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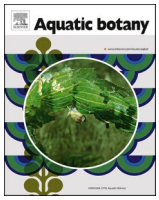

How Characean algae take up needed and excrete unwanted ions – An overview explaining how insights from electrophysiology are useful to understand the ecology of aquatic macrophytes

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ABSTRACT

Characean algae have been a model plant for electrophysiology for many decades, due to the large size of the internodal cells and their robust recovery from invasive manipulation. The information gained from them has provided a template for understanding the electrophysiology of many plant groups. The relative ability to take up or export ions, including nutrients and toxins, can be part of the explanation as to why certain macrophytes occur preferably in nutrient-rich or oligotrophic habitats, why some macrophytes can grow in brackish water or only in freshwater, or why growth is limited to a particular range of pH. The electrical characteristics of the macrophyte's cells play a determining factor in these transport properties, yet electrophysiological results are seldom cited in ecological publications, perhaps due to difficulties of communication between fields with different research approaches and terminology. We here present main electrophysiological findings on the transport of ions in and out of cells, in a way that is more accessible to ecologists. We examine the mechanism by which Characean algae generate the electrical voltage difference across their membrane, its effect on the transport of ions, and the mechanisms by which ions can be moved against the gradients that determine passive movements. Finally, we use the example of salinity tolerance to show what we learn about the evolution of salt tolerance in plants by using electrophysiological techniques.

1. Introduction

Characean algae have a complex morphology and relatively large cells. They are closely related to modern land plants (Nishiyama et al., 2018). While most Characean algae occur in freshwater, they can tolerate salinities from freshwater up to hypersaline conditions, although they are not known to occur in marine ecosystems (Schneider et al., 2015). They can build up large biomasses, and contribute to a number of ecosystem services, including removal of nutrients from water, storage of carbon and nutrients in biomass and sediments, possible phytoremediation of organic chemicals and heavy metals from water, as well as provision of habitat and food for a number of organisms (Schneider et al., 2015).

All biological organisms generate electrical potential differences across their cell membranes; this is called the membrane potential. Due to the large size of the internodal cells and their robust recovery from invasive manipulation, Characean algae have been a model plant for electrophysiology for many decades. Electrophysiology is the study of the electrical properties of bio-membranes and provides information on how charged ions and molecules move in and out of living cells. The electrical potential difference across the cell membrane has a profound effect on the transport of ions. For plants and algae, it is important in the transport of charged nutrients, such as PO_4^{3-}, NO_3^- or NH_4^+ into the cells, to prevent accumulation of "unwanted" ions such as toxic trace metal elements (e.g., As, Cd, Cr, Hg, Pb), and to keep the concentrations of some ions, such as Ca^{2+} and Na^+, low inside. An understanding of the mechanisms needed to take up "wanted" and exclude "unwanted" compounds can therefore provide insights into how algae evolved to live in particular habitats, and may help explain why certain species preferably occur in nutrient-rich or brackish waters, while others are restricted to nutrient-poor habitats, or water with a particular pH range or low salinity. In other words: electrophysiology of Characean cells may help explain the ecology of Characean algae.

Salinization, caused by factors such as irrigation, storm surges,

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application of de-icing salts, hydrologic alterations and climate change, is a widespread and increasing threat to inland and coastal wetlands (Herbert et al., 2015). Since Characean algae frequently grow in such habitats (e.g., Lirman et al., 2008; Rodrigo et al., 2013), it is important to understand how their growth is limited and what strategies they have evolved to cope with the stress of excess Na^+ and accompanying ions. Yet the electrophysiological results which are crucial to understanding this are seldom cited in ecological publications, perhaps due to difficulties of communication between fields with such different approaches. We here attempt to bridge this gap, by presenting the electrophysiological results in a way that is more accessible to ecologists, by putting them into an ecological context, and by describing some situations where electrophysiological research is useful for understanding charophyte ecology. We focus on Characean algae, since they have been well studied physiologically, but the general principles are applicable for a wider range of plants and algae.

In this paper, we will answer the following questions in the following chapters:

- Why is the transport of ions across plant cell membranes important, but energy-consuming?
- How do ions move across the membrane and how are they maintained out of equilibrium? That is, how are cations, which are drawn into the cell by the negative voltage inside, kept from accumulating too much? And how are anions, which are repelled by this negativity, brought into the cell? What is the proton pump and why is it important for moving ions across cell membranes?
- How do Characean algae take up macronutrients and carbon?
- What is necessary for tolerance to changes in salinity? Most Characean algae occur in freshwater, but there are several cases of salt tolerant species throughout the group. What are the mechanisms by which salt tolerance is achieved?
- How may these salt tolerance mechanisms have evolved?

We shall see how electrophysiological insights have guided answers to the questions, and how we can approach the inevitable additional questions that arise from our answers.

2. Why is the transport of ions across plant cell membranes important, but energy-consuming?

Living systems, both animal and plant, employ a huge range of electrochemical processes. Many nutrients exist as ions (e.g., K^+, Na^+, Ca^{2+}, $Fe^{2+\text{ or }3+}$, $Cu^{1+\text{ or }2+}$, PO_4^{3-}, NO_3^-, etc.). Toxic elements like Al^{3+} and other metals, such as lead and cadmium, also carry a charge. Charge must also be considered when we think about concentrations of H^+ and OH^-, which determine the pH level.

