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Facile processing of *Microchloropsis salina* biomass for phosphate recycle

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ABSTRACT

Algal biomass is a proposed feedstock for sustainable production of petroleum displacing commodities. However, production of 10% of US demand for liquid transportation fuel from algae would require a 60 150% increase over current agricultural demand for phosphorus fertilizers. Without efforts to recycle major nutrients, algal biomass production can be expected to catalyze a food versus fuel crisis. We have developed a novel and simple process for efficient liberation of phosphate from algal biomass and have demonstrated recycling at both laboratory and pilot scale, of up to 70% of total cellular phosphate from osmotically-shocked but non-denatured *Microchloropsis salina* biomass using a range of mild incubation conditions. The phosphate released in this process is bioavailable, can support the same level of algal growth as standard nutrients, and does not contain any growth inhibitory compounds as evidenced by its ability to support multiple sequential cycles of growth and remineralization.

1. Introduction

Microalgae have been successfully utilized for many different applications including biofuels, methane production, human consumption, cosmetics, pigments and fishmeal replacements [1,2]. In particular, algal biofuels are part of the United States Department of Energy (DOE) roadmap to sustainability with a target of at least 36 billion gal of renewable fuel desired by 2022 as set by the Energy Independence Act of 2007 [3]. Microalgae's attractiveness lies in the fact that it does not require arable land or freshwater and can generate carbon-neutral biofuels to replace fossil fuels with large greenhouse gas emissions [4 6]. One major challenge facing industrial microalgae production is the availability of the nutrients required to cultivate quantities conducive to large scale needs [7 9]. As with most crops, microalgae require the application of essential nutrients (primarily nitrogen, phosphorus, and carbon) to reach desirable densities. Limitation of nutrients adversely affects algae production. In addition, fertilizer costs are among the largest operating expense burdens to large scale microalgae production [7,10,11].

Although nitrogen fertilizer can be produced commercially as ammonia through the Haber-Bosch process, phosphorus is a non-renewable resource that is mined from finite reserves. While determining the timeframe for exhaustion of total mineral phosphate varies widely and is not of immediate concern, it is agreed that worldwide phosphorus consumption will increase causing major price increases [12 14]. Furthermore, phosphate mining in the future could increasingly become reliant on one country, Morocco, which could create market instability [15]. US demand for liquid fuels in 2008 was approximately 300 billion gallons per year (BGY). Assuming algal biomass is 0.5% phosphorus and contains 20 50% lipid, algal lipid production sufficient to replace 10% of the US petroleum demand (30 BGY) would require 62 154% of 2006 US phosphate demand [9]. Therefore, it is imperative to find avenues for phosphorus recycle and re-use to allow for sustainable algal biofuel production.

Phosphorus has been successfully recycled from various sources of wastewater with municipal wastewater garnering the most interest due to its overall quantity and existing infrastructure [16]. While the technology exists to reclaim the phosphorus from municipal wastewater,

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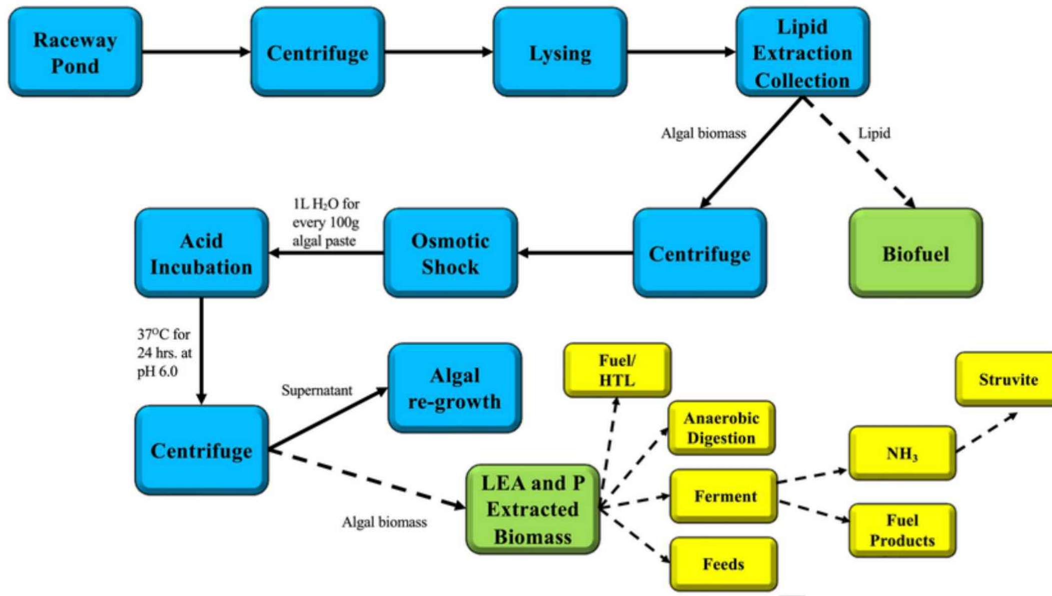

Fig. 1. Flow diagram of pilot-scale algal growth, harvest, lysis, osmotic shock, and algal re-growth process. Dashed lines indicate potential downstream processing steps not conducted in this study.

