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Decrypting stable isotope ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) variability in aquatic plants

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Summary

1. Variability in C and N stable isotopes has been acknowledged to hinder their use as tracers of food sources in the study of trophic interactions in ecosystems. This is particularly so whenever benthic primary production is substantial (variability in $\delta^{13}\text{C}$) and the ecosystem under study is affected by human impacts (variability in $\delta^{15}\text{N}$) in aquatic ecosystems.
2. In this study we aim to better understand the large and often unexplained variability in the natural abundance of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures of aquatic plants by analyzing the isotopic composition of plants from 81 lentic systems from NE Spain in relation to extrinsic (alkalinity, pH, nutrient concentrations, water body typology and basin land use) and intrinsic (functional group, carbon assimilation metabolism, elemental composition) predictors.
3. We have encountered a large plasticity of aquatic plants in isotopic signatures associated to the variation in local conditions at regional scale. The $\delta^{13}\text{C}$ signature varied from -43.1‰ to -7.5‰ (35.7‰ range) and drivers were both intrinsic and extrinsic. The functional group was the most important factor as it is influenced by different carbon sources. Aquatic plants with leaves in contact with the atmosphere (helophytes, free floating and floating attached; -34.8 ‰ to -14.6 ‰) responded in a similar way as terrestrial plants, which contrasted with the enriched mean values of rooted submerged plants (-16.7 ‰ to -10.5 ‰) that were more enriched than the described terrestrial C3 range (-34 ‰ to -22 ‰) and completely overlapped the terrestrial C4 range (-20 ‰ to -8 ‰). Concentration of DIC and pH also emerged as important extrinsic factors driving $\delta^{13}\text{C}$ variability.

- 40 4. The $\delta^{15}\text{N}$ signature ranged from -5.2‰ to 20.1‰ (25.2‰ range) and the
41 variability was mostly associated with extrinsic factors such as water body type
42 and basin land use, as they influence both the $\delta^{15}\text{N}$ signature and concentration
43 of the dissolved inorganic nitrogen in the aquatic ecosystems.
- 44 5. Only one multifactorial model including the functional group (with the largest
45 contribution), the DIC and pH was selected as the best model explaining the
46 variability in $\delta^{13}\text{C}$ signatures of aquatic plants. The final model had a relatively
47 large explained deviance and was consistent with the previous unifactorial
48 results. Two different models were selected as the best models explaining
49 variability in $\delta^{15}\text{N}$ signatures of aquatic plants. The models included the
50 geomorphological type of water body as the variable with the largest
51 contribution, and the percentage of either natural or agricultural coverage in the
52 basin. These results are summarized in a conceptual model showing the
53 predictors and their range and direction of variation.
- 54 6. This study shows that extrinsic factors are of major importance in influencing
55 the stable isotope signatures of aquatic plants compared to terrestrial plants
56 because of varied sources and an often limited isotopic discrimination.

57   **Introduction**

58   Aquatic plants are an important element of the underwater landscape in stagnant and
59   slow moving waters. They provide shelter for associated species and contribute to the
60   regulation of organic matter and nutrient cycling. At the base of the food chain, aquatic
61   plants can be grazed by invertebrates, fish, waterfowl and some mammals (e.g. Bakker
62   *et al.*, 2016). If not consumed directly, they also contribute to the dead autochthonous
63   organic matter pool (i.e. Obrador & Pretus 2012) that is used by decomposers and
64   detritivores (e.g. shredder and collector invertebrates, Covich *et al.*, 1999).

65   Stable isotope analysis (SIA), mainly carbon (C) and nitrogen (N), has been widely used
66   to study ecological processes and particularly trophic interactions in aquatic ecosystems
67   (Boecklen *et al.*, 2011; Middelburg, 2014; Kluijver *et al.*, 2015). Specifically, $\delta^{13}\text{C}$ is a
68   powerful tool to identify organic matter flows from basal resources to consumers while
69     $\delta^{15}\text{N}$ traces trophic positions of organisms in food webs. Stable isotope signatures from
70   freshwater ecosystems have been relatively well studied (Boecklen *et al.*, 2011)
71   although aquatic plants (algae, bryophytes and vascular plants) have often not been
72   considered in food web studies because both isotopic signatures ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) show
73   large and often unexplained variability, which complicates the interpretation of the
74   trophic relationships (Finlay & Kendall, 2007; Dethier *et al.*, 2013). However, aquatic
75   plants are critical compartments to understand organic matter origin (autochthonous vs.
76   allochthonous, littoral vs. planktonic) and recycling in aquatic systems, and thus, should
77   be included in trophic studies (Watson & Barmuta, 2011).

78   The $\delta^{13}\text{C}$ signature of the C source for aquatic plants is variable as C can be obtained
79   from the air, the water column and/or the sediment pore water (Winkel & Borum, 2009).

80   The air has a rather constant isotopic signal for carbon (ca. -8‰) and therefore its

variability is essentially related to the C-uptake and assimilation mechanisms (Bender, 1971; Winter & Holtum, 2002) plus stomatal conductance (Farquhar, 1983; Farquhar *et al.*, 1989; Diefendorf *et al.*, 2010) as shown for terrestrial plants. In the water, dissolved inorganic carbon (DIC) may originate from diffused air CO₂, the weathering of minerals in the basin (+1‰, Marcé *et al.*, 2015) and respiration from both the basin and local ecosystem (ca. -30‰, Keeley & Sandquist, 1992). Further, stable isotope fractionation of ¹³C during assimilation is affected by factors such as the use of different forms of carbon (CO₂ or HCO₃⁻) for aquatic plant photosynthesis which in turn is modulated by the ambient pH. The assimilation of HCO₃⁻ in addition to CO₂, results in δ¹³C signatures 7-11‰ more enriched (Mook *et al.*, 1974) than plants exclusively using CO₂.

