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Carbon acquisition mechanisms in *Chara tomentosa*

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Abstract

Carbon uptake mechanisms of the stonewort *Chara tomentosa* from the brackish Baltic Sea were studied by recording changes in pH, alkalinity and inorganic carbon concentrations of the seawater medium during photosynthesis in a closed system. The use of inhibitors identified three mechanisms: (1) a vanadate-sensitive P-type H^+-ATPase (proton pump) was involved in carbon uptake. This was previously shown for perfused cells of *Chara corallina*, but not for living cells. (2) Periplasmic carbonic anhydrase that catalyses the dehydration of HCO_3^- into CO_2 outside the cell membrane was highly active during carbon uptake, also at high pH (>9). (3) At high pH, there was direct uptake of HCO_3^- with the help of an anion exchange protein, which previously has not been shown in *Chara*. We also document here the occurrence of charasomes in the cell membrane of *C. tomentosa*, always with mitochondria located in their direct vicinity. The simultaneous high periplasmic carbonic anhydrase and proton pump activities and the occurrence of charasomes 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 alga. Probably, this occurs in acidic bands in *C. tomentosa* in which we found a banding pattern of $CaCO_3$ incrustations (alkaline bands). The results were compared with a similar study on the green alga *Cladophora glomerata* from the same area, which had very low carbonic anhydrase activity (almost negligible), no structures isolating the periplasm from the bulk seawater medium analogous to charasomes and no $CaCO_3$ incrustation.

Keywords: *Chara tomentosa*; Characean; Proton pump; Carbonic anhydrase; Anion exchanger; Charasomes; Photosynthesis; pH; Alkalinity; Carbon uptake mechanisms

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1. Introduction

The carbon acquisition strategies of aquatic photosynthetic organisms have wide-reaching ecological implications as uptake rates of inorganic carbon are directly involved in the growth and survival of a species in its environment. At natural seawater pH (ca. 8.2), HCO_3^- is the dominating carbon species with a concentration of ca. 2 mM whereas [CO_2] is only ca. 10 μM. Algae can utilise HCO_3^- in two ways: (a) HCO_3^- acts as an external reservoir for CO_2 production and CO_2 is the DIC-species that is transported over the cell membrane, and (b) HCO_3^- is transported over the cell membrane and is later transferred into CO_2 inside the cell. When HCO_3^- acts as an external reservoir for CO_2, periplasmic carbonic anhydrases (CA) catalyse the dehydration of HCO_3^- into CO_2 (Badger and Price, 1994). From the observation that the photosynthetic DIC assimilation at high pH proceeded without changes in alkalinity, Axelsson and Uusitalo (1988) suggested membrane transport of HCO_3^- via a HCO_3^-/OH^- antiport system for the green marine macroalga *Ulva* sp. Larsson et al. (1997) suggested that such transport is achieved with the help of an anion exchange protein.

Active (energy-consuming) uptake of DIC has also been suggested to occur in algae. H^+-ATPase-driven DIC uptake has been identified in a number of microalgae (Thielmann et al., 1990; Karlsson et al., 1994), in the characean *Chara corallina* Klein ex. Willd. em. R.D.W. (=*Chara australis* R. Br.) (Mimura et al., 1993), the red macroalga *Coccotylus truncatus* (Pall.) M.J. Wynne and J.M. Heine (Snoeijs et al., 2002), the brown macroalgae *Laminaria digitata* (Huds.) Lamour. and *Laminaria saccharina* (L.) Lamour. (Klenell et al., in press), and the green macroalga *Cladophora glomerata* (L.) Kütz. (Choo et al., 2002). A P-type H^+-ATPase (proton pump) is a membrane protein that pumps protons out of the cell, thus creating a gradient of pH and electrical potential differences across the plasma membrane (Taiz and Zeiger, 1998). This 'proton motive force' can drive a secondary transport of different substances (ions, metabolites, etc.) into and out of the cell, either by uniport, antiport or symport mechanisms (Michelet and Boutry, 1995). H^+-ATPases can also contribute to the driving forces for diffusion through ion channels, or create H^+ extrusion areas where dehydration of HCO_3^- into CO_2 can take place because of low pH just outside the algal cells (Smith, 1988).

Among the macroalgae, the freshwater characean *C. corallina* is one of the best-studied species with respect to DIC uptake. Its giant cells can easily be perfused in the laboratory, and previous knowledge on carbon uptake in *Chara* is primarily based upon results obtained by this method of emptying cells and refilling them with experimental solutions (Mimura et al., 1993). In the present paper, we investigate the DIC transport mechanisms across the plasma membrane in intact, living cells of *Chara tomentosa* L. This widespread freshwater species is common in calcareous lakes in the Swedish county of Uppland, but it is also a dominant macrophyte in shallow bays of the atidal northern Baltic Sea with salinity below ca. 5 practical salinity units (psu) (Snoeijs, 1999). It occurs in shallow water (down to a depth of ca. 2 m) from June to September, often in dense stands. $CaCO_3$ incrustation which is typical for this species is thought to be of major importance in DIC acquisition (McConnaughey, 1998). In the Baltic Sea, *C. tomentosa* has much thinner $CaCO_3$ incrustations than in freshwater lakes close to the Baltic Sea coast.

