delta^{15}N$ and N concentration were analysed in the herbarium specimens. As the $\delta^{15}N$ value of a plant sample is determined primarily by the isotope ratio of the nitrogen source (Nadelhoffer & Fry 1994; Peñuelas & Estiarte 1997), especially when the N supply rate is low in relation to plant demand (Högberg *et al.* 1999) as it is the case in Spanish ecosystems (Rodà *et al.* 2000), leaf $\delta^{15}N$ was used to make inferences on the N source and on the relative activity of the different processes of the N cycle (supplies, retention, and losses) in the surrounding system. Long-term measurements of precipitation nitrogen in Europe, Africa and North America suggest that both ammonium and nitrate inputs to terrestrial ecosystems are relatively depleted in ^{15}N , with values for these inputs ranging from roughly -10 – 0 ‰ (Nadelhoffer & Fry 1994). The $\delta^{15}N$ in nitrate from alpine and polar ice cores has been found to decrease together with increasing nitrate concentrations after 1950, when anthropogenic emissions of nitrogen started to increase rapidly in the Northern Hemisphere (Freyer *et al.* 1996). Based on these facts, it was hypothesized that the increase of anthropogenic N inputs would be translated into a decrease of plant $\delta^{15}N$, especially if this N is retained instead of being lost from terrestrial ecosystems. The P, K and S concentrations of the herbarium specimens were measured to test whether ecosystem inputs of other nutrients from fossil fuel combustion, fertilisers, industrial sources, or urban wastewater (e.g. detergents) were also increasing.

Materials and methods

Herbarium specimens

The vascular plants studied were: *Acer opalus*, *Arbutus unedo*, *Borago officinalis*, *Cistus albidus*, *Chenopodium ambrosoides*, *Erica multiflora*, *Fagus sylvatica*, *Fraxinus excelsior*, *Hedera helix*, *Juniperus oxycedrus*, *Laurus nobilis*, *Lonicera implexa*, *Phillyrea angustifolia*, *Pinus sylvestris*, *Plantago major*, *Populus alba*, *Quercus coccifera*, *Q. ilex*, *Rhamnus alaternus*, *Rhododendron ferrugineum*, *Rosmarinus officinalis*, *Ruscus aculeatus*, *Salvia officinalis*, and *Thymus vulgaris*; and the bryophytes studied were *Grimmia laevis*, *Hypnum cupressiforme*, and *Pleurochaete squarrosa*. Leaves had been stored in the herbaria of the 'Unitat de Botànica de la Facultat de Farmàcia de la Universitat de Barcelona' and the 'Unitat de Botànica de la Universitat Autònoma de Barcelona'. The studied her-

barium specimens were collected in Spain (mostly northeastern rural regions). The older specimens had been fumigated with DDT and most specimens had been submitted to temporary freezing temperatures as conservation procedures. Material was sampled from four different decades; for the most recent one specimens already collected and stored for more than 5 years were sampled in order to avoid possible disturbances by initial processes of decomposition. The same locations and seasons for each species were sampled throughout the studied periods. When no samples from the same location could be found throughout the century in the herbarium collections, the closest possible geographical location was sampled. Leaves from the same terminal position of the herbarium specimen were analysed to avoid, as much as possible, variability resulting from leaf age, position and growth light environment of the collected herbarium specimens. Analyses from 1 to 10 different herbarium specimens were conducted for each vascular plant species at each sampling decade (Table 1). Analyses from 4 to 9 different herbarium specimens were conducted for each bryophyte species at each sampling decade (Table 2). Each analysis was conducted with 3–6 bulked leaves (40–60 for bryophytes) of the same stage of development. The drying procedure was similar for all specimens. They had been herbarium pressed when collected, and prior to the current analyses bryophyte specimens were freed by hand of extraneous material still adhering to the leaves by using plastic tweezers and disposable plastic gloves. Thereafter, they were washed with deionized water, oven-dried at 60 °C to constant weight (dry weight) and ground to a fine powder in a mill.