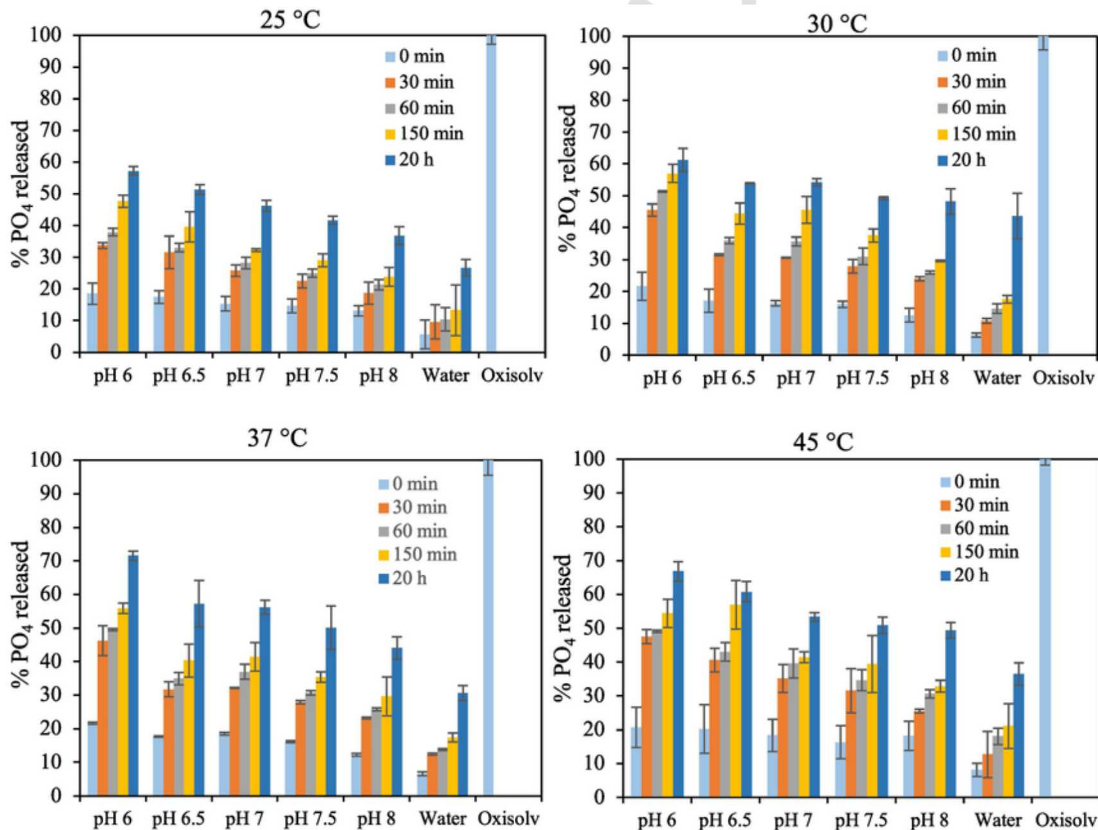

Fig. 2. Effect of temperature and pH on rate of phosphate remineralization from *M. salina* biomass. At each time point soluble phosphate was determined. As a control, total cellular phosphate was determined after Oxisolv digestion of original biomass.

only about 1% of the US petroleum demand could be satisfied if all municipal wastewater in the US were used for algae cultivation assuming 4.5×10^{13} L of wastewater per year [17], 7 mg P/L [18], and algae with 0.5% phosphorus and 25% lipid. Thus, other avenues for phosphorus recovery must be explored beyond wastewater to achieve large-scale algae for fuels production capacity.

In addition to using phosphorus from waste streams, the phosphorus demand for algae cultivation can be reduced by recycling the phosphorus contained in the microalgae itself. Nucleic acids (RNA and DNA) and phospholipids constitute the primary pools of cellular phosphate in eukaryotic microalgae. There have been prior attempts to recover this phosphorus from algae by collecting it from the products of anaerobic di-

