Nutrient Removal by Algae Grown in CO₂-Enriched Wastewater over a Range of Nitrogen-to-Phosphorus Ratios

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Abstract

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In conventional wastewater treatment, biological nutrient removal (BNR) depends on bacterial assimilation for phosphorus removal and nitrification+denitrification for nitrogen removal, with the resulting loss of the fixed nitrogen resource. Alternatively, treatment by microalgae allows for assimilative removal of both phosphorus (P) and nitrogen (N) thereby avoiding the oxygen demand of nitrification and preserving fixed N for fertilizer use. Paddle wheel mixed high-rate ponds have much higher algal productivity than typical oxidation ponds, but even high-rate ponds often cannot grow sufficient algae to completely assimilate the N and P in domestic wastewater. Algae growth in high-rate ponds is usually limited by the inorganic carbon concentration. Addition of carbon dioxide to high-rate ponds, for example from flue gas, eliminates this limitation and accelerates algae growth and nutrient assimilation. This laboratory study explored the extent to which soluble N and P are removed simultaneously by CO₂-enriched algae cultures.

Algal polycultures were grown on diluted domestic wastewater media that were manipulated to obtain a wide range of N:P ratios (2.5:1 to 103:1). In addition, two levels of trace metal concentrations were studied. Media feeding was semi-continuous. The variables monitored included N and P removals, the range of N:P ratios in the algal biomass, biomass production, and alkalinity. To achieve removal of total N and P, suspended solids also must be removed prior to discharge. Since flocculation and settling is a preferred method of algae removal, the effects of low dissolved nutrient concentrations and media composition on algae sinking potential (settleability) were also investigated.

The low trace metal cultures achieved >99% total ammonia nitrogen (TAN) removal for N:P ratios 2.5 through 30 and >98% dissolved reactive phosphorus (DRP) removal for N:P ratios 2.5 through 60 (with one exception at N:P-20). This removal was due to the growth of 180-500 mg/L algal volatile solids. Effluent concentrations were <0.1 mg/L TAN for N:P 2.5 through 30, and <0.5 mg/L TAN for N:P-60. DRP effluent concentrations were ≤0.02 mg/L DRP. After 24 hours of settling in beakers, nearly all cultures had total suspended solids (TSS) concentrations <40 mg/L. Alkalinity consumption increased with increasing N:P ratios.

For cultures with the higher trace metal concentrations, nutrient removal was similar: >96% of TAN and >95.9% DRP removal for all N:P conditions. However, settling with these media was poor. TSS concentrations after 24-h of settling were >100 mg/L. No clear relationship for alkalinity was found for these cultures.

N:P ratios in the algal biomass correlated with the N:P ratios in the media, except for control cultures that did not receive wastewater. No relationship was found between settling and the N:P ratios of the media or biomass. Nitrogen-fixing algae thrived in media containing N:P ratios of 2.5:1 and 5:1.

Algae were found to be plastic in their cellular N:P ratios (4.6 to 63, with wastewater media) which allowed them to simultaneously remove both dissolved N and P to low levels, while growing settleable biomass. These results indicate that CO₂-enriched high rate pond systems would be useful in simultaneously removing N and P from wastewaters with a wide range of N:P ratios.

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Introduction

With the increase in CO₂ in the atmosphere (IPCC, 2007), increases in oil prices (CEC, 2008) and food prices (FAO, 2008), and continued impairment of surface and ground waters (USEPA, 2002; UNEP, 2006), there is increasing impetus to develop low-cost, energy-efficient methods of wastewater treatment. One such method uses shallow, stirred high-rate ponds (HRPs) that produce algae for wastewater oxygenation and nutrient removal (Nurdogan and Oswald, 1995; Downing *et al.*, 2002). Since algae might be exploited as an energy crop (Oswald and Golueke, 1960; Sheehan *et al.*, 1998), algal HRPs are a form of solar energy collector in addition to performing the work of wastewater treatment. Although large-scale HRPs have been used for wastewater treatment since the 1960s (Oswald *et al.*, 1970), their use for nutrient removal has been limited by the low dissolved CO₂ concentrations available for assimilation (Goldman *et al.*, 1974), by nutrient-rich suspended solids in the effluent, and, in winter, by low insolation and temperature (Oswald, 1981). Removing the CO₂ limitation has been shown to be beneficial to treatment. Sparging of CO₂ into wastewater algae cultures accelerates algae growth and nutrient removal (Woertz *et al.*, 2009).

At full-scale, this CO₂ could originate from flue gas or other industrial processes (Benemann, 2003). Flue gas exchange with HRP waters is performed at commercial algae production facilities (e.g., Cyanotech Corporation, Hawaii and Seambiotic, Israel) by gas sparging in the ponds. Alternatively, CO₂ can be stripped from flue gas using technologies such as wet scrubbers and concentrated for delivery to remote HRPs

(Dooley *et al.*, 2008). Large-scale use of HRP to fix CO₂ and produce algae has been researched extensively by governments and commercial interests over the years (e.g., Sheehan *et al.*, 1998; Negoro *et al.*, 1991; Nakamura *et al.*, 2005).

The primary objective of this research was to determine the plasticity of the N:P ratio in algae performing nutrient removal from wastewaters with a wide range of influent N:P concentrations and to measure the effluent dissolved nutrient concentrations. To eliminate carbon limitation, CO₂ was sparged into the cultures.

Once grown, algae need to be removed from the treated wastewater to complete the nutrient removal and meet suspended solids discharge limits. One of the difficulties of algal treatment is removal of the microalgae due to their colloidal nature (Eisenberg *et al.*, 1981). The stress of low nutrient concentrations may promote algae settling (Kjørboe *et al.*, 1990); thus an effective nutrient removal process could also improve algae removal. This possibility was tested as a secondary objective of this research by comparing the settleability of cultures with different levels of nutrient removal and different N:P ratios.

These objectives have implications for the treatment of municipal wastewater as well as agricultural and industrial wastewaters with extreme N:P ratios. The nutrient content and thus the nutrient requirements of algal cells and algae productivity under low nutrient conditions are also important considerations in any scheme for algae production for biofuel.

Background

Eutrophication is one of the main water quality problems throughout the world (UNEP, 2006). In many U.S. waterways, nutrients are the leading cause of impairment. The nutrient sources include discharges of treated industrial and municipal wastewaters (USEPA, 2002). While many wastewater treatment plants have been upgraded over the past two decades to use bacterial nitrification, converting ammonia to nitrate, these upgrades have not decreased significantly the overall nitrogen discharge to receiving waters near metropolitan areas in the U.S. (USGS, 1999).

Current biological nutrient removal (BNR) technologies typically use bacterial nitrification followed by denitrification to remove the bulk of the nitrogen, and bacterial assimilation and/or chemical precipitation to remove phosphates. These methods, especially in activated sludge processes, are effective but consume considerable amounts of energy. For nitrogen removal plants, energy intensity ranges widely from 0.7 to 2.1 kWh/kg bCOD (biodegradable Chemical Oxygen Demand) removed (de Haas and Hartley, 2004). Capital costs are also high. For example, the nitrogen removal upgrade of a 24 million gallon per day activated sludge plant cost \$2 per gallon per day capacity (Brown *et al.*, 2007). Similarly, phosphorus removal by biological or chemical means adds cost. For example, Jiang *et al.* (2005) estimated that upgrades to a 20 MGD activated sludge plant to decrease effluent phosphorus concentration from 2 mg/L to 0.05 mg/L, using a combination of biological and chemical means, would have a total annualized cost of about \$0.50 per gallon per day capacity. In addition to being

expensive, traditional BNR systems may not effectively treat industrial wastewaters with low N:P ratios (e.g., corn, bakery, and textile processing) or those with high N:P ratios (e.g., leather tanning and petroleum refining) (Gerardi, 2002).

A potential limitation of any assimilative nutrient removal processes would be an imbalance of N:P in the wastewater compared to the N:P ratio in the cell tissues. Using algae for example, if the classic Redfield atomic ratio of 16:1 N:P was considered unchangeable, then P would remain in solution when algae were used to treat wastewater with a N:P ratio lower than 16:1. However, Klausmeier *et al.* (2004a) state that the Redfield ratio should be seen as an effect of the N:P levels in the media examined, not as a result of the organisms' capabilities or optimal growing conditions. In addition, Rhee and others have found that optimum N:P ratios were species specific (Rhee, 1978; Rhee and Gotham, 1980). Further, Bulgakov and Levich (1999) found that environmental N:P ratios affect phytoplankton community structure, shifting it from chlorophytes at higher N:P ratios to cyanophytes (which can fix nitrogen) at lower N:P ratios.