Variation in the natural abundance of δ¹⁵N in terrestrial plants is controlled by both extrinsic (nitrogen sources) and intrinsic (e.g. N-fixing or non-N-fixing plants, variable discrimination during assimilation) factors (Evans, 2001; Robinson, 2001). Most of the variation of δ¹⁵N in aquatic plants thriving in streams is related to extrinsic factors such as the nitrogen sources and nutrient concentration (Pastor *et al.*, 2013; Peipoch *et al.*, 2014b). The main nitrogen sources in aquatic systems are atmospheric deposition, mineralization of organic matter, human waste waters, chemical fertilizers and animal waste. The δ¹⁵N signature of these sources is variable and depends on factors such as basin land use, human disturbances, the presence of N-fixing plants in the watershed, the presence of reducing conditions and local processes (e.g. assimilation, nitrification, denitrification and mineralization; Finlay & Kendall, 2007). Furthermore, aquatic plants use inorganic nitrogen forms, mainly NH₄⁺ and NO₃⁻ (Barko *et al.*, 1991; Peipoch *et al.*, 2014a) and organic nitrogen (e.g. amino acids) that may have substantially different δ¹⁵N signatures.

Some studies have emphasized the wide ranges of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in aquatic plants, particularly in continental waters (Keeley & Sandquist, 1992; Finlay & Kendall, 2007; Milligan *et al.*, 2010), and to a minor extent in marine angiosperms (Hemminga & Mateo, 1996; Raven *et al.*, 2002; Walton *et al.*, 2016). However, to our knowledge there have been no attempts to examine the relative influence of environmental conditions (extrinsic factor) and of functional groups (intrinsic factor) on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ variability in continental aquatic plant species from stagnant water bodies. Actually, SIA has been rarely used to understand intrinsic properties of aquatic plant functioning due to difficulties and restrictions associated with the aquatic environment. Only a few studies have been published (Boon & Bunn, 1994; Yoneyama *et al.*, 2001; Peipoch *et al.*, 2014a), which contrast with the wider use in terrestrial physiology (e.g. Collister *et al.*, 1994; Evans, 2001; Kalcsits *et al.*, 2014).

In this study we aimed at understanding the variability in the natural abundance of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ stable isotope signatures of aquatic plants thriving in stagnant systems and at examining the relevance of intrinsic (i.e. elemental composition, metabolism and functional group) vs. extrinsic (environmental) factors that might influence such variability to improve its use as a food tracer and better understand ecological processes. We hypothesized that extrinsic factors are more important than intrinsic factors in determining the isotope signatures because variability in the sources should be much larger than the partitioning associated to physiological and metabolic processes. We analyzed a relatively large and varied sample of aquatic plants (charophytes and 5 functional groups of vascular plants) thriving in a wide range of stagnant water bodies from a highly diverse (both species richness and ecosystem typology) Mediterranean region.

Methods

A total of 169 aquatic plant samples were collected at 81 lentic water bodies in North-eastern Spain (Fig. 1, Table S1. Supporting Information) from 2005 to 2009 (extended study area description in Chappuis *et al.*, 2011a, b). Water bodies belonged to 8 different geomorphological types: alpine lakes, alpine reservoirs, karstic lakes, coastal lagoons, permanent ponds, temporary pools, reservoirs and meanders [see extensive description in Chappuis *et al.*, 2014 as well as detailed information on environmental factors (carbon system and nutrients)]. Plants were collected during their growing period which is spring in most of the localities and water bodies. There were however two exceptions; vernal pools that were visited a bit earlier (winter or early spring) to ensure the inundation period, and Pyrenean lakes that were visited in summer due to the delay caused by the winter ice cover.

Leaves from different individuals (> 10) of each aquatic plant species were manually collected by snorkeling, SCUBA diving or with waders. Only abundant species were sampled at each water body, which resulted in a median of 2 species per water body (range from 1 to 8). A total of 49 different taxa were analyzed corresponding to 6 functional groups: helophytes (only the plant base is submerged, e.g. *Typha* sp.), free floating (the plant is not attached to the sediment and floats on the water surface, e.g. *Lemna minor*), free submerged (the plant is not attached to the sediment and floats in the water column, e.g. *Utricularia vulgaris*), floating attached (rooted plants with leaves floating on the water surface, e.g. *Potamogeton natans* and *Ranunculus aquatilis*), rooted submerged vascular plants (completely submerged and rooted to the sediment, e.g. *Potamogeton crispus* and *Ruppia cirrhosa*) and submerged stoneworts (algae completely submerged and anchored to the sediment by rhizoids, e.g. *Chara* spp. and *Nitella* spp.). Water samples were collected at -0.5 m at the central part of each water body, filtered (Whatman GF/F glass microfiber filter, 0.7µm pore size) and kept frozen

until analysis. Light was not measured as all plants were collected in the photic zone that frequently reached the bottom. Similarly, depth was not measured as aquatic plant species thrive at different depths in each water body.

