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Running title: Sulfate and evolution of chlorophyll a+c phytoplankton

ABSTRACT

During the Mesozoic Era, dinoflagellates, coccolithophorids and diatoms became prominent primary producers in the oceans, succeeding an earlier biota in which green algae and cyanobacteria had been proportionally more abundant. This transition occurred during an interval marked by increased sulfate concentration in seawater. To test whether increasing sulfate availability facilitated the evolutionary transition in marine phytoplankton, the cyanobacterium *Synechococcus* sp., the green alga *Tetraselmis suecica*, and three algae containing chlorophyll a+c (the diatom *Thalassiosira weissflogii*, the dinoflagellate *Protoceratium reticulatum*, and the coccolithophorid *Emiliana huxleyi*) were grown in media containing 1, 5, 10, 20 or 30 mM SO_4^{2-} . The cyanobacterium and the green alga showed no growth response to varying $[\text{SO}_4^{2-}]$. In contrast, the three chlorophyll a+c algae showed improved growth with higher $[\text{SO}_4^{2-}]$, but only up to 10 mM. The chlorophyll a+c algae, but not the green alga or cyanobacterium, also showed lower C:S with higher $[\text{SO}_4^{2-}]$. When the same experiment was repeated in the presence of a ciliate predator (*Euplotes* sp.), *T. suecica* and *T. weissflogii* increased their specific growth rate in most treatments, whereas the growth rate of *Synechococcus* sp. was not affected or decreased in the presence of grazers.

In a third experiment, *T. suecica*, *T. weissflogii*, *P. reticulatum*, and *Synechococcus* sp. were grown in conditions approximating modern, earlier Paleozoic and Proterozoic seawater. In these treatments, sulfate availability, nitrogen source, metal availability and P_{CO_2} varied. Monospecific cultures exhibited their highest growth rates in the Proterozoic treatment. In mixed culture, *T. weissflogii* outgrew other species in modern seawater and *T. suecica* outgrew the others in Paleozoic water. *Synechococcus* sp. grew best in Proterozoic seawater, but did not outgrow eukaryotic species in any treatment. Collectively, our results suggest that secular increase in seawater $[\text{SO}_4^{2-}]$ may have facilitated the evolutionary expansion of chlorophyll a+c phytoplankton, but probably not to the exclusion of other biological and environmental factors.

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INTRODUCTION

In the productive waters that bathe continental shelves, three groups of algae with chloroplasts containing chlorophyll a and chlorophyll c dominate photosynthesis: diatoms, dinoflagellates, and coccolithophorids. Despite their abundance and diversity, these taxa are relative newcomers to marine ecosystems (Falkowski *et al.*, 2004). Both conventional fossils and preserved lipids suggest that in Proterozoic oceans cyanobacteria and other photosynthetic bacteria fueled ecosystems, whereas cyanobacteria and green algae were major primary producers in Paleozoic oceans (Knoll *et al.*, 2007; Kodner *et al.*, 2008; Johnston *et al.*, 2009). Dinoflagellates may have been the first of the major chlorophyll a+c phytoplankton to appear; purported dinocysts have been reported from Paleozoic rocks, and low abundances of dinosterane, the geologically stable form of dinosterol synthesized by dinoflagellates, have been reported from Cambrian deposits (Molodowan & Talyzina, 1998). Despite this, unambiguous dinocysts and shales with abundant dinosterane occur only in Triassic and younger successions, indicating, at the very least, that photosynthetic dinoflagellates assumed a globally important and persistent role in marine primary production only at this time (Knoll *et al.*, 2007). The fossil record of diatoms extends only to the latest Jurassic Period (Harwood *et al.*, 2004), and the group rose to ecological prominence only in later Cretaceous and Paleogene oceans (Kooistra *et al.*, 2007). Similarly, the oldest coccoliths occur in later Triassic rocks, and coccolithophorids became widespread only during the Cretaceous Period (Bown *et al.*, 2004).

At least three classes of hypothesis are available to explain the observed stratigraphic succession of primary producers. Perhaps seawater chemistry has changed through time in a way that favored successive phytoplankton groups. Quigg *et al.* (2003), for example, proposed that decreasing iron availability associated with the increasing ventilation of deep oceans provided a competitive edge to the chlorophyll a+c algae that dominate modern shelf production, due to the lower requirement of their plastids for Fe compared to that green algae. Alternatively, we might hypothesize that a Mesozoic increase in grazing pressure by micrograzers favored the modern phytoplankton biota – sort of a microscopic version of Vermeij's (1977) hypothesis that escalating predation drove marked evolutionary changes in Mesozoic marine animals. It is also possible that changing phytoplankton composition principally reflects the individual adaptations of photosynthetic clades rather than ecosystem-wide causes and effects. Kooistra *et al.* (2007),

for example, have enumerated cell biological adaptations that favor diatom abundance, while Bowler *et al.* (2010) have discussed much the same thing from a genomic perspective. The hypotheses, of course, are not mutually exclusive.