In the present study, we investigated the hypotheses that (1) three mechanisms, periplasmic CA, a proton pump and an anion exchange protein are involved in the DIC uptake of *C.*

tomentosa in brackish water, and (2) *C. tomentosa* possesses charasomes (=plasmalemmasomes), cellular structures consisting of intricate networks of anastomosing membrane tubules that are continuous with the plasma membrane (Franceschi and Lucas, 1980) and which are thought to be involved in DIC uptake (Price et al., 1985).

2. Materials and methods

2.1. The algal material

Specimens of *C. tomentosa* were collected from a depth of 1–2 m on 14 September 1998, 28 September 2000 and 13 September 2001 at Skatviken (60°28′N, 18°05′E; salinity 3.2, 4.8 and 4.7 psu, respectively). Skatviken is a shallow coastal lagoon situated in the atidal southern Bothnian Sea, northern Baltic Sea. Prior to the experiments, *C. tomentosa* was cultivated as 7–12 cm long apical tips (including the uppermost two nodes with branches) at 14 °C and 290 μmol photons PAR $m^{-2}\,s^{-1}$ in a 12 h photoperiod in natural seawater from the sampling site (NSW) for several weeks. Stock cultivation (several years) of *C. tomentosa* collected in 1998 was performed in 40 l aquaria filled with site water and with the algae planted in sediment from the sampling site. The aquaria were kept at 14 °C and 34 μmol photons PAR $m^{-2}\,s^{-1}$ in a 12 h photoperiod. The NSW in all cultivations was exchanged every one to two weeks and no extra nutrients were added.

2.2. Inhibitors of carbon uptake mechanisms

The inhibitors used were acetazolamide for periplasmic CA (AZ, $pK_a = 7.2$, Sigma; Moroney et al., 1985), 4,4′-diisothiocyano-stilbene-2,2′-disulfonate for a HCO_3^--transporting anion exchange protein (DIDS, pK_a below 1.3, Sigma; Keifer et al., 1982, Smith and Bidwell, 1989) and orthovanadate dissolved as Na_3VO_4 for P-type H^+-ATPases (VAN, pK_a ca. 8.7, Sigma; Gilmour et al., 1985). A proton buffer, Trizma base (TRIS, $pK_a = 8.15$, Sigma), was used to study its effect on the rate of pH change due to the absorption of H^+. No stock solutions were used; all inhibitors and the buffer were added to the NSW in the final concentrations used in the experiments, except for AZ, which was pre-dissolved in 0.2 ml 0.5 M NaOH before adding 500 ml of seawater. In solution of pH 3–13, VAN occurs as $H_2VO_4^-$ and HVO_4^{2-}(Harris, 1999). After addition of the inhibitors to the NSW, the pH was adjusted to 8.00 with HCl.

2.3. Buffering capacity of inhibitors

The pH of the NSW is closely related to [DIC], and a pH increase in light follows photosynthetic DIC uptake and changes in pH can be used to investigate the DIC uptake of algae (Axelsson and Uusitalo, 1988; Maberly, 1990; Granbom and Pedersén, 1999). The buffer capacity of inhibitors may interfere with this process. Therefore, we compared the buffer capacity of filtered NSW (4.8 psu) with that of filtered NSW with 0.2 mM AZ, 0.2 mM VAN, 0.3 mM DIDS or 0.2 mM TRIS by titration of 50 ml NSW sample with 0.1 M NaOH, using an automatic titrator (719 S Titrino, Metrohm™). Titration curves were made for

the pH interval 8.00–10.20, with pH recordings for each 0.01–0.05 pH unit. Three replicate samples were measured for each treatment. The buffer capacity was calculated as the number of moles of OH^- added to achieve 1 pH unit change and then expressed as titre slope in mM H^+ pH $unit^{-1}$.

2.4. DIC–alkalinity–pH experiments

To investigate if processes other than DIC uptake by the algae and the DIC buffer system of NSW would influence alkalinity and [DIC] during pH-drift, we performed DIC–alkalinity–pH experiments during which all three parameters were measured simultaneously. Apical pieces of *C. tomentosa* collected in 2001 were incubated by placing 0.3 g of fresh weight (FW) in each of twenty-four 50 ml Erlenmeyer flasks with 60 ml of NSW, leaving a small head space of air. The flasks were sealed with rubber stoppers and put on a shaker table with a speed of 200 rpm at 23–24 °C and 500 μmol photons PAR m^{-2} s^{-1}. Three flasks were randomly taken out every 1.5 h over 12 h, and [DIC], alkalinity and pH of the NSW were analysed. Four treatments were applied: without addition of an inhibitor (REF), with 0.2 mM AZ, with 0.3 mM DIDS and with 0.2 mM VAN. [DIC] was analysed by a method modified after Bidwell and McLachlan (1985) and Lignell and Pedersén (1986) with an Infrared Gas Analyser (IRGA; Series 225 Gas Analyser, the Analytical Development Co. Ltd.™, UK). Total alkalinity was analysed according to Almgren et al. (1983) using an automatic titrator (719 S Titrino, Metrohm™, Switzerland).

