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COMMENTARY

Active water transport in unicellular algae: where, why, and how

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Abstract

The occurrence of active water transport (net transport against a free energy gradient) in photosynthetic organisms has been debated for several decades. Here, active water transport is considered in terms of its roles, where it is found, and the mechanisms by which it could occur. First there is a brief consideration of the possibility of active water transport into plant xylem in the generation of root pressure and the refilling of embolized xylem elements, and from an unsaturated atmosphere into terrestrial organisms living in habitats with limited availability of liquid water. There is then a more detailed consideration of volume and osmotic regulation in wall-less freshwater unicells, and the possiblity of generation of buoyancy in marine phytoplankton such as large-celled diatoms. Calculations show that active water transport is a plausible mechanism to assist cells in upwards vertical movements, requires less energy than synthesis of low-density organic solutes, and potentially on a par with excluding certain ions from the vacuole.

Key words: Active water transport, aquaporins, contractile vacuoles, diatoms, water vapour uptake, xylem refilling.

Introduction

Water transport in biology is, in general, energetically downhill, from a site with a high water potential to a site with lower water potential, where water potential is as defined (Milburn 1979; Jones 2013) in equation 1.

$$\psi_w = \psi_p + \psi_\pi + \psi_g + \tau \quad (1)$$

where ψ_w=water potential, ψ_p=pressure potential, ψ_π=osmotic potential, ψ_g=gravitational potential, τ=matric potential, and all units are J m^{-3} (=N m^{-2}=Pa).

Osmosis involves movement of water from the side of the membrane where solutes are more dilute (high water potential from high osmotic potential) to the side of the membrane where solutes are less dilute (low water potential from low osmotic potential, noting that osmotic pressure and osmotic potential have opposite algebraic signs) (Jones, 2013). Pressure-driven water transport occurs from a place where water is at high pressure (high water, as pressure, potential) to a place where it is at low pressure (low water, as pressure, potential) (Jones, 2013). Gravitational potential and matric potential are also important in some situations, but pressure and water potentials are the main determinants of the phenomena discussed here. A parallel case is transpiratory water loss where water vapour transport occurs from a gas phase (intercellular gas spaces) where the water vapour concentration is high to a gas phase (the bulk atmosphere beyond the diffusion boundary layer) where the water vapour concentration is low (Jones, 2013). These downhill movements of liquid and gaseous water are deemed adequate by most commentators to account for the observed phenomena of water transport in the soil–plant–atmosphere system (Milburn, 1979; Jones, 2013). Downhill water transport

also accounts for many processes that are not a core component of the transpiratory water flux from the soil to the atmosphere, for example cell expansion driven by an osmotic potential difference (Milburn, 1979; Jones 2013), and nastic movements (Milburn, 1979), including (re)setting of the bladder of the submerged carnivorous flowering plant *Utricularia* (Sydenham and Findlay, 1975), and the trap in the wetland carnivorous plant *Dionea* (Forterre *et al.*, 2005) by storing energy in the deformed trap (see review by Dumais and Forterre, 2012).

While there can be no dissent from the adequacy of energetically downhill movement of water vapour in the atmospheric component of the transpiratory pathway, there is some evidence suggesting that a purely downhill water flow is an incomplete explanation of root pressure phenomena, including its natural manifestation as guttation, and that active water transport—the energized net flux of water from a site of low water free energy to a site of higher water free energy—is involved (e.g. Pickard, 2003*a*, *b*). Suggestions that the cohesion–tension hypothesis is not the complete explanation of the mass flow of liquid water within the plant during transpiration and that active water transport is involved (Zimmerman *et al.*, 2004, 2007) have met with significant opposition (Angeles *et al.*, 2006; Jones, 2013). Some of these arguments are presented and discussed in the recent paper in this journal by Wegner (2014).

In this contribution, we begin by considering the arguments of Wegner (2014), and then progress to the involvement of active water transport in the uptake of water vapour from the atmosphere, in contractile vacuole function in wall-less freshwater unicells, and possibly in generating buoyancy in some marine phytoplankton.

Is active water transport involved in root pressure and in refilling embolized xylem elements?

Wegner (2014) indicates that there have been several reports of active water transport being involved in root pressure exudation, from Oertli (1966) to Pickard (2003*b*). He outlines that there have been attempts to 'explain' the apparent active water transport in terms of longitudinal (Anderson *et al.*, 1970) or radial (Taura *et al.*, 1988) localized phenomena, while still allowing essentially downhill (osmotic) water flow. However, attempts to establish the occurrence of these localized osmotic phenomena have not been successful to date (Wegner, 2014). At the moment, then, active water transport is plausible as a mechanism, under some circumstances, to cause root pressure and the associated phenomenon of guttation.

As to the mechanism, there is little support for primary active transport of water; that is, the provision of metabolic energy to a membrane-located transporter that moves water alone against an energy gradient (i.e. from the side of the membrane with low water free energy to the side with high water free energy). The mechanism is generally thought to involve secondary active water transport, in which water transport is coupled to the movement of some other solute or solutes across the membrane (fig. 1 of Wegner (2014); Zeuthen and Zeuthen, 2007; Zeuthen, 2010; Zeuthen and McAaulay, 2012). In certain metazoan epithelia, the members of the cation chloride co-transporter (CCC) superfamily move up to 500 molecules of water out of epithelial cells per ion pair moving out of epithelial cells in model a of fig. 6 of Zeuthen (2010; see Zeuthen and McAaulay, 2012; Wegner, 2014). Since there is no direct supply of metabolic energy to CCCs, in steady state the water flux is energized by primary active transport of ions, where one or other of the ion pairs is recycled into the epithelial cells with the other ion moving down the electrochemical potential difference generated by the primary active ion transport. Crucially, the return flux of ions into the cells from the side to which water is secreted must have a lower ratio of water moved per ion pair transported than is the case for the CCC. The lower the moles of water transported per mole of the ion pair moving during the return influx of ions, the more energetically efficient is the net water flux out of the cells. Equally crucially, a high energetic efficiency of active water transport requires that there is minimal downhill water flux back into the cells from the compartment to which active water transport occurs.

Minimizing the water conductivity of the lipid bilayer component of the plasmalemma is presumably subject to constraints on the lipid composition of the membrane, related to, for example, membrane fluidity. Minimizing downhill water entry catalysed by transmembrane proteins such as aquaporins requires either the absence of these proteins, or minimizing their catalytic capacity by post-translational regulation. A final crucial point is that the requirements for maintaining a high water flux with a high energetic efficiency for active water transport are not the same as those for osmotic water flux. Active water transport needs a minimal downhill water flux back into the cells from the compartment to which there is a net water flux, while osmotically driven water transport needs a significant downhill water flux from the cells into this extracellular compartment. These opposing requirements make it difficult to conceive of active water transport as a supplement to (i.e. operating in parallel with) osmotic water movement. This is also the case for the operation of diffusive carbon dioxide entry in parallel with a carbon dioxide-concentrating mechanism (Hepburn *et al.*, 2011; Raven *et al.*, 2012). While not expanded upon here, Wegner (2014, p. 385) considers the case of osmotic water flux and CCC-driven water flux operating in the same direction.

Wegner (2014) suggests a mechanism for CCC-based active water transport in root pressure (his Fig. 1). This is based on (i) the known proton:chloride secondary active transport of chloride ions; (ii) the passive movement of potassium through an inwardly rectifying potassium channel (i.e. one that passes positive charge more easily into the cell than out of the cell), from the external medium to the cytosol; (iii) the primary active proton efflux across the plasmalemma via the P-type proton ATPase; and (iv) the probable occurrence of members of the CCC superfamily in the plasma membrane of cells in vascular tissue. These components would yield a functional active water transport system, although, as Wegner (2014) mentions, more work is needed to test the proposed mechanism.