Once at the laboratory, epiphytes and mineral deposition were manually removed from the fresh leaves' surface. Leaves were frozen, dried in a freeze dryer TELSTAR Cryodos and manually ground to a fine homogeneous powder using an agate mortar and pestle. Finally, two aliquots of about 3 mg of each sample were weighed, and encapsulated for SIA. A sub-sample of species with calcareous depositions, such as *Chara* sp. or some specimens of *Stuckenia pectinata*, were acidified following Serrano *et al.*, (2008) to remove inorganic carbon that could bias the $\delta^{13}\text{C}$ values of the organic carbon (Schlacher & Connolly, 2014). Two aliquots of each acidified sample were encapsulated following the same steps used for non-treated samples. Elemental and isotopic carbon and nitrogen composition of the samples were analyzed at the Stable Isotope Facility of the University of California-Davis by EA-IRMS (Elemental Analyzer – Isotope Ratio Mass Spectrometer) using a PDZ Europa ANCA-GSL elemental analyzer interfaced to PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd, Cheshire, UK). Water chemical analyses were carried out according to standard methods (The MOLAR Water Chemistry Group, 1999) and are described in detail in Chappuis *et al.*, (2014). Water column samples were analysed for pH (measured in situ with an Orion electrode mod. 231), conductivity (measured in situ with a PTI-10 conductivity meter), alkalinity, dissolved carbon (TOC, DOC and DIC), total phosphorus, ammonium, nitrite and nitrate.

Land uses were measured for the whole basin and for a 50 m buffer around the water body using the software ArcMap 10.2.1. Detailed topographic digital maps were obtained from the Cartographic Institute of Catalonia (ICC, www.icc.es) and the land

use map was downloaded from the Land Cover Map of Catalonia website (4th edition, 2009, <http://www.creaf.uab.es/MCSC/usa/index.htm>). The catchment area for each water body was manually drawn in ArcMap as the watershed delineation tools included in the software did not give accurate results. The area of each land use was calculated within each basin and the percentage of natural, agricultural and urban land use was subsequently determined. Furthermore, a 50 m buffer was drawn around each water body within the catchment area. Wider buffers were discarded as a large number of basins are small and the buffer zone was equal to the whole basin. The percentages of natural, agricultural and urban land use within the 50 m buffers were calculated with the same procedure described for basins.

Statistical analysis

The isotopic ranges and variability of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were first explored among taxa and water bodies. Secondly, the relationship between isotope signatures and intrinsic factors (7 variables included in Table 1) and extrinsic factors (17 variables included in Table 2) were analyzed both individually and through multifactorial models. Generalized Additive Models (GAM, Hastie & Tibshirani, 1990) were performed to explore unifactorial relationships as complex non-linear responses were expected. GAM analyses were done with the R package “mgcv” using a Gaussian distribution and smoothing the quantitative explanatory variables with penalized regression splines. The variable goodness-of-fit was measured by the explained deviance (D^2).

Generalized Linear Models (GLM) were performed to build multifactorial models. An exhaustive search was done with the R package “glmulti”. All possible models were calculated using the explanatory variables that had previously shown significant relationship with the corresponding response variable. A confidence set was defined as

all those models that differed up to 2 AICc (Akaike information criterion corrected for finite sample sizes) units from the model with the lowest AICc value. Models within a confidence set are essentially indistinguishable in terms of their fit to the data. The significance of each explanatory variable of the selected models was calculated with the package “vegan” and the relative importance of each variable was estimated by r^2 partitioning using package “relaimpo”. Non-significant variables (p -value > 0.01) were removed and/or the final model was ordered (from high to low contribution variables) accordingly. All statistical analysis and graphs were performed in the software R (R Core Team, 2014).

Results

Variability in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ among aquatic plants

Values of $\delta^{13}\text{C}$ showed a unimodal distribution and ranged from -43.1 to -7.5‰ with a median of -23.7‰ (Fig. 2). Values of $\delta^{15}\text{N}$ also show a unimodal distribution and ranged from -5.2 to 20.1‰ with a median of 3.5‰ (Fig. 2).

Variability was large for $\delta^{13}\text{C}$ among species and often within species (Fig. S1 Supporting Information). The average range per taxon was of 9.3‰ and the largest species range corresponded to *Myriophyllum spicatum* (n=10) (-32.9‰ to -13.8‰) and *Ranunculus trichophyllus* (n=7) (-34.8‰ to -15.7‰). The range was independent from the number of samples (p -value > 0.05, see Table S2 Supporting Information for the number of samples). Some species, such as *Potamogeton nodosus* (n=8) and *Ranunculus trichophyllus* (n=7), showed a wide range of $\delta^{13}\text{C}$ values. In contrast, other species, such as *Ruppia cirrhosa* (n=8) showed narrow $\delta^{13}\text{C}$ ranges. The widest range corresponded to Characeae (-43.1‰ to -10.5‰) that included 20 samples of the genera *Chara* and *Nitella*. Similarly, the variability was large for $\delta^{15}\text{N}$ among species and often within

species (Fig. S1 Supporting Information). The average range per taxon was 7.1‰. *Ruppia cirrhosa* (n=8) showed the widest range for $\delta^{15}\text{N}$ values (0.4‰ to 20.1‰) in contrast with the narrow range for $\delta^{13}\text{C}$. No relationship was found between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures (Pearson correlation of 0.01, p-value > 0.05).

In addition, when considering the stable isotope signatures of the different plants developed in each water body, we found that variability was large for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ among water bodies and often within water bodies (Fig. S2 Supporting Information). The average range per water body was 6.1‰ for $\delta^{13}\text{C}$ and 3.9‰ for $\delta^{15}\text{N}$, which was lower than the average range observed among different aquatic plant species.

