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Growth of microalgae on struvite as a nutrient source

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Highlights

- **N/P supplementation by crude struvite from wastewater treatment performs as well as, if not better than established media for cultivation of the algae production strains, *P. tricornutum* and *N. salina.***
- **More efficient utilization of P per unit biomass from struvite.**
- **Metals present in crude struvite fulfill the trace metals requirement for *N. salina* and *P. tricornutum*.**
- **Culture of alga on sturvite result in elevated per cell pigment concentration compared to the conventional medium formulation.**

Abstract

Providing the major nutrients, nitrogen and phosphorus, has been identified as a major cost and sustainability concern for algae production scale-up for fuels and other biobased commodities. In this work, we investigated the suitability of crude and purified struvite ($MgNH_4PO_4$), a common precipitate from wastewater streams, as a renewable replacement for conventional N/P sources for microalgae cultivation. Crude struvite obtained in the form of wastewater stone was characterized for soluble N/P, trace metals, and biochemical components and compared to the purified mineral form. Cultivation trials using struvite as a major nutrient source were conducted using two microalgae production strains, *Nannochloropsis salina* and *Phaeodactylum tricornutum*, in both lab and outdoor pilot-scale raceways in a variety of seasonal conditions. In both experimental settings, crude and purified struvite were found to result in biomass productivities as high or higher than established media formulations. Furthermore, analysis of nutrient uptake by the alga suggest that struvite provides increased nutrient utilization efficiency, and that crude struvite satisfies the trace metals requirement and results in increased pigment productivity for the two microalgae strains under investigation. Based on these results, struvite should serve as an effective mineral form for nutrient recycling concomitant with algae processing to fuels and biobased commodities.

1. Introduction

Cultivation of microalgae presents the possibility for sustainable production of bioproducts, feeds, and fuels. To achieve this goal, a number of technical challenges must be met for reducing the costs of algae biomass production and processing, including balancing space needs, productivity and biomass concentrations, and energy and nutrient requirements (Greenwell et al., 2010). In a recent lifecycle analysis of full scale algae production for fuels, the major nutrients - nitrogen and phosphorus - were identified as the single largest cost contributor (>30% of the total) (Liu et al., 2013). On a national scale, displacement of ~10% of the U.S. transportation fuels demand by algae was calculated to require a significant fraction of current domestic fertilizer use (Pate et al., 2011), presenting the likelihood of food versus fuels crises and therefore limiting the ultimate sustainability of algae-based fuels if nutrient recycling is not included in the process.

Low-energy intensity fertilizer regeneration and methods for precision application have been identified as critical scientific and technological breakthroughs required for sustainable global development (Buluswar et al., 2014). Although ammonia can be synthesized from atmospheric nitrogen in the Haber-Bosch process, the manufacture is dependent on the availability of natural gas resources. Phosphate is a non-renewable (mined) resource. The results of multiple resource assessment studies indicate that current phosphorus reserves will be depleted in the 21st century (Abelson, 1999; Lougheed, 2011); demand for biomass-based energy is expected to accelerate this phenomenon (Neset & Cordell, 2012). In addition to cost and sustainability concerns, uncontrolled phosphate release is a major contributor to eutrophication (Smil, 2000) and subsequent ecological collapse in waterways (e.g. the Gulf of Mexico "Dead Zone") (Diaz & Rosenberg, 2008). Extraction of phosphorus-rich materials from waste processing provides a means for phosphate management and recycling (Dong et al., 2014; Gilbert, 2009; Shu et al., 2006; Tchobangolous et al., 2003) that could minimize downstream impacts and depletion of strategic reserves. In concentrated waste streams, such as animal feeding operations and wastewater treatment facilities, phosphate precipitates spontaneously to form struvite (NH_4MgPO_4) and calcium phosphate salts, resulting in pipe fouling and increased maintenance costs (Fattah, 2012). Multiple technical approaches have been explored for high efficiency (up to 90%) recovery of struvite from wastewaters, including collection of crystallized precipitates (Marti et al., 2010), and by employing optimized fluidized bed reactors (Bhuiyan et al., 2008) and regenerative ion exchange materials (Sengupta, 2013). A case study investigating the potential for using digester recovered struvite concluded that the levels of heavy metal and micropollutants were within acceptable tolerances for use as an agricultural fertilizer (Uysal et al., 2010).

In this study, we sought to determine whether struvite could serve a suitable replacement for the nitrogen and phosphorous requirements for algae cultivation. We investigated purified and crude struvite sources on the productivity of two microalgae production strains (*Nannochloropsis salina* and *Phaeodactylum tricornutum*) in both lab and outdoor trials, with comparison to established growth medium formulations. The struvite sources were characterized for total soluble

nitrogen and phosphorus, trace metals, and potential organic carry-overs, including sugars and proteins. Various nutrient replacement regimes were investigated to evaluate the potential for slow release effects and other modified nutrient uptake phenomena and how these impact biomass yield (Klausmeier et al., 2004). All of the algal growth studies were conducted in batch, and in addition to biomass productivity, phenotypic variables including cell size, number, and chlorophyll content were assessed. Pilot-scale algae raceway studies of mono- and mixed cultures of *Nannochlropsis* and *Phaeodactylum* were conducted to identify seasonal effects. These data include supporting information incorporating solar flux, temperature, wind speed, precipitation, and culture pH and salinity. The results should be applicable for advancing sustainable development of algal culture for biobased products and fuels.

2. Materials and Methods

2.1 Microalga strains

Nannochloropsis salina (CCMP 1776) and *Phaeodactylum tricornutum* (CCMP 632) were obtained from the National Center for Marine Algae and Microbiota (NCMA) at Bigelow Laboratory for lab-scale cultivation assays. *N. salina* (CCMP 1776) and a local isolate of *Phaeodactylum tricornutum* (isolated from the Laguna Madre, Corpus Christi, TX) were employed for outdoor cultivation at Texas Agrilife, Corpus Christi, TX, in 557L raceway systems.

2.2 Media compositions and struvite sources

For lab-scale assays, the control culture medium was L1 enriched artificial seawater adjusted to pH 8.0; the specific formulation is shown in Table 1 (Davis et al., 2014). For these trials, experimental media compositions using struvite as a partial or complete N or P source were generated using artificial seawater, varying levels of struvite with supplementation of sodium nitrate and dibasic sodium phosphate as necessary, and the trace metals and vitamins, except where denoted otherwise. For pilot-scale outdoor growth trials, the control culture medium was composed of local filtered seawater, 2.0 mM nitrogen (from ammonium sulfate, 0.13 mM phosphorus (from phosphoric acid) (16:1 N:P ratio) and 0.07 mM iron (from iron sulfate). For these trials, experimental media compositions using struvite as a partial or complete N or P source were generated using local seawater, varying levels of struvite with supplementation of ammonium sulfate and phosphoric acid as necessary. Struvite was acquired as the purified hydrate ($NH_4MgPO_4 \bullet 6H_2O$, Sigma-Aldrich and Multiform Harvest, Inc., Seattle, Washington), and in crude form (as wastewater stone) from a Boise, Idaho based wastewater treatment facility managing effluent from a concentrated animal feeding operation for cattle (the generous gift of Gary Westlund, Guard Products, Inc.).

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Table 1. Control medium formulation (L1) used for comparison to struvite-based microalgael growth medium.

