THE ROLE OF ALGAL SPECIES ON PHOSPHORUS BIOAVAILABILITY IN SECONDARY WASTEWATER EFFLUENTS

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The Role of Algal Species on Phosphorus Bioavailability in Secondary Wastewater Effluents

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ABSTRACT

Phosphorus (P) is a limiting nutrient responsible for the eutrophication of surface waters and marine coasts. Wastewater treatment technologies are being stressed to achieve low P effluents and national effluent P limits are expected in the coming years. This research investigates how different algae, as individual species and a mixed culture, utilize bioavailable phosphorus (BAP) when examined by the standard algal species *Raphidocelis subcapitata* along with *Chlorella vulgaris* and *Chlamydomonas reinhardtii* as additional test species. It also investigates how different P-species contribute to BAP using the standard algal species. Results show there is no significant difference in %(BAP/total dissolved P) among the cultures studied and that all P-species contributed to BAP. BAP is an important parameter in determining effluent contributions to eutrophication; this research demonstrates that the current standard algal species is a reliable species for BAP bioassays and that certain P-species may be used to determine BAP estimates.

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DEDICATION

I would like to dedicate this research study first and foremost to God. I would also like to dedicate this to my loving wife, Kristi Swanson, and to my sister, Elena Swanson.

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LIST OF ABBREVIATIONS

AHP.....Acid Hydrolysable Phosphorus

AMP.....Adenosine Monophosphate

ANOVA.....Analysis of Variance

BAP.....Bioavailable Phosphorus

BNR.....Biological Nutrient Removal

BOD.....Biochemical Oxygen Demand

C.....Carbon

DI.....Deionized Water

DO.....Dissolved Oxygen

DOP.....Dissolved Organic Phosphorus

EBPR.....Enhanced Biological Phosphorus Removal

HCl.....Hydrochloric Acid

HPOAS.....High Purity Oxygen Activated Sludge

IAP.....Immediately Available Phosphorus

MBBR.....Moving Bed Biofilm Reactor

MBR.....Membrane Bioreactor

N.....Nitrogen

NRP.....Non-Reactive Phosphorus

OP.....Organic Phosphorus

P.....Phosphorus

PAHP.....Particulate Acid Hydrolysable Phosphorus

PAP.....Potentially Available Phosphorus

POP .....Particulate Organic Phosphorus

PP .....Particulate Phosphorus

RMSE.....Root Mean Square Error

RO .....Reverse Osmosis

RP.....Reactive Phosphorus

SAHP .....Soluble Acid Hydrolysable Phosphorus

SNRP.....Soluble Non-Reactive Phosphorus

SRP .....Soluble Reactive Phosphorus

TDN .....Total Dissolved Nitrogen

TDP .....Total Dissolved Phosphorus

TP.....Total Phosphorus

WEFTEC.....Water Environment Federation Technical Exhibition and Conference

WWTP .....Wastewater Treatment Plant

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1. GENERAL INTRODUCTION

1.1. Background

Eutrophication is a process where excess nutrients, such as nitrogen (N) and phosphorus (P), accumulate in water bodies promoting plant and algal growth, and ultimately resulting in hypoxic conditions or dead zones. Eutrophication is a leading cause of impairment for many freshwater and coastal marine ecosystems. While eutrophication is a natural process, its extent and growth rate have increased due to human activities from point-source and non-point source loading. The mediated damage by eutrophication in the U.S. is estimated at $2.2 billion annually (Dodds et al., 2009).

Eutrophication causes lake and sea-side recreational revenue losses, fishing industry revenue losses, decreased conservation values, and decreased aesthetic values. Eutrophication can lead to toxic algal blooms that result in fatalities and livestock losses, water purification problems, and increased water treatment costs. Lake Winnipeg has experienced a steady increase of blue-green algae growth over the past 30 years, resulting in larger floating mats of algae over the years. These blooms affect the 5.5 million people that rely on the health of the lake. It also affects the tourist and fishing industries of the lake that combined account for $125 million per year. The Gulf of Mexico has been experiencing a hypoxic dead zone, where low oxygen levels can no longer support the life of aerobic organisms, since the 1950's. The shores, beaches, and wildlife support a $20 billion tourist industry and marine animals support the Gulf States in a $1 billion fishing industry (EPA GMPO, 2014).

Among these two major nutrients, P is typically found in lower concentrations than N in water bodies and therefore, is a limiting nutrient for biological growth. Certain phytoplankton have been found to be capable of fixing N from the atmosphere into the water body, thereby

circumventing N management practices (Schindler et al., 2008). Often being a major source of P to surface water through their effluent discharges, wastewater treatment plants (WWTPs) are required, in some states, to employ various types of P removal processes. However, present and potential future regulations on effluent P concentrations (requiring as low as <10 μg/L) have stressed existing treatment technologies and are expected to be implemented at a national level.

While current effluent discharge regulations are based on total phosphorus (TP), studies have shown that only a portion of TP is bioavailable to support the growth of algae (Gu et al., 2011). Bioavailable phosphorus (BAP) is the P that algae utilize for their growth, metabolic maintenance, and reproduction. While there is no standard test to determine BAP, algal assays have proven to be a reliable method. Current methods of testing the bioavailability of P with *Selenastrum capricornutum* may not reliably estimate BAP, as enzymatic activity varies depending on the species of algae (Kwon et al., 2011).