Only through careful experimentation can we begin to sort out these views of long term phytoplankton evolution. In this paper, we take a first step in this direction, reporting the outcome of experiments designed to test the specific hypothesis that increasing concentration of seawater sulfate favored chlorophyll a+c algae with low C to S ratios. As detailed in the following section, this hypothesis is motivated by the stratigraphic correlation between the taxonomic and ecological expansion of chlorophyll a+c phytoplankton and an increase of marine sulfate levels to their current, historically high levels. In our experiments, single species stand in for entire divisions, undoubtedly a gross oversimplification. Nonetheless, they provide starting points for experimental tests of physiological links between phytoplankton evolution and environmental history.

GEOCHEMICAL AND PHYSIOLOGICAL BACKGROUND

Sulfate abundance in time and space

If phytoplankton composition has changed through time, so, too, has the chemical state of the oceans that support its growth. Sulfate abundances in ancient seawater have been inferred in several ways: experimentally observed correlation between S isotopic fractionation and sulfate abundance at very low $[\text{SO}_4^{2-}]$, dynamic models of observed secular variations in the isotopic composition of sulfate in seawater, and, in younger records, reconstruction of seawater chemistry from fluid inclusions in evaporite minerals. While all of these proxies are subject to error, they collectively provide a consistent picture of seawater $[\text{SO}_4^{2-}]$ through geologic history. Sulfate abundance was probably less than 200 μM in the Archean oceans that sustained the first marine cyanobacteria (Habicht *et al.*, 2002), and $[\text{SO}_4^{2-}]$ increased to no more than 1-5 mM in the Proterozoic oceans within which green algae rose to ecological prominence (Shen *et al.*, 2002; Canfield, 2004; Kah *et al.*, 2004). Sulfate abundance may have reached values as high as 15 mM in later Ediacaran oceans, but declined again into the Cambrian (Horita *et al.*, 2002; Petrychenko

et al., 2005). Analyses of fluid inclusions in salt crystals indicate Cambrian $[\text{SO}_4^{2-}]$ in the range of 3-12 mM; models based on C and S isotopic variations suggest $[\text{SO}_4^{2-}]$ near the lower end of this range (Gill *et al.*, 2011).

Sulfate abundance appears to have remained below about 10 mM until the Carboniferous Period, when it increased to >15 mM (Gill *et al.*, 2007; Fig. 1). Sulfate levels may have declined transiently after that time (Luo *et al.*, 2010; Newton *et al.*, 2011). For the most part, however, fossils, biomarker molecules and molecular clocks place the rise of diatoms, coccolithophorids, and photosynthetic dinoflagellates in late Paleozoic to early Mesozoic oceans, where $[\text{SO}_4^{2-}]$ lay in the range of 13-27 mM. In today's oceans, $[\text{SO}_4^{2-}]$ is 28 mM, at or near its historic maximum. Of course, $[\text{SO}_4^{2-}]$ varies in space as well as in time, in the ocean, and not all aquatic environments on Earth have $[\text{SO}_4^{2-}]$ as high as in the oceans. Freshwater environments have $[\text{SO}_4^{2-}]$ values of 0.01 to 1 mM (Holmer & Storkholm, 2001; Giordano *et al.*, 2008).

Sulfur use by algae

How might changing sulfate availability affect phytoplankton growth? Sulfur is an essential element for all organisms (Schultze *et al.*, 1992; Marrs, 1996; Grossman & Takahashi, 2001). Algae acquire sulfur from the environment as SO_4^{2-} , and SO_4^{2-} uptake is regulated by sulfate availability itself (Yildiz *et al.*, 1994; Pollock *et al.*, 2005).

The sulfur assimilated by algae finds functional use in three main classes of biomolecule. There is a universal requirement for sulfur in proteins. The S-bearing amino acid cysteine forms disulfide bridges used to stabilize protein conformation, and a second S-bearing amino acid, methionine, is the starting material for protein synthesis (Adams & Capecchi, 1966, Webster *et al.*, 1966). There are indications that the abundance of S-amino acids can be modulated in response to changes in environmental S availability (Giordano *et al.*, 2000). Iron-sulfur clusters and thiol groups lie at the heart of molecular function in many enzymes and electron transport systems, and thiols also mediate the interaction of biomolecules with metals (Kawakami *et al.*, 2006). Sulfur occurs, as well, in sulfolipids, found in membranes. Sulfolipids are obligate constituents of the thylakoid membranes in chloroplasts (Goss & Wilhelm 2009) and can, in some instances, substitute facultatively for membrane phospholipids in P-limited environments (Van Mooy *et al.*, 2006).