2.5. pH-drift experiments

Apical parts of *C. tomentosa* (1.5–3.5 g FW) were placed in each of three 50 ml Erlenmeyer flasks that were filled with 60 ml filtered NSW. The flasks were sealed airtight with rubber stoppers through which electrodes were immersed into the water. Three identical pH electrodes with temperature measurement (Pt 1000, Metrohm™) attached to three pH meters (Model 713, Metrohm™), using the NBS scale and two-point calibrated with Radiometer™ buffers pH 7 and 9, were used. The water in the flasks was continuously stirred and pH evolution was measured every minute for 10 h. Each experiment lasted for 5–7 days of which 3 were measuring days. Between the measuring days the algae were kept for 36–60 h in new NSW in a culture room at 14 °C. On the first measuring day a control curve of the pH-drift was made (new NSW + alga). On the second measuring day the pH-drift of the treatment was recorded (new NSW + alga + inhibitor or buffer). On the third measuring day a post-control pH-drift of the algae was recorded (new NSW + alga). A test without algae confirmed that the pH of the medium stayed at pH 8.00 for 10 h. The treatments included 0.2 mM AZ, 0.3 mM DIDS, 0.2 mM VAN, 0.2 mM TRIS and 0.2 mM AZ + 0.3 mM DIDS (AZDIDS treatment). Temperature was 23–24 °C and illumination was 290 μmol photons PAR m^{-2} s^{-1} during all experiments.

2.6. Calculations and statistics

The results of the pH-drift experiments were expressed as rates of pH change (pH unit $kg\,FW^{-1}$ min^{-1}) for each 0.01 pH unit between pH 8.0 and 10.2 for each control, treatment

and postcontrol run. The contribution to the pH change by buffering of AZ, VAN and TRIS was calculated from the titer slope curves as [(μM H^+ pH unit^{-1} inhibitor − μM H^+ pH unit^{-1} NSW without inhibitor)/(μM H^+ pH unit^{-1} inhibitor)] × 100% for each 0.01 pH unit between pH 8.0 and 10.2. The rates of pH change of the AZ, VAN and TRIS treatments were corrected with the help of these percentages for each 0.01 pH unit between pH 8.0 and 10.2. The rate of DIC uptake by the alga (μmol DIC kg FW^{-1} min^{-1}) was calculated from the rates of pH change by using the equilibria of pH, temperature, salinity, alkalinity, total DIC and CO_2 in seawater in the computer model of Turner (Göteborg) by assuming stable alkalinity of 1.3 mM. The justification of the use of stable alkalinity in the model is verified by calculations presented in Sections 3 and 4. Statistical tests were performed with the program MINITAB^{TM}, Version 13.1. In the DIC–alkalinity–pH experiments, one-way ANOVA was used to test for the effect of incubation time. Throughout this paper significance is accepted at $P < 0.05$.

2.7. Microscopy

C. tomentosa was studied by transmission electron microscopy (TEM) of algal material from Skatviken, both fresh material collected in 2000 (salinity 4.8 psu) and material cultivated in the laboratory for about two years (collected in 1998, salinity 3.2 psu) were used. Pieces of *C. tomentosa*, ca. 2 cm in length and ca. 0.2 cm in diameter were taken from the apical parts of different plants. All pieces consisted of one central cell with bark cells around it. The algal pieces were fixed in chilled 2.5% glutaraldehyde with 15 mM sucrose buffered with a 50 mM sodium cacodylate buffer at pH 7.2, and later treated with 2% osmium tetroxide at 4 °C for 2 h. The algae were embedded in Epon and 100 nm thick cross-sections were double stained with lead citrate and uranyl acetate. The sections were placed on formvar films covered nylon grids and coated with a conducting carbon layer. Analyses were performed with a Phillips^{TM} CM-10 transmission electron microscope. Calcification of *C. tomentosa* was documented with a Nikon^{TM} light microscope with photographic equipment.

3. Results

3.1. Buffer capacity

Addition of AZ, VAN or TRIS increased the buffer capacity of NSW, but DIDS did not. The buffer capacity of 0.2 mM AZ was always higher than that of 0.2 mM VAN. At pH 8.0, the buffer capacity of 0.2 mM TRIS was the same as that of 0.2 mM AZ, but with higher pH the buffer capacity of TRIS decreased and was even slightly less than that of the control (REF: no inhibitor added) at pH 10.2. The R^2-values of trigonomal regressions with pH as predictor variable and titer slope as response variable were between 0.93 and 0.95.

3.2. Alkalinity and carbon concentrations

In the REF experiment of the DIC–alkalinity–pH experiments, the pH had increased to 9.75 after 12 h, while DIC simultaneously decreased from 1.3 to 0.9 mM and alkalinity from

