

Phosphorus-Zinc Interactive Effects on Growth by *Selenastrum capricornutum* (Chlorophyta)

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■ Culturing experiments in chemically defined growth media were conducted to observe possible Zn and P interactions on *Selenastrum capricornutum* Printz growth indexes. Elevated Zn concentrations (7.5×10^{-8} and 1.5×10^{-7} M [Zn^{2+}]) were highly detrimental to algal growth, affecting lag, exponential, and stationary growth phases. P behaved as a yield-limiting nutrient with maximum cell densities increasing linearly with total P. This yield limitation was intensified at elevated Zn concentrations. Although calculated cellular phosphorus concentrations increased markedly with Zn ion activity, elevated Zn concentrations had no apparent effect on rates of phosphorus uptake estimated for *Selenastrum* during exponential growth. Results indicated that P-Zn interactions were significant in describing *Selenastrum* cell yield results and are consistent with previous Zn studies on chlorophytes. These P-Zn interactions and the observed inhibitory growth effects of submicromolar Zn concentrations suggest that in nature an apparent P yield-limiting condition may result from elevated Zn concentrations.

Introduction

The degree to which soils and natural waters may support nutritional needs of biota is dependent on numerous interactive processes. Direct field application of results from laboratory experiments that monitor biological response to various concentrations of a single nutrient or toxicant may often be inappropriate because significant nutrient interactions are not understood. A growing body of information indicates that nutrient availability to biota involves interactions with physical, chemical, and biological parameters (1-4). An understanding of how and to what

extent these interactions affect organisms is essential in developing models that describe and predict environmental impacts.

Interactions involving micronutrient availability, for example, have generated increasing environmental concerns (2, 3). There may be only submicromolar differences between micronutrient concentrations that generate beneficial effects and those that impose growth (yield or rate) inhibition. Since Eiler (5) first demonstrated that zinc is essential for growth of *Stichococcus bacillaris* (chlorophyta), Zn^{2+} has consistently been added to algal culturing media. Biochemical significance of zinc as a cofactor in numerous enzyme systems and in indoleacetic acid synthesis has been well established (6). Cases of zinc deficiency in algae have been cited by numerous workers (7-9). This essential micronutrient, however, may also induce toxic response at submicromolar activity (9, 10). *Macrocystis pyrifera* (phaeophyta) gametophytic growth is inhibited at a computed Zn ion activity of approximately 10^{-10} M (11). One micromolar total Zn added to seawater (background total Zn $\sim 10^{-7}$ M) reduced growth rates of *M. pyrifera* juveniles (12). Bartlett et al. (10) reported a noticeable increase in lag-phase duration of the freshwater chlorophyte *Selenastrum capricornutum* due to elevated trace metal (viz., copper, zinc, and cadmium) concentrations. Total Zn additions in the micromolar range to synthetic algal nutrient medium (SANM) (13) reduced 14-day cell yield of *S. capricornutum* by as much as an order of magnitude (14). This toxic Zn addition represented a Zn ion activity of $\sim 10^{-8}$ M in SANM.

Some suggestions in the literature indicate phosphorus (P), an essential macronutrient that often limits algal biomass in nature (15, 16), may have an interactive effect

Table I. *Selenastrum* Culturing Data with 95% CI ($n = 3$ Treatment Replicates) for Nine Treatments at Various Initial P and Zn Concentrations

