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PRIMARY RESEARCH PAPER

Sharp differences in the $\delta^{13}C$ values of organic matter and carbonate encrustations but not in ambient water DIC between two morphologically distinct charophytes

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Abstract The stable carbon isotope composition of the carbonate encrustations ($\delta^{13}C_{CARB}$), organic matter ($\delta^{13}C_{ORG}$) and the dissolved inorganic carbon ($\delta^{13}C_{DIC}$) in the ambient lake waters were analysed for two common but morphologically different and, thus, representing different growth forms of *Chara* species. We hypothesized that the relationships between $\delta^{13}C_{CARB}$, $\delta^{13}C_{ORG}$ and $\delta^{13}C_{DIC}$ are species specific and related to the different growth forms of the studied charophytes. For each species (*Chara tomentosa* and *Chara globularis*), 10 individuals and water samples from above the macrophytes were collected in five lakes at three sites per lake in mid-summer. Opposing shifts were found between $\delta^{13}C_{CARB}$ and $\delta^{13}C_{DIC}$ values with ^{13}C enrichment in *C. tomentosa* and ^{13}C depletion in *C. globularis*. In addition, *C. globularis* exhibited more negative values of $\delta^{13}C_{ORG}$ than *C. tomentosa*, even under similar conditions. The $\delta^{13}C_{CARB}$ and $\delta^{13}C_{ORG}$ values were positively correlated in both species, but $\delta^{13}C_{CARB}$ and $\delta^{13}C_{DIC}$ as well as $\delta^{13}C_{ORG}$ and $\delta^{13}C_{DIC}$ were positively correlated in *C. tomentosa* only. The differences found result from the different proportions between ^{13}C and ^{12}C in DIC used as a CO_2 source for photosynthesis, which is linked to the different growth forms represented by the two studied charophytes and, thus, are species specific as we hypothesized.

Keywords Stable isotopes · C · O · Characeae · Organic matter · Carbonate · Encrustation

Introduction

Charophytes (Charales; Charophyta) are aquatic macroscopic green algae of the family Characeae, whose

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members are distributed around the world and are found in various types of aquatic environments, including brackish and freshwaters, standing and floating water, and permanent and ephemeral waters (e.g., Hutchinson, 1975; Wade, 1990). However, charophytes prefer freshwater lakes, where they grow along a wide depth gradient depending on light availability (e.g., Martin et al., 2003). Charophytes can significantly modify the conditions of their environments, influencing both abiotic (e.g., pH, hardness, O_2 concentration and water clarity) and biological (e.g., the structure of plankton community) components of aquatic ecosystems, particularly when they form dense and extensive meadows (Kufel & Kufel, 2002; Pentecost et al., 2006; Apolinarska et al., 2011; Pełechaty et al., 2013). The precipitation of $CaCO_3$ as encrustations on the thalli surface, a feature typical of these macroalgae, results from photosynthetic CO_2 assimilation from soluble bicarbonates (McConnaughey, 1998). According to different authors, the amount of $CaCO_3$ on encrusted charophyte species may range from approximately 30% to over 80% of their dry mass (e.g., Pentecost, 1984; Krolikowska, 1997; Kufel & Kufel, 2002; Pełechaty et al., 2010; Urbaniak, 2010; Pełechaty et al., 2013). Thus, dense and extensive charophyte meadows contribute significantly to calcium carbonate precipitation and the deposition of lake marl sediments (Pełechaty et al., 2013).

The presence of carbonate encrustations promotes the preservation of charophyte remains in sediments and sedimentary rocks. The oldest charophyte fossils found date back to the Upper Silurian period (Croft, 1952). Thus, these macroalgae represent a potential archive for use in palaeoenvironmental studies (Apolinarska et al., 2011). Information regarding the precipitation of carbonate encrustation in *Chara* marl lakes and its possible application has increased over the last two decades. Coletta et al. (2001) postulated that the oxygen and carbon stable isotope values recorded in carbonate deposits on thalli could contain a record of the environmental conditions under which these carbonates precipitated. Furthermore, $\delta^{13}C$ and $\delta^{18}O$ measurements along the charophyte stem—the so-called isotope age gradient—can indicate seasonal changes in environmental conditions, including changes in the water temperature, the evaporative influence on the $\delta^{18}O$ value of water or changes in the $\delta^{13}C$ value of dissolved inorganic carbon (DIC) in water due to the photosynthetic activity of primary producers (Pentecost et al., 2006). In temperate climates, most calcification takes place during the summer months (Pentecost et al., 2006; Pełechaty et al., 2010). Therefore, stable isotope values tend to peak during the growing season. The use of charophyte carbonate $\delta^{13}C$ and $\delta^{18}O$ values as an environmental record is possible only if the relation between the carbon and oxygen isotope values of modern charophyte encrustations, as well as $\delta^{18}O_{WATER}$ and $\delta^{13}C_{DIC}$, have been characterized.

Very little is known about the $\delta^{13}C$ values of charophyte organic matter and their relationships with the $\delta^{13}C$ values of DIC in surrounding waters and of carbonates precipitated by charophytes. Pentecost et al. (2006) defined the $\delta^{13}C$ values of *Chara hispida* Linné 1753 organic matter but found only a weak and statistically insignificant correlation between the $\delta^{13}C$ values of organic matter and of carbonates. Nuñez et al. (2002) studied the stable carbon isotope composition of bulk organic matter in sediment cores taken from a marl lake (Malham Tarn, NW England) and in peat and modern *Chara* samples for comparison. The authors emphasized the use of $\delta^{13}C$ data biomarkers for the elucidation of environmental changes in lakes and their catchment areas.

In this study, we examine two *Chara* species widely distributed, *Chara tomentosa* Linné 1753 and *Chara globularis* Thuillier 1799, to determine whether there are species-related differences between the $\delta^{13}C$ values of organic matter ($\delta^{13}C_{ORG}$), carbonate encrustations ($\delta^{13}C_{CARB}$) and ambient dissolved inorganic carbon ($\delta^{13}C_{DIC}$). *Chara tomentosa* is a tall branchy species that forms thick but sparser stands compared to *C. globularis*, a slender charophyte that grows in compact carpets close to the sediment. We hypothesized that there would exist species-specific relationships between the $\delta^{13}C$ values of the above components resulting from the contrasting growth forms of the studied charophytes.

Materials and methods

Study sites

The study was carried out in seven lakes located in western Poland (Fig. 1), six in the Lubuskie Lake District and one in the Myśliborskie Lake District. For each of the studied species, *C. tomentosa* and *C. globularis*, individual thalli were sampled in five lakes and at three sites per lake (*Chara* stands). In three lakes, the two species co-occurred. In the other four lakes, the species occurred separately, each in two lakes. Altogether, the study was carried out at 30 sites (15 sites in five lakes for each of the studied species). The lakes differed in terms of morphology, water flow and catchment area (Table 1). This study included both shallow, small, polymictic lakes and deep, large, stratified water bodies (Table 1). The residence time of the water varied from approximately 0.5, 2.8 and 3.3 years in Lakes Malcz, Jasne and Karskie Wielkie, respectively, to about 5 years in other lakes (no reliable data exist for Lake Męcko Duże). With the exception of Lake Karskie Wielkie, which is slightly eutrophicated, the studied lakes are mesotrophic bodies of water. All lakes are characterized by well-developed aquatic vegetation dominated by charophyte meadows (Table 1) and as such represent the *Chara*-lake group. Furthermore, the studied lakes are characterized by high water clarity, with the highest Secchi depth values exceeding 6 m in Lake Pierwsze (Pełechaty et al., 2007) and Lake Męcko Duże (this study). Water clarity exceeding 5 m was measured in Lakes Jasne, Niesłysz and Złoty Potok (Pełechaty et al., 2007). Five of the investigated lakes have a direct catchment area covered mostly by forest. In contrast, the catchments of Lake Niesłysz and Lake Karskie Wielkie are primarily used for agricultural and recreational purposes. Three of the studied lakes, Lake Pierwsze, Lake Męcko Duże and Lake Malcz, are located near the Wędrzyn Military Training Ground. In this territory, human activity is regulated by the armed forces and is therefore substantially restricted.