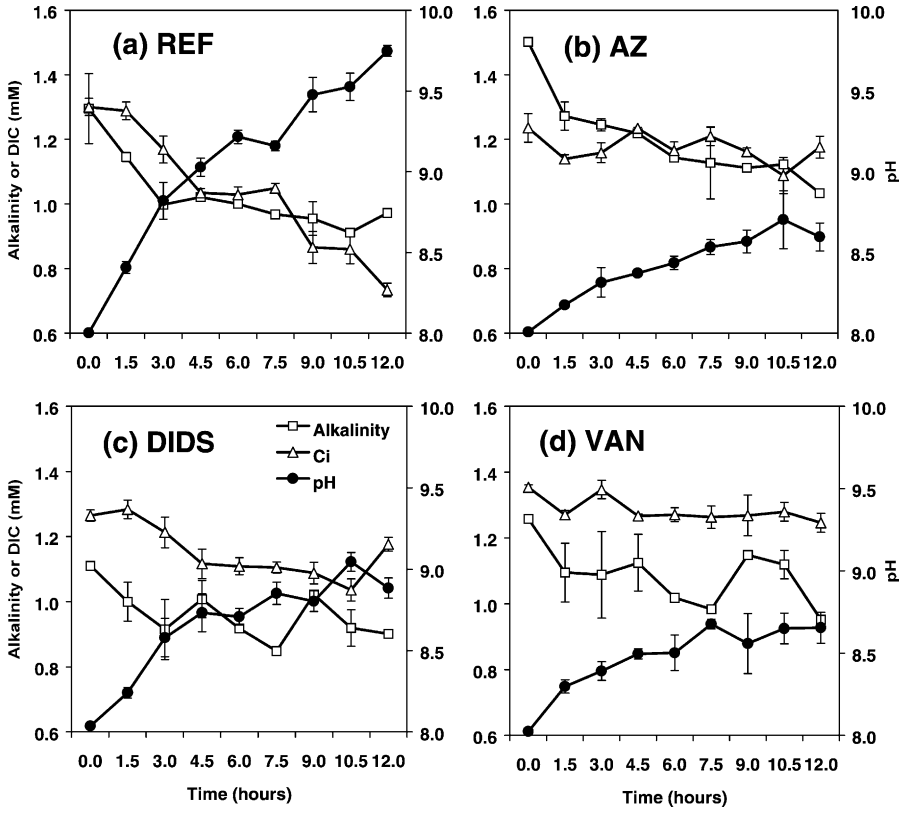

Fig. 1. Changes of pH, alkalinity and [DIC] in the NSW medium during photosynthesis of *Chara tomentosa* in a closed system. (a) REF (no inhibitor); (b) 0.2 mM AZ; (c) 0.3 mM DIDS; (d) 0.2 mM VAN. Error bars: 1 S.E. (not visible when very small).

1.3 to 0.7 mM (Fig. 1a). All three inhibitors (AZ, DIDS and VAN) had negative effects on the pH increase and the DIC decrease (Fig. 1b–d). The pH increase with time was significant for all four treatments, but the DIC decrease with time was only significant for REF and DIDS (ANOVA, $P > 0.05$; Fig. 1a and c). The 4.7 psu NSW (REF treatment) had a mean alkalinity ($\pm$S.D., $n = 3$) of 1.3$\pm$0.2 mM. The addition of 0.2 mM AZ (negative ions) raised alkalinity by ca. 0.2 mM and, and the addition of 0.3 mM DIDS slightly lowered alkalinity. The alkalinity of the NSW decreased significantly in the REF and AZ experiments (ANOVA, $P < 0.05$; Fig. 1a and b), but not in the DIDS and VAN experiments (ANOVA, $P > 0.05$; Fig. 1c and d).

We compared our results from the DIC–alkalinity–pH experiments with the theoretical equilibria in seawater (Fig. 2a) at ambient temperature and salinity (23.5 °C, 4.7 psu). When calculated from measured [DIC] and pH, alkalinity did not decrease (ANOVA, $P > 0.05$), but when calculated from measured alkalinity and pH, [DIC] decreased more than our measured values. The latter is not possible because we measured all DIC in the samples by adding a strong acid so that all carbon would appear in the form of CO_2 to be able to