Medium component	Quantity
Artificial seawater	
NaCl	362.7 mM
Na_2SO4	25.00 mM
KCl	8.030 mM
$NaHCO_3$	2.067 mM
KBr	0.725 mM
H_3BO_3	0.433 mM
NaF	0.0657 mM
$MgCl_2•6H_2O$	47.18 mM
$CaCl_2•2H_2O$	9.134 mM
$SrCl_2•6H_2O$	0.0214 mM
Nutreints	
$NaNO_3$	0.882 mM
Na_2HPO_3	0.0362 mM
Trace metals	
$Na_2EDTA•2H_2O$	11.7 µM
$FeCl_3•6H_2O$	11.7 µM
$MnSO_4•4H_2O$	0.909 µM
$ZnSO_4$	0.080 µM
$CoSO_4$	0.050 µM
$CuSO_4•5H_2O$	0.010 µM
$Na_2MoO_4•2H_2O$	0.082 µM
Na_2SeO_3	0.010 µM
$NiSO_4•6H_2O$	0.010 µM
Na_3VO_4	0.010 µM
K_2CrO_4	0.010 µM
Vitamins	
Thiamine	296 nM
Vitamin B12	0.369 nM
Biotin	2.05 nM

2.3 Culture conditions

2.3.1 Lab-scale growth assays

Cultures of *N. salina* (CCMP 1776) and *P. tricornutum* (CCMP 632) were inoculated with late-log phase axenic cultures to achieve starting near-IR optical densities of ~0.05 – 0.10 in a MC1000 multicultivator apparatus (Photon Systems Instruments). The system was configured for constant air bubbling in each culture tube (200 cm^3/min) and a light/dark dirunal cycle of 16/8 hours, with a sinusoidal light cycle corresponding to a photosynthetically active radiation (PAR) irradiance maximum of 1,000 µmol photons/m^2/sec and minimum of <25 µmol photons/m^2/sec. The temperature of the chamber was calibrated to 21°C during the dark cycles and gradually increased to 26°C (±0.5°C) following the peak of the sinusoidal light cycle. For phosphorus replacement assays, experimental treatments (n=3 each) were supplemented with purified or crude struvite to replace 33, 67 and 100% of the phosphorus in the control treatment with and without the addition of trace metals and vitamins.

2.3.2 Pilot-scale outdoor growth assays

Cultures of *N. salina* (CCMP 1776), *P. tricornutum* (local isolate), or a mixture of the cultures (1:1 based on ash-free dry weight, AFDW, for phosphate replacement; 4:1 based on cell count for nitrogen replacement) were stocked into 12 outdoor raceways (557 L at 20 cm depth) at an initial stocking density of ~0.15 g/L AFDW at

5 cm. Seawater used in the trials was pumped from the Laguna Madre, Corpus Christi, TX. Incoming seawater was filtered through a diatomaceous filter (Pentair Pool Products, Sanford, NC), chlorinated to 15 ppm (minimum duration of 30 minutes) and then stored until use. Each raceway was fitted with a paddlewheel to provide a water flowrate of 50-60 cm/second and a CO_2 injection system. The control raceways (n=3) were supplemented with our standard nutrient blend (modified from Zmora and Richmond, 2004) to achieve 2.0 mM nitrogen (from ammonium sulfate), 0.13 mM phosphorus (from phosphoric acid) and 0.07 mM iron (from iron sulfate) at 16:1 N:P ratio for the phosphorus replacement trials and 1:1 N:P ratio for nitrogen and phosphorus replacement trials. For the nutrient replacment trials, experimental treatments (n=3 each) were supplemented with struvite (Multiform Harvest; Seattle, WA) to replace 33, 67 and 100% of the nutrients in the control treatment. The experimental raceways were then supplemented with ammonium sulfate, phosphoric acid (if necessary) and iron sulfate to balance nutrient levels with those in the controls. Water depth in each raceway was gradually increased to a final depth of 20 cm. Raceways were monitored daily for solar radiation, rainfall, wind-speed, pH, temperature, salinity, AFDW, ammonia and phosphate. Trials conducted were terminated after 15 days of culture.

2.4 Analytical methods

2.4.1 Struvite characterization

The struvite samples were analyzed for elemental composition, soluble ammonium and phosphate, trace metals, the presence of proteins and carbohydrates, and for crystallographic confirmation of the struvite mineral lattice. Independent elemental analyses were performed by Mirco Analysis, Inc. and New Jersey Feed Laboratory, Inc. Quantitative analysis of soluble ammonium was performed on saturated solutions of struvite in artificial seawater using the modified Berthelot colorimetric assay (Abcam, Inc) in 96-well format using an ammonium chloride standard dissolved in artificial seawater, with absorbance measured at 670 nm. Quantitative analysis of soluble phosphate was performed on saturated solutions of struvite in artificial seawater using a malachite green colorimetric assay (Abcam, Inc) in 96-well format using a dibasic sodium phosphate standard in artificial seawater, with absorbance measured at 670nm. Absence of proteins was confirmed colorimetrically from saturated solutions of the crude material using the bicinchoninic acid assay (MicroBCA, Pierce) in 96-well format using a BSA standard in artificial seawater, with absorbance values measured at 562nm. Absence of carbohydrates was confirmed by the phenol-sulfuric acid assay using a D-glucose standard in artificial seawater, with absorbance measured at 480 nm (Taylor, 1995). Semi-quantitiative analysis of the presence of trace metals in the crude struvite was performed using SEM-EDS (FEI Quanta 3D FEG SEM with an Apollo 40 EDAX EDS detector).

2.4.2 Lab-scale spectrophotometric monitoring and cytometry

For lab-scale growth assays, chlorophyll and near-IR optical densities (OD) were collected by the MC1000 multicultivator apparatus at 10 minute intervals during

each assay. The spectrophotometric data was despiked to correct for refractive artifacts resulting from aeration bubbles by adapting a method previously described for correcting noise imparted on velocimeter data in bubbly flow (Mori et al., 2007). The data was then used to generate growth curves for comparing yields from the various media formulations. At various time points during the algal growth phase, aliquots of the cultures were serially diluted and dark-adapted for subsequent hemacytometry, UV-VIS (400-800nm) spectrophotometry, and flow cytometry. This approach allowed determination of cell-counts from the spectrophotometric data by cross-correlation with hemacytometry. The UV-VIS absorption data was also used for comparison of the relative pigment abundance among the various medium formulations by normalization of the major PSII absorption peaks (420nm and 680nm) by the near-IR scatter. Flow cytometry (Accuri C6, forward scatter cutoff = 100, flow rate= 35µL/min, sheath diameter= 16µm) was performed to evaluate cell-size and per-cell chlorophyll fluorescence. Calibration of the forward scatter detector channel was performed using the following sizes of fluorescent polystyrene beads (FluoSpheres, Invitrogen, USA) suspended in double distilled filtered (20 nm, Whatman, USA) water: 2.0 µm, 7.52 µm, 9.7 µm, and 15.41 µm. Calibration of the fluorescence detector channels was performed using constant diameter (3 µm), 8-intensity level fluorescent validation beads (Spherotech, BD Bioscience). Analysis of the data was performed using software provided by Accuri or by the FloJo software package (Tree Star, Inc.). The flow cytometry measurements were also cross-validated with hemacytometry in order to obtain accurate cell counts.

2.4.3 Monitoring of outdoor cultures

Outdoor cultures were monitored 2 times daily for salinity, temperature and pH using a YSI Model 650 handheld meter attached to a YSI 600XL multiparameter sonde (YSI, Inc. Yellow Springs, OH). Cultures were monitored daily for afdw (Standard Methods, 1995), phosphorus (FIAlab 2600, FIAlab Instruments Inc, Bellevue, W) and ammonia (Bower & Bidwell, 1978; Solarzano, 1969; Spotte, 1972a,b). Site conditions were monitored continuously for solar radiation, rainfall, wind speed and air temperature using an onsite weather station which is part of the Texas AgriLife crop weather monitoring program (http://cwp.tamu.edu). Each raceway was also monitored continuously for water temperature by the weather station. The pH in each raceway was maintained at ~7.8 using a PinPoint pH controller (American Marine Inc. Ridgefield, CT) and CO_2 injection system. Carbon dioxide was added to each raceway through a ceramic air diffuser (Sweetwater, Apoka, FL).