Chara tomentosa and *Chara globularis* stand structure

Environmental samples were collected from two different types of macrophyte stands dominated either by *C. tomentosa* or by *C. globularis*. According to a preliminary study performed prior to sample collection, both types of stands were well developed in the form of extensive charophyte meadows. In five out of the 15 *Chara tomentosa* stands studied, cover reached 100%, whereas at seven stands it was at least 70%. In the other three stands, the species coverage was 50% (Table 2). Regardless of the exact coverage of *C. tomentosa* in the stands studied, the species was the dominant taxon, with minor or negligible contributions by vascular plants and mosses.

Compared to *Chara tomentosa*, *Chara globularis* coverage was in many cases more extensive. In nine out of the 15 studied stands, this species covered 100% of the studied stand area, and in five stands the coverage reached or even exceeded 70%. At one site, the coverage was only 40% (Table 2). Similar to *C. tomentosa*, *C. globularis* was always the dominant

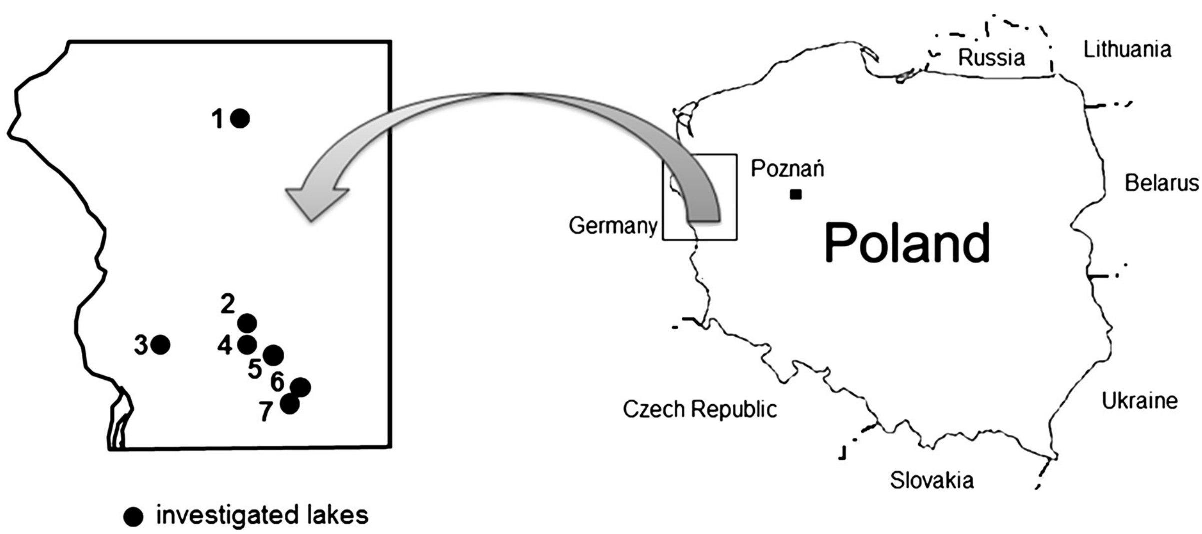

Fig. 1 Locations of the lakes in which *Chara tomentosa* and *Chara globularis* stands were investigated. *1* Lake Karskie Wielkie; *2* Lake Pierwsze; *3* Lake Jasne; *4* Lake Męcko Duże; *5* Lake Malcz; *6* Lake Niesłysz; *7* Lake Złoty Potok

Table 1 Selected habitat characteristics of investigated lakes

Lake	Geographical coordinates	Area (ha)	Max. depth (m)	Mean depth (m)	Character of flow	No. of charophytes forming extensive stands
Męcko Duże[a,b]	52°22′0″N, 15°11′2″E	40.9	22.7	9.2	Closed	7
Karskie Wielkie[a,c]	52°55′4″N, 15°04′8″E	150	17.6	6.2	Outflow	4
Jasne[a,d,e]	52°17′7″N, 15°03′06″E	15.1	9.5	4.3	Closed	7
Niesłysz[a,f]	52°13′9″N, 15°23′8″E	486.2	34.7	7.8	Throughflow	5
Złoty Potok[a,f]	52°13′0″N, 15°22′5″E	32.8	13.7	5.9	Closed	5
Malcz[a,b]	52°21′1″N, 15°13′3″E	36.2	7.3	3.4	Throughflow	4
Pierwsze[a,f]	52°23′11″N, 15°09′18″E	19.3	10.7	4.7	Closed	6

Data sources [a] Jańczak (1996), [b] Pełechaty & Pukacz (2006), [c] Cyrwus (2008, unpublished data), [d] Pełechaty et al. (2010), [e] Pukacz & Pełechaty (2013), [f] Pełechaty et al. (2007)

species in a milieu containing far less abundant vascular plants and mosses.

The water quality parameters measured during the study period (Table 3) confirmed the results of earlier studies, which characterized the investigated lakes as hardwater ecosystems with moderate nutrient availability and high transparency (Pełechaty et al., 2007 and references therein; Kraska, 2009; Cyrwus 2008, unpublished data; Pełechaty et al., 2015). The aforementioned environmental conditions promote charophyte dominance in the form of submerged vegetation (Dąmbska, 1964; Hutchinson, 1975; Krause, 1981, 1997; Blindow, 1992a, b), including extensive meadows formed by both of the species studied.

As the smaller, slenderer and more densely growing charophyte species, *Chara globularis* formed very compact patches concentrated near the bottoms of the lakes studied (depths of 3–4 m). Thus, the species came into contact with deeper waters rather than the more homogeneous waters above the charophyte stand. In contrast, the taller, thicker and branched thallus *Chara tomentosa* formed communities, which, compared to *C. globularis* patches, contacted more surface water and was less influenced by near-sediment conditions. Moreover, *C. tomentosa* patches were not as compact as those of *C. globularis*, allowing waters from above the patch to penetrate (stand depths were 2–3 m).

In published studies, both species are characterized by a high and comparable productivity. Pentecost (1984) showed that the biomass produced by *C. globularis* can exceed 1.1 kg DW m^{-2} (0.89 kg DW m^{-2} on average), although lower biomass values with a maximum of 0.305 kg DW m^{-2} were also recorded (Fernández-Aláez et al., 2002). For *C. tomentosa*, Blindow et al. (2002) recorded values between 0.7 and 0.8 kg DW m^{-2}, which are comparable to those of *C. globularis* recorded by Pentecost (1984). However, Krolikowska (1997) determined a *C. tomentosa* biomass of 1.2 kg DW m^{-2} in a compact monospecific community. The similarity of the two species in terms of biomass production, despite their morphological differences, emphasizes the greater density of *C. globularis* stands compared to those formed by *C. tomentosa*.