Separating charges across a resistor can result in an electrical voltage. Because membranes in living cells are primarily lipids, they are hydrophobic, and hydrophilic molecules, like ions, cannot easily cross. Therefore, the lipid part of membranes functions as a resistor (Coster and Smith, 1974). Proteins located within a membrane generally form a local hydrophilic region through which ions can more easily pass, although some resistance still needs to be overcome. While significant differences in ion concentrations can occur between the interior and exterior of the cell (e.g., Hope and Walker, 1975), the sum of positive charges is very nearly equal to the sum of negative charges on each side of the membrane. This is called "macroscopic electroneutrality," because the concentrations are measured in millimolar (mM) amounts. However, as different ions penetrate the membrane at different rates, very small imbalances, around 10^{-12} to 10^{-14} M, can occur. These small differences cannot be measured chemically against a background of 10^{-1} to 10^{-3} M, but they can generate a significant electrical potential. Any ion moving through the membrane will lead to a change in the membrane potential. Because of this, electrophysiological techniques are a very sensitive way of measuring ion movements.

In general, we use the term "membrane potential" when we mean the difference in voltage between the cytoplasm and the external solution (Klejchova et al., 2021). For Characeae, the "external solution" is the water in the habitat where the specimen grows. The membrane potential is always measured as a relative difference, inside voltage minus external voltage. Usually, the cytoplasm has a miniscule excess of negative charge that generates a more negative membrane potential than the external solution (e.g., the water of the stream or pond). Characean algae have a very negative membrane potential difference. It is frequently −200 mV or even more negative, whereas animal cells and marine algae typically have membrane potentials of about −50 to −60 mV (see for instance: https://en.wikipedia.org/wiki/Membrane_potential, or Gutknecht, 1970). In land plants, the membrane potentials range from −100 to −200 mV. The negative membrane potential in Characean cells is set up mainly by the "proton pump" (see below).

What is the significance of this electrical potential? We think of solutes diffusing down their concentration gradients, but for ions you must factor in the electrical driving force, which can have a much greater effect on the energy than the concentration. We use the term "electrochemical potential difference" to account for the two types of driving forces on ion movement. For instance, K^+ concentration inside the plant (more specifically, in the cytoplasm) is often 100 or more times the concentration in the external medium (100 mM or more in the cytoplasm, while external concentrations in freshwater generally are 1 mM or less). This >100:1 ratio of internal to external concentration would suggest that cells are constantly tending to lose K^+ (assuming a pathway exists for K^+ movement, which is generally the case). But a *Chara* cell has a large negative voltage, which pulls cations into the cell. The relationship between the force of concentration ratios and the force of electrical differences is given by the Nernst equation (see Appendix 1 and 2). It takes roughly −60 mV to counteract a 10:1 concentration gradient of cations. This means that if K^+ is present in a 10:1 ratio, its chemical tendency to diffuse out will just be balanced by a −60 mV membrane potential, and there will be no net driving force on K^+; we say it will be in equilibrium at −60 mV, with equal inward and outward driving forces. Therefore, the membrane potential, which generates this equilibrium state, is called the equilibrium potential. The relation is logarithmic (see Appendix 1), so that it will take about −120 mV to balance a 100:1 gradient and about −180 mV to balance a 1000:1 gradient. Since Characean algae have a membrane potential more negative than −200 mV, that means that, counter-intuitively, K^+ will move inward, not outward; even though there is a substantial outward concentration gradient, the inwardly directed electrical gradient is even bigger. This means that *Chara* is having to work constantly to keep K^+ from achieving higher concentrations inside the cell. Na^+, on the other hand, has a lower concentration in the cytoplasm than K^+, perhaps 10 mM, so that if the external concentration is 1 mM, as may typically occur in freshwater habitats, the total inward driving force will be even greater. In saline media, where the external concentration can be >100 mM, the problem is even worse. Now both the concentration gradient and the electrical potential difference are working to accumulate Na^+ inside the cell.

For anions, the argument is opposite, since they have a negative charge that will be repelled by the negative interior of the cell. This is a significant concern for the uptake of anionic nutrients, such as PO_4^{3-} or NO_3^-, and the more negative the anion is, the more difficult it is. These anions must therefore be taken up by active transport mechanisms, as we shall discuss in more detail below.

3. How do ions move across the membrane and how are they maintained out of equilibrium?

3.1. The importance of the proton pump for moving ions across the (plasma) membrane and maintaining their concentrations out of equilibrium

Ion transporters, often quite selective to a particular type of ion, move the charged particles across the cell membrane. Some transporters (ion channels) simply provide a pathway for passive transport across the non-conductive lipid membrane. Others require energy input to move ions against their electrochemical potential gradients. In plants, including Characean algae, the "proton pump" plays a central role for transport of ions across the cell membrane.

Proton pumping is mediated by an ATPase, i.e., an enzyme that uses energy in the form of ATP (adenosine triphosphate) to pump H^+ out of the cell, creating a proton concentration gradient (fewer protons inside) and a negative membrane potential (Shimmen and Tazawa, 1977, Fig. 1). Kitasato (1968) suggested that the proton pump generates most of the negative membrane potential difference (down to −250 mV). Beilby (1984) inferred a pump stoichiometry of $1H^+$/ATP, i.e., one ATP is needed to pump one H^+ out of the cell (one of the pump modeling equations is included in Appendix 1D). Since both electrical and concentration gradients affect the movement of ions across membranes (see above), the process of pumping protons out of the cell is affected by the pH of the external medium. At low pH, where the concentration of H^+ in the external medium is high, the proton pump is less effective in moving protons out. The maximum negative membrane potential occurs at an external pH of 7.5. Above that pH, the membrane potential again becomes less negative. The main reason for this is suggested to be the increase in OH^- transport (see Section 4.3), although we cannot discount the possibility of other transport systems contributing. A less negative membrane potential makes import of nutrients and export of wastes more difficult for the cell, because the membrane potential set up by the proton pump provides the energy for transporting other ions across the membrane (see Section 3.2.2 below). For this reason, the pH of the habitat in which Characean algae grow is of great importance for the uptake of needed ions into the cell, and the export of excess or dangerous ions. Most *Chara* species occur in calcareous habitats (Krause, 1997), suggesting a preference for habitats with pH above 7, while species of the related genus *Nitella* also occur in calcium-poor habitats. Only few species of Characean algae, mainly within the genus *Nitella*, are "acidophilic", e.g., *Nitella translucens*, a species for which a lower pH limit of 5.8 is reported (Walker and Sanders, 1991; Krause, 1997). Wood and Imahori (1965), in their worldwide monograph, report that Characeae occur in "mildly acid and alkaline conditions", and mention pH limits of about 5.2 and 9.8. Giving exact pH limits for Characean algae in their natural habitats is notoriously difficult, however, due to considerable daily variations in water pH, which are caused by intense photosynthesis and respiration (Christensen et al., 2013).

