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RESEARCH ARTICLE

Do stable carbon and nitrogen isotope values of *Nitella flexilis* differ between softwater and hardwater lakes?

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Abstract

The composition of stable carbon and nitrogen isotopes ($\delta^{13}C$ and $\delta^{15}N$) is widely used for tracking the origin of organic matter (OM) present in lacustrine sediments. These isotopes also define the evolution of OM in food web loops. Charophyte species *Nitella flexilis* C. Agardh, 1824 can be found in different aquatic environments where it contributes significantly to sediment formation and influences biota function. Therefore, it is crucial to study more about $\delta^{13}C$ and $\delta^{15}N$ in different lake types. Here, we present the results of the first comprehensive study of *N. flexilis* $\delta^{13}C$ and $\delta^{15}N$, which add to the knowledge of the C and N isotope records of charophytes. We obtained the $\delta^{13}C$ and $\delta^{15}N$ records of *N. flexilis* OM from hardwater and softwater lakes and checked for differences between these records. We also analyzed the differences in physical and chemical parameters. Finally, we compared the $\delta^{13}C$ and $\delta^{15}N$ records with physical and chemical parameters to identify the variables that have the highest influence on *N. flexilis* $\delta^{13}C$ and $\delta^{15}N$ values. Our study showed that both $\delta^{13}C$ and $\delta^{15}N$ did not differ significantly in the two types of lakes, although the lakes had significant differences in several physical and chemical parameters (pH, Ca^{2+}, dissolved inorganic carbon, total phosphorus, conductivity). However, we observed that $\delta^{13}C$ values were influenced by light conditions (photosynthetic active radiation, depth, dissolved OM), while $\delta^{15}N$ values were influenced by the total nitrogen concentration in water.

Keywords $\delta^{13}C$ · $\delta^{15}N$ · Macrophytes · Charophytes · Organic matter · Water chemistry

Introduction

Charophytes (Charales; Charophyta) are macroscopic green algae distributed in various aquatic environments, including freshwater wetlands, brackish ecosystems, running and standing water, and permanent and ephemeral waters (Pukacz et al. 2013; Rodrigo et al. 2016; Torn et al. 2019; Brzozowski and Pełechaty 2020; Romanov et al. 2022). However, these organisms mainly prefer freshwater ecosystems, especially lakes with good lighting (high water transparency) (Pukacz et al. 2013; Brzozowski and Pełechaty 2020). *Nitella flexilis* C. Agardh, 1824 is an example species with a broad ecological amplitude; however, it forms dense underwater meadows covering the bottom in oligo-mesotrophic softwater lakes characterized by a low but more neutral water pH (Bociąg et al. 2011). Nonetheless, this charophyte species can be found as well-developed communities in more hardwater and eutrophic lakes (Urbaniak and Gąbka 2014). After their decay, these macrophytes might significantly contribute to the formation of organic

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sediments. Literature data indicate that charophytes from the *Chara* genus participate in the deposition of carbonate-rich sediments as a high amount of calcium carbonate precipitates from their heavily encrusted thallus (Pełechaty et al. 2013). However, *Nitella* spp. may be slightly encrusted or unencrusted, depending on the calcium concentration in water (Apolinarska et al. 2011; John and Rindi 2015). Thus, *N. flexilis* occurring in different lake habitats, ranging from softwater to hardwater, could be an excellent study material to analyze the stable carbon and nitrogen isotope signatures (δ^{13}C and δ^{15}N) of their OM and the variables influencing them. In this work, we investigated the δ^{13}C and δ^{15}N records of this charophyte species because knowledge of its relationship with physical and chemical water parameters may help to conduct further paleoecological research, for example, to track the vegetation changes or the evolution of lakes from oligotrophic, softwater lakes to mesotrophic, more hardwater lakes based on sediment deposition by this species.

The concentration of calcium ions is not the only factor that differentiates softwater and hardwater lakes, as their names might suggest (Murphy 2002). Other important physical and chemical parameters, namely conductivity and pH (Murphy 2002), which differentiate aquatic vegetation with stable carbon and nitrogen isotopes, also differ between these lakes. According to researchers, the δ^{13}C and δ^{15}N values of aquatic plants in different aquatic ecosystems might be related to multiple parameters of water and sediment chemistry, such as pH and nutrient concentration (King et al. 2009; Matuszak et al. 2011; Apolinarska et al. 2016; Pronin et al. 2016, 2019; Chappuis et al. 2017; Zhang et al. 2021; Liu et al. 2022). Moreover, literature reports on the δ^{13}C values of the organic matter (OM) of charophytes are limited (Pentecost et al. 2006; Sensuła et al. 2006; Matuszak et al. 2011; Apolinarska et al. 2016; Pronin et al. 2016; Rodrigo et al. 2016; Chappuis et al. 2017; Morkūnė et al. 2022), and even less in the case of δ^{15}N values (King et al. 2009; Matuszak et al. 2011; Chappuis et al. 2017; Morkūnė et al. 2022).

Due to the environmental plasticity of *N. flexilis*, it is an excellent material to verify if the differences in the water chemistry of softwater and hardwater lakes have an impact on the δ^{13}C and δ^{15}N values of the OM of *N. flexilis*. It is worth emphasizing that, to our knowledge, this is the first study to analyze the δ^{13}C and δ^{15}N values of *N. flexilis*. The study also attempted to identify the water parameters that have the highest influence on the isotope values of *N. flexilis*.