Field sampling

At each study site, 10 individual charophytes from an area of 4 m^2 were collected, and water from the immediate environment was sampled for isotopic analyses in July 2012. Prior to charophyte sampling, the basic physical and chemical properties of the water just above each studied charophyte patch were measured (water temperature, oxygen concentration, conductivity, pH) using portable field measurement equipment (Elmetron CX-401; Elmetron Sp. j., Zabrze, Poland, CyberScan 200 and CyberScan 20; Eutech Instruments Europe BV, Nijkerk, The Netherlands, respectively). Water samples for laboratory analyses were collected from above the macrophytes using a bathometer in a one-litre plastic bottle and preserved with chloroform. Water samples for stable carbon isotope analysis of DIC were collected in 10-ml glass septa test tubes and preserved with two drops of $HgCl_2$. All charophyte samples were collected by diving. In addition to the aforementioned

Table 2 Carbon stable isotope composition of DIC ($\delta^{13}C_{DIC}$), carbonates ($\delta^{13}C_{CARB}$) and organic matter ($\delta^{13}C_{ORG}$) of *Chara tomentosa* (CT) and *Chara globularis* (CG)

Lake	Stand	Depth (m)	Coverage (%)	$\delta^{13}C_{DIC}$ (‰ V-PDB)	$\delta^{13}C_{CARB}$ (‰ V-PDB)	$\delta^{13}C_{ORG}$ (‰ V-PDB)
Męcko Duże	CT1	3	50	0.20	1.75	−15.75
	CT2	3	50	−0.06	1.71	−15.38
	CT3	3	80	−0.04	1.54	−15.09
	CG1	4	100	−0.02	−2.45	−23.40
	CG2	4	100	0.19	−3.14	−24.50
	CG3	4	100	−0.02	−2.15	−23.60
Karskie Wielkie	CT1	3	70	−5.27	−3.58	−20.91
	CT2	3	80	−5.11	−3.83	−21.85
	CT3	3	80	−5.12	−3.53	−20.47
	CG1	3	70	−5.53	−6.69	−27.90
	CG2	3	70	−5.53	−6.56	−27.60
	CG3	3	40	−4.97	−6.90	−27.60
Jasne	CT1	3	100	0.17	0.91	−14.72
	CT2	3	100	−0.03	1.54	−13.64
	CT3	3	100	0.10	1.33	−13.59
	CG1	4	100	0.01	−10.59	−35.40
	CG2	4	100	−0.05	−7.78	−30.20
	CG3	4	100	−0.07	−7.32	−29.20
Niesłysz	CT1	2	80	−1.30	−0.35	−14.28
	CT2	2	80	−1.51	−0.44	−14.44
	CT3	2	50	−1.47	−0.59	−13.90
Złoty Potok	CT1	2	90	−3.24	−1.69	−17.53
	CT2	2	100	−2.93	−1.64	−18.30
	CT3	2	100	−3.21	−1.44	−17.98
Malcz	CG1	3	80	0.28	−1.51	−22.20
	CG2	3	80	0.31	−2.04	−21.60
	CG3	3	80	0.30	−0.20	−20.70
Pierwsze	CG1	4	100	−1.26	−3.93	−20.10
	CG2	4	100	−0.69	−2.12	−19.80
	CG3	4	100	−1.11	−2.51	−18.50

The depth and vegetation coverage of the studied stands are also provided

in situ measurements, water visibility was determined by Secchi disc at a vegetation-free pelagic region in each lake studied.

Laboratory work and analyses

The air-dried *Chara* samples were kept on paper sheets until further treatment. The thalli of charophytes with calcite encrustations were homogenized using a mortar and pestle. Prior to the isotope analysis of *Chara* organic matter ($\delta^{13}C_{ORG}$), the samples were decalcified in 15% HCl for 2 h to remove carbonates and then rinsed with distilled water. The procedure was repeated three times. Finally, after centrifugation and water removal, the samples were dried at a temperature of 50°C for 12 h and then homogenized and pulverized using an agate mortar.

The stable C isotope composition of charophyte carbonates ($\delta^{13}C_{CARB}$) and dissolved inorganic carbon ($\delta^{13}C_{DIC}$) were measured at the Isotope Dating and

Table 3 Physical and chemical characteristics of water sampled above *Chara tomentosa* (CT) and *Chara globularis* (CG) stands studied in July 2012

Lake	Stand	Temperature (°C)	Oxygen (mg l^{-1})	pH	Conductivity (μS cm^{-1})	Alkalinity (mmol l^{-1})	Total hardness (mg $CaCO_3$ l^{-1})	HCO_3^- (mg l^{-1})
Męcko Duże	CT	18.97 ± 0.05	7.50 ± 0.29	8.75 ± 0.04	261.00 ± 1.41	1.77 ± 0.05	109.67 ± 0.47	107.77 ± 2.88
	CG	19.07 ± 0.21	7.33 ± 0.09	8.70 ± 0.08	264.33 ± 3.86	1.70 ± 0.00	109.73 ± 1.32	103.70 ± 0.00
Karskie Wielkie	CT	20.33 ± 0.05	7.50 ± 0.29	8.53 ± 0.05	595.00 ± 5.35	2.77 ± 0.05	234.80 ± 3.22	168.77 ± 2.88
	CG	20.00 ± 0.08	7.73 ± 0.09	8.50 ± 0.08	586.67 ± 13.12	2.73 ± 0.05	232.67 ± 4.11	166.73 ± 2.88
Jasne	CT	21.90 ± 0.08	6.27 ± 0.17	8.50 ± 0.08	294.33 ± 4.03	1.53 ± 0.05	130.53 ± 387	93.53 ± 2.88
	CG	21.77 ± 0.24	5.80 ± 0.42	8.50 ± 0.00	314.00 ± 9.42	1.63 ± 0.05	129.53 ± 2.76	99.63 ± 2.88
Niesłysz	CT	22.17 ± 0.15	8.17 ± 0.58	8.57 ± 0.15	317.67 ± 18.50	1.90 ± 0.00	131.40 ± 4.08	115.90 ± 0.00
Złoty Potok	CT	22.06 ± 0.11	6.97 ± 0.20	8.57 ± 0.06	332.00 ± 5.56	2.17 ± 0.06	148.13 ± 1.90	132.17 ± 3.52
Malcz	CG	23.10 ± 0.30	5.30 ± 0.10	8.47 ± 0.12	266.00 ± 6.60	1.87 ± 0.06	106.47 ± 1.10	113.87 ± 3.52
Pierwsze	CG	21.40 ± 0.44	6.97 ± 0.21	8.97 ± 0.06	158.30 ± 5.77	1.17 ± 0.06	65.07 ± 1.68	71.17 ± 3.52

