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# Factors Influencing Luxury Uptake of Phosphorus by Microalgae in Waste Stabilization Ponds

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Phosphorus removal in waste stabilization ponds (WSP) is highly variable, but the reasons for this are not well understood. Luxury uptake of phosphorus by microalgae has been studied in natural systems such as lakes but not under the conditions found in WSP. This work reports on the effects of phosphate concentration, light intensity, and temperature on luxury uptake of phosphorus by WSP microalgae in continuous culture bioreactors. Increasing temperature had a statistically significant "positive effect" on intracellular acid-insoluble polyphosphate concentration. It is likely that elevated temperature increased the rate of polyphosphate accumulation, but because the biomass was not starved of phosphate, the stored acid-insoluble polyphosphate was not utilized. Increasing light intensity had no effect on acid-insoluble polyphosphate but had a "negative effect" on the acid-soluble polyphosphate. A possible explanation for this is that the faster growth rate at high light intensity results in this form of polyphosphate being utilized by the cells for synthesis of cellular constituents at a rate that exceeds replenishment. The variability in the phosphorus content of the microalgal biomass shows that with this new understanding of the luxury uptake mechanism there is the potential to optimize WSP for biological phosphorus removal.

## Introduction

Small communities around the world rely on waste stabilization ponds (WSP) for wastewater treatment. WSP are simple to construct, have very low operating costs, and offer effective treatment in terms of organic carbon and pathogen removal. However, phosphorus removal in WSP is highly variable, with an average removal of between 15 and 50% (*1*–*3*). Phosphorus removal from wastewater is important because effluents high in phosphorus can cause algal blooms and eutrophication in receiving waters.

In the future, WSP will need to be upgraded for more effective phosphorus removal. Currently the most common method for upgrading WSP for phosphorus removal relies on dosing of chemicals to precipitate out the phosphate. Chemical dosing is expensive, and it produces a chemical sludge that must be disposed of. Another technology that could be used to upgrade pond systems is the enhanced biological phosphorus removal (EBPR) form of the activated sludge process. However, this process is expensive and increases the complexity of the wastewater treatment system. Ideally a new low-cost solution is needed so that WSP can be upgraded to effectively remove phosphorus from wastewater while maintaining their relative simplicity.

Both chemical and biological mechanisms are known to contribute to phosphorus removal that naturally occurs in WSP. During growth, microalgae consume inorganic carbon. If this consumption exceeds replenishment via absorption from the atmosphere and bacterial oxidation of organic waste, the pH increases. An elevated pH can cause phosphates to precipitate by complexation with metal ions such as calcium, magnesium, and iron present in the wastewater. Phosphorus is also removed biologically. Growth of microalgae consumes phosphorus as an essential element needed for cellular constituents such as phospholipids, nucleotides, and nucleic acids (*4*). A second biological mechanism is luxury uptake of phosphorus. Luxury uptake is the storage of phosphorus within the biomass in the form of polyphosphate. Polyphosphate can be present as acid-soluble or acid-insoluble polyphosphate. Acid-soluble polyphosphate is actively involved in metabolism, while acid-insoluble polyphosphate is stored for when the external phosphate concentration becomes limiting (*4*). Until recently, luxury uptake by microalgae was not considered a significant mechanism in WSP. While luxury uptake of phosphorus by microalgae has been studied in the natural environment, barely any information exists on possible luxury uptake under the conditions found in WSP. For example algal growth in freshwater systems such as lakes may be limited by phosphate availability; however, in WSP phosphate is available in amounts excess to requirements for growth.

We have used three methods to detect if luxury uptake in WSP microalgae occurs to any significance (*5*). These methods included staining of biomass for polyphosphate granules, measuring the percentage phosphorus in the microalgal biomass, and using chemical extraction techniques to measure the polyphosphate contained in the biomass. Luxury uptake of phosphorus has been shown to occur under conditions relevant to WSP (*5*). Screening batch experiments have further identified the key variables that affect biological phosphorus removal in WSP microalgae (*6*). These variables are the phosphate concentration, light intensity, and temperature (*6*). While prior batch studies identified the factors that influenced phosphate removal, they did not establish how these factors influence luxury uptake. The present study uses continuous culture reactors operated under controlled conditions to assess the impact of previously identified factors on luxury uptake.