Isotope and elemental analyses

The $\delta^{15}N$ ratios were measured on a Delta C mass spectrometer (Finnigan Mat, Bremen, Germany) operated in continuous flow mode after combustion of the samples in a C and N elemental analyser (NA1500, Series 1, Carlo Erba Instrumentazioni, Milan, Italy). Results of ^{15}N natural abundance were expressed in δ -values (Nadelhoffer & Fry 1994). The $\delta^{15}N$ is expressed in parts per thousand relative to atmospheric N_2 : $\delta^{15}N = (R_{\text{sample}}/R_{\text{atmosphere}} - 1) \times 1000$ ‰, in which R_{sample} and $R_{\text{atmosphere}}$ are the $^{15}N/^{14}N$ ratios of the sample and the atmospheric N_2 standard, respectively. The analytical precision was in general better than 0.2 ‰.

Leaf concentrations of P, K and S were measured on subsamples (about 100 mg) of ground leaves. Samples were digested in a microwave Whirlpool AVM 635 using open fluorinated ethylene propylene flasks (Nalge Company, Rochester) with an acid solution of 2:1 nitric and 60% perchloric acids. Once samples were efficiently

Table 1 Changes in leaf $\delta^{15}\text{N}$ and leaf N concentration in 24 vascular plant species collected in Spain in four different decades throughout the XX century. Values are means, with SEM in parentheses. Period values not significantly different at the $P < 0.05$ level are followed by the same letter (anova followed by Tukey's test). n- number of samples

Species	Period	$\delta^{15}\text{N}$ (‰)	N (% DW)	n
<i>Acer opalus</i>	1920–30	0.97 (2.71)a	2.53 (0.01)a	3
	1945–55	-0.43 (0.38)a	2.33 (0.19)a	4
	1960–70	-2.05	2.60	1
	1985–95	-2.15	2.01	1
<i>Arbutus unedo</i>	1945–55	0.70 (0.92)a	1.76 (0.09)a	5
	1960–70	-1.63	1.83	1
	1985–95	-5.87 (0.19)b	1.14 (0.01)b	8
<i>Borago officinalis</i>	1920–30	5.20 (1.74)a	2.43 (0.13)a	4
	1945–55	2.60 (0.46)a	2.15 (0.12)b	4
	1985–95	0.00 (2.20)a	2.65 (0.16)a	4
<i>Chenopodium ambrosoides</i>	1920–30	9.20 (1.84)a	3.12 (0.14)a	4
	1945–55	9.80 (2.27)a	4.70 (0.56)b	4
	1985–95	1.00 (1.05)b	2.81 (0.25)a	4
<i>Cistus albidus</i>	1920–30	1.90 (0.16)a	1.62 (0.01)a	8
	1945–54	0.79 (0.07)b	1.50 (0.07)a	8
	1960–70	-0.22	0.67	1
	1985–95	-4.07 (0.27)c	1.84 (0.15)a	6
<i>Erica multiflora</i>	1920–30	0.08 (1.96)a	1.23 (0.09)a	4
	1945–55	4.40 (0.58)a	1.00 (0.06)a	3
	1960–70	2.67 (1.82)a	1.24 (0.57)a	2
	1985–95	-0.45	0.97	1
<i>Fagus sylvatica</i>	1920–30	0.30 (0.20)a	3.79 (0.06)a	4
	1945–55	-0.50 (0.30)a	3.22 (0.16)b	4
	1985–95	-5.10 (1.81)b	2.95 (0.14)b	4
<i>Fraxinus excelsior</i>	1920–30	-0.50 (0.22)a	3.22 (0.11)a,b	4
	1945–55	-0.50 (0.29)a	3.00 (0.13)a	7
	1985–95	-0.07 (0.04)a	3.40 (0.13)b	8
<i>Hedera helix</i>	1920–30	4.80 (1.84)a	2.00 (0.14)a	4
	1945–55	4.50 (1.45)a	2.10 (0.17)a	4
	1985–95	2.90 (0.20)a	1.65 (0.15)a	4
<i>Juniperus oxycedrus</i>	1920–30	-2.90	1.20	1
	1945–55	1.55 (2.82)a	0.90	2
	1985–95	-4.27 (0.57)a	1.84 (0.05)	3
<i>Laurus nobilis</i>	1920–30	6.50 (1.33)a	3.39 (0.57)a	4
	1945–55	1.30 (0.46)b	3.20 (0.49)a	4
	1985–95	0.00 (1.58)b	1.94 (0.08)b	4
<i>Lonicera implexa</i>	1920–30	0.10 (0.75)a	1.73 (0.12)a	3
	1945–55	-1.55 (2.65)a,b	1.45 (0.25)a	2
	1985–95	-3.20 (0.59)b	1.28 (0.02)a	2
<i>Phillyrea angustifolia</i>	1920–30	0.15 (0.75)a	1.30 (0.20)a	2
	1945–55	1.83 (0.33)a	1.40 (0.21)a	3
	1960–70	2.39 (3.48)a,b	1.46 (0.37)a	3
	1985–95	-5.01 (0.75)b	1.04 (0.08)a	4
<i>Pinus sylvestris</i>	1945–55	-2.70	2.00	1
	1985–95	-4.58 (2.08)	1.43 (0.17)	2
<i>Plantago major</i>	1920–30	4.20	1.90	1
	1945–55	6.53 (3.72)a	2.37 (0.54)a	3
	1960–70	0.89	2.27	1
	1985–95	2.50 (0.40)a	1.55 (0.65)a	2
<i>Populus alba</i>	1945–55	4.75 (0.35)a	3.10 (0.40)a	2
	1985–95	1.07 (0.28)b	1.46 (0.13)b	2
<i>Quercus coccifera</i>	1920–30	4.20	1.40	1
	1945–55	5.90	1.10	1
	1960–70	-1.24 (2.08)	1.38 (0.08)	3
<i>Quercus ilex</i>	1920–30	2.32 (0.23)a	1.62 (0.02)a	7

