frontiers | Frontiers in Freshwater Science

TYPE Original Research
PUBLISHED 27 September 2024
DOI 10.3389/ffwsc.2024.1421114

OPEN ACCESS

EDITED BY
Peter Goethals,
Ghent University, Belgium

REVIEWED BY
Evangeline Fachon,
Woods Hole Oceanographic Institution,
United States
Leandro E. (Steve) Miranda,
US Geological Survey and Mississippi State
University, United States

*CORRESPONDENCE
Gerli Albert
✉ gerli.albert@ut.ee

RECEIVED 21 April 2024
ACCEPTED 10 September 2024
PUBLISHED 27 September 2024

CITATION
Albert G, Pajusalu L, Pritchard DW, Hepburn CD, Torn K, Paalme T, Põllumäe A and Martin G (2024) Comparison of carbon uptake strategies between *Chara aspera* and *Chara tomentosa* growing in the brackish Baltic Sea and in the freshwater lakes of Estonia. *Front. Freshw. Sci.* 2:1421114.
doi: 10.3389/ffwsc.2024.1421114

Comparison of carbon uptake strategies between *Chara aspera* and *Chara tomentosa* growing in the brackish Baltic Sea and in the freshwater lakes of Estonia

Gerli Albert[1]*, Liina Pajusalu[1], Daniel W. Pritchard[2], Christopher D. Hepburn[2,3], Kaire Torn[1], Tiina Paalme[1], Arno Põllumäe[1] and Georg Martin[1]

[1]Estonian Marine Institute, University of Tartu, Tallinn, Estonia, [2]Coastal People Southern Skies, Centre of Research Excellence, Dunedin, New Zealand, [3]Department of Marine Science, University of Otago, Dunedin, New Zealand

The carbon acquisition strategies of aquatic photosynthetic organisms play a key role in the growth and survival of a species. There is much research indicating that the predicted changes (e.g., the balance between carbonate species: CO_2, HCO_3^-, and CO_3^{2-}) in the seawater carbonate chemistry, due to ocean acidification, could affect benthic primary producers and their communities. However, considerably less is known about brackish water (e.g., the Baltic Sea), and even less about the possible effects of acidification on freshwater biota. This study aimed to compare the carbon uptake strategies of two dominant charophyte species: *Chara aspera* and *Chara tomentosa* growing in freshwater lakes of Estonia and in the brackish NE Baltic Sea. This could indicate how they might respond to the predicted increasing CO_2 concentration linked to climate change scenrios. Carbon use strategies in charophytes were determined by analysing natural carbon isotope signatures ($\delta^{13}C$), pH drift experiments and photosynthesis *vs.* dissolved inorganic carbon curves. The study showed that freshwater and brackish water *C. aspera* and *C. tomentosa* likely use different carbon uptake mechanisms. Our results indicated that freshwater charophytes preferentially use CO_2 and brackish water charophytes HCO_3^-, likely due to their local acclimatization to different growth environments. Also, *C. tomentosa* and *C. aspera* from the studied lakes are likely carbon saturated (photosynthetic processes are operating at their maximum efficiency due to the availability of dissolved inorganic carbon in their environment) and probably will not gain photosynthetic advantages from acidification. However, the predicted increase in CO_2 concentration may positively affect the growth of the charophytes in the brackish Baltic Sea.

KEYWORDS

charophytes, carbon physiology, ocean acidification, macrophytes, increasing

1 Introduction

Ocean acidification (OA) is a well-known phenomenon, a direct consequence of increasing carbon dioxide (CO_2) concentration in the atmosphere. The understanding of the effects of OA on ocean biota at organism, community, and ecosystem levels has increased in the last decade (Gattuso and Hansson, 2011; Doney et al., 2020; Terhaar et al., 2020). However, considerably less is known about brackish water (e.g., the Baltic Sea), and even less about the possible effects of acidification on freshwater biota. The future OA scenarios for the Baltic Sea could be more severe than predicted for oceans because of the low buffering capacity of brackish water (Omstedt et al., 2012; Melzner et al., 2013), the high freshwater input with high levels of dissolved organic carbon (DOC) and low alkalinity (Melzner et al., 2013). Also, the extent of acidification could markedly vary between different areas of the Baltic Sea due to geological, biogeochemical, and riverine input differences. It is estimated that the Baltic Sea surface water pH levels could decline by 0.25–0.5 units by the end of the century (Omstedt et al., 2010, 2012; Kuznetsov and Neumann, 2013).

Lakes could act as a source or sink of CO_2 depending on the chemical, physical, and biological processes (McKinley et al., 2011; Shao et al., 2015). Many small lakes are known to be net CO2 emitters (Hanson et al., 2004; Alin and Johnson, 2007; Cole et al., 2007). However, it does not mean that acidification due to increasing CO_2 could not affect these lakes (Phillips et al., 2015). Rising CO_2 levels can lower the pH of lake water, potentially disrupting aquatic ecosystems, affecting the solubility of nutrients and metals, and harming organisms sensitive to changes in acidity. In general, it is difficult to assess the degree to which inorganic carbon dynamics affect pCO_2 (partial pressure of carbon dioxide) in the lakes because there is usually a lack of data about the carbon cycles and lake-atmosphere CO_2 fluxes (Eadie and Robertson, 1976; Atilla et al., 2011; Bennington et al., 2012; Vesala et al., 2012). However, it is generally known that diel cycles of productivity, driven by photosynthesis during the day and respiration at night, can lead to significant fluctuations in pH and CO_2 in different aquatic systems, e.g., in lake and marine environments. These cycles cause pH to rise during the day as CO_2 is consumed and decrease at night as CO_2 is produced (Andersen et al., 2017). Also, the impact of catchments e.g., the delivery of dissolved inorganic carbon (DIC) and dissolved organic carbon (DOC) play a big role in especially small lakes alkalinity and carbon budgets (McConnaughey et al., 1994; Einsele et al., 2001; Stets et al., 2009).