Sulfonium compounds are positively charged molecules in which a single sulfur atom is linked to three functional groups. Algae and higher plants synthesize a variety of sulfonium compounds, of which 3-dimethylsulfoniumpropionate, or DMSP (Keller *et al.*, 1989; Ratti & Giordano, 2008), is the best known. Many marine algae (and some halophytic angiosperms) produce DMSP; intracellular concentrations range from 50 to 400 mM, comprising a measurable proportion of all organic S in the cell (Matrai & Keller, 1994; Keller *et al.*, 1999; Wolfe, 2000; Yoch, 2002; Ratti & Giordano, 2008).

Several functions have been attributed to DMSP. Inspired by the Gaia hypothesis, Charlson and colleagues (1987) suggested that DMS released to the atmosphere by DMSP catabolism governs a feedback mechanism between algal growth and global temperature (see also Andreae & Crutzen, 1997). Other authors have focused attention on the role played by DMSP within the cell (Giordano *et al.*, 2008; Ratti & Giordano, 2008). DMSP has been proposed to act as an osmoprotectant (Stefels, 2000; Van Bergeijk *et al.*, 2003), a cryoprotectant (Karsten *et al.*, 1996; Rijssel & Gieskes, 2002), an antioxidant (Sunda *et al.*, 2002; Bucciarelli & Sunda, 2003) and a molecular defense against grazing, thanks to the toxicity of acrylate produced during DMSP catabolism (Wolfe *et al.*, 1997; Wolfe, 2000). There is also evidence that intracellular DMSP concentration increases under conditions of nitrogen depletion (Bucciarelli & Sunda, 2003), perhaps because DMSP is a structural analogue of glycine betaine, an ammonium compound that acts as a compatible solute in many marine algae (Rhodes & Hanson, 1993).

Elemental composition has been assayed for relatively few algae (e.g. Ho *et al.*, 2003; Quigg *et al.* 2003, 2011), but data in hand suggest that chlorophyll a+c algae have lower C:S than green algae and cyanobacteria (Norici *et al.*, 2005). Why this should be so remains conjectural, although one study reported that the freshwater diatom *Cyclotella meneghiniana* contains significantly more sulfolipid than the freshwater green alga *Chlamydomonas reinhardtii* (Vieler *et al.*, 2007). To the extent that lower C:S is a general feature of chlorophyll a+c algae (Goss & Wilhelm, 2009), it provides a potential physiological link between Phanerozoic increase in marine sulfate concentrations and the evolutionary expansion of diatoms, coccolithophorids and dinoflagellates.

MATERIALS AND METHODS

Cultures

For our first experiment, on the impact of sulfate concentration *per se*, semi-continuous cultures of the cyanobacterium *Synechococcus* sp. (UTEX LB 2380), the green alga *Tetraselmis suecica* (PCC 305), the diatom *Thalassiosira weissflogii* (CCAP 1085/1), the coccolithophorid *Emiliana huxleyi* (PML 92/11), and the dinoflagellate *Protoceratium reticulatum* (PRA 0206) were grown axenically in 150 mL glass tubes (19 cm x 3.5 cm) filled with 100 mL of ESAW (Enriched Seawater Artificial Medium; Berges *et al.*, 2001) buffered with 10 mM Tris-HCl, pH 8.0. Each species was cultured in the presence of 1 mM, 5 mM, 10 mM, 20 mM or 30 mM Na₂SO₄ in order to mimic differing marine [SO₄²⁻] from the Proterozoic to the present. The osmolarity of the medium was kept constant at 0.45 mol L⁻¹ using NaCl.

Cultures were maintained at 20°C under a continuous photon flux density (PFD) of 120 µmol photons·m⁻²·s⁻¹, provided by cool white fluorescent tubes. All experiments were carried out on cells in the exponential growth phase allowed to grow at the given SO₄²⁻ concentration for at least 4 generations prior to any measurement.

For a second experiment, designed to assess the impact of grazers on the response to changes in sulfate concentrations, the ciliate *Euplotes* sp. was added to monospecific algal cultures in a proportion of about 0.05-0.1 µg of algal C per ciliate cell. Growth conditions for these cultures were the same as for the first experiment. We were unable to grow *E. huxleyi* in the presence of ciliates, and so this species was omitted from subsequent experiments.