2.4.4 Analysis of N and P concentrations in algae biomass

Determination of total nitrogen and phosphate uptake in algae biomass was performed by oxisolv (potassium persulfate, EMD Millipore) digestion (Nydahl, 1978) followed by colorimetric analysis. Specifically, algae biomass samples were lyophilized and resuspended in sterile filtered double distilled water to achieve 0.5% solids loading. The samples were then sealed and placed on a shaker incubator at 40˚C, 325 rpm for 4 hours, followed by agitation for 16 hours at room temperature. Freshly prepared aqueous oxisolv solutions (50 mg/mL) were then

combined with the samples at a 1:1 volume ratio. The oxidation reaction was then performed in an autoclave (121˚C, 15 psi, 30 mins). After cooling, the samples were centrifuged and the supernatant collected for colorimetric analyses. The concentration of phosphate was obtained using the malachite green assay described in section 2.4.1 with the addition of 25 mg/mL oxisolve to the phosphate standards. Total nitrogen in the biomass was determined from the concentration of nitrate using the Griess reaction kit (Promega, Inc), with 25 mg/mL oxisolv added to the sodium nitrate standard solutions. Briefly, 50µL of sample was combined with 50 µL of 1% solution of sulfanilamide in 5% phosphoric acid in a multiwall plate and incubated at room temperature with protection from light for 10 minutes. Immediately following, 50 µL of a 0.1% solution of N-1-napthylenediamine dihydrochloride in water was added to the sample wells and incubated at room temperature with protection from light for an additional 10 minutes, and the absorbance measured at 540nm.

3. Results

3.1 Chemical analysis of struvite

Comparison of the chemical properties of reagent grade struvite (Sigma Aldrich) and wastewater stone ("crude struvite") samples obtained from a bovine concentrated animal feeding operation was performed. Following drying, (105˚C , 4 hrs) elemental analysis of the samples indicated the presence of 44.04% and 42.64% H_2O equivalents for the pure and crude samples, respectively, consistent with the hexahydate form of the mineral. The total nitrogen was 3.63% (crude) and 5.72% (pure), phorphorus was 6.37% (crude) and 12.62% (pure), and magnesium was 3.47% (crude) and 9.90% (pure). Total carbon in the crude struvite was found to 3.81%, however, there was no indication of soluble protein or carbohydrate materials in the samples. Saturated solutions of pure and crude struvite in artificial seawater yielded 0.206 mM and 0.087 mM soluble phosphate, respectively. Saturated solutions of pure and crude struvite in artificial seawater yielded 0.386 mM and 0.227 mM soluble ammonium, respectively. SEM-EDS analysis of the crude struvite samples indicated the presence of significant quantities of Fe, Si, K, Ca, Al, S, Cl, and Ti (see Fig. 1); additional trace metals, including Cr, Mn, Cu, Ni, V, and Zn were below the limit of detection. XRD measurements (Bruker D8 X-ray diffractometer, 10-90 degree scan with steps of 0.02 degrees) confirmed the presence of the struvite mineral lattice (orthorhombic space group (Pmn2(1)): repeat unit lengths of a = 6.945, b = 11.208, c = 6.1355) (see S2); other magnesium phosphates, including apatite, phosphate apatites, dittmarite, collinsite, and baricite were not detected. However, the spectra confirm the previously reported hemimorphic nature of crude struvite crystal (Abbona & Boistelle, 1979).

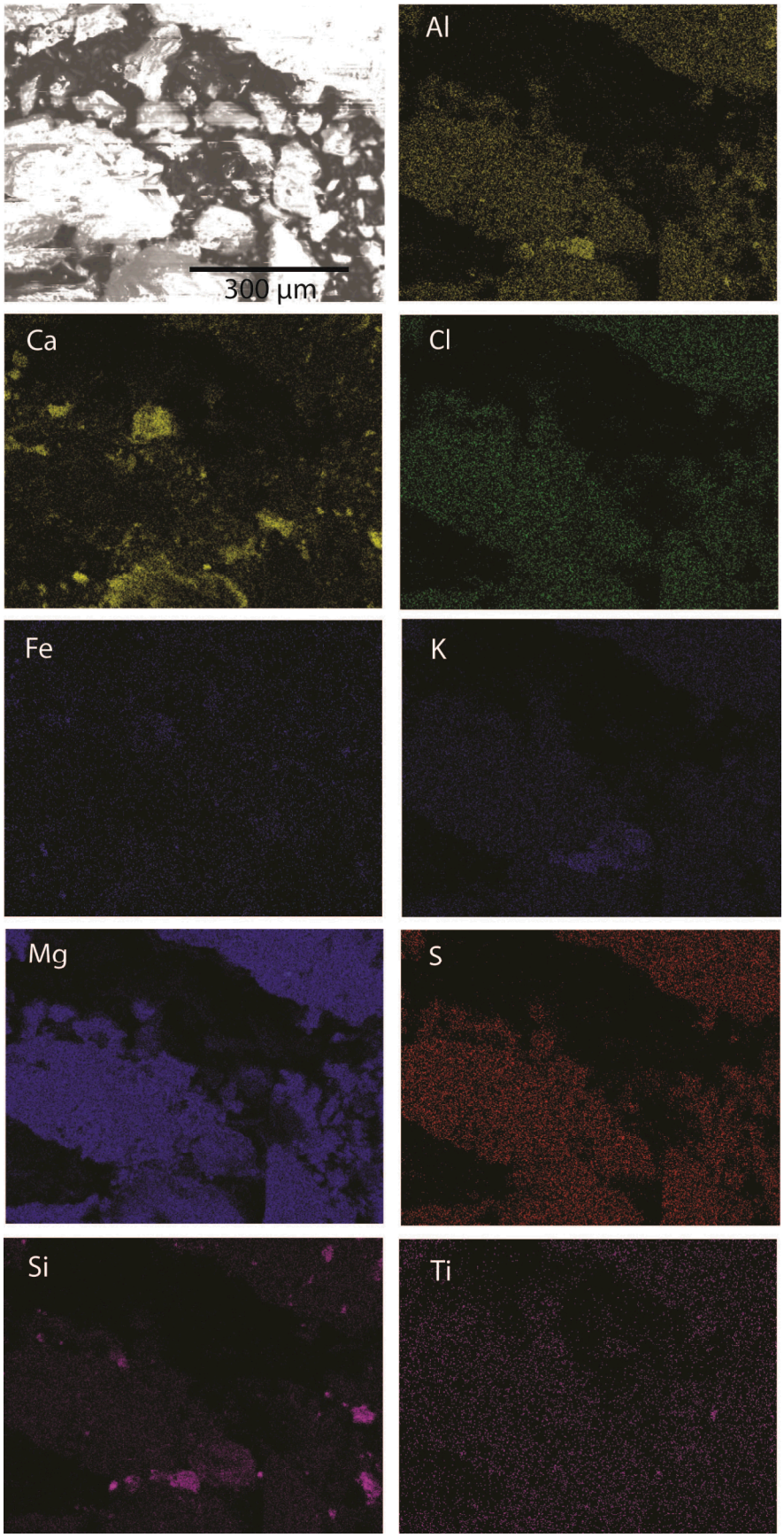
300 µm
Al
Ca
Cl
Fe
K
Mg
S
Si
Ti

Figure 1. SEM-EDS images of crude struvite indicating the relative abundance of various trace metals.