Estonian lakes, due to their unique geological and hydrological characteristics, exhibit distinct patterns of carbon dynamics (Ott and Kõiv, 1999; Simm et al., 2006; Nõges et al., 2010). Could be expected that the presence of limestone and other carbonate-rich substrates can contribute to higher alkalinity levels, enhancing the lakes' buffering capacity and pH stability. The relatively shallow nature of many Estonian lakes (Simm et al., 2006; Nõges et al., 2010) can lead to more pronounced diel variations in pH and CO_2 levels, as sunlight impacts the entire water column directly. However, each Estonian lake has unique properties, making it difficult to generalize or compare carbon cycling processes even within the region, complicating the comparison with lakes in other regions, this emphasizes the need for site-specific studies to understand carbon dynamics accurately.

This study focuses on charophytes, which are a globally widespread group of submerged algae with well-developed complex thalli and morphology. They grow in freshwater lakes, ponds, streams, brackish environments, and even in hypersaline water bodies to a limited extent (Krause, 1997). Charophyte communities are important habitats for various invertebrate species, and they provide feeding and nursery areas for many fish species (Blindow and van de Weyer, 2016). In the Baltic Sea, charophytes inhabit sheltered soft-bottom coastal areas where their distribution pattern is primarily controlled by exposure, sediment type, and salinity regime (Schubert and Yousef, 2001; Torn et al., 2004). While some euryhaline charophyte species are found all over the Baltic Sea, the majority of the species are growing below salinity 7 PSU (Practical Salinity Unit; Schubert and Blindow, 2003).

The genus *Chara* grows predominantly in calcium-rich waters and only with a few exceptions in oligotrophic soft-waters conditions (Torn et al., 2015; Blindow and van de Weyer, 2016). The *Chara* species are capable of forming very dense canopies in oligotrophic, calcareous lakes and ponds as well as in sheltered bays in the Baltic Sea (Schubert and Blindow, 2003). Charophytes have been reported to be able to utilize bicarbonate as a carbon source (McConnaughey et al., 1994; Herbst and Schubert, 2018) and have a higher affinity to HCO_3^- than vascular macrophytes (Van den Berg et al., 1998). Internodal cells of *Characean* are able to generate spatially separated bands of high and low pH in the medium adjacent to their cell surface (Spear et al., 1969; Lucas and Smith, 1973). When exposed to light, the internodes show alkaline regions with pH values between 8.5 and 9.5 and acid regions with a pH of about 5.5. The bands disappear when photosynthesis is inhibited and the pH at the cell surface becomes uniform with values of about pH 6.0 (Lucas and Smith, 1973). During the pH banding, *Characean* cell walls calcify in the alkaline regions where the pH increases and consequently shift the chemical equilibrium toward the formation of carbonate ions (Stumm and Morgan, 1970; McConnaughey and Falk, 1991), which precipitate with calcium in the form of calcite. This process itself significantly depletes bicarbonate ions, which has been observed within a dense *Chara* cover (Van den Berg et al., 1998). The extent of calcification, however, depends on pH and the concentrations of bicarbonate and calcium (McConnaughey and Whelan, 1997). Also, on individual characteristics of the charophyte species (Andersen et al., 2017).

The study focuses on two charophyte species: *Chara aspera* Willd. and *Chara tomentosa* L. growing in freshwater lakes and in the brackish Baltic Sea. *C. aspera* has slender, small to medium size (5–20 cm) thalli. The species has a very broad tolerance to environmental conditions and therefore, it is the most widespread charophyte species in the coastal seas and freshwater lakes of Estonia. *C. tomentosa* is fairly large (25–60 cm) in size compared to *C. aspera* and is also a widely distributed species in Estonia, mostly occurring sparsely or forming patches within communities dominated by other charophytes (Torn et al., 2015).

The aim was to compare the carbon uptake strategies of the two species, which could indicate how they might respond to the predicted increasing CO_2 concentration associated with climate change. The mechanisms of DIC uptake and carbon requirements for macroalgal growth are species-specific (Hepburn et al., 2011; Albert et al., 2020; Pajusalu et al., 2020). It is thought that the species that lack a carbon concentrating mechanism (CCM)—relying solely on CO_2, CCMs with low affinities for DIC, and CCMs that can be downregulated, could benefit from increasing CO_2 concentration (Giordano et al., 2005). Species with efficient CCMs that are not capable of taking up additional CO_2 or species with CCMs which could not be downregulated are thought to be unaffected by OA or the benefit could be considerably lower

(Beardall and Raven, 2004; Cornwall et al., 2017). In this study the carbon use strategies in charophytes were determined by analysing natural carbon isotope signatures ($\delta^{13}C$), pH drift experiments and photosynthesis *vs.* dissolved inorganic carbon (P *vs.* DIC) curves. We hypothesized that there is a significant difference between the freshwater and brackish water *C. aspera* and *C. tomentosa* carbon uptake strategies. Also, based on our earlier work (Pajusalu et al., 2015) the brackish Baltic Sea charophytes are carbon limited during the active vegetation period under the current DIC availability and therefore benefit from increasing CO_2 concentration.