For a third set of experiments, phytoplankton species were grown in reconstructed paleoenvironments. Three media were used, all based on ESAW: one was designed to mimic the typical composition of today's ocean; another was intended to approximate conditions in earlier Paleozoic oceans, and a third had a composition thought to mirror the composition of Proterozoic oceans. Sulfate levels in our Paleozoic and Proterozoic media are based on those shown in Figure 1 (see also Shen *et al.*, 2002; Kah *et al.*, 2004 for Proterozoic [SO₄²⁻]). Nitrogen, iron, and molybdenum values are based on estimates in Anbar and Knoll (2002), Saito *et al.* (2003), Scott *et al.* (2008), and Canfield *et al.* (2008); while single values stand in for abundances through long time intervals, the media we prepared faithfully reflect the view that nitrogen in Proterozoic photic zones was predominantly ammonium, and both Fe and Mo levels were higher than in younger oceans. P_{CO₂} of the air in equilibrium with our Paleozoic medium was based on

model estimates by Berner (e.g. Berner & Kothvala, 2001); Proterozoic P_{CO_2} was based on the estimate by Kaufman & Xiao (2003). Table 1 shows the ESAW modifications and P_{CO_2} in equilibrium with these media for each treatment. In these experiments, we grew monospecific cultures of *Synechococcus* sp., *Tetraselmis suecica*, *Thalassiosira weissflogii*, and *Protoceratium reticulatum*, as well as mixed cultures initially containing equal cell numbers of each species.

The mixed cultures were started from semicontinuous monospecific cultures, acclimated to each growth regime for at least 4 generations. The cultures were diluted daily at the maximum rate of the slowest growing species (*P. reticulatum*), to ensure that the selection of species was not due to the fact that the slowest growing species were washed out. The cultures were maintained for at least 15 days, prior to sampling; this time was determined to be sufficient for the cultures to reach equilibrium (i.e. in all case no further change in relative cell abundance occurred after this time; in fact, equilibration usually occurred after about 5-10 days).

Growth rate, cell size and dry weight

Cell concentrations of *Synechococcus* sp., *T. weissflogii*, *T. suecica*, and *E. huxleyi* were estimated with a Burker hemocytometer, while the number of the larger *P. reticulatum* cells was measured using a Sedgwick-Rafter chamber (McAlicie, 1971). Specific growth rates, μ , were derived from daily counts of exponentially growing cells in batch cultures, carried out on a minimum of three distinct cultures for each treatment. These growth rate were used to establish the dilution rates of semicontinuous cultures.

The volume of *T. weissflogii* cells was calculated from microscope measurements of the longitudinal and transverse axes of cells, using the equation $V = \pi d^2 h / 4$ (Hillebrand *et al.*, 1999). *T. suecica* cell volume was calculated from measurements of the longitudinal and transverse axes of the cells using the volume equation for a prolate spheroid $V = \pi d^2 h / 6$ (Hillebrand *et al.*, 1999). *E. huxleyi* and *P. reticulatum* cell volumes were estimated from measurements of the transverse axis, using the equation $V = \pi d^3 / 6$ (Hillebrand *et al.*, 1999). Measurements were conducted on three different cultures; 60-100 cells were measured for each culture. *Synechococcus* sp. cell volume was measured using a CASY TT cell counter (Innovatis AG, Reutlingen, Germany).

For dry weight determination, cells were washed with an ammonium formate solution isosmotic to the culture media and dried at 100°C until weight stabilized. Measurements were conducted for at least three independent cultures.

Elemental Composition: C:N:S and C, N, S quotas

Cellular abundances of C, N and S were determined using an elemental analyzer (EA1108, Carlo Erba Instruments, Milan, Italy) on 1-6 mg of cells (dry weight) washed twice with an ammonium formate solution isosmotic to the culturing media and dried at 80°C until the weight stabilized. To facilitate combustion in the reactor of the elemental analyzer, a small amount of vanadium peroxide was added to each sample. Sulphanilamide (C:N:S= 6:2:1) was used as a standard. Elemental quotas were calculated as pico- or femto-grams per cell, normalized to cell dry weight and to cell volume. The oven temperature was set 1020°C, the column temperature at 70°C, and the detector temperature at 190°C. The He flow was 100 mL min⁻¹; the reference He flow was 40 mL min⁻¹; and the O₂ flow was 20 mL min⁻¹. Data acquisition and analysis were performed with the software EAS-Clarity (DataApex Ltd. 2006, Czech Republic). All measurements were repeated for four independent cultures.

DMSP measurement

A relationship between C:S and DMSP has been proposed (Norici *et al.*, 2005; Takahashi *et al.*, 2011). We therefore measured DMSP in all growth regimes to assess if such relationship existed. Cells were filtered on glass fiber filters (GF/F; Whatman, Maidstone, England) and transferred into 12 mL glass vials; 6 mL of ice cold 5N NaOH were added, and the vials were quickly closed using screw caps with rubber septa. Samples were incubated overnight in the dark, at 4 °C (Steinke *et al.* 1998). DMS generated by the alkaline hydrolysis of DMSP was measured with a modification of the method described by van Bergeijk and Stal (1996). A 25 µL gas aliquot was taken from the headspace of the vials using a gas-tight syringe and injected into a gas chromatograph (Focus, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a capillary column made of a polystyrene-divinylbenzene copolymer (RT-Q PLOT; 0.53 mm; 30 m, Restek, PA, USA) and a flame ionization detector (FID). The retention time of DMS was ~4.7 minutes. DMSP standards (Research Plus Inc, Barnegat, NJ, USA) were treated as the samples and used for calibration. Spectra were acquired and elaborated using the Chrom-Card

Software (Thermo Fischer Scientific, Waltham, MA, USA), with measurements repeated for at least three independent cultures.