Lake	Stand	Ca^{2+} (mg l^{-1})	Mg^{2+} (mg l^{-1})	TP (mg l^{-1})	TN (mg l^{-1})	SO_4^{2-} (mg l^{-1})	Cl^- (mg l^{-1})	SI	Visibility (m)
Męcko Duże	CT	40.29 ± 0.30	2.22 ± 0.30	0.05 ± 0.00	1.35 ± 0.00	15.00 ± 0.00	6.33 ± 0.47	0.87 ± 0.05	6.5
	CG	40.48 ± 0.58	2.13 ± 0.46	0.04 ± 0.00	1.35 ± 0.00	15.00 ± 0.00	6.57 ± 0.47	0.81 ± 0.08	
Karskie Wielkie	CT	78.42 ± 1.26	9.51 ± 1.50	0.04 ± 0.00	1.86 ± 0.03	40.00 ± 0.00	25.67 ± 0.94	1.11 ± 0.06	3.1
	CG	77.19 ± 0.17	9.74 ± 0.97	0.05 ± 0.00	1.82 ± 0.03	35.00 ± 4.08	23.00 ± 1.41	1.06 ± 0.08	
Jasne	CT	47.78 ± 1.59	2.76 ± 0.58	0.05 ± 0.00	1.65 ± 0.03	16.67 ± 2.36	7.67 ± 0.47	0.67 ± 0.11	3.0
	CG	47.00 ± 0.49	2.98 ± 0.49	0.05 ± 0.00	1.63 ± 0.06	20.00 ± 0.00	8.33 ± 0.47	0.69 ± 0.01	
Niesłysz	CT	43.96 ± 0.23	5.29 ± 0.96	0.08 ± 0.01	1.80 ± 0.06	16.67 ± 2.36	12.33 ± 0.47	0.79 ± 0.13	4.2
Złoty Potok	CT	49.73 ± 1.01	5.85 ± 0.22	0.06 ± 0.00	1.16 ± 0.07	30.00 ± 0.00	9.33 ± 0.47	0.89 ± 0.06	4.3
Malcz	CG	38.27 ± 0.30	2.67 ± 0.30	0.05 ± 0.02	1.91 ± 0.05	10.00 ± 0.00	7.00 ± 0.00	0.65 ± 0.10	3.8
Pierwsze	CG	22.58 ± 0.24	2.12 ± 0.50	0.06 ± 0.01	1.32 ± 0.12	10.00 ± 0.00	7.67 ± 0.47	0.72 ± 0.07	5.4

Means ± standard deviations ($N = 3$). Visibility was measured at macrophyte-free pelagic sites

SI saturation index

Environment Research Laboratory in Warsaw, Poland. Carbonates were dissolved in 100% phosphoric acid (density 1.9) at 75°C using a Kiel IV online carbonate preparation line connected to a ThermoFinnigan Delta + mass spectrometer. All values are reported per mil relative to V-PDB by assigning a $\delta^{13}C$ value of 1.95‰ to NBS19. The reproducibility was tested by replicate analysis of laboratory standards and was found to be better than ±0.03‰.

The $\delta^{13}C_{DIC}$ analyses were conducted using a GasBench-II headspace autosampler connected to a Finnigan MAT 253 isotope ratio mass spectrometer (IRMS). During the $\delta^{13}C_{DIC}$ determination procedure, septum-sealed sample vials were filled with 3–4 drops of phosphoric acid (98%) and then flushed with a continuous flow of He to replace the air in the vials. Then, the samples were injected into the vials with a syringe, and CO_2 was released by contact with acid. The CO_2 and He mixture was left to equilibrate for 18 h, and after purification (removal of water vapour) and separation on a GC column, CO_2 was measured in the IRMS using a sample or a reference gas as a comparison. To ensure the precision of the results, four international carbonate standards were measured in each series of samples: NBS 18, NBS 19, LSVEC and IAEA-CO-9.

The *Chara* organic matter ($\delta^{13}C_{ORG}$) samples were analysed at the Institute of Geosciences, Goethe University in Frankfurt, Germany. Carbon isotope analysis of organic matter was performed using a Flash Elemental Analyser 1112 (ThermoQuest) connected to the continuous flow inlet system of a MAT 253 gas source mass spectrometer (ThermoQuest). In addition to IAEA and NBS reference materials (NBS 18, NBS 19, LSVEC), a USGS 24 standard was analysed along with the samples in order to prove accuracy and precision. The reproducibility of both samples and standards was tested and was found to be better than ±0.2‰ for $\delta^{13}C_{ORG}$ (van de Schootbrugge et al., 2008).

The water samples used for hydrochemical analyses were stored in the refrigerator until further treatment. The total alkalinity was determined by titration of a 0.1 mol l^{-1} HCl-acidified water sample against the indicator methyl orange. The bicarbonate concentration was calculated by multiplying the alkalinity by 61 $g mol^{-1}$ (the molar mass of HCO_3^-). Total water hardness and Ca^{2+} concentration were determined using the versenate method, while Mg^{2+} concentration was calculated as the difference between the total hardness and the concentration of Ca^{2+} ions. The anions Cl^- and SO_4^{2-} were determined by ion chromatography using the Thermo Scientific Dionex IonPac AS22 Carbonate Eluent Anion-Exchange Column (Thermo Scientific, USA). Total nitrogen was determined using a TOC-L Shimadzu analyser with a TNM-L unit via catalytic thermal decomposition and chemiluminescence methods (Shimadzu, Japan). Total phosphorous was determined using an ICP-OES 9820 (Shimadzu, Japan).

The calcite saturation index (SI) was calculated for each study site in order to determine whether conditions of HCO_3^- ion supersaturation occurred. The following formula was applied (Kelts & Hsü, 1978):

$$SI = \log IAP/K_C,$$

where IAP is the Ca^{2+} and CO_3^{2-} ion activity product and K_C is the equilibrium constant for the following reaction:

$$CaCO_3 \leftrightarrow Ca^{2+}_{(aq)} + CO^{2-}_{3\,(aq)}.$$

The study results were assessed statistically using Statistica 10 software (StatSoft Inc., Tulsa, OK, USA). A scatter plot of the relation between $\delta^{13}C_{CARB}$, $\delta^{13}C_{DIC}$ and $\delta^{13}C_{ORG}$ was used for data presentation. Additionally, the Pearson's correlation between $\delta^{13}C_{CARB}$ and $\delta^{13}C_{ORG}$, $\delta^{13}C_{CARB}$ and $\delta^{13}C_{DIC}$ as well as $\delta^{13}C_{DIC}$ and $\delta^{13}C_{ORG}$ was calculated and presented in a scatter biplot. Differences in isotopic composition between the studied species were determined via *t* test. For all the statistics, $P < 0.05$ was used to determine significance.

Results

The $\delta^{13}C_{DIC}$ values above *Chara tomentosa* and *Chara globularis* stands

The $\delta^{13}C_{DIC}$ values of the waters above *Chara globularis* stands were in many cases similar to the $\delta^{13}C_{DIC}$ values observed in *C. tomentosa* stands, especially in lakes where both species co-occurred (any differences were statistically insignificant; *t* test, $P > 0.05$, Fig. 2a). The most negative values for both investigated species were found in Lake Karskie Wielkie (Fig. 3; Table 2), while the most positive values were found in Lake Malcz for *C. globularis* and in Lakes Jasne and Męcko Duże for both species (Fig. 3; Table 2).

The δ^{13}C values of *Chara tomentosa* and *Chara globularis* organic matter and carbonate encrustations

The carbonate encrustations of *C. tomentosa* had substantially higher δ^{13}C values in comparison to *C. globularis* (Table 2). In lakes where both species co-occurred, the difference was statistically significant (*t* test, $P < 0.001$, Fig. 2b). The greatest differences in the $\delta^{13}C_{CARB}$ values of the studied species were noted in Lake Jasne, where the most negative values for *C. globularis* and the second most positive values for *C. tomentosa* were observed (Fig. 3a, b; Table 2).