treatments ^a		growth indexes \pm 95% CI ^b			
initial phosphate concn	initial zinc concn	L	G	S	P _T /S
(2.0 \times 10 ⁻⁶ , 1 \times 10 ⁻¹¹)	(<10 ⁻⁶ , <10 ⁻⁹)	-	1.9 \pm 0.2	1.1 \pm 0.4	1.9 \pm 0.6
(4.0 \times 10 ⁻⁶ , 2 \times 10 ⁻¹¹)	(<10 ⁻⁶ , <10 ⁻⁹)	-	1.9 \pm 0.1	2.1 \pm 0.3	1.9 \pm 0.3
(6.0 \times 10 ⁻⁶ , 5 \times 10 ⁻¹¹)	(<10 ⁻⁶ , <10 ⁻⁹)	0.2 \pm 0.1	1.9 \pm 0.3	3.1 \pm 0.6	2.0 \pm 0.4
(2.0 \times 10 ⁻⁶ , 1 \times 10 ⁻¹¹)	(7.5 \times 10 ⁻⁶ , 7.5 \times 10 ⁻⁹)	1.0 \pm 0.4	1.6 \pm 0.4	0.9 \pm 0.3	2.0 \pm 1.4
(4.0 \times 10 ⁻⁶ , 2 \times 10 ⁻¹¹)	(7.5 \times 10 ⁻⁶ , 7.5 \times 10 ⁻⁹)	0.9 \pm 0.2	1.7 \pm 0.3	1.6 \pm 0.4	2.0 \pm 0.5
(6.0 \times 10 ⁻⁶ , 5 \times 10 ⁻¹¹)	(7.5 \times 10 ⁻⁶ , 7.5 \times 10 ⁻⁹)	0.9 \pm 0.4	1.8 \pm 0.3	2.3 \pm 0.5	2.0 \pm 0.6
(2.0 \times 10 ⁻⁶ , 1 \times 10 ⁻¹¹)	(1.5 \times 10 ⁻⁷ , 1.5 \times 10 ⁻⁷)	6.9 \pm 0.4	0.7 \pm 0.2	0.2 \pm 0.1	16.6 \pm 7.5
(4.0 \times 10 ⁻⁶ , 2 \times 10 ⁻¹¹)	(1.5 \times 10 ⁻⁷ , 1.5 \times 10 ⁻⁷)	6.8 \pm 0.5	0.9 \pm 0.2	0.5 \pm 0.3	8.4 \pm 4.8
(6.0 \times 10 ⁻⁶ , 5 \times 10 ⁻¹¹)	(1.5 \times 10 ⁻⁷ , 1.5 \times 10 ⁻⁷)	6.6 \pm 0.3	1.0 \pm 0.2	0.9 \pm 0.3	6.4 \pm 2.3

^a Molar phosphorus and Zn concentrations are given in the following format: (total concentration, computed free ion concentration).
^b Symbols L, G, and S denote growth lag phase (in days), growth rate (in doublings-day⁻¹), and stationary phase cell density (in 10⁶ cells·mL⁻¹), respectively. Column labeled P_T/S (10⁻¹⁵ mol of P/cell) is the ratio representing an estimate for cellular P concentration during exponential growth. Symbol (-) indicates that lag phase was not significantly greater than 0 by Student's *t* test.

with Zn. Bates et al. (17) observed a reduction in cell yield by *Chlamydomonas variabilis* (chlorophyta) in response to elevated Zn concentrations and attributed this effect to incomplete use of intracellular P. Conversely, Rana and Kumar (18) observed a mitigation of Zn toxicity to the chlorophyte *Chlorella vulgaris* and the blue-green *Plectononema boryanum* at elevated phosphate concentrations. Preliminary culturing experiments in chemically defined algal growth medium (S-3) indicated that, unlike previous copper studies on *S. capricornutum* (19, 20), elevated Zn concentrations significantly reduced stationary phase cell density. The fact that the cell yield (stationary phase cell density, S) in S-3 medium is P limited (21) prompted this study to closely examine P-Zn effects on *S. capricornutum* growth indexes.

Experimental Section

Selenastrum cells were batch cultured in a chemically defined medium designed to produce a virtually Zn-free (<1 nM total Zn) basal medium (S-3) (21). Previous studies (21) indicated that cell yield (S) in S-3 was limited by P. All nutrient stock solutions, with the exception of trace element solutions, were eluted through Chelex-100 (100-200 mesh; Bio-Rad Laboratories, Richmond, CA) to remove cationic impurities (22). Trace elements were then added to produce desired initial micronutrient concentrations. S-3 media were finally filter sterilized (0.45 μ m) (MF-type; Millipore Corp., Bedford, MA). Clean techniques and culturing methodology described by Kuwabara and North (23) were consistently employed in all phases of experimentation to avoid trace metal contamination of culturing media. Product names are for identification purposes only and do not constitute endorsement by the U.S. Geological Survey.

Initial total P (P_T) at 6, 4, and 2 μ M and initial Zn free ion concentration ([Zn²⁺]₀) at 1.5 \times 10⁻⁷, 7.5 \times 10⁻⁸, and <10⁻⁹ M (i.e., no Zn addition to basal S-3) were used in a 3² factorial experimental design with three replicates for each of the nine treatments (Table I). Concentrations for P and Zn were selected such that stationary growth phase would be attained within the 14-day culturing period and so that P and Zn effects would be discernible over the selected concentration ranges. Total concentrations of other S-3 chemical constituents were not varied between treatments (Table II).