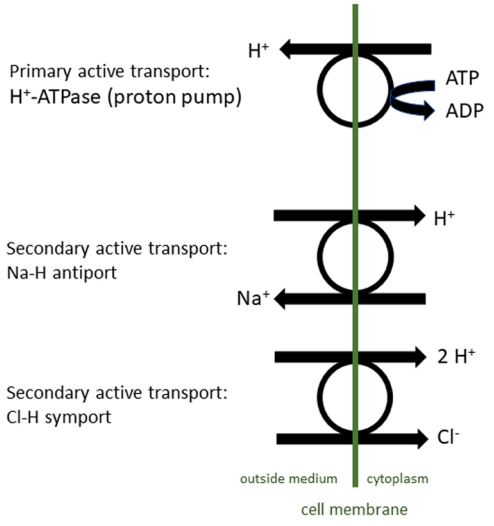

Fig. 1. Examples of cotransport systems energized by the H-ATPase ("proton pump"): Na/H antiporter and 2H/Cl symporter (for details see Beilby and Casanova, 2014).

3.2. How are other ions transported across the membrane?

3.2.1. Non-selective cation channels

These channels are coded for by a gene called HKT (high affinity K^+ transporters - Riedelsberger et al., 2021). The large negative membrane potential, generated by the proton pump, sets up a significant gradient for the uptake of cations. Therefore, active transport is generally not needed for the uptake of cations into the cell, even if they move against their concentration gradients. However, because the lipid membrane is a barrier for movement, there needs to be a protein that penetrates the membrane to permit cations to cross. One can think of a ball rolling downhill that encounters a wall. Even though there is sufficient energy in the gravitational gradient to bring the ball further downhill, it cannot proceed unless there is a hole in the wall through which it can move. For many cations, including Ca^{2+}, Mg^{2+}, and many micronutrients, one uptake pathway is the non-selective cation channel (Demidchik and Maathuis, 2007).

Electrochemical measurements can be used to quantify the flow of ions through these channels, and an important characteristic is the reversal potential, i.e. that potential at which there is zero net flow of ions through the channel, which is the potential at which the electrical and chemical forces are equal and opposite. This is also called the equilibrium potential. If we only look at one type of ion, for example K^+, the net movement of that ion would be zero when it is at equilibrium, that is, when the membrane potential is equal to the equilibrium potential for that ion. For instance, for K^+, the equilibrium potential is generally close to −170 mV (for cytoplasmic K^+ at 100 mM and room temperature of 20 °C), so when the membrane potential is −170 mV, there will be no net flow of K^+ through the channel. When the membrane potential is more negative than −170 mV there is a net inward current, meaning a net inward movement of K^+ through this channel. When the membrane potential is less negative there will be a net outward movement of K^+.

In reality, however, more than one type of cation passes through the non-selective cation channels, including, for example, K^+, Na^+, Ca^{2+}, and Mg^{2+}, each driven by its own electrochemical gradient. In this case, the reversal potential, i.e., the potential where there is zero net flow of ions across the membrane, will be somewhere between the most negative and the least negative equilibrium potential of these ions (see Appendix 1C for a quantitative treatment). Generally, the equilibrium potential for K^+ (around −170 mV, see above) is the most negative of the major ions. For instance, at a concentration of 0.1 mM K^+ in the outside medium, the reversal potential was found to be at ~−100 mV (Beilby, 1985). Since the membrane potential of Characean algae typically is more negative than −100 mV, the net effect of the non-selective cation channel will be to admit most cations into the cell. K^+, however, will only flow into the cell when the membrane potential is more negative than −170 mV. Zero net flow of ions, however, does not mean that nothing is happening. At the reversal potential, which in our above example was at −100 mV, ions are flowing in and out, depending on their equilibrium potentials, which in turn depend on the concentrations on each side of the membrane. This means that individual permeable ions are carrying current across the membrane, but the sum of these currents is equal to zero at −100 mV. At potentials more negative, the inward currents predominate, while at potentials more positive, the outward currents are dominant.

3.2.2. Co-transport systems

Because of the negative membrane potential, the uptake of anions generally needs active transport, and the same is true for the transport of cations out of the cell. We often think of active transport as being directly powered by ATP hydrolysis, as in the case of the proton pump. However, many of the active transport systems, which transport ions against concentration and/or electrical gradients, are co-transport systems. This means they are not directly powered by ATP hydrolysis, but depend on the proton gradient. Once the proton pump has set up an electrochemical gradient for protons, comprised of both the concentration difference and the electrical gradient, it can be used to transport other ions across the membrane. When the cell opens a pathway for H^+ from a region of high energy (high concentration or positive potential in the external medium) to a region of low energy (low concentration or negative potential inside the cell), H^+ will move from high to low energy spontaneously. As it loses energy, this energy can be captured and used to transport another substance actively against its gradient, just as a flowing river can be channeled through a water wheel that can be used to turn a mill stone. There are two types of co-transport, symport and antiport (Box 1, Fig. 1).