A mathematical model by Klausmeier *et al.* (2004b) predicts that, at slow growth rates, cellular N:P ratios match media input ratios, between N:P atomic ratios of 5 and 80. Rhee (1978) showed that with N:P atomic ratios in the media between 5 and 80, nitrogen and phosphorus were removed below detection levels. He suggested these levels of nitrogen and phosphorus left in the surrounding media were likely only detectable when the N:P atomic ratio in the feed was lower than the minimum cellular N:P (e.g., the minimum cellular N:P for *Scenedesmus* sp. is about 4:1), or higher than the maximum cellular N:P

(e.g., the maximum cellular N:P for *Scenedesmus* sp. is about 142:1). However, neither Klausmeier nor Rhee used wastewater in their experiments.

For strictly assimilative nutrient removal from any wastewater, a carbon (C) source must be available, or added as needed (Jewell et al., 1992). Most algal species cannot use bicarbonate directly, so the addition of CO₂ to the medium provides the algae with an accessible carbon source which can increase growth rates even when total inorganic carbon concentrations are high (Azov *et al.*, 1982). When pond surface area for natural CO₂ dissolution from the atmosphere is sufficient, algae can be effective in removing nutrients from domestic wastewater (Nurdogan and Oswald, 1995). For autotrophic algal treatment, complete BNR may be accomplished with inorganic carbon addition (Woertz *et al.*, 2009; Feffer, 2007), but the capability of nutrient assimilation by algae will also depend on factors such as light, pond depth, temperature, algae grazers, and the wastewater constituents (N:P ratios, micronutrients, and inhibitors).

Where land availability and climate allow, pond-based wastewater treatment requires less energy and expense than activated sludge, in part due to photosynthetic oxygenation by algae. Downing *et al.* (2002) estimated the total cost of pond based wastewater treatment to be about 1/3 that of activated sludge wastewater treatment for secondary treatment plus filtration and partial nutrient removal.

Algal biofuel production is another area of interest. Algae oil production rates from mixed-cultures of native algae are projected to be between 1,200 and 2,200 gal/acre/yr,

about 20 to 40 times higher than soy (Woertz *et al.*, 2009). Thus, production of biodiesel from an algal pond system treating wastewater could reduce net operational costs, while treating wastewater and offsetting fossil fuel CO₂ emissions.

A common problem with treatment ponds is excessive total suspended solids (TSS) in the effluent, primarily colloidal algae. Although algae removal can be accomplished with the aid of chemical coagulation, algal bioflocculation (the spontaneous flocculation of cells due to biological activity) followed by sedimentation is potentially a lower-cost alternative, but one that is still under development (Eisenberg *et al.*, 1981; Garcia *et al.*, 2000). Nutrient limitation promotes bioflocculation of algae in seawater (Kjørboe *et al.*, 1990), and thus stress caused by complete uptake of one or more nutrients in CO₂-supplemented wastewater media may improve flocculation, especially in media with extreme initial N:P ratios.

Considering the literature, it is expected that a mixed culture of algae should be able to effectively treat wastewaters with a wide range of N:P ratios, removing nitrogen and phosphorus to low levels, assuming other nutrients and trace materials are sufficient. Further, when these low levels are reached, algae may flocculate and settle, decreasing or eliminating the need for chemical coagulation of wastewater pond effluent. In addition, those algal species that have optimum growth N:P ratios similar to the initial media N:P ratio are likely to dominate in the culture.

Methods

In three experiments (termed A, B, and C), partially-treated domestic sewage was diluted and fortified with N or P to create media with specific N:P ratios and concentrations. These media were fed to duplicate cultures in Roux bottles sparged with an air-CO₂ mixture. The bottles were inoculated with a mixture of green and blue-green microalgae and cultured between rows of fluorescent lamps. Fresh media were fed daily to maintain a 3-day hydraulic residence time in each bottle. Total and volatile suspended solids (TSS and VSS) concentrations were determined twice per week, and pH and temperature were measured once per week. Samples of culture bottle effluent were analyzed for nutrient content, as described below, on a weekly basis. Data from these analyses were used to characterize nutrient removal and cell nutrient content only after the cultures had reached steady state (in this case, defined as three consecutive measurements of VSS within 20% of each other).

Culture Bottles and Lighting

The 1-L Pyrex[®] Roux bottles were filled with medium to 900 mL and stirred by 2.5-cm, TFE-coated magnetic bars, spinning at approximately 300 rpm. The light:dark cycle was 16 h:8 h, with each bottle receiving 4,300 lux from full-spectrum lamps, as described in Feffer (2007). Each bottle was sparged with 0.17 L gas/L medium/min of air containing about 0.5% CO₂ to maintain a pH of 7.0 to 8.0. A photograph of the setup is in Appendix B Additional Methodology. Previous work demonstrated that NH₃ volatilization was negligible from Roux bottles at these pH values (Feffer, 2007).

The algal inoculum originated from earlier laboratory cultures (Feffer, 2007) and contained a variety of species collected from wastewater ponds and streams. The primary genera were *Chlorococcum*, *Chlorella*, *Scenedesmus*, and *Ankistrodesmus*. Members of the family Dictyosphaeriaceae and various diatoms were also present (See Appendix A Micrographs). A polyculture was chosen to more closely mimic wastewater treatment ponds, which typically contain a variety of algae species.

Media

For Experiments A and B, a 55-L sample of wastewater was collected on October 13, 2007, from a primary sedimentation tank effluent weir channel at the San Luis Obispo municipal wastewater treatment plant. For Experiment C, a 35-L sample of wastewater was collected on April 11, 2008 at the same location (Table 1). In both cases, the wastewater was mixed thoroughly and filtered through 190-micron paint filters into HDPE jugs, which were frozen at -20°C for later use. Before use, the wastewater was thawed at room temperature, and then thoroughly mixed before being added to the Roux bottles. Between feedings, thawed wastewater was stored at 2°C.

The feed nutrient ratios in this study are denoted as “N:P-X,” where X is the N:P mass ratio in the feed. The cultures that received only defined medium are appended with “DM.” Each N:P condition was tested in duplicate bottles.

At high cell concentrations, self shading can cause light limitation. To prevent light limitation from affecting the results, the wastewater had to be diluted to achieve target cell concentrations of less than 600 mg/L TSS. For Experiments A and B, each day the feed added to the wastewater cultures contained 75 mL of primary effluent wastewater and 225 mL of one of three defined media with N or P added to achieve the desired N:P ratio. The defined medium-only culture received 300 mL of a fourth defined medium with a specific N:P ratio: for Experiment A, this was an N:P of 20 (N:P-20DM), and for Experiment B, this was an N:P of 103 (N:P-103DM). See Appendix B for defined media recipes.

The micronutrient and macronutrient solutions for the defined media of Experiments A and C were based on algal growth potential medium (hereafter referred to as APHA medium) (APHA, 1995; Appendix B Additional Methodology). Because the cultures in APHA medium without wastewater did not grow well, the defined medium for Experiment B was changed to the WC Medium (Andersen, 2005; Appendix B Additional Methodology), which was found to support more rapid growth but resulted in unsettleable cultures. Since such poor settling was uncharacteristic of outdoor pilot studies using 100% wastewater media (Lundquist, personal communication, April 7, 2008), APHA medium was used once again for Experiment C. The WC media included the N-containing buffer, Tris HCl. This nitrogen was considered in the N:P ratios reported.

Several modifications were made to the formulations of the APHA and WC media. Silica was omitted from all media to moderate diatom growth since primary wastewater ponds are typically dominated by chlorophytes rather than diatoms (Dor, 1999). For all experiments, the concentration of NaHCO_3 in the feed was increased from the levels specified in the media recipe to 672 mg/L to buffer against large pH variations which may occur during the light cycle (Tadesse *et al.*, 2004). The WC medium had much higher concentrations of trace metals than the APHA media, presumably the cause of the differences in culture appearance, settling behavior, and nutrient removals described in the Results and Discussion section. See Appendix B Additional Methodology for details on trace metal concentrations.

In all experiments, NH_4Cl was substituted for NaNO_3 in the media, as oxidized nitrogen is typically absent from untreated domestic wastewater, and ammonium is generally 60% of total Kjeldahl nitrogen (TKN) in untreated wastewater (Barnes and Bliss, 1983).

K_2HPO_4 was the form of phosphorus used in all media (APHA, 1995; Andersen, 2005).

Both media are fully specified in Appendix B Additional Methodology.

To promote adaptation of the inoculum algae to conditions of the current experiments, 10 mL of the previously mentioned algae inoculum was placed into each of two Roux bottles with APHA growth medium for two weeks of batch growth prior to the beginning of the experiments. One bottle contained 225 mL wastewater and 665 mL defined medium; the other bottle contained only defined medium; both bottles had N:P media ratios of 20:1.