Substantially higher δ^{13}C values for organic matter were also revealed by *C. tomentosa*. The most negative values for *C. tomentosa* were found in Lake Karskie Wielkie and for *C. globularis* in Lake Jasne (Fig. 3; Table 2). Conversely, in Lake Jasne and Lake Męcko Duże, *C. tomentosa* revealed the most positive $\delta^{13}C_{ORG}$ values, while for *C. globularis* the most positive $\delta^{13}C_{ORG}$ values were noted in Lake Pierwsze (Fig. 3; Table 2). Again, the highest contrast in the carbon stable isotope signal was noted in Lake Jasne. In lakes where both *Chara* species co-occurred, the difference in the $\delta^{13}C_{ORG}$ values between the studied

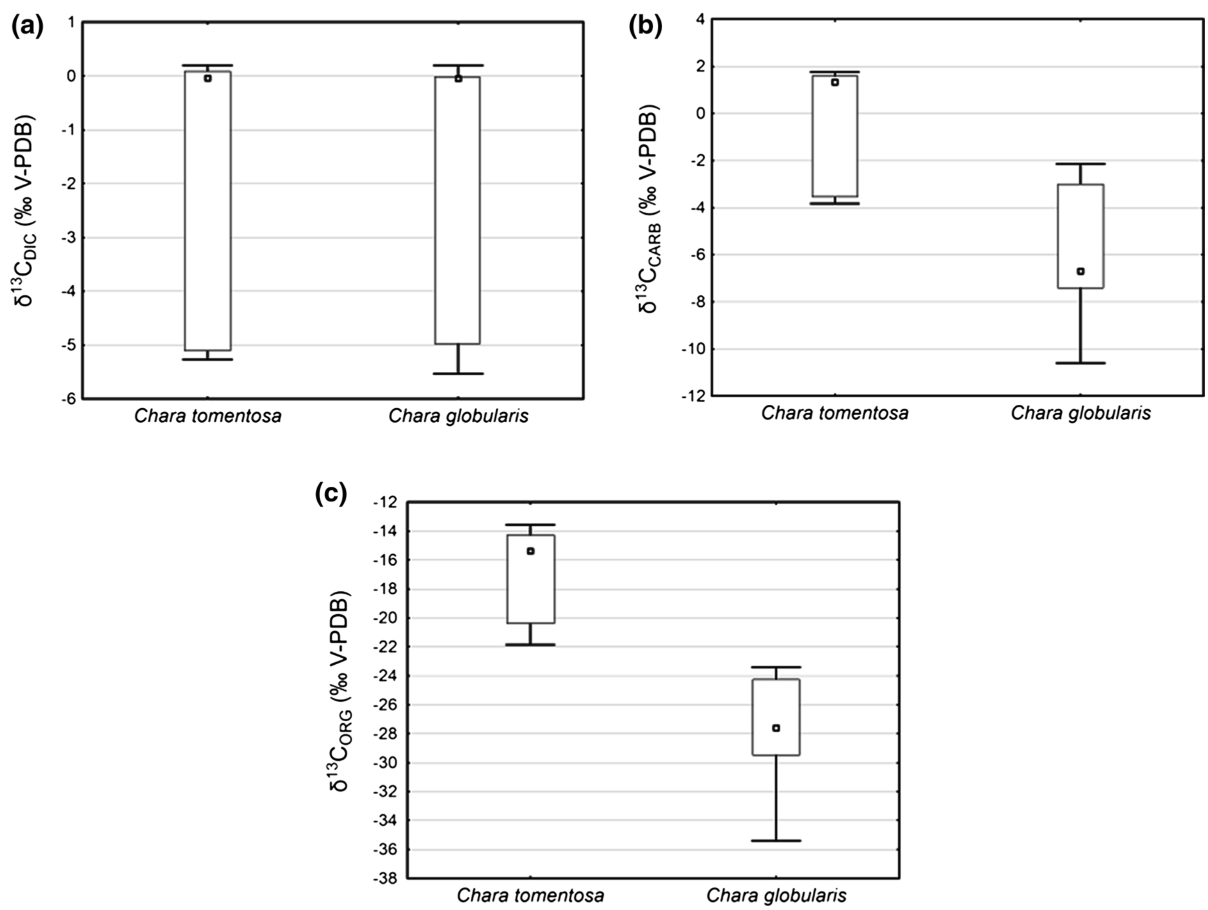

Fig. 2 Differences in carbon stable isotope values between **a** DIC (dissolved inorganic carbon) in the *C. tomentosa* and *C. globularis* stands, **b** carbonate encrustations of *C. tomentosa* and *C. globularis*, and **c** organic matter of *C. tomentosa* and *C. globularis.* Carbon stable isotope values were measured in nine stands ($N = 9$) for each species in the lakes where both species co-occurred. Median, box 25–75% and min–max; except for $\delta^{13}C_{DIC}$, the differences between species were statistically significant (*t* test, $P < 0.05$)

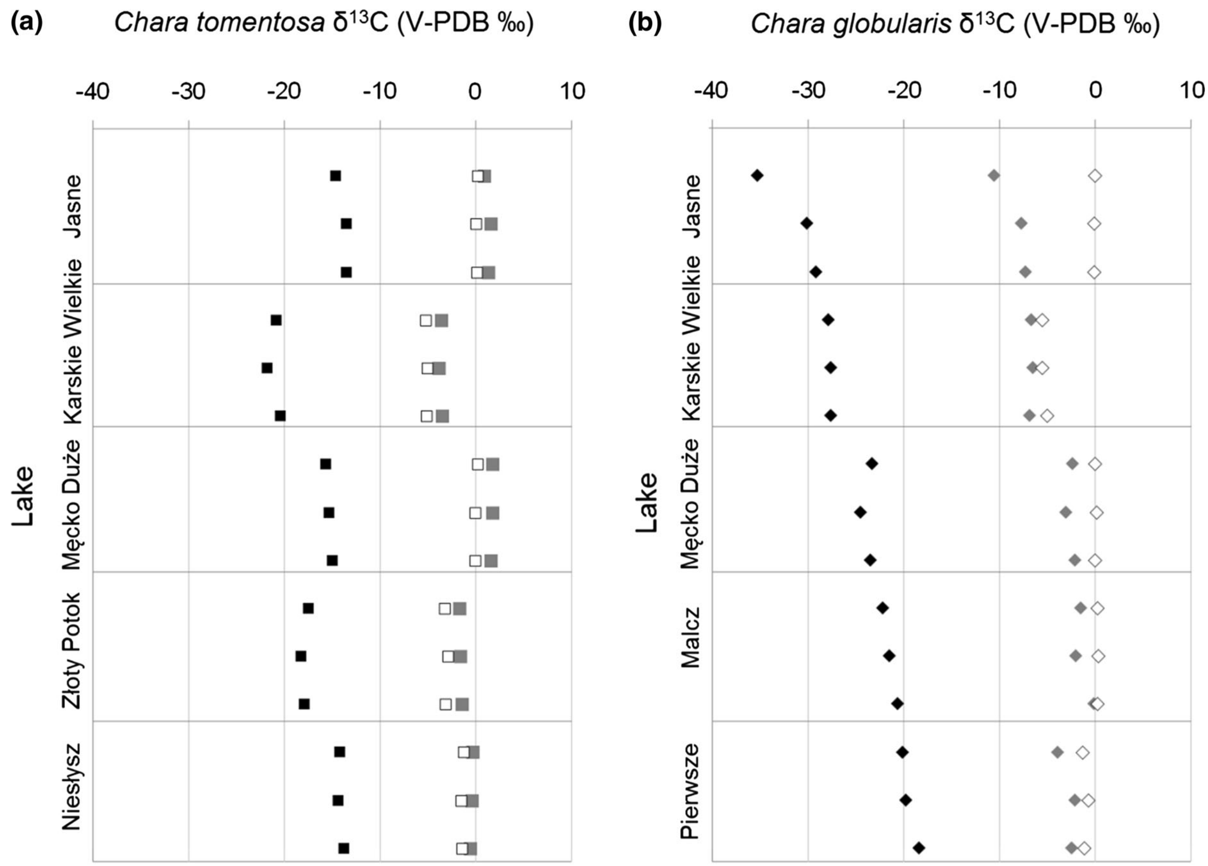

Fig. 3 Relation between the $\delta^{13}C$ values of charophyte carbonates (*grey squares* and *rhombuses*), $\delta^{13}C$ values of DIC (*white squares* and *rhombuses*) and $\delta^{13}C$ values of organic matter (*black squares* and *rhombuses*) for **a** *Chara tomentosa* stands ($N = 15$) and **b** *Chara globularis* stands ($N = 15$)

species was statistically significant (*t* test, $P < 0.00001$; Figs. 2c, 3a, b).