4. How do Characean algae take up macronutrients and carbon?

Plants, including Characean algae, need large amounts of carbon, nitrogen and phosphorus for growth. In fresh and saltwater ecosystems, phosphorus is generally negatively charged ($H_2PO_4^-$, HPO_4^{2-}), nitrogen can be positively charged (NH_4^+), negatively charged (NO_3^-), or electrically neutral (NH_3), while carbon can be negatively charged (HCO_3^-) or neutral (H_2CO_3/CO_2). As we have seen above, charge has a major effect on the transport of substances across the cell membrane.

In fungi and land plants, there is evidence that NO_3^- and $H_2PO_4^-$ are brought in by symports with more than one H^+, since they carry a net positive current (Blatt et al., 1997; Chen et al., 2008). This has not been measured directly in Characean algae, but the same may be true for them. More knowledge exists, however, on the uptake of ammonium and carbon. The uptake of these substances is affected by the pH of the external medium, i.e. of the habitat in which the algae grow, because the pH affects both the charge on the substrate and the energy gradient for protons.

4.1. Ammonia/ammonium

At a pH above 9.3, significant amounts of NH_3/NH_4^+ will be available as the neutral ammonia, NH_3. This molecule is sufficiently lipophilic that it can simply diffuse through the lipid portion of the membrane. Rapid metabolism to organic forms can keep the NH_3 concentration in the cytoplasm low, sufficient to drive continued uptake down the concentration gradient. However, below pH 9.3, more of the nitrogen is in the form of ammonium, NH_4^+. At pH 7.3, for instance, the ratio of NH_4^+ to NH_3 will be 100:1. The hydrophilic NH_4^+ cannot cross the lipid portion of the membrane, but there is a channel in the membrane that permits its entry. Although we do not know the molecular identity of this channel, there is evidence that it is expressed only in N-starved cells (Walker et al., 1979a,b). When the NH_4^+ channel is present, the negative membrane potential is sufficient to drive uptake of NH_4^+. Indeed, uptake of both NH_3 and NH_4^+ may be relevant for Characean algae, because photosynthesis in dense charophyte beds can cause daily variations in water pH between 7.5 and 9.5 (Christensen et al., 2013), i.e. the pH range where nitrogen uptake is likely to shift between NH_4^+ and NH_3. In addition, typically there are considerable differences in NH_3/NH_4^+ concentrations between the water surrounding Characean algae, and the sediment underneath them, with generally higher concentrations in the sediment (Schneider and Melzer, 2004). Characean algae are able to take up NH_4^+ from their belowground parts (Box, 1987; Vermeer et al., 2003). Consequently, pH in water and sediment, together with available concentrations of NH_3/NH_4^+ determine whether or not NH_4^+ channels are expressed.

4.2. Inorganic carbon

The form that inorganic carbon takes is also dependent on pH. In water, CO_2 rapidly equilibrates with the hydrated form, carbonic acid, H_2CO_3. Both CO_2 and H_2CO_3, are sufficiently hydrophobic that they can diffuse across the lipid part of the membrane. However, above pH 6.4, more of the carbon will be present as bicarbonate ion. The ratio of bicarbonate ion to carbonic acid will be 10:1 at pH 7.4 and 100:1 at pH 8.4. Therefore, in neutral to alkaline waters, there is an advantage for photosynthetic organisms that are able to take up bicarbonate, HCO_3^-. For uptake of bicarbonate, channels that mediate passive uptake will not

Box 1
Symport and antiport.

Symport ("carry with").

In symport systems, transport of the driving ion (for example H^+ going down its gradient) is in the same direction as that of the driven ion (for example Cl^- going against its gradient). In this case, both are entering the cytoplasm from the external medium. As a proton enters the cell, the transporter that mediates that entry captures the energy lost by the H^+ and uses it to bring Cl^- in along with it. A single H^+ does not carry enough energy to bring a Cl^- ion against its large gradient, so two H^+ enter the cell with every Cl^- (Fig. 1; Beilby and Walker, 1981). Thus, the net movement, 2 H^+ plus 1 Cl^- carries a net positive charge. To prevent the cytoplasm from becoming less negatively charged and too acid, the proton pump will have to actively pump the excess H^+ out of the cell again.

Symport can also be achieved with other ions than H^+. As mentioned above, both the membrane potential and the concentration gradient set up a large driving force for the inflow of Na^+ into the cell, and it has been shown that the energy of Na^+ inflow can be harnessed for co-transport of useful ions and nutrients into the cell (Smith and Walker, 1989; Walker and Sanders, 1991). However, most of the Na^+ that enters the cell must be removed again. This is generally achieved with an antiport.

Antiport ("carry against").

In antiport systems, movement of H^+ into the cell powers the export of another cation. The most important example of this is the export of Na^+ (Fig. 1). Because of the negative membrane potential, there is a large driving force for the movement of Na^+ into the cell, but the cytoplasmic concentration of Na^+ must be limited because many important enzymes become dysfunctional with Na^+ (Maathuis and Amtmann, 1999; Wakeel et al., 2011). Consequently, the cell needs to work continuously to keep Na^+ out of equilibrium. Plants, including Characean algae, typically utilize a Na/H antiport, which powers the export of Na^+ with the energy lost by a H^+ entering the cell. In most cases, it can be shown that a single H^+ does have enough energy to move a single Na^+. This is consistent with the findings that no current has ever been shown associated with this transport (Kiegle and Bisson, 1996; Hunte et al., 2005).