Chlorophyll Fluorescence

The chlorophyll fluorescence associated to PSII was determined to obtain a general indication of the health conditions of the algae and of the functional status of their photosynthetic apparatus, under the various growth regimes. For these measurements, we used a Dual-PAM-100 fluorometer provided with an optical unit ED-101US/MD for suspensions (Walz GmbH, Effeltrich, Germany; Quigg *et al.*, 2006). Cells were collected by filtration (*P. reticulatum*) or centrifugation (*T. suecica*, *T. weissflogii*, *Synechococcus* sp. and *E. huxleyi*), washed and resuspended in fresh medium. The samples were dark adapted for 15 min, an interval previously determined to be sufficient for complete oxidation of the electron transport chain. A 1.5 mL aliquot of cell suspension was used for the measurement. The measuring beam was supplied at an irradiance of $24 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and a frequency of 20 KHz. The saturating pulse had an intensity of $10,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and a duration of 300 ms. The actinic light was provided at a photon flux density similar to that used for growth ($126 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). The maximum fluorescence yield and the dark fluorescence yield of dark adapted (F_m and F_o , respectively) and illuminated (F_m' and F_o') cells were determined. The maximal quantum yield of PSII and the quenching parameters qP , qL , qN and NPQ were derived from these measurements according to Schreiber *et al.* (1986) and Kramer *et al.* (2004). Data acquisition and analysis were conducted using the Dual-PAM v1.8 software (Walz GmbH, Effeltrich, Germany).

Statistics

Data are reported as mean $\pm$ standard deviation for measurements obtained from at least three distinct cultures. Statistical significance of differences among the means was determined by analysis of variance (ANOVA) and Tukey's post-hoc test, using GraphPad Prism 4.03 software (GraphPad Software, San Diego, CA, USA), with the level of significance set at 95%.

RESULTS

Experiment 1: Sulfate availability and microalgal growth

In our initial experiment, we simply varied the amount of sulfate in growth media, holding other environmental parameters constant. Our 1 mM to 5 mM $[\text{SO}_4^{2-}]$ treatments probably bracket levels actually experienced by early cyanobacteria and green algae radiating in Neoproterozoic and earlier Paleozoic oceans; whereas the 10 mM to 20 mM $[\text{SO}_4^{2-}]$ levels approximate sulfate abundances in the late Paleozoic and Mesozoic oceans that supported early dinoflagellates, coccolithophorids and diatoms (Fig. 1). Many physiological parameters show no statistically significant variation with changing $[\text{SO}_4^{2-}]$; these data are reported as Supplementary Information. One parameter that does show significant variation is specific growth rate (μ = proportional increase in biomass per day during exponential growth phase). Barton *et al.* (2010) argue that high growth rate is the most appropriate measure of organismic fitness in phytoplankton. Certainly, differential growth rate is a property we need to track in experimental tests of evolutionary hypotheses.

Neither the cyanobacterium, *Synechococcus* sp., nor the green alga, *Tetraselmis suecica*, showed any consistent response to increasing sulfate availability (Fig. 2). In contrast, the specific growth rates of all three chlorophyll a+c algae increased with increasing sulfate availability, two of them (the dinoflagellate *Protoceratium reticulatum* and the coccolithophorid *Emiliana huxleyi*) significantly (Fig. 2). Our chlorophyll a+c species all grew most slowly in the 1 mM sulfate treatment; indeed, *Protoceratium reticulatum* did not grow at all. All showed improved growth at 5 mM, and two, *P. reticulatum* and *E. huxleyi*, grew faster still at 10 mM. No increase in growth rate was observed at sulfate abundances above 10 mM. Thus, these species showed increased growth up to the sulfate levels experienced by their early ancestors, but not beyond that level. Consistent with these results, non-photochemical quenching, a physiological measure of stress (*sensu lato*), is most pronounced in the chlorophyll a+c species grown at low $[\text{SO}_4^{2-}]$ (see Supplementary Information).

C:S mirrors these contrasting growth patterns. *Synechococcus* sp. and *Tetraselmis suecica* show no variation in C:S with increasing sulfate abundance, whereas the three chlorophyll a+c algae (especially *P. reticulatum*) do (Fig. 3). Increased S content does not relate to increasing DMSP, as DMSP:S quota did not vary with $[\text{SO}_4^{2-}]$ in any of the three species that produced this

compound (Fig. 3; the diatom *Thalassiosira weissflogii* did not produce DMSP). C:N also shows no strong relationship with $[\text{SO}_4^{2-}]$ (Fig. 3).