Chemical speciation of growth media was calculated by using the computer program MINEQL (24). α,α' -(1,2-Ethanediyldiimino)bis[2-hydroxybenzene]acetic acid (EDDHA), a particularly strong iron chelator (25), was added to S-3 at a concentration equal to the total iron

Table II. Molar Concentrations of Those Macronutrients Micronutrients, and EDDHA Held Constant in S-3 for All Nine Experimental Treatments

nutrient or chelator	total concn	computed free ion concn	major species (%)
metals			
Fe ³⁺	6.0 \times 10 ⁻⁷	9 \times 10 ⁻²⁰	FeEDDHA (100)
Mn ²⁺	2.1 \times 10 ⁻⁶	2 \times 10 ⁻⁸	Mn ²⁺ (97)
Cu ²⁺	<2 \times 10 ⁻⁶	<1 \times 10 ⁻¹⁰	Cu(OH) ₂ ⁰ (82)
Co ²⁺	6.0 \times 10 ⁻⁶	6 \times 10 ⁻⁸	co ²⁺ (97)
ligands			
NO ₃ ⁻	3.0 \times 10 ⁻⁴	3 \times 10 ⁻⁴	NO ₃ ⁻ (100)
MoO ₄ ²⁻	3.0 \times 10 ⁻⁶	3 \times 10 ⁻⁶	MoO ₄ ²⁻ (100)
B(OH) ₃ ⁻	3.0 \times 10 ⁻⁶	9 \times 10 ⁻⁶	HB(OH) ₂ (97)
EDDHA ⁴⁻	6.0 \times 10 ⁻⁷	1 \times 10 ⁻²²	FeEDDHA (100)

concentration to prevent ferric hydroxide precipitation. Stability constants for EDDHA reactions with Fe, Zn, Cu, Mg, and Ca (26) were added to the MINEQL thermodynamic data for computer calculations. In addition, log association constants for the one to one complexation of Co, Mn, and Cu with EDDHA were approximated at 17 (equal to the log association constant for ZnEDDHA). Results from these calculations indicated that chemical speciation of micronutrients besides iron in S-3 was controlled by inorganic complexation (Table II).

Teflon and linear polyethylene were used for all surfaces in contact with growth media to reduce wall sorption losses during the culturing period. Algae were batch cultured in 250 mL of S-3 at 24 \pm 0.05 $^{\circ}$ C with an initial algal density of 2 \times 10⁴ cell·mL⁻¹. Filtered air was supplied at 125 mL·min⁻¹ to mix media. Nonsynchronous cultures were continuously illuminated at approximately 14 W·m⁻² by cool-white fluorescent lamps. Over 14 culturing days, daily progress in each of the 27 cultures was monitored by measuring cell density using an electronic particle counter. Cells were also examined under light microscope to detect morphological changes. Regression of cell counts during exponential growth with time was used to determine growth rate (G) and lag-phase duration (L). The regression model used was log (c) = at + b, where c represents cell count at a given time, t refers to time elapsed after cell inoculation, and a and b are the calculated slope and intercept, respectively. This exponential growth model was employed to iteratively screen daily cell count data by analysis of variance (i.e., examining the change in residual mean square by addition of another day's cell counts), to avoid inclusion of cell counts during lag and stationary phase, and to provide consistency in growth rate determinations. Slope a and intercept b are related to G and