Active water transport from an unsaturated atmosphere into terrestrial multicellular organisms

What seems like an improbable phenomenon has been convincingly demonstrated for a number of terrestrial arthropods from the Crustacea (some terrestrial isopods) and Insecta (some representatives of several Orders), both insects and crustaeans belonging to the (super) Class Pancrustacea, and some mites and ticks from the Chelicerata (Ramsay, 1954; Machin, 1979; Wright and Machin, 1990) (Table 1). Although the anatomical location of active water influx varies, with involvement of one or other end of the gut in the insects and mites, and a ventral pleon in the isopods, the mechanism seems to be the same in all cases. An epithelium bathed by the haemocoel on the inside and by the atmosphere on the outside has ion transporters which cycle ions across the epithelium in what is overall a process energized by metabolism. Movement of the ions into the haemocoel is accompanied by secondary active transport of water from the low water potential of a surface solution in equilibrium with the unsaturated atmosphere into the higher water potential of the haemocoel (Ramsay, 1954; Machin, 1979; Wright and Machin, 1990). The return of the ions from the haemocoel to the outer surface involves no such coupling with a water flux (Ramsay, 1954; Machin, 1979; Wright and Machin, 1990). Many of the arthropods showing active water transport live in habitats with an ample supply of organic matter as an external energy source but have a very limited supply of liquid water; examples are dry exfoliated mammalian skin, dry wood, and stored cereals, including both grain and processed materials such as flour (Machin, 1979; Wright and Machini, 1990).

There is also a possible role for active water transport in terrestrial photosynthetic organisms, namely poikilohydric and desiccation-tolerant green algae, both free-living and lichenized. Photosynthesis and growth can occur at quite negative water potentials, for example measurable photosynthesis at a water potential as low as –29 MPa (Lange, 1969; Lange *et al.*, 2001; Pintado and Sancho, 2002) in species of the lichen *Ramalina* whose photobiont is the trebouxiophycean green alga *Trebouxia.* These values of water potential for the lichen can be compared with the good growth at –30 MPa of the halophilic chlorophycean green alga *Dunaliella* from the Great Salt Lake (Brock, 1975) and probably similar values for this alga in the Dead Sea (Oren *et al.*, 1995), and 10% of the maximum rate of photosynthesis at a relative humidity equivalent to –37 MPa for the aerophilic trebouxiophycean green alga *Apatococcus* (Bertsch, 1966; see also Raven, 1993). The possible role of active water transport in the growth of algae in very low water potential habitats (air of low relative humidity; hypersaline habitats) could enable growth at a lower external water potential than is tolerated internally in terms of its equivalent in the concentration of compatible and other solutes. Here active water influx could permit growth with a rather lower intracellular osmolarity than that which corresponds to the external water potential. Any active water transport here would, as with the arthropods, involve active water transport into the cytosol from the medium; the other cases in Table 1 involve transport out of the cytosol and into the endomembrane lumen.

Active water transport in effectively wall-less freshwater cells

The metabolism of freshwater organisms requires that the osmolarity of their intracellular compartments exceeds the very low value in their immediate environment (Kitching, 1956; Raven, 1982, 1995). For cells surrounded by a complete, turgor-resisting cell wall, the expansion of cell volume is countered by the turgor-resisting cell wall. In this case, a non-growing cell has

Table 1. *Summary of the occurrence (organisms and environments), function, and mechanism of active water transport*

Organisms	Environment	Function	Mechanism
Terrestrial and halotolerant algae[a]; some terrestrial arthropods[b]	Terrestrial habitats of low relative humidity (algae); hypersaline habitats (algae); dry habitats with ample energy supply (arthropods)	Potentially allow algae to grow with an intracellular osmolarity equivalent to a water potential higher than that in the medium; allows arthropods to grow in very high food energy density but no liquid water	Presumably energized ion cycling across a plasma membrane with the ions carrying more water in the direction of active water transport (i.e. into the cells) than on the return journey
Wall-less or effectively wall- less freshwater cells[b]	Habitats with significantly lower osmolarity than that of the cell	Maintain cell volume by exporting excess water that has entered osmotically	As above, but active water transport from cytosol into an endomembrane vesicle (the contractile vacuole) with subsequent expulsion of fluid from the contractile vacuole to the medium
Large marine phytoplankton cells, e.g. large diatoms[a]	Surface ocean; sufficient light for photosynthesis, high enough external density to permit cells to have a lower density than the medium without involvement of gas vesicles	Maintain vacuole at a density lower than that of seawater	As above, but active water transport from the cytosol into the central vacuole

[a] Hypothetical active water transport.
[b] Demonstrated active water transport.

an osmotic potential equal and opposite to the pressure (turgor) potential, so there is no water potential difference across the plasmalemma and no tendency for water to enter or leave. Cell growth in a walled cell with the possibility of cell wall plasticity [i.e. an organic wall rather than the silicified wall (frustule) of diatoms] involves osmotic water entry down an osmotic potential gradient driving the plastic expansion of the cell which, at the end of cell growth, sets the size of the cell at a given turgor pressure. Changes in intracellular osmolarity allow a change in cell volume dictated by the extent of the change in osmolality and the bulk elastic modulus of the cell wall (Jones, 2013).

The situation is different for a freshwater cell with no cell wall, or for a walled flagellate cell such as those of *Chlamydomonas* (Raven, 1982, 1995) (Table 1). For these walled flagellated cells, the requirement that the flagella execute the bending implicit in their role in motility means that the flagella cannot be surrounded by a turgor-resistant cell wall, even though such a wall could be very thin when surrounding a structure of very small radius such as a flagellum (Laplace's principal: see Raven, 1987). Here the osmotic entry of water would cause the cell to burst unless there was an efflux of water equal in magnitude to the osmotic water entry. In this case, the removal of water from cells is critical to cell survival, and, since the water efflux is from a lower (cell contents) to a higher (freshwater medium) water potential, it is by definition a case of active water transport (Kitching, 1956; Raven, 1982, 1995). This active water transport involves contractile vacuoles or some analogous structures (Kitching, 1956).

While there are clearly constraints on how low an intracellular osmolarity can be and yet be compatible with metabolism, Raven (1982, 1995) points out that the minimum energy requirement for active water efflux is, all else being equal, proportional to the osmolarity difference (inside minus outside) squared. This is because the water influx is, for a given hydraulic conductivity of the plasmalemma, linearly proportional to the osmotic potential difference across the membrane, while the minimum energy needed to transport a mole of water out of the cell is also a linear function of the osmotic potential difference. This energetic argument is consistent with the observed very low intracellular osmolarity of many wall-less (or functionally wall-less) freshwater cells (Raven, 1982, 1995). It is also consistent with the generally low hydraulic conductivity of the plasmalemma of (effectively) wall-less freshwater algal cells (Raven, 1982, 1995). A higher hydraulic conductivity has been proposed for the plasmalemma of the green freshwater flagellate *Mesostigma viride* (Streptophyta: Charophyceae) on the basis of high contractile vacuole activity, although without distinction of hydraulic conductivity and osmolarity difference in determining the rate of water entry (Buchman and Becker, 2009).

As to the mechanism of active water efflux in these wall-less freshwater cells (Table 1), much of the recent work has focused on the mechanism of fluid expulsion rather than how the low-osmolarity fluid is generated (e.g. Komsic-Buchmann *et al.*, 2012; Plattner, 2013, and references therein). Knowledge of the mechanism of fluid expulsion is insufficient to quantify the energy cost, and so the minimum energy cost of volume regulation in these effectively wall-less freshwater cells remains as calculated by Raven (1982, 1995).

Turning to the mechanism of active water transport, the initial assumption is that the contractile vacuole solution expelled to the medium is hypo-osmotic to the cytosol (House, 1974; Raven, 1982, 1995). One possibility for generation of a contractile vacuole solution hypo-osmotic (to the cytosol) is that there is secondary active transport of water with solutes (generally thought to be ions) into the growing contractile vacuole by movement through the same transporter rather than osmotically, with resorption of solutes into the cytosol prior to expulsion of the hypo-osmotic solution. The other possibility is active transport of solute (ions), with no direct coupling to water, into the growing contractile vacuole, with water following osmotically, followed by resorption of the solute prior to expulsion of the hypo-osmotic solution. Both of these possibilities are consistent with the sort of energetic analysis carried out by Raven (1982, 1995), and both can account (with appropriate regulatory properties) for the increase in volume of contractile vacuoles in the filling phase of the cycle.