Experiment 2: Sulfate availability and microalgal growth in the presence of a grazer

In actual oceans, predation can exert a strong influence on net growth rates. To examine whether the growth relationships observed in our first experiment are robust when grazers are present, we ran the experiment again, but with a ciliate, *Euplotes* sp., present. Our dinoflagellate, *P. reticulatum*, formed a protective cyst upon introduction of the ciliate predator and so was not considered further. Also, rates of predation on *E. huxleyi* outstripped growth rate, in time eliminating the algal population. Thus, we were only able to compare our cyanobacterium, green alga and diatom across the full spectrum of sulfate abundances (Fig. 4). For *T. suecica* and *T. weissflogii*, specific growth rate increased for all treatments when *Euplotes* sp. was present. In this experiment, however, *T. suecica* did show differences in growth rate at different $[\text{SO}_4^{2-}]$, whereas *T. weissflogii* did not. Specifically, when grown in the presence of *Euplotes* sp., *T. suecica* showed no increase in specific growth rate relative to pure culture at 1 mM $[\text{SO}_4^{2-}]$, but did at higher sulfate concentrations. An opposite result was observed in *Synechococcus* sp.; when the cyanobacterium was cultured in the presence of grazers the specific growth rate was not affected (at 5 mM or 30 mM $[\text{SO}_4^{2-}]$) or decreased significantly (1 mM, 10 mM and 30 mM $[\text{SO}_4^{2-}]$; Fig 4). Except at the lowest sulfate level, *T. suecica* grown with the ciliate had a C quota (amount of carbon per cell) about twice that of cells grown in pure culture (Fig. 3). As cell volume is unchanged in these treatments (Supplementary Information), the amount of organic material per cell must be higher in cells subject to grazing. C:S did not vary among treatments for our experimental green alga (Fig. 3), suggesting a relatively tight linkage between C and S uptake. Perhaps sulfate limitation at 1 mM $[\text{SO}_4^{2-}]$ prevents the physiological response observed in other treatments with predators present. Our diatom species shows no similar response at low $[\text{SO}_4^{2-}]$. The presence of *Euplotes* sp. in the *Synechococcus* sp. culture resulted in a decrease in C:N at lower $[\text{SO}_4^{2-}]$ (1 mM, 5 mM and 10 mM) and a decrease of about 50% in C:S at all $[\text{SO}_4^{2-}]$.

Experiment 3: Sulfate availability and microalgal growth when multiple environmental parameters are allowed to vary.

While experiments on axenic cultures of single species identify changes in growth rate with changing $[\text{SO}_4^{2-}]$, they do not directly measure competitive ability. For this reason, we completed a third set of experiments, asking how our experimental species fare when grown together. Under culture conditions designed to emulate present day seawater (“modern” in Table 1 and Figs. 5 and 6), our diatom species not only had the highest growth rates in axenic culture, but also the highest growth rate in mixed culture, identifying it as the best competitor under the specified culture conditions.

A complicating issue is that sulfate was not the only environmental variable to change systematically through Earth history. The availability of trace metal micronutrients has changed as a function of marine redox state (Anbar & Knoll, 2002; Saito *et al.*, 2003), and P_{CO_2} has decreased through time (albeit not monotonically) as well (Berner & Kothavala, 2001). Thus, in our third set of experiments we grew photosynthetic microorganisms in a series of culture conditions designed to mimic chemical environments in Proterozoic, Paleozoic and modern oceans. As shown in Table 1, our Paleozoic treatment differs from present day conditions in having lower sulfate (14 mM) and higher P_{CO_2} (2500 ppm); the Proterozoic treatment has still lower sulfate (3 mM), higher P_{CO_2} (5000 ppm), lower metal concentrations, and ammonia rather than nitrate as the source of nitrogen.

Most species exhibited higher growth rates under the two “ancient” seawater conditions, although not always significantly so (Fig. 5). *T. weissflogii* doubled its specific growth rate in the Proterozoic treatment, perhaps as a function of iron availability. Growth of *P. reticulatum* scaled with P_{CO_2} . Only the cyanobacterium *Synechococcus* sp. did not show monotonic increases in growth with successively “older” treatments. For reasons we cannot explain, *Synechococcus* sp. grew most poorly under Paleozoic conditions. Note that relative specific growth rates observed in the present day treatment of this experiment are consistent with those found in the 30 mM $[\text{SO}_4^{2-}]$ treatment in experiment 1.