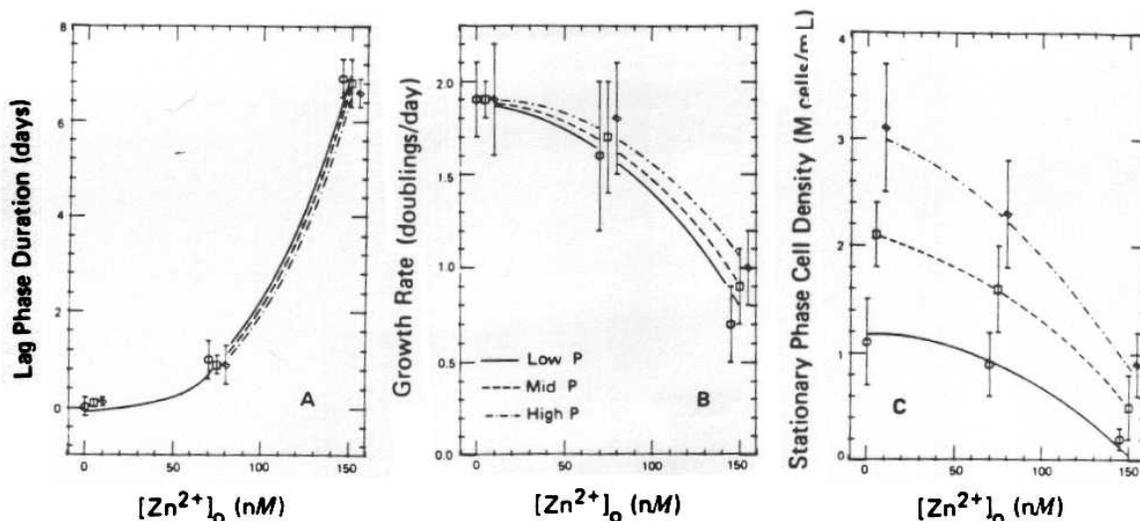


Figure 1. Zn and P effects on *Selenastrum*: (A) Lag-phase duration (days), (B) exponential growth rate (doublings-day⁻¹), and (C) stationary phase cell density (10⁶ cells·mL⁻¹). Circle, square, and diamond symbols represent growth indexes measured at low, middle, and high initial total P (P_{T_0}) concentrations, respectively. Confidence intervals ($p < 0.05$, $n = 3$ treatment replicates) as well as symbols have been horizontally spaced such that measurements shown for the same $[Zn^{2+}]_0$ do not overlap. Solid, dashed, and dash-dotted curves represent least-squares fits of experimental data for low (2 μ M), middle (4 μ M) and high (6 μ M) P_{T_0} , respectively. Equations defining these curves are listed along with a brief discussion of their derivation in the Appendix.

L , respectively, in the following way: $a = G \cdot \log 2$, and $b = \log c_0 - (a \cdot L)$, where c_0 represents initial cell density (27). Confidence intervals ($p < 0.05$, $n = 12$ daily cell counts per treatment) for both growth rate and lag phase duration were determined by assuming Student's t distribution (28).

Trace metal contamination is of particular concern when media like S-3 is used where trace metal activities (except for Fe) are poorly buffered. For example, total Cu concentration in S-3 (Table II) was undetectable by our graphite furnace atomic absorption spectroscopy methods (<2 nM total Cu). Without further addition of a synthetic chelator or defined particles to buffer Cu ion activity, the computed upper limit for free Cu ion activity (1×10^{-10} M) in S-3 is well within the activity range that may induce algal toxic response (29). As previously mentioned, considerable care was taken during all phases of experimentation to avoid trace metal contamination. Brown and Button (30) determined a maximum specific growth rate for *Selenastrum* of 1.2 day⁻¹ (or 1.7 doublings-day⁻¹) using a metal-buffered medium at 20 °C. Kuwabara et al. (21) observed a 1.8 doubling-day⁻¹ growth rate in control (i.e., not trace metal stressed) cultures at 24 °C using an ethylenediaminetetraacetic acid (EDTA) metal-buffered medium. The fact that a comparable growth rate of 1.9 doublings-day⁻¹ was achieved in S-3 cultures at low initial Zn suggests that precautionary measures used here to avoid trace contamination effects were successful.

Results and Discussion

Algal growth phase indexes (L , G , and S) determined for the nine experimental treatments are graphically represented in Figure 1 along with least-squares fits of the culturing data for each of the three initial total P concentrations. Results for all three growth phase indexes indicate that Zn was detrimental to *Selenastrum* over the concentration range used here. During lag phase, cells in high $[Zn^{2+}]_0$ took on an enlarged, granular appearance, reaching nearly twice their initial cell volume ($\sim 100 \mu$ m³). These apparently Zn-stressed cells appeared to slowly revert back to normal morphology upon initiation of exponential growth.

In contrast to Zn effects, P effects were only pronounced on S (Figure 1C). Yield limitation by P was noted at low initial Zn concentration with S varying linearly with P_{T_0} [approximately 5×10^6 cells·mL⁻¹·(μ M P_{T_0})⁻¹]. Although P effects were still evident at elevated Zn concentrations, yield limitation within the same P_{T_0} treatment group intensified. Cell yields (S) observed at 1.5×10^{-1} μ M $[Zn^{2+}]_0$ ranged from 0.2×10^6 to 0.9×10^6 cells·mL⁻¹ compared to a range of 1.1×10^6 to 3.1×10^6 cells·mL⁻¹ at low (no added) Zn activity. An interference (antagonistic interaction) on S was evident between P and Zn. Analysis of variance of S data indicated that independent P effects, for example, were insufficient in describing the extent to which S was enhanced by increasing P_{T_0} over the $[Zn^{2+}]_0$ range. Note how data as well as curve fits for S (Figure 1C) at a given $[Zn^{2+}]_0$ seem to separate more with decreasing $[Zn^{2+}]_0$.

