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When to say when: can excessive drinking explain silicon uptake in diatoms?

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Diatoms are the single most important drivers of the oceanic silicon biogeochemical cycle. Due to their considerable promise in nanotechnology, there is tremendous interest in understanding the mechanism by which they produce their intricately and ornately decorated silica-based cell wall. Although specific proteins have been implicated in some of the key steps of silicification, the exact mechanisms are poorly understood.
Silicon transporters, identified in both diatoms and silicoflagellates, are hypothesized to mediate silicon uptake. Recently, macropinocytosis, the non-specific engulfment of extracellular fluid, was proposed as a more energetically favorable uptake mechanism, which can also explain the long-observed effect of salinity on frustule morphology. We explore the bioenergetic, membrane recycling, and vacuolar volume requirements that must be satisfied for pinocytosis-mediated silicon uptake. These calculated requirements contrast starkly with existing data on diatom physiology, uptake kinetics, growth, and ultrastructure, leading us to conclude that pinocytosis cannot be the primary mechanism of silicon uptake.

Keywords: diatoms; pinocytosis; silicification; silicon uptake

Introduction

Diatoms are unicellular, eukaryotic phytoplankton that are estimated to be responsible for over 40% of marine primary productivity.[1] Because most diatoms have an obligate growth requirement for silicon (Si), silicon uptake is tightly coupled to the cell cycle, occurring only during specific stages.[2,3] At oceanic pH, soluble silicon exists predominantly (97% of total dissolved silicon) as silicic acid ($Si(OH)_4$), with the remaining portion as silicate ($SiO(OH_3^-)$). Diatoms, together with radiolarians, silicoflagellates, and sponges, convert 6.7 Gtons of this soluble silicon into biogenic silica (SiO_2) annually.[2] The mechanism by which diatoms take up silicon from a relatively silicon-deplete ocean and deposit and mold it into their cell wall is unknown. The details of silicification have been reviewed elsewhere,[3,4] but the major steps of the process are depicted in Figure 1. The first step of silicon uptake requires diatoms to overcome a steep outward facing concentration gradient (generally <1 to 10 μM in seawater compared to 19–340 mM inside the cell).[3] Silicon transporters (SITs) have been identified in numerous diatoms as well as some silicified flagellates.[5–8] These transporters have been shown, in a heterologous system, to directly transport $Si(OH)_4$ in a sodium-dependent manner[9] consistent with the observation that uptake in marine diatom cells is coupled to a Na^+ gradient.[10] Intracellular silicic acid is somehow maintained at concentrations that exceed the solubility of silica.[11] Precipitation of macromolecules from the soluble silicon pool of ^{31}Si-radiolabeled diatoms recovered a significant fraction of the added ^{31}Si suggesting stabilization of silicic acid may occur through complexation with organic molecules.[12] Formation of the new cell wall occurs in a slightly acidic vesicle called the silica deposition vesicle, or SDV,[13] but the mechanism by which silicic acid enters the SDV is unknown. Within the SDV, silaffins and polyamines play a role in polymerization of silica,[14–16] while cytoplasmic actin and microtubules aid in shaping the mature frustule.[17] The precise reproduction of silica structures from generation to generation strongly suggests a genetic basis for pattern formation.

It has recently been suggested that the first step of silicon acquisition by diatoms may not be mediated by SITs, but rather by macropinocytosis,[18] a non-specific, wholesale mechanism by which extracellular fluids are engulfed *via* plasma membrane invagination. Vrieling and colleagues (2007) observed nanostructural differences between diatoms grown in low and high salinity (20 and 33 on the practical salinity scale, respectively; hereafter referred to as psu). They conclude that these salinity-dependent phenomena are likely due to the simultaneous uptake of silicic acid and other external ions *via* pinocytosis. Fusion of pinocytotic vesicles with the silicalemma (the membrane surrounding the SDV) would provide a direct route for silicic acid (as well as other ions) to enter the SDV and serve as the precursor for the new cell wall. Although such vesicles were not observed in their study, the presence of small vesicles ($\sim$35 nm) fusing with the SDV have been observed in other studies including those of *Ditylum brightwellii*,[19] *Gomphonema parvulum*,[20] and *Stephanopyxis turris*.[21] In addition, 30–40 nm vesicles were