3.2 Algae productivity

In the outdoor phosphorus replacement trials the ability of struvite to replace phosphorus was assessed using 1) monocultures of *N. salina* (CCMP 1776), 2) monocultures of *P. tricornumtum* (local isolate), 3) mixed cultures of *N. salina* and *P. tricornutum* (under seasonal conditions favoring *N. salina* growth; mean PM water temperature of 30.5±2.4^{o}C), and 4) mixed cultures of *N. salina* and *P. tricornutum* (under seasonal conditions favoring *P. tricornumtum* growth; mean PM water temperature of 12.85±7.0^{o}C). Daily maximum and cumulative biomass productivities (g AFDW/m^2/day) of trials cultivated with phosphorus replacement (% of control) using commercial struvite is presented in Fig 2A. Daily maximum and cumulative biomass productivities of trials cultivated with nitrogen replacement (% of control), e.g. N:P 1:1 based on the stoichiometric ratio of N and P in struvite, is presented in Fig 2B. There were few significant differences in biomass productivity between the treatments during the course of the four phosphorus replacement trials. The data from these trials suggests struvite was able to completely replace phosphorus in the nutrient mix without any adverse effect on the average biomass yield of mono- and mixed cultures of *N. salina* and *P. tricornumtum*. For the nitrogen replacement regime (N:P 1:1), there were several instances of increased biomass productivity by the use of struvite as source for both N and P. For *N. salina* in particular, use of struvite as a major nutrient replacement resulted in a significant increase in cumulative productivity versus controls for summer monoculture cultivation and for winter co-culture. Analysis of the species composition of the co-cultures (N:P replacement "winter" and "spring") revealed that *N. salina* was the dominant algae species in both of the control cultures (44.4:1 in "spring" and 56.5:1 in "winter). In the experimental struvite-based cultures, however, this species dominance was either greatly reduced (6.8-10.3:1 in "spring") or eliminated (0.68-2.6:1 in "winter"), without detrimental impact on yield. In order to assess the potential impact of "slow nutrient release" of the N/P from struvite related to solvation kinetics and nutrient uptake by the algae cells, the assays were extended well beyond the anticipated log-phase growth regime. The extended production data suggests the "slow release" of the struvite nutrients did not impact microalgae growth or raceway contamination based on microscopic evaluation.

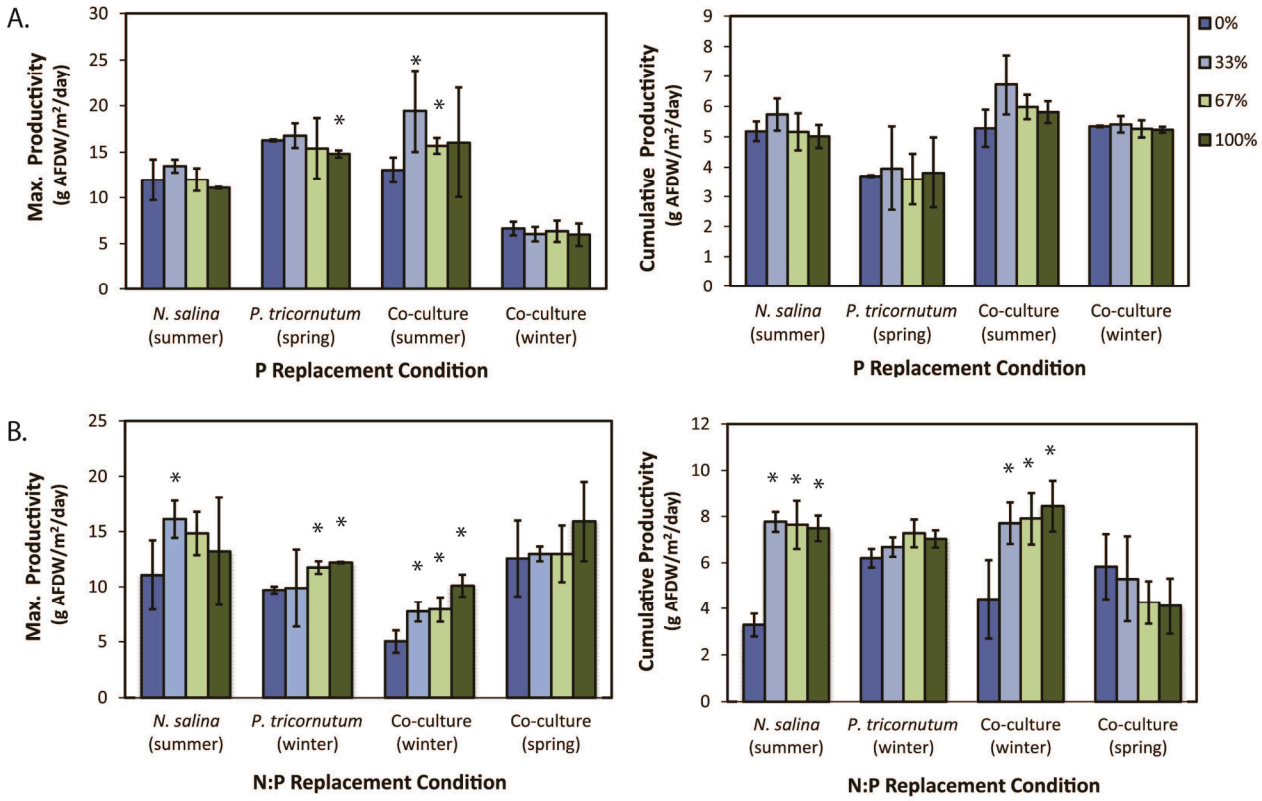

Figure 2. Maximum and cumulative ash-free biomass productivity (dry weight) of *N. salina*, *P. tricornutum*, and co-cultures cultures obtained from 15 day outdoor raceway growth trials performed from October 2013 through April 2015 in Corpus Christi, TX. Nutrient conditions depicted in (A) correspond to various phosphate replacement regimen by struvite according to the Redfield ratio (16:1 N:P). Nutrient conditions depicted in (B) correspond to various nitrogen replacement regimen by struvite, resulting in 1:1 N:P, e.g. excess phosphate. Data labeled with an asterisk indicates significant difference from the control medium formation. *N. salina* P replacement "summer" cultures were terminated Oct 14, 2013; P. tricornutum P replacement "spring" cultures were terminated April 9, 2015; co-culture P replacement "summer" cultures were terminated June 14, 2014; co-culture P replacement "winter" cultures were terminated Feb 7, 2014; *N. salina* N:P replacement "summer" cultures were terminated Oct 15, 2014; *P. tricornutum* N:P replacement "winter" cultures were terminated Feb 7, 2015; co-culture N:P replacement "winter" cultures were terminated Feb 26, 2015; co-culture N:P replacement "spring" cultures were terminated April 8, 2015. See supplemental material (S1) for details including solar radiation intensity, temperature, precipitation, wind speed, and culture pH and salinity.