Influence of intrinsic factors on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ variability

Of the studied intrinsic factors, only the functional group of the different aquatic plants showed an important and significant relationship with $\delta^{13}\text{C}$ signatures (Table 1). The group of free submerged plants displayed the lowest values of $\delta^{13}\text{C}$ along with the groups of helophytes and free floating plants that also showed relatively low values (Fig. 3). Floating attached plants, which are rooted but have floating leaves in contact to the atmosphere, displayed intermediate values of $\delta^{13}\text{C}$. Groups including plants completely submerged, like stoneworts and rooted submerged plants, showed the most enriched $\delta^{13}\text{C}$ values and widest range of variability (see Fig. 3). Signatures of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ did not show any significant relationship with the elemental composition of leaf tissue, except for a very weak relationship between $\delta^{15}\text{N}$ signatures and percentage of phosphorus (positive), and C:N ratio (negative) of the leaves (Table 1).

The photosynthetic pathway was known for only 10 species within the data set and, consequently, could not be included in the modelling analysis. Emergent species that were C3 showed $\delta^{13}\text{C}$ signatures within the range described for C3 terrestrial plants (Fig.

2): *Polygonum amphibium* (-29.0‰), *Eleocharis acicularis* (-25.9‰, C3+C4) and *Ranunculus aquatilis* (range from -20.1‰ to -28.1‰). In contrast, submerged C3 species showed more positive $\delta^{13}\text{C}$ signatures, which better fitted within the range described for C4 terrestrial plants (Fig. 2): *Elodea canadensis* (-20.1‰), *Ranunculus aquatilis* (-14.6‰ and -16.7‰), *Potamogeton berchtoldii* (-10.5‰ and -15.4‰), *Potamogeton lucens* (-15.4‰) and *Potamogeton alpinus* (-16.4‰). The difference between emergent and submerged plants of *Ranunculus aquatilis* was striking. We also found less negative signatures in submerged (-19.3‰) than in floating (-26.2‰) leaves within a population of *Potamogeton nodosus*. Three species of the data set display Crassulacean Acid Metabolism (CAM): *Isoetes setacea* (-26.8‰), *Isoetes lacustris* (range from -21.1‰ to -22.7‰) and *Isoetes echinospora* (range from -21.0‰ to -24.6‰). The observed $\delta^{13}\text{C}$ values for these species were within the range for terrestrial CAM species (Fig. 2).

Influence of extrinsic factors on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ variability

Of the measured environmental factors, water pH and DIC concentration showed the strongest relationships with $\delta^{13}\text{C}$ of plants (Table 2). The relationship of $\delta^{13}\text{C}$ with pH was complex, rather positive and showed the maximum variability between pH 7.5 and 8, which is the pH range where CO_2 is still present but at really low concentration and HCO_3^- is dominant (Fig. 4). Considering all data together, the signature of $\delta^{13}\text{C}$ displayed a significant negative relationship with DIC (Table 2, Fig. 4). However, if we analyzed this relationship for each functional group separately, only the groups with species completely submerged showed a significant negative linear relationship with DIC of the water (submerged stoneworts: $R^2=0.64$, p-value=0.000; rooted submerged vascular plants: $R^2=0.22$, p-value=0.000) while groups of plants that had tissues in contact with the air showed no significant relationship (floating attached: p-

value=0.083; helophyte: p-value=0.851). Groups for free floating and free submerged species were not analyzed because of small sample size ($n < 6$). In addition, $\delta^{13}\text{C}$ signatures were related with altitude of the water bodies (Table 2), which could be explained by the fact that water pH and DIC correlated with altitude (Pearson correlation of -0.42 and -0.51, respectively). Signatures of $\delta^{13}\text{C}$ also showed significant negative relationships with the concentration of dissolved nitrogen forms (especially with nitrite and nitrate), phosphate and the molar ratio between dissolved inorganic nitrogen and phosphate (Table 2). The signature of $\delta^{13}\text{C}$ also showed some significant relationship with land use, especially with the agricultural percentage of the basin and in the 50 m buffer around the water body (Table 2). Actually, agricultural land use correlated with DIC in the water (Pearson correlation of 0.40).

Significant relationships were found between $\delta^{15}\text{N}$ and the water alkalinity and DIC (Table 2). Actually, both environmental variables correlated well with the basin agricultural and natural land use (Pearson correlation with agriculture: 0.41 for alkalinity and 0.40 for DIC; Pearson correlation with natural land use: 0.45 for alkalinity and 0.44 for DIC). Among the dissolved inorganic nitrogen (DIN) forms, only nitrite concentration showed some significant relationship with $\delta^{15}\text{N}$ (Table 2). No significant relationships were found with phosphate concentration or the molar ratio between dissolved inorganic nitrogen and phosphate.

The geomorphological type of the water body showed a significant relationship with $\delta^{15}\text{N}$ (Table 2). Typologies from higher altitudes and subjected to less human pressure (e.g. alpine reservoirs and lakes) showed lower $\delta^{15}\text{N}$ mean signatures and narrower ranges (Fig. 5). In contrast, typologies at medium and low altitudes (e.g. coastal lagoons, ponds, temporary pools and karstic lakes) exhibited generally larger mean signatures and wider ranges (Fig. 5). Some geomorphological types correlated well with basin land

use. For example, alpine lakes, alpine reservoirs and temporary pools had between 90 and 100% of natural coverage and no or little agricultural coverage.