To start Experiment A, 10 mL of the adapted inoculum, wastewater, and defined medium (with the appropriate amounts of nitrogen and phosphorus needed for each condition) were mixed and distributed to the Roux bottles. After three days, the daily feeding schedule began.

At the end of Experiment A, all bottles were intermixed. This mixture was used as inoculum in Experiment B, with 600 mL being added to each new bottle. The same procedure was followed to startup Experiment C. Before reuse, the bottles were scrubbed with phosphate-free detergent, rinsed with hot water, and tripled-rinsed with DI water.

For Experiment C, new primary effluent wastewater was collected since the wastewater from the previous collection had already been consumed. This new wastewater had nutrient concentrations that differed from the wastewater used in the previous experiments. To make the Experiment C media, the volume of wastewater added to the bottles was decreased to match the total phosphorus (TP) concentration in the N:P-20 medium of Experiment A. Then three defined media were prepared that created N:P ratios of 2.5, 20, and 60 when mixed with wastewater. Thus, each wastewater culture bottle received 32.5 mL of wastewater and 267.5 mL of defined medium for each feeding. The defined medium-only culture received 300 mL of a fourth defined medium with an N:P of 20 (N:P-20DM). Table 5 and Table 6 (Results Section, page 18) show the final conditions in the feed for all experiments. The amounts of wastewater and dilution media used in each experiment are listed in Table 2 through Table 4.

Table 1 Characteristics of Wastewater Used in Experiments A, B, and C

Parameter	Wastewater used in Experiments A and B (collected October 18, 2007)	Wastewater used in Experiment C (collected April 11, 2008)
sCOD (mg/L)	174	96
TSS (mg/L)	87	98
VSS (mg/L)	45	87
NH _x (mg/L as N)	38	32
TKN (mg/L as N)	56	63
TP (mg/L as P)	2.8	6.5

Note: sCOD is soluble chemical oxygen demand.

Table 2 Wastewater Feed and Dilution Water Volumes, Experiment A

Bottle Name	Ingredient	Amount
N:P-5,20,30	Primary Wastewater	75.0 mL
	Defined Medium (APHA) with supplemental N and P	225 mL
N:P-20DM	Defined Medium (APHA) with N and P	300 mL

Table 3 Wastewater Feed and Dilution Water Volumes, Experiment B

Bottle Name	Ingredient	Amount
N:P-12,82,102	Primary Wastewater	75.0 mL
	Defined Medium (WC) with supplemental N and P	225 mL
N:P-103DM	Defined Medium (WC) with N and P	300 mL

Table 4 Wastewater Feed and Dilution Water Volumes, Experiment C

Bottle Name	Ingredient	Amount
N:P-2.5,20,60	Primary Wastewater	32.5 mL
	Defined Medium (APHA) with supplemental N and P	267.5 mL
N:P-20DM	Defined Medium (APHA) with N and P	300 mL

Nutrient Analyses

Nutrients were determined in effluent samples on a weekly basis beginning one week after each experiment started. Total ammonia, nitrate, nitrite, total Kjeldahl nitrogen, dissolved reactive phosphorus, and total phosphorus were determined according to the

methods described below. Matrix spike samples were analyzed in every batch of nutrient analyses. More than 80% of matrix spike recoveries were between 85% and 115%.

Ammonia and TKN samples were preserved by adding H₂SO₄ until pH 2 was reached, then refrigerated in plastic sample bottles. Samples were analyzed for total ammonia within 7 days, and TKN samples were analyzed within 28 days. Prior to nitrite/nitrate analysis, samples were filtered to 0.22 µm and stored at -20°C in 5-mL Dionex Polyvials, generally undergoing analysis within 28 days. Prior to measuring DRP, samples were filtered to 0.45 µm and refrigerated in glass sample bottles. DRP samples were analyzed within 7 days. Total phosphorus samples were preserved by adding H₂SO₄ until pH 2 was reached, then refrigerating in plastic sample bottles. TP samples were analyzed within 28 days.

Total ammonia (NH₃+NH₄⁺, or NH_x) concentrations were determined using the Ammonia-Selective Electrode Method (APHA, 1995), except for the final six sampling dates. For these, NH_x was determined by a fluorometric method (Protocol B by Holmes *et al.*, 1999).

To help determine if nitrification was occurring, both nitrite (NO₂⁻) and nitrate (NO₃⁻) were determined by ion chromatography (Dionex DX 120).

Organic nitrogen was measured to estimate the N content of the algal cells. The Macro-Kjeldahl Method was used for this purpose (APHA, 1995). The initial ammonia

distillation step of this procedure was omitted since NH_x was determined separately.

Organic N was calculated as the difference of TKN and NH_x . For the TKN titration, 0.01-N H_2SO_4 was used instead of 0.02 N to more precisely determine the endpoint of titration.

The Ascorbic Acid Method (APHA, 1995) was used to measure both dissolved reactive phosphorus (DRP) and total phosphorus (TP). A 0.5-cm cuvette was used for DRP samples in Experiment A. Due to low concentrations in samples, this path length was increased to 5-cm for Experiments B and C (see Appendix B Additional Methodology for details on path length).

The nitrogen content of the cells (%N) was estimated by dividing the difference between TKN and $\text{NH}_x\text{-N}$ by the volatile suspended solids concentration (VSS). The phosphorus content (%P) was determined by dividing the difference between total phosphorus and dissolved reactive phosphorus by the VSS, as follows:

$$\%N = \frac{TKN - \text{NH}_x}{VSS} * 100 \qquad \%P = \frac{TP - \text{DRP}}{VSS} * 100$$

In order to estimate the total nitrogen and total phosphorus concentrations in supernatant (as these were not directly measured), VSS concentrations in the supernatant after 24 hours of settling were multiplied by the initial %N and %P in VSS for those samples.

Suspended Solids and Settling

Because settling is a preferred method to harvest algal biomass and achieve low effluent TSS concentrations, the settleability of the algae in the bottle effluents was measured by performing settling tests in 100-mL Griffin beakers. TSS was measured immediately after sampling from the bottles (0 hours), and after 2 hours and 24 hours of settling.

Settling tests were performed twice per week. Solids samples were refrigerated and analyzed within 7 days. A TSS standard (ULTRACheck) was used to verify method. VSS concentrations were used to estimate algal biomass concentrations (see comments in the following paragraph). TSS and VSS were determined using Method 2500 (APHA, 1995).

Other Analyses

Alkalinity was determined weekly using Method 2320B (APHA, 1995), using a 50-mL sample size. Dissolved oxygen, pH, and temperature were measured occasionally to verify stable conditions. Two weeks after the start of each experiment, algae were identified microscopically with the aid of Prescott *et al.* (1978), and genera dominance was determined qualitatively at 1000X magnification. When identifying algal cells, any matter that appeared to originate from the wastewater was noted. These observations were made once per experiment. Microscopy revealed the majority of biological matter as algae. A minor bacterial component was present, mainly in the form of rod-shaped bacteria adhered to the sides of algae. No zooplankton were observed. As a result, algal concentration was estimated as VSS.

Results and Discussion

Water quality measurements for all experiments were made on samples from duplicate culture bottles following a period of time for steady-state to be achieved. The error bars in the time series graphs (Figure 10 through Figure 14), indicate the standard deviation of only the duplicate bottles. Error bars in the remaining figures indicate the aggregated standard deviation of samples over time from duplicate bottles. As these standard deviations were small in most cases, the reported results shown in Table 5 and Table 6 are the average over the entire steady-state time period. For Experiment A, data from eight sampling dates were used, with duplicates from each N:P ratio. For Experiments B and C, data from four sampling dates were used, also with duplicates from each N:P ratio.

Operating Conditions and Cultures

Dissolved oxygen levels remained above 7 mg/L and often were above 10 mg/L throughout all experiments. Temperature remained $25\pm 2^{\circ}\text{C}$, with the exception of five consecutive days during Experiment A, when cultures reached 32°C due to a building air conditioning malfunction. The higher temperature did not have any observed effect on the cultures.

The cultures were dominated by spherical *Chlorococcum*- and *Chlorella*-like cells, with *Scenedesmus* and members of the family Dictyosphaeriaceae as the second most common organisms. Cyanobacteria (with morphology similar to *Anabaena*, including the presence of heterocysts) were observed in cultures with N:P ratios of 20:1, but significantly more

were found growing in the N:P-2.5 cultures. However, cyanobacteria were never present in greater numbers than chlorophytes. Representative photomicrographs are shown in Appendix A Micrographs.