## Materials and Methods

**Experimental Setup.** The effect of phosphorus concentration, light intensity, and temperature on the percentage phosphorus and polyphosphate in the biomass were assessed using a full factorial experimental design requiring a total of eight reactors. The experimental matrix is shown in Table 1.

The continuous culture reactors (Figure 1) used for all the experiments had a volume of three liters and a hydraulic retention time of ten days. Culture depth in the reactors was approximately 70 mm. The rectangular reactor vessels were 170 mm wide and 250 mm long. The reactors were fed with synthetic wastewater according to Davis and Wilcomb (*7*). This synthetic wastewater contained the metal ion-chelating agents EDTA (ethylenediaminetetraacetate) and sodium citrate to prevent phosphorus precipitation and enable the research to focus on the biological phosphorus mechanisms.

The reactors were mixed using magnetic stirrers (50 mm stir bars). Lighting was provided by fluorescent daylight lamps

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**TABLE 1. Experimental Design Used for the Continuous Reactors**

| reactor | temp (°C) | light intensity ($\mu$E/m$^2$ s) | phosphate (mg P/L) |
|---|---|---|---|
| A | 15 | 60 | 5 |
| B | 15 | 60 | 15 |
| C | 15 | 150 | 5 |
| D | 15 | 150 | 15 |
| E | 25 | 60 | 5 |
| F | 25 | 60 | 15 |
| G | 25 | 150 | 5 |
| H | 25 | 150 | 15 |

(Philips, 36 W) set on timers to simulate day and night (15 h light and 9 h dark). The light intensity was monitored at the surface of the reactors using a PAR (photosynthetically active radiation) irradiance sensor (QSL-2101; Biospherical Instruments, San Diego, CA). All experiments were conducted in a controlled temperature room. At the start of the experiments the reactors were inoculated with broth from a continuous culture inoculum reactor dominated by *Scenedesmus* spp. Details of this inoculum reactor have been reported previously (*5*).

**Analytical Methods.** The phosphate and solids concentration of the reactor effluent was monitored to determine when quasi-steady-state had been reached. A quasi-steady-state was reached because of the changing day/night cycle. The quasi-steady-state was defined to occur when three consecutive measurements were within ±10%. The phosphate concentration was measured using ion chromatography (Dionex ICS-2000; Dionex Corporation, Sunnyvale, CA). Dionex IonPac AS11-HC (4 × 250 mm) and Dionex IonPac AG11-HC columns were used with a 25 $\mu$L sample loop. The eluent concentration was 32 mM KOH at a flow rate of 1 mL/min, and the operating temperature was 30 °C. A Suppressed Conductivity ASRS-ULTRA suppressor was used in recycle mode. The biomass was recovered on a 0.45 $\mu$m membrane filter and measured as dry weight (*8*).

Once a quasi-steady-state had been reached in the continuous culture reactors, the analysis of the different forms of phosphorus was undertaken. The forms of phosphorus analyzed included the phosphate in the biomass-free liquid, the total phosphorus (TP) in the biomass-free liquid, and the TP of the biomass. The TP was measured according to standard methods (*9*) using the nitric acid and sulfuric acid digestion and analysis using the ascorbic acid method.

The polyphosphate was analyzed using the chemical extraction technique described by Aitchison and Butt (*10*) and Kanai et al. (*11*). This methods involves a series of extractions using trichloroacetic acid, ethanol, ethanol/ether (3:1 by volume), and potassium hydroxide.

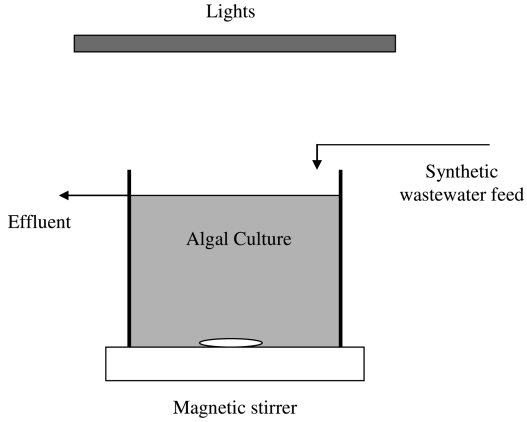


**FIGURE 1. Continuous culture reactor.**

**Statistical Analysis.** The results were analyzed using the statistical software MINITAB (Minitab Inc., State College, PA). Variables are reported as significant at either 95% confidence ($p$-value less than or equal to 0.05) or 90% confidence ($p$-value between 0.05 and 0.10).