be of much help, because the negative membrane potential will tend to drive the negatively charged bicarbonate ion out of the cytoplasm. Therefore, it is often postulated that there must be an active transport mechanism, although to our knowledge no active transport mechanism has yet been unequivocally identified. Since the equilibrium between the uncharged carbonic acid and the negatively charged bicarbonate is pH dependent, however, an alternative way to take up carbon does exist: acidification of the external medium to force the equilibrium state towards CO_2 and H_2CO_3. The plant does not have to drive the whole environment acidic, it only needs to make the water very near the surface sufficiently acidic. There are several levels of morphology and anatomy that support the possibility of local lower pH values. The first is that Characean algae often grow in dense stands, and the entanglement of the plants due to the presence of branchlets means that there are large unstirred layers between the plants, which could serve to concentrate protons. Second, the cell wall itself can serve as an unstirred layer, buffered by uronic acids in the structural hemicelluloses and pectins (Brett and Waldron, 1996; Sorenson et al., 2012; Domozych et al., 2014; Herburger et al., 2019). However, measurement of pH in these regions seldom detected a pH much less than 7, let alone below 6.4 where more than half of the carbon would be present as carbonic acid. However, in many *Chara* species, the plasma membrane generates an elaborately invaginated structure called a charasome (or plasmalemmasome, since some other related genera may also have them). These structures are associated with light and photosynthesis (Chau et al., 1994; Foissner et al., 2015) and have been shown to be associated with ATPase activity (Price and Whitecross, 1983). Their structure provides an increase in surface area for transport, and their convoluted shape can provide a tortuous path to the bulk external surface, allowing the build-up of a low pH, although their small size has to date precluded the ability to measure this directly. As we have seen above, the proton pump becomes less effective at a pH in the external medium below 7.5, and it is important to uphold a negative membrane potential. For this reason, the existence of specialized areas in the plasma membrane where outside pH can be lowered while the remaining parts of the plasma membrane uphold a slightly alkaline medium, makes sense. Also, the presence of carbonic anhydrase, that catalyzes dehydration of HCO_3^- into CO_2, suggests most of the import is as CO_2 in the acid zones (Ray et al., 2003).

4.3. OH^-

Irrespective whether the external medium is acidified to create carbonic acid, or bicarbonate is taken up, either mechanism will cause the cytoplasm to become more alkaline (Beilby and Bisson, 2012). If proton pumping is required to acidify the external medium and generate CO_2, the proton pump will extract H^+ from H_2O and leave behind OH^-. If HCO_3^- is taken up directly, when CO_2 is extracted for photosynthetic fixation, OH^- will be left behind. To regulate cytoplasmic pH, the plant will need to either take up H^+ from the external medium or export OH^- from the cytoplasm. In order to be able to acidify the outside medium to force the equilibrium towards CO_2, but at the same time prevent alkalization of the cytoplasm, the transport of H^+ and OH^- out of the cell are spatially separated. This results in a characteristic banding pattern along the internodes of Characean algae, where acid and alkaline regions alternate (Fig. 2). When the algae are photosynthetically active, H^+ is transported out of the cell in the acidic parts, and OH^- transporters are activated in the alkaline parts of the internodes (Beilby and Bisson, 1992, 2012). These OH^- transporters are probably channels, as the currents can be quite large (Beilby, 2015). Technically, a similar effect would be reached by transporting OH^- out or H^+ in. It is most likely OH^- out, however, because at the maximal activation of the transporter (extracellular pH_o 10 – 11), there are too few H^+ in the outside medium to provide observed inward currents (see Beilby and Casanova, 2014, for an overview). The process of transporting OH^- out will result in alkalinization of the medium in which *Chara* is growing, which is often noticed in laboratory culture, where the external medium can be driven to pH 9 (personal observation). Likewise, it has been shown that photosynthesis in ecosystems with dense *Chara* vegetation can drive water pH from 7.5 to 9.5 (Christensen et al., 2013).

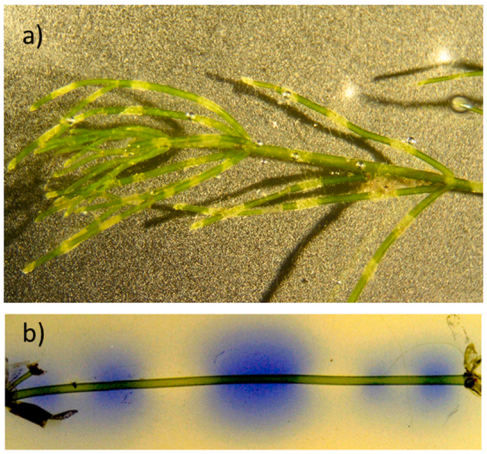

Fig. 2. pH banding (a) Calcification in the alkaline bands of *Nitella flexilis;* (b) An isolated internode of *C. australis* is placed in a medium containing bromthymol blue pH indicator, which turns blue above pH 7.6 and yellow below pH 6.0. The internode diameter of both species is approximately 1 mm. Picture (a) is a courtesy of Sven Dahlke, picture (b) comes from Beilby and Casanova (2014) with publisher permission.

5. Salt tolerance in Characean algae: a case study

In the following, we use the example of salt tolerance to show what we learn about the evolution of plants to tolerate stressful conditions by using an electrophysiological approach. Since Characean algae are primarily freshwater algae, it is natural to think of high salt as being more stressful. However, we should keep in mind that for algae that are adapted to high salt conditions, a decrease in salinity can also be stressful. There are several stresses involved in varying salt conditions. There are chemical changes, often thought of as increases in Na^+, but other cations and anions also increase. This change in salt concentrations will also have osmotic consequences, resulting in an increase or decrease in turgor. Turgor regulation requires the movement of many ions in order to change the ion concentrations by significant amounts, while the membrane potential is already affected by the movement of relatively few ions. We also need to keep in mind that, due to the negative membrane potential, cations may flow passively into the cell, while anions generally flow out rather than in. Regulating both turgor and membrane potential, therefore, is a significant challenge.