Materials and methods

Study sites

The study included 12 hardwater lakes (Ca^{2+} concentration from 21.9 to 47.4 mg/l, Table 1) and 11 softwater lakes (Ca^{2+} concentration from 2.2 to 12.4 mg/l, Table 1). The hardwater lakes were investigated in the middle of the growing season of 2008–2010 and softwater lakes in July 2020 (Fig. 1). The classification of softwater lakes followed the recommendation of Murphy (2002). Moreover, to determine the character of the direct catchment, a 100-m belt was created around each lake using ArcGIS 10.7 software (Esri) and Geoportal (https://www.geoportal.gov.pl/) and CORINE Land Cover maps (CLC). This helped identify the main land cover types, which are listed in Table 1. We used ArcGIS software and Geoportal to calculate the total area of each investigated lake, as shown in Table 1. To assess the trophy state of the lakes, we calculated the Carlson Trophy State Index (TSI) based on the available data of total phosphorus (TP) and Secchi disk visibility (Carlson 1977). The TSI is listed in Table 1 as an average of these two components.

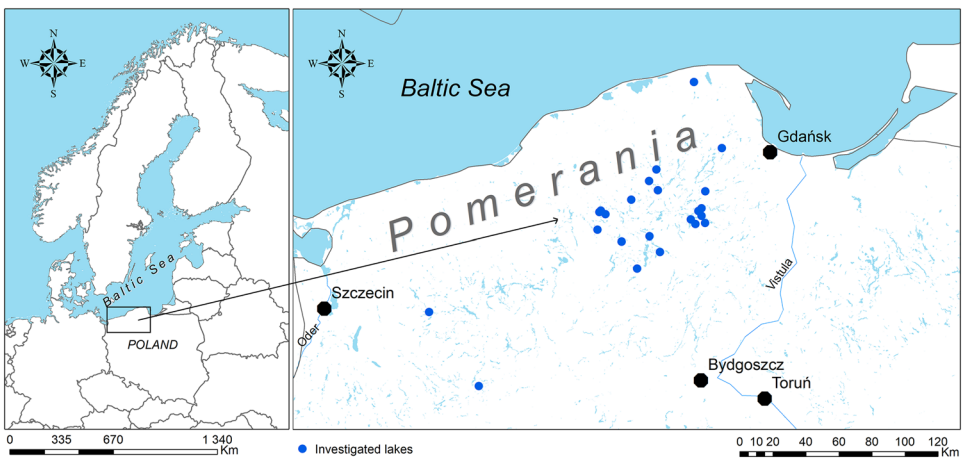


Fig. 1 Localization of the investigated lakes

Table 1 Characteristics of two types of investigated lakes

Lake	Localization	Total surface (ha)	Group	$\delta^{13}C$ (‰ V-PDB)	$\delta^{15}N$ (‰ AIR)	Depth (m)	pH	Conductivity (µS cm^{-1})	DIC (mg C/l)	DOC (mg C/l)	Ca^{2+} (mg Ca/l)	TN (mg N/l)	TP (mg P/l)	PAR (%)	$O_{2\,(\%)}$	C/N	*N. flexilis* cover (%)	TSI[a]	Main catchment type
Ińsko	53° 26′ 40.80″ N, 15° 32′ 23.71″ E	589.9	Hard-water	−20.05	−7.69	2.8	7.92	211.0	16.25	1.17	35.06	1.10	0.15	22.20	123.8	11.80	50	40.2	Forest
Piaszno	54° 5′ 37.94″ N,17° 21′ 36.89″ E	62.7	Hard-water	−21.97	0.61	4.6	7.70	189.5	15.22	1.66	32.95	0.50	0.15	11.78	116.0	12.11	100	40.2	Forest
Dymno	53° 55′ 31.75″ N, 17° 3′ 18.70″ E	77.0	Hard-water	−23.28	−3.51	6.0	8.39	175.0	15.53	1.41	43.02	0.66	0.15	9.80	102.5	10.82	100	38.7	Forest/ agricultural
Choczewskie	54° 44′ 28.18″ N, 17° 55′ 25.62″ E	169.7	Hard-water	−22.54	1.16	1.5	8.01	166.1	13.15	2.75	26.37	2.13	0.29	19.56	122.3	16.58	50	51.4	Forest
Gardliczno	53° 53′ 50.21″ N, 17° 32′ 8.08″ E	44.5	Hard-water	−30.06	2.89	4.5	7.87	187.9	14.78	2.24	32.71	1.23	0.15	14.58	106.7	8.23	50	40.8	Forest
Strupino	54° 2′ 18.63″ N, 17° 58′ 57.07″ E	41.7	Hard-water	−20.99	−1.18	6.3	7.77	157.6	10.63	0.90	27.90	0.74	0.20	1.40	109.3	13.30	15	40.8	Forest

Table 1 (continued)