Feffer (2007) was able to grow cultures inoculated with wastewater pond algae on pure defined media of APHA DM in batch mode, but he noted that growth was slower than in wastewater media. In the present study, several unsuccessful attempts were made to propagate algae on pure APHA DM (the lower trace metal media used in Experiments A and C), including re-inoculation, initial periods of batch growth, and addition of yeast extract and B vitamins. In contrast, growth on pure WC DM (the higher trace metal media used in Experiment B) was successful and could be maintained with a 3-day hydraulic residence time.

Nutrient Removal

Removal of both dissolved N and P was nearly complete for most media N:P ratios. Total ammonia removal was $\geq 99.6\%$ in Experiments A and C except in the N:P-60 culture, which had 98.8% removal (Table 5). Similarly, for Experiment B, ammonia removal was $\geq 99.7\%$ except for N:P-102, with 96.6% removal. Residual total ammonia concentrations averaged <0.1 mg/L as N for most experiments (detection limit: 0.01 mg/L). Nitrate concentrations averaged <0.02 mg/L as N (detection limit: 0.01 mg/L as N), and nitrite was always less than the detection limit (0.01 mg/L as N) for all experiments. Phosphorus removal averaged $>98\%$ for Experiments A and C, except for the N:P-20 (in Experiment A) culture, which achieved 96.8% removal (Table 6). Phosphorus removal was $\geq 95.9\%$

for Experiment B. Remaining dissolved phosphorus concentrations averaged <0.03 mg/L as P for all experiments, with a detection limit of 0.01 mg/L as P.

Table 5 Nitrogen Feed and Effluent Concentrations in All Experiments

N:P Mass Ratio in Feed	Feed				Effluent (NOx remained below detection limit)		%N in Cells
	Total N in Feed (mg/L N)	Particulate N in Feed (mg/L N)	NH _x in Feed (mg/L N)	Tris N in Feed* (mg/L N)	NH _x Out (mg/L N)	% Removal	
2.5 (C)	6.83	1.6	5.2	--	0.03	99.6	6.2
5 (A)	14.0	4.5	9.5	--	0.04	99.7	4.8
12 (B)	57.3	4.5	9.5	43.3	0.16	99.7	12.1
20 (A)	14.0	4.5	9.5	--	0.05	99.6	4.8
20 (C)	14.0	3.4	10.6	--	0.03	99.8	5.0
30 (A)	21.0	4.5	16.5	--	0.09	99.6	4.3
60 (C)	42.0	3.4	38.6	--	0.49	98.8	14.5
82 (B)	57.3	4.5	9.5	43.3	0.05	99.9	13.5
102 (B)	71.2	4.5	37.5	43.3	2.43	96.6	16.1**
103 DM(B)	71.8	0.0	14.0	57.8	0.07	99.9	21.9**

* Tris buffer in WC media contained N. ** Unusually high N contents; analytical error suspected, although quality control tests did not detect errors. See text. In the leftmost column of Table 5 and Table 6 (A), (B), or (C) indicate the experiment code.

Table 6 Phosphorus Feed and Effluent Concentrations in All Experiments

N:P Mass Ratio in Feed	Phosphorus			%P in Cells
	TP In Feed (mg/L P)	DRP Effluent (mg/L P)	% Removal	
2.5 (C)	2.72	<0.01	99.6	1.35
5 (A)	2.80	0.02	99.2	0.88
12 (B)	5.60	0.03	99.6	1.04
20 (A)	0.70	0.02	96.8	0.51
20 (C)	0.70	<0.01	99.9	0.25
30 (A)	0.70	0.01	98.6	0.31
60 (C)	0.70	<0.01	99.9	0.24
82 (B)	0.70	0.03	95.9	0.33
102 (B)	0.70	0.01	98.1	0.29
103 DM(B)	0.70	0.01	98.2	0.19

Nutrients Remaining after Sedimentation

Aliquots of effluent were allowed to settle for 24 h, with sampling at 2 h and 24 h (only 24-h settling data are shown for brevity). Supernatant TSS and VSS were determined, and supernatant particulate nutrient concentrations were estimated using the cell nutrient

contents measured in the unsettled samples. For Experiments A and C, total nitrogen concentrations in the 24-h supernatant were estimated to be <2 mg/L as N, except for N:P-60, in which about 7 mg/L remained. Total phosphorus was estimated to be <0.5 mg/L as P for all conditions. For Experiment B, total nitrogen in the 24-h supernatant was estimated to range from 13 to 32 mg/L as N, and total phosphorus ranged from 0.29 to 1.2 mg/L as P, due to poor settling. Algae settling results are discussed further later in this section.

Cellular and Residual Nutrient Concentrations

As described previously, the different experiments used different defined media for dilution, which affected the settling rate, nutrient assimilation, and cellular nutrient contents. Thus, results from Experiments A and C, which used the APHA medium, are described together; results from Experiment B, which used the WC medium, are described separately.

Nutrient Content of Cells – Experiments A and C

For those bottles receiving feed with 14 mg/L total N, the cellular nitrogen content was around 5%, with the exception of N:P-2.5, for which it was about 6%. Cellular nitrogen levels in the N:P-30 cultures were 4.3%, even though the N:P-30 culture received nitrogen concentrations 1.5-times higher than the lower N:P cultures (Table 5). The N:P-60 medium contained nitrogen concentrations three times higher than the N:P-2.5 through N:P-20 media. The resulting cellular nitrogen level was also around three times higher, at 14.5% (Figure 1). Pure cultures of *Chlorella* grown by Milner (1976) in media with a variety of nutrient levels most often contained 5.2-9.5% N, with occasional examples as

low as 1% and as high as 14% N.

Figure 1 Nitrogen Content in Cells during Experiments A and C

In the current experiments, cellular P content ranged from 0.25-1.4% in the cultures receiving media with N:P ratios of 2.5-20 (Figure 2). At higher N:P ratios, the P content ranged from 0.24-0.31%. Cellular P content generally decreased with increasing N:P input ratios, even with the same concentration of phosphorus in the feed (Figure 2). In comparison, cellular phosphorus in *Chlorella* grown by Milner (1976) ranged from 1.2-1.8%.

Figure 2 Phosphorus Content in Cells during Experiments A and C

Effluent Residual Nutrients – Experiments A and C

In the effluent samples from Experiments A and C, nitrite concentrations were below the detection limit of 0.01 mg/L as N, and nitrate concentrations averaged <0.02 mg/L as N (not graphed). The total ammonia concentrations remaining in cultures during Experiments A and C averaged <0.1 mg/L as N, except in the N:P-60 culture, which contained 0.5 mg/L as N (98.8% removal) (Figure 3). For the experiments with the same influent total ammonia concentration of 14 mg/L as N (N:P-2.5, -5, and -20), the residual total ammonia concentrations were similar (0.03-0.16 mg/L as N).

Figure 3 Total Ammonia Residual during Experiments A and C

DRP remaining in the samples for Experiments A and C averaged <0.025 mg/L as P for all conditions, with levels in Experiment A higher than those in Experiment C (Figure 4).

The residual dissolved N and P concentrations did not correlate with cell N content or cell P content, respectively.

Figure 4 Dissolved Reactive Phosphorus Residual during Experiments A and C

Nutrient Content of Cells – Experiment B

For dilution water and the defined media control cultures of Experiment B, the WC medium was used, which had higher trace metal concentrations than the APHA medium. In Experiment B, cultures that received wastewater followed the same general pattern of nutrient content as the Experiment A and C cultures. However, the linear regression of %N-to-media-N:P-ratio had a lower slope than it did in Experiments A and C (Figure 5). The N content of the cells in Experiment B were generally higher than all the cultures of Experiments A and C (Figure 1 and Figure 5). For Experiment B, the N content range was 12-22%, with the highest N content in the N:P-103 DM culture. The 22% value is higher than seen in the literature; however, quality control results did not indicate any analytical errors for these analyses. Alternative explanations are discussed in the Conclusion section.

Figure 5 Nitrogen Content of Cells during Experiment B

The P content of the cells in Experiment B (0.19-1.0%) were similar to those of Experiments A and C (0.24-1.4%) (Figure 2 and Figure 6). Cellular P content was significantly higher for N:P-12 but was fairly constant for N:P ratios from 82 to 103 (Figure 6).

Figure 6 Phosphorus Content of Cells during Experiment B

Effluent Residual Nutrients – Experiment B

As in the other Experiments, nitrite was not detected in any Experiment B samples, and nitrate concentrations averaged <0.02 mg/L as N. Residual total ammonia concentrations were also low (Figure 7) and similar to those of Experiments A and C. However, the mean total ammonia concentration remaining in the N:P-102 cultures in Experiment B was nearly five-times the amount remaining in N:P-60 cultures in Experiment C (2.43 vs. 0.49 mg/L as N, respectively).