## Results and Discussion

Results of the continuous culture experiments are reported here as the percentage phosphorus in the biomass and the polyphosphate content of the cells. Implications of these measurements for phosphorus removal in WSP are discussed.

**Percentage Phosphorus in Biomass.** The average percentage phosphorus in the biomass for each reactor is shown in a cube plot in Figure 2. A cube plot is used to enable the effects of each of the variables tested to be identified. The average percentage phosphorus in the biomass was 1.27% with a maximum of 3.16% and a minimum of 0.41%. In the absence of luxury uptake, microalgae typically contain 1% phosphorus (*12, 13*). These results suggest that luxury uptake was occurring (*12*). The highest percentage phosphorus occurred (Figure 2) at high temperature (25 °C) and low light intensity (60 $\mu$E/m$^2$ s). Some of the conditions tested resulted in the biomass containing less than 1% phosphorus. The lowest percentage phosphorus occurred at low temperature (15 °C) and low phosphorus concentration (5 mg/L). To determine which effects are significant, a statistical analysis is required.

Table 2 shows the results of the statistical analysis and reports the $p$-values for the variables. Light intensity was found to have a negative effect on the phosphorus content of the biomass (Table 2), meaning that the reactors held at low light intensity had a higher amount of phosphorus in their biomass compared with those at higher light intensity. This was unexpected because phosphorus uptake is known to be an energy-requiring process (*13–16*) and microalgae receive their energy via photosynthesis that is generally enhanced with increasing intensity of light. Nonetheless, the observed negative effect of light intensity on phosphorus content was consistent with other published reports. For example, Hessen et al. (*14*) reported that the phosphorus-to-carbon ratio in green microalga *Selenastrum* increased with reducing light intensity. Similar results were observed by Martinez et al. (*15*) in studies of phosphorus metabolism of *Scenedesmus*. Possible reasons for this negative effect will be discussed in later sections of the paper.

The temperature had a positive effect on the percentage phosphorus in the biomass (Table 2). Temperature is known to not only affect the rate of biological reactions but also the cell composition. Temperature has been reported to affect the fatty acid composition (*16*), protein concentration (*17*), and the nitrogen-to-carbon ratio (*18*).

The effect of phosphate concentration on the percentage phosphorus in the solids was not statistically significant (Table 2). In other studies (*10*), the phosphate concentration has been reported to profoundly affect the phosphorus content of microalgal biomass, but this apparently applies to natural environments in which cells are starved of phosphate for periods of time before being re-exposed. This situation does not generally occur in WSP because phosphate concentration typically exceeds the growth limiting level. The levels of phosphate tested in the present experiments (5 and 15 mg/L) were within the range of phosphate concentration typically found in WSP. These results (Table 2) suggest that at the levels found in WSP the phosphate concentration does not have a direct effect on the percentage phosphorus in the microalgal biomass.

Interactive effects of the variables were tested (Table 2). An interaction occurs when the effect of a variable is dependent on another. Significant interactions occurred between phosphate concentration and light intensity and