Finally, there was a significant relationship between $\delta^{15}\text{N}$ and the land use, especially with the percentage of natural and agricultural use (Table 2). Relationships were stronger for the land use at the basin level than for the 50 m buffer, except for the urban use. The more natural the coverage was, the more negative the $\delta^{15}\text{N}$ signature was, and vice versa for agricultural coverage.

Multifactorial models

Only one multifactorial model was selected as the best model explaining the variability in $\delta^{13}\text{C}$ signatures of the aquatic plants. The final model had a relatively large explained deviance and included the functional group (with the largest contribution), DIC and pH (Table 3). This model was consistent with the previous unifactorial results, as it included the variables with the largest explained deviance.

Two different models were selected that best explained variability in $\delta^{15}\text{N}$ signatures of aquatic plants. The models included the geomorphological type of water body as the variable with the largest contribution, and the percentage of either natural or agricultural coverage in the basin (Table 3). The first model, which included the natural coverage, performed slightly better than the second model.

Discussion

Aquatic plants showed broad ranges of both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures in the water bodies of the studied area. These ranges were similar to those reported from compilations of data for aquatic plants worldwide (e.g. Keeley & Sandquist, 1992; Finlay & Kendall, 2007), which indicated a large plasticity in isotopic signatures of

aquatic plants associated with the variation in local conditions at regional scale. The main extrinsic and intrinsic factors driving $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures in aquatic plants are discussed in the following sections and represented in a conceptual model (Fig. 6). In general, we found that variability in $\delta^{13}\text{C}$ signatures was associated with both intrinsic (functional group) and extrinsic factors, whereas, variability in $\delta^{15}\text{N}$ signatures was mostly associated with extrinsic factors. Nevertheless, we also found some interplay between intrinsic and extrinsic factors in determining isotopic variability among aquatic plants.

Variation of $\delta^{13}\text{C}$ in aquatic plants from stagnant water bodies

The most important factor determining $\delta^{13}\text{C}$ variability was the functional group as this determines the origin of potential carbon sources, and thus the $\delta^{13}\text{C}$ of the source. Aquatic plants with leaves in contact with the atmosphere (i.e. helophytes, free floating plants and floating plants attached to the substrata) exhibited relatively lower mean $\delta^{13}\text{C}$ signatures because they obtained most of the carbon from atmospheric CO_2 and consequently, showed narrow ranges in $\delta^{13}\text{C}$ with lower mean signatures similarly as terrestrial plants. In contrast, submerged plants used dissolved carbon from the water column and often from the sediment (Winkel & Borum, 2009). These carbon sources exhibit large $\delta^{13}\text{C}$ variability and fractionation during DIC uptake and assimilation also differs among species. Consequently, submerged aquatic plant species exhibited a wide range in $\delta^{13}\text{C}$ signatures and less negative $\delta^{13}\text{C}$ mean values since the aquatic environment generally decreases fractionation.

The type of photosynthetic pathway is the most important factor defining variability in $\delta^{13}\text{C}$ signatures among terrestrial plants (Marshall *et al.*, 2007). Our data from emergent aquatic plant species was consistent with this finding. In contrast, the $\delta^{13}\text{C}$ range for

rooted submerged C3 plants was more positive (from -16.7 ‰ to -10.5 ‰) than the terrestrial C3 range (from -34 ‰ to -22 ‰, Benedict, 1978; Smith & Walker, 1980; Finlay & Kendall, 2007) and completely overlapped the C4 range in terrestrial plants, which suggested carbon limitation. Rooted submerged CAM species in aquatic ecosystems showed rather negative signatures in agreement with the use of photorespiratory CO₂ and/or CO₂ from the sediment (Madsen & Sand-Jensen, 1991; Winkel & Borum, 2009).

Besides the $\delta^{13}\text{C}_{\text{DIC}}$ signature, which was not directly assessed in this study, the two most important extrinsic factors related to variability in $\delta^{13}\text{C}$ signature of aquatic plants were the DIC concentration and the pH in the water column because they determine the total amount of inorganic carbon and the concentrations of the different forms (i.e. CO₂ and HCO₃⁻). The larger the availability in DIC the more the plants can discriminate against the heavy isotope, which resulted in more depleted $\delta^{13}\text{C}$ signatures. Water pH determines the relative proportions of the different inorganic carbon forms. Dissolved CO₂ is predominant at low pH and absent at pH greater than 8. Between pH 7 and 8 most of the carbon is in the form of HCO₃⁻ but some dissolved CO₂ is still available. At this range, plants that uptake only CO₂ can still use this form for photosynthesis, but in some cases they may be carbon limited and isotope discrimination may be low. Furthermore, plants that can also assimilate HCO₃⁻ (Madsen & Sand-Jensen, 1991; Yin *et al.*, 2016) are using both C forms of DIC. Concordantly, this was the pH range with a major variability of $\delta^{13}\text{C}$ signature in our data set.

An additional extrinsic factor that is especially important in stagnant water ecosystems is the ambient diffusional resistance (Keeley & Sandquist, 1992). The thicker the boundary layer, the lower the DIC diffusion and the larger $\delta^{13}\text{C}$ values.