Lake	Localization	Total surface (ha)	Group	$\delta^{13}C$ (‰ V-PDB)	$\delta^{15}N$ (‰ AIR)	Depth (m)	pH	Conductivity (µS cm^{-1})	DIC (mg C/l)	DOC (mg C/l)	Ca^{2+} (mg Ca/l)	TN (mg N/l)	TP (mg P/l)	PAR (%)	$O_{2\,(\%)}$	C/N	*N. flexilis* cover (%)	TSI[a]	Main catchment type
Kotel	54° 0′ 45.19″ N, 18° 0′ 46.75″ E	28.1	Hard-water	−20.26	−3.50	1.2	7.92	178.0	13.89	1.00	28.46	0.41	0.24	40.80	122.4	9.20	5	48.3	Forest
Czyste	53° 58′ 4.12″ N, 17° 57′ 29.57″ E	33.5	Hard-water	−26.02	−2.48	2.1	8.11	195.0	16.68	1.41	37.30	0.75	0.10	13.40	134.1	12.07	5	42.3	Forest
Białe	54° 22′ 38.03″ N, 18° 11′ 34.18″ E	50.5	Hard-water	−25.62	−0.51	5.2	8.02	131.1	9.37	0.52	21.96	1.15	0.35	8.71	84.4	11.84	100	46.1	Forest/ agricultural
Prusionek	53° 58′ 25.86″ N, 18° 2′ 48.15″ E	5.98	Hard-water	−22.99	−1.15	1.4	7.90	148.0	11.33	1.93	27.90	1.38	0.35	21.10	138.8	12.46	5	51.2	Forest
Szczuczarz	53° 3′ 16.68″ N, 16° 1′ 11.69″ E	138.2	Hard-water	−23.81	−3.53	3.0	8.00	266.0	20.05	1.32	43.37	0.38	0.21	20.87	115.5	9.93	1	46.0	Forest
Wdzydze	53° 59′ 31.66″ N, 17° 54′ 50.85″ E	954.6	Hard-water	−26.22	−3.29	3.1	7.88	243.0	23.44	7.23	47.37	2.60	0.23	4.80	83.1	9.75	1	46.4	Forest

Table 1 (continued)

Lake	Localization	Total surface (ha)	Group	$\delta^{13}C$ (‰ V-PDB)	$\delta^{15}N$ (‰ AIR)	Depth (m)	pH	Conductivity (µS cm^{-1})	DIC (mg C/l)	DOC (mg C/l)	Ca^{2+} (mg Ca/l)	TN (mg N/l)	TP (mg P/l)	PAR (%)	$O_{2\,(\%)}$	C/N	*N. flexilis* cover (%)	TSI[a]	Main catchment type
Dobrogoszcz	54° 8′ 49.62″ N, 18° 2′ 30.012″ E	54.3	Softwater	−23.10	3.18	1.9	8.16	194.0	11.08	1.61	12.38	2.92	0.07	0.50	69.5	13.85	10	44.7	Forest/agricultural
Jeleń	54° 11′ 52.46″ N, 17° 31′ 14.75″ E	81.1	Softwater	−14.75	−0.09	2.3	8.09	56.6	7.78	0.58	7.29	3.33	0.03	10.40	88.8	12.48	35	28.9	Forest
Kamień	54° 1′ 39.39″ N, 17° 3′ 51.89″ E	45.5	Softwater	−16.43	−2.30	0.6	6.80	48.6	9.00	4.48	5.89	2.46	0.08	31.20	83.3	11.38	25	38.8	Forest
Krasne	53° 52′ 5.31″ N, 17° 16′ 52.93″ E	28.8	Softwater	−27.09	−1.67	2.2	5.87	24.3	9.64	3.83	3.25	0.55	0.03	3.30	82.4	13.11	100	35.3	Forest
Moczadło	53° 48′ 49.87″ N, 17° 38′ 6.39″ E	4.0	Softwater	−22.81	−0.79	5.5	5.97	29.7	5.70	0.75	3.05	0.64	0.02	34.30	91.1	11.04	15	24.3	Forest
Obrowo Małe	54° 15′ 40.13″ N, 17° 35′ 12.42″ E	9.5	Softwater	−20.08	−1.98	2.5	6.74	29.6	4.84	0.56	3.81	3.83	0.03	12.50	66.7	10.61	2	34.1	Forest
Okoń Duży	53° 43′ 14.83″ N, 17° 25′ 34.12″ E	5.7	Softwater	−23.95	−1.01	2.3	7.20	44.9	6.91	2.74	6.01	4.97	0.04	21.00	90.1	8.96	1	36.4	Forest

Table 1 (continued)

Lake	Localization	Total surface (ha)	Group	$\delta^{13}C$ (‰ V-PDB)	$\delta^{15}N$ (‰ AIR)	Depth (m)	pH	Conductivity (µS cm^{-1})	DIC (mg C/l)	DOC (mg C/l)	Ca^{2+} (mg Ca/l)	TN (mg N/l)	TP (mg P/l)	PAR (%)	$O_{2\,(\%)}$	C/N	*N. flexilis* cover (%)	TSI[a]	Main catchment type
Piasek	54° 0′ 46.71" N, 17° 7′ 4.50" E	40.8	Softwater	−26.29	−5.36	7.5	6.83	50.8	8.68	2.20	8.46	0.74	0.07	7.60	79.4	9.52	10	31.5	Forest
Smołowe	54° 1′ 56.96″ N, 17° 4′ 48.84″ E	35.6	Softwater	−28.73	−5.49	2.5	6.27	26.5	5.95	2.73	2.16	0.60	0.03	9.50	85.5	9.66	1	34.3	Forest
Zakrzewie	54° 3′ 14.86″ N, 18° 0′ 39.54″ E	10.2	Softwater	−18.97	−2.26	1.1	9.12	69.0	7.99	1.01	10.30	2.57	0.02	46.80	102.5	11.72	15	31.7	Forest
Łąkie	54° 8′ 53.37″ N, 17° 36′ 13.05″ E	21.8	Softwater	−33.13	2.11	4.3	6.52	45.7	7.58	5.71	5.65	4.15	0.04	0.90	48.1	10.39	40	33.6	Forest/agricultural