Let us deal first with the transport of water into the growing contractile vacuole on the same transporter as the solute (Zeuthen and Zeuthen, 2007; Zeuthen, 2010; Zeuthen and McAaulay, 2012; Wegner, 2014). As mentioned above, the upper limit on the water transported per ion pair transported is 500 (Zeuthen and Zeuthen, 2007; Zeuthen, 2010; Zeuthen and McAaulay, 2012), which is close to the 'mechanistic' value of Raven (1982, 1995) derived by other means. This value is, as indicated by Raven (1982), an underestimate to the extent that no account is taken of recouping of solutes leaking into, and water out of the hypo-osmotic contractile vacuole. The more significant of these leaks is water moving back into the cytosol. The significance of this can be estimated from the area of the contractile vacuole as a fraction of the cell surface area. From the measurements of Luykx *et al.* (1997) on *Chlamydomonas reinhardtii*, the surface area of a 5 μm radius cell is 100 times the area of a single contractile vacuole of 0.5 μm radius at its maximum size. Since there are two contractile vacuoles per cell (Luykx *et al.*, 1997), this ratio is taken to apply to the mean surface area of the two contractile vacuoles through the contractile vacuole filling–expulsion cycle. Assuming the same hydraulic conductivity of the contractile vacuole membrane as for the plasmalemma, and an osmolarity in the contractile vacuole equal to that of the medium (as in Raven, 1982), leakage of water from the contractile vacuole only adds 1% to the 'mechanistic' cost of the contractile vacuole activity of Raven (1982). Failing a parallel secretion (co-transport with water) and resorption (without water) of solutes, resorption must occur prior to expulsion of the contents of the contractile vacuole to the medium, without significant withdrawal of water during the solute resorption phase. Since, *ex hypothesis*, the contractile vacuole lumen is hypo-osmotic to the cytosol throughout the filling and expulsion cycle, or at least in the solute resorption phase, water transport by specialized aquaporin transporters in the contractile vacuole membrane (Plattner, 2013) would only act to short-circuit this mechanism of removing water

from the cytosol whenever the contractile vacuole is hypo-osmotic to the cytosol. As indicated below, there is as yet no evidence of the occurrence of water–solute co-transporters in the contractile vacuole membrane. It must be emphasized that there is no evidence of CCC transporters in microalgae with contractile vacuoles. This mechanism is shown in Fig. 1.

An alternative method of producing a hypo-osmotic (to the cytosol) solution in the contractile vacuole is the influx of solutes into the contractile vacuole, independently of water, with osmotic water entry presumably via aquaporins. This would initially generate a hyperosmotic (to the cytosol) contractile vacuole fluid, with withdrawal of solutes (but not water) before expulsion of the then hypo-osmotic solution to the medium (e.g figs 3 and 4 of Docampo *et al.*, 2013). Achieving this would involve a switch from solute entry to solute removal, and a parallel very large decrease (Chaumont *et al.*, 2005) in aquaporin activity. This model for the production of a hypo-osmotic solution in the contractile vacuole appears consistent with some morphological studies on contractile vacuoles indicating a two-stage process (Allen and Naitoh, 2002). In both this model and the co-transporter model described above, the transport of solutes into and/or out of the contractile vacuole lumen must be energized, as it involves an uphill gradient, and either mechanism could underlie the analysis of energetic analysis of Raven (1982, 1995)

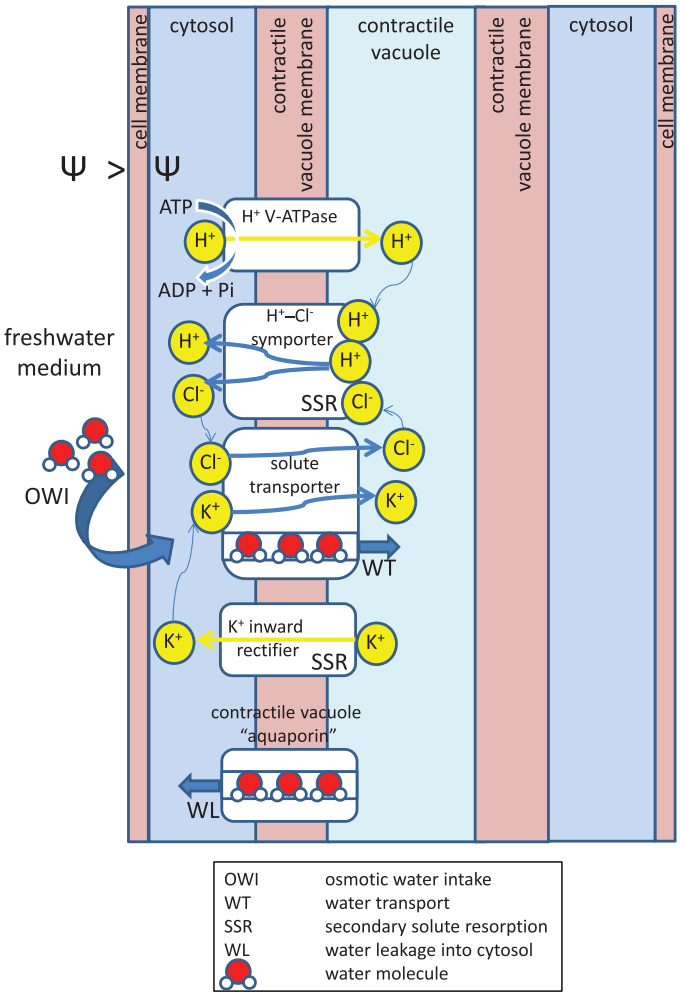

Fig. 1. Hypothesized mechanism of active water efflux from wall-less freshwater microalgal cells following osmotic water entry. The freshwater medium has a higher water potential than the cytosol, so water enters the cell osmotically down the water potential gradient (OWI = osmotic water intake) across the plasmalemma. Water molecules are transported (WT) into the contractile vacuole (or functional equivalent) in a process that couples water and ion transport via a co-transporter that translocates K+ and Cl– together with a fixed number of water molecules. The ions are at least partly recycled from the vacuole lumen into the cytosol through secondary solute resorption (SSR) involving a K+ inward-rectifying channel and a Cl––2H+ symporter, respectively, with very little water transported with the ions. These processes are energized by the activity of an H+ VATPase that generates and maintains the higher H+ electrochemical potential in the contractive vacuole than in the cytosol. The combined effects of the influx and efflux of the ions across the contractile vacuole membrane is active water transport into the contractile vacuole. Expulsion of the contents of the contractile vacuole to the medium completes active water efflux from the cell. Aquaporin-like transporters (proteins that enhance water conductance of membranes) are present in the contractile vacuole membrane, but would only act to short-circuit the mechanism shown here, since the water potential difference across the contractile vacuole membrane means that the spontaneous direction of water transport is from the contractile vacuole to the cytosol, leading to water efflux from the contractile vacuole to the cytosol. This is the same as occurs across the plasmalemma from the external medium to the cytosol, and results in the need for active water efflux. Aquaporins do have a role in other hypothesized mechanisms of active water efflux. For more details, see the text.