The relationships between the $\delta^{13}C_{DIC}$, $\delta^{13}C_{CARB}$ and $\delta^{13}C_{ORG}$ values of the studied species are presented in scatter plots (Fig. 3). Compared to DIC, carbonate encrustations of *C. tomentosa* were enriched in ^{13}C, while those involving *C. globularis* were ^{13}C depleted (Fig. 3a, b). This shift was apparent in all studied lakes. In both studied species, the organic matter was ^{13}C depleted relative to both DIC and carbonates, a trend that was valid for all lakes (Fig. 3a, b).

A close relationship between the $\delta^{13}C_{CARB}$ and $\delta^{13}C_{ORG}$ values was reflected in their high correlation coefficients (Fig. 4a). We also found a close relationship between the $\delta^{13}C_{CARB}$ and $\delta^{13}C_{DIC}$ values for *C. tomentosa*, while the relationship between the $\delta^{13}C_{CARB}$ and $\delta^{13}C_{DIC}$ values of *C. globularis* was weak and statistically insignificant (Fig. 4b). Similar observations were made concerning the relationship between $\delta^{13}C_{ORG}$ and $\delta^{13}C_{DIC}$ values: a high correlation coefficient was obtained for *C. tomentosa* but not for *C. globularis* (Fig. 4c).

Discussion

Main factors controlling the composition of carbon stable isotope for DIC, charophyte encrustation and organic matter

Taking into account the complexity of lake ecosystems, a number of fractionation factors that influence the carbon stable isotope composition of DIC can be specified. The isotope composition of inflowing waters (i.e., surface and ground waters), CO_2

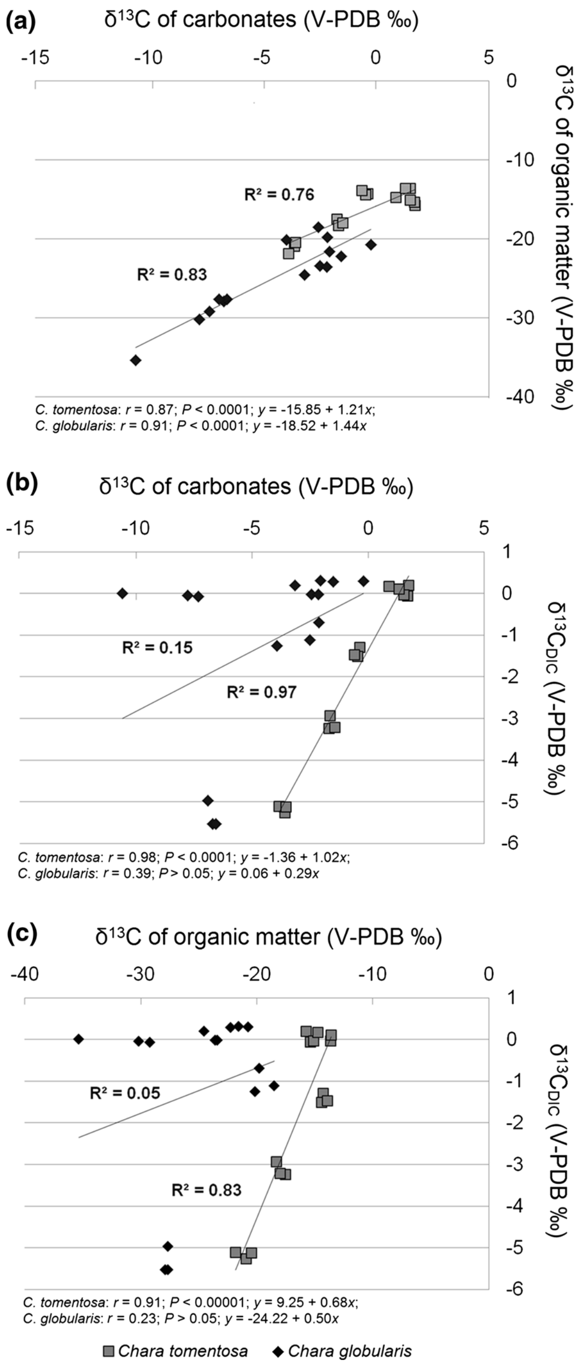

Fig. 4 Correlations between the stable isotope values of **a** carbonate encrustations and organic matter, **b** carbonate encrustations and dissolved inorganic carbon, and **c** organic matter and dissolved inorganic carbon of *Chara tomentosa* (*grey squares*, $N = 15$) and *Chara globularis* (*black rhombuses*, $N = 15$) stands

exchange with the atmosphere and the time of water exchange in a lake are of prime importance for the $\delta^{13}C_{DIC}$ values (Leng & Marshall, 2004 Myrbo & Shapley, 2006 and references in both cited papers). Additionally, $\delta^{13}C_{DIC}$ values are related to the rate and intensity of photosynthesis because aquatic autotrophic organisms preferentially uptake $^{12}CO_2$, leaving ambient DIC ^{13}C enriched (e.g., Pentecost & Spiro, 1990; Andrews et al., 1997; McConnaughey & Whelan, 1997; Leng & Marshall, 2004 and references therein). Therefore, $\delta^{13}C_{DIC}$ values vary both horizontally (e.g., shallow littoral waters vs. deep pelagic regions) and vertically within the water column, where the epilimnetic layer is usually ^{13}C enriched compared to hypolimnetic waters. Under aerobic conditions, the near-bottom waters are ^{13}C depleted due to $^{12}CO_2$ release resulting from sedimentary organic matter decomposition (Myrbo & Shapley, 2006 and reference therein). Thus, aquatic macrophytes, including charophytes at deeper sites or concentrated near lake-floor sediment can utilize this ^{13}C-depleted source of CO_2 for photosynthesis. Under anaerobic conditions, $\delta^{13}C_{DIC}$ in pore and near-bottom water is strongly dependent on the degradation of sedimentary organic matter stored in carbon-rich sediments (Gu et al., 2004). Bacterial methane production, depending on the resultant product, CH_4 or CO_2, may cause the ^{13}C depletion or ^{13}C enrichment of pore and near-bottom water, respectively. However, the lakes included in this study are not significantly fertile (i.e., clear-water mesotrophic lakes, and only Lake Karskie Wielkie is slightly eutrophicated) and contain abundant submerged vegetation dominated by charophytes (indicative of a low trophy state; Krause, 1981), out of which many species can overwinter. In addition, charophytes are characterized by low decomposition ratios compared to vascular plants (Kufel & Kufel, 2002). Therefore, methanogenesis, in our opinion, can be considered a less significant ^{13}C fractionation factor in these lakes.