We observed a number of responses when the species were grown in mixed rather than pure culture. *T. suecica* showed the same pattern of differential growth among treatments as in the pure culture experiment, but at lower absolute specific growth rates. In mixed culture

Synechococcus sp. grew more slowly in the present day treatment than its growth in pure culture would have predicted; this may be a direct response to the growth of *T. weissflogii*, which showed a much higher growth rate in mixed culture than would be predicted from the pure culture experiments. Evidently, *T. weissflogii* is a superior competitor in our modern seawater experiment. *P. reticulatum* did not grow at all in the mixed cultures.

Whereas our diatom grew faster than other species in the mixed culture experiment in modern seawater, consistent with diatom prominence in present day primary production, our green alga outgrew the other taxa in Paleozoic seawater. Our cyanobacterium grew best in Proterozoic seawater, but, as in all other treatments, grew more slowly than the two algal species in the mixed culture.

DISCUSSION

Our experiments were designed to test an evolutionary hypothesis inspired by an observed stratigraphic correlation between seawater chemistry and phytoplankton composition through earth history. Existing data on the chemical composition of phytoplankton cells indicate that green algae and cyanobacteria, dominant primary producers in Proterozoic and Paleozoic shelf waters, have higher C:S than diatoms, dinoflagellates, and coccolithophores, dominant shelf phytoplankton since the later Mesozoic Era. As more species are analyzed, this distinction may turn out to be statistical rather than absolute, but then the fossil record is a statistical digest of phytoplankton composition through time. The changing taxonomic composition of the phytoplankton parallels long terms changes in the abundance of sulphate in seawater, motivating the hypothesis that increasing $[\text{SO}_4^{2-}]$ might have facilitated the expansion of algae with relatively low C:S. The only way to test this or any other hypothesis relating evolution and environmental change is by physiological experiment (see Zerkle *et al.*, 2006, and Glass *et al.*, 2009, for pioneering examples).

In our initial experiment, our dinoflagellate and coccolithophorid species grew poorly at the low sulphate concentrations likely to have characterized Proterozoic and early Paleozoic oceans, but showed a significant increase in growth rate at sulphate levels thought to have been reached only in late Paleozoic and younger oceans. Our experimental diatom also showed

increased growth at higher $[\text{SO}_4^{2-}]$, but not with statistical significance. In contrast, our green and cyanobacterial species showed no directional growth response to increasing $[\text{SO}_4^{2-}]$.

These results are consistent with the sulphate facilitation hypothesis, but they do not rule out other influences. For example, the observation that *T. weissflogii* grew more rapidly than *T. suecica* in all treatments is consistent, as well, with Kooistra and colleagues' (2007) view that diatom success owes much to clade-specific adaptations. Differences among experimental organisms in growth at very low $[\text{SO}_4^{2-}]$ also correlate with the relative success of different photosynthetic clades in freshwater. Most green algal and cyanobacterial species live in lakes, rivers or soils, as do nearly half of diatom species, but only a small proportion of dinoflagellates and no coccolithophorids (Falkowski *et al.*, 2004).

Our second experiment, in which a predator was introduced to algal cultures, demonstrates that growth responses to changes in the physical environment can be modified by changes in biological environment. The relative specific growth rates between our green alga and diatom species grown at differing $[\text{SO}_4^{2-}]$ were retained in experiment 2, but within species responses to sulfate availability were not. In continuing experiments we hope to pursue this further, including experimental treatments that add metazoan micrograzers such as copepods.

In our third set of experiments, when the experimental algae were grown individually under culture conditions designed to simulate modern, Paleozoic and Proterozoic seawater, all species exhibited higher growth rates under more "ancient" conditions, perhaps reflecting the fertilizing effects of elevated iron and CO_2 . When species were grown in mixed culture, the species that grew best in the modern and paleozoic treatments were those that were most prominent in the modern and Paleozoic records – diatoms for the present day and green algae for the Paleozoic Era. Thus, our experimental results are all consistent with the hypothesis that increasing sulfate played a role in the large scale pattern of phytoplankton succession observed in the geologic record. Moreover, they are consistent with the additional observation that green algae were unusually prominent phytoplankton components during the two Mesozoic intervals when sulphate levels are thought to have declined transiently to low levels (Payne & van de Schootbrugge, 2007; Luo *et al.*, 2010; Newton *et al.*, 2011).

That stated, when taken as a whole, our experiments caution against simplistic interpretation of these results. Through geologic time, as $[\text{SO}_4^{2-}]$ increased, P_{CO_2} decreased, and so did the abundance of redox sensitive metals such as Fe. Thus, decreasing iron availability

might have had the evolutionary consequences postulated by Quigg *et al.* (2003), although physiological effects of changing Fe on phytoplankton growth and competitive ability have yet to be tested directly by experiments. (Our results in experiment 3 show that the diatom, *T. weissflogii*, actually grew best in the iron-rich Proterozoic medium, suggesting that the Fe hypotheses might usefully be restated to postulate that chlorophyll a+c algae grow less poorly than greens when iron is scarce). Our results also suggest that grazing affects growth rates under different nutrient conditions and does so differently for different taxa. Calanoid copepods and other pelagic planktivores have a scant fossil record (Selden *et al.*, 2010), but may well have invaded the pelagic realm on time scales relevant to the rise of armored chlorophyll a+c phytoplankton (Bradford-Grieve, 2002). Finally, the relatively high growth rates achieved by our diatom species over a wide range of culture conditions are consistent with Kooistra *et al.* (2007) hypothesis that adaptations specific to diatoms explain their extraordinary ecological and evolutionary radiation.