The biochemical energization of ion transport is, as is common in the endomembrane system, by proton transport by V-ATPase and/or proton pyrophosphatase (Fig. 1; Robinson *et al.*, 1998; Clarke *et al.*, 2002; Nishihara *et al.*, 2008; Komasic-Buchmann *et al.*, 2012; Docampo *et al.*, 2013; Plattner, 2013). The nature of the solute involved in secondary active water transport has been suggested to be bicarbonate on the basis of an absence of acidification of the contractile vacuole by the proton V-ATPase and/or the proton pyrophosphatase (Robinson *et al.*, 1998; Tominaga *et al.*, 1998). The absence of acidification suggested to these authors that bicarbonate has titrated the protons to yield carbon dioxide, some of which could leak out during the filling phase of the contractile vacuole cycle. Such a conversion would require the presence of carbonic anhydrase within the contractile vacuole lumen to obtain complete conversion of protons and bicarbonate to the equilibrium carbon dioxide concentration (Raven, 1984, 1997), given the half-time of the uncatalysed conversion within the contractile vacuole and the cycling time of the contractile vacuole. There is evidence of carbonic anhydrase in the contractile vacuole, with slowing of the water inflow–outflow cycle when the activity is inhibited (Marchesini *et al.*, 2002; fig. 3 of Docampo *et al.*, 2013). However, there are other reasons why the contractile vacuole lumen does not acidify despite the operation of the proton V-ATPase. The contractile vacuole lumen is ~80 mV positive relative to the cytosol (Grønlien *et al.*, 2002), so there is an inside positive electrochemical potential difference for protons across the contractile vacuole membrane so electrically driven ion fluxes and secondary active transport processes with protons as the driving ion could still occur across the membrane despite the absence of a pH difference.

Other suggestions as to the solutes driving secondary active water transport or osmotic water into the contractile vacuole lumen are phosphate (plus a cation such as ammonium) (Ruiz *et al.*, 2001; Montalvetti *et al.*, 2004; Rohloff *et al.*, 2004; fig. 3 of Docampo *et al.*, 2013) or potassium and chloride (Stock *et al.*, 2001, 2002), based on the contents of the contractile vacuole, or ammonium bicarbonate related to the occurrence of carbonic anhydrase in the contractile vacuole lumen (fig. 4 of Docampo *et al.*, 2013). Potassium and chloride as the solutes would agree with activity of the potassium variant of the CCC (Zeuthen and McAulay, 2012; Wegner, 2014). However, there seems to be no evidence so far of such a transporter in unicells using contractile vacuoles, although there is gene sequence evidence for occurrence of the transporters in the marine green prasinophycean flagellate *Micromonas* (Chan *et al.*, 2011) which is probably isosmolar with seawater (Raven, 1984) and lacks contractile vacuoles. The motile male gametes of mosses occur in low osmolarity environments, and presumably regulate their volume by active water transport (Raven, 1982, 1995). There is genome sequence evidence of several CCCs in the moss *Physcomitella patens* whose male gametes swim through fresh water and so must have a volume regulation mechanism.

The occurrence of significant concentrations of solutes in contractile vacuoles, in this case potassium chloride at a higher concentration than in the cytosol, has been demonstrated (Stock *et al.*, 2001, 2002). Depending on what stage in the vacuole filling process these measurements represent, this may also represent the composition of the secreted fluid. If this is the case, then as many (isosmolar solution) or more (hyperosmolar solution) moles of solute per mole of water are expelled to the medium than the moles of solute:moles of water ratio in the cytosol, and involves minimal resorption of solutes (without significant water) from the contractile vacuole fluid to the cytosol. Such a loss of solutes to the medium must, in the steady state, involve re-accumulation of the solutes (in this case potassium chloride) from the medium into the cytosol without co-transport with water, at a similar energetic cost to resorption from the contractile vacuole (Raven, 1982, 1984, 1995; Raven *et al.*, 2014).

The possibility of recouping solutes across the plasmalemma rather than from the contractile vacuole prior to expulsion of the (then hypo-osmolar) contents was rendered unlikely the results of Luykx *et al.* (1997) and Montalvetti *et al.* (2004) who showed that contractile vacuole function was not inhibited, but rather increased, as predicted from the need for increased water expulsion rate at lower external osmolarities, by incubation of cells in pure water. This causes extreme dilution of any solutes in the expelled fluid and hence an extreme quantitative problem with recouping the solutes. There are data relevant to the possibility of external recycling in Malhotra and Glass (1995) who investigated tracer potassium influx and potassium content in potassium-replete *C. reinhardtii.* From fig. 5 of Malhotra and Glass (1995) the tracer ($^{42}K^+$) influx is sufficient to double the potassium content in 2 h. Although Malhotra and Glass (1995) do not cite growth rates, these are unlikely (at 25 °C) to yield a doubling time of <6 h (Raven *et al.*, 2013), so the potassium influx is three times greater than is needed for one doubling. While this may seem a rather small value for the external recycling of ions assuming an external osmolarity of 2 osmol m^{-3} (Raven, 1982), the osmolarity of the growth medium (Polley and Doctor, 1985) used by Malhotra and Glass (1995) is, assuming an osmotic coefficient of 0.9 for ions, 72 osmol m^{-3}, which is slightly lower than the 85 osmol m^{-3} for the intracellular osmolarity of *Chlamydomonas* cited by Raven (1982). This suggests that no contractile vacuole activity occurs in the work of Malhotra and Glass (1995). More focused experiments are needed to determine if potassium (and chloride) external recycling can account for contractile vacuole activity.

In summary, while active water efflux in freshwater unicells clearly occurs through the contractile vacuole apparatus (or its functional equivalent in effectively wall-less freshwater unicells), the details of its mechanism are still unclear. Since the arguments above on the mechanism of active water transport by contractile vacuoles used data from a range of organisms, it is possible that more than one combination of (i) solute:water co-transport stoichiometry; (ii) osmotic water movement into contractile vacuoles involving aquaporins; and (iii) internal and external solute recycling to the cytosol can occur in different organisms.

Could active water transport be involved in buoyancy generation in marine phytoplankton?

From the above discussion it appears that energy-requiring water transport is critical to address the physiological needs of certain freshwater flagellates, whose functioning and survival would not be possible unless water was, respectively, being actively taken up by, or removed from, cells. However, active water transport may also be a mechanism for planktonic cells to adjust their buoyancy. In an environment where light and nutrients are often spatially separated, vertical adjustment relative to the water surface may be a competitive strategy to gain access to nutrients at depth (Doblin *et al.*, 2006; Villareal *et al.*, 2014).

Most phytoplankton organisms lack flagellar motility, or other means of locomotion depending on mechano-chemical transducers. Accordingly, their vertical motion is dictated, as shown quantitatively by Stokes' Law, by the density difference between the organism and the surrounding water, the temperature-dependent viscosity of the medium, and the size and shape of the organism (Denny, 1995; Raven and Waite, 2004; Walsby and Holland, 2006; Holland, 2010). Stokes' Law assumes spherical organisms, and deviations from this shape are dealt with empirically. Most non-motile phytoplankton sink relative to the surrounding water because they are denser than their immediate environment. The higher density comes about from the organism volume and density difference-weighted excess of dense components (e.g. high molecular mass organic compounds such as most proteins and polysaccharides, inorganic solids such as silica and calcite, and low molecular mass compounds yielding a dense solution) relative to less dense components (e.g. lipids, low molecular mass compounds yielding a low-density solution, and, in some cyanobacteria, gas vesicles): Gross and Zeuthen (1948); Walsby (1994); Villareal and Carpenter (2003); Woods and Villareal (2008); Raven and Knoll (2010). Organisms that are buoyant relative to the medium have a relative excess of the lower density components (Boyd and Gradmann, 2002; Woods and Villareal, 2008).

A fraction of phytoplankton organisms that are denser than the medium and cannot swim will be lost from the photic zone each day; this is a loss factor from the population although it may be advantageous under some conditions (Raven and Waite, 2004). For picophytoplankton (≤2.0 μm equivalent spherical diameter) the sinking rate is very small relative to the depth of the photic zone, and net population growth rates under natural conditions of resource supply and grazing are achievable with even small mixing depths. For larger cells, the potential for growth to exceed sinking becomes less likely as the specific growth rate generally declines with increasing cell size (generally a small effect for phytoplankton: Beardall *et al.*, 2009; Finkel *et al.*, 2010) and, more significantly, the sinking rate increases (Stokes' Law) for larger cells.

To counteract the tendency to sink out of the euphotic zone, there are three currently recognized options for biologically mediated upward movement: (i) flagellar motility; (ii) density decrease by gas vacuoles; and (iii) low-density vacuolar osmolyte solutions. Active water transport is only an alternative for option (iii); we outline the reasoning for this below.