In aquatic environments, inorganic C can be assimilated in two forms, CO_2 and HCO_3^- (Smith & Walker, 1980). As evidenced for the marine environment, CO_2 is ^{13}C depleted compared to other components of DIC (H_2CO_3, HCO_3^-, CO_3^{2-}) by typically 8–12‰ (Mook et al., 1974). The difference between the stable isotopic composition values of HCO_3^- and CO_2 is temperature dependent. The $\delta^{13}C$ values of CO_2 are 12‰ lower than those of HCO_3^- at 0°C and 8.4‰ lower at 30°C (Mook et al., 1974). For this reason, Fry (1996) assumed that under isotopic equilibrium the cells using CO_2 should have $\delta^{13}C$ values 8–12‰ lower than cells using HCO_3^-. Thus, the $\delta^{13}C$ values of ambient DIC depend not only on the character of a lake but also on the variable in time and space, temperature, and the pH-dependent proportions of CO_2, HCO_3^- and CO_3^{2-} (Smith & Walker, 1980; Zhang et al., 1995). Therefore, in addition to lake morphology and the related residence time of water, other environmental conditions, including water physical and chemical properties, should be taken into consideration. In this study, no direct effects of the lake morphology and the rate of water exchange on the $\delta^{13}C_{DIC}$ values were identified. In addition, the pH values of waters, almost equal in all lakes studied, exceeded 8 at each study site (Table 3), permitting the assumption that HCO_3^- ions were the form of inorganic carbon in all waters sampled above charophyte beds. This was unequivocally confirmed by C species modelling performed with MINEQL 4.6 software (Environmental Research Software, Hallowell, ME, USA; since the developed model is of minor relevance to the study aim, we have avoided it). Additionally, HCO_3^- ion accessibility for autotrophs in the studied lakes was reflected in the abundant encrustation present on the charophyte thalli and in the calculated values of the calcite saturation index (SI, Table 3). The SI values indicate supersaturation with respect to HCO_3^- ions, which further indicates favourable conditions for calcium carbonate precipitation in all studied lakes. What differentiates the lakes is the character of flow (Table 1). Compared to other lakes, the most negative $\delta^{13}C$ DIC values were found in Lake Karskie Wielkie (Table 2), the only outflow lake included in this study (Table 1). In addition to the flow character, the waters of Lake Karskie Wielkie revealed distinctive chemical properties (i.e., much higher conductivity, alkalinity and hardness as well as Ca^{2+}, SO_4^{2-} and Cl^- concentrations) compared to the other lakes. Importantly, the second most negative $\delta^{13}C_{DIC}$ values were measured in Lake Złoty Potok (Table 2), which, similar to Lake Karskie, exhibited a higher alkalinity, hardness and SO_4^{2-} concentration than the other lakes (Table 3), in which the $\delta^{13}C_{DIC}$ values were relatively similar. The aforementioned chemical water characteristics are related to the ground waters that feed the lakes. According to the data available, including the most negative $\delta^{18}O$ values in the group of studied lakes (unpublished data), it is reasonable to suggest that ground waters significantly contribute to the water budget of Lakes Karskie Wielkie and Złoty Potok, and the $\delta^{13}C$ DIC values are strongly dependent on the $\delta^{13}C$ DIC values of inflowing waters. The moderately higher fertility of Lake Karskie Wielkie and the agricultural character of its catchment basin may also point to a contribution of surface inflow from arable fields to the water chemistry and isotopic composition of this lake.

Apart from the $\delta^{13}C$ values of ambient DIC, the so-called 'vital effect' is regarded as an important factor to control the $\delta^{13}C$ values of biogenically precipitated carbonates in lakes (Leng & Marshall, 2004 and references therein). Above the charophyte stands, DIC is strongly ^{13}C enriched by the photosynthetic activity of these macroalgae (Coletta et al., 2001; Pełechaty et al., 2010; Apolinarska et al., 2011). As a consequence, elevated $\delta^{13}C_{DIC}$ values are recorded in the carbonate encrustations of these charophytes.

The $\delta^{13}C$ values of organic matter ($\delta^{13}C_{ORG}$) depend on the carbon source utilized (e.g., CO_2(aq), HCO_3^-), the isotope effects associated with the assimilation of carbon, the photosynthesis pathway performed by the photosynthesizing organism and the cellular carbon budget (Hayes, 1993). For aquatic environments, many authors have recorded different $\delta^{13}C_{ORG}$ values of autotrophic organisms such as marine phytoplankton or freshwater macrophytes and found smaller and larger offsets of $\delta^{13}C_{ORG}$ within the same investigated groups of organisms (e.g., Keeley, & Sandquist, 1992 and reference therein; Rau et al., 1992; Fry, 1996 and reference therein; Rau et al., 2001; Herzschuh et al., 2010; Mendonça et al., 2013). In the following discussion, we will try to explain the observed differences between the $\delta^{13}C_{CARB}$, $\delta^{13}C_{ORG}$ and $\delta^{13}C_{DIC}$ values of the two studied charophyte species in light of CO_2 source used for their photosynthetic activity, ^{13}C-enriched DIC from well-mixed waters above and penetrating *Chara tomentosa* stand and, for *Chara globularis*, ^{13}C-depleted DIC from near-bottom waters with a higher proportion of ^{12}C released by organic matter decomposition and carbonate dissolution in surface sediments. The proportion between ^{13}C and ^{12}C in C taken up by the two charophytes seems related to their growth forms.

$\delta^{13}C_{CARB}$ versus $\delta^{13}C_{DIC}$

This study revealed opposing tendencies in the shift between the $\delta^{13}C_{CARB}$ and the $\delta^{13}C_{DIC}$ values of the studied species (Fig. 3a, b). We postulate that this contradictory shift results from the different growth forms represented by the studied species. *C. tomentosa* is a bigger and more highly branched *Chara* species with a much longer thallus compared to *C. globularis* (Dąmbska, 1964; Krause, 1981, 1997; Torn et al., 2006; Pełechaty & Pukacz, 2008). The length of *C. tomentosa* thallus can exceed 140 cm, whereas *C. globularis* thalli are usually 20 cm long. *C. tomentosa* is often found at more shallow depths than *C. globularis*. The latter species forms more compact stands near the bottom of lakes compared to the less compact stands of *C. tomentosa*. Considering these characteristics, we suggest that the encrustation of *C. globularis* was ^{13}C depleted compared with the $\delta^{13}C$ values measured in DIC due to the assimilation of carbon from waters near the bottom of the lake rather than the well-mixed water above the stands. The near-bottom water is usually enriched with ^{12}C derived from organic matter decomposition in the surface sediment. The lack of correlation between $\delta^{13}C_{CARB}$ and $\delta^{13}C_{DIC}$ values in *C. globularis* stands (Fig. 4b) seems to confirm this hypothesis. In contrast, a strong correlation between the $\delta^{13}C$ values of *C. tomentosa* encrustations and those of DIC (Fig. 4b) emphasizes a significant relationship between C_{CARB} and C_{DIC} in homogenous ambient water. In addition, this strong correlation may suggest that in contrast to *C. globularis*, *C. tomentosa* precipitated encrustation in or close to an isotopic equilibrium with DIC from the immediate environment.