3.3 N/P nutrient uptake

Biomass samples collected at regular intervals during the extended outdoor cultivation trials were analyzed to determine the uptake of nitrogen and phosphate

under various P-replacement regimes using struvite. Time course data showing the total N and phosphate concentrations of the biomass for both microalgae strains is presented in Fig 3. For *P. tricornutum*, the maximum phosphate accumulation occurs in control medium after day 8 until the end of the growth trial; in samples where struvite was used as a replacement for N/P, maximum phosphate accumulation occurs after day 8 but decreases by day 15, with the effect being more pronounced at higher struvite P-replacement regimes. For *N. salina*, the maximum phosphate accumulation also occurs in control medium between day 4 and day 15 of the trial; in samples where struvite was used as a replacement for N/P, phosphate accumulation remains largely static during the extended growth trial. For both *P. tricornutum* and *N. salina*, the total nitrogen accumulated in the biomass reached a peak between day 4 and day 12 of the trial, with some indication of N-enrichement in samples with nutrient replacement by struvite. For both *P. tricornutum* and *N. salina*, the N:P ratios roughly followed the N-accumulation trends. However, samples with higher levels of nutrient replacement by struvite had the highest N:P, albeit at different growth intervals. For *P. tricornutum*, N:P was consistently higher in the late growth stages (following day 12) in struvite-containing samples than in controls. In this case, the average N:P was 17-24:1 in samples with 100% phosphorus replacement by struvite and 6-8:1 in control medium at day 12 and day 15. For *N. salina*, N:P was consistently higher in the mid-growth stages (day 4 through day 8) in struvite-containing samples than in controls. In this case, the average N:P was 44-47:1 in samples with 100% phosphorus replacement by struvite and 10-26:1 in control medium at day 4 and day 8.

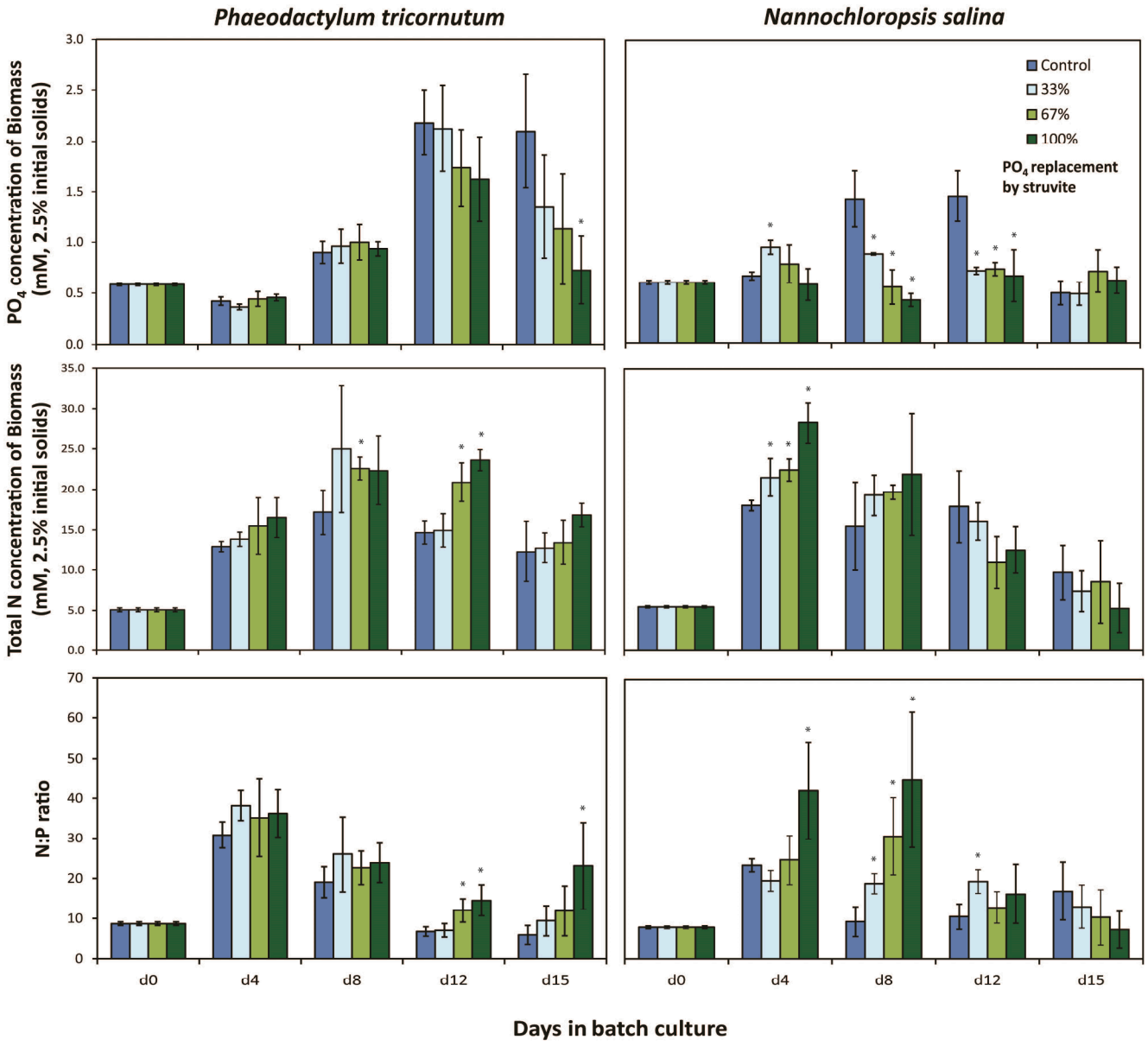

Figure 3. Concentrations of major nutrients in harvested *P. tricornutum* and *N. salina* biomass cultivated with struvite in outdoor raceways. Top panels: total cellular phosphate concentration versus time in batch culture. Middle panels: total cellular nitrogen versus time in batch culture. Bottom panels: Ratio of nitrogen and phosphorus versus time in batch culture. Data labeled with an asterisk indicates significant difference from the control medium formation.

3.4 Physiological parameters of algae grown on struvite

In addition to biomass yield, two key physiological parameters of interest were monitored based on cultivation of *N. salina* and *P. tricornutum*using struvite as a nutrient source. These parameters included the pigment yield as indicated by chlorophyll fluorescence and the effective mean cell diameter, each measured by flow cytometry. Finally, correlation of the in situ optical density values at 800nm

(OD800) to cell counts obtained by hemacytometry allowed accurate determination of cell counts from the spectrophotometric method. For *N. salina*, cell counts corresponding to 2.362×10^7 per OD800 (-2.328×10^6 intercept) were obtained; for *P. tricornutum*, cell counts corresponding to 1.610x107 per OD800 (-1.080×10^6 intercept) were obtained, indicating a cell count per OD800 ratio of 1.467 of *Nannochloropsis* compared to *P. tricornutum*for OD800 values <2. Flow cytometry data indicated an average forward scatter ratio of 0.868 of *N. salina* relative to *P. tricornutum*, corresponding to a relative cell volume of 0.71. Combining the relative cell count per OD800 and normalized by the relative cell volume gives approximately unity (1.04), consistent with our previous measurements for biomass estimation from near IR spectrophotometry of microalgae of varying composition (Davis et al., 2015).

Figure 4 depicts the biomass productivity, effective mean cell diameter, and the chlorophyll A concentration per cell normalized to cell volume (V_b) for *N. salina* and *P. tricornutum* cultured using crude and purified struvite for full phosphorus replacement as compared to control (L1) cultures. The total biomass productivity in these studies was found to be either insignificantly different or elevated for the struvite-based cultures, in agreement with the results of the outdoor raceway growth trials. Furthermore, cultures containing crude struvite for 100% phosphorus replacement but lacking the addition of trace metals (see Table 1) were found to be equally productive as cultures grown on L1 medium, suggesting that crude struvite not only provides major N/P nutrients, but also fulfills the trace metals requirement for these algae strains. The effective mean diameter of the cells was found to be larger for both algae strains when cultivated in L1 versus crude struvite at stationary phase, but no significant size differences were observed between the L1 and purified struvite cultures . The mean ChlA pigment concentration per cell (adjusted for mean cell volume) was found to be elevated in struvite-based medium compared to L1 for both strains; significantly elevated pigment concentrations were detected in both the purified and crude struvite media formulations for *N. salina*, but only in the crude struvite formulation for *P. tricornutum*. Overall reduction in per cell pigment levels were observed for all strains and media at stationary phase (day 8), however the effect was more exaggerated in L1 medium.