Cyclic vertical migration of phytoplankton

Positive buoyancy, like upward movement using flagella, is used by some phytoplankton organisms as part of cyclic vertical migrations that appear to be related to resource acquisition, particularly where there are inverse vertical gradients of photosynthetically active radiation (PAR) and nutrients, with limiting nutrient concentrations near the surface (Raven and Richardson, 1984). This has been tested by examining C:N:P:Fe of cells at different parts of their diel cycle (or longer, for cells whose vertical migrations take place over longer periods; Villareal *et al.*, 1999, 2007; McKay *et al.*, 2000; Doblin *et al.*, 2006).

Such migrations can only occur, and could only enhance overall resource acquisition, when there is a small vertical component of water movement in the photic zone relative to the rate at which organisms can move through the water. In such environments there is only a low rate at which nutrients regenerated in the dark depths are mixed into the photic zone, and cyclic vertical migration permits organisms to obtain nutrients at the lower part of the cycle and to undergo PAR (daylight permitting) in the upper part of the cycle. Such cyclic migrations are known on a diel basis for flagellates such as dinoflagellates and the freshwater colonial chlorophycean *Volvox*, or on longer time scales for oceanic phytoplankton such as *Ethmodiscus*, *Rhizosolenia*, *Halosphaera*, and *Pyrocystis* (Villareal and Lipschultz, 1995; Doblin *et al.*, 2006; Beardall *et al.*, 2009).

The origin of the low-density solution in the vacuole in the positively buoyant, ascending phase of the vertical migration is partly a result of the relative concentrations of the major inorganic ions from seawater that are major solutes in the vacuole. The ions that form dense solutions (divalent cations, sulphate, potassium) are generally excluded relative to those (sodium, chloride) that form less dense solutions (Anderson and Sweeney, 1978; Boyd and Gradmann, 2002; Woods and Villareal, 2008). The difference between the osmolarity contributed by the inorganic ions and the total vacuolar osmolarity (equal to, or higher than, that of seawater) is assumed to be contributed by organic solutes.

While there are measurements on smaller phytoplankton cells (Dickson and Kirst, 1987), there seems to be no complete osmotic balance for large, positively buoyant cells (Woods and Villareal, 2008). Boyd and Gradmann (2002) point out that tri- and tetramethyl ammonium cations are organic solutes that produce solutions of particularly low density, although no anions are known that form solutions of similarly low density. Furthermore, there is no published evidence as to the occurrence of tri- and tetramethyl ammonium in these phytoplankton cells although it is known that trimethyl ammonium acts in promoting buoyancy in a marine planktonic crustacean (Sanders and Childress, 1998).

Transfer of certain ions from seawater into the vacuole at the expense of other ions to generate buoyancy is an

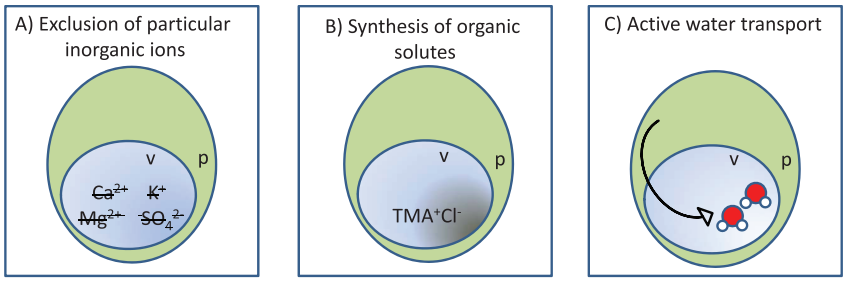

Fig. 2. Three possible mechanisms to create positive buoyancy in marine phytoplankton where the negative intracellular water potential equals the negative water potential in the medium. All involve decreasing the density of the vacuole (v) which comprises a significant portion of the cell protoplast (p) volume. (A) Exclusion of seawater ions yielding dense solutions and preferentially accumulating ions that yield less dense solutions; (B) Replacement of some inorganic ions in the vacuole by organic ions (e.g. trimethylammonium chloride, TMA+Cl–) that yield solutions that have similar density to pure water, i.e. less dense than seawater; and (C) Replacement of some of the vacuolar solutes that generate a fraction of the negative vacuolar water potential though contribution of their osmotic potential by active water accumulation that provides the same fraction of the water potential. Note that organic solute accumulation and active water transport options are estimated to give identical decreases in vacuolar density and that the energy cost of the active water transport option can be less that than of the organic solute accumulation. For more details, see the text.

energy-requiring process (Raven, 1984, 1995; Andrews *et al.*, 2005; see below). The synthesis of organic osmolytes in generating buoyancy is more energetically expensive (and slower) than ion transport on the basis of generating a given osmolarity in the vacuole (Raven, 1995; Andrews *et al.*, 2005). As would be expected from these energy requirements, generation of positive buoyancy (or reduction in negative buoyancy) is restricted or essentially eliminated in the presence of metabolic inhibitors and is frequently decreased in darkness relative to the presence of PAR (Anderson and Sweeney, 1978; Waite *et al.*, 1992, 1997). Given that adjustment of buoyancy is potentially beneficial for cells and that synthesis of organic osmolytes is a relatively slow process, another mechanism to fulfil the cellular requirement for rapid vertical motion is active water transport (Fig. 2).

Characteristics of diatoms that are consistent with active water transport

Since most of the cases of positive buoyancy in phytoplankton not generated by gas vesicles concern diatoms, the discussion to follow emphasizes these algae (Table 1). Diatoms exhibit two phenomena which suggest that the accepted view of the water relations of walled cells does not always apply to them. First, plasmolysis; that is, water loss from the protoplast of a walled diatom cell upon exposure to increased external osmolarity using a variety of external solutes (Bogen and Follman 1955; Lewin and Guillard 1963), shows that the decrease in protoplast volume following the increase in external osmolarity as a result of net osmotic water efflux is followed by rapid deplasmolysis (water uptake leading to protoplast expansion). 'Rapid' here means faster than expected from the uptake rate of the applied solutes by other organisms. While such large solute fluxes are not impossible, Follman (1957) could not find evidence of cellular uptake of the applied osmotic agents using cytochemical techniques; however, more sensitive and quantitative measurements of solute entry are required. The alternative to rapid entry of the applied solutes as an explanation for this rapid deplasmolysis is active water influx (Bogen and Follman, 1955; Follman, 1957). The finding that rapid deplasmolysis in some diatoms is prevented by respiratory inhibitors is consistent with active (energy-requiring) water influx, but is also consistent with active influx of solutes.

The second observation concerns cell expansion in the marine diatom *Chaetoceros decipiens* (Pickett-Heaps, 1998*a*, *b*). The growth of the spines on the cells before they silicified could not, from physical principles, be based on cell turgor. Furthermore, the absence of significant turgor at other times in the cell cycle suggests that cell expansion, involving the relative movement of the two valves of the frustule and increasing the volume they enclose prior to cell division, might be driven by a mechanochemical motor rather than turgor pressure. Expansion of cells of heterokontophytes (=ochristans or ochrophytes) other than diatoms can also occur in the absence of turgor pressure (Money and Harold, 1993). These observations could have relevance to buoyancy in growing cells: it is not always necessary for cells to be turgid for growth to occur, thereby economizing on the resource inputs needed to achieve buoyancy either by synthesis of solutes forming low-density solutions or by active influx of water.