Due to the higher proportion of $^{12}CO_2$ assimilated preferentially during photosynthesis, the remaining water DIC becomes ^{13}C-enriched DIC source for the calcite precipitated as an encrustation by charophytes (cf. Pentecost & Spiro, 1990; Andrews et al., 1997). This relationship has been reported also by Pełechaty et al. (2010) for *C. rudis*, a species morphologically similar to *C. tomentosa*. In addition, the $\delta^{13}C_{CARB}$ values of *C. tomentosa* collected in this study from Lake Jasne were comparable to those shown by Pełechaty et al. (2010) for *C. rudis* in the same lake. By contrast, the $\delta^{13}C_{CARB}$ values recorded for *C. globularis* in Lake Jasne were significantly different from those reported for *C. tomentosa* (^{13}C depleted), exceeding 12‰ at one stand, and this tendency was also valid for other studied lakes. Pukacz et al. (2014) reported the horizontal homogeneity of physical and chemical water properties in Lake Jasne, with no significant differences between pelagic open waters and charophyte stands. Thus, it can be assumed that the relatively homogeneous waters of Lake Jasne should counteract the difference in isotopic values between the studied species. The existence of this difference, therefore, seems to confirm that the $\delta^{13}C_{CARB}$ of *C. globularis* was ^{13}C depleted, because this species uses the DIC of the near-bottom waters rather than of the water above the studied stands (which was sampled in this study). In summary, by creating a dense community just off the bottom sediment, *C. globularis* appears to use a DIC stock enriched in ^{12}C derived from decomposition in sediments. As a result, the *C. globularis* $\delta^{13}C_{CARB}$ value reveals significant ^{13}C depletion compared not only to *C. tomentosa* but also to DIC in water sampled above *C. globularis* stands.

$\delta^{13}C_{ORG}$ of charophytes as an indicator of C source

The carbon stable isotope values of organic matter were higher in *C. tomentosa* compared to *C. globularis* (Figs. 2e, 3a, 4a; Table 2). These differences were significant in all lakes in which the two species co-occurred and exceeded 20‰ in Lake Jasne (Fig. 3a, b; Table 2).

This study presents strong and statistically significant positive correlations between the $\delta^{13}C_{CARB}$ and $\delta^{13}C_{ORG}$ values for both species (Fig. 4a). In contrast to our findings, Pentecost et al. (2006) recorded a week and insignificant positive correlation between the *C. hispida* $\delta^{13}C$ values of encrustation and organic matter. In that study, the $\delta^{13}C_{ORG}$ values of *C. hispida*, recorded in July and August, ranged between approximately −19.0 and −17.0‰, but the mean value over the investigated period (from February to August) was −19.7‰. In our study, the mean $\delta^{13}C_{ORG}$ value of *C. tomentosa,* the species most similar to *C. hispida* in terms of size and morphology (i.e., −16.5‰), corresponded to the above data for *C. hispida*, while the mean $\delta^{13}C_{ORG}$ value of *C. globularis* (i.e., −24.8‰) emphasized the difference between this species and the above two large *Chara* species.

Interestingly, the $\delta^{13}C_{ORG}$ values obtained for *C. tomentosa* are similar to those of ^{13}C-enriched vascular macrophytes, which have an average value of −13.5‰ (Mendonça et al., 2013). On the other hand, the cited authors provide a value of −27.2‰ as the average $\delta^{13}C$ value of ^{13}C-depleted plants, similar to our results for *C. globularis*. Thus, the difference in the $\delta^{13}C$ values for organic matter between *C. tomentosa* and *C. globularis* is similar to that between ^{13}C-enriched C_4 plants, with $\delta^{13}C$ values ranging from −16 to −10‰, and C_3 plants, with $\delta^{13}C$ values ranging from −32 to −22‰ (e.g., O'Leary et al., 1992; Hayes, 1993; Fry, 1996 and reference therein). Because both studied *Chara* species are members of the same genus and exhibit similar physiological photosynthetic patterns, the aforementioned comparisons point to the possible risk of misinterpreting the carbon source in palaeoecological studies. In terrestrial plants, the differing $\delta^{13}C$ values of organic matter are the result of the photosynthetic pathways used for CO_2 fixation and the isotopic signature of the atmospheric CO_2 used for photosynthesis (Fry, 1996 and references therein; Mendonça et al., 2013). In C_3 plants, large fractionation associated with the enzyme ribulose bisphosphate carboxylase/oxygenase (Rubisco), which catalyses the first step in CO_2 fixation, are observed (e.g., O'Leary et al., 1992; Hayes, 1993; Fry, 1996 and reference therein). During photosynthetic carbon fixation in the C_4 pathway, phosphoenolpyruvate carboxylase catalyses the first carbon fixation step, which is associated with a smaller carbon isotope fractionation compared to that of Rubisco (e.g., O'Leary et al., 1992; Hayes, 1993). For aquatic plants, the carbon source (CO_2 vs. HCO_3^-) seems to be a key isotope discrimination factor. The average difference in the $\delta^{13}C_{ORG}$ values between *C. tomentosa* and *C. globularis* (8.3‰) is close to the difference reported by Mook et al. (1974) between the $\delta^{13}C$ values of HCO_3^- and the $\delta^{13}C$ values of dissolved CO_2, falling within the range between 8 and 12‰ and reaching 9‰ at 25°C. These data prompt us to suggest that ^{13}C-enriched HCO_3^- from above- and within-stand waters is the main source of C for *C. tomentosa*, while $^{12}CO_2$ released from organic matter decomposing in lake bottom sediments and ^{13}C-depleted HCO_3^- ions are likely to serve as C sources for *C. globularis*. Thus, the C source utilized is species specific and appears to be inseparably related to the growth form of each species. Additional detailed field investigations of a range of species representing different growth forms and a range of conditions above, within and beneath a charophyte stand as well as laboratory testing the exact CO_2 acquisition for photosynthesis and membrane transport mechanisms (e.g., Ray et al., 2003; Beilby & Casanova, 2014), and respiration effects would explicitly verify our assumption. This is particularly important in the context of the aforementioned use of $\delta^{13}C$ values in palaeoecological studies as an indication of the source of matter in sediment deposits.

Conclusions

In the present study:

- We found opposing shifts between $\delta^{13}C_{CARB}$ and $\delta^{13}C_{DIC}$ values:
  - ^{13}C enrichment in *Chara tomentosa*
  - ^{13}C depletion in *Chara globularis*.
- A strong positive correlation was found between $\delta^{13}C_{CARB}$ and $\delta^{13}C_{DIC}$ as well as between $\delta^{13}C_{ORG}$ and $\delta^{13}C_{DIC}$ for *C. tomentosa* but not for *C. globularis*.
- The $\delta^{13}C_{CARB}$ and $\delta^{13}C_{ORG}$ values were strongly positively correlated in both species.
- *C. globularis* samples exhibited more negative values of $\delta^{13}C_{ORG}$ than did *C. tomentosa*, particularly in lakes where the two species co-occurred.
- The differences between the isotope values of carbonate encrustations and the organic matter of the studied charophyte species were statistically significant.
- The differences in isotope signatures were repetitive at each site and in each investigated lake.
- The proportion between ^{13}C and ^{12}C used for photosynthesis from ^{13}C-enriched DIC for *C. tomentosa* and ^{13}C-depleted DIC for *C. globularis* is postulated to be a key factor influencing the isotope values recorded in the carbonates and organic matter of the species and may be useful in future palaeoecological studies.

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