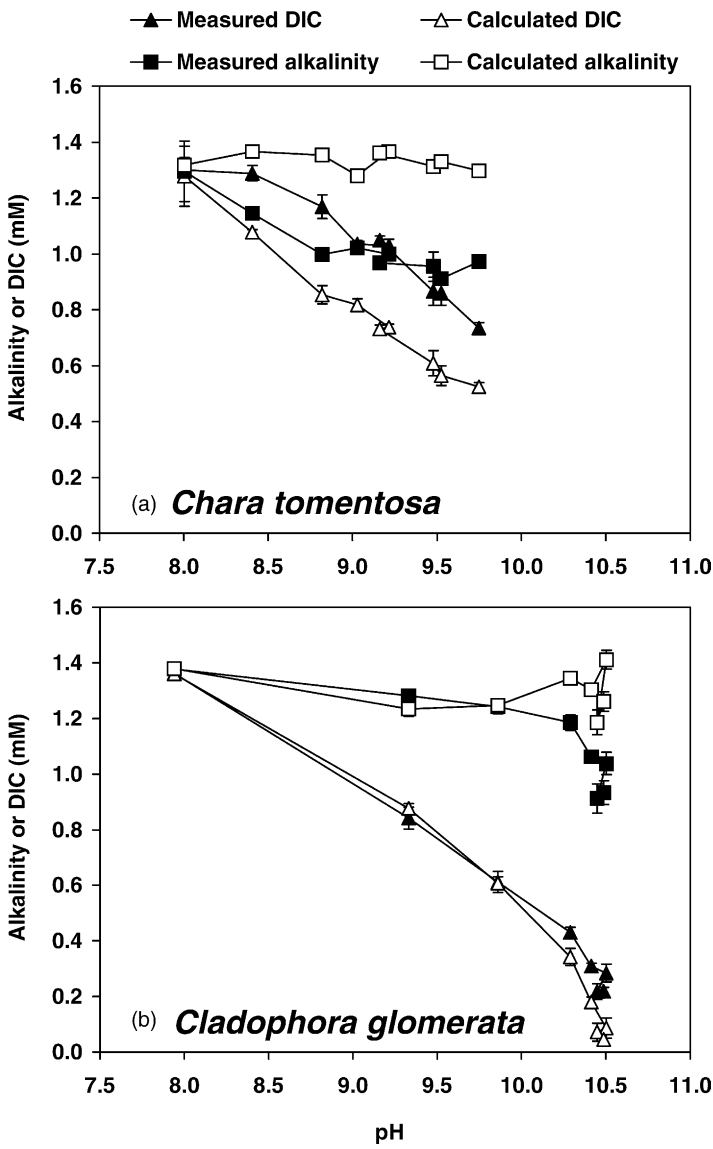

Fig. 2. Comparisons of measured and calculated alkalinity (calculated from [DIC], pH, salinity and temperature) and comparisons of measured and calculated [DIC] (calculated from alkalinity, pH, salinity and temperature) for (a) *Chara tomentosa* and (b) *Cladophora glomerata*. Error bars: 1 S.E. (not visible when very small).

measure [DIC]. Fig. 2b shows the result from the same experiment with the green alga *C. glomerata*. For this alga, measured and calculated values agreed below pH 10, while above pH 10 a similar deviation as in *C. tomentosa* was observed, most probably caused by chemical precipitation of $Mg(OH)_2$ at high pH (Turner, personal communication). Microscopic

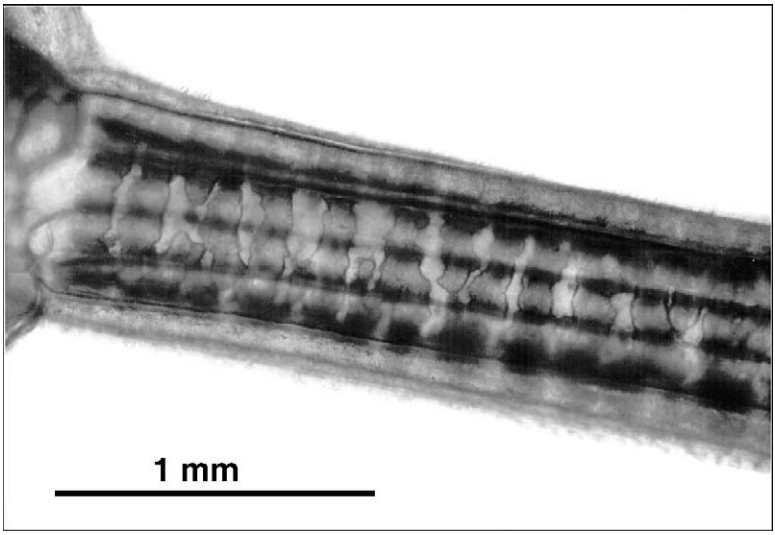

Fig. 3. LM micrograph of *Chara tomentosa* (main stem), showing $CaCO_3$ precipitation in bands perpendicular to the main stem and the bark cells.

observations showed that the cell walls of *C. tomentosa* from Skatviken did possess $CaCO_3$ incrustations (Fig. 3).

3.3. *pH-drift*

In pH-drift experiments a pH compensation point is reached where the uptake of DIC from the medium is equal to the release of DIC from the alga to the medium. In our pH-drift experiments a compensation point was approached after 10 h of pH-drift, but not yet reached. Some tests were made with longer incubation periods and it appeared that the pH can still increase after 24 h of pH-drift of *C. tomentosa*, albeit extremely slowly. This suggests a strong adaptation to carbon limitation in *C. tomentosa*. In the six control runs starting from pH 8.00 (Fig. 4) the pH varied between 9.56 ± 0.08, and 10.18 ± 0.04 after 10 h. This relatively large difference is probably caused by different algal biomass, e.g. 1.5 g for the TRIS experiment and 3.5 g for the AZDIDS experiment. The TRIS experiment was carried out with algae and water sampled in 2001, while the other experiments used material from 1998. In the REF experiment, the DIC uptake rates of *C. tomentosa* slightly increased on the second and third days relative to the first day (Fig. 4a). In the following pH-drift experiments using inhibitors (Fig. 4b–f), the results were corrected for these effects to obtain the net inhibitor effects.