Species	Period	$\delta^{15}\text{N}$ (‰)	N (% DW)	<i>n</i>
<i>Rhamnus alaternus</i>	1945–55	1.72 (0.50)a	1.54 (0.01)b	10
	1960–70	–2.21 (0.82)b	1.26 (0.04)c	4
	1985–95	–0.57 (0.13)b	1.71 (0.02)d	6
	1945–55	1.00 (0.38)a	1.93 (0.20)a	3
<i>Rhododendron ferrugineum</i>	1960–70	1.02	1.40	1
	1985–95	–2.60 (0.84)b	2.10 (0.24)a	5
	1945–55	0.18 (0.66)	1.38 (0.08)	4
<i>Rosmarinus officinalis</i>	1985–95	–0.92	1.36	4
	1920–30	0.50 (3.30)a,b	1.15 (0.15)a,b	2
	1945–55	3.15 (0.19)a	1.43 (0.09)a	4
	1960–70	–1.98	0.97	1
<i>Ruscus aculeatus</i>	1985–95	–1.32 (1.16)b	1.00 (0.13)b	4
	1920–30	–2.00 (1.40)a	1.32 (0.14)a	4
	1945–55	0.50 (0.22)a	1.94 (0.22)a	4
	1985–95	2.40 (0.22)b	2.04 (0.14)a	4
<i>Salvia officinalis</i>	1920–30	2.20 (1.64)a	2.53 (0.30)a	4
	1945–55	2.80 (1.56)a	2.57 (0.49)a	4
	1985–95	2.60 (2.29)a	2.40 (0.22)a	4
	1920–30	3.30 (1.30)a	1.70	2
<i>Thymus vulgaris</i>	1945–55	4.35 (4.25)a	1.40 (0.10)a	2
	1985–95	–0.93 (1.27)a	1.62 (0.33)a	2
	1920–30	3.30 (1.30)a	1.70	2

Table 2 Changes in leaf $\delta^{15}\text{N}$ and leaf N, P, K and S concentrations in 3 bryophyte species collected in Spain in four different decades throughout the XX century. Values are means, with SEM in parentheses. Period values not significantly different at the $P < 0.005$ level are followed by the same letter (anova followed by Tukey's test). *n*, number of samples