Results presented here are distinct from previous algal micronutrient studies in two respects. First, elevated Zn concentrations primarily affected S rather than L or G in *Chlamydomonas variabilis* (chlorophyta) (17). Second, observed copper (Cu) effects on laboratory-cultured chlorophytes indicate G and S are insensitive toxicity indexes relative to lag-phase duration (10, 19, 31). Although both Zn and Cu are among essential micronutrients, algal response to elevated concentrations of these trace metals is different. These observations suggest that different toxicity mechanisms may exist between algal species for the same metal toxicant and within an algal species for different metal toxicants. It is, however, evident from this study and previous micronutrient studies on other phytoplankton species (9, 10, 18, 32) and Zn ion activities that have induced toxic algal response (10^{-9} – 10^{-7} M as Zn^{2+}) are similar to those determined for both polluted and unpolluted natural waters (9, 33). Zn input into receiving waters especially with low dissolved organic carbon concentrations (i.e., low Zn complexing capacity) may therefore inhibit algal growth or yield.

After the algal culturing period, dissolved phosphate was measured by isobutyl acetate extraction (34) in high P_{T_0} media (6 μ M P) for both high and low $[Zn^{2+}]_0$. Concen-

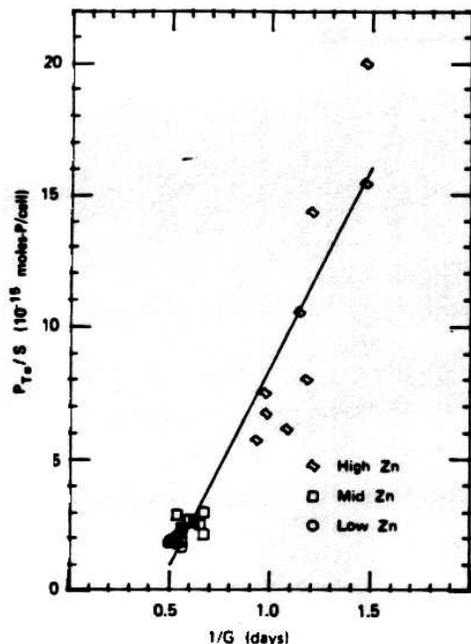


Figure 2. Cellular P concentrations for *Selenastrum* in relation to division time for all 27 cultures in the experimental design. Cellular P concentrations are expressed as the ratio P_{T_0}/S (10^{-15} mol of P/cell) where P_{T_0} is the initial total P concentration and S is the stationary phase cell density. Division time is expressed as $1/G$ (days), the reciprocal of the doubling rate. Circle, square, and diamond symbols represent data from low, mid-value, and high $[Zn^{2+}]_0$, respectively. The line drawn represents a least-squares fit of the data and is defined by the equation $P_{T_0}/S = 15.0(1/G) - 6.5$, with a χ^2 goodness of fit parameter of 8.8 for the 27 plotted points ($p > 0.995$). The slope of the line, and its 95% confidence interval [$(15.0 \pm 2.1) \times 10^{-15}$ mol of P-cell $^{-1}$ -day $^{-1}$], is an estimate of the P uptake rate for *Selenastrum* during exponential growth.

trations were $<0.1 \mu\text{M}$ dissolved phosphate in both samples, suggesting an efficient depletion of P during the algal culturing period. This result and the direct dependence of S on P_{T_0} support the hypothesis of yield limitation by P in S-3. In a P yield-limiting medium, the test organism exhausts the P supply to attain cell yield (S). The ratio of P_{T_0} to S therefore represents an average cellular P concentration. If this P_{T_0}/S ratio is calculated for the nine treatments (see Table I), there is close agreement between P_{T_0}/S values at low Zn ion activity but discernibly higher P_{T_0}/S values at elevated Zn activity (Figure 2). There is also a significant positive correlation ($r = 0.95$) between P_{T_0}/S and $1/G$ (the division time) for the 27 algal cultures (Figure 2). If one considers the possibility of a linear relationship between P_{T_0}/S and $1/G$, then the slope of that relationship represents an estimate of the P uptake rate averaged over a division time during exponential growth. From P growth kinetic experiments in continuous culture at 20°C , Brown and Button (30) observed P uptake rates for *Selenastrum* between 7 and $105 (\times 10^{-15})$ mol of P-cell $^{-1}$ -day $^{-1}$ over an external phosphate concentration range of 0.1 – $3.0 \mu\text{M}$. The P uptake rate estimated here (approximately 15×10^{-15} mol of P-cell $^{-1}$ -day $^{-1}$; Figure 2) falls within the range observed by Brown and Button (30). The range of cellular P content observed by Brown and Button [$(1.4$ – $4.1) \times 10^{-15}$ mol of P/cell] for *Selenastrum* is also similar to cellular P concentrations estimated here for low value and mid-value $[Zn^{2+}]_0$ cultures [$(1.8$ – $2.6) \times 10^{-15}$ mol of P/cell]. Cellular P estimates for the high $[Zn^{2+}]_0$ cultures [$(6.7$ – $10.0) \times 10^{-15}$ mol of P/cell] were, however, higher than observed by Brown and Button (30).