Variation of $\delta^{15}\text{N}$ in aquatic plants from stagnant water bodies

Our results showed that variability in aquatic plant $\delta^{15}\text{N}$ signatures were mainly influenced by extrinsic factors (e.g. geomorphological type and land use) as observed for aquatic plants from stream ecosystems (Pastor *et al.*, 2013; Peipoch *et al.*, 2014b). Basin land use and human disturbances associated with the different land uses are major factors determining variability in $\delta^{15}\text{N}$ signatures of DIN, consequently, $\delta^{15}\text{N}$ in aquatic plants may respond to this variability since DIN is the major N source for them (e.g. Cole *et al.*, 2005; Peipoch *et al.*, 2012; Pastor *et al.*, 2014). Peipoch *et al.* (2012) found that the $\delta^{15}\text{N}$ signature of DIN and aquatic plants increases from freshwater ecosystems draining mainly forested basins to those draining agricultural and horticultural basins and shows the largest values in urban basins. Our results supported this trend as the geomorphological typology correlated with human pressure and altitude. Aquatic plants in water body types located at high altitudes and having low human pressures had the lowest $\delta^{15}\text{N}$ signatures and the narrowest ranges of these values. In contrast, aquatic plants located in water bodies at mid and low altitudes, which may have high human pressures, exhibited high $\delta^{15}\text{N}$ values and large ranges of these values.

Another important extrinsic factor influencing the variability in $\delta^{15}\text{N}$ in aquatic plants, basically among freshwater bodies, is the DIN concentration (Jones *et al.*, 2004; Serret *et al.*, 2008). The larger the DIN concentration in the water column the more plants can discriminate against the heavy isotope and the more negative are $\delta^{15}\text{N}$ signatures.

Surprisingly, we found no relationship between $\delta^{15}\text{N}$ in aquatic plant tissue and water column DIN concentration. One reason could be that most samples were from rooted aquatic plants, which mainly used DIN from the sediment pore water (Barko *et al.*, 1991), whose concentration did not necessarily correlate with water column DIN (Gacia *et al.*, 2009). The concentration of other nutrients and their stoichiometry can also be

important factors (King *et al.*, 2009; Pastor *et al.*, 2013). For example, plants thriving in phosphorus limited ecosystems tends have a lower demand relative to the nitrogen source and discrimination is likely to occur, which results in low plant $\delta^{15}\text{N}$ (King *et al.*, 2009). On the contrary, discrimination does not occur in nitrogen limited ecosystems and plant $\delta^{15}\text{N}$ tend to be larger the stronger the limitation is (King *et al.*, 2009).

The studied intrinsic factors showed no or little influence on the variability in $\delta^{15}\text{N}$ in aquatic plants. Nevertheless, other studies highlight a few intrinsic factors such as functional groups, discrimination, plant production and presence of mycorrhizae (Michelsen *et al.*, 1998; Evans, 2001) that control some of the $\delta^{15}\text{N}$ variability. Species that exclusively use nitrogen from the water column (e.g. rootless vascular plants such as *Utricularia* spp. and mosses) show lower average $\delta^{15}\text{N}$ values than species that use nitrogen from the sediment pore water (King *et al.*, 2009). No differences were seen among functional groups in our dataset due to a lack of species primarily using nitrogen from the water column, since most species also obtained nitrogen from the sediment pore water, and probably to a large and uncoupled $\delta^{15}\text{N}$ variability of DIN in water and pore water.

Some aquatic plants exhibit discrimination against the heavy isotope (e.g. De Brabandere *et al.*, 2007) while others do not (e.g. Cohen & Bradham, 2010), as observed for terrestrial plants (Evans, 2001; Dawson *et al.*, 2002). Ultimately, aquatic plant production can be controlled by extrinsic factors such as nutrients and light availability that indirectly influence $\delta^{15}\text{N}$ signatures (Von Schiller *et al.*, 2007; Peipoch *et al.*, 2014b).

Conclusions

This study shows that extrinsic factors are of major importance in influencing the stable isotope signatures of aquatic plants compared to terrestrial plants because they are subject to additional partitioning factors such as the concentration and the isotopic signal of the source and to diffusional resistances that increase the variability in isotopic composition for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. Furthermore, the functional group of aquatic plants emerges as the most important intrinsic factor as it segregates aquatic plants with tissues in contact with the atmosphere that perform more like terrestrial plants from the submerged plants subject to more extrinsic limiting factors.

Ultimately, being aquatic plants at the base of the food web, the large variability found in stable isotopic signatures and the associated factors influencing them provided in this study should be taken into consideration when using stable isotopes in these ecosystems to infer food trophic interactions.

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618 **Tables**

619 **Table 1.** Results from Generalized Additive Models (GAM), which indicate the
 620 unifactorial relationships between stable isotope signatures and all intrinsic
 621 factors considered.

INTRINSIC VARIABLES	$\delta^{13}\text{C}$		$\delta^{15}\text{N}$	
	D ²	p-value	D ²	p-value
Functional group	28.5	<0.001	-	ns
% of C in leaf tissue	-	ns	-	ns
% of N in leaf tissue	-	ns	-	ns
% of P in leaf tissue	-	ns	7.1	<0.005
C:N in leaf tissue	-	ns	9.1	<0.005
N:P in leaf tissue	-	ns	-	ns
C:P in leaf tissue	-	ns	-	ns

622 The explained deviance (D²) corresponds to the percentage of deviance explained
 623 by the variable compared to the null model. ns – non-significant (p-value > 0.05)

624

625 **Table 2.** Results from Generalized Additive Models (GAM), which indicate the
626 unifactorial relationships between stable isotope signatures and all environmental
627 factors considered.