CONCLUSIONS

The experiments described in this paper provide but a first step toward understanding the influence of changing marine environments on phytoplankton evolution through time. Further experiments are necessary to show to what extent the species we have chosen are representative of larger clades. Nonetheless, the results permit some first order tests of our motivating hypothesis.

While more experiments need to be run, it is not too early to suggest that existing hypotheses to explain the geologic succession of primary producers in the oceans are not so much wrong as incomplete. The rise of chlorophyll a+c phytoplankton may not reflect the influence of sulfate availability *vs.* Fe availability *vs.* increasing predation pressure *vs.* individual adaptations so much as the synergistic influences of *multiple* physical and biological factors that have covaried through time. Secular variation in sulfate abundance will play a role in continuing explanations, but not to the exclusion of other influences.

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FIGURE LEGENDS:

Figure 1. Sulfate concentration and different taxa phytoplankton radiation over geological time. Paleobiologic data from Falkowski *et al.* (2004); data on sulphate abundances from Gill *et al.* (2007, 2011), Warren (2010), and sources therein.

Figure 2. Specific growth rate of *T. suecica*, *T. weissflogii*, *P. reticulatum*, and *E. huxleyi* cells acclimated to 1 mM, 5 mM, 10 mM, 20 mM or 30 mM [SO₄²⁻]. Error bars indicate standard deviations about the mean (n ≥ 3).

Figure 3. Panel A: C:N ratio in *T. suecica*, *T. weissflogii*, *Synechococcus* sp., *P. reticulatum*, and *E. huxleyi* cells cultured in the presence of five different sulfate concentrations. Panel B: C:N ratio in *T. suecica*, *T. weissflogii* and *Synechococcus* sp. cells cultured in the presence of five different sulfate concentrations and the ciliate predator *Euplotes* sp. Panel C: C:S ratio in *T. suecica*, *T. weissflogii*, *Synechococcus* sp., *P. reticulatum*, *E. huxleyi* cells acclimated to five different sulfate concentrations. Panel D: C:S ratio in *T. suecica*, *T. weissflogii* and *Synechococcus* sp. cells acclimated to five different sulfate concentrations and to the presence of *Euplotes* sp. Panel E: Ratio of DMSP cell content to cell S quota in *T. suecica*, *T. weissflogii*, *P. reticulatum*, and *E. huxleyi* cells acclimated to five different sulfate concentrations. Panel F: DMSP cell content; cell S quota in *T. suecica* cells acclimated to five different sulfate concentrations in the presence of *Euplotes* sp. Error bars indicate standard deviation about the mean (n ≥ 3).

Figure 4. Effect of the presence of *Euplotes* sp. on the specific growth rates of *T. suecica*, (panels A, B) *T. weissflogii* (panels C, D) and *Synechococcus* sp. (panels E, F) cells cultivated at five different sulfate concentrations. Error bars indicate standard deviations about the mean (n = 3).

800

801 **Figure 5.** Specific growth rate (μ) of *T. suecica*, *T. weissflogii*, *P. reticulatum* and
802 *Synechococcus* sp. cells cultivated individually or in mixed cultures in media mimicking the
803 chemical compositions of Proterozoic, Paleozoic or present day seawater, binned by species.
804 Error bars indicate standard deviations about the mean ($n = 3$).

805

806 **Figure 6.** Specific growth rate (μ) of *T. suecica*, *T. weissflogii*, *P. reticulatum* and
807 *Synechococcus* sp. cells cultured in media mimicking the chemical compositions of Proterozoic,
808 Paleozoic or present day seawater; binned by treatment. Error bars indicate standard deviations
809 about the mean ($n = 3$).

810

811 **Table 1.** Pure and mixed cultures were grown in media constructed to approximate aspects of
 812 modern, Paleozoic and Proterozoic seawater. See text for justification of parameters.

813

	Present Day	Paleozoic	Proterozoic
P_{CO_2}	384 ppm	2500 ppm	5000 ppm
NO_3^-	10 μ M	10 μ M	-
NH_4^+	-	-	10 μ M
$SO_4^{=}$	28 mM	14 mM	3 mM
Fe	50 nM	50 nM	380 nM
Zn	100 nM	100 nM	-
Mo	105 nM	105 nM	10.5 nM

814