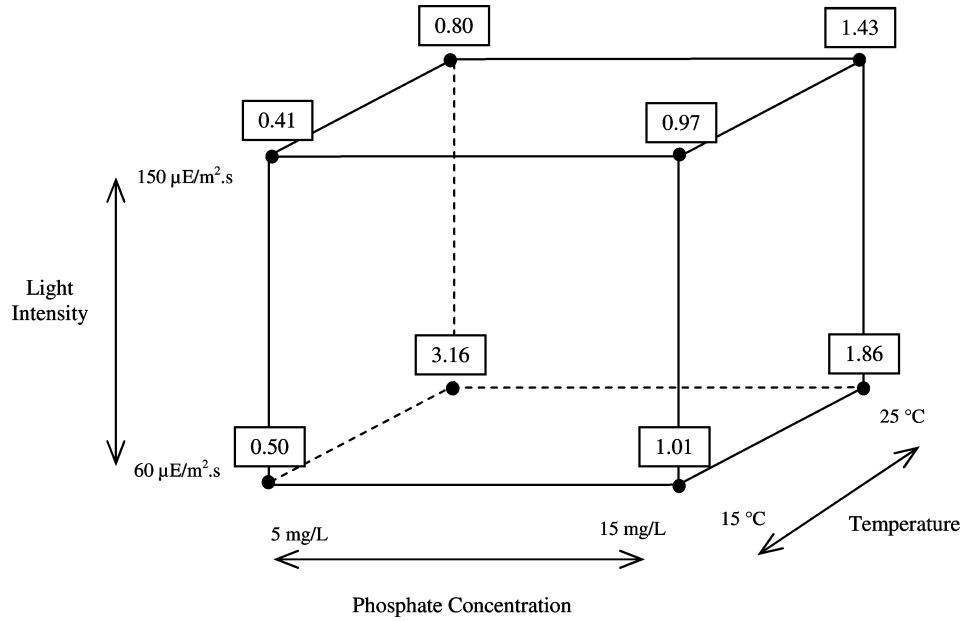


**FIGURE 2. Percentage phosphorus in biomass at quasi-steady-state.**

**TABLE 2. Statistical Analysis of the Phosphorus Content of Biomass**

| variable | *p*-value | significance | effect |
|---|---|---|---|
| phosphate concentration | 0.634 | not significant | N/A[a] |
| light intensity | 0.006 | 95% confidence | negative |
| temp | 0.000 | 95% confidence | positive |
| phosphate × light intensity | 0.036 | 95% confidence | positive |
| phosphate × temp | 0.059 | 90% confidence | negative |
| light intensity × temp | 0.009 | 95% confidence | negative |

[a] Not applicable.

also between light intensity and temperature (Table 2). The interaction between phosphate concentration and temperature was significant at 90% confidence (Table 2). These results show that in the ranges examined, the phosphate concentration alone does not significantly affect the percentage phosphorus in the biomass, but it is an important variable because of its interactions with both light intensity and temperature.

These results reveal that in continuous culture the principal factors that affect the phosphorus content of WSP microalgae are the light intensity and temperature. The phosphate concentration does not have any direct affect in the 5−15 mg/L range, but it is an important variable because of interactions with the other factors. During luxury uptake, microalgae accumulate phosphorus as polyphosphate. Therefore, to confirm luxury uptake, direct measurement of polyphosphate in the cells is necessary, as discussed next.

**Polyphosphate in Biomass.** Once a quasi-steady-state had been reached, polyphosphate was measured in the biomass samples from continuous culture reactors held under various specified conditions. To correct for any differences in solid concentration between the reactors, the polyphosphate is reported as the quantity present per unit of total dry mass. The acid-soluble and acid-insoluble polyphosphate for each reactor is shown in Figure 3. Most of the polyphosphate is in the form of acid-soluble polyphosphate (Figure 3) which is actively involved in metabolism (*19*).

Table 3 shows the *p*-values along with the effect of each variable on the acid-soluble and acid-insoluble polyphosphate. The phosphate concentration in the synthetic wastewater had no significant affect on the amount of polyphosphate in the biomass (Table 3). An increase in temperature from 15−25 °C increased the amount of acid-insoluble polyphosphate per unit cell dry weight but had no significant effect on the acid-soluble polyphosphate (Table 3). Barely any acid-soluble polyphosphate was present in the biomass at the low temperature (Figure 3).

According to Miyachi and co-workers (*4, 20, 21*), acid-insoluble polyphosphate was stored in microalgal biomass for use when the external phosphate level becomes limiting for growth. In contrast, acid-soluble polyphosphate is mostly used for the synthesis of cellular constituents under normal conditions. Temperature is known to affect the rate of biological reactions therefore it is likely that higher temperature resulted in an increase in polyphosphate accumulation. However, because the cells were not starved of phosphate the acid-insoluble polyphosphate was not utilized.

