Culturing *Selenastrum capricornutum* (Chlorophyta) in a synthetic algal nutrient medium with defined mineral particulates

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Abstract

Algal nutrient studies in chemically-defined media typically employ a synthetic chelator to prevent iron hydroxide precipitation. Micronutrient-particulate interactions may, however, significantly affect chemical speciation and hence biovailability of these nutrients in natural waters. A technique is described by which *Selenastrum capricornutum* Printz (Chlorophyta) may be cultured in a medium where trace metal speciation (except iron) is controlled, not by organic chelation, but by sorption onto titanium dioxide. Application of this culturing protocol in conjunction with results from sorption studies of nutrient ions on mineral particles provides a means of studying biological impacts of sorptive processes in aquatic environments.

Introduction

Trace metal-particulate interactions play an important role in controlling chemical speciation of micronutrients and toxicants in natural waters (Li, 1981; Kuwabara, 1982; Luoma & Davis, 1983), and may strongly affect the availability of these trace materials to algae. In chemically-defined media used for algal culturing, a synthetic chelator (e.g. ethylenediaminetetraacetic acid [EDTA], citric acid or nitrilotriacetic acid [NTA]) is typically added to the medium to control the concentration of biologically available forms of metals, and to allow the use of solute speciation models to compute speciation of growth media constitutents (Morel *et al.*, 1979). This paper describes a method for employing titanium dioxide (TiO_2) particles instead of a synthetic chelator to control micronutrient availability to algae under controlled conditions, allowing careful examination of the effects of micronutrient-mineral particulate interactions on algal growth.

Sorption of metals on metal oxides has been well studied (James & Parks, 1982), but direct application of this knowledge to algal studies is complicated by a number of methodological problems. Turbidity of the growth medium may be increased due to the presence of particulates, thus decreasing light intensity. Particles may induce cell clumping due to changes in surface charge when particles adhere to cell walls. Algal growth media are chemically more complex than electrolyte solutions typically used in adsorption experiments (i.e. there may be competition between ions for adsorbent sites (Dempsey & Singer, 1980)). Finally, micronutrient concentrations in culturing media (submicromolar total concentrations) are low compared to typical adsorbate concentrations used in laboratory adsorption studies. We discuss herein a method by which these difficulties may be overcome.

Materials and methods

A chemically defined culturing medium, Synthetic Algal Nutrient Medium (SANM) employing EDTA to control metal availability (Miller *et al.*, 1978) was used in original and in modified form for this work. Ethylenediaminedihydroxyphenylacetic

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Table 1. Chemical speciation of trace metals and phosphate in the original SANM formulation, SANM modified by using EDDHA instead of EDTA as the organic chelator (S-2), and S-2 modified by lowering total Mn, Co and Zn to achieve the same free ion concentrations as in the original SANM formulation (S-3). Ionic strength and pH of these media were 1.4 mmol and 7.5, respectively. Symbol '-' denotes that chelator was not added to the medium. Total Cu concentrations indicate that trace metal contamination was undetectable by atomic absorption spectrophotometric analysis (i.e. Cu was not added as a micronutrient).

Nutrient or chelator	Analytical concentration (nmol)			Free ion concentration (nmol)			Major species (%)		
	SANM	S-2	S-3	SANM	S-2	S-3	SANM	S-2	S-3
Metals									
Fe	5.9×10^{2}	5.9×10^{2}	5.9×10^{2}	1×10^{-10}	3×10^{-11}	3×10^{-11}	FeEDTA (100)	FeEDDHA (100)	FeEDDHA (100)
Mn	2.1×10^{3}	2.1×10^{3}	2.1×10^{3}	7×10^{2}	2×10^{3}	2×10^{3}	MnEDTA (66)	Mn^{2+} (96)	Mn^{2+} (96)
Cu	<2	<2	<2	$<3 \times 10^{-5}$	$<1 \times 10^{-1}$	$<1 \times 10^{-1}$	CuEDTA (100)	$Cu(OH)_2$ (95)	$Cu(OH)_2$ (95)
Zn	2.4×10^{1}	2.4×10^{1}	4×10^{2}	4×10^{2}	2×10^{1}	4×10^{2}	ZnEDTA (100)	Zn^{2+} (93)	Zn^{2+} (93)
Co	6.0	6.0	6.0	2×10^{2}	6	6	CoEDTA (100)	Co^{2+} (97)	Co^{2+} (97)
Ligands									
PO_4^{3-}	6.0×10^{3}	6.0×10^{3}	6.0×10^{3}	5×10^{2}	5×10^{2}	5×10^{2}	HPO_4^{2-} (58)	Same as SANM	
EDTA	2.0×10^{3}			1×10^{-7}			MnEDTA (69)		
EDDHA		5.9×10^{2}	5.9×10^{2}		2×10^{-13}	2×10^{-13}	-	FeEDDHA (100)	FeEDDHA (100)