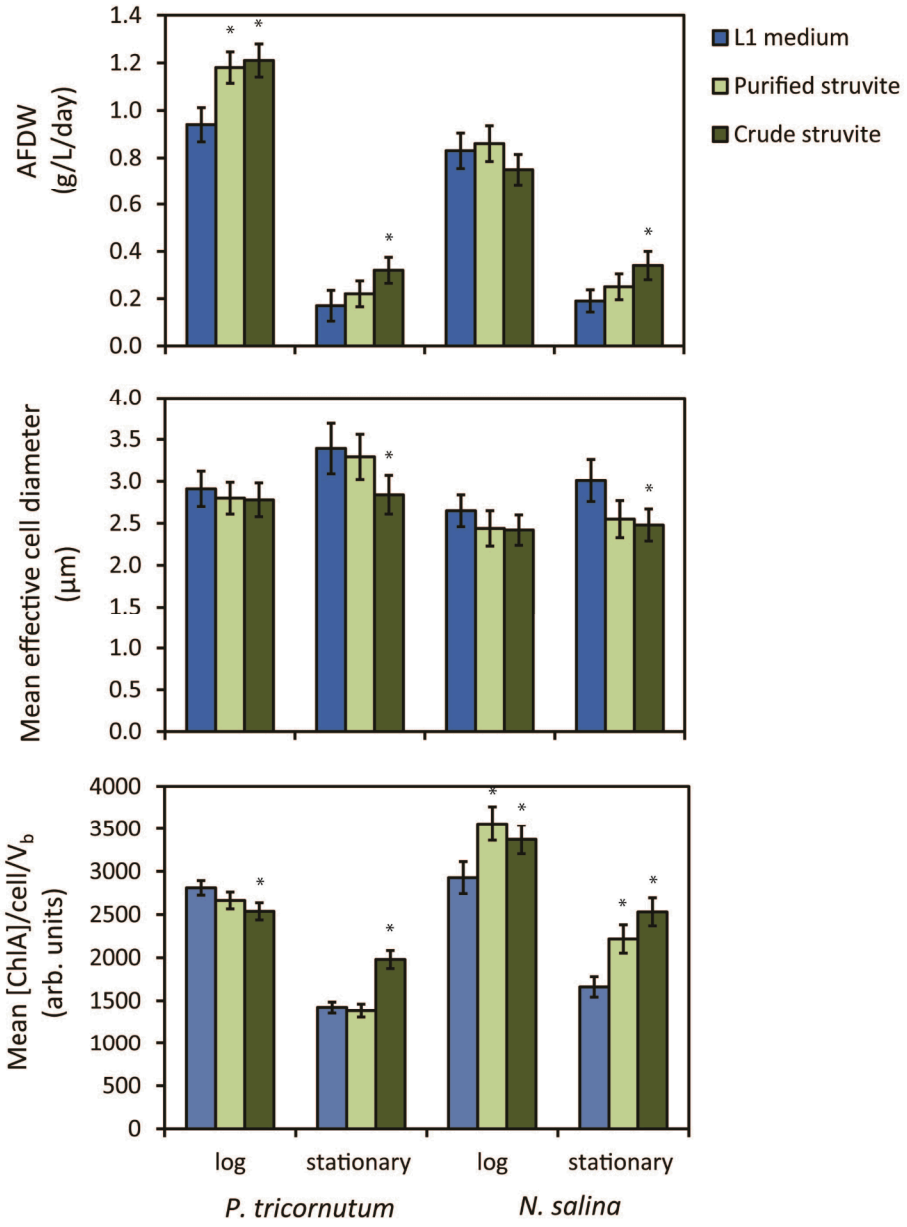

Figure 4. Productivity and physiological parameters of lab cultures cultivated with the full phosphate requirement provided by control medium (L1), purified struvite, and crude struvite. Biomass yield, in terms of ash-free dry weight (AFDW), mean effective cell diameter, and mean chlorophyll concentration per cell per unit biovolume (V_b) (estimated from flow cytometry) are depicted for *N. salina* and *P. triconorutm* monocultures for day three of cultivation, representing log-phase growth, and day eight of cultivation, representing stationary phase. Data labeled with an asterisk indicates significant difference from the control medium formation.

4. Discussion

A key challenge for achieving sustainable and cost-competitive biofuels and biobased commodities from microalgae is the ability to effectively recycle the major N/P nutrients. Phosphate in particular is non-renewable resource, and deemed "life's bottlekneck" by the renowned biochemist and author Isaac Asimov (Asimov, 1974). In this study we sought to investigate the potential for using struvite (NH_4MgPO_4) for culture of microalgae. Struvite commonly forms as a problematic precipitate in wastewater treatment systems where sufficient quantities of soluble phosphate, ammonia, and magnesium are present at near neutral pH. Based on this phenomenon, struvite is an attractive candidate for recapturing remineralized nitrogen and phosphorus from biomass processing if it can subsequently be used for high productivity algal culture. Struvite for the trials was sourced in both purified and crude forms and analyzed for elemental and biochemical composition, verification of the struvite crystal lattice, and solubility of the major nutrients in marine medium. These characterization data indicate that the wastewater stone used for our algae growth trials was composed of ~48% struvite with a solubility coefficient, K_{sp} ~$2x10^{-14}$, corresponding to ~90% of values reported at similar temperatures but significantly lower osmotic strengths from a recent study of the thermodynamic solubility of struvite (Bhuiyan et al., 2007), and consistent with the commonly cited literature on the physico-chemical properties of struvite (Bube, 1910; Snoeyink & Jenkins, 1980; Taylor et al., 1963). Mass contributions from other metals, especially silicon, calcium, iron, aluminum, potassium and sulfur were evident from SEM-EDX analysis of the crude material. Although carbon was detected in the samples there was no indication of biological carbon sources (e.g. proteins or carbohydrates) that may have otherwise contributed to heterotrophic growth of the microalgae (Perez-Garcia et al., 2011) or inhibited the initial formation of struvite crystals (Wierzbicki et al., 1997). These data suggest that the elemental carbon that was detected was likely from carbonate salts (e.g. calcium carbonate and bicarbonate) related to the hardness of the source waters.

In order to evaluate the potential for replacing conventional algae nutrients with struvite, lab-based and outdoor pilot scale growth trials were conducted using two promising marine microalgae production species, *Nannochloropsis salina* and *Phaeodactylum tricornutum*. Yields from batch culture of the two algae species were compared with established enriched natural seawater formulations in the outdoor pilot scale trials and enriched artificial seawater medium in the lab trials, with similar trends observed in the two cultivation systems. For both culture systems, cultivation yields were either unaffected or increased when struvite was used for replacement of the phosphorus or nitrogen requirement compared to the control medium. The highest outdoor maximum and cumulative biomass productivities were observed in summer co-culture with struvite as the dominant source of phosphate. Extended growth trials were employed to identify the potential impacts of slow dissolution of ammonium and phosphate during the growth trials. In these studies, no significant impact was observed in the outdoor trials, however, in the lab trials, late stage growth of both *N. salina* and *P.*

tricornutum was more robust using crude struvite equivalents (from wastewater stone) in place of the established nutrient formulation for L1 medium (Berges et al., 2001). The fact that this effect was not significant for the growth trials that utilized pure struvite may relate to the presence of potentially limiting trace metals present in the crude struvite source.