Figure 7 Total Ammonia Residual during Experiment B

DRP concentrations averaged <0.03 mg/L as P for Experiment B (Figure 8), but they were generally higher than those in Experiments A and C.

Figure 8 Dissolved Reactive Phosphorus Residual during Experiment B

Temporal Cell and Nutrient Variations in Cultures

To give an indication of the temporal variation in cell and nutrient concentrations during the experiments, time-series graphs from Experiment A are provided below. As described in the methods section, the cultures in Experiment A were started with 10 mL of inoculum added to 890 mL of growth medium. The error bars represent the standard deviation of duplicate cultures.

Volatile suspended solids ranged from 200-600 mg/L for Experiment A, with the N:P-30 cultures generally having higher VSS concentrations than the lower N:P ratio cultures (Figure 9).

Figure 9 VSS Time Series - Experiment A

Removal of dissolved nutrients was excellent and relatively consistent. After the first week of operation, total ammonia nitrogen concentrations stabilized at <0.5 mg/L (Figure 10), and dissolved phosphorus remained <0.08 mg/L (Figure 11).

Figure 10 Residual Total Ammonia Nitrogen Time Series - Experiment A

Figure 11 Residual Dissolved Reactive Phosphorus Time Series - Experiment A

Total nitrogen (Figure 12) and total phosphorus (Figure 13) concentrations reveal mass balances indicating experimental error. The total nitrogen concentrations should have been nearly constant and equal to the influent total nitrogen. Similarly, total phosphorus also should have been constant and equal to the influent total phosphorus.

The total nitrogen input was 14 mg/L for N:P-5 and -20, and 21 mg/L for N:P-30. For N:P-20 and -30, the influent concentrations was close to or within the error bars for Weeks 7-9, once steady state was reached. The increased nitrogen content in N:P-5 may be due to nitrogen fixation by organisms such as *Anabaena*. In addition, such imbalances might be due to variation in particulate nutrient concentration among the frozen wastewater containers, analytical error, or contaminated reagents (See Table 5 and Table 6 for input N and P concentrations).

Figure 12 Total Nitrogen Time Series - Experiment A.
Influent concentrations were 14 mg/L for N:P-5 and N:P-20, and 21 mg/L for N:P-30.

Figure 13 Total Phosphorus Time Series - Experiment A.
Influent concentrations were 2.8 mg/L for N:P-5, and 0.7 mg/L for N:P-20 and N:P-30.

The ratio of particulate N to particulate P (cellular N:P) averaged about five for the cultures grown in N:P-5 medium, but cellular N:P ratios were lower than the media ratios

for N:P-20 and N:P-30 (Figure 14). The variability of the ratios was also greater for the higher media ratios.

Figure 14 Cellular N:P (on a mass basis) Time Series - Experiment A

Solids and Settleability – Experiments A, B, and C

Examining the data from all the experiments, no relationship was observed between suspended solids concentrations (initial or final) and media N:P ratios (Figure 15 and Figure 16) or dissolved nutrient concentrations. Cultures in Experiment C had lower average TSS and VSS concentrations than those in Experiment A (Figure 15 and Figure 16). The VSS contribution of the influent wastewater feed was minor at 11 mg/L for Experiment A and 9 mg/L for Experiments B and C. For the duration of all experiments, the TSS/VSS ratio was nearly unity, indicating that the majority of suspended solids was biomass. All flocs observed were less than 1 mm in size.

Figure 15 Total Suspended Solids Concentrations Initially and after 24 h of Settling during Experiments A and C

Figure 16 Volatile Suspended Solids Concentrations during Experiments A and C

After settling for 2 h, cultures in Experiments A and C achieved 30-60% suspended solids removal, while the 2-h removal efficiency for Experiment B was generally only 20-40%. After settling for 24 h, all conditions for Experiments A and C resulted in TSS

concentrations <40 mg/L (TSS removal >88%), with the exception of N:P-60, which had 44 mg/L in the supernatant (TSS removal 86%) (Figure 15).

Experiment B cultures did not settle well, with TSS after 24 h of settling ranging between 111 and 144 mg/L (Figure 17). The removal fractions for Experiment B were lower than that for Experiments A and C (Figure 19).

The TSS in Experiment B differed from those of the other experiments in another way. The Experiment B cultures were prone to clog the TSS analytical filters more rapidly¹ than samples from Experiments A and C, and they gave the filter pad a shiny appearance immediately after filtering, as discussed further in the General Discussion section.

The 100% defined medium cultures did not grow as well as the culture containing some wastewater. This point was illustrated clearly in the DM-only culture (N:P-103DM) in Experiment B. Despite having nearly the same media N:P ratio, the DM-only culture had substantially lower TSS concentrations than the N:P-102 culture that received wastewater as a component of its feed (Figure 17 and Figure 18).

¹ An average of 6 mL passing through the filter caused a slow drip in Experiment B, while cultures in Experiments A and C took at least twice that volume to achieve a slow drip. This increased clogging occurred despite the TSS and VSS concentrations in Experiment B being similar to Experiments A and C..

Figure 17 Total Suspended Solids Concentrations Initially and after 24 h of Settling during Experiment B

Figure 18 Volatile Suspended Solids Concentrations during Experiment B

Improved settleability due to nutrient stress was not clearly demonstrated even in cultures with extreme media N:P ratios (2.5 and 103) or low cell N and P contents (minimum P was 0.2% in N:P-103DM and minimum N was 4% in N:P-30). These results indicate that either nutrient stress was not achieved (i.e., sufficient cellular nutrients were present), that

nutrient stress does not increase settleability for the cultures tested, or for Experiments A and C, settleability induced by nutrient stress had already reached a maximum for all N:P ratios.

Figure 19 Total Suspended Solids Removal Percentages after 24 Hours of Settling

Residual nutrient concentrations in the extreme N:P cultures were still generally 50-100% of half-saturation constants (K) found in literature (Table 7). These K values suggest that cell growth was not nutrient limited in the experiments despite the low effluent concentrations.

Table 7 Half Saturation Constants

Source	Ammonia (mg/L as N)	Phosphate (mg/L as P)
Various freshwater and marine genera (Klausmeier <i>et al.</i> , 2004a)	0.0784	0.0062
Various freshwater and marine genera (micro and macroalgae) (Hein <i>et al.</i> , 1995)	0.01	N/A
<i>Scenedesmus</i> sp. (Rhee, 1974)	N/A	0.0186

Alkalinity Consumption

In Experiments A and C, higher N:P ratios resulted in higher alkalinity consumption (Figure 20). However, N concentration removal did not correlate well with alkalinity consumption (not shown).

Figure 20 Alkalinity Consumed during Experiments A and C

In Experiment B, however, no clear pattern emerges (Figure 21).

Figure 21 Alkalinity Consumed during Experiment B

For all experiments, NO_2^- and NO_3^- concentrations did not increase significantly during the course of treatment, therefore alkalinity loss due to nitrification was unlikely. Brewer and Goldman (1976) suggested uptake of NH_4^+ , or some other cation without its associated anion, may be responsible for decreases in alkalinity during algae growth². This theory matches the trend of the data in Figure 20, but not in direct proportion to N concentration removal as would be expected.

On the nitrogen-sparse end of the spectrum, N:P-2.5 cultures had much higher cyanobacteria concentrations than the cultures given media with higher N:P ratios, presumably because cyanobacteria can fix nitrogen and therefore compete more effectively at low concentrations of available nitrogen³.

² Alkalinity loss may also be due to byproducts of photosynthesis, such as glycolic acid. Though Brewer and Goldman did not find a correlation between glycolic acid production and biomass in their study, they noted in a later study (Goldman and Brewer, 1980) that some other weak acid or a salt of a strong organic acid may have been produced but not measured. It is worth noting that in Hellebust's study (1965), *Chlorococcum* sp. excreted nearly 20% of its fixed carbon, with greater than 10% of that in the form of glycolic acid. Tolbert and Zill (1956) found that *Chlorella* sp. excreted 3-10% of its carbon as glycolic acid. Production of biomass in the current experiments was not significantly higher with a higher feed nitrogen, but the composition of biomass or the rate of excretion may have changed, and these factors were not tested. Further research should focus on determining these parameters and their effects, as this affects large-scale treatment ponds.

³ This increased population of cyanophytes may explain the higher cellular nitrogen in N:P-2.5 cultures seen in Figure 22 and the increased vibrancy of green in N:P-2.5 over N:P-20 cultures.