Species	Period	$\delta^{15}\text{N}$ (‰)	N (% DW)	K (mg g ⁻¹ DW)	P (mg g ⁻¹ DW)	S (mg g ⁻¹ DW)	<i>n</i>
<i>Hypnum cupressiforme</i>	1920–30	4.72 (4.50)a	0.96 (0.19)a	1.83 (0.69)a	0.37 (0.08)a	0.80 (0.24)a	6
	1945–55	4.40 (1.28)a	0.98 (0.08)a	1.98 (0.43)a	0.42 (0.08)a	0.73 (0.08)a	6
	1960–75	3.40 (2.43)a	0.98 (0.13)a	3.58 (0.37)b	0.50 (0.06)b	0.95 (0.07)a	6
	1985–90	2.38 (1.61)a	1.12 (0.09)a	4.46 (0.54)b	1.13 (0.15)b	0.99 (0.14)a	9
<i>Pleurochaete squarrosa</i>	1945–55	1.47 (0.35)a	1.10 (0.12)a	1.80 (0.22)a	0.48 (0.08)a	0.93 (0.04)a	5
	1960–75	–0.60 (2.68)a	1.19 (0.17)a	3.54 (0.73)a,b	0.58 (0.18)a	1.22 (0.18)a	7
	1985–90	–3.11 (1.89)a	1.52 (0.14)a	3.34 (0.18)b	0.79 (0.19)a	1.52 (0.27)a	6
<i>Grimmia laevigata</i>	1945–55	–0.25 (0.53)a	1.55 (0.06)a	3.00 (0.09)a	0.74 (0.10)a	1.10 (0.04)a	4
	1960–75	–1.65 (1.89)a	1.25 (0.26)a	4.00 (0.99)a,b	0.66 (0.11)a	1.29 (0.25)a,b	4
	1985–90	1.77 (1.37)a	1.33 (0.12)a	6.16 (1.32)b	1.00 (0.04)b	1.62 (0.07)b	4

oxidized (0.5 cm³ remaining), they were diluted with 20 mL of 3% perchloric acid. Flasks were stoppered and shaken by hand for thorough mixing of the contents. The sample solution was poured into a polystyrene sample cuvette, placed in an autosampler carousel and analysed for the above mentioned elements by ICAP-AES (inductively coupled argon plasma emission spectrometer) Polyscan Thermo Jarrel ASH Model 61 E (Peñuelas & Matamala 1993). Quality control was ensured by taking a reference sample (Pine needles, NIST 1575) after every 10

samples analysed. Readings were accepted when reference samples were within a 5% range of known values.

Statistics

Statistical analyses were conducted using a one-way anova with sampling decade as the main effect and using the Tukey's significant difference at 0.05 level between species means for the periods 1920–30, 1940–50, 1960–70 and 1985–95, after testing for normality and homogeneity

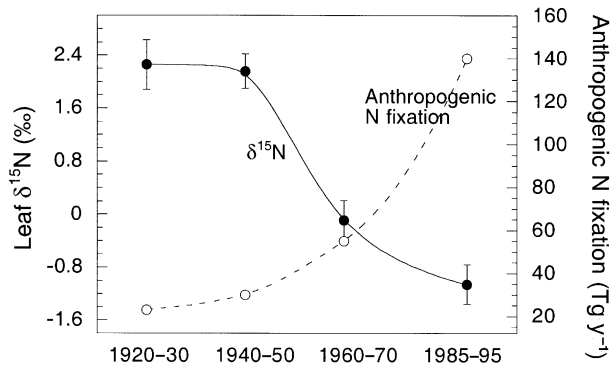


Fig. 1 Trend of the leaf $\delta^{15}\text{N}$ overall average for 27 plant species collected in Spain in four different decades throughout the XX century. It is depicted together with the trend of global anthropogenic N fixation (Vitousek 1994). Bars indicate \pm SE. $n=27$ species mean values. One to 10 herbarium specimens were analysed per decade and species (Table 1).

of variance. Regression models of the studied variables across the decades were conducted with the species mean values, and therefore with values of known associated variance. Thus, as a model I regression was not strictly appropriate, a model II regression with the reduced major axis method was used (Sokal & Rohlf 1995). Statistical analyses were conducted using the statistical program package SYSTAT 5.2 (SYSTAT Inc., Evanston, IL)