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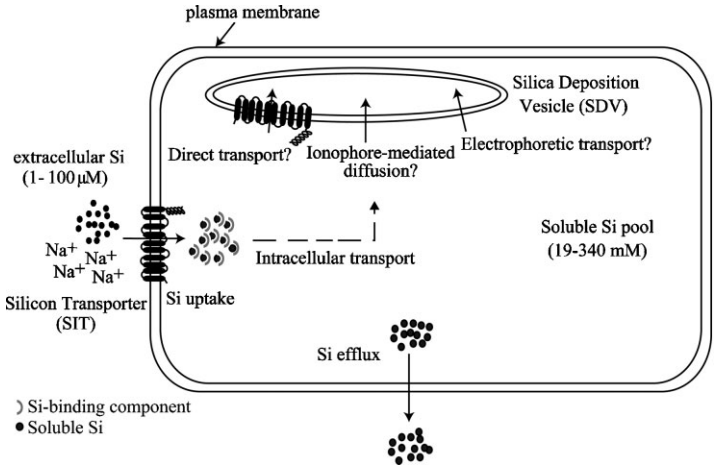

Figure 1. Schematic of diatom silicon metabolism. Extracellular silicon (black dots), present predominantly as silicic acid, is co-transported with sodium (Na^+) across the plasma membrane *via* silicon transporters (SITs). Supersaturating levels of silicic acid are hypothesized to be maintained in soluble form by binding to an intracellular Si-binding component (horseshoe-shaped structures). Silicic acid is transported through the cytoplasm to the silica deposition vesicle (SDV) by an unknown mechanism. Silicic acid crosses the silicalemma into the SDV, possibly though direct transport by SITs, ionophore-mediated diffusion, or electrophoretic transport. Silicic acid efflux also occurs, allowing further control over intracellular silicon levels.

observed in *Thalassiosira eccentrica* and designated silicon transport vesicles (STVs).[22] However, despite their name, STVs, like all other small vesicles studied, have not been experimentally shown to contain any form of silicon. In fact, more recent work using electron spectroscopic imaging in *T. pseudonana* showed silicon is more or less evenly distributed throughout the cytoplasm, with the notable absence of localization in membrane enclosed structures.[23] It is more likely that the observed vesicles are involved with expansion of the SDV by providing membrane material.[19] To evaluate the prospects for pinocytosis-mediated silicic acid uptake in diatoms, we (1) calculated the required volume of Si-containing vesicles relative to total cellular volume, (2) determined the surface area of vesicular membrane required to meet the Si demand, and (3) compared existing data on the energetics of silicon uptake to the bioenergetics of transporter-mediated and pinocytosis-mediated silicon uptake.

Membrane recycling and kinetics

To determine whether pinocytosis is a metabolically reasonable route for silicon uptake, rates of pinocytosis and the required surface area of vesicular membrane to that of the cell surface were calculated using equations 1–4.

$$V_{ss} = \mu_{Si} \times Q_{BSi} \quad (1)$$

A range of steady-state Si uptake rates (Equation 1; V_{ss}; fmol $Si \cdot cell^{-1} \cdot d^{-1}$) was compiled from specific growth rates (μ_{Si}; d^{-1}) and biogenic silica quotas (Q_{BSi}; fmol $Si \cdot cell^{-1}$) previously reported for several diatom species (Table 1). The rate of seawater "drinking" required to meet the observed silicon requirement was calculated using Equation 2,

$$R_{cell} = \frac{V_{ss}}{[Si]} \quad (2)$$

in which R_{cell}, the volume seawater $\cdot cell^{-1} \cdot h^{-1}$, was calculated from V_{ss} (Equation 1) and the external $Si(OH)_4$ concentration, [Si].

A number of assumptions were made in the subsequent calculations. First, the extracellular silicic acid concentration used was 100 μM, which is much higher than that found in surface waters, but only slightly higher than the oceanic average. Second, the net uptake rate, represented by V_{ss}, closely approximated the gross uptake rate. Third, a diatom was assumed to be engaged in pinocytosis (*i.e.*, drinking) 24 hours per day. In regards to pinocytosis, these assumptions are generous given that most diatoms live in environments containing <10 μM $Si(OH)_4$,[2] net and gross uptake rates are not necessarily equal,[24] and uptake only occurs in distinct phases of the cell cycle.[25] Thus, the required rate of seawater "drinking" is underestimated to the extent which these assumptions deviate from reality.

From Equation 2 and Table 1, a cell volume-specific pinocytosis rate (R_{vol}; h^{-1}) was calculated by Equation 3.

$$R_{vol} = \frac{R_{cell}}{\text{cell volume}} \quad (3)$$

Table 1. Rate of pinocytosis, vesicle volume and membrane processing required to sustain observed biogenic silica quotas in a 100 μM $Si(OH)_4$ ocean.