General Discussion

The nitrogen and phosphorus content of cells in Experiments A and C (Figure 22) followed a similar pattern as that observed by Rhee (1978), namely, nitrogen content was similar in cultures with media up to an N:P-30 atomic ratio (13.5 mass ratio). Higher N:P in the feed led to higher cellular nitrogen. In the present study, cellular nitrogen appears to be similar for N:P-2.5 through N:P-30 mass ratio cultures, with a higher cellular N content at N:P-60.

Figure 22 Nitrogen and Phosphorus Content of the Cells during Experiments A and C

The N:P ratios in the feed correlated with the cellular N:P ratios. The correlation of media and cell N:P ratios for Experiments A and C (Figure 23) are similar to the low dilution rate cultures of Klausmeier *et al.* (2004b). Note that the slope in Figure 23 is close to 1, indicating that in this range, communities of algae appear to be able to assimilate nearly all the nitrogen and phosphorus in the media. These results are

consistent with various critiques of the Redfield Ratio, such as Klausmeier *et al.* (2004a), who showed that internal algal nutrient concentrations are a function of nutrients in their environment.

Figure 23 N:P Ratios of the Cells During Experiments A and C

For those cultures fed wastewater in Experiment B, the N:P ratios of the cells also correlated with the N:P ratios in feed. However, the DM-only culture did not follow this pattern (Figure 24). In fact, exceptionally high N contents (up to 30.5%) were measured in many of the Experiment B cultures. These values are higher than seen in the literature for intracellular content. However, algae are known to excrete nitrogen in the form of amino acids and organic nitrogen (Hellebust, 1965) outside of the cell, which may be adsorbed onto portions of extracellular polysaccharides (EPS) (Engel and Passow, 2001). As no EPS extraction was performed, this extracellular nitrogen would have been counted as intracellular nitrogen. In addition, no analytical errors could be detected via

the matrix spikes, standards, and blanks processed with these analytical batches. TKN analyses were conducted on Experiment A and B samples simultaneously in a sixteen-place digestion unit, and Experiment B and C samples were conducted within the same day, using the same reagents. Thus, any measurement errors would have likely occurred in samples from both, but the Experiment A and C samples had more typical cellular N contents of 4.3 to 14.5%.

It is uncertain why TKN results were high. One possibility is evaporation and distillation of some component of the WC medium interfered with resulting pH or the color indicator reagents used. Another possibility is the presence of a large number of nitrogen-fixing organisms. However, microscopy revealed only a small number of cyanobacteria in each sample. Finally, the amine group of the tris buffer used in the WC medium might not have been detected by the ammonia electrode, but it was degraded in the TKN digestion. These circumstances would have led to an overestimate of organic N.

Figure 24 N:P Ratios of the Cells during Experiment B

Effluent nitrogen and phosphorus concentrations remained low in all three experiments, but in the N:P-102 bottles, the effluent concentrations were much higher, suggesting a “breakthrough” effect. Further testing should be done to learn more precisely what concentration this occurs.

A clear relationship of settleability to N:P ratios in feed was not seen, but the settleability of cultures in Experiment B was much less than that of those in Experiments A and C. The poor settling in Experiment B suggests that the trace metal-rich Experiment B dilution water affected settleability.

The rapid clogging of the suspended solids filter pads and the shiny appearance of the filtered solids during Experiment B suggests an explanation for the poor settling: a greater concentration of EPS may have been present, excreted by algae or bacteria.

Higher EPS concentrations could have been caused by the higher multivalent cation concentration of the Experiment B dilution water (Table 19). Such an effect has been suggested by Wilén *et al.* (2003) for activated sludge cultures. Further, Wilén *et al.* suggested that EPS can cause poor settling, such as observed in Experiment B. Liao *et al.* (2001) suggest that factors such as surface properties and composition of EPS may affect bioflocculation more than the amount of EPS.

Experiments A and B used 75 mL of wastewater per 300 mL of feed. Whereas, Experiment C used only 32.5 mL per 300 mL. VSS seemed to depend on the amount of wastewater added (Figure 16 and Figure 18, pages 31 and 33), more than the concentration of N or P in the bottle influent. In Experiment C, VSS concentrations were lower (average 230 mg/L) than in Experiment A (average 390 mg/L), which were in turn lower than in Experiment B (480 mg/L). The VSS for N:P-103DM (no wastewater) in Experiment B was lower than those cultures with wastewater, and algae in N:P-20DM (no wastewater) in Experiments A and C could not grow quickly enough to prevent wash out. This result suggests that there may be some factors in wastewater, perhaps higher concentrations of trace metals, that support faster growth. If the factor in wastewater is additional trace metals, it may also explain why Experiment B had a higher VSS in general: Experiment B media had higher metals concentrations. The higher VSS in the cultures that received more wastewater was not due mainly to more wastewater VSS addition because the wastewater VSS additions were minor (9-11 mg/L) compared to the VSS grown in the culture bottles (200-500 mg/L).

Conclusion

When enriched with CO₂, polycultures of algae are able to almost completely remove both dissolved nitrogen and phosphorus from diluted wastewater media over a great range of nitrogen-to-phosphorus mass ratios, from at least 2.5:1 to 102:1. Low N:P feed ratios led to observably higher cyanobacteria concentrations than the higher N:P ratios, presumably because the cyanobacteria fix nitrogen and can compete better with chlorophytes under these conditions.

High N:P ratios in the feed correlated with high N:P ratios inside algal cells, except for those cultures that did not receive wastewater as part of their feed. No relationship was found between N:P ratios and settling rates. However, algae in media with low trace metal concentrations settled better than in media with higher trace metals concentrations.

Because the amount and type of EPS excreted by algae and bacteria is known to affect flocculation, and multivalent cations affect EPS production, the presence of trace metals and other constituents in the medium may affect settling. Further work should be done on trace constituents to determine which factors are important to settleability.

This study confirms, for wastewater cultures, the plasticity of cell nutrient ratios for polycultures containing primarily green algae. These results support the continued development of CO₂-supplemented pond systems for simultaneous removal of both dissolved nitrogen and phosphorus from wastewaters, even in cases of extreme wastewater N:P ratios, such as occurs with some industrial and agricultural wastewaters.

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Appendix A Micrographs

The following micrographs are labeled with a tentative identification and the sample location or culture.

Genus: *Chlorella* (Defined Medium Inoculum)

Genus: *Scenedesmus* (Wastewater Inoculum). See Page 10 for discussion of media adaptation.

Unknown filamentous species, possibly *Anabaena* or *Nodularia* (N:P-12(B))

Genus: *Ankistrodesmus* (N:P-20(C))

Family: Dictyosphaeriaceae (Wastewater Inoculum)

Family: Dictyosphaeriaceae (Wastewater Inoculum)

Genus: *Scenedesmus* (N:P-12(B))

Genus: *Anabaena* (N:P-2.5(C))

Genus: *Scenedesmus* (N:P-60(C))

Genus: *Scenedesmus* (Wastewater Inoculum)

Genus: *Closterium* (N:P-12(B))

Genus: *Nitzschia* (Wastewater Inoculum)

Floc (N:P-2.5(C))

Floc (N:P-20(C))

Floc (Wastewater Inoculum)

Genus: *Scenedesmus* (Wastewater Inoculum)

Appendix B Additional Methodology

For Experiments A and B, a sample of wastewater was collected on October 13, 2007, by dipping 5-gallon plastic buckets into the primary effluent weir channel in the San Luis Obispo municipal wastewater treatment plant. For Experiment C, the sample of wastewater was collected from the same location on April 11, 2008. The wastewater was then mixed thoroughly, filtered through 190-micron paint filters, placed into HDPE jugs, and frozen at -20°C for later use. Before using, the mixture was left at room temperature until completely thawed, then thoroughly mixed before adding to experimental bottles. Between feedings, thawed wastewater was stored at 2°C.

The culture bottles used were 1-L Pyrex Roux bottles, on magnetic stirrers. TFE-coated 2.5-cm magnetic stir bars were placed inside the bottles and spun at approximately 300 rpm.

Before inoculating the bottles used for experimental conditions, 10 mL of algae inoculum from a previous experiment by Feffer (2007) was grown in batch mode by placing the inoculum into two 1-L Pyrex[®] Roux bottles of growth medium for two weeks in order to allow the algae to adapt to the medium. One bottle of growth medium contained 225 mL wastewater and 665 mL defined medium; the other bottle of growth medium was only defined medium (prepared according to Table 8, reproducing the N:P-20 conditions).

The wastewater, with an N:P mass ratio of 20:1, was diluted to about 25% of original strength for Experiments A and B and about 10% of original strength for Experiment C to avoid light limitations; this was supplemented with nitrogen or phosphorus to achieve the desired N:P ratios. For constituents in collected wastewater, refer to Table 1.