acid (EDDHA), a particularly strong Fe chelator (Schroder, 1964) was used in place of EDTA in the modified media to prevent Fe hydroxide precipitation (Fe was in ferric form in aerated algal media), while speciation of Zn, Co and Mn was controlled by inorganic complexation (Table 1). This modified medium will henceforth be referred to as S-2. A third solution designated S-3 was formulated like S-2, except with less Zn. A comparison between speciation of SANM and S-2 constituents (Table 1) shows that trace metal speciation in SANM is controlled by EDTA complexation, while EDDHA in S-2 only controls Fe speciation. Chemical speciation of media constitutents was calculated using the computer program MINEQL (Westall *et al.*, 1976) with stability constants included for metal-EDDHA and metal-EDTA reactions (Martell & Smith, 1974).

Stringent control of experimental conditions (Kuwabara & North, 1980) was employed to avoid trace metal contamination of culturing media. All nutrient stock solutions, with the exception of trace element solutions, were passed through columns of Chelex-100 resin (100–200 mesh, Bio-Rad Laboratories, Richmond, Calif., U.S.A.) to remove cationic impurities. Background trace metal concentrations before trace element stock addition were undetectable by atomic absorption spectrophotometric analysis (see Table 1, Cu background concentrations). Algae were cultured in fabricated linear polyethylene culturing vessels and fluoroethylenepolymer (FEP) aerators (Kuwabara, 1980). Changes in cell density were monitored daily using a Model Z_{BI} Coulter Counter and MCV computer (Coulter Electronics, Hialeah, FL, U.S.A.).

TiO_2 was use in this study because of its low solubility in the algal medium, its white color minimized light absorption, and because Ti has neither

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been demonstrated as an essential nutrient nor as a toxicant to any alga. Titanium dioxide (TiO_2) used in this study was prepared by Tioxide International Ltd. (Stockton-on-Tees, England) and was composed of 78% rutile and 22% anatase (sample CLDD 1492/2). The primary particle size for this sample was 13 nm, although some larger particles were present. The TiO_2 was washed in 0.1 mol redistilled HCl and rinsed four times with deionized water (18 mΩ or mOhm resistance) before use.

All experiments employed the unicellular, planktonic green alga *Selenastrum capricornutum* Printz.

Results and discussion

Coagulation of algae and TiO_2 particles

In preliminary culturing experiments with TiO_2 particles added to SANM, *Selenastrum* exhibited a tendency to form cell clusters (typically 3–4 cells but some clusters had as many as 50 cells). This resulted in an underestimation of cell density and overestimation of cell size using the electronic counting apparatus. When clusters were separated by sonication alone, clusters quickly (i.e. within the sampling and counting period) reformed.