Box 1

Decreasing the density of the vacuole of large marine phytoplankton cells through changes in the vacuolar composition are subject to the constraint that the vacuole has the same water potential as the rest of the protoplast (the cytoplasm and its compartments) and the cell is surrounded by seawater. The three alternative mechanisms, as indicated in Fig. 2, are as follows: (i) exclusion of seawater ions yielding dense solutions and accumulating those that yield less dense solutions; (ii) replacement of some inorganic ions in the vacuole by organic ions that yield less dense solutions; and (iii) replacement of some of the vacuolar solutes that generate a fraction of the vacuolar water potential though contribution of their osmotic potential by active water accumulation that provides the same fraction of the water potential. Table 2 shows the inputs (rows 1–5 and 8) into these calculations, and the outcomes of the calculations in the computed energy costs (lines 6–7) and the density (lines 9–11) for alternatives (ii) and (iii) above. The conclusions are that the organic solute accumulation and active water transport options give identical decreases in vacuolar density (rows 9–10) and that the energy cost of the active water transport option can be less that than of the organic solute accumulation.

Table 2. *Energetics of buoyancy generation by accumulation of solutes yielding low-density solutions in the vacuole and by active water transport in the vacuole*

	Cell property	Value
1	Dimensions of cylindrical cell	Radius 4×10^{-4} m, length 8×10^{-4} m, area 1.4×10^{-5} m^2, volume 4.02×10^{-10} m^3
2	Specific growth rate of cell	0.5 m^3 cell volume d^{-1} instantaneous increase in cell volume
3	Seawater osmolarity	1013 osmol m^{-3}
4	Vacuolar osmolarity for buoyancy generated by trimethylammonium chloride	1013 osmol m^{-3}, of which 128 osmol m^{-3} is contributed by trimethylammonium chloride
5	Vacuolar osmolarity for buoyancy generated by active water transport	885 osmol m^{-3}
6	Energy (for active transport of solutes and biochemistry) required for trimethylammonium chloride production	4.10×10^{-7} W $cell^{-1}$
7	Energy required for active water transport	7.15×10^{-10} W $cell^{-1}$ for net active water transport; 4.84×10^{-8} W $cell^{-1}$ for gross active water transport, compensating for water leakage down the water potential gradient
8	Density of seawater	1024.6 kg m^{-3}
9	Density of vacuole with buoyancy generated by trimethylammonium chloride	1017.9 $kg.m^{-3}$
10	Density of vacuole with buoyancy generated by active water transport	1017.6 $kg.m^{-3}$

Cell dimensions are from Boyd and Gradmann (2002); see Appendix 1 for details of calculations.

The plasmolytic data consistent with active water transport in diatoms, and the absence of turgor at some stages in the cell cycle, can be combined to suggest a role for active water influx in generating positive buoyancy of marine diatoms. For a large, vacuolate cell with a lower concentration of measured intracellular (vacuolar, inorganic) osmolytes than is required to make the protoplast isosmolar with the surrounding seawater, the assumption of additional organic intracellular (vacuolar) osmolytes to make the cell isosmolar with the seawater (Boyd and Gradmann, 2002) could be replaced with the assumption of active water influx from the cytosol to the vacuole. An important comparative aspect is that this location for active water transport (i.e. into the lumen of the endomembrane system) is topologically the same as the active water transport into contractive vacuoles considered above. As with the presence of organic osmolytes, this could make the energetic state of water in the vacuole equal to that in the cytosol and in the surrounding seawater, yet result in the cell being less dense than the surrounding medium (Boyd and Gradmann, 2002). As with the synthesis of osmolytes that yield a low-density solution, active water influx could enable the protoplast to fill the space within the frustules with a low-density protoplast. There are, of course, differences. Some organic osmolytes [e.g. $(CH_3)_3NH_4^+$ trimethylammonium cation] generate solutions isosmolar with seawater that are less dense than even pure water if they could exist without a counter-ion (Boyd and Gradmann, 2002); when the counter-ion Cl^- (the least dense option) is included, the solution is denser than pure water but less dense that seawater. Active water transport generates a contribution to effective osmolarity that has the same density as pure water; that is, is marginally less dense that trimethylammonium chloride.

Why was it assumed that the cytoplasm (cytosol, plastids, mitochondria, and non-vacuolar components of the endomembrane system) is isosmolar with seawater in considering the possibility of active water transport into the vacuole in generating buoyancy? The reason is that a cell with turgor generated osmotically (increased osmolarity due to accumulation of osmolytes), and a wall with a very low potential for elastic expansion and without increased overlap of the valves of the inextensible silicified diatom cell wall, prevents increasing cell volume with increasing turgor. Thus, any additional turgor as a result of active (non-osmotic) water influx does not cause a significant increase in cell volume: the active water influx is essentially all balanced by pressure-driven water efflux rather than increasing the volume of the protoplasm (cytoplasm plus vacuole) volume and diluting the osmotically active contents (*ex hypothesis* with a greater density than seawater), thereby decreasing overall cell density. The alternative possibility is that the cell is isosmolar with seawater before the onset of active water transport: in this case starting active water transport into the vacuole requires a decreasing content of osmolytes so that the volume of the vacuole does not tend to increase and, hence, if the cell wall cannot increase in area, pressure (and water potential) would increase, with water efflux which would short-circuit active water entry.

To compare the energy costs of producing organic solutes, or of active water transport, in generating buoyancy for a marine diatom, calculations are summarized in Box 1 and are expanded on in Appendix 1.

Quantitative aspects of generating low-density vacuoles by active water transport and by producing low-density organic osmolytes.

The calculations in Appendix 1, summarized in Table 2 and Box 1, suggest that active water transport into the vacuole is a possible alternative to generation of organic osmolytes that yield low-density solutions. A third mechanism could be to change the inorganic ionic composition of the vacuole

to minimize the content of the ions yielding dense solutions, namely Ca^{2+}, K^+, Mg^{2+}, and SO_4^{2-}. This could at first sight not involve any energy inputs other than those used in producing the normal, higher density vacuoles, especially since the two ions of choice for a low-density vacuole (Na^+ and Cl^-) are the two most common ions in seawater (Boyd and Gradmann, 2002). However, some additional energy would be needed because K^+ is invariably at higher concentrations than is Na^+ in the cytoplasm (cytosol, plastid stroma, plus mitochondrial matrix), so there are significant free energy differences for these monovalent cations across both the plasmalemma and the tonoplast in cells with low-K^+, high-Na^+ vacuoles (Raven, 1984). Even if the high-Na^+ vacuole could be achieved with no additional energy input compared with the low-Na^+ vacuole, using Na^+ and Cl^- to generate the vacuolar osmolarity needed for buoyancy, it would not make as great a contribution to decreasing vacuolar density as either of the other mechanisms (Boyd and Gradmann, 2002).

Accepting that active water transport cannot be ruled out *a priori* as a means of generating low vacuolar osmolarity, how do the predictions from this mechanism, and from the alternatives, fit with observed properties of large-celled diatoms? Active water transport requires a continuous energy input. Cessation of active water influx to (for example) the vacuole as a result of energy shortage or other cause would lead to osmotic water efflux from the vacuole and vacuolar shrinkage. This water would cause swelling of the cytoplasm and a decrease in its osmolarity, but the surrounding seawater would also withdraw water osmotically from the protoplast so that the overall effect would be protoplast shrinkage, with the vacuole shrinking relatively much more than the cytoplasm. Under a microscope, this would resemble plasmolysis. In terms of buoyancy, the total of osmotically active solutes in the protoplast would not be changed by the water movements, but they would be in a smaller volume. Even if these solutes were still less dense than seawater there would still be an increased protoplast density as a result of the cessation of active water influx, and for the cell as a whole the density would become closer to that of seawater as a result of the seawater that now occupies the volume within the rigid cell wall that corresponds to the decrease in protoplast volume. However, protoplast shrinkage following decreased energy input could also result from osmolyte efflux down free energy gradients in excess of energy-dependent intracellular synthesis (organic osmolytes) or energy-dependent accumulation from the medium (inorganic ions).