Successful salt tolerance requires the maintenance of low Na^+ and high K^+, since many of the enzymatic processes in the cytoplasm require K^+ and are inhibited by Na^+ (Maathuis and Amtmann, 1999; Wakeel et al., 2011). All cells must work to maintain low cytoplasmic Na^+, even in fresh water, because of the positive charge on the ion and negative membrane potential, as mentioned above. The main mechanisms for this in Characean algae in particular and plants in general are shown in Fig. 3. One option for reducing Na^+ entry is to decrease the permeability to Na^+. This has been shown for example for *C. australis* (Hoffmann et al., 1989). However, decreasing the permeability for Na^+ can slow the entry, but Na^+ will still enter, and active transport processes are needed to remove it from the cytoplasm. Although active sequestration of Na^+ in the vacuole has been shown in angiosperms (Fukuda et al., 2004; Epimashko et al., 2006), this process is not important in Na^+ regulation in Characean algae (Whittington and Bisson, 1994). Active export across the plasma membrane in plants, including Characean algae, generally is achieved by an antiport (Fig. 3; Horie and Schroeder, 2004). Export by a Na-ATPase, a transport enzyme that is directly powered by ATP rather than indirectly via the H^+-gradient, has never been shown in

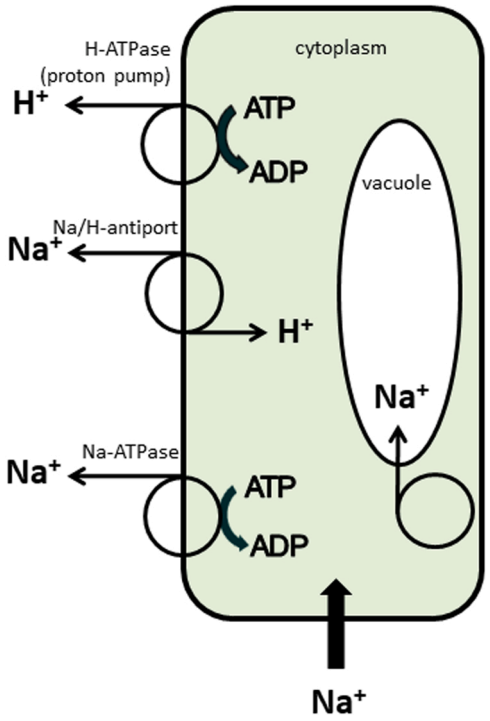

Fig. 3. Na^+ transport at single cell level. Na^+ moves passively into the cytoplasm through non-selective cation channels in both salt sensitive and salt tolerant charophyte species, and could theoretically be stored in the vacuole, as observed in some angiosperms. All Characeae contain the H-ATPase ("proton pump"), which powers the Na/H antiporter. At least one salt tolerant species, *C. longifolia,* also contains a putative Na-ATPase, which could export Na^+ directly.

angiosperms. However, it has been reported in bryophytes (Benito and Rodriguez-Navarro, 2003), the alga *Tetraselmis* (Balnokin et al., 1997; Matalin et al., 2021) and in the salt tolerant *Chara longifolia* (Phipps et al., 2021a). Not all Characean algae, however, possess a Na-ATPase, since it has not been found in the more salt sensitive *C. australis* and *C. braunii* (Nishiyama et al., 2018; Phipps et al., 2021a,b).

To explain the processes involved in salt tolerance, we will examine comparative studies on two species of *Chara*, the freshwater species *C. australis* R. Br. (synonym *C. corallina* Klein ex Willd.) and the salt tolerant *C. longifolia* Rob. (synonym *C. hornemannii* f. *longifolia* (Rob.) R. D.W.), as well as some *Lamprothamnium* species, where all known species are tolerant of salinity (Casanova, 2013). *Chara longifolia* is sometimes regarded as freshwater species but there are several records from brackish water, and Beilby and Casanova (2014: page 26) describe *C. longifolia* as brackish water species. Comparative studies on salt tolerance have also been done on other Characean species (e.g., Winter and Kirst, 1991).

5.1. Differences in salt tolerance among the studied species

Although *C. australis* is found naturally only in freshwater, in the laboratory it may tolerate some salinity (Tufariello et al., 1988). However, *C. australis* does not regulate osmotically (Bisson and Bartholomew, 1984), and the loss of turgor that follows increased salinity decreases the growth rate. In contrast, *C. longifolia* can be maintained in culture in freshwater, in artificial seawater with high NaCl, or in a solution mimicking Waldsea Lake, from which the species was collected, which has high concentrations of Na^+, Mg^{2+}, Cl^-, and SO_4^{2-} (Hoffmann and Bisson, 1986). The *C. longifolia* culture we worked with grows most rapidly and densely in freshwater cultures, even though it was collected originally in a saltwater lake. This apparent tolerance to different salinities may explain why some authors regard *C. longifolia* as freshwater species, while others describe it as brackish (see above). However, after about 5 years, freshwater cultures will die off. When grown in fresh water, its osmotic pressure is similar to that of *C. australis,* but its vacuolar contents have a much lower Na^+ concentration (2 mM vs. 50 mM; Hoffmann and Bisson, 1986). This suggests that *C. longifolia* has evolved a more powerful way to export Na^+ out of the cell, than *C. australis* has.

The studied *Lamprothamnium* species (mainly *L. beilbyae*) were collected from coastal lake systems around Sydney. The lakes are separated from the ocean by sand bars, which can be washed away by storms and high tides, and salinity fluctuates from less than ½ seawater to full seawater. *Lamprothamnium* grows well in aquaria in a range of salinities, although freshwater plants seem thinner and smaller than those in higher salinities. The media of 1/6 and 1/3 seawater support the most vigorous plants. The vacuolar concentrations of K^+ and Cl^- increase with salinity to keep the turgor pressure steady, while Na^+ concentration remains almost constant (Bisson and Kirst, 1980).