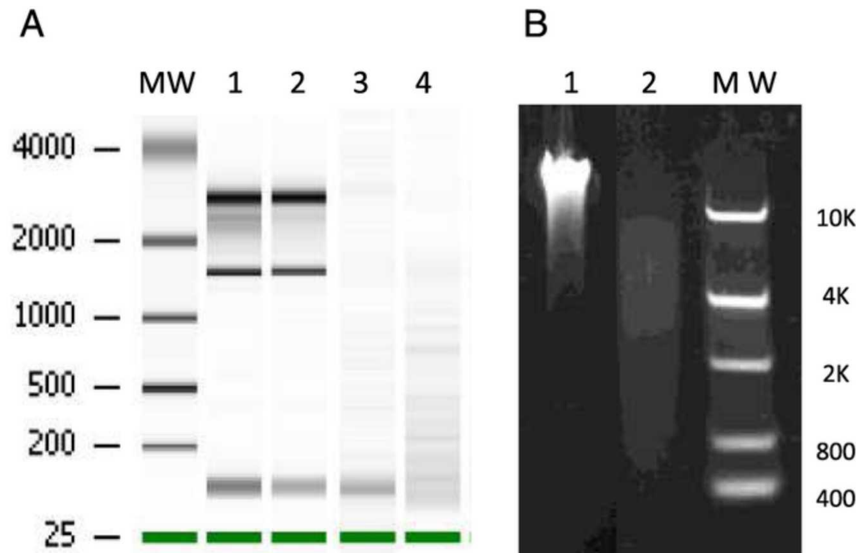

Fig. 3. Degradation of *M. salina* RNA pool during phosphate remineralization. Lanes 1 and 2 show total RNA prior to remineralization, while lane 3 and 4 show total RNA after remineralization. B. Degradation of *M. salina* genomic DNA during phosphate remineralization. Lane 1 shows genomic DNA prior to remineralization. Lane 2 shows genomic DNA after remineralization (25 times more material was loaded in lane 2 than in lane 1).

gestion or thermochemical conversion. Anaerobic digestion typically yields a product with total ammonia amounts that would inhibit microalgae growth and therefore must be diluted [19]. In addition, while it may be possible to overcome, sodium can inhibit cellular metabolism of bacteria during the process [20,21]. This problem may be unavoidable at large scale due to the need to utilize species that require saltwater so as not to compete with crop irrigation and water for human consumption. Phosphorus liberated through thermochemical conversions, including catalytic hydrothermal gasification [7,22] and hydrothermal liquefaction (HTL) [23,24], produces a nutrient rich aqueous phase that can be recycled as growth media. Unfortunately, catalysts (such as nickel) used in the conversion processes may reduce the microalgae's ability to uptake nutrients when directly recycled into the growth media [23,25]. Rösch et al. [22] even considered recovering nutrients from the manure of animals fed residual algal biomass, but this approach seems unfeasible for adoption into most livestock production.

The goal of this study was to develop a simple osmotic shock and digestion technique to remove phosphorus from harvested *Microchloropsis salina* biomass and recycle it for cultivation of new cultures of *M. salina*. Lab-scale trials were conducted testing different temperature and pH levels to determine their effect on phosphorus removal and subsequent lab-scale cultures were grown on the liberated phosphorus. Pilot-scale outdoor trials were also conducted using phosphorus liberated from lipid-extracted algal biomass (LEA) using a solventless process. A concomitant benefit of the solventless lipid extraction and phosphorus recycling procedure described here is that proteins are left intact, making the LEA usable for generating revenue streams in other industries, such as aquaculture.

2. Materials and methods

Two lab-scale experiments and two pilot-scale experiments were conducted for this study. The lab-scale experiments include 1) evaluation of the impact of time, temperature, and pH during incubation on phosphate remineralization rate and 2) the impact of recycling phosphate multiple times on algal growth rate in small cultures. The pilot-scale experiments (illustrated in Fig. 1) included two trials and evaluated the impact of using recycled phosphate on algal growth rate in mid-scale outdoor cultures. Cultivation and processing methods are described below.

2.1. Lab-scale phosphate recycling experiments

2.1.1. Algal strain and culture conditions

Axenic cultures of *M. salina* (CCMP 1776) were obtained from the National Center for Marine Algae and Microbiota (Boothbay Harbor, ME). Stock cultures were maintained in ESAW medium [26] under constant light and temperature (100 mol photons m ^{2} s ^{1} and 21 °C). Cell numbers, in both stock and experimental cultures, were determined by direct counting using a Coulter Z2 particle counter (Beckman Coulter Inc., Fullerton, CA).

2.1.2. Phosphate remineralization

For phosphate remineralization studies, 900 mL cultures, in 1 L bottles, of *M. salina* were grown in ESAW medium supplemented with 44.8 M phosphate (2×) and 1.65 mM nitrate (3×) under constant light and temperature (100 mol photons m ^{2} s ^{1} and 21 °C) and constant aeration. This medium was empirically determined to be phosphate limited (data not shown) and should limit the storage accumulation of polyphosphate in the final biomass. Cultures were inoculated with 1×10^6 cells/mL and harvested by centrifugation in early stationary phase (3×10^7 cells/mL). Harvested biomass (approximately 20% solids) was osmotically shocked by rapid resuspension, to 2% solids, in ice cold 40 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH6 8) and incubated at either 25, 30, 37, or 45 °C with shaking (120 rpm). Triplicate 5 mL samples, from each culture, were incubated at each combination of temperature and pH. For each time point (0 min, 30 min, 60 min, 150 min, and 24 h), 3 × 0.5 mL subsamples were removed from each incubation sample, solids were pelleted by centrifugation at 4 °C, and soluble phosphate was determined for each supernatant. Samples of the starting biomass were retained for determination of total cellular phosphate.