Assessment of nutrient uptake time courses and the associated N:P ratios for the two algae strains indicate some potentially impactful differences for the different major nutrient sources . The general trend that emerged for both algae species was the tendency to accumulate less phosphate and more nitrogen with increasing replacement of conventional major nutrient sources by struvite (see Fig 3). This effect led two substantially higher N:P ratios in the biomass in certain growth stages for algae cultivated on struvite compared to L1 medium. For *P. tricornutum* the effect is evident only very late in the extended growth trial, whereas for *N. salina*, the effect is evident in mid- to late-log phase. High levels of phosphorus accumulation in the biomass using control medium never resulted in increased biomass productivity. This effect suggest that replacement of more soluble forms of phosphate common in standard media formulations with struvite may prevent luxury uptake of phosphorus (Powell et al., 2011). It has been previously observed that *P. tricornutum* in particular is very efficient in depleting available phosphorus in algae cultivation media (Ansell et al., 1964; Kuenzler & Ketchum, 1962). Although this is desirable for purposes of wastewater remediation, more efficient phosphorus resource utilization could have a dramatic benefit for reducing the overall nutrient costs incurred for commercial culture of algae biomass (Liu et al., 2013). The fact that nitrogen resource utilization efficiency appears to have increased in struvite-based media suggests that the protein content of the biomass is elevated compared to that grown in control medium, as proteins are the dominant nitrogen sink (80-90% of biological N) in algae (Barsanti & Gualtieri, 2014).

Based on the expectation of phenotypic and biochemical variations under variable nutrient regimes (Geider & MacIntyre, 2002), basic physiological parameters and the pigment content of the biomass were assessed to establish the potential of using struvite for algae culture for biofuel and bioproduct applications. Accurate measurement of cell counts, approximate cell volumes, and pigment concentrations in the algae cells was achieved using previously described spectroscopic and cytometric methods (da Silva et al., 2009; Davis et al., 2015; Davis et al., 2012). In these experiments, cultures grown on conventional algae medium (L1) had reduced per cell concentration of pigments compared to those grown in struvite-based medium. Previous studies suggest that differences in the nitrogen source can have substantial impacts on the growth rate of algae (Ludwig, 1938). Specifically for *Nannochloropsis*, ammonium (in this case, as partially contributed by struvite) was been found to have a positive impact on the algae growth rate but not the maximum cell density (Hii et al., 2011). In this work we observed not only that the nutrient source could increase overall yield, but that it had a large impact on the final species composition of co-cultures where struvite provided the full nitrogen (and phosphorus) requirement. In another study, the authors concluded that trace metals are a dominant factor in physiological changes in algal cells (Cakmak et al.,

2014). Our data comparing crude and purified struvite nutrient sources to control medium suggest that metals present in the crude material (wastewater stone) fulfill the trace metals requirement for these algae production strains. Furthermore, cultivation of algae using struvite, especially in crude form, was found to have a beneficial role for increasing pigment accumulation in the biomass.

A potential concern for utilization of struvite as a major nutrient source for algae cultivation is the apparent mismatch between the stoichiometric equivalents of N and P in struvite versus the typical, Redfield ratio-based culture medium formulations (Geider & La Roche, 2002; Redfield et al., 1963). In this work, we either supplemented the struvite growth medium with sodium nitrate to achieve total N equivalency between the control and experimental media or struvite was used to achieve the full N-requirement (resulting in N:P 1:1, e.g. excess P). The fact that complete replacement of the phosphate requirement by struvite resulted in significantly elevated N:P in the biomass suggest that significant deviations from the Redfield ratio may be achieved while maintaining high biomass productivities. This is corroborated by our N:P replacement data and previous observations that relative to the standard Redfield ratio, decreased N:P ratios are supportive of algal blooms in ecological nutrient cycling (Arrigo, 2005). Furthermore, in a recent study on cultivation of *Nannochloropsis sp.*, the authors observed an increase in biomass productivity of up to ~47% for N:P of 3:1 relative to 20:1(Encarnacao et al., 2012).

In this study we have demonstrated growth of mono- and mixed cultures of *Nannochloropsis salina* and *Phaeodactylum tricornutum* using struvite as a replacement for the major nutrients, nitrogen and phosphorus. Trends observed in lab cultures grown on synthetic media with diurnal light cycling and the associated temperature variations were found to have strong predictive value for trends observed in subsequent outdoor culture in pilot-scale raceway systems. The results suggest not only that struvite is a suitable replacement for conventional sources of N and P, but that in some cases the struvite-based medium outperforms conventional medium in terms of biomass productivity and nutrient utilization efficiency, and may serve as an effective mineral form for nutrient recycling concomitant with biomass processing to fuels and biobased commodities.

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Abbona, F., Boistelle, R. 1979. Growth Morphology and Crystal Habit of Struvite Crystals (Mgnh4po4.6h2o). *Journal of Crystal Growth*, **46**(3), 339-354.

Abelson, P.H. 1999. A potential phosphate crisis. *Science*, **283**(5410), 2015.

Ansell, A.D., Coughlan, J., Lander, K.F., Loosmore, F.A. 1964. Studies on the mass culture of *Phaeodactylum*. IV. Production and nutrient utilization in outdoor mass culture. *Limnol. Oceanogr.*, **9**(3), 334-342.

Arrigo, K.R. 2005. Marine microorganisms and global nutirent cycles. *Nature*, **437**, 349-355.

Asimov, I. 1974. *Asimov on Chemistry*. Doubleday, New York.

Barsanti, L., Gualtieri, P. 2014. *Algae: Anatomy, Biochemistry, and Biotechnology, Second Edition*. CRC Press, Boca Raton, FL.

Berges, J.A., Franklin, D.J., Harrison, P.J. 2001. Evolution of an artificial seawater medium: Improvements in enriched seawater, artificial water over the last two decades. *Journal of Phycology*, **37**(6), 1138-1145.

Bhuiyan, M.I., Mavinic, D.S., Beckie, R.D. 2007. A solubility and thermodynamic study of struvite. *Environ Technol.*, **28**(9), 1015-26.

Bhuiyan, M.I., Mavinic, D.S., Koch, F.A. 2008. Phosphorus recovery from wastewater through struvite formation in fluidized bed reactors: a sustainable approach. *Water Sci Technol*, **57**(2), 175-81.

Bube, K. 1910. Uber magnesium ammonium phosphate. *Z. Anal. Chem.*, **49**, 525-596.

Buluswar, S., Friedman, Z., Mehta, P., Mitra, S., Sathre, R. 2014. 50 Breakthroughs: Critical scientific and technological advances needed for sustainable global development. in: *LIGTT*, LBNL Institute for Globally Transformative Technologies. Berkeley, CA, USA.

Cakmak, Z.E., Olmez, T.T., Cakmak, T., Menemen, Y., Tekinay, T. 2014. Induction of triacylglycerol production in Chlamydomonas reinhardtii: Comparative analysis of different element regimes. *Bioresour Technol*, **155**, 379-387.

da Silva, T.L., Reis, A., Medeiros, R., Oliveira, A.C., Gouveia, L. 2009. Oil production towards biofuel from autotrophic microalgae semicontinuous cultivations monitorized by flow cytometry. *Appl Biochem Biotechnol*, **159**(2), 568-78.

Davis, R., Kinchin, C., Markham, J., Tan, E., Laurens, L.M., Sexton, D., Knorr, D., Schoen, P., Lukas, J. 2014. Process Design and Economics for the Conversion of Algal Biomass to Biofuels: Algal Biomass Fractionation to Lipid- and Carbohydrate-derived Fuel Products.