A further test is the effect of treatments that alter metabolic rates. Positive buoyancy is known to be decreased, or reversed, by changes in energy supply for cells. A rapid effect would be expected for inhibition of active transport of water. The energized fluxes of ions would be expected to take longer, while generation of buoyancy by synthesis (and reversal by breakdown) of compounds such as trimethylammonium might take even longer (at least for synthesis). How these qualitative orderings of probable time courses relate to the observed rate at which inhibitors alter buoyancy (Anderson and Sweeney, 1978; Waite *et al.*, 1992, 1997) is not clear in the absence of additional data on lipid solution hydraulic conductivity (L_p), rates of ion movements across membranes, and rates of synthesis, breakdown, and efflux of organic osmolytes. However, an additional test is the 'obvious' one of measuring intracellular osmolarity or, with greater cumulative errors, the sum of all intracellular solute concentrations corrected for the osmotic coefficient. This should be referred to the intracellular volume of the original cells, so that any plasmolysis as a result of the cessation of active water transport is taken into account. A 10% contribution to the vacuolar water potential by active water transport would mean only 90% of the intracellular volume would be occupied by the protoplast when active water transport had ceased. If there is a shortfall in the measurable solutes, then active water transport is a possible explanation. However, an unmeasured solute could have been involved, and the cumulative errors in measuring all the solutes could give a total osmolarity not distinguishable from the extracellular osmolarity even if some solute was omitted.

Conclusions

Active water transport can have roles in the soil–plant–atmosphere transpirational continuum in vascular plants, and it clearly occurs in some terrestrial arthropods as in water vapour uptake from an unsaturated atmosphere. Here we show that active water transport also plays a role in volume regulation in wall-less, or effectively wall-less, freshwater algal cells, and, on present evidence, could be a component of buoyancy generation in larger marine diatom cells.

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Appendix 1 Some specimen calculations on buoyancy regulation in marine diatoms

The cell dimensions and composition of the centric marine diatom *Ethmodiscus rex* are taken from Boyd and Gradmann (2002). The surface area of the cylindrical cell is 2.01×10^{-6} m^2 and the volume is 4.02×10^{-10} m^3 (radius 4×10^{-4} m, length 8×10^{-4} m) Table 2, row 1). The total cell osmolarity is 1195 osmol m^{-3}; in the vacuole, 681 osmol m^{-3} is inorganic ions with an excess of anions over cations (measured), 439 osmol m^{-3} is the zwitterionic glycine betaine (assumed), and 75 osmol m^{-3} is trimethylammonium$^+$ (TMA^+; assumed) as the cation balancing the excess of inorganic anions over inorganic cations (Boyd and Gradmann, 2002). In calculating (below) the effect on vacuolar density of replacing TMA^+Cl^- with the equivalent in terms of vacuolar water potential generated by active water influx, it is necessary to know the osmolarity of the ion pair TMA^+Cl^- for a TMA concentration of 60 mol m^{-3}.

The osmolarity generated by 60 mol m^{-3} TMA (activity coefficient 1.245 at the concentration of ions in the vacuole: table 5 of Boyd and Gradmann, 2002) is 75 osmol m^{-3}, while the 60 mol m^{-3} (activity coefficient 0.88 at the

concentration of ions in the vacuole: table 5 of Boyd and Gradmann, 2002) generates 53 osmol m^{-3}; that is, a total of 75+53=128 osmol m^{-3}. The seawater osmolarity of standard seawater (salinity=34.32‰, density=1024.3 kg m^{-3}; Table 2, line 8) is 1013 osmol m^{-3} (p. 611 of Boyd and Gradmann, 2002; Table 2, row 4); that is, the cell turgor expressed in terms of internal minus external osmolarity is 182 osmol m^{-3}. Since the mechanism of buoyancy generation by active water transport is most readily considered (and is most energetically efficient) in the occurrence of turgor, it is assumed that turgor is zero (see comments in the main text on the absence of turgor at some life cycle stages in some marine diatoms).

To make the vacuolar solution isosmolar with seawater and so decrease the turgor to zero, the values for vacuolar concentration in Boyd and Gradmann (2002) are modified to have 182 osmol m^{-3} less glycine betaine (257 rather than 439 osmol m^{-3} glycine betaine), a total of 1013 osmol m^{-3}, which is the same as the osmolarity of standard seawater (Boyd and Gradmann, 2002) (Table 2, row 3). The specific growth rate is taken to be 0.5 d^{-1}, or 5.79×10^{-6} s^{-1}, which is at the high end of the observed range (Lin and Carpenter, 1995) (Table 2, row 2).

The composition used above is now used to compute the energy cost of the generation of buoyancy by synthesis of the solute TMA. For the energy cost of generating a water potential (–0.307 MPa at 20 °C) equivalent to the osmolarity of TMA^+Cl^- (128 osmol m^{-3}) by active transport of water into the vacuole from the cytosol (and the membrane-bound compartments it contains, other than the vacuole) isosmolar with the seawater medium. In this calculation, it is assumed that the 128 osmol m^{-3} generated by TMA^+Cl^- (see previous paragraph) in the vacuole is replaced by the generation of 128 osmol m^{-3} of osmolarity equivalent in terms of water potential [0.307 MPa at 20 °C (Raven, 1982, 1984)] using active water transport, that is a vacuolar osmolarity of 885 osmol m^{-3} (Table 2, row 5). The energy required is computed in W $cell^{-1}$, using the assumptions made by Raven (1982).

The biosynthetic pathway of TMA^+ is not yet defined; the assumption is made that it is produced by the conversion of glycine betaine [$(CH_3)_3CH_2COO^-$] into TMA^+ and acetate. The synthetic pathway of glycine betaine is taken as the decarboxylation of serine to ethanolamine, thence by transmethylation to choline that is then oxidized to betainealdehyde and then glycine betaine. The synthesis of glycine betaine needs 15 mol of NADPH (220 kJ per mol of NADPH) and 8 mol of ATP (55 kJ per mol of ATP) to produce 1 mol of glycine betaine from 3 mol of CO_2 and 1 mol of NO_3^-; that is, 3740 kJ per mol of glycine betaine ($15\times220+8\times55$). The metabolism of a mole of acetate produced in the conversion of a mole of glycine betaine to a mole of TMA^+ generates 11 mol of ATP, equivalent to 605 kJ per mol of acetate. The synthesis of TMA^+ then costs 3135 kJ per mol (3740–605). With an osmotic coefficient of 1.245 for TMA^+, the 75 osmol per m^3 TMA^+ is equivalent to 60 mol m^{-3} TMA^+ (75/1.245=60.24; see above).

For the charge balancing of 60 mol m^{-3} of chloride, the minimum energy cost of moving 1 mol of chloride from seawater into the vacuole is not more than 16 kJ, with a vacuolar potential of greater than –160 mV relative to the seawater (Boyd and Gradmann, 1999) and the vacuolar chloride concentration rather less than that in seawater (Boyd and Gradmann, 2002). This could be readily supplied by 1 mol of ATP (55 kJ) in moving 1 mol of chloride across the plasmalemma and then the tonoplast (55 kJ per mol of chloride). This is very small relative to the 3135 kJ per mol for TMA^+ synthesis. With a cell volume of 4.02×10^{-10} m^3, there is 2.41×10^{-8} mol of TMA^+ per cell (60 mol $m^{-3}\times4.02\times10^{-10}$ m^3). With the growth rate of 5.79×10^{-6} s^{-1}, the required rate of TMA^+ synthesis is 1.40×10^{-13} mol $cell^{-1}$ s^{-1} (2.41×10^{-8} mol $cell^{-1}\times5.79\times10^{-6}$ s^{-1}); that is, an energy cost of 4.37×10^{-7} W $cell^{-1}$. With the addition of the energy used in chloride influx, the energy cost is increased to 4.10×10^{-7} W $cell^{-1}$ (Table 2, row 6).