2.1.3. Phosphate determination

Dissolved inorganic phosphate was determined with Abcam Phosphate Assay (ab65622) per manufacturer's instructions. Total cellular phosphate was determined by the oxidation of the biomass prior to phosphate analysis. Algal biomass was diluted to 0.5% solids in water. Oxisolv (EMD-Millipore, Billerica, MA; #112936) was added to 25 mg/mL final concentration. Samples were incubated at 99 °C for 3 h then cooled to room temperature. Samples were centrifuged before assaying for phos-

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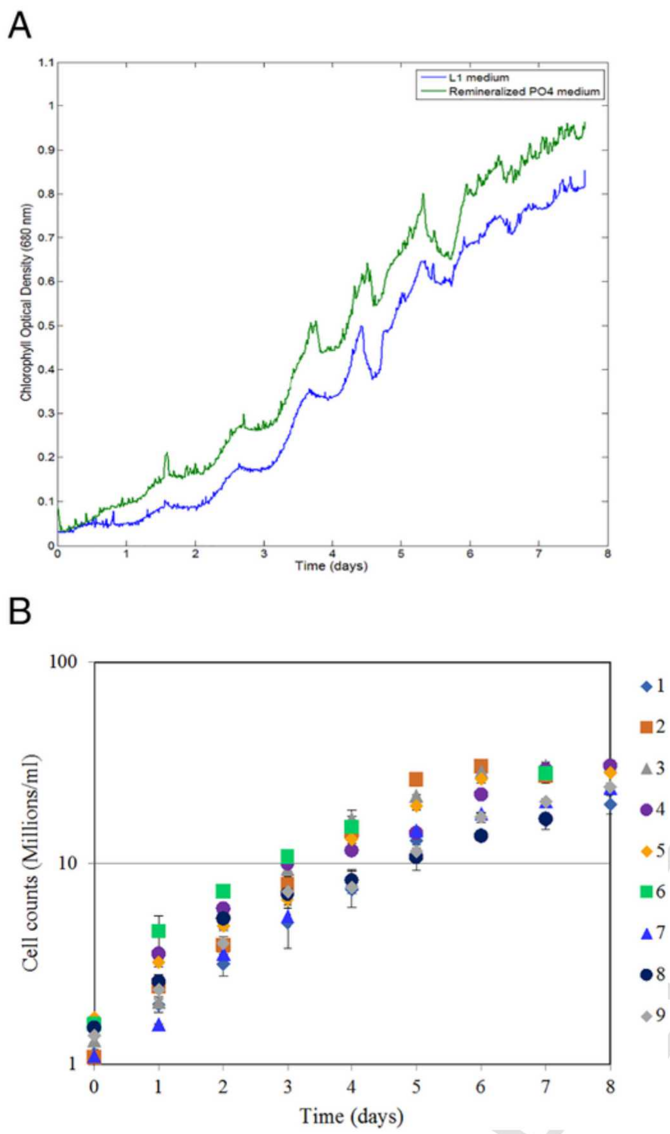

Fig. 4. A) Representative growth curve of *M. salina* using standard nutrients (L1 medium) and phosphate recycled from *M. salina* biomass. B) 9-week sequential recycle of phosphate in cultures of *M. salina*.

phate (Abcam ab65622) and nitrate (Millipore Spectroquant Nitrate assay 114773).

2.1.4. Nucleic acid extraction and analysis

To determine the fate of cellular phosphate pools contained in the nucleic acids, *M. salina* paste was re-suspended in cold H_2O at a final concentration of 2% solids. MES buffer was added to a final concentration of 25 mM at a pH 6. For digestion, samples were incubated at 37 °C for 20 h with gentle shaking. RNA and DNA were extracted from control and digested samples using Zymo ZR Bacterial/Fungal MiniPrep Kits (R2014 (RNA) and D6005 (DNA), Zymo Research, Irvine CA). Extracted RNA was analyzed on a Bioanalyzer RNA Nano 6000 chip (5067-1511, Agilent Technologies, Santa Clara CA) and DNA on a 0.8% E-gel agarose gel (G5018-08, ThermoFisher, Waltham MA).

2.1.5. Laboratory scale phosphate recycling and biomass regrowth experiments

To demonstrate the growth of *M. salina* on remineralized phosphate derived from algal biomass, triplicate 2 liter cultures of *M. salina* were grown in ESAW supplemented with 2× phosphate and 3× nitrates at 100 mol photons m ^{2} s ^{1}, 21 °C, and bubbled with air. Cultures were inoculated with 1×10^6 cells/mL and harvested by centrifugation in early stationary phase (3×10^7 cells/mL). Phosphate was remineralized from algal biomass by resuspension in MES buffer pH 6 at 2% solids and incubation overnight at 37 °C. Soluble phosphate was determined as described above.