Light intensity only affected the amount of acid-soluble polyphosphate (Table 3). Acid-soluble polyphosphate is used by microalgal cells for producing DNA and phosphoprotein (*4*). Therefore, the rate of growth and the amount of acid-soluble polyphosphate in the cells are linked. At a light intensity of 150 $\mu$E/m$^2$ s the growth rate is expected to be high compared with the rate at a light intensity of 60 $\mu$E/m$^2$ s; therefore, more acid-soluble polyphosphate is likely consumed in metabolism at the higher light level and less accumulates.

The observed amount of polyphosphate in the biomass is a consequence of the combined effects of luxury uptake and metabolic consumption. Depending on how a combination of variables influences the rates of luxury uptake and consumption, biomass may have different levels of total phosphate and different proportions of the two types of polyphosphates.

In view of these observations, a better understanding of luxury uptake requires analysis of not only the mass fraction of total phosphorus in the biomass but also of the proportion present as acid-soluble and acid-insoluble polyphosphate. It is interesting to note that even at 1% phosphorus which indicates normal metabolism, some polyphosphate is present (Figures 2 and 3). This shows that the presence of polyphosphate does not necessarily mean that luxury uptake is

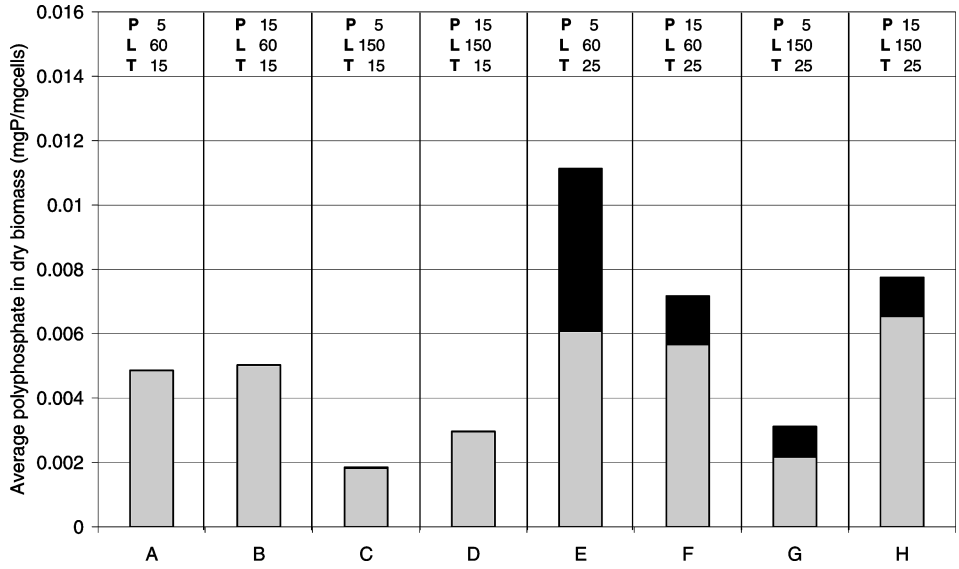


**FIGURE 3. Polyphosphate fractions in the biomass for the eight continuous culture reactors. The phosphate concentration (P), light intensity (L), and temperature (T) for each experiment indicated at top each bar. Light colored bars are for acid-soluble polyphosphate. Dark bars denote acid-insoluble polyphosphate.**