For active water transport, the gradient of 128 osmol m^{-3} (from the tonoplast to the protoplast) is equivalent to 0.307 MPa or 0.307 MJ m^{-3} at 20 °C. The assumptions made about the water potential gradient that lead to this driving force preclude the occurrence of net water influx in growth down a water potential gradient, since the water potential gradient is such that it moves water out of the cell. This means that all the water entering in growth must move by the energized (active) transport mechanism, most plausibly a potassium chloride:water co-transporter (Zeuthen and Zeuthen, 2007; Zeuthen, 2010; Zeuthen and McAulay, 2012; fig. 1 of Wegner, 2014) of the kind discussed in the text in the context of xylem function and contractile vacuoles. However, there is as yet no evidence of such a co-transporter in diatoms. The net rate of water influx in growth (m^3 $cell^{-1}$ s^{-1}) can be calculated as the product of the values cited above for the (instantaneous) cell volume (4.02×10^{-10} m^3 $cell^{-1}$) and the specific growth rate (5.79×10^{-6} s^{-1} as $4.02\times10^{-10}\times5.79\times10^{-6}$ or 2.33×10^{-15} m^3 $cell^{-1}$ s^{-1}). The water potential gradient against which this occurs is 3.07×10^5 J m^{-3} (from above), so the energy required to bring about the water influx during growth is more than the rate at which water enters (2.33×10^{-15} m^3 $cell^{-1}$ s^{-1}) times the water potential gradient (3.07×10^5 J m^{-3}) or 7.15×10^{-10} J $cell^{-1}$ s^{-1} ($=7.15\times10^{-10}$ W $cell^{-1}$), since the calculation assumes thermodynamic equilibrium (Table 2, row 7).

To this must be added the additional influx needed to balance the efflux of water down the 0.307 MJ m^{-3} gradient. The efflux is calculated using equation (A1)

$$J_v = L_p \times \Delta\psi_w \quad \text{(A1)}$$

where J_v=volume (water) influx, m^3 m^{-2} membrane area s^{-1}; L_p=lipid solution hydraulic conductivity, m^3 m^{-2} membrane area s^{-1} Pa^{-1}; $\Delta\psi_w$=difference in water potential between the inside and outside (low inside), Pa (=N m^{-2} or J m^{-3})

Even with the high estimate of L_p, namely 10^{-14} m^3 m^{-2} s^{-1} Pa^{-1} (table 3 of Raven 1982) the efflux is 3.07×10^{-9} m^3 m^{-2} s^{-1}; using the cell surface area from above (2.01×10^{-6} m^2 $cell^{-1}$) this is equivalent to $2.01\times10^{-6}\times3.07.10^{-9}$ or 6.17×10^{-15} m^3 $cell^{-1}$ s^{-1}. This passive leakage of water out of the cell of

6.17×10^{-15} m^3 cell^{-1} s^{-1} must be balanced by an equal and necessarily active influx during growth, so the total active influx is net active water influx of 2.33×10^{-15} m^3 cell^{-1} s^{-1} plus the leak recovery of 6.17×10^{-15} m^3 cell^{-1} s^{-1} to give a total of 8.5×10^{-15} m^3 cell^{-1} s^{-1}. The gradient against which the total influx occurs is 3.07×10^5 J m^{-3}, so the total energy required is $>3.07 \times 10^{-9}$ J cell^{-1} s^{-1} or 3.07×10^{-9} W cell^{-1}. A mechanistically based energy cost of active water transport from Raven (1982) is 100 mol of ATP per m^3 water transported against the 0.307 MJ m^{-3} gradient. At 55 kJ per mol of ATP, the energy cost is 10^2 mol of ATP times 5.5×10^4 J per mol of ATP, so the energy cost for active water influx of 8.8×10^{-15} m^3 cell^{-1} s^{-1} is 4.84×10^{-8} J cell^{-1} s^{-1} or 4.84×10^{-8} W cell^{-1} (Table 2, row 7).

These calculations show that active water transport (mechanistic cost) involves a lower energy cost (4.84×10^{-8} W cell^{-1}) than that of TMA^+ synthesis plus chloride influx (4.10×10^{-7} W cell^{-1}) and could therefore be a plausible mechanism to adjust buoyancy in marine diatoms. A slower rate of cell growth would involve more leakage of water relative to net active water influx, with a corresponding increased energy cost per cell doubling. While there might also be leakage of TMA, this is likely to be much less significant than water leakage.

To put these energy costs into the context of decreasing the density of the vacuolar solution, the contribution to overall cell density from 60 mol m^{-3} each of TMA^+ and of chloride is computed from the densities of 1000 mol m^{-3} solutions of the two ions (Boyd and Gradmann, 2002), assuming a linear relationship between excess density of the solution (the difference between the density of the solution and the density of pure water, in kg m^{-3}) and ion concentration. For TMA^+, the density of a 1000 mol m^{-3} solution is 992.7 kg m^{-3}; that is, the solution has an excess density of –7.3 kg m^{-3} relative to pure water, so for a 60 mol m^{-3} solution the excess density is –0.438 kg m^{-3}. For chloride, the density of a 1000 mol m^{-3} solution is 1011.8 kg m^{-3}; that is, an excess density of +11.8 kg m^{-3} relative to pure water, so for a 60 mol m^{-3} solution the excess density is +0.708 kg m^{-3}. The overall excess density contribution of 60 mol m^{-3} of each of the ion pair TMA^+Cl^- is (–0.438+0.708) or 0.270 kg m^{-3}. This is a very small contribution to the overall vacuolar density of 1019.8 kg m^{-3} (table 3 of Boyd and Gradmann 2002).

Before comparing the values for the isosmolar vacuole with the vacuolar density of 1019.8 kg m^{-3} for turgid cells cited by Boyd and Gradmann (2002) the vacuolar density must be corrected for the assumption that there is 128 osmol m^{-3} less glycine betaine than in the calculations of Boyd and Gradmann (2002). Using the osmotic coefficient of 1.25 for glycine betaine in table 5 of Boyd and Gradmann (2002), the concentration of glycine betaine is decreased by 102 mol m^{-3}; 1000 mol m^{-3} of glycine betaine has an excess density of 18.8 kg m^{-3}, so, again assuming linearity of excess density and concentration, 102 mol m^{-3} glycine betaine has an excess density of +1.92 kg m^{-3}. This would decrease the density of the vacuole cited by Boyd and Gradmann (1019.8 kg m^{-3}), and the excess density of +19.8 kg m^{-3} to an excess density of +19.8–1.92 or 17.9 kg m^{-3}. This gives a vacuolar density of 1017.9 kg m^{-3}, compared with the seawater density cited by Boyd and Gradmann of ~1024.6 kg m^{-3}. The density of 1017.9 kg m^{-3} using TMA^+Cl^- (Table 1, rows 8 and 9) in a vacuole isosmosmolar with seawater would be further reduced to 1017.6 kg m^{-3}; that is, by the excess density of the TMA^+Cl^- component (+0.27 kg m^{-3}), by replacing this osmolyte by active water transport producing a density of 1017.6 kg m^{-3} (Table 1, row 10). There are two important points here. One is the similarity (identity) of the decrease in density resulting from TMA^+ synthesis and from active water transport, and the other is that the calculations presented here suggest that active water transport is less energically costly, at least during cell growth, than is TMA^+Cl^- accumulation.

A further consideration is the implications of the assumed L_p value for the plasmalemma/tonoplast of *E. rex* for growth of cells if there was no active transport. Despite the low surface area:volume quotient, it is likely that the assumed L_p value could account for the observed growth rate in the absence of active transport (e.g. during the descent phase of periodic vertical migration). It is unlikely that, despite its large size and low surface area per unit volume, *E. rex* in its natural environment (open-ocean waters) is ever subjected to rapid changes in external osmolarity such as would require a higher L_p such as could be provided by aquaporins (membrane proteins that increase membrane conductance to water). There are no unequivocal signs of aquaporins in the genomes of the two completely sequenced diatoms, though Scala *et al.* (2002) refer to sequences with 'twilight zone' (i.e. of doubtful significance) similarities to aquaporins in the genome of *Phaeodactylum tricornutum.* Furthermore, Petrova *et al.* (2013) found molecular genetic evidence of an aquaporin-like protein in the diatom *Synedra aucsi.* It should be noted that the occurrence of aquaporins in a membrane does not mean that there is always a higher L_p than in a comparable but aquaporin-free membrane, since aquaporin functioning can be regulated post-transcriptionally (Chaumont *et al.*. 2005).

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