AZ, VAN and TRIS reduced the DIC uptake rates throughout the experiments (Fig. 4b and d–f), but DIDS reduced DIC uptake rates only above pH ca. 8.8 (Fig. 4c). About 50% of the reductions of the DIC uptake rates by AZ and TRIS were caused by the buffering effects of these compounds, but in the case of VAN the buffering effect was of minor importance. In the postcontrol run of AZ the DIC uptake rates recovered to the levels of the control, but in the post-controls after the DIDS, AZDIDS, VAN and TRIS treatments they stayed low

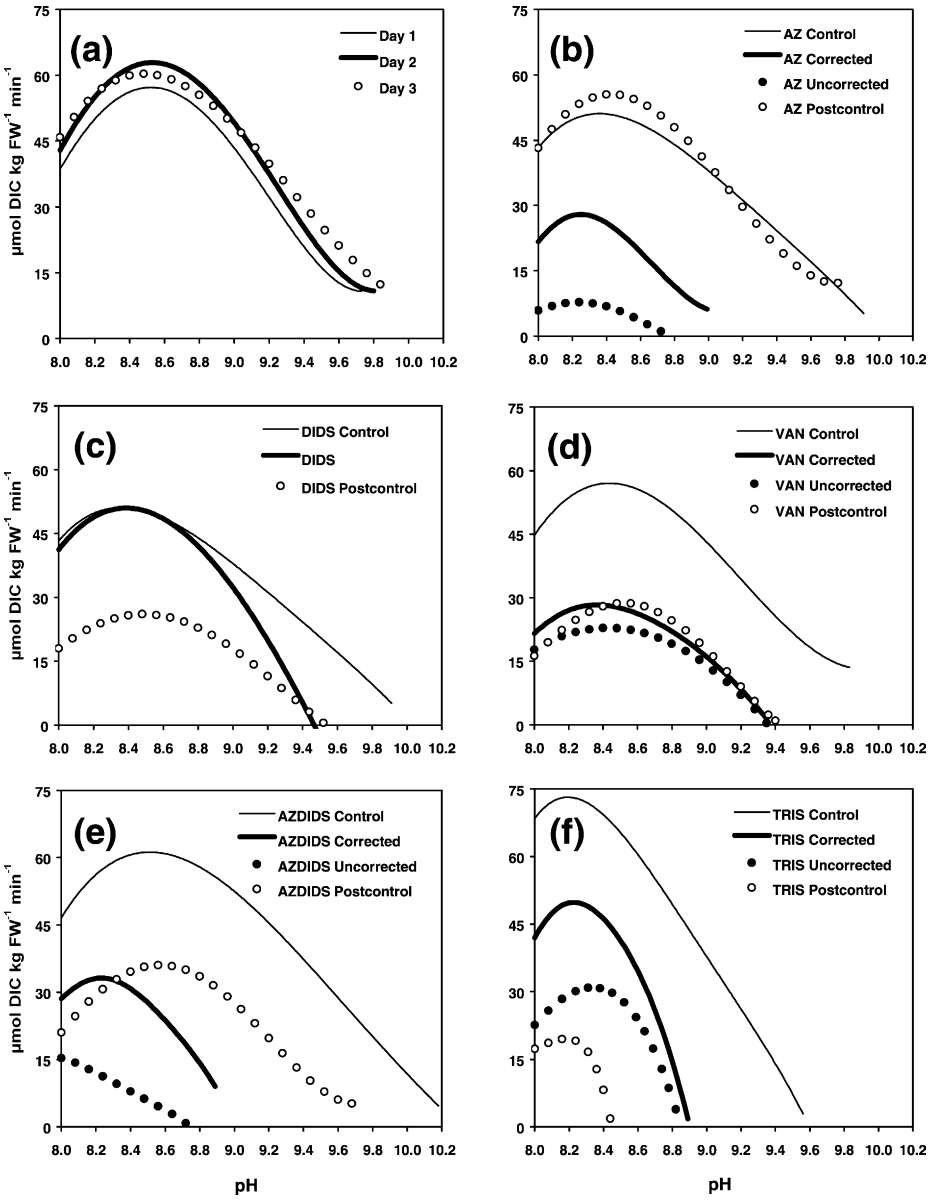

Fig. 4. DIC uptake rates at different pH in *Chara tomentosa* calculated from the pH-drift experiments. Each experiment was carried out during three measuring days: first day, control; second day, treatment; third day, postcontrol. Six treatments were applied: (a) without inhibitor; (b) 0.2 mM AZ; (c) 0.3 mM DIDS; (d) 0.2 mM VAN; (e) 0.2 mM AZ + 0.3 mM DIDS; (f) 0.2 mM TRIS. The coefficient of variation of the original pH measurements was <2% ($n = 3$).