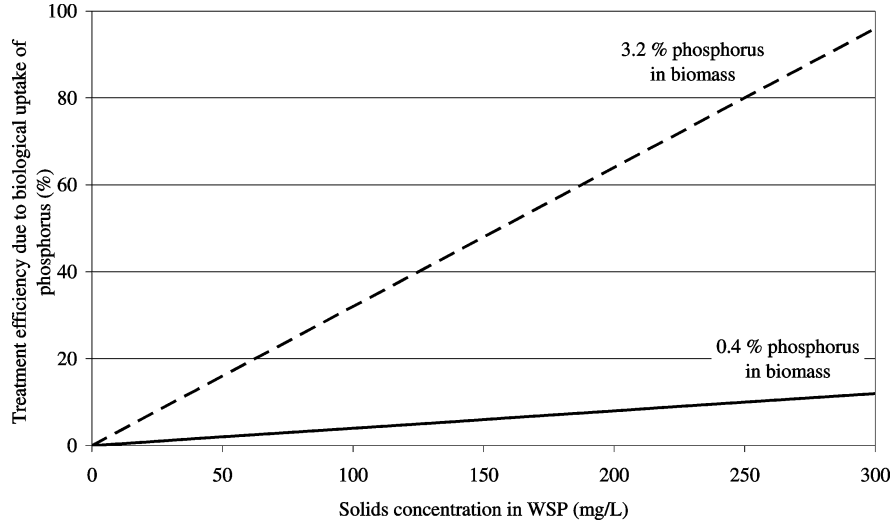


**FIGURE 4. Potential biological phosphorus removal in WSP for wastewater with an initial phosphate concentration of 10 mg/L.**

**TABLE 3. Statistical Analysis of the Polyphosphate Content Per Unit Dry Biomass**

| | acid-soluble polyphosphate | | | acid-insoluble polyphosphate | | |
|---|---|---|---|---|---|---|
| variable | *p*-value | significance | effect[a] | *p*-value | significance | effect[a] |
| phosphorus | 0.176 | not significant | N/A | 0.329 | not significant | N/A |
| light intensity | 0.043 | 95% confidence | negative | 0.211 | not significant | N/A |
| temp | 0.141 | not significant | N/A | 0.022 | 95% confidence | positive |

[a] Not applicable.

occurring in the biomass. The percentage phosphorus shows how much accumulation has occurred, and the polyphosphate extraction shows that it is in fact luxury uptake and not another removal mechanism such as chemical precipitation.

**Phosphorus Removal in WSP.** These findings help to explain the highly variable phosphorus removal in WSP. More phosphorus was accumulated in the biomass at warmer temperature (25 °C) (Figures 2 and 3). This explains at least some of the seasonal variation in phosphorus removal that has been documented in WSP. For example, Picot et al. (*1*) reported that, in a full-scale WSP, phosphate removal varied between 15% in the winter and 30% in the summer.

Luxury uptake has not previously been identified as a significant mechanism of phosphorus removal in WSP. If it is indeed the case that luxury uptake is as significant in full-scale systems as it was in this laboratory research, then it potentially offers an opportunity to develop a new technique for biological phosphorus removal via microalgae. The work presented in this paper clearly shows that investigating the effects of light intensity and ways to manipulate this variable is important for improving biological phosphorus removal in WSP. Light intensity varies seasonally and also decreases exponentially throughout the depth of the pond according to Beer's Law. Therefore variables such as the amount of vertical mixing can have important implications for phos-

phorus removal. Vertical mixing in WSP occurs naturally because of turbulent diffusion, action of wind, thermal convection, and release of biogas from the anaerobic sludge layer. The degree of vertical mixing determines the frequency of microalgal movement between the relatively better illuminated surface layer and the poorly illuminated interior of the WSP. Phosphate removal may be influenced also by the predominant algal species in the WSP. For example, motile algae have the ability to move within the pond depth in response to the light intensity.

Biological phosphorus removal by microalgal biomass is dependent on both the algal solids concentration and the amount of phosphorus that can be accumulated in the biomass. Figure 4 shows the potential biological phosphorus removal from wastewater containing a phosphate concentration of 10 mg/L. The percent removal in Figure 4 has been calculated for a range of concentrations of microalgal solids which reflects the typical range found in pond systems. The percentage phosphorus levels of 0.4% and 3.2% were used as these are the minimum and maximum values found in this study (Figure 2). To achieve this phosphorus removal, the microalgae need to be harvested from the WSP. There are a number of established technologies that could effectively remove the algae including dissolved air floatation, microfiltration, sand filtration, and sedimentation (*22*).

Clearly, Figure 4 reveals a tremendous potential for biological phosphorus removal in WSP if operation is optimized to enable maximal phosphorus uptake by the microalgae. To improve biological phosphorus removal the biomass concentration and the amount of phosphorus in the biomass need to be maximized.

## Acknowledgments

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