Species	μ (d^{-1})	Q_{Bsi} (fmol Si · $cell^{-1}$)	V_{ss} (fmol Si · $cell^{-1} \cdot day^{-1}$)	R_{cell} (pL · $cell^{-1} \cdot h^{-1}$)	Cell volume (μm^3)/ surface area (μm^2)	R_{vol}(h^{-1})	$Vesicle_{SS}$ (vesicle · $cell^{-1}$)	SA ratio (vesicle SA/ cell SA)	Volume ratio (vesicle vol/cell vol)
Thalassiosira weissflogii[a]	1.4[c]	1600	2240	933	700/380	1340	6.70×10^8	8900	32
T. pseudonana[b]	2.5[c]	43–56	108–140	48–58	14/28	3170–4130	3.21×10^7–4.19×10^7	5700–7400	76–99
T. pseudonana[c]	1.6[b]	31.3	50	21	29/45	726	1.50×10^7	1700	17
Cerataulina pelagica[c]	1.4[b]	1470	2060	858	950/470	903	6.16×10^8	6600	22
Skeletonema costatum[c]	1.8[b]	44.4	79.9	33	34/50	994	2.39×10^7	2400	24
Pseudo-nitzschia heimii type 1[d]	1.4[b]	2690	3770	1570	1140/1160	1380	1.13×10^9	4900	33
P. dolorosa[d]	1.0[b]	570	570	238	370/490	645	1.70×10^8	1700	16
P. multiseries[d]	0.7[b]	1070	749	312	640/690	491	2.24×10^8	1600	12

Only data in which cells exhibited exponential, steady state growth rates and where silicic acid concentrations were relatively invariant were considered. Growth rate (μ) from batch cultures or chemostat data (denoted b and c, respectively) and biogenic silica quota (Q_{BSi}) were obtained from published data. Steady state Si uptake rate (V_{ss}) was calculated (Equation 1) as $\mu_{Si} \times Q_{BSi}$. The volume of seawater necessary to sustain silica quotas *via* a pinocytosis-mediated mechanism, R_{cell}, was calculated (Equation 2) as V_{ss}/[Si]. For centric diatoms, cell volume and surface area were calculated using standard formula for spheres, whereas the data of Marchetti and Harrison (2007) were used for pennate diatoms. R_{vol}, the cell volume-specific rate of pinocytosis (h^{-1}), was calculated (Equation 3) as R_{cell}/cell volume. Steady state vesicle number, $Vesicle_{ss}$, was calculated (Equation 4) as (R_{cell}/vesicle volume)/(ln(2)/$\tau_{1/2}$).

[a]See ref. (25)

[b]See ref. (53), assuming $d = 3$ μm

[c]See ref. (51), cell width = 3.8, 12.2, and 4.0 μm for *T. pseudonana*, *C. pelagica*, and *S. costatum*, respectively

[d]See ref. (52)

These calculations suggest that in order to achieve the observed Si quota, the volume of seawater processed through pinocytosis each hour must be 500–4,000 fold greater than the cell volume (Table 1). To put this in perspective, the pinocytosis uptake volume for two obligate phagotrophs, *Dictyostelium discoideum*, and *Acanthomoeba castellanii*, is 7 and 1.3 fold greater than the cell volume, respectively.[26]

In addition to a large requisite cell volume-normalized seawater flux, pinocytosis-mediated silicic acid uptake would also require a flux of cell surface membrane into the cytoplasm. This required flux, under steady state conditions, was used to calculate the steady state vesicle number per cell ($Vesicle_{ss}$; vesicle · $cell^{-1}$) and therefore the vesicular surface area (SA) and volume per cell using Equation 4.

$$Vesicle_{ss} = \frac{(R_{cell}/\text{vesicle volume})}{(\ln(2)/\tau_{1/2})} \quad (4)$$

The minimum required vesicle surface area and vesicle volume were obtained assuming a vesicle diameter of 40 nm, the upper range of that observed for diatoms. The shortest reported half life ($\tau_{1/2}$) of a vesicular membrane is 1 min.[27] While there are no data derived from diatoms, the fundamental constraints on intracellular vesicular transport and re-adsorption to the plasmalemma are not assumed to differ appreciably in eukaryotes. Based on these calculations, depending on the species, $vesicle_{ss}$ ranged from 1.5×10^7–1.1×10^9 vesicle · $cell^{-1}$. For *T. weissflogii*, this represents 3.4×10^6 μm^2 of plasmalemma membrane (compared to the $\sim 3.8 \times 10^2$ μm^2 found at the cell surface). This almost 9,000-fold greater vesicular surface area relative to that of the cell surface is inconsistent with diatom C:P ratios given the high phosphorus content of phospholipid bilayers. The molar ratio of C:P in phosphoglycerides is about 38:1,[28] and the elevated membrane quantity required here would result in a cellular C:P ratio much lower than typically observed in marine phytoplankton. In addition, this required vesicle number per cell corresponds to a steady state vesicle volume 32-fold greater than the cell volume, which is inconceivable. Alternatively, if vesicles concentrate silicic acid, stabilization in soluble form would be required through either elevated pH (*i.e.*, high [OH^-]) or the presence of Si-specific organic binding ligands. Either stabilizing agent would seem to pose further bioenergetic constraints on silica formation, as they too would be dumped into the SDV upon vesicular fusion.