Remineralized phosphate was used to replace reagent phosphate in experimental cultures grown in L1 medium [27]. To obtain precise specific growth rates and capture potential effects involved with diurnal cycling, axenic cultures of *M. salina* were inoculated at late-log to achieve starting chlorophyll (680 nm) optical densities of ~0.05 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 diurnal cycle of 16/8 h, with a sinusoidal light cycle corresponding to a photosynthetically active radiation (PAR) irradiance maximum of 1000 mol photons m ^{2} s ^{1} and minimum of < 25 mol photons m ^{2} s ^{1}, measured using a US-SQS/L spherical micro-quantum sensor positioned inside the medium-filled culture tubes. 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. Chlorophyll (680 nm) and scatter-based (720 nm) optical densities of the cultures were measured on-line at 10 minute intervals to obtain growth curves.

To demonstrate multiple subsequent rounds of phosphate remineralization and reuse, triplicate 2 liter cultures were grown in ESAW supplemented with 2× phosphate and 3× nitrate at 100 mol photons m ^{2} s ^{1},

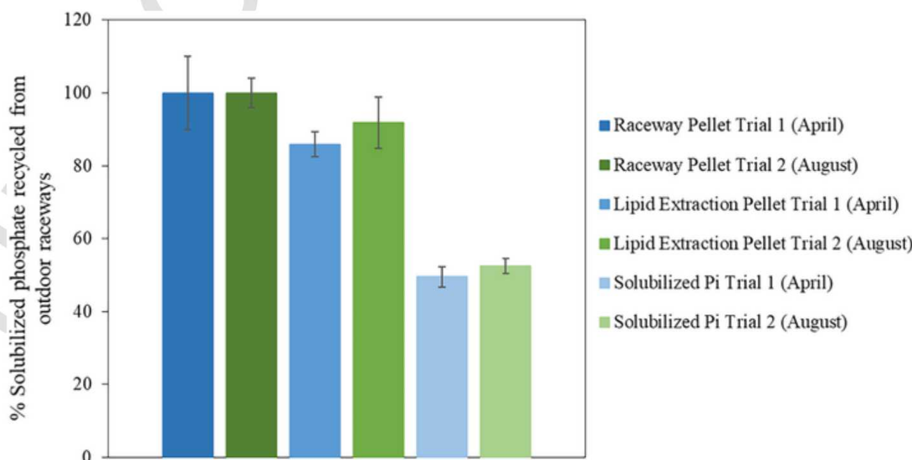

Fig. 5. Total solubilized phosphate available for recycle back into raceways for algal culture. Raceway and lipid extraction pellets are before incubation and solubilized phosphate is after incubation at 37 °C with stirring for 24 h.

Table 1
Environmental data from two pilot-scale outdoor raceway trials.

Trial	Raceway medium temp. (°C)		Salinity (ppt)	pH	Solar radiation (kcal/cm²/day)	Air temp. (°C)	Total precipitation (cm)	Wind speed (mph)
	A.M.	P.M.						
Trial 1	21.2 ± 1.7	27.2 ± 1.3	32.3 ± 1.2	7.6 ± 0.3	399.6 ± 149.5	24.8 ± 1.0	0.13	6.3 ± 1.2
Trial 2	26.9 ± 0.9	31.4 ± 3.7	29.9 ± 1.4	7.6 ± 0.3	502.6 ± 24.9	31.4 ± 0.5	0.33	5.4 ± 0.9

Table 2
Maximum and cumulative productivities (g/L ADFW/m²/day), final densities (g/L AFDW), and 4-day growth rate for both outdoor trials featuring control vs. recycled phosphorus treatments.

Trial	Treatment	Maximum productivity (g AFDW/m²/day)	Cumulative productivity (g AFDW/m²/day)	Final density (g/L AFDW)	4-day growth rate
Trial 1	Control	8.93 ± 0.71	7.99 ± 0.20	0.45 ± 0.01	0.28
	Recycled P	10.03 ± 1.18	8.72 ± 0.45	0.49 ± 0.02	0.29
Trial 2	Control	9.31 ± 1.06	5.91 ± 0.47	0.36 ± 0.02	0.18
	Recycled P	10.51 ± 1.41	5.71 ± 0.62	0.35 ± 0.03	0.18