To start Experiment A, 10 mL of the inoculum (wastewater-grown inoculum for the bottles receiving wastewater, and DM-grown inoculum for those bottles receiving only DM) were added to the bottles. Also added was wastewater (for those cultures receiving wastewater), and defined medium (with the appropriate amounts of nitrogen and phosphorus needed for the condition), with the amount in each bottle totaling 900 mL. Bottles with N:P-5, 20, and 30 received 225 mL of wastewater, and 665 mL of defined medium. N:P-20DM bottles received 890 mL of defined medium. After three days of batch growth, the daily feeding schedule began.

Cultures were fed once a day at the end of the light period by removing 300 mL from the bottle and replacing with the appropriate medium.

The constituents of the defined medium for Experiment A are listed in Table 8, with micronutrient and macronutrient solutions being prepared as in APHA 8010:IV A and B (Page 57) .

Table 8 Defined Medium, Experiment A

Defined Medium Condition	Ingredient	Amount
N:P-5	Macronutrient Solution	1.00 mL
	Micronutrient Solution	1.00 mL
	KH ₂ PO ₄	2.80 mL of 1000 mg/L as P
	NH ₄ Cl	None
	Sodium Bicarbonate Buffer	100 mL of 6.72 g/L NaHCO ₃
	Deionized Water	895 mL
N:P-20	Macronutrient Solution	1.00 mL
	Micronutrient Solution	1.00 mL
	K ₂ HPO ₄	None
	NH ₄ Cl	None
	Sodium Bicarbonate Buffer	100 mL of 6.72 g/L NaHCO ₃
	Deionized Water	898 mL
N:P-20DM	Macronutrient Solution	1.00 mL
	Micronutrient Solution	1.00 mL
	KH ₂ PO ₄	0.70 mL of 1000 mg/L as P
	NH ₄ Cl	14.0 mL of 1000 mg/L as N
	Sodium Bicarbonate Buffer	100 mL of 6.72 g/L NaHCO ₃
	Deionized Water	883
N:P-30	Macronutrient Solution	1.00 mL
	Micronutrient Solution	1.00 mL
	K ₂ HPO ₄	None
	NH ₄ Cl	9.25 mL of 1000 mg/L as N
	Sodium Bicarbonate Buffer	100 mL of 6.72 g/L NaHCO ₃
	Deionized Water	889 mL

These solutions were then added to the bottles for each feeding as in Table 9:

Table 9 Feed, Experiment A

Bottle Name	Ingredient	Amount
N:P-5,20,30	Primary Wastewater	75.0 mL
	Defined Medium with supplemental N & P	225 mL
N:P-20DM	Defined Medium with N & P	300 mL

At the end of Experiment A, all bottles were mixed together and 600 mL of this mixture was added to each new bottle, ready for the new medium for Experiment B. The constituents of the defined medium for Experiment B are listed in Table 10, which follows the “WC Medium” listed in Algal Culturing Techniques (Andersen, 2005). Silica

was omitted, and nitrogen and phosphorus were as listed below. The medium was pH adjusted to 7.6 - 8.0 before use.

Table 10 Defined Medium, Experiment B

Defined Medium Condition	Ingredient	Amount
N:P-12	CaCl ₂ Solution	1.00 mL
	MgSO ₄ Solution	1.00 mL
	Tris HCl	650 mg
	Trace Metal Solution	1.00 mL
	Vitamin Solution	1.00 mL
	K ₂ HPO ₄	6.50 mL of 1000 mg/L as P
	NH ₄ Cl	None
	Sodium Bicarbonate Buffer	100 mL of 6.72 g/L NaHCO ₃
	Deionized Water	890 mL
N:P-82	CaCl ₂ Solution	1.00 mL
	MgSO ₄ Solution	1.00 mL
	Tris HCl	650 mg
	Trace Metal Solution	1.00 mL
	Vitamin Solution	1.00 mL
	K ₂ HPO ₄	None
	NH ₄ Cl	None
	Sodium Bicarbonate Buffer	100 mL of 6.72 g/L NaHCO ₃
	Deionized Water	896 mL
N:P-103DM	CaCl ₂ Solution	1.00 mL
	MgSO ₄ Solution	1.00 mL
	Tris HCl	650 mg
	Trace Metal Solution	1.00 mL
	Vitamin Solution	1.00 mL
	K ₂ HPO ₄	0.70 mL of 1000 mg/L as P
	NH ₄ Cl	14.0 mL of 1000 mg/L as N
	Sodium Bicarbonate Buffer	100 mL of 6.72 g/L NaHCO ₃
	Deionized Water	881 mL
N:P-102	CaCl ₂ Solution	1.00 mL
	MgSO ₄ Solution	1.00 mL
	Tris HCl	650 mg
	Trace Metal Solution	1.00 mL
	Vitamin Solution	1.00 mL
	K ₂ HPO ₄	None
	NH ₄ Cl	18.5 mL of 1000 mg/L as N
	Sodium Bicarbonate Buffer	100 mL of 6.72 g/L NaHCO ₃
	Deionized Water	877.5 mL

These solutions were then added to the bottles for each feeding as in Table 11.

Table 11 Feed, Experiment B

Bottle Name	Ingredient	Amount
N:P-12, 82, 102	Primary Wastewater	75.0 mL
	Defined Medium with supplemental N & P	225 mL
N:P-103DM	Defined Medium with N & P	300 mL

At the end of Experiment B, all bottles were mixed together and 600 mL of this mixture was added to each new bottle, ready for the new medium for Experiment C. The constituents of the defined medium for Experiment C are listed in Table 12, with micronutrient and macronutrient solutions being prepared as in APHA 8010:IV A and B. Medium pH was not adjusted before addition to bottles.

Table 12 Defined Medium, Experiment C

Defined Medium Condition	Ingredient	Amount
N:P-2.5	Macronutrient Solution	1.00 mL
	Micronutrient Solution	1.00 mL
	K ₂ HPO ₄	2.30 mL of 1000 mg/L as P
	NH ₄ Cl	None
	Sodium Bicarbonate Buffer	100 mL of 6.72 g/L NaHCO ₃
	Deionized Water	896 mL
N:P-20	Macronutrient Solution	1.00 mL
	Micronutrient Solution	1.00 mL
	K ₂ HPO ₄	None
	NH ₄ Cl	8.0 mL of 1000 mg/L as N
	Sodium Bicarbonate Buffer	100 mL of 6.72 g/L NaHCO ₃
	Deionized Water	890 mL
N:P-20DM	Macronutrient Solution	1.00 mL
	Micronutrient Solution	1.00 mL
	K ₂ HPO ₄	0.70 mL of 1000 mg/L as P
	NH ₄ Cl	14.0 mL of 1000 mg/L as N
	Sodium Bicarbonate Buffer	100 mL of 6.72 g/L NaHCO ₃
	Deionized Water	883 mL
N:P-60	Macronutrient Solution	1.00 mL
	Micronutrient Solution	1.00 mL
	K ₂ HPO ₄	None
	NH ₄ Cl	39.5 mL of 1000 mg/L as N
	Sodium Bicarbonate Buffer	100 mL of 6.72 g/L NaHCO ₃
	Deionized Water	858.5 mL

These solutions were then added to the bottles for each feeding as in Table 13. For Experiment C, new primary effluent wastewater was collected. As this new wastewater had nutrient concentrations that differed from the wastewater used in the previous experiments, the volume of wastewater added to the bottles was decreased to match the TP concentration in the N:P-20 medium of Experiment A. Then three defined media were prepared that created N:P ratios of 2.5, 20, and 60 when mixed with wastewater. Thus, each wastewater culture bottle received 32.5 mL of wastewater and 267.5 mL of defined medium for each feeding. The defined medium-only culture received 300 mL of a fourth defined medium with an N:P of 20 (N:P-20DM).

Table 13 Feed, Experiment C

Bottle Name	Ingredient	Amount
N:P-2.5,20,60	Primary Wastewater	32.5 mL
	Defined Medium with supplemental N & P	267.5 mL
N:P-20DM	Defined Medium with N & P	300 mL

APHA Medium

Adapted from APHA 8010 (APHA, 1995)

Prepare culture medium for freshwater algae by adding reagent-grade macronutrients (8010:IV.A) and micronutrients (8010:IV.B) to deionized water.