A growth half-saturation constant for *Selenastrum* of $48 \text{ nM } P_T$ was also determined from their uptake experiments (30). The fact that a linear equation approximates the relationship between P_{T_0}/S and $1/G$ suggests that P uptake by *Selenastrum* during exponential growth (estimated by the slope) was similar for all cultures. Although cellular P may have been affected by Zn ion activity, the estimated rate of P uptake over the initial Zn activity range used here was apparently not greatly affected. In contrast to recent nutrient uptake experiments, an estimate for P uptake rate was calculated here as an average over the exponential growth phase (i.e., short-term P uptake was not directly measured). However, these crude observations from batch culturing data are consistent with previous growth kinetic results for *Selenastrum* (30) and suggest a toxicological mechanism, involving intracellular Zn interference of complete P utilization. This conclusion could be confirmed by more sophisticated uptake experiments.

Bates et al. (35) hypothesized that Zn is complexed and stored intracellularly in polyphosphate bodies. As cells metabolize P for growth, the organically complexed Zn is dissociated and released and eventually reaches a threshold intracellular level, and growth terminates without complete use of intracellular P. This mechanism is consistent with results presented here in that elevated Zn concentrations intensified P yield limitation and conversely elevated P concentrations mitigated Zn inhibition of S . The fact that this mechanism does not explain inhibitory Zn effects on L and G is understandable because Bates et al. (35) observed a fairly constant L (~ 1 day) and only a slight decrease in G over a 0 – $10 \mu\text{M}$ Zn activity range. Authors suggested that the test strain of *Chlamydomonas variabilis* may have been Zn tolerant (17). Toxicity to Zn-sensitive algal species like *Selenastrum capricornutum* must therefore involve other toxicity mechanisms. On the basis of Cu and $\text{Si}(\text{OH})_4$ uptake experiments using the diatom *Thalassiosira pseudonana*, Rueter et al. (36) hypothesized that Cu competes with $\text{Si}(\text{OH})_4$ (an essential macronutrient for diatoms) for the same transport site and inhibits the functioning of that site. Furthermore, it was suggested that *Thalassiosira* growth rates were also inhibited by intracellular Cu resulting in an accumulation of intracellular Si even at reduced Si uptake rates. Zn effects on both *Selenastrum* growth rate and cell yield suggest plausible toxicity mechanisms that work jointly involving P-Zn interactions: (1) Zn-P analogues to the Cu-Si mechanisms affecting growth rate proposed by Rueter et al. (36) and (2) an eventual complete suppression of P metabolism by intracellular release of Zn from polyphosphate bodies resulting in incomplete use of intracellular P and hence yield inhibition (35). The dominant Zn effect on lag phase (Figure 1A) relative to P does not suggest a strong P-Zn interactive toxicity mechanism.

Although the cell yield results indicate that S is inhibited at elevated Zn activities, the direct dependence of S on P_{T_0} is generally preserved over the $[Zn^{2+}]_0$ range (Figure 1C). A similar result was observed by Rana and Kumar (18) using another P yield-limiting medium on *Chlorella vulgaris* and *Plectonema boryanum*. The chemical complexity of natural waters along with the diversity of transport processes controlling nutrient flux creates conditions where different nutrients may limit biomass [e.g., nitrate N at certain coastal sites (37) and P in some fresh waters (15, 16, 38)]. Growth-phase response described herein by *S. capricornutum* to various P and Zn concentrations, along with work by others, suggests that in nature growth or yield limitation by an essential nutrient other than P or Zn (e.g., Nn) could shift to an apparent P-lim-