Fusion of high pH vesicles would alter the acidity of the SDV,[13] requiring pumping of additional H^+ into the SDV to promote silica polymerization.

Bioenergetics

The ATP requirement for Si uptake is estimated to be ~0.5–1 ATP per Si.[29] The energy requirement for pinocytosis is proposed to be less than that required for specific transport and intracellular stabilization of silicic acid.[18] Therefore, we compared the empirically derived energetics of silicic acid uptake with the theoretical costs of transporter-mediated and pinocytosis-mediated silicic acid uptake. In marine diatoms, silicic acid and Na^+ uptake are coupled in a 1:1 ratio.[10,30] In this scenario, if it is assumed that each Na^+ co-transported with $Si(OH)_4$ is pumped out *via* a Na^+K^+ATPase, the calculated ATP demand would be 0.33 ATP per Si transported (based on 1 ATP/3 Na^+), consistent with the ATP estimate of Raven (1983). In the case of pinocytosis, the cost to remove each Na^+ by the Na^+K^+ATPase is ~1600 ATP per Si (assuming $[Na^+] = 0.48$ M, equivalent to a salinity of 36 psu, and $[Si(OH)_4] = 100\ \mu M$). For a C:Si ratio of 7:1, this translates into an ATP cost of ~230 mol ATP · mol net C fixed^{-1} indicating an additional ~38 mol C respired · mol C fixed^{-1}. This is more than 100-fold higher than the calculated respiratory demand for C fixation.[31,32] This seems particularly disadvantageous compared to non-siliceous cells and would suggest a strong effect of salinity on the bioenergetics of diatoms. It is also unclear how the well-documented Na^+-dependency of $Si(OH)_4$ uptake[6,10,30] would be explained with this variant of a pinocytosis-mediated mechanism.

Conclusions

Based on the calculations above and those performed by others,[29] the concept of pinocytosis-mediated silicic acid transport is difficult to conceptualize from either a materials flux or bioenergetics perspective. Regardless, Vrieling et al. (2007) present compelling data showing that cells grown under low salinity (psu 20) have denser frustules, increased surface roughness, and smaller pore size compared to those grown at psu 33. There is little debate that external ionic strength influences diatom silicification, as this phenomenon has been documented by several investigators.[33–35] However, there are alternative mechanisms by which salinity could elicit the observed effects on silicification that warrant discussion and consideration.

Diatoms, like all living organisms, adapt to osmotic stress through a wide range of adaptations at the molecular, cellular, and organismal levels. Organisms are affected by changes in salinity in three ways: (1) changes in cellular water potential due to osmotic stress, (2) increased uptake or loss of ions due to ionic stress and (3) changes in intracellular ionic ratios.[36] Indeed, diatoms are capable of responding to changes in osmolarity by increasing intracellular levels of calcium, which is characteristic of the activation of signal transduction pathways.[37] The alteration of silicified structures observed in response to low salinity may be an indirect effect of the cell's acclimatization to its new environment.

Intracellular Na^+ concentrations are relatively invariant in diatoms with respect to extracellular salinity,[38] but the total osmolyte concentrations are in slight excess of extracellular concentrations.[39] This suggests that the ratio of intracellular Na^+: organic osmolyte may be a function of salinity, with a lower ratio at high salinity as cells increase organic osmolyte levels.[40] Therefore, if the osmolyte pools in the SDV and cytosol have the same relative proportions of inorganic and organic osmolytes, the concentration of organic osmolytes in the SDV would be higher under higher salinity (Figure 2). Organic osmolytes, such as amino acids, betaine, and dimethylsulfoniopropionate (DMSP), could affect silica morphology. Indeed, *in vitro* studies demonstrate that amino acids with nitrogen-containing side chains produce larger silica particles while hydroxyl-containing or hydrophobic amino acids generate smaller particles.[41] Determining the steady state intracellular distribution of organic and inorganic osmolytes, including Na^+, will allow quantitative comparisons among cells grown under different salinity regimes and perhaps shed some light on the effect of osmolytes on silicification. Measuring Na^+ and organic osmolytes occluded in the frustule would also be informative and may soon be possible using the ultra-clean frustule cleaning and extraction methods that are currently being developed.[42]