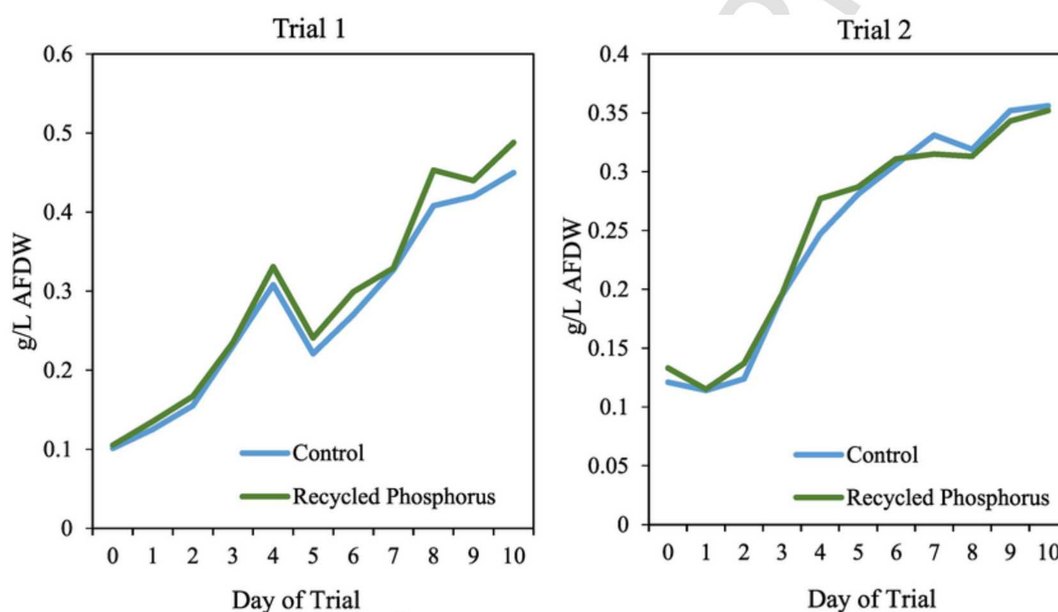

Fig. 6. Exponential phase growth rates (0.28 vs. 0.29 and 0.18 vs. 0.18 respectively) and maximum final densities (0.45 ± 0.01 vs. 0.49 ± 0.02 and 0.36 ± 0.02 vs. 0.35 ± 0.03 g/L AFDW respectively) for control vs. recycled phosphorus treatments for two trials with *M. salina* in outdoor pilot-scale raceways featuring a standard nutrient control vs. a recycled phosphorus treatment terminated after 10 days of culture.

21 °C, and bubbled with air and as described above. Cultures were inoculated with 1×10^6 cells/mL and harvested by centrifugation, in early stationary phase, after 8 days of incubation. Phosphate was remineralized from the harvested biomass by rapid resuspension in MES buffer, pH 6 and incubation at 37 °C overnight. Remineralized phosphate was determined and used to replace up to 70% of the phosphate used for the next round of incubation. This process of biomass growth, harvest, and phosphate remineralization was repeated for 8 subsequent rounds of cultivation. In each subsequent round of cultivation, remineralized phosphate was determined and used to replace up to 70% of the phosphate.

2.2. Pilot-scale phosphate recycling experiments

2.2.1. Algal strain and culture conditions

Cultures of *M. salina* (CCMP 1776) were obtained from the National Center for Marine Algae and Microbiota (Boothbay Harbor, ME). Cultures were scaled up and stocked into 6 (Trial 1) or 12 (Trial 2) outdoor raceways (557 L each at 20 cm depth) at an initial stocking density of ~0.15 g/L AFDW at 5 cm depth. Water depth in each raceway was gradually increased to a final depth of 20 cm over a period of two days. Seawater used in the trials was pumped from the Laguna Madre, Corpus Christi, TX, filtered through a diatomaceous filter (Pentair Pool Products, Sanford, NC), chlorinated to 15 ppm (minimum duration of 30 min) and then stored until use. Experimental raceways were outfitted with a paddlewheel to provide a water flowrate of 50 60 cm/s and a CO_2 injection system which maintained the pH at ~7.8.

2.2.2. Algal biomass harvesting and phosphate remineralization

The sequence of events for algal growth, harvesting, lysing, and algal re-growth is included in Fig. 1. *M. salina* biomass was harvested from six raceway ponds in four batches totaling 2600 L of primary culture volume (Trial 1) and twelve raceway ponds in two batches totaling 6684 L of primary culture volume (Trial 2). Harvesting by centrifugation (WVO Designs, North Charleston, SC) occurred over a two-day period for Trial 1 and a four-day period for Trial 2. For each batch, the recovered biomass paste was re-suspended in natural seawater to roughly 10 L of volume for lysing. After suspension, algal slurry was lysed using the OpenAlgae electromechanical pulsing method [28,29]. Pulses were applied with

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8 kV/cm field strength, 1400 Hz, and 1.5 s pulse width. Each fluid unit volume was exposed to at least 8 pulses. Conductivity was measured to range between 40 and 50 mS/cm for the re-suspended concentrate and the lysing process caused a temperature increase of roughly 11 °C in all batches (initial culture temperature varied from 18 °C to 29 °C depending on time of day). Samples were collected before and after lysing. Analysis of the phosphate content of the biomass before and after electrolysis indicated little effect of the lysing method on cellular phosphate levels (data not shown).