Table 14 Modified Macronutrient Solution used in Experiments A and C, based on APHA 8010:IV.A

Compound	Concentration (mg/L)	Element	Resulting Conc. of element (mg/L)
MgSO ₄ · 7H ₂ O	14.7	S Mg	1.91
MgCl ₂ · 6H ₂ O	12.16		2.90
CaCl ₂ · 2H ₂ O	4.41	Ca	1.20

Table 15 Modified Micronutrient Solution used in Experiments A and C, based on APHA 8010:IV.B

Compound	Concentration ($\mu\text{g/L}$)	Element	Resulting Conc. of element ($\mu\text{g/L}$)
H_3BO_3	186	B	32.5
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	414	Mn	115
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	6.9	Zn	1.57
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	1.42	Co	0.354
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	0.011	Cu	0.004
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	7.26	Mo	2.88
FeCl_3	96.0	Fe	33.0
$\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$	300	-	-

WC Medium (Wright's Cryptophytes Medium)

From Algal Culturing Techniques (Andersen, 2005)

Create stock solutions for all components below, other than Tris buffer. Into 900 mL of deionized water, dissolve Tris buffer. Add remaining components from the table below.

Dilute to 1L with DI water, adjust pH to 7.6 – 8.0 before use.

Table 16 Modified Medium used in Experiment B, based on WC Medium

Component	Stock Solution (g/L)	Quantity Used	Concentration in Final Medium (M)
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	36.76	1 mL	2.50×10^{-4}
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	36.97	1 mL	1.50×10^{-4}
Vitamin Solution	(see Table 17)	1 mL	-
Trace Metals Solution	(see Table 18)	1 mL	-

Make primary stock solutions of Vitamin H and Vitamin B₁₂. Into a 1-L volumetric flask, add 950 mL of DI water. Add remaining components in table below. Dilute to 1L.

Table 17 Vitamin Solution for WC Medium used in Experiment B

Component	Stock Solution (g/L)	Quantity Used	Concentration in Final Medium (M)
Thiamine HCl (Vitamin B ₁)	-	100 mg	2.96×10^{-7}
Biotin (Vitamin H)	0.5	1 mL	2.05×10^{-9}
Cyanocobalamin (Vitamin B ₁₂)	0.5	1 mL	3.69×10^{-10}

Make primary stock solutions listed below. To 1-L volumetric flask, add 950 mL of DI water. Add stock solutions and other components as listed in table below. Dilute to 1 L.

Table 18 Modified Trace Metal Solution used in Experiment B, based on WC Medium

Component	Stock Solution (g/L)	Quantity Used	Concentration in Final Medium (M)
Na ₂ EDTA · 2H ₂ O	-	4.36 g	1.17 x 10 ⁻⁵
FeCl ₃	-	1.90 g	1.17 x 10 ⁻⁵
CuCl ₂ · 2H ₂ O	6.83	1 mL	4.01 x 10 ⁻⁸
ZnSO ₄ · 7H ₂ O	22.0	1 mL	7.65 x 10 ⁻⁸
CoCl ₂ · 6H ₂ O	10.0	1 mL	4.20 x 10 ⁻⁸
MnCl ₂ · 4H ₂ O	180.0	1 mL	9.10 x 10 ⁻⁷
Na ₂ MoO ₄ · 2H ₂ O	6.0	1 mL	2.48 x 10 ⁻⁸
H ₃ BO ₃	-	1.00 g	1.62 x 10 ⁻⁵

A comparison of the trace metal concentrations in each medium is found in Table 19.

Table 19 Trace Metal Constituents in the Media Used for Wastewater Dilution and in the Defined Medium Cultures

Constituent	Experiments A and C, APHA (mol/L)	Experiment B, WC (mol/L)	Ratio of media WC/APHA
Ca ²⁺	1.1 x 10 ⁻¹⁰	1.0 x 10 ⁻³	9.1 x 10 ⁶
Mg ²⁺	8.4 x 10 ⁻¹⁰	1.5 x 10 ⁻⁴	1.8 x 10 ⁵
Fe ³⁺	5.9 x 10 ⁻¹⁰	1.2 x 10 ⁻⁵	2.0 x 10 ⁴
Cu ²⁺	6.3 x 10 ⁻¹⁴	4.0 x 10 ⁻⁸	6.4 x 10 ⁵
Zn ²⁺	2.4 x 10 ⁻¹¹	7.7 x 10 ⁻⁸	3.2 x 10 ³
Co ²⁺	6.0 x 10 ⁻¹²	4.2 x 10 ⁻⁸	7.0 x 10 ³
Mn ²⁺	2.1 x 10 ⁻⁹	9.1 x 10 ⁻⁷	4.4 x 10 ²
Mo ²⁺	3.0 x 10 ⁻¹¹	2.5 x 10 ⁻⁸	8.3 x 10 ²

Experiment Conditions

Experiment A ran for two months, Experiment B ran for 1 month, and Experiment C ran for one and a half months.

Sampling was performed by removing the air line and stopper from the bottle and scrubbing the insides of the bottle with a plastic bottle brush. The bottle brush was shaken

inside the bottle to remove large particles of biomass, then rinsed with deionized water before use in another bottle. The bottle was shaken vigorously to homogenize before sampling.

The algae received 16 hours of daylight and 8 hours of darkness. Using a LX-101 Lux Meter (Lutron Corporation), it was determined that 4,300 lux was provided to each Roux bottle from full-spectrum bulbs, with ambient fluorescent room lighting during the day averaging 804 lux. Heat was removed from the area by a household fan (Figure 25). For details on the full-spectrum fluorescent lamp fixtures used, see Feffer, 2007.

Figure 25 Experimental Setup with room lights on and culture lamps off (left) and room lights off and culture lamps on (right).

Each bottle had a 3-day hydraulic residence time (HRT), and was sparged with 1.25 L/min air, enhanced with 0.5% CO₂ using a Matheson Model 603/610A gas mixer. The

CO₂ was supplied with a 50-lb tank of 99.97% CO₂, and a Maxima Model A-805 2.5-psi aquarium air pump was used to supply the air. Valves were adjusted manually for each bottle to supply each bottle with approximately 0.17 L gas/min-L algae suspension.

Ammonia and TKN samples were preserved by adding H₂SO₄ until pH 2 was reached, then refrigerating in plastic sample bottles. Ammonia samples were analyzed within 7 days, and TKN samples were analyzed within 28 days. Prior to nitrite/nitrate analysis, samples were filtered to 0.22 µm and stored at -20°C in 5-mL Dionex Polyvials, generally undergoing analysis within 28 days. Prior to measuring DRP, samples were filtered to 0.45 µm and refrigerated in glass sample bottles. DRP samples were analyzed within 7 days. Total phosphorus samples were preserved by adding H₂SO₄ until pH 2 was reached, then refrigerating in plastic sample bottles. TP samples were analyzed within 28 days.

Ammonia levels were determined using the Ammonia-Selective Electrode Method (APHA 4500-NH₃ D), but using a pre-prepared ISA solution (Spectro-Pure). A Hanna Instruments 209 pH Meter and Electrometer ammonia probe with an Orion 95-12 Ammonia-selective electrode were used to read ammonia levels. A Corning 355 pH/ion analyzer with a Sensorex Combination pH probe was used to ensure the pH of the sample during the ammonia testing reached at least 11. A Fisher Thermix Model 120 MR Stirrer with a TFE-coated stirbar was used to mix the solution during testing. Due to equipment failure, ammonia was determined by using the fluorometric method, Protocol B, outlined in Holmes *et al.* (1999) for the last six sampling dates.

A Dionex DX 120 Ion Chromatograph (with G9-HC IonPac Guard Column, AS9-HC 4mm IonPac IC column, DS4-1 Detection Stabilizer, and AS40 Automated Sampler) was used to measure NO_3^- and NO_2^- levels in the water. Samples were filtered to 0.22 micron, placed in 5-mL Dionex Polyvials. Samples were frozen to preserve them.

To determine the amount of nitrogen inside algal cells, the Macro-Kjeldahl Method (APHA Method 4500- N_{org}) was performed on an 18-station Kjeldahl apparatus (Figure 26). The ammonia removal step was not performed. To more accurately determine the endpoint of titration, 0.01N H_2SO_4 was used. After distillation, the Kjeldahl apparatus was rinsed as detailed in Feffer, 2007.

The Ascorbic Acid method for phosphorus determination (APHA 4500-P E) was used to measure DRP and TP in the cultures. Prior to testing, samples were filtered to 0.45 microns. DRP samples for Experiment A used a 0.5-cm path length cuvette, which was shorter than recommended for their low concentrations. A 5-cm path length cuvette was obtained, and used for Experiments B and C.

Dissolved oxygen, pH, and temperature were checked periodically to verify stable conditions. Dissolved oxygen was measured using a YSI 58 DO meter. A cursory identification and enumeration was performed on cultures in all three experiments, at 1000x magnification, with and without phase contrast. Microscopy was performed with an Olympus CX41 optical light microscope; images were captured using an integrated

Infinity 2 digital camera. Measurements were calibrated by using a 50 μ m-square hemacytometer.

Figure 26 Kjeldahl Apparatus