2 Materials and methods

Charophytes from the brackish Baltic Sea were collected in July 2018 by Scuba diving from the Väike Strait Northern Gulf of Riga (58.5155°N, 23.2035°E), at a depth of 2 m. At this depth, *C. aspera* is the dominant species and *C. tomentosa* grows in patches. The strait is part of the Väinameri Sea and is characterized by lots of islets and shoals. The strait is 2–4 km wide and generally <3 m deep. Salinity in Väike Strait ranges from 3.7 to 6.3 PSU, during the sampling period, the salinity was 6 PSU. In Estonia, charophytes from freshwater (salinity below 0.2 PSU) were collected with a hook from two lakes 16 km apart. *C. aspera* was collected in July 2018 from Seljajärv (58.9199°N, 26.2753°E), which is originally an old sand quarry, however, not been used for several decades and now characterized by limestone bedrock and dense charophyte mat, where the species *C. aspera* forms a monodominant community in shallower depths (0.3–0.5 m). *C. tomentosa* was collected in July 2018 from Äntu Sinijärv (59.0621°N, 26.2404°E) at a depth of 0.4 m where the species grows sparsely in a dense community formed by *Chara papillosa* Kütz (Figure 1). Äntu Sinijärv is a calcareous lake with a high visibility down to ∼15 m, pH of 7.4–8.0 and an HCO_3^- content of 262–274 mgL^{-1} (Laumets et al., 2014). The lake is fed by direct precipitation and by groundwater from a limestone aquifer that discharges into the lake through at least seven submerged spring vents (Saarse and Liiva, 1995). Throughout the paper, *C. tomentosa* collected from Äntu Sinijärv and *C. aspera* collected from Seljajärv are referred to as freshwater charophytes.

For background information, the daily natural fluctuations of pH levels, photosynthetically active radiation (PAR), and oxygen (mg l^{-1}) were measured in shallow water (ca 2 m). Measurements were carried out in Väike Strait during the active vegetation period in July (58.5126°N, 23.1981°E; Figure 2). The coverage of the species were *C. aspera* 100%, *C. tomentosa* 1%, and *C. connivens* 1% in the study area. Measurements were performed using an optical CO_2 sensor (AMT Analysenmesstechnik GmbH, Germany) attached to a CTD sensor system (Sun & Sea Technology GmbH, Germany), which was placed among vegetation at a depth of 2 m during a full 24-h cycle with a frequency of 20 min in July 2018. The irradiance at a depth of 2 m was measured as PAR using an ODYSSEY PAR Logger.

2.1 Carbonate system calculations

For the calculation of carbonate chemistry, we used total alkalinity (TA), pH_{NBS} (National Bureau of Standards scale), temperature, and salinity values. These parameters were input into the CO_2SYS software (Lewis and Wallace, 1998) to compute the carbonate chemistry (Table 1). For alkalinity values, the water samples (250 ml each) were collected from Väike Strait, Seljajärv, and Äntu Sinijärv at a depth of 0.5 m using laboratory glass bottles, which were filled carefully to avoid any air bubbles. The samples were preserved at 4°C until the analysis of TA, following the HELCOM draft guidelines for sampling and determination of TA (HELCOM, 2016). Triplicate subsamples were analyzed for TA using potentiometric titration with a Metrohm 848 Titrino Plus. The pH_{NBS} value used for the carbonate system calculations is the year-round average pH of the site, and the temperature is the summer average of that year when the alkalinity samples were collected.

2.2 Laboratory experiments

For the laboratory experiments, all specimens collected from Väike Strait, Äntu Sinijärv, and Seljajärv were placed in coolers containing water collected at the site and transported to the laboratory immediately. P vs. DIC curves and pH drift experiments were carried out using the laboratory facilities of the Kõiguste field station at the Estonian Marine Institute, the University of Tartu in Saaremaa Island. Using forceps the collected specimens were carefully cleaned of all macroscopic epiphytes before the start of the experiments.

2.2.1 pH drift experiments

The aim of the pH drift experiment was to determine the relative HCO_3^- affinity of charophyte species. When algae are capable of raising the pH above 9 in seawater (≈35 PSU and 2,300 µmol/L carbonate alkalinity) then they are theoretically able to use bicarbonate (HCO_3^-), because this is the point when CO_2 is virtually absent in the medium or appreciably low and should limit the photosynthesis for obligate CO_2 users (Maberly, 1990). Due to differences in salinity and DIC availability between the seawater and the brackish and freshwater used in this study, revised pH compensation points were calculated from data from the sites (Table 1) using the R package *seacarb* (Gattuso et al., 2022). In this study, the pH compensation point was taken to be 9.2 for brackish water (Väike Strait) and 9.8 for freshwater lakes (Seljajärv and Äntu Sinijärv). To detect whether charophytes *C. aspera* and *C. tomentosa* collected from brackish water and freshwater lakes are capable of using bicarbonate as an external carbon source for photosynthesis the pH was measured before and after the incubation of the algae (Maberly, 1990; Raven et al., 2005; Hepburn et al., 2011; Cornwall et al., 2017).

Before the start of the experiments, three individuals ($n = 3$) of both charophyte species from brackish Baltic Sea and freshwater lakes were cut ∼0.5–0.6 g wet weight apical parts and acclimatized in water collected from the site for 24 h. In the experiment, the acclimatized specimens were incubated in 60 ml sealed, transparent containers filled with brackish water (pH 8.15; salinity 6 PSU) and freshwater (pH 8.10; salinity 0.2 PSU). Waters used for experiments were sterilized with an AquaCristal 18W ultraviolet sterilizer and

FIGURE 1
Geographic locations of the studied sites in Estonia, including Väike Strait, Seljajärv, and Äntu Sinijärv, with corresponding DIC (µmol kg^{-1}) values calculated based on pH and total alkalinity measurements.

passed through a Glass Microfiber filter (GF/F, Ø 47 mm). All replicates were placed randomly onto a shaker table under a continuous irradiance of 80 µmol m^{-2} s^{-1} of PAR at 15°C. The PAR was measured with an LI-COR 250 light meter.