A microporous hollow-fiber membrane contactor was used to separate neutral lipids from the lysed biomass. The slurry was passed through the contactor for seven passes and samples were collected before and after extraction. Analysis of the phosphate content of the biomass before and after lipid extraction indicated little effect of the method on phosphate content of the biomass (data not shown). The lipid-extracted algal slurry was then dewatered by centrifugation (WVO Designs, North Charleston, SC). Lipid extracted algal paste was then osmotically shocked with deionized water at ~1 L water to 100 g paste. The mixture was then pH adjusted to 6.0 with 1 M citric acid solution and incubated for 24 h at 37 °C with constant stirring (as previously determined by lab-scale trials to result in effective phosphate remineralization). After incubation, the algal slurry was centrifuged to obtain the phosphate rich supernatant. Total phosphate of the supernatant subsample was measured using FiaLab (FIAlab 2600, FIAlab Instruments Inc., Bellevue, WA). This supernatant was applied directly to outdoor algae cultures as described below.

2.2.3. Experimental trials utilizing recycled phosphorus

Two experimental trials were conducted with lysed, lipid extracted, osmotically shocked, and digested *M. salina* supernatant. Trial 1 control raceways (n = 3) were supplemented with a standard nutrient blend (Zmora and Richmond, 2007) adjusted to an N:P ratio of 10:1 at 0.49 mM nitrogen (as ammonium sulfate), 0.05 mM phosphorus matched to the molar equivalent of the determined cellular released phosphate for the treatment raceways (phosphoric acid), and 0.018 mM iron (iron sulfate). Recycled phosphorus treatment raceways (n = 3) were supplemented with recycled phosphorus (liberated cellular phosphorus), nitrogen (ammonium sulfate), and iron (iron sulfate) to match equivalent molar levels as those in the control raceways. Trial 2 control raceways (n = 6) were supplemented with a standard nutrient blend (Zmora and Richmond, 2007) to an N:P ratio of 13:1 at 1.04 mM nitrogen (ammonium sulfate), 0.08 mM phosphorus matched to the molar equivalent of the determined cellular released phosphate for the treatment raceways (phosphoric acid), and 0.02 mM iron (from iron sulfate).Recycled phosphorus treatment raceways (n = 6) were supplemented with recycled phosphorus (liberated cellular phosphorus), nitrogen (ammonium sulfate), and iron (iron sulfate) to match equivalent molar levels as those in the control raceways. Trials were terminated after 10 days.

2.2.4. Outdoor culture monitoring

Cultures were monitored twice daily (AM and PM) for salinity, temperature, and pH using a YSI PRO DO 2030 handheld meter and a YSI pH100A handheld meter (YSI, Inc., Yellow Springs, OH). Raceways were sampled daily for AFDW [30], total phosphorus and ammonia (FIAlab 2600, FIAlab Instruments Inc., Bellevue, WA). Daily algal productivity (g AFDW/m^2/day) values were tested for normality and equality of variance prior to ANOVA statistical analysis using R-Studio (V. 0.98.1091). Tukey's HSD was used to determine statistical differences with error rate set at p 0.05. Outdoor raceways were maintained at a pH of ~7.8 using a PinPoint pH controller (American Marine Inc. Ridgefield, CT) attached to a solenoid with CO_2 delivered via a ceramic air diffuser (Sweetwater, Apoka, FL). Environmental parameters (solar radiation, rainfall, wind speed, and air temperature) were monitored continuously using an on-site weather station which is part of the Texas AgriLife crop monitoring program (http://cwp.tamu.edu.com).

3. Results and discussion

3.1. Lab-scale

3.1.1. Phosphate remineralization and recycle

Fig. 2 demonstrates that phosphorus release increased with time and decreasing pH for all time-temperature-pH combinations tested with osmotically shocked but otherwise non-denatured *M. salina*. The amount of phosphate released also generally increased with increasing temperature, but this trend was not monotonic. We found that the endogenous rate of phosphate remineralization at pH 6 and 37 °C was sufficient to release 70% of total cellular phosphate over a period of 24 h. Both RNA and genomic DNA appeared to be substantially degraded during the remineralization process (Fig. 3), but phospholipids remained largely intact (data not shown).

Remineralized phosphate used to completely replace total phosphate in standard algal growth medium L supported the growth of *M. salina* (Fig. 4). The overall culture yield was slightly higher using recycled nutrients, Growth curve data from the multicultivator and diel cycle incubator growth trials are depicted in Fig. 4A. Representative growth curves from 8-day trials are depicted, and clearly indicate that the cultures grown on recycled P-based medium perform at least as well as control medium with no adverse consequences during light or dark cycles, despite appreciable respiration loss in both the control and experimental trials. The data further suggest some improvement of biomass titers can be achieved using recycled P, which might be due to mixotrophic growth on dissolved organic carbon carried over with the soluble phosphate.

Multiple rounds of phosphate recycle and regrowth were carried out in modified ESAW medium as described above in which the ratio of phosphate to nitrate was demonstrated to result in phosphate limitation. As shown in Fig. 4B, although there was some variation, in each round of incubation we did not see a dramatic decrease in either growth rate or final culture yield over the course of the trials. This is not an unexpected result. Phosphate is remineralized from harvested biomass and therefore is not subject to accumulation of inhibitory compounds such as that which can occur during the recycle of culture water (for review see [31]).