[a]Trophy State Index calculated there as an average of TSI TP and TSI SD

Field study

Through an experienced SCUBA diver, ten individuals were collected at each plant study site for further analyses of $\delta^{13}C$ of OM ($\delta^{13}C_{ORG}$) and $\delta^{15}N$ of total nitrogen ($\delta^{15}N_{TN}$). Before plant collection, field measurements of pH, oxygen concentration, and conductivity were performed from a boat using a multivariate sonde YSI 650 MDS with 6600 V2 probe in 2020 and a WTW 320/SET1 pH meter with glass METTLER electrode and SENTIX 97T electrode in 2008–2010. Photosynthetic active radiation (PAR) was measured using a Licor LI-250 Light Meter and expressed here as percentage of the light reaching the water surface (based on the obtained values for the water surface and just above-investigated plants), and the depth of the stands was also recorded. In addition, water from the surroundings of the plants was collected in a 0.5-l plastic bottle for chemical analyses to determine the concentrations of nutrients [total nitrogen (TN) and total phosphorus (TP)], calcium (Ca^{2+}), dissolved inorganic carbon (DIC), and dissolved organic carbon (DOC).

Laboratory analyses, including $\delta^{13}C_{ORG}$ and $\delta^{15}N_{TN}$ analysis

The concentration of dissolved forms of inorganic carbon (CO_2, HCO_3^-, and CO_3^{2-}) was assessed in the collected water samples by titration. Ca^{2+} concentration was measured using a complexometric method with EDTA disodium salt in the presence of calconcarboxylic acid sodium salt as an indicator. TN and TP concentrations were determined by photometric methods using a MERCK Spectroquant cuvette on a UV–Vis spectrophotometer. TP was analyzed after mineralizing the water samples using a mixture of nitric and sulfuric acid (2:1). Mineralization was done in a Mars 5 CEM microwave digestion system (USA). After estimating %C and %N concentrations in plant samples (Flash Smart EA, Thermo Scientific USA), the C/N ratios were calculated.

The samples of plants were washed in the field, and epiphytes and other contaminations (sand, sediments, and others) were removed. Then, the samples were dried at 60 °C for 48 h and stored. The plant materials collected from 2008 to 2010 were stored dry in envelopes but dried again before further treatment. Next, dry plant samples were powdered using a mixer mill (MM 400 Retsch Germany) or, if the amount was low, an agate mortar. Afterward, with the use of highly concentrated HCl, *N. flexilis* samples from both lakes were checked for the presence of carbonates. No carbonates were found in the samples from softwater lakes, while samples from hardwater lakes were decarbonated using the desiccator method with 37% of HCl in a desiccator by creating an acidic mist with which the carbonates reacted. The samples in small glass vials were allowed to stay in the desiccator for 48 h. Then, they were placed under a fume cupboard for 24 h for evaporation, dried in the oven at 40 °C for 24 h, and homogenized again using an agate mortar. Finally, the powder was transferred to tiny capsules and weighed. The $\delta^{13}C$ and $\delta^{15}N$ analyses of plants OM were performed in 23 samples (triplicated as a laboratory standard procedure—comprehensive analyses of 69 samples) in the GISMO platform in the Biogéosciences Laboratory of the University of Burgundy (Dijon, France). The analyses were done on a Flash Smart EA elemental analyzer (Thermo Scientific, USA) coupled to a Delta V stable isotope ratio mass spectrometer (Thermo Scientific, USA). The standard USG40 (glutamic acid, $\delta^{13}C = -26.39‰$, $\delta^{15}N = -4.5‰$) and the standard Wheat Flour B2157 (Elemental Microanalysis) certified reference materials were used for calibration and as control. The $\delta^{13}C$ and $\delta^{15}N$ values were expressed in ‰ relative to V-PDB standard for carbon and atmospheric N_2 for nitrogen. The precision of the analysis was validated by external reproducibility of replicate standard analyses (USG40 and B2157) and found to be better than ±0.15‰ for $\delta^{13}C$ and ±0.20‰ for $\delta^{15}N$ (2σ).

Statistical analysis

All the values of stable isotopes and other analyzed physical and chemical variables were checked for the normality of distribution by applying the Shapiro–Wilk test using the Statistica 13.0 software (StatSoft Inc., Tulsa, OK, USA). As the analyzed variables were not normally distributed, nonparametric analyses were used. The data of all variables were not additionally normalized. The Mann–Whitney *U* test was applied for comparing the values of $\delta^{13}C$ and $\delta^{15}N$ of *N. flexilis* and other water parameters in the two types of investigated lakes. In addition, Spearman rank correlations were used to determine the relationships between the investigated variables in all lakes. Moreover, the variables before the principal component analysis (PCA) were standardized as *z*-score to avoid scale effect. All these analyses were performed using the Statistica 13.0 software and visualized by R.4.0.3 software (R Core Team 2022) using the ggplot2 package (Wickham 2009). For all the statistics, $p < 0.05$ was used to determine significance. Correlation heat maps were created in the corrplot R package (Wei and Simko 2021). The PCA was performed using the FactoMineR package (Lê et al. 2008), and the obtained results were visualized using the factoextra and ggplot2 packages (Lê et al. 2008; Wickham 2009).