In the containers, the pH$_{NBS}$ readings were taken after 24, 32, and 48 h. After the final pH measurements, the algal material was removed and the containers with brackish and freshwater were left open for 48 h. Then the pH was measured again to determine whether the brackish and freshwater had re-equilibrated with the atmosphere. The pH was measured using a METTLER TOLEDO InLab® Expert Pro-ISM-IP67 pH-electrode (accuracy: ±0.001 and resolution: ±0.002) connected to a METTLER TOLEDO model Seven2Go^{TM} pro S8 pH/Ion Meter and calibrated against the NBS buffers.

2.2.2 Photosynthesis vs. dissolved inorganic carbon

The aim of photosynthesis vs. dissolved inorganic carbon experiment was to provide characteristics of carbon acquisition for *C. aspera* and *C. tomentosa* from brackish water and freshwater lakes under different DIC concentrations by using the oxygen evolution rates. Before the start of the experiments, three individuals of both charophyte species from brackish Baltic Sea and freshwater lakes were cut ∼0.5–0.7 g wet weight apical parts and acclimatized in brackish and freshwater for 24 h. Then the waters used for the experiment were filtered and UV-treated, as described in detail above.

To maintain the pH constant throughout the experiment Tris buffer solution was used. The effect of the Tris buffer on the photosynthetic ability was tested on both species and both habitats –brackish water ($n = 3$) and freshwater ($n = 3$). There was no significant difference in photosynthesis rate between the trials with and without Tris (final concentration of 15 mM, data not shown).

For the experiments, DIC-free brackish and freshwater were created following the methods of Beardall and Roberts (1999). Briefly, the pH of Tris-buffered seawater was lowered to 3 using a 1 M HCl solution, sparged with nitrogen gas for 2 h and then raised to the experimental pH of 8.1 using 1 M NaOH. Photosynthetic experiments were carried out at 10°C in 245 mL airtight chambers under photosynthetically saturating irradiance (500 µmol photons m^{-2} s^{-1} of PAR). The water temperature was maintained at 10°C by immersing the chamber in a water bath, with a magnetic stir bar (separated from the algal tissue with a mesh screen) to

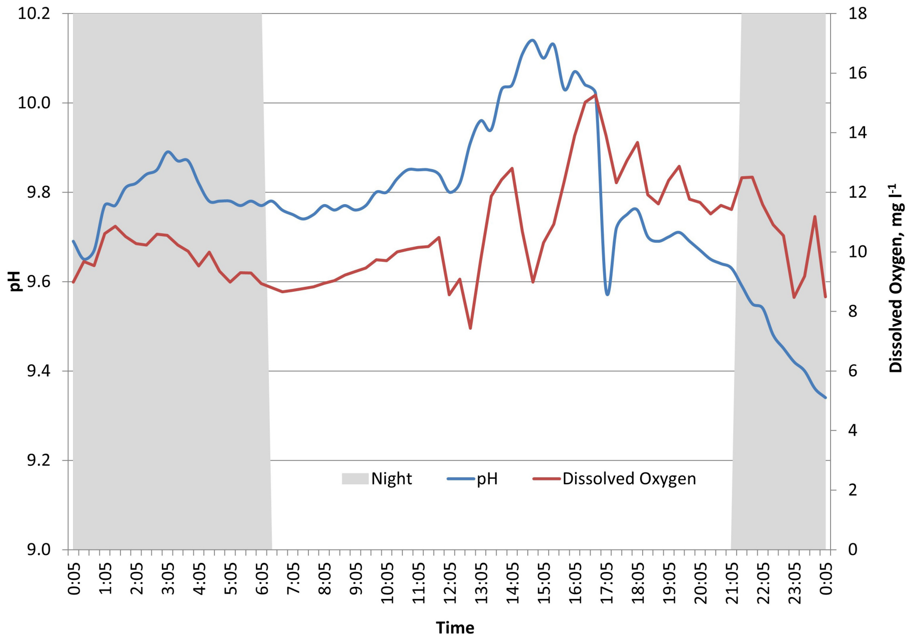

FIGURE 2
Natural fluctuations of brackish water pH and dissolved oxygen (mg l^{-1}) measured among charophytes habitat at the depth of 2 m, during an active vegetation period in July, in the Väike Strait (continuous recordings within 24 h). The gray area represents night (PAR < 50 μmol m^{-2} s^{-1}).

TABLE 1 Carbonate chemistry of Väike Strait (brackish Baltic Sea), Seljajärv (a freshwater lake), and Äntu Sinijärv (a freshwater lake).

Sampling site	Väike Strait	Seljajärv	Äntu Sinijärv
pH_{NBS}	8.32 ± 0.05	8.00 ± 0.03	7.88 ± 0.04
TA (μmol kg^{-1})	1,600 ± 50	3,016 ± 60	4,048 ± 70
pCO_2 (μatm)	288 ± 15	1,387 ± 30	3,727 ± 50
HCO_3^- (μmol kg^{-1})	1,528 ± 45	2,995 ± 50	4,034 ± 65
CO_3^{2-} (μmol kg^{-1})	32 ± 2	10 ± 1	7 ± 1
CO_2 (μmol kg^{-1})	21 ± 1	107 ± 5	287 ± 10
DIC (μmol kg^{-1})	1,582 ± 40	3,112 ± 55	4,329 ± 60

The values represent mean + SD.
pCO_2, HCO_3^-, CO_3^{2-}, CO_2, and DIC were calculated based on TA and pH_{NBS} using CO_2sys software.

provide water motion inside the chamber. Approximately every 7–10 min, 0.3 M $NaHCO_3^-$ solution was injected into the chamber to increase the DIC concentrations sequentially to 0.2, 0.6, 1.2, 2, 3, 4.5, 7, and 10 mM. The oxygen evolution was measured using an Ocean Optics′ NeoFox-TP oxygen probe connected to Ocean Optics′ NeoFox Viewer software. Probes were calibrated using a two-point calibration, achieved by bubbling DIC-free brackish and freshwater with nitrogen and ambient air. Three replicate curves of all studied species were conducted. After the experiments, samples were dried at 60°C until a constant weight was reached. Dry weights were used to normalize the photosynthetic rate (μmol O_2 $hour^{-1}$ gDW^{-1}) of the individuals at each DIC concentration.