5.2. Electrophysiological responses to changes in salinity

Many Characean species, for example *L. papulosum,* occur in shallow ponds, including saltwater habitats, e.g., coastal wetlands (Krause, 1997). Evaporation during dry and warm summer months will drive salinity in these habitats up, and so will storms that bring seawater into a coastal pond. Conversely, when rain falls on such a small water body, its salt concentration will be rapidly diluted. Consequently, after a heavy rainfall, Characean algae that grow in such habitats need to rapidly decrease their internal ionic concentrations in order to prevent large concentration differences between the internal and external medium. The immediate danger of a much higher internal ion concentration than in the outside medium is that the difference in osmotic pressure may pull water into the cell, resulting in a pressure high enough to burst the cell. Therefore, for salt-adapted cells, lowering the salinity is more stressful than increasing the salinity, since increased turgor could result in cell wall rupture, whereas decreased turgor is generally less damaging. Rapid response is therefore more important to a decrease in salinity than an increase.

Many Characean algae adjust to a rapidly decreasing ion concentration in the outside medium by using high conductance K^+ channels. These are channels through which K^+ rapidly flows out, such that the cell loses K^+ (Beilby and Shepherd, 1996). However, as long as the membrane potential remains more negative than the equilibrium potential for K^+, the net driving force on K^+ will be inward, and simply opening the channels will bring K^+ into the cells instead of out. For this reason, Characean algae first will depolarize the membrane potential to a value less negative than that which brings K^+ in. This was measured, for example, in *C. longifolia*, where the response to a decrease in salinity from 375 to 225 mOsmol kg^{-1} triggered a prompt decline in the membrane potential to a value that is not negative enough to counter the tendency of K^+ to diffuse out (Hoffmann and Bisson, 1990). Second, the K^+ channels will only open when the membrane potential has reached this less negative value, assuring that K^+ will only be allowed to flow out. This was shown in *L. beilbyae* cells, which also first depolarized upon exposure to decreased salinity, followed by opening up high conductance K^+ channels (Beilby and Shepherd, 1996).

However, opening only a cation channel is insufficient to reduce turgor significantly. This is because even a small number of cations leaving the cell will make the membrane potential more negative (see Section 2), and a more negative membrane potential will in turn, once having reached the point where it is sufficiently negative to just balance the concentration gradient, prevent further K^+ from moving out. To restore the depolarized state, another cation could be allowed to move in. However, this would again increase the osmotic pressure, and nothing would have been achieved. Allowing an anion to move out, however, will both reduce the osmotic pressure and depolarize the membrane, thus allowing more K^+ to move out. In *L. beilbyae,* the

decrease in membrane potential is achieved by rapid opening of Ca^{2+}-activated Cl^- channels (Beilby and Shepherd, 1996). The K^+ channels open after the membrane potential allows K^+ outflow. Cl^- and K^+ effluxes continue until the turgor is regulated (usually within few hours).

When the salinity of the outside medium increases, the non-selective cation channels become more conductive, and the influx of cations will restore turgor, but also increase Na^+ concentration in the cytoplasm. Salt-sensitive algae cannot remove the Na^+ in sufficient amounts, because the proton pump is inhibited and therefore the Na^+/H^+ antiporter fails (Whittington and Bisson, 1994). The salt-tolerant *C. longifolia* (Hoffmann and Bisson, 1987, 1990) and *L. beilbyae* (Beilby and Shepherd, 2001) have a less negative membrane potential with increasing salinity, due to the opening of the non-selective cation channels that allow cations to enter. As with the salt sensitive algae, this will help maintain turgor, at the expense of increased Na^+. Anions must also be pumped in to maintain turgor, and this is achieved by a symport. However, the energy in the proton gradient will be decreased due to the decreased membrane potential, making the symport and antiport of ions less effective. This, together with the energy demands of Na^+ export, may be a reason why even salt tolerant Characean algae often grow more slowly in saltwater (as observed in *C. longifolia* in laboratory cultures).

5.3. Preventing toxic accumulation of sodium

Under all conditions, algae must work to prevent the intracellular accumulation of sodium to toxic levels, and the effort is greater at higher salinities. In *L. beilbyae* an increase in pump activity was observed at higher salinities (Beilby and Shepherd, 2001). If this activity is due to the H-ATPase, it would increase the energy available to export Na^+ by an Na-H antiport. However, electrophysiological results cannot distinguish between pumping due to the H- and Na-ATPase, as in both cases singly charged positive ions are pumped out. For this reason, the increased pump activity might be directly related to Na^+ export. Molecular analyses are needed for this distinction. Such analyses were performed in *C. longifolia*.

As mentioned above, when grown in fresh water, the vacuolar contents of *C. longifolia* have a much lower Na^+ concentration than those of *C. australis*, indicating that *C. longifolia* has evolved a more powerful way to reduce Na^+ in the cell than *C. australis*. This was shown to be due to differences in Na^+ export, rather than different restriction on entry or sequestration in the vacuole (Hoffmann et al., 1989; Whittington and Bisson, 1994). In freshwater cultures at pH 7, Na^+ efflux in *Chara australis* was about 9 nmol m^{-2} s^{-1}, while efflux from *C. longifolia* was 25 nmol m^{-2} s^{-1}, and *C. longifolia* from salt culture was 265 nmol m^{-2} s^{-1} (Whittington and Bisson, 1994; Kiegle and Bisson, 1996). There are three lines of evidence that suggest that a Na-ATPase is responsible for the Na^+ export in *C. longifolia*, rather than a Na/H-antiport:

1) Na^+ export still was observed at pH 9; at such a high pH, the energy in the proton gradient would not be sufficient to power a 1:1 Na-H-antiport (Kiegle and Bisson, 1996).
2) A gene showing strong sequence similarities to the Na-ATPase of moss (ENA) was found in *C. longifolia* but not in *C. australis* (Phipps et al., 2021a).
3) The expression of this gene greatly increased in response to an increase in salt (Phipps et al., 2021b).