Results

The results showed no statistically significant differences between the calculated $\delta^{13}C$ and $\delta^{15}N$ values of *N. flexilis* collected from the two types of lakes (Fig. 2). Furthermore, the $\delta^{13}C$ values in softwater lakes showed more variations (ranging from −33.12‰ to −14.75‰; Table 1, Fig. 2a) compared with the values in hardwater lakes (from −30.06‰ to −20.05‰; Table 1, Fig. 2a). On the other hand, the $\delta^{15}N$ values only slightly differed in the investigated groups of lakes (−7.69‰ to 2.88‰ in hardwater and −5.49‰ to 3.18‰ in softwater lakes; Table 1, Fig. 2b). Comparing the results of selected investigated variables in the two types of lakes, we observed statistically significant differences (Mann–Whitney *U* test: $p < 0.05$) in pH, Ca^{2+} concentration, TP concentration, DIC concentration, and conductivity (Fig. 3a–e). There were also differences in TN concentration, where a wide range of values was detected in the softwater lakes, but in the hardwater lakes, the variability of TN concentrations was narrower (Fig. 2f); however, these differences were statistically insignificant (Mann–Whitney *U* test: $p > 0.05$). The above-mentioned differences were also reflected by the PCA results (Fig. 4), where the two groups of investigated lakes were clearly segregated. Among the investigated variables, pH, Ca^{2+}, conductivity, DIC, TP, and TN differed the most in the two groups of lakes and correlated with the first axis of PCA (Fig. 4). Moreover, *N. flexilis* values of $\delta^{13}C$ were more closely related to the second PCA axis, which correlated with PAR, depth, and DOC. TN concentration was also found to be a crucial factor affecting *N. flexilis* values of $\delta^{15}N$ (Fig. 4). The first two PCA axes explained 51.5% of the total variance. We found several relationships when comparing the obtained $\delta^{13}C$ and $\delta^{15}N$ values of *N. flexilis* with the physical and chemical parameters of water. The values of $\delta^{13}C$ were positively related to PAR and negatively correlated with the depth and concentration of DOC. In turn, $\delta^{15}N$ values showed a moderate positive relationship with TN and a low negative relationship with Ca^{2+} concentration (Fig. S1 in Supplementary Materials 1).

Discussion

The $\delta^{13}C$ and $\delta^{15}N$ values of aquatic plants range widely among different aquatic ecosystems. The $\delta^{13}C$ values of macrophytes range from −50‰ to 0.4‰ (Herzschuh et al. 2010), and that of $\delta^{15}N$ from −15‰ to 20‰ (Douglas et al. 2022). Our study showed that the $\delta^{13}C$ and $\delta^{15}N$ values of *N. flexilis* OM also fall in the above-mentioned ranges. Moreover, the values of $\delta^{13}C$ reported by us are in line with the range of values previously reported for charophytes (Pentecost et al. 2006; Apolinarska et al. 2016; Pronin et al. 2016; Rodrigo et al. 2016; Chappuis et al. 2017). Similarly, the values of $\delta^{15}N$ determined in this study were in line with those reported for charophytes in the limited available literature (King et al. 2009; Matuszak et al. 2011; Chappuis et al. 2017; Morkūnė et al. 2022).

Stable carbon isotope composition of *N. flexilis*

Compared with hardwater lakes, the concentration of DIC is limited in softwater lakes, and the water pH is usually lower. Thus, we expected significantly lower $\delta^{13}C$ values in the studied softwater lakes. We made this assumption by changing the proportion of C in water according to the variability of pH. In water with pH below 4.5, CO_2 is the only form of inorganic carbon. On the other hand, in water with a neutral pH, HCO_3^- starts to be the dominant form (the highest concentration is in pH about 8.5), whereas if pH is higher than 10.5, CO_3^{2-} is the main C form. The forms of C differ in $\delta^{13}C$ values; in CO_2, ^{13}C is depleted by 8–12‰ compared with HCO_3^- (Mook et al. 1974; Shmit and Walker 1980). This assumption was confirmed in the case of Jeleń and Zakrzewie lakes, where water pH was high and the $\delta^{13}C$ values were also among the highest (Table 1). On the other

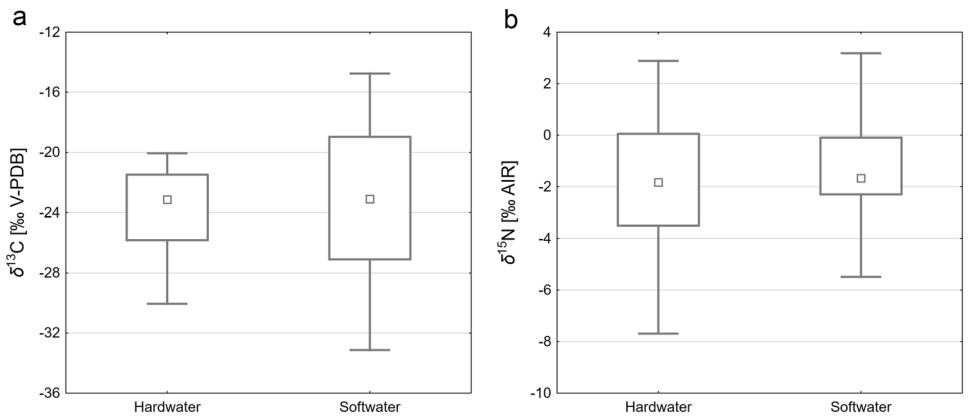


Fig. 2 Comparison of *N. flexilis* in hardwater ($N = 12$) and softwater ($N = 11$) lakes: **a** $\delta^{13}C$ values; **b** $\delta^{15}N$ values. Median, box 25–75% and min–max. Mann–Whitney *U* test: $p > 0.05$ in both cases