2.2.3 Natural carbon isotope ($\delta^{13}C$) analysis

The goal of the carbon isotope ($\delta^{13}C$) analysis was to detect the presence or lack of a carbon CCM operation for *C. aspera* and *C. tomentosa* from brackish water and freshwater lakes. When the value of $\delta^{13}C$ is between −30 and −10‰ it indicates the presence of CCM and the uptake of both carbon forms (CO_2 and HCO_3^-) for macroalgal photosynthesis (Raven et al., 2002). The $\delta^{13}C$ values above −10‰ indicate the presence of CCM and primarily the use of HCO_3^-. The $\delta^{13}C$ values below −30‰ indicate that there is no CCM present and macroalgae rely purely on diffusive CO_2 supply (Raven et al., 2002).

Three individual ($n = 3$) specimens of both species from brackish Baltic Sea and freshwater lakes were cut ~0.5–0.6 g wet weight sections and were dried at 60°C until a constant weight was reached, ground into a fine powder using a mortar and pestle and stored in microcentrifuge tubes. Three replicates of both studied species from both habitats were analyzed using an elemental analyzer (FlashEA 1112 HT, Thermo Scientific) interfaced through a ConFlo IV dilution device (Thermo Scientific) with an isotope ratio mass spectrometer (Delta V Plus, Thermo Scientific). About 1 mg of the sample was weighed into tin capsules for determination of $\delta^{13}C/\delta^{12}C$. The C isotope composition is reported per mill respective to Vienna Pee Dee Belemnite (V-PDB) and calibrated using international IAEA standards IAEA-CH-3 and IAEA-CH-6. Long-term reproducibility precision and accuracy were ±0.1‰. $\delta^{13}C$ isotopic analysis of DIC was conducted from both studied lakes. Waters containing DIC were analyzed by Continuous Flow Isotope Ratio Mass Spectrometry (CF-IRMS) using a Thermo Finnigan GasBench coupled to a DeltaVPlus.

2.3 Statistics

Statistical analysis was conducted using the R statistical software platform (R Core Team, 2017). For the pH drift experiment, the final (48 h) pH values for *C. aspera* and *C. tomentosa* from brackish water and freshwater were compared using a two-way ANOVA *post-hoc*-test and two-tailed *t*-test. For the P vs. DIC experiment, a Michaelis-Menten curve (Johnson and Goody, 2011) was fitted to plots of photosynthetic rate vs. DIC concentration. The Michaelis-Menten equation is $P = P_{max}/DIC + K_{0.5}$, where P_{max} is the point at which the maximum photosynthetic rate of the organism is reached and $K_{0.5}$ is the concentration of DIC at which the photosynthetic rate of the organism is half of P_{max} (Johnson and Goody, 2011). A maximum likelihood, non-linear mixed-effects modeling was approached using the lme4 package in R (Bates et al., 2015). This approach allowed a single model to be used, whilst accounting for species-level (fixed factor, *C. aspera* and *C. tomentosa* from brackish water and freshwater) and individual-level (random factor, replicate incubation id) variability in P_{max} and $K_{0.5}$. Within-species comparisons of P_{max} and $K_{0.5}$ were made using simultaneous *t*-tests, using the R package multcomp (Hothorn et al., 2008).

3 Results

The pH compensation points of *C. aspera* and *C. tomentosa* were significantly higher in brackish than in freshwater (two-way ANOVA: $f = 17.39$, $p = 0.003$). In brackish water, the pH compensation points of *C. aspera* and *C. tomentosa* were 10.51 ± 0.06 and 10.51 ± 0.04 (mean ± SE; $n = 3$), respectively. In fresh water, the pH compensation points of *C. aspera* and *C. tomentosa* were 8.99 ± 0.14 ($n = 3$) and 9.45 ± 0.10 ($n = 3$), respectively. Also, there was no significant difference between *C. aspera* and *C. tomentosa* collected from the freshwater lakes (two-tailed *t*-test: $t = 1.1043$, $p = 0.3316$) and from the brackish Baltic Sea (two-tailed *t*-test: $t = 4.7058$, $p = 0.112$).

The value of $\delta^{13}C$ for brackish water *C. aspera* was −13.11‰ ± 0.26 (mean ± SE, $n = 3$), for brackish water *C. tomentosa* −13.88‰ ± 0.22 ($n = 3$), indicating the presence of CCM operation (uptake of both CO_2 and HCO_3^-). The carbon isotope value for freshwater *C. aspera* was −30.23‰ ± 0.27 ($n = 3$) and for freshwater *C. tomentosa* −32.33‰ ± 0.90, indicating the reliance of mainly on CO_2 (Figure 3). The differences in $\delta^{13}C$ in DIC values between Seljajärv (−9.24‰) and Äntu Sinijärv (−12.93‰) suggest variations in carbon sources or processes affecting DIC in these lakes.

P_{max} of *C. tomentosa* from brackish water was 114.68 ± 12.39 (RATE ± SE, µmol O_2 $hour^{-1}$ gDW^{-1}), was significantly higher than *C. tomentosa* from freshwater 52.14 ± 7.57 (RATE ± SE, µmol O_2 $hour^{-1}$ gDW^{-1}; *post-hoc* test; $p = 0.002$) and *C. aspera* from freshwater 58.68 ± 6.65 (RATE ± SE, µmol O_2 $hour^{-1}$ gDW^{-1}; *post-hoc* test; $p = 0.005$). There were, however, no significant differences between the $K_{0.5}$ values for these curves ($p = 0.915$ and 0.999 respectively), as well as no other significant differences between P_{max} and $K_{0.5}$ values for any other comparisons (Figure 4; Table 2).