Davis, R.W., Carvalho, B.J., Jones, H.D.T., Singh, S. 2015. The role of photo-osmotic adaptation in semi-continuous culture and lipid release from *Dunaliella viridis*. *Journal of Applied Phycology*, **27**, 109-123.

Davis, R.W., Volponi, J.V., Jones, H.D., Carvalho, B.J., Wu, H., Singh, S. 2012. Multiplex fluorometric assessment of nutrient limitation as a strategy for enhanced lipid enrichment and harvesting of *Neochloris oleoabundans. Biotechnol Bioeng*, **109**(10), 2503-12.

Diaz, R.J., Rosenberg, R. 2008. Spreading dead zones and consequences for marine ecosystems. *Science*, **321**(5891), 926-9.

Dong, B., Ho, N., Ogden, K.L., Arnold, R.G. 2014. Cultivation of *Nannochloropsis salina* in municipal wastewater or digester centrate. *Ecotoxicology and Environmental Safety*, **103**, 45-53.

Encarnacao, T., Burrows, H.D., Pais, A.C., Campos, M.G., Kremer, A. 2012. Effect of N and P on the uptake of magnesium and iron and on the production of

carotenoids and chlorophyll by the microalgae *Nannochloropsis sp*. *Journal of Agricultural Science and Technology* **2**, 824-832.
Fattah, K.P. 2012. Assessing struvite formation potential at wastewater treatment plants. *International Journal of Environmental Science and Development*, **3**(6), 548-552.
Geider, R.J., La Roche, J. 2002. Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis. *European Journal of Phycology*, **37**, 1-17.
Geider, R.J., MacIntyre, H.L. 2002. Physiology and biochemistry of photosynthesis and algal carbon acquisition. in: *Phytoplankton Productivity: Carbon Assimilation in Marine and Freshwater Ecosystems*, (Eds.) P.J.l.B. Williams, D.N. Thomas, C.S. Reynolds, Blackwell. Oxford, UK, pp. 44-77.
Gilbert, N. 2009. The disappearing nutrient. *Nature*, **461**, 716-718.
Greenwell, H.C., Laurens, L.M., Shields, R.J., Lovitt, R.W., Flynn, K.J. 2010. Placing microalgae on the biofuels priority list: a review of the technological challenges. *J Roy Soc Interf*, **7**(46), 703-26.
Hii, Y.S., Soo, C.L., Chuah, T.S., Mohd-Azmi, A., Abol-Munafi, A.B. 2011. Interactive effect of ammonia and nitrate on the nitrogen uptake by *Nannochloropsis sp. Journal of Sustainability Science and Management*, **6**(1), 60-68.
Klausmeier, C.A., Litchman, E., Daufresne, T., Levin, S.A. 2004. Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. *Nature*, **429**, 171-174.
Kuenzler, E.J., Ketchum, B.H. 1962. Rate of phosphorus uptake by *Phaeodactylum tricornutum*. *Bio. Bull.*, **123**(1), 134-145.
Liu, X., Saydah, B., Eranki, P., Colosi, L.M., Mitchell, B.G., Rhodes, J., Clarens, A.F. 2013. Pilot-scale data provide enhanced estimates of the life cycle energy and emissions profile of algae biofuels produced via hydrothermal liquefaction. *Bioresour Technol*, **148**, 163-171.
Lougheed, T. 2011. Phosphorus paradox: Scarcity and overabundance of a key nutrient. *Environmental Health Perspectives*, **119**(5), A208-A213.
Ludwig, C.A. 1938. The availability of different form of nitrogen to a green alga. *Journal of Botany*, **25**, 448-458.
Marti, N., Pastor, L., Bouzas, A., Ferrer, J., Seco, A. 2010. Phosphorus recovery by struvite crystallization in WWTPs: influence of the sludge treatment line operation. *Water Research*, **44**(7), 2371-9.
Mori, N.T., Suzuki, T., Kakuno, S. 2007. Noise of acoustic Doppler velocimeter data in bubbly flow. *Journal of Engineering Mechanics*, **133**(1), 122-125.
Neset, T.-S.N., Cordell, D. 2012. Global phosphorus scarcity: identifying synergies for a sustainable future. *Journal of the Science of Food and Agriculture*, **92**(1), 2-6.
Nydahl, F. 1978. On the peroxodisulphate oxidation of total nitrogen in waters to nitrate. *Water Research*, **12**, 1123-1130.
Pate, R., Klise, G., Wu, B. 2011. Resource demand implications for US algae biofuels production scale-up. *Appl Energ*, **88**(10), 3377-3388.
Perez-Garcia, O., Escalante, F.M.E., de-Bashan, L., Bashan, Y. 2011. Heterotrophic cultures of microalgae: Metabolism and potential products. *Water Research*, **45**, 11-36.

Powell, N., Shilton, A., Pratt, S., Chisti, Y. 2011. Luxury uptake of phosphorus by microalgae in full-scale waste stabilisation ponds. *Water Sci Technol*, **63**(4), 704-9.

Redfield, A.C., Ketchum, B.H., Richards, F.A. 1963. The influence of organisms on the composition of sea-water. in: *The Sea*, (Ed.) M.N. Hill, Interscience Publications. New York, NY, pp. 26-77.

Sengupta, S. 2013. Macronutrient removal and recovery from tertiary treatment of wastewater. in: *Novel Solutions to Water Pollution*, (Eds.) S. Ahuja, K. Hristovski, Oxford University Press. Northamptonshire, G.B., pp. 167-187.

Shu, L., Schneider, P., Jegatheesan, V., Johnson, J. 2006. An economic evaluation of phosphorus recovery as struvite from digester supernatant. *Bioresource Technology*, **97**(17), 2211-6.

Smil, V. 2000. Phosphorus in the environment: Natural flows and human interferences. *Annu Rev Energy Envinron*, **25**, 53-88.

Snoeyink, V.L., Jenkins, D. 1980. *Water chemistry*. Wiley, University of Michigan.

Taylor, A.W., Frazier, A.W., Gurney, E.L. 1963. Solubility product of magnesium ammonium. *Trans. Faraday Soc.*, **59**, 1580-1589.

Taylor, K.A.C.C. 1995. A modification of the phenol/sulfuric acid assay for total carbohydrates giving more comparable absorbances. *Applied Biochemistry and Biotechnology*, **53**(3), 207-214.

Tchobangolous, G., Burton, F.L., Stensel, H.D. 2003. *Wastewater Engineering: Treatment and Reuse*. *4 ed*. McGraw-Hill, New York, NY.

Uysal, A., Yilmazel, Y.D., Demirer, G.N. 2010. The determination of fertilizer quality of the formed struvite from effluent of a sewage sludge anaerobic digester. *Journal of Hazardous Material*, **181**(1-3), 248-54.

Wierzbicki, A., Sallis, J.D., Stevens, E.D., Smith, M., Sikes, C.S. 1997. Crystal growth and molecular modeling studies of inhibition of struvite by phosphocitrate. *Calcified Tissue International*, **61**(3), 216-222.

Cycle 12 *Phaeo* P replacement stock April 9, 2014

Maximum and final biomass productivity (g AFDW/m^2/day) of *Phaeodactylum tricornutum* (local isolate) cultures with phosphorus replacement using commercial struvite

Phosphorus Replacement Level (%Control)	Maximum Biomass Productivity (g AFDW/m^2/day)	Final Biomass Productivity (g AFDW/m^2/day)
Control	16.26±0.00[a]	3.70±0.03[a]
100	14.80±0.38[a]	3.95±1.17[a]
67	15.40±3.26[a]	3.59±0.85[a]

33	16.77±1.32[a]	3.81±1.39[a]