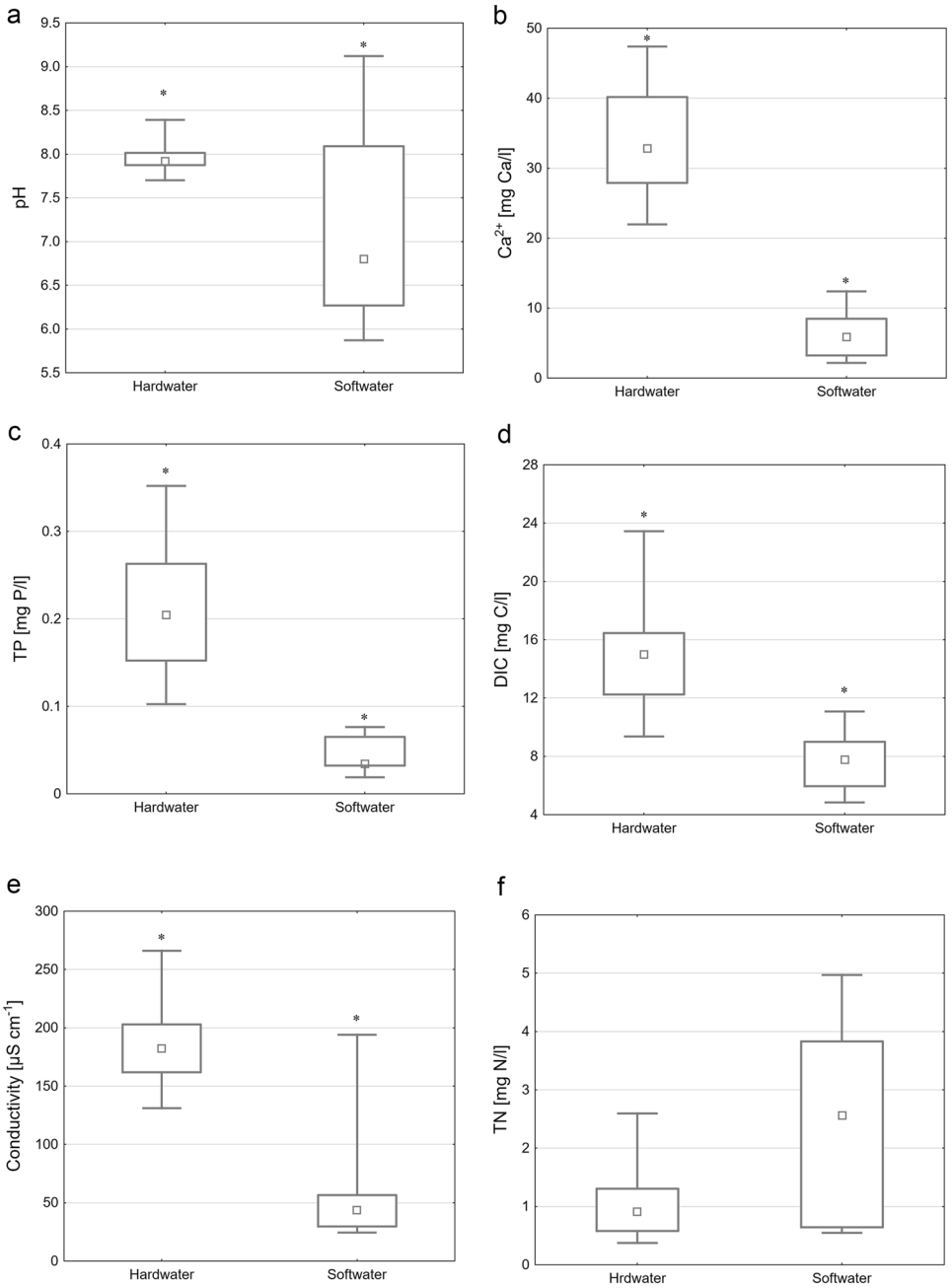


Fig. 3 Comparison of the physical and chemical variables of water in hardwater ($N = 12$) and softwater ($N = 11$) lakes with *N. flexilis* stands. Median, box 25–75%, and min–max. Mann–Whitney *U* test: $^{*}p < 0.05$

hand, in Lake Kamień, where water pH was almost neutral, the values of $\delta^{13}C$ were also high (Table 1). Unfortunately, in this study, we did not investigate the $\delta^{13}C$ values of DIC, which would indicate the isotope value of inorganic carbon in water. However, we found significant positive correlations between pH and DIC (Fig. S1 in Supplementary Materials 1). This relationship might partly explain the high variability in the obtained $\delta^{13}C$ values of *N. flexilis*, especially in softwater lakes, because *N. flexilis* could use both CO_2 and HCO_3^- forms of DIC for photosynthesis (Shmit and Walker 1980; Chmara et al. 2021). Furthermore, a study on a Mediterranean pond near shallow lagoons in Spain, which is richer in Ca^{2+}, showed that $\delta^{13}C$ values of *Nitella hyalina* ranged from −26‰ to −20‰ (Rodrigo et al. 2016). We assumed that the $\delta^{13}C$ values of hardwater lakes would not differ much due to more consistent water pH and the fact that the water in these lakes has higher buffer capability compared with that in softwater lakes. Although the range of $\delta^{13}C$ values in hardwater lakes was lower than that of softwater lakes, it was still significant (Fig. 2). These results indicate that other variables may also have a more significant influence on the $\delta^{13}C$ values of *N. flexilis* OM.