4 Discussion

Over a decade of research into ocean acidification illuminates various responses by oceanic biota (Gattuso and Hansson, 2011; Mercado and Gordillo, 2011; Terhaar et al., 2020). However, in the Baltic Sea, there is a lack of data to predict the response of macrophyte communities to acidification and in freshwater lakes (all over the world), this information is basically absent. In this study, we expected the same charophyte species growing in different habitats will likely use different carbon uptake mechanisms.

Our results from the pH drift experiment showed that *C. aspera* and *C. tomentosa* growing in brackish water likely possess a CCM. Both species raised the pH well above the pH compensation point. The freshwater *C. aspera* and *C. tomentosa* pH drift values stayed under the calculated thresholds, indicating the reliance on solely CO_2. Furthermore, the pH compensation points for brackish *C. aspera* and *C. tomentosa* were significantly higher compared to freshwater *C. aspera* and *C. tomentosa*. The brackish water pH drift results presented here are also compatible with our measurements from the Väike Strait, which show charophytes ability to raise pH above 10 in their natural community in summer conditions. This indicates an effective CCM operation since at pH 9.2 the concentration of CO_2 is too low to support photosynthetic activity for obligate CO_2 users. Ray et al. (2003) documented the occurrence of charasomes in the cell membrane of *C. tomentosa* growing in the northern Baltic Sea, the simultaneous high periplasmic carbonic anhydrase and proton pump activities suggest proton-pump driven H+ extrusion and membrane transport of CO_2 derived from HCO_3^- as the major form of DIC acquisition in this species. In general, macrophytes having the ability to use HCO_3^- are better adapted to environments with high pH and low CO_2 e.g., the brackish Baltic Sea (Maberly and Spence, 1983; Ray et al., 2003).

Our pH drift experiment results of brackish water charophytes are also consistent with the carbon isotope ($\delta^{13}C$) values which were between −30 and −10‰ indicating the presence of CCM and the uptake of both carbon forms (CO_2 and HCO_3^-) for photosynthesis. However, we acknowledge that the baseline $\delta^{13}C$ values for DIC in different areas of the Baltic Sea may vary due to the nature of the brackish environment. Given the $\delta^{13}C$ values of −13.11‰ and −13.88‰ for *C. aspera* and *C. tomentosa* in our study, these values fit within the expected range for a mixed marine and freshwater system, indicating the presence of CCMs and utilization of both CO_2 and HCO_3^- for photosynthesis (Rolff and Almesjö, 2009; Emeis et al., 2014). The brackish *C. aspera* and *C. tomentosa* had relatively more positive $\delta^{13}C$ values than freshwater charophytes, indicating the uptake of different DIC species for photosynthesis: primarily the use of HCO_3^- in the brackish Baltic Sea and CO_2 in freshwater lakes. Furthermore, we found that the studied freshwater lakes have significantly higher pCO_2 concentrations than the brackish Baltic Sea.

Seljajärv's average $\delta^{13}C$ value of −9.24‰ suggests an enriched $\delta^{13}C$ signature, likely due to significant bicarbonate contributions from limestone or other mineral sources, and perhaps a minimized role of organic matter decomposition. In contrast, Äntu Sinijärv's $\delta^{13}C$ value of −12.93‰ indicates a more depleted $\delta^{13}C$, pointing toward a greater impact from organic decomposition or

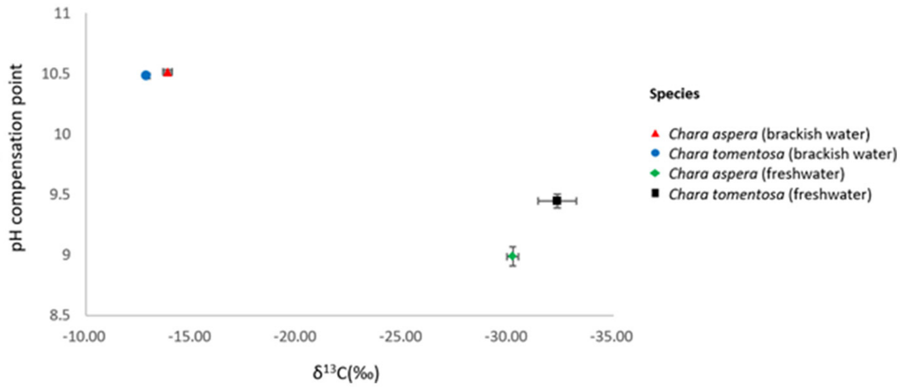

FIGURE 3
pH compensation points vs. carbon stable isotope ($\delta^{13}C$) values of the charophyte species *C. aspera* and *C. tomentosa* from freshwater and brackish Baltic Sea ±SE ($n = 3$).