Results and discussion

The overall species $\delta^{15}\text{N}$ values decreased consistently from 2.26‰ in 1920–30 and 2.16‰ in 1940–50, to –0.09‰ in 1960–70, and to –1.06‰ in 1985–95 (Fig. 1). As widely recognised (Handley & Scrimgeour 1997), many environmental and geographical factors affect foliar ^{15}N abundance, but this strong ^{15}N signal provides, at least, a clear indication of an altered N cycle in Spanish ecosystems. The decreasing pattern was general in almost all species without significant differences between vascular plants and bryophytes (Tables 1 and 2). This pattern is strikingly coincident with the pattern for nitrate $\delta^{15}\text{N}$ in alpine and polar ice cores (Freyer *et al.* 1996), and it is also in agreement with preliminary herbarium studies (Peñuelas & Estiarte 1997). It confirmed our hypothesis: nitrogen used by plants is becoming poorer in ^{15}N . These results indicate that increasing nitrogen inputs in Spanish terrestrial ecosystems are in accord with increasing N emissions from increased fossil fuel combustion and livestock production in the region (Anonymous 1991) and in the globe (Vitousek 1994). They also suggest a predominance of these inputs over outputs because otherwise the latter

(denitrification, ammonia volatilization, and nitrate leaching) would have caused a $\delta^{15}\text{N}$ enrichment of the remaining available nitrogen (Högberg *et al.* 1995). This predominance of inputs over outputs has been confirmed with actual measurements of atmospherically deposited N being retained within these Mediterranean ecosystems (Avila *et al.* 1999; Rodà *et al.* 2000).

The present results provide an interesting contrast with some studies in Sweden (Näsholm *et al.* 1997) and the USA (Garten 1993), where the $\delta^{15}\text{N}$ signatures of foliage have been observed to increase rather than decrease with N availability. As fractionation during uptake seems greater when N is not limiting growth (Högberg *et al.* 1999), larger fractionation during uptake, as a consequence of the increased availability of N, may have been present in those northern ecosystems. N supply rate to Spanish terrestrial ecosystems is still low in relation to plant demand, in contrast with those cold-temperature ecosystems, as indicated by low N losses in streamwater ($<0.1 \text{ kg N ha}^{-1} \text{ y}^{-1}$, Avila *et al.* 1999). Moreover, Mediterranean ecosystems have drier soils and are more dependent on the atmospheric N component than cold-temperate ecosystems. Atmospheric N supply has been calculated to represent a very high proportion (36–53%) of the N incorporated into biomass in Mediterranean ecosystems of NE Spain (Rodà *et al.* 2000). Other possible explanations for these contrasting results between Northern and Mediterranean ecosystems are less plausible. For example, a different relative importance of ammonium (usually ^{15}N enriched: Garten 1992; Koopmans *et al.* 1997) and nitrate (usually ^{15}N depleted: Garten 1992; Koopmans *et al.* 1997) in deposition can be discarded because there were no clear differences between Spain (Rodà *et al.* 2000) and these other countries (Garten 1993; Koopmans *et al.* 1997; Näsholm *et al.* 1997) in the relative contribution of ammonium and nitrate to total precipitation. Moreover, both positive and negative values for precipitation ammonium-N have been reported (Garten 1993; Koopmans *et al.* 1997)

There are also other factors influencing plant $\delta^{15}\text{N}$ such as ecosystem water availability (Handley *et al.* 1999), but the drier conditions of the last decades in NE Spain (Piñol *et al.* 1998) would have had to induce a reverse trend to the one found here. In fact, leaf $\delta^{15}\text{N}$ values have been found to increase in NE Spain Mediterranean ecosystems in response to recent droughts (Peñuelas *et al.* 2000). The most likely explanation of this strong ^{15}N signal seems thus to be an increased predominance of N inputs over outputs.