One of these variables might be related to the ecology of *N. flexilis*, as this species forms more complex and dense

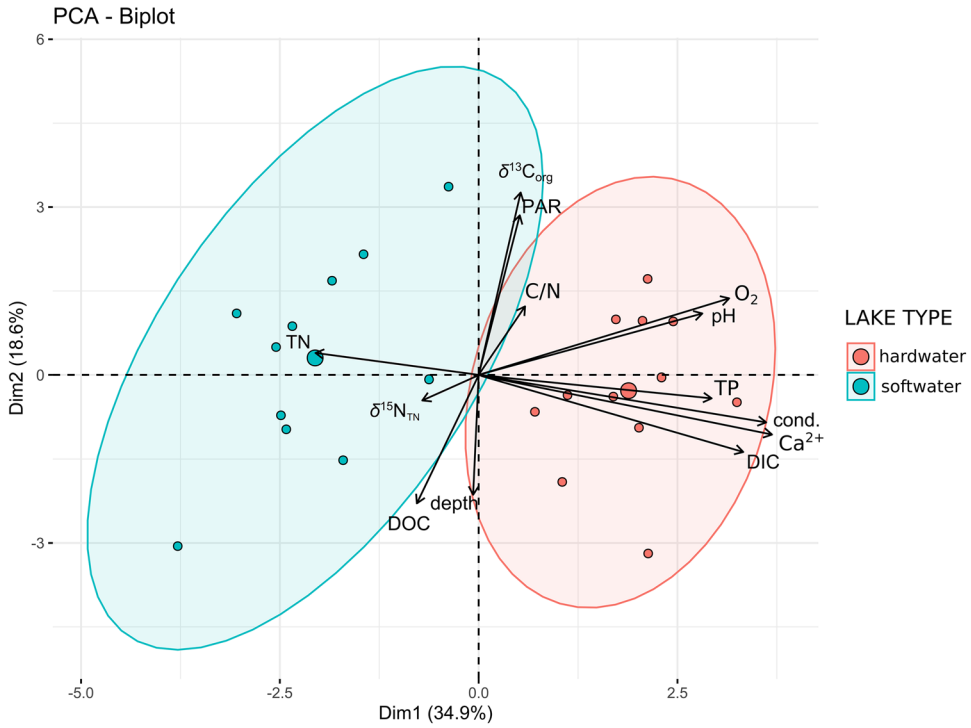


Fig. 4 PCA of the investigated variables of hardwater and softwater lakes. The ellipse represents 95% contribution of sites in the selected groups

underwater meadows in hardwater lakes (Urbaniak and Gąbka 2014; Table 1). These meadows perform many functions in lakes, one of which is the accumulation of biomass (Kufel and Kufel 2002; Pełechata et al. 2023) that might release a higher amount of $^{12}CO_2$ during decomposition. Moreover, such dense meadows of charophytes stimulate more CO_2 release from the interstitial water, which might have higher $^{12}CO_2$ than the ambient water. These assumptions align with the significant differences observed by Pronin et al. (2016) between two morphologically different *Chara* species. The authors suggested that differences between the $\delta^{13}C$ values of OM of those two species were due to the use of different proportions of C sources for photosynthesis: CO_2 and partly HCO_3^- by *Chara globularis* Thuillier 1799 and mostly HCO_3^- by *Chara tomentosa* L. This might also partly explain the high variability in the $\delta^{13}C$ values of the two groups of lakes investigated here. However, other researchers who investigated charophytes as a whole group (including *Nitella* and *Chara* genera together) found extensive variability in $\delta^{13}C$ values in the OM of charophytes (from −40‰ to −10‰; Chappuis et al. 2017), which could not be explained only by the use of different isotopic signals of C source for photosynthesis. Similarly, our findings, especially those observed for softwater lakes, are outside the range of differences of $\delta^{13}C$ values between CO_2 and HCO_3^-; thus, this might only partly explain this variability.

The above statement is in line with the results of Liu et al. (2022), who suggested that the differences in $\delta^{13}C$ values between CO_2 and HCO_3^- only partly explain the observed differences between the two macrophytes (*Potamogeton* sp. and *Cladophora* sp.) investigated in their study. The rest of the high variability in $\delta^{13}C$ values (average 14–16‰, which was close to 10‰ in hardwater lakes and 18‰ in softwater lakes reported by us) between these two species was probably caused by biosynthesis fractionation (Liu et al. 2022). Our results might also support this statement due to the relationships of $\delta^{13}C$ with the lake's light conditions (PAR, depth, and DOC concentration; Figs. 4 and S1). Light availability increases the efficiency of photosynthesis (Van Den Berg et al. 1998), and plants preferentially uptake ^{12}C (O'Leary et al. 1992). Thus, we can assume that under more intensive photosynthesis, plants might use more ^{13}C due to rapidly decreasing ^{12}C, and as a result, $\delta^{13}C$ of their OM increases.

Furthermore, a study on Lake Constance in Germany showed high negative relationships between the $\delta^{13}C$ values of *Chara* spp. and depth (Matuszak et al. 2011). Our results on the $\delta^{13}C$ values of *N. flexilis* also support this observation. However, this relationship was only noticeable and not very high (Fig. S1 in Supplementary Materials 1). This might be due to the fact that diverse lake ecosystems were included in our study. On the other hand, Liu et al. (2022) did not find such relationships when they investigated two plant species along a depth gradient in three Tibetan Plateau lakes in China. Thus, we believe that all types of related isotopic studies concerning

macrophytes, including charophytes, are essential to better understand the complexity of $\delta^{13}C$ in plant OM.