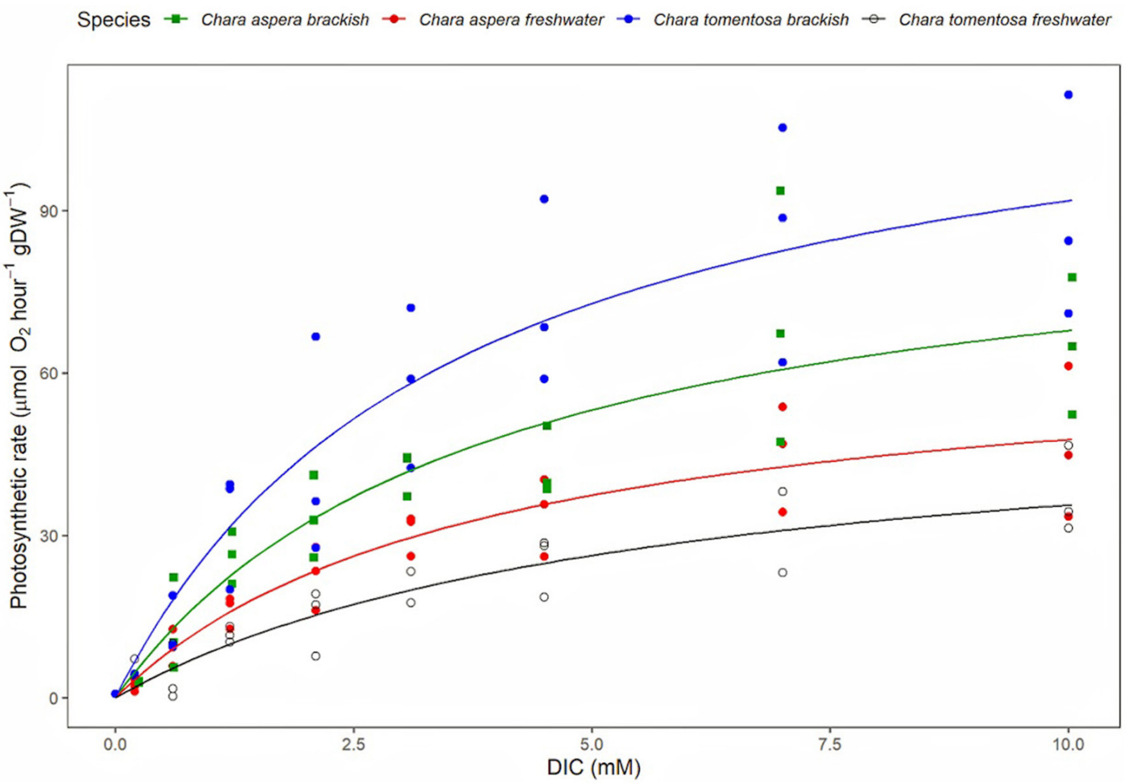

FIGURE 4
The photosynthetic rates of *C. aspera* and *C. tomentosa*, from freshwater ($n = 3$) and brackish Baltic Sea ($n = 3$), at different concentrations of dissolved inorganic carbon (mM).

atmospheric CO_2 mixing into the lake's carbon pool. Notably, bicarbonate from mineral sources, as documented in literature (Ott, 2011), contributes to both lakes, suggesting a common geochemical influence. The disparity in $\delta^{13}C$ between the charophytes and their respective lake's DIC could illuminate the effectiveness of the CCMs in harnessing available carbon. More proficient CCMs are expected to show minimal deviation from the ambient DIC $\delta^{13}C$, indicating a strategic adaptation to local carbon sources. Therefore, the reason for the different carbon uptake mechanisms between brackish and freshwater charophytes is driven by the environments where they grow—the freshwater charophytes probably do not need to use energetically expensive HCO_3^- since the availability of energetically inexpensive CO_2 is in abundance.

Also, the P vs. DIC experiment results show the freshwater charophytes are likely carbon-saturated, meaning their photosynthetic processes are operating at their maximum

TABLE 2 The half-saturation point ($K_{0.5}$) and maximum photosynthetic rate (Pmax) of brackish and freshwater *C. aspera* and *C. tomentosa* ±SE ($n = 3$).

Species	Brackish Baltic Sea	Freshwater lakes
	$K_{0.5}$ (mM) Pmax (μmol O_2 hour^{-1} gDW^{-1})	$K_{0.5}$ (mM) Pmax (μmol O_2 hour^{-1} gDW^{-1})
Chara aspera	3.41 ± 0.74	3.09 ± 0.35
	83.56 ± 13.04	58.68 ± 6.65
Chara tomentosa	2.82 ± 0.40	5.311 ± 0.92
	114.68 ± 12.39	52.14 ± 7.57

efficiency due to the availability of DIC in their environment. In Seljajärv, the DIC concentration ($3{,}112 \pm 55$ μmol kg^{-1}) is close to the $K_{0.5}$ for *C. aspera* ($3{,}090 \pm 350\,\mu$M) but significantly lower than the $K_{0.5}$ for *C. tomentosa* ($5{,}311 \pm 920\,\mu$M). This suggests that C. aspera is likely well-adapted to the available DIC levels in Seljajärv, while *C. tomentosa* might be less efficient due to its higher $K_{0.5}$. In the brackish Baltic Sea, the $K_{0.5}$ values for both species (*C. aspera*: $3{,}410 \pm 740\,\mu$M, *C. tomentosa*: $2{,}820 \pm 400\,\mu$M) are higher than the DIC concentration ($1{,}582 \pm 40$ μmol kg^{-1}). This suggests both species might be operating below their optimal DIC saturation levels, indicating potential limitations in their carbon uptake efficiency in this environment. *C. tomentosa* has a lower $K_{0.5}$ than *C. aspera*, indicating a higher affinity for DIC, but given that both $K_{0.5}$ values exceed the available DIC, both species may still face DIC limitation, with *C. tomentosa* being slightly more efficient under these suboptimal conditions.