Differential centrifugation successfully separated algal cells (and clumps) from TiO_2 particles. The following pretreatment technique allowed effective monitoring of cell density changes. Twelve ml of algal suspension was centrifuged for 10 min., at 13 r.p.m. Seven ml supernatant was removed and the algal pellet in the bottom of the centrifuge tube was then resuspended by adding 7 ml of medium without particles and the centrifugation was repeated. This resuspension procedure was repeated twice. Most titania particles (ca. 82% per treatment) remained suspended after centrifugation. Although a slower rotational velocity would maintain more TiO_2 in suspension, and more than 7 ml supernatant could be removed to increase the efficiency of particle-cell separation after centrifugation, the above treatment produced a dense algal pellet and undetectable cell loss during supernatant removal for the 50 mg l^{-1} titania concentration used. The resulting resuspended pellet was then sonicated (5 s, 100 W) using a thin probe sonicator and then counted. No evidence of cell fragmentation due to sonication was observed. Tests of this sampling treatment indicated good cell recovery (i.e. agreement between hemacytometer cell density counts before sample pretreatment and Coulter Counter measurements after pretreatment), and no reclumping in pretreated samples after 24 h. With the cell clumping problem overcome, difficulties in growth medium formulation were then investigated.

Particle effects on algal growth

An experiment was conducted to test the S-2 medium and to observe possible interactions of Zn with TiO_2 particles. Zn was selected because: (1) it is an essential algal nutrient capable of inducing toxic response at micromolar total concentrations, and (2) Zn in the EDDHA media remains virtually uncomplexed (i.e. free to react with particle surfaces added to the medium, see Table 1). Five treatments were used: (1) S-2 medium only, (2) S-2 with 50 mg l^{-1} TiO_2 added, (3) S-2 with 0.1 μmol total Zn added, (4) S-2 with both TiO_2 and Zn added, and (5) a control with SANM. Results of these experiments are summarized in Table 2.

Addition of Zn without TiO_2 reduced growth

Table 2. Growth parameters with 95% confidence intervals observed for *Selenastrum* in various synthetic algal culturing media. Medium S-2 referes to a modified SANM (Miller *et al.*, 1978) formulation with EDDHA replacing EDTA as the organic chelator (see discussion in text). Medium S-3 is an S-2 modification with total Zn lowered such that computed free Zn ion concentration is similar to those computed for SANM. Designations 'w/TiO_2' and 'w/Zn' refers to the addition of 50 mg l^{-1} TiO_2 and 0.1 μmol total Zn, respectively, to the medium. Symbol '-' denotes that the lag phase was not statistically significant. Abbreviations 'n' and 'd' denote number of samples and time in days, respectively.

Culturing medium	Lag phase (d, n = 12)	Growth rate (dblgs/d, n × 12)	14-d Stationary phase density (10^6 cells/cells ml^{-1}) (n × 3)
SANM (control)	-	1.80 ± 0.03	4.2 ± 0.1
SANM w/TiO_2	-	1.75 ± 0.03	4.0 ± 0.1
S-2	2.0 ± 0.4	1.40 ± 0.06	1.8 ± 0.1
S-2 w/TiO_2	0.5 ± 0.1	1.69 ± 0.04	2.3 ± 0.1
S-2 w/Zn	5.9 ± 0.1	1.16 ± 0.07	0.7 ±0.1
S-2 w/Zn and TiO_2	3.2 ± 0.2	1.56 ± 0.03	1.9 ± 0.1
S-3	-	1.89 ± 0.04	4.0 ± 0.1
S-3 w/TiO_2	-	1.79 ± 0.10	2.9 ± 0.1

below that of other treatments (Table 2). Addition of TiO_2 to S-2 with added Zn showed improved growth response, suggesting that adsorption onto TiO_2 in treatment 4 reduced Zn toxicity observed in treatment 3. Growth was, however, inhibited in S-2 medium alone (treatment 1 above) relative to growth in the SANM control. Addition of TiO_2 to S-2 mitigated this inhibition (growth response from treatment 2 was only slightly poorer than in SANM (Table 2).