EXTRINSIC VARIABLES	$\delta^{13}\text{C}$		$\delta^{15}\text{N}$	
	D ²	p-value	D ²	p-value
Water-column chemistry				
pH	34.1	<0.001	20.6	<0.001
Alkalinity	22.2	<0.001	41.6	<0.001
DIC	31.1	<0.001	40.0	<0.001
NH ₄	8.6	<0.005	-	ns
NO ₂	25.6	<0.001	16.6	<0.001
NO ₃	19.1	<0.001	-	ns
DIN	9.4	<0.005	-	ns
PO ₄	11.8	<0.005	-	ns
DIN:PO ₄	22.0	<0.001	-	ns
Water body characteristics				
Geomorphological type	10.9	<0.01	27.0	<0.001
Altitude	28.4	<0.001	15.9	<0.001
Basin land use				
Natural (%)	-	ns	41.3	<0.001
Agricultural (%)	24.7	<0.001	40.0	<0.001
Urban (%)	-	ns	13.5	<0.005
50 m buffer land use				
Natural (%)	26.3	<0.001	33.0	<0.001
Agricultural (%)	27.7	<0.001	28.4	<0.001
Urban (%)	-	ns	25.2	<0.001

628 The explained deviance (D²) corresponds to the percentage of deviance explained
629 by the variable compared to the null model. ns – non-significant (p-value > 0.05)

630

631 **Table 3.** Best multifactorial models for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures.

$\delta^{13}\text{C}$ model			$\delta^{15}\text{N}$ model		
Variables	D ² / contribution	p-value	Variables	D ² / contribution	p-value
Model 1	55.3		Model 1	35.3	
1 st - Functional group	39.7	<0.001	1 st - Geomorphological type	59.4	<0.001
2 nd - DIC	34.0	<0.001	2 nd – Natural coverage	40.6	<0.001
3 rd - pH	26.3	<0.001	Model 2	32.1	
			1 st - Geomorphological type	67.7	<0.001
			2 nd – Agricultural coverage	32.3	<0.005

632 The explained deviance (D²) corresponds to the percentage of deviance explained
633 by the model compared to the null model. Contribution is the relative importance
634 of each predictor within the model.

635

636 **Figure legends**

637 **Fig. 1.** Location of the 81 sampled water bodies in Catalonia (North-eastern Spain).

638 **Fig. 2.** Frequency histograms of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures in aquatic plants thriving
639 in the sampled water bodies. Described ranges for freshwater macrophytes,
640 terrestrial plants and marine macrophytes based on the ranges published by
641 different authors (Benedict, 1978; Smith & Walker, 1980; Keeley & Sandquist,
642 1992; Hemminga & Mateo, 1996; Raven *et al.*, 2002; Finlay & Kendall, 2007;
643 Milligan *et al.*, 2010; Mancinelli *et al.*, 2013; Walton *et al.*, 2016) are depicted below
644 each stable isotope axis.

645 **Fig. 3.** Range of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures by functional form groups. Each box
646 shows the median (black line), the interquartile range (box) and the outliers (open
647 circles).

648 **Fig. 4.** Carbon stable isotope signature ($\delta^{13}\text{C}$) in relation to water body pH and DIC
649 concentration (ppm). Different symbols are used to show the functional group of
650 each sample. Only significant regressions are represented: the dotted line
651 corresponds to submerged charophytes and the solid line corresponds to rooted
652 submerged vascular plants. Note that $y = \delta^{13}\text{C}$ and $x = \text{DIC}$.

653 **Fig. 5.** Range of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures by geomorphological type. Each box
654 shows the median (black line), the interquartile range (box) and the outliers (open
655 circles).

656 **Fig. 6.** Conceptual models showing the main factors that control $\delta^{13}\text{C}$ signatures of
657 submerged plants and $\delta^{15}\text{N}$ signatures of aquatic plants. See the text for detailed
658 explanations. Arrows indicate the usual effect of an increased amount of the

659 specified factor. Ranges or approximate values are shown for some factors (e.g.
660 photosynthetic pathway). ^a: grey lines indicate described ranges for terrestrial
661 plants; black lines indicate the range found for aquatic plants in this study. ^b:
662 mycorrhizae effect is detectable only in oligotrophic environments. ^c: ambient
663 diffusional resistances are important for plants that obtain N only from the water
664 (rootless vascular plants and mosses).

665

Fig . 1 Location of the 81 sampled water bodies in Catalonia (North-eastern Spain).

Fig. 2 Frequency histograms of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures in aquatic plants thriving in the sampled water bodies. Described ranges for freshwater macrophytes, terrestrial plants and marine macrophytes based on the ranges published by different authors (Benedict 1978, Smith and Walker 1980, Keeley and Sandquist 1992, Hemminga and Mateo 1996, Raven et al. 2002, Finlay and Kendall 2007, Milligan et al. 2010, Mancinelli et al. 2013) are depicted below each stable isotope axis.

Geomorphological type

Geomorphological type

SUBMERGED AQUATIC PLANTS

AQUATIC PLANTS

Supporting Information

Table. S1. Geomorphological type and location (UTM, datum ED50) of the 81 studied water bodies.