3.2. Pilot-scale

3.2.1. Phosphate remineralization

Both pilot-scale trials resulted in ~50% remineralized phosphate available for recycle back into culture water (Fig. 5) which is less than the 70% obtained in lab scale trials even though the same processing conditions were utilized. Some of the reduction can be accounted for via biomass losses that occurred during centrifuging events and possible biomass loss during lipid extractions stage. Also, the larger volumes of re-suspended algal biomass (30 L for Trial 1 and 60 L for Trial 2) compared to lab-scale may have taken longer to reach the desired incubation temperature of 37 °C during the 24 h incubation process which could have resulted in less remineralization time. This can easily be solved by pre-heating the water to be used for the incubation process.

The pilot-scale results highlight the potential for phosphate recycling with a method that does not require dangerous or potentially co-product damaging chemicals. Protein capabilities of lipid and phosphorus-extracted algal biomass were evaluated in an enzyme viability in-vitro test of *Litopenaeus setiferus* (Gulf White Shrimp) according to the methods in Ali et al. [32]. This trial verified that the proteins were still intact and available for use by the shrimp (data not shown). Thus, the lipid extraction and phosphorus remineralization processes in this study did not significantly alter the proteins or phosphorus as evidenced by the shrimp and algae's ability to utilize them to support growth. This allows for the

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protein fraction of the post-extraction biomass to be suitable for use for other industries and the remineralized phosphate available for recycle back to the culture.

3.2.2. Growth trials utilizing recycled phosphate

The outdoor regrowth trials were conducted to determine the potential for utilizing recycled phosphate from lipid-extracted algae as the sole phosphorus source for *M. salina* cultures. Outdoor raceway environmental parameters for both trials are listed in Table 1. When comparing the control vs. recycled phosphorus raceways (Table 2), both trials resulted in statistically similar maximum productivity (Trial 1: 8.93 ± 0.71 (control) vs. 10.03 ± 1.18 and Trial 2: 9.31 ± 1.06 (control) vs. 10.51 ± 1.41) and cumulative productivity (Trial 1: 7.99 ± 0.2 (control) vs. 8.72 ± 0.45 and Trial 2: 5.91 ± 0.47 (control) vs. 5.71 ± 0.62) (g AFDW/m^2/day). Likewise, Trials 1 and 2 both exhibited statistically similar final algal densities to controls (Trial 1: 0.45 ± 0.01 (control) vs. 0.49 ± 0.02 and Trial 2: 0.36 ± 0.02 (control) vs. 0.35 ± 0.03 g/L AFDW) and growth rates after 4-days of culture (Trial 1: 0.28 (control) vs. 0.29 and Trial 2: 0.18 (control) vs. 0.18 respectively) for control vs. recycled phosphorus treatments (Fig. 6). This result demonstrates the ability to use recycled phosphate from lipid extracted algae and a simple osmotic shock incubation procedure to successfully culture *M. salina* outdoors in raceways. No additional processing of the liberated phosphorus was necessary unlike that from digestion or thermochemical conversion, which can result in a product that needs further dilution or processing to be suitable for algae cultivation. While this experiment was conducted in a batch process to demonstrate the ability of *M. salina* to utilize only recycled phosphorus, it is expected that semi-continuous outdoor cultures could also be operated with entirely recycled phosphorus or with limited phosphorus addition. Microscopic evaluation of cultures revealed no differences in grazers or competing algal species between the control and regrowth cultures; both control and treatment cultures exhibited a limited number of grazers and did not inhibit the introduction of competing algal species. There was an initial concern that cell-derived organic carbon introduced into the raceways with the recycled phosphorus would contribute to bacterial blooms (outdoors, non-axenic cultures). Utilization of different nitrogen levels for the two trials was intended as a test of a potential mechanism to control such bacterial growth. However, no obvious bacterial blooms were noted for either trial.

Current methods of algae production are still cost prohibitive [7,33]. New and innovative methods of lipid extraction and co-product conversion can help alleviate the cost associated with mass algal culture. For fuel-only pathways, such as digestion and thermochemical conversion, remineralizing phosphate from lipid-extracted algae with the process described in this study prior to conversion would effectively recycle the phosphate while still yielding a residual biomass product that is suitable for conversion to fuels. Thus, this technology should be compatible with existing processing methods to enable phosphorus recycling without reducing fuel yields.

We have demonstrated, at laboratory and pilot pond scale, that in the algal biofuel species, *Microchloropsis salina* it is possible to remineralize 50 70% of cellular phosphate from osmotically shocked, non-denatured algal biomass through a simple incubation process. This released phosphate is bioavailable, will support normal algal growth and does not appear to contain any inhibitory agents as evidenced by the repeated recycle. There are two advantages to recycling nutrients as the first step in biomass processing for the production of fuels. This process does not require any additional treatment of the phosphate prior to utilization and is compatible with a variety of lipid extraction or biomass processing methods.

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Declarations of interest

The authors have no conflicts of interest.

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