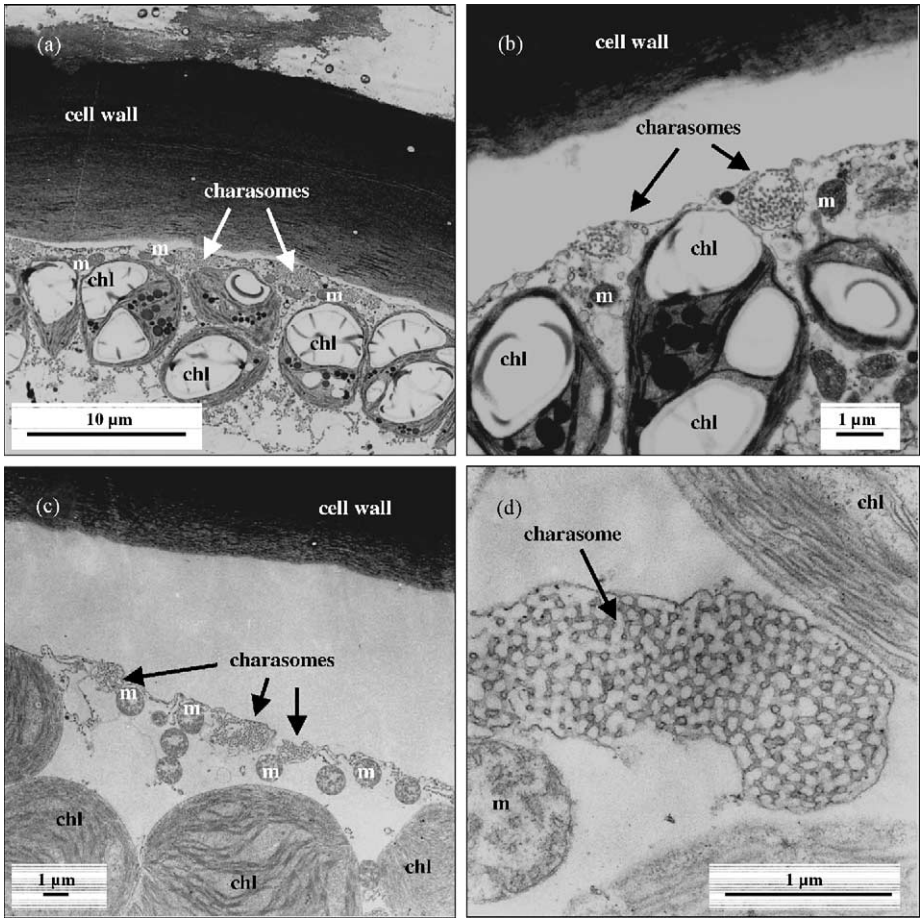

Fig. 5. TEM micrographs of *Chara tomentosa* from Skatviken (a, b) wild material collected 28 September 2000; (c, d) material collected in 1998 and aquarium-cultivated for two years. (a, c) Cell wall detached from cell membrane by the preparation procedure. Chl: chloroplast; m: mitochondrion.

(Fig. 4). This confirms that AZ did not penetrate the cell membrane and that CA activity was located in the periplasm.

3.4. Charasomes

Fig. 5 shows TEM micrographs of *C. tomentosa*. Three sequences of ca. 1 cm in length and 0.2 cm in diameter, each consisting of one central cell with bark cells around it, were investigated. Numerous charasomes occurred along the outer cell membranes of the bark cells along the whole sequence of each sliced sequence. In the direct vicinity of a charasome, one or more mitochondria were always present. No charasomes were observed in the membranes bordering other bark cells or the central cell.

4. Discussion

The present study on *C. tomentosa* and our previous study on *C. glomerata* (Choo et al., 2002) together show that algae taken from the same habitat can have very different strategies for carbon acquisition. The two species have in common that both are freshwater green-algal species that are dominant primary producers in the upper littoral of the northern part of the brackish Baltic Sea, *C. tomentosa* on soft bottoms and *C. glomerata* on rocky substrates. Both *C. tomentosa* and *C. glomerata* use a VAN-sensitive proton pump and a DIDS-sensitive anion exchange protein for DIC uptake. Fundamental differences are found in the activity of periplasmic CA; this enzyme is highly active in *C. tomentosa*, even in NSW with pH > 9.0, but only of marginal importance in *C. glomerata*. A large DIC uptake at high pH with periplasmic CA blocked by AZ suggests direct HCO_3^- transport over the cell membrane in *C. glomerata*, probably using a secondary membrane HCO_3^-/H^+ co-transport energised by a proton pump (Choo et al., 2002). The strategy of *C. tomentosa* is more likely that of H^+ extrusion with the help of a proton pump (creating low pH outside the cell membrane), in combination with CA, and membrane transport of CO_2. This seems unrealistic; especially at high medium pH the buffer system of the seawater would continuously counteract the action of CA. However, we found intricate networks of anastomosing tubules of the plasma membrane in *C. tomentosa* (charasomes), similar to those described for *C. corallina* (Franceschi and Lucas, 1980). Charasomes create a separation of the periplasmic space from the bulk seawater medium, which makes DIC utilisation less sensitive to high medium pH than a system that utilises only a planar periplasmic space (such as *C. glomerata*). This strategy of *Chara* species is analogous to that of some aquatic phanerogams. Elzenga et al. (1989) and van Ginkel and Prins (1998) found that the cells of the lower surface of *Elodea* and *Potamogeton* leaves contain H^+-ATPase pumps that act to acidify the wall. Wall ingrowths or specific transfer areas of the epidermis cells (Rascio et al., 1999) help retain the protons within the walls where low pH will induce the dehydration of HCO_3^- into CO_2. Inhibition of the H^+-ATPase pumps with VAN or erythrosin B led to a significant acidification of the cell sap of *Elodea*, which showed that H^+ extrusion was inhibited (Beffagna and Romani, 1988). This is also in agreement with the view of a regulatory role for the plasma membrane H^+ pump with regard to the intracellular pH.