The majority of papers suggest the genus *Chara* ability to use HCO_3^- for photosynthesis (Price et al., 1985; Van den Berg et al., 2002; Ray et al., 2003). Van den Berg et al. (1998) found that *C. aspera* had a high photosynthetic rate over a wide range of HCO_3^- concentrations, suggesting efficient bicarbonate use. Pronin et al. (2016) studied seven different lakes in Poland and found *C. tomentosa* carbon isotope values varied on average between −15‰ to −21‰, suggesting HCO_3^- is the main source of carbon for *C. tomentosa.* Furthermore, Osmond et al. (1981) found that at pH 7.0 and 7.5, and high CO_2 levels the submerged macrophyte *Stuckenia pectinata* L. stable carbon isotope values were −25.0‰ and −25.7‰. However, in shallow lakes when CO_2 concentration approaches zero, under such conditions, *Stuckenia pectinata* relies solely on HCO_3^- uptake as inferred from pH-drift experiments under comparable conditions (Sand-Jensen, 1983). In the recent study by Pronin et al. (2023) they examined the δ^{13}C values of the charophyte *Nitella flexilis* in both softwater and hardwater lakes and reported the δ^{13}C values did not significantly differ between the two types of lakes but showed more variation in softwater lakes. They concluded that the variability is attributed to different proportions of carbon forms (CO_2 and HCO_3^-) available for photosynthesis, influenced by pH. These results indicate high variability of carbon uptake mechanisms between different growth environments of the same species. Based on this study, we cannot exclude the possibility that the studied freshwater charophytes have physiological mechanisms available for the operation of CCM when the concentration of CO_2 is too low for photosynthesis. However, this needs further investigation using different experimental approaches.

Studies have shown that macrophytes have different types of CCMs with varying energetic efficiencies (Giordano et al., 2005; Hepburn et al., 2011; Cornwall et al., 2017). The sheltered nature of charophyte habitats likely plays an important role in DIC acquisition. Our measurements in the brackish Baltic Sea indicate that CO_2 concentration is virtually absent during the active growth period in charophyte habitats. Therefore, the studied charophytes have likely developed an effective CCM, enabling them to raise the pH above 10 in the Väike Strait. However, based on our P vs. DIC experiment results the brackish Baltic Sea *C. tomentosa* and *C. aspera* might be limited by current DIC availability (∼1.5 mM). Furthermore, brackish *C. tomentosa* P_{max} was significantly higher compared to freshwater charophytes indicating an effective DIC uptake. This is consistent with Pajusalu et al. (2015) who found that *C. tomentosa* exhibited a significant increase in net primary production rates when the CO_2 concentration was elevated until 2,000 μatm. Also, *C. aspera* showed only a slight response to elevated pCO_2, possibly due to differences in the ecophysiology and life strategy of the species (Pajusalu et al., 2015). Furthermore, our P vs. DIC experiment results refer to a possible scenario when the atmospheric CO_2 concentration increases by 150–250% predicted by IPCC (2022), the brackish Baltic Sea *C. tomentosa* and *C. aspera* might be able to downregulate CCM operation e.g., switch from an HCO_3^- to CO_2 based metabolism. This could mean that lowered pH will have a positive impact on sheltered parts of the Baltic Sea where charophytes grow.

Another possible explanation why the brackish water charophytes could be DIC limited might be related to the extent of calcification. Calcite-encrusted charophytes are usually associated with base-rich waters associated with limestone in the catchment (Coletta et al., 2001). In this study, we found that alkalinity is much higher in freshwater lakes compared to brackish water. Charophytes are generally thought to be efficient calcifiers in freshwater (McConnaughey and Whelan, 1997; Lucas and Berry, 1985), which ensures the conversion of HCO_3^- to free CO_2 for continued photosynthesis without increasing pH to inhibiting levels ($Ca^{2+} + 2HCO_3^- \rightarrow CaCO_3 + CO_2$; Andersen et al., 2017). However, in the northern Baltic Sea, *C. tomentosa* has much thinner $CaCO_3$ incrustations than in freshwater lakes close to the Baltic Sea coast (Ray et al., 2003). HCO_3^- use without calcification increases pH ($HCO_3^- \rightarrow CO_2 + OH^-$) which may inhibit photosynthesis (Andersen et al., 2017). There is a lack of research available about the mechanisms and peculiarities of *Chara* calcification in the brackish Baltic Sea compared to freshwater environments, however, the species calcification in the Baltic Sea could be minor due to low calcium conditions.

4.1 Summary statement

The study showed that freshwater and brackish water *C. aspera* and *C. tomentosa* likely use different carbon uptake mechanisms. Our results indicated that freshwater charophytes preferentially use CO_2 and brackish water charophytes HCO_3^-, likely due to their local acclimatization to different growth environments. At present, the high pCO_2 concentrations in Äntu Sinijärv and Seljajärv indicate that these lakes might be functioning as CO_2 sources rather than sinks. Therefore, the freshwater *C. tomentosa* and *C. aspera* are likely carbon saturated in these lakes and probably will not gain photosynthetic advantages from acidification. However, the predicted increase in CO_2 concentration may positively affect the growth of the charophytes in the brackish Baltic Sea.

5 Future research needs

This study opened an understanding of the carbon uptake strategies of *C. aspera* and *C. tomentosa* growing in the brackish Baltic Sea and in the freshwater lakes of Estonia. The comparison underscores the importance of considering both biological and geochemical perspectives to fully appreciate the nuances of aquatic plant ecology and the carbon cycle. However, for a more detailed understanding of the CCM operation in these species, the next step will be to carry out the pH banding experiments e.g., identifying the alkaline and acid zones to determine the level of HCO_3^- transport. Additionally, gathering more background information about the freshwater lakes will be important. This includes measuring daily natural fluctuations of pH levels, photosynthetically active radiation (PAR), and oxygen levels among the vegetation, similar to the studies carried out in the brackish Baltic Sea.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

GA: Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. LP: Conceptualization, Data curation, Supervision, Writing – review & editing. DP: Formal analysis, Methodology, Writing – review & editing. CH: Conceptualization, Writing – review & editing. KT: Conceptualization, Investigation, Writing – review & editing. TP: Conceptualization, Investigation, Writing – review & editing. AP: Data curation, Formal analysis, Writing – review & editing. GM: Funding acquisition, Supervision, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. Funding for this study was provided through the basic research and infrastructure fund of the University of Tartu Estonian Marine Institute.

Acknowledgments

The authors would like to acknowledge those who have aided us in the completion of this work.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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