The reactions occurring in the SDV depend on pH, where the slight acidity promotes silica polymerization[13]. This acidity is probably maintained by SDV proton pumps, but it is unclear how these are regulated and which ions, if any, are co-transported in the process. Moreover, silaffins (lysine rich polycationic peptides associated with silica deposition) and polyamines affect the morphology of silica precipitates in a pH-dependent manner,[14,15] with the ratio of silaffins to polyamines also having an effect on structure.[14] If salinity somehow alters this ratio and/or alters the activity of SDV proton pumps—two broad possibilities that have not yet been investigated—this may explain the effect of salinity on silica morphology.

A mechanistic understanding of the silicification/salinity relationship observed over the last three decades would have profound implications on the understanding of coastal and estuarine Si biogeochemical cycling and would provide new prospects for manipulating diatoms to produce novel silica nanostructures. This understanding may be advanced by exploring how SDV size, ultrastructure or internal osmolyte

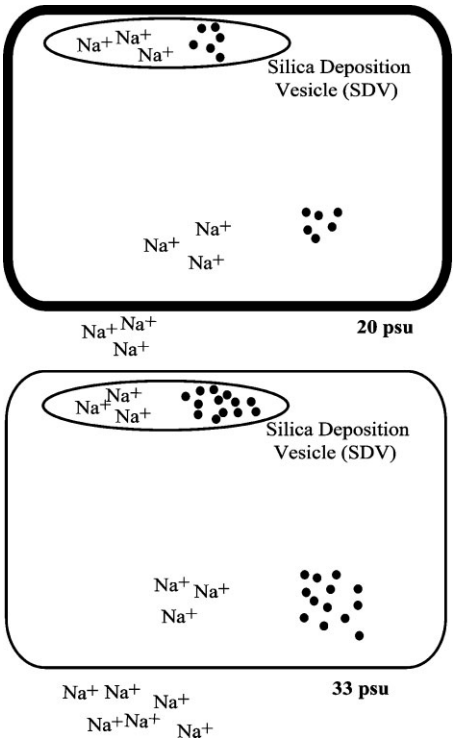

Figure 2. Maintenance of intracellular osmolarity. Cells grown under low salinity (20 psu) produce a thicker frustule (top cell, denoted by a thicker line). Diatoms maintain relatively similar intracellular Na^+ concentrations irrespective of extracellular salinity. Under high salinity (bottom cell), organic osmolytes (black dots) are likely increased to maintain homeostasis. The levels of inorganic and organic osmolytes will be similar in the cytosol and in the SDV, suggesting that under high salinity, more organic osmolytes are present in the SDV. Could these osmolytes affect the silica morphology observed in response to changes in salinity?

composition respond to differences in salinity. The SDV is formed by the coalescence of smaller vesicles[19,22] and undirectional expansion of the SDV is well-documented.[22,43,44] Acute decreases in salinity lead to water influx in some marine algae,[36,45] and it is intriguing to speculate whether the SDV might swell over short time frames, providing more space for production of a thicker frustule through silica deposition. However, it might be naïve to expect that such a simple mechanism could adequately explain the observed differences in silicification, simply because the differences in frustule morphology are achieved under steady state conditions in which transient changes in osmotic pressure are presumably minimal.

Our knowledge and understanding of the complex process of biosilicification would be greatly advanced by the isolation of the SDV and its constitutive components. However, the SDV has remained elusive despite over 50 years of attempts at biochemical purification. The recent availability of genomic information[46,47] and modern proteomic analyses has nonetheless led to the isolation and identification of cell wall-associated proteins that do offer insight into the genetic control of diatom structure formation.[48] These proteins include those involved in polyamine biosynthesis and signal transduction, as well as cytoskeletal components.[48] A suite of proteins were also found to be coordinately regulated with silaffins, suggesting their involvement in silica polymerization.

While progress has been made over the last 10 years, much more work is required to completely understand the mechanism of biosilicification. In this regard, the centric diatom, *T. pseudonana* is an ideal model organism. *T. pseudonana* has a relatively simple cell wall architecture, has a complete genome sequence available[46], is one of only a few diatoms capable of being genetically transformed[49], and can be grown in synchronous culture[50]. In addition to the silaffins, other cell-wall associated proteins have been identified. Further characterization of these proteins, including their cellular localization and changes in expression in response to changing environmental conditions, will provide new insight into biosilicification. Perhaps some of these proteins will prove useful as tractable markers of the SDV, enabling us to evaluate responses of the SDV and/or its constituents,to changing environmental conditions.

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