Taken together, these findings suggest that a Na-ATPase plays a critical role in *C. longifolia*'s ability to maintain lower cytoplasmic Na^+ concentrations and thus to survive in higher salinity environments.

6. How may these salt tolerance mechanisms have evolved?

How did the gene coding for the Na-ATPase evolve? Is it an ancestral gene that is repressed or lost in salt-sensitive algae, but expressed in salt tolerant? Is the gene present in all salt tolerant species, or have some salt tolerant species evolved different mechanisms? The only whole genome sequences we have for Characean species is for the mildly salt tolerant *C. braunii,* and this gene is not present in its genome (Nishiyama et al., 2018; Phipps et al., 2021a).

Some phylogenetic trees suggest that the trait of salt tolerance is polyphyletic (i.e. salt tolerant species occur in several clades; Phipps et al., 2021b), but more work needs to be done on evolutionary relations within the group to verify this. A factor in the evolution of salt tolerance might lie in whole genome duplications (WGD), which have occurred frequently in *Chara* species (Casanova, 2015). WGD are powerful facilitators of evolution (De Bodt et al., 2005), as duplicate genes can assume new functions. WGD are less common in salt sensitive species (4 out of 20 species studied, or 20 %), occur in 4 out of 6 moderately salt tolerant (67 %) and 7 out of 7 (100 %) highly salt tolerant species (Phipps et al., 2021b). Since the salt tolerant *C. longifolia* has 28 chromosomes, while the salt-sensitive *C. australis* has 14, you could expect that *C. longifolia* also has twice as many forms for each gene as *C. australis.* This is, however, not true of ATPases. The H-ATPase (i.e., the proton pump) and Na-ATPase both belong to a group of ATPases known as P-type ATPases, along with calcium-transporting ATPases, and many others (Axelsen and Palmgren, 1998). Although we predict in general that *C. longifolia* should have twice as many copies of each gene, it has fewer genes for Ca- and H-ATPases than *C. australis*, but two expressed genes for Na-ATPases, while *C. australis* has none (Table 1). This suggests that the genes for Na-ATPase could have evolved from either of the other two P-type ATPases, although they are more similar to Ca-ATPase (Phipps et al., 2021b).

Why may the evolution of a gene coding for salt tolerance in Characean algae be important for ecology? Climate change has been predicted to increase the number of extreme weather events, including heavy rainfall as well as hot and dry periods (Coumou and Rahmstorf, 2012). This will, among many other effects, lead to more and more rapid changes of salinity in habitats of Characean algae. Polyploid species, with a reservoir of duplicate genes that can provide raw fodder for evolution of new functions, may be well placed to adapt to these and other environmental perturbations, and be more likely to survive.

Much more research also needs to be done in this system. For instance, the regulation of turgor is as important as Na^+ efflux. We know what ion fluxes occur but have not identified molecular candidates for the transporters carrying the ions. We do not know, for either Na^+ export or turgor regulation, how the plants measure the changes in turgor or ion concentration that initiate the response. Channels that sense membrane tension have been suggested as initiators for osmotic regulation in land plants (Falke et al., 1988; Cosgrove et al., 1991), although the molecular identity of these channels is not clear. In Characean algae, no such channels have been unambiguously identified, i.e., by using single channel analysis. No clear line of signal transduction has been shown, although it is clear from a number of studies that Ca^{2+} influx is likely to be involved, as it is in so many other signaling pathways (Stento et al., 2000; Kaneko et al., 2009), but the identity of the Ca^{2+} transport systems involved is not clear. Phipps et al. (2021b) identified signal transduction elements that are induced by salt stress prior to the induction of relevant transport systems, and therefore are likely candidates to be involved in the process.

Table 1
Number of P-type cation ATPases present in the transcriptome of *C. longifolia* and *C. australis* (Phipps et al., 2021a).

Number of genes expressed	*C. longifolia* (28 chromosomes)	*C. australis* (14 chromosomes)
Ca-ATPase	2	4
H-ATPase	1	2
Na-ATPase	2	0

7. Concluding remarks

Finally, why are these physiological findings important for ecology? We have explained why it is complicated to take up nutrients and carbon from an aquatic medium, why even freshwater algae need to continuously work to export Na^+, why changing salinity can be dangerous and difficult to deal with, and how pH of the surrounding water interacts with the transport of ions across the cell membrane. One important thing to keep in mind is the inter-relatedness of proton transport and the transport of other ions, either through H-cotransport systems or through effects of pH on the membrane potential. At higher external pH, as often occurs in charophyte-rich saline habitats such as Waldsea Lake (Hammer, 1978), charophyte-rich habitats that are prone to salinity changes such as the Albufera de Valencia lagoon (Calero et al., 2015), or other shallow charophyte-rich ponds (Christensen et al., 2013), the membrane potential is less negative (Beilby, 1984; Beilby and Bisson, 1992; Shepherd et al., 2002). This means that the driving force for H^+ uptake is less. This, in turn, means that the H-Na antiport system for Na export is less capable of preventing Na^+ accumulation. The evolution of a Na^+-ATPase can enable the alga to have a high-efficiency Na^+ export that is not sensitive to variations in the external pH, or other factors that might cause the strength of the electrochemical proton gradient to decrease. In other words: species that have developed a Na^+-ATPase can better cope with salinity changes, irrespective of changes in water pH.

CRediT authorship contribution statement

MJB: Conceptualization, Writing – original draft, Writing – review & editing, MAB: Conceptualization, Writing – original draft, Writing – review & editing, SCS: Conceptualization, Writing – original draft, Writing – review & editing.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aquabot.2022.103542.

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