In *Chara* cells, the presence of a plasma membrane-mediated HCO_3^- acquisition mechanism driven by an ATP-dependent H^+ extrusion has previously been reported from studies with perfused cells of *C. corallina* (Lucas, 1983). In this species, the electrogenic membrane potential across the plasma membrane in light and with excess supply of bicarbonate ions usually falls within the range −190 to −250 mV (Keifer et al., 1982). It is the inside of the plasma membrane that is negatively charged. Intracellular addition of VAN lowered the electrogenic membrane potential of *Nitellopsis* down to −100 mV (Shimmen and Tazawa, 1982). This was considered proof for the involvement of a proton pump in the creation of the electrogenic membrane potential, and this pump was identified as a P-type H^+-ATPase because it was inhibited by VAN. Mimura et al. (1993) found that *Chara* cells possess both CO_2 and HCO_3^- transporting systems that are supported by a VAN-sensitive H^+-ATPase at the plasma membrane. In intact cells, VAN has been shown to inhibit the proton pump in both microalgae (e.g. Thielmann et al., 1990), in red, green and brown macroalgae (Choo et al., 2002; Snoeijs et al., 2002; Klenell et al., in press) and in the phanerogam *Elodea*

(Beffagna and Romani, 1988). Different aspects of the use of VAN in DIC uptake studies were discussed by Snoeijs et al. (2002).

Price et al. (1985) and Chau et al. (1994) convincingly argued that the principal role of charasomes is that of HCO_3^- utilisation, but that a secondary role of Cl^- uptake is possible. A HCO_3^--utilising function of charasomes may explain why *Chara* tends to be found in more alkaline environments where other charophytes such as some *Nitella* species, which lack charasomes, are not found. Walker et al. (1980), Lucas et al. (1983) and Price et al. (1985) discussed possible models of DIC uptake in *C. corallina* in connection with the occurrence of charasomes: H^+/HCO_3^- co-transport, passive entry of $[H^+]$-generated H_2CO_3 and passive entry of CA- and $[H^+]$-generated CO_2. We have shown that *C. tomentosa* possesses external CA which catalyses the dehydration of HCO_3^- into CO_2 in periplasmic space. This is the first positive identification of a periplasmic CA in a *Chara* species. Price et al. (1985) reported that ethoxyzolamide (EZ), a CA inhibitor that penetrates cell membranes, inhibited photosynthesis in *C. corallina* at alkaline pH, but not at acidic pH. As EZ inhibits both periplasmic and intracellular CA, they could not localise CA and decide which form of DIC is transported over the cell membrane, HCO_3^-, H_2CO_3 or CO_2. We used AZ, which is known not to penetrate the cell membrane (Moroney et al., 1985). This is also supported by the complete recovery of DIC uptake in our postcontrol run after the AZ treatment. Our findings of simultaneous large periplasmic CA activity and P-type H^+-ATPase activity, and the location of ATP-generating mitochondria close to the charasomes in *C. tomentosa*, suggest proton-pump driven H^+ extrusion and membrane transport of CO_2 derived from HCO_3^- from the medium as the major form of DIC acquisition in this alga.

Aquatic plants and algae can extract H^+ from solution or manufacture H^+ using a process such as calcification ($Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + H^+$; McConnaughey, 1998). Therefore, many HCO_3^--using aquatic plants (e.g. *Potamogeton*), microalgae (e.g. coccolithophorids), macroalgae (e.g. *Chara*, deep-growing marine red algae), and photosynthetic symbioses (e.g. corals) are highly calcareous. We found discrepancies between calculated and measured [DIC] and alkalinity, for *C. tomentosa* at pH > 8, but for *C. glomerata* only at pH > 10. This is most probably caused by alga-mediated and chemical precipitation of $CaCO_3$ and $Mg(OH)_2$, respectively. Supersaturated and precipitated $CaCO_3$ in the medium through algal activity were probably included in our total carbon measurements and caused the discrepancy between the measured and the calculated values. This implies that the total carbon left in the medium in our experiments would reflect the carbon that was taken up by the algae or became incorporated in the cell wall of *C. tomentosa* as $CaCO_3$. We showed that alkalinity calculated from measured [DIC] and pH was constant in the DIC–alkalinity–pH experiments, and therefore it was possible for us to use the theoretical model to calculate [DIC] from pH at constant alkalinity in the pH-drift experiments. Our observations suggest that the decreasing alkalinity in the experiment with *C. tomentosa* is due to loss of HCO_3^- and CO_3^{2-} ions (to $CaCO_3$) from the medium mediated by the alga already from pH 8. Our microscopic observations verified that the cell walls of *C. tomentosa* (from Skatviken) indeed possess $CaCO_3$ incrustations. Partial isolation of the calcifying regions appears to be important in achieving localised $CaCO_3$ supersaturation. The diffusion of H^+ from the medium requires alkaline zones along plant surfaces that are spatially separated from the acidic zones where H^+ extrusion and DIC uptake take place. McConnaughey and Falk, 1991 confirmed (by ^{14}C labelling) that most of the carbon precipitating at the alkaline surface

of *Chara* was absorbed by the cell in its non-calcified acidified zones and that buffers competing with HCO_3^- for proton acceptance at the acidic surface inhibit calcification. The alkaline zones in *Chara* specialise in proton uptake, relying on calcification to generate protons, and the acidified zones specialise in proton secretion and bicarbonate utilisation (McConnaughey, 1998).

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