Growth inhibition observed in S-2 may have been due to: (1) toxicity of EDDHA to *Selenastrum*, (2) Mn and Co deficiency in S-2 due to oxidation of Mn(II) and Co(II) promoted by EDDHA (Martell & Smith, 1974) to a valence more difficult for *Selenastrum* to assimilate, or (3) Zn, Mn or Co overabundance in S-2, because organic chelation no longer controlled speciation of these metals (Table 1). The first two explanations do not seem as plausible as the third. It is doubtful that EDDHA is toxic because the compound has been used for decades as a fertilizer ingredient for alkaline soils where Fe chlorosis (a deficiency symptom) is prevalent (Holmes & Brown, 1955). EDDHA was added to S-2 as the FeEDDHA complex (log K = 33.9) in a 1:1 ratio with Fe so that other metals would not interact with EDDHA. Because EDDHA typically forms much weaker complexes with divalent metals (e.g. log $K_{ZnEDDHA} = 16.8$, log $K_{MgEDDHA} = 8.0$) than with Fe, EDDHA should have control Fe speciation only. Unfortunately, stability constants for MnEDDHA and CoEDDHA complexes are not available because EDDHA promotes Co(II) and Mn(II) oxidation. Nevertheless, available stability constants for other divalent metals and for Fe with EDDHA indicate that significant Mn and Co complexation with EDDHA is unlikely. The FeEDDHA complex is generally much stronger than divalent metal-EDDHA complexes, and negligible concentrations of free EDDHA should be available to interact with other metals. Also, if Mn and Co deficiencies caused growth inhibition in S-2, then addition of TiO_2 (i.e. a surface for Mn and Co adsorption) should have intensified rather than mitigated this inhibition. The possibility of trace metal toxicity in S-2 was therefore explored.

A 1 μmol total Zn concentration in SANM (ca. 3 nmol as free ion) produces significant reduction in 14-day maximum yield, a measure of static phase density, in *Selenastrum* (Greene *et al.*, 1975). Computed Zn activity in S-2 was ca. 20 nmol (Table 1), almost an order of magnitude higher than in the Zn-toxic medium studied by Greene *et al.* (1975). Addition of 50 μmol uncomplexed Mn in SANM reduces 13-day total algal cell volume (another measure of static phase cell density) by 50% (Christensen & Scherfig, 1979). Mn activity in S-2 was 2 μmol, an order of magnitude lower than computed for Christensen's Mn-toxic medium. Thus Mn toxicity in S-2 was unlikely. No investigations revealing Co toxicity to any chlorococci were found. Zn toxicity is therefore the most likely cause for the observed growth inhibition in S-2.

Modified growth medium formulation

Total Zn concentration in S-2 was lowered to compensate for changes in Zn speciation with EDDHA. Free Zn ion concentration in the modified formulation (henceforth referred to as S-3) was similar to that computed for the original SANM formulation with EDTA. Algal growth in S-3 was similar to that observed in SANM, supporting the contention that inhibition in S-2 was primarily due to Zn overabundance (Table 2). Addition of TiO_2 to S-3 retarded algal growth slightly. Inhibition of growth in S-3 with TiO_2 by turbidity or other physical effects is unlikely. The culturing system used here with lighting at 14 W-m^2 (>2 times the intensity used by Miller *et al.* (1978)), and all white construction would presumably maintain light saturated conditions. Furthermore, TiO_2 addition to SANM (where trace metal speciation was EDTA controlled) did not significantly change algal growth relative to SANM controls. At decreased Zn concentrations in S-3, addition of TiO_2 may have removed Zn or phosphate (PO_4) from solution by adsorption, creating a deficiency of one or both of these nutrients.

Conclusion

Techniques presented here represent an initial step toward quantitatively examining effects of particulates on micronutrient speciation and hence availability to aquatic organisms. The growth medium formulation (S-3) and culturing techniques discussed provide a means of culturing *Selenastrum*

in a medium where trace metal (except Fe) speciation may be controlled not by organic chelation, but by sorptive processes. With data describing Zn and PO_4 sorption onto TiO_2 (or any other defined solid) in S-3, it is conceiveable that algal response may then be modeled in this colloidal suspension just as researchers now model growth in culturing media containing organic chelators that suppress sorptive processes. Importance of these heretofore suppressed processes in the speciation and cycling of micronutrients in natural waters may be better quantified. This culturing protocol overcomes turbidity and cell clumping problems. In addition, S-3 formulation allows sorption studies to be conducted in a chemically defined growth medium with minimal chelator interference.

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