Stable nitrogen isotope composition of *N. flexilis*

Similar to $\delta^{13}C$ values, our results on the $\delta^{15}N$ values of *N. flexilis* were in line with those reported in the literature. King et al. (2009) showed that the $\delta^{15}N$ values of *Nitella* sp. varied from −9.94‰ to 6.50‰, which are higher than that reported by us (−7.69‰ to 3.18‰). Similar values were also reported by Chappuis et al. (2017) for charophytes; however, the range was shifted to ^{15}N-enriched values (from about −2.5‰ to 9.9‰). Thus, our results do not differ much from those reported for various lakes, ranging from coastal lakes to mountain oligotrophic lakes (Chappuis et al. 2017) and nutrient-limited upland lakes (King et al. 2009). Our findings confirmed the high variability of $\delta^{15}N$ in macrophytes, especially charophytes, previously reported in several studies (King et al. 2009; Matuszak et al. 2011; Chappuis et al. 2017; Morkūnė et al. 2022). The high variability of $\delta^{15}N$ macrophytes and charophytes might be related to their use of different N sources for tissue development (Chappuis et al. 2017). According to studies, macrophytes might use different forms of N dissolved in water, namely NO_3^-, NH_4^+, and N_{ORG}, for the development of their structures (Schuurkes et al. 1986; Peipoch et al. 2012, 2014; Pastor et al. 2014).

Furthermore, the fractionation of $\delta^{15}N$ during the development of plant tissues tends to favor ^{14}N (Evans 2001). However, this might be less important when the plant N demand exceeds plant N availability (Pennock et al. 1996; Waser et al. 1998; Jones et al. 2004). Unfortunately, in our study, we obtained only complete data on TN for both types of investigated lakes. Therefore, we can assume that the recorded $\delta^{15}N$ variability of *N. flexilis* was related to a different form of dissolved N in water. This hypothesis might be supported by the moderate positive correlations found between $\delta^{15}N$ and TN (Fig. S1 in Supplementary Materials 1). However, this might also be related to the impact of N load from the catchment of investigated lakes. Chappuis et al. (2017) observed this relationship in their study on 81 aquatic ecosystems across Catalonia (northeastern Spain). The lakes investigated in our study were mainly located in the forest-dominated catchment (Table 1); thus, we can conclude that the variability of N input was relatively low. Our comparison showed that the difference in TN concentration between hardwater and softwater lakes was statistically insignificant; however, higher concentrations were recorded in softwater lakes (Fig. 3f). This might be related to the potential C or P limitation of these lakes due to significantly lower TP and DIC concentrations compared with hardwater lakes (Fig. 3c and d). Therefore, the slight variability in $\delta^{15}N$ between hardwater and softwater lakes observed in this study might be due to N demand and availability than other factors, as evidenced by King et al. (2009). On the other hand, the pH variability (Fig. 3a) in softwater lakes may favor the occurrence of NH_4^+ and NO_3^- forms in water, which can be available for uptake by *N. flexilis*.

$\delta^{13}C$ and $\delta^{15}N$ of plant OM and C/N ratio as a potentially helpful marker in different types of studies

In many types of studies, the authors support their research using stable isotope methods. In the past two decades, $\delta^{13}C$ and $\delta^{15}N$ values of plant OM have more often been used in source mixing models, for example, to estimate the proportional contribution of OM in lacustrine sediments (Guo et al. 2020; Duan et al. 2022; Wu et al. 2022; Douglas et al. 2022). Recently, some authors studied macrophytes, including charophytes, to track food sources in the food web loop in aquatic environments (Morkūnė et al. 2022). The C/N results presented by us are in line with those reported in the literature for some charophyte species (Rodrigo et al. 2016). As Meyers (1994) suggested, parameters such as the C/N ratio of plants and sediments are a good indicator of the origin of material that forms sediments and are also included as an additional proxy in source mixing models with isotopic data (Duan et al. 2022). Thus, to the best of our knowledge, the first complete information on the $\delta^{13}C$ and $\delta^{15}N$ values and C/N ratio of *N. flexilis* collected from different lake types, presented in this paper, might be helpful to validate better the isotopic-oriented models used for the above-mentioned purposes. Furthermore, our results indicated that it could be difficult to track vegetation changes and shifts in lake trophic conditions on the basis of *N. flexilis* $\delta^{13}C$ and $\delta^{15}N$ values and C/N ratio in the OM deposited in the sediments of the studied lakes. This is due to the non-statistically significant differences in $\delta^{13}C$ and $\delta^{15}N$ values and C/N ratios between the two investigated groups of lakes and the relatively higher variability of the obtained results, especially in softwater lakes.

Conclusion

Although the two studied groups of lakes (softwater and hardwater) showed differences in Ca^{2+}, pH, conductivity, DIC, and TP, the $\delta^{13}C$ and $\delta^{15}N$ values of *N. flexilis* OM were not statistically different. We found some relationships indicating the influence of light conditions on the $\delta^{13}C$ values of the investigated green macroalgae and a moderate relationship between the $\delta^{15}N$ values and TN concentration. Our results suggest that an attempt to identify the factors differentiating $\delta^{13}C$ and $\delta^{15}N$ in plant OM should be cautiously approached. The findings presented by us suggest the need for more in-depth studies, especially experimental ones

with thoughtful factor gradient settings, to determine the factors that significantly shape the $\delta^{13}C$ and $\delta^{15}N$ values of submerged plants. The information from such studies might be helpful, for example, to identify the source of OM in lacustrine sediments and to better interpret food webs with charophytes as primary producers.

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Author contributions EP: conceptualization, supervision, methodology, project administration, funding acquisition, formal analysis, writing—original draft, writing—review and editing, visualization. KB: methodology, writing—review and editing. RC: methodology, writing—review and editing. MM: methodology, writing—review and editing. RR: methodology, writing—review and editing. ALS: methodology, writing—review and editing. OM: methodology, writing—review and editing

Data availability Most data generated or analyzed during this study are included in this article. The rest of the included data are available from the authors on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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