Water body ID	Geomorphological type	UTM-x	UTM-y	Water body ID	Geomorphological type	UTM-x	UTM-y
1	Karstic lake	295026	4656654	53	Pond	420003	4592431
2	Karstic lake	334894	4688647	54	Pond	404636	4658131
3	Karstic lake	336455	4667776	55	Pond	442356	4636112
4	Karstic lake	336201	4667886	56	Reservoir	523617	4685635
5	Alpine reservoir	347340	4716146	57	Coastal lagoon	512132	4678739
6	Alpine reservoir	343278	4724649	58	Reservoir	315281	4634559
7	Alpine lake	329901	4722925	59	Pond	292280	4605107
8	Alpine reservoir	317770	4731871	60	Pond	397827	4656619
9	Alpine reservoir	318289	4707416	61	Pond	450030	4649571
10	Alpine reservoir	315315	4702168	63	Pond	431348	4594358
15	Reservoir	484461	4626073	64	Coastal lagoon	318738	4507102
17	Karstic lake	479366	4663159	65	Coastal lagoon	320619	4509437
21	Pond	437083	4683363	66	Pond	317673	4509355
22	Pond	444337	4682001	67	Coastal lagoon	301678	4501883
23	Pond	459406	4666103	68	Coastal lagoon	308575	4502599
24	Pond	502218	4645976	69	Karstic lake	296787	4505582
25	Pond	511792	4681532	70	Karstic lake	296885	4505232
26	Pond	511641	4681356	71	Coastal lagoon	509249	4674333
27	Temporary pool	492338	4677562	72	Karstic lake	510457	4679585
28	Pond	323695	4603395	73	Meander	494281	4627289
29	Meander	306676	4614599	74	Reservoir	296111	4618066
30	Pond	312875	4624978	87	Temporary pool	306243	4599722
32	Meander	506668	4670232	90	Temporary pool	525043	4685381
33	Pond	472987	4631578	91	Temporary pool	495297	4692265
35	Reservoir	444762	4633624	168	Pond	290853	4582440
36	Pond	442153	4632992	170	Temporary pool	492150	4694497
38	Reservoir	437603	4629742	172	Temporary pool	498359	4694128
39	Reservoir	437396	4629696	173	Temporary pool	496658	4692330
40	Pond	405284	4693038	174	Temporary pool	496703	4692092
41	Reservoir	420516	4655264	175	Alpine lake	455791	4709030
42	Pond	429326	4637275	176	Alpine lake	396488	4704748
43	Coastal lagoon	515884	4653238	177	Alpine lake	395632	4704377
44	Reservoir	493307	4628062	178	Alpine lake	384468	4701669
45	Karstic lake	293670	4513448	179	Alpine lake	362726	4730191
46	Karstic lake	296380	4504620	180	Alpine lake	363422	4730367
47	Pond	267590	4515274	181	Alpine lake	330254	4721070
48	Meander	264463	4514555	182	Alpine lake	321985	4724904
49	Pond	279644	4536202	183	Alpine lake	310844	4731811
50	Karstic lake	296698	4505583	184	Alpine lake	309949	4732266
51	Coastal lagoon	368792	4557322	185	Alpine lake	321072	4731050
52	Coastal lagoon	426621	4572903				

Fig. S1. Box plot of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures by taxa. Each box shows the median (black line), the interquartile range (box) and the outliers (open circles). Species abbreviations: Algae - algae, Ali pla - *Alisma plantago-aquatica*, Cal - *Callitriche* sp., Cal bru - *Callitriche brutia*, Cal pal - *Callitriche palustris*, Cer dem - *Ceratophyllum demersum*, Characeae - characeae, Ele aci - *Eleocharis acicularis*, Elo can - *Elodea canadensis*, Equ pal - *Equisetum palustre*, Het ren - *Heteranthera reniformis*, Iso ech - *Isoetes echinospora*, Iso lac - *Isoetes lacustris*, Iso set - *Isoetes delilei*, Jun - *Juncus* sp., Lem gib - *Lemna gibba*, Lem min - *Lemna minor*, Lud gra - *Ludwigia grandiflora*, Lud pal - *Ludwigia palustris*, Lur nat - *Luronium natans*, Men - *Mentha* sp., Moss - moss, Myr alt - *Myriophyllum alterniflorum*, Myr spi - *Myriophyllum spicatum*, Myr ver - *Myriophyllum verticillatum*, Naj mar - *Najas marina*, Nym alb - *Nymphaea alba*, Pol amp - *Polygonum amphibium*, Pot alp - *Potamogeton alpinus*, Pot ber - *Potamogeton berchtoldii*, Pot col - *Potamogeton coloratus*, Pot cri - *Potamogeton crispus*, Pot den - *Groenlandia densa*, Pot luc - *Potamogeton lucens*, Pot nat - *Potamogeton natans*, Pot nod - *Potamogeton nodosus*, Pot pec - *Stuckenia pectinatus*, Pot pol - *Potamogeton polygonifolius*, Pot tri - *Potamogeton trichoides*, Ran aqu - *Ranunculus aquatilis*, Ran tric - *Ranunculus trichophyllus*, Rup cir - *Ruppia cirrhosa*, Spa ang - *Sparganium angustifolium*, Spa ere - *Sparganium erectum*, Sub aqu - *Subularia aquatica*, Utr aus - *Utricularia australis*, Ver bec - *Veronica beccabunga*, Zan pal - *Zannichellia palustris*, Zos nol - *Zostera noltii*.

Fig. S2. Box plot of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures by water body. Each box shows the median (black line), the interquartile range (box) and the outliers (open circles). Check Table S1 for water body geomorphological type and location.