Leaf N concentrations of vascular plants decreased in the second half of the century (Fig. 2) as expected from the increased atmospheric CO_2 concentrations and greater C availability (McGuire *et al.* 1995), and also in

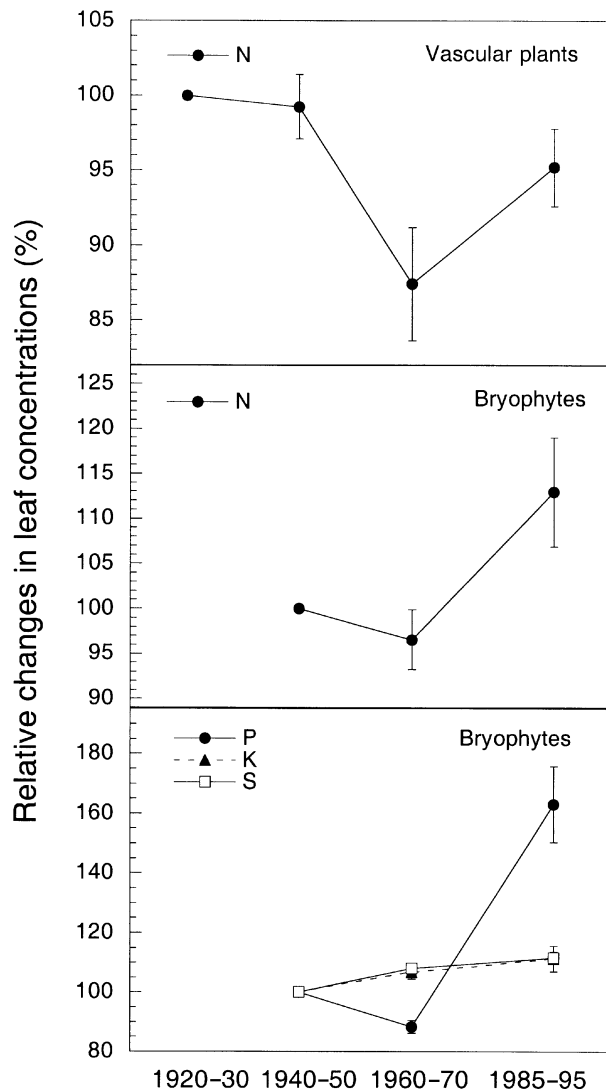


Fig. 2 Relative changes in the overall leaf concentration of N in vascular plants and of N, P, K and S in bryophytes collected in Spain in four different decades throughout the XX century. The values are relative to the 1920–30 species average values in vascular plants and to the 1940–50 species average values in bryophytes. Bars indicate \pm SE. In vascular plants $n=24$ species mean values 1–10 herbarium specimens were analysed per decade and species). In bryophytes $n=3$ species mean values (4–9 herbarium specimens analysed per decade and species, Table 2).

agreement with our previous herbarium studies (Peñuelas & Matamala 1990). However, there was a slight trend towards recovery. Coinciding with the increase in anthropogenic N inputs (Anonymous 1991 and Fig. 1) there was a 9% increase in N concentrations in 1985–95 relative to 1960–70 (Fig. 2). Bryophyte species, which are more exposed to the direct effects of atmospheric N inputs, were also found to have increased N

concentrations (1985–95 concentrations were 17% higher than in 1960–70; Fig. 2). Changes relative to 1920–30 values for each species of vascular plant and to 1940–50 values for each species of bryophyte were used in the data analyses to standardize the variability resulting from species genetic differences and to focus on the environmental variability.

In bryophytes P, K, and S concentrations in 1985–95 were 65%, 13%, and 12% higher than in 1940–50 (Fig. 2, Table 2), providing further evidence of increasing general eutrophication of Spanish terrestrial ecosystems. However, the concentrations of these nutrients in vascular plants did not present any clear pattern (data not shown), probably because they are less sensitive than bryophytes to aerial deposition, and their nutrient uptake and mobilization is more complexly regulated.

Human alteration of nutrient cycles represents an ongoing global change and the plant chemical changes presented here provide new evidence for this alteration. They indicate that added anthropic N and nutrients are retained, at least in part, in the terrestrial ecosystems of rural regions of Spain which, although not pristine, are not near to very highly industrialized or urbanized areas. Profound consequences may be foreseen for ecological processes at all levels of organization, from organism to global levels, including, for example, effects on community biodiversity, vegetation distribution, or even on enhanced carbon sink (Vitousek *et al.* 1997). This increased eutrophication could be partly responsible of the global increase in biospheric activity inferred from the increase in the amplitude of the atmospheric CO₂ annual oscillation during the period 1960–94 (Keeling *et al.* 1996).

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