Algal blooms are known to occur at different P levels with different species of algae. In Australia, algal blooms were found occur at P levels as low as 10 μg/L whereas in European water bodies, algal blooms tend to occur at relatively much higher levels (Shaw et al., 2001). Moreover, algal blooms also differed with the dominance of specific types of algal species. Red tides (e.g. *Alexandrium catenella*) are common algal blooms in the east and west coasts as well as Florida and the Gulf of Mexico, while green tides in many coastal regions occurred by *Enteromorpha spp.* and *Codium isthmocladum* outcompeting seagrass and coral reefs. Brown tides by the pelagophytes are commonly seen in the northeast and mid-Atlantic US estuaries, while blue-green algal blooms by Cyanobacteria are a major issue in fresh water lakes such as Lake Winnipeg and Lake Erie (Environment Canada, 2011).

1.2. Research Problem Statement

BAP is the sum of immediately available P (IAP) and potentially available P (PAP). IAP is the portion that can be taken up by biota within a few hours, while PAP is the portion transformed, after longer durations, to BAP forms by physical (e.g. desorption), chemical (e.g. dissolution), and/or biological (e.g. enzymatic degradation) processes (Schoumans et al., 2013). To understand P uptake, it is helpful to know the different forms of P. P-speciation is based on filtration and reactivity. Filtration separates particulate P from soluble P; however, particulate P is effectively removed from wastewaters by current treatment technologies and therefore soluble forms of P are more problematic. Reactivity separates soluble reactive P (SRP) from soluble non-reactive P (SNRP). Non-reactive P can be further separated via weak acid hydrolysis separating soluble acid hydrolysable P (SAHP) and dissolved organic P (DOP). Studies have overlooked this last analytical distinction in their investigations of P-species and BAP, in attempts to relate P-species concentrations to BAP (Gao et al., 2014; Oladeji et al., 2008; Nausch and Nausch, 2011; Li and Brett 2011, 2012, 2013; Ekholm and Krogerus, 1998, 2003; Saavedra and Delgado, 2006).

In order to measure BAP algal bioassays are utilized. *Raphidocelis subcapitata* (formerly known as *S. capricornutum*) is the standard algal species used for the determination of BAP in bioassays (Miller et al., 1978; Li and Brett, 2013). However, current methods of testing the bioavailability of P with *R. subcapitata* may not reliably estimate BAP, as numerous algae exist in water environment and their enzymatic activity varies depending on the species (Kwon et al., 2011). Enzymatic activity can cause the dissolution and/or hydrolysis of some forms of SAHP and DOP, in receiving waters, transforming non-BAP to BAP.

It is important to understand what P-species are contributing to BAP in WWTPs by testing all P-species possible. This will provide operators with knowledge of which P-species to focus on removing. With the future implementation of P limits on WWTPs, it is also important to know whether the current algal species used in bioavailability tests provides a conservative estimate of BAP (a higher BAP estimate compared to other algal species) or whether another algal species could provide a higher, more conservative value.

1.3. Research Goal, Objectives, and Hypotheses

The goal of this research is to determine the effect of algal species and P-species on BAP measurements. The research objectives and respective hypotheses are as follows.

1. To determine the BAP from three different algal species when present individually and as a mixed culture.

   Hypothesis: BAP will differ when analyzed via different algal species.

2. To analyze phosphorus-speciation prior to and after the bioavailability assays for the standard algal species, *R. subcapitata.*

   Hypothesis: All P-species of secondary wastewater effluents contribute to the BAP concentration.

1.4. Research Approaches

To achieve the first objective, the algal assay bottle test is utilized to determine the BAP of samples as measured with different algal species, as individual species and a mixed culture. The algal species used for this objective are *Raphidocelis subcapitata, Chlorella vulgaris,* and *Chlamydomonas reinhardtii*. Total dissolved P is determined through persulfate digestion and the ascorbic acid method. BAP is determined through the difference in total P prior to and after the incubation period and through a growth curve method. For the second objective, in order to

determine the distribution of P-species in wastewater samples, the ascorbic acid method is utilized along with pretreatment methods to differentiate the P-species. The ascorbic acid method is used to determine the soluble reactive P content of the wastewater samples. Acid hydrolysis and the ascorbic acid method are used to determine the combined soluble reactive P and soluble acid hydrolysable P. Persulfate digestion and the ascorbic acid method are used to determine the total dissolved P. These values are then be used to determine the individual soluble reactive P, soluble acid hydrolysable, and dissolved organic P concentrations of the wastewater samples. BAP is determined through the algal assay bottle test.

1.5. Dissertation Organization

This thesis is divided into 5 chapters. This chapter includes background information; research problem statement; research goal, objectives and hypotheses; research approaches; and dissertation organization. A review of key literature is presented in Chapter 2 to provide an understanding of the topic and identify existing gaps that support the need for this research. Chapter 3 presents the work related to the first objective, to determine the BAP from three different algal species when present individually and as a mixed culture. The work in Chapter 3 has been published in the proceedings of Water Environment Federation Technical Exhibition and Conference (WEFTEC) 2015 and will be submitted to a peer review journal. Chapter 4 presents the work related to the second objective, to analyze P-species prior to and after the bioavailability assays. The work in Chapter 4 will be submitted to a peer review journal. The conclusions and recommendations for future work are presented in Chapter 5.

2. LITERATURE REVIEW

2.1. Eutrophication

Eutrophication is a leading cause of impairment in many freshwater and coastal ecosystems around the world. It is the accumulation of nutrients, particularly N and P in water bodies promoting algal growth that can result in floating mats of algae called algal blooms. Eventually the algae die, sink, and decompose where they are consumed by other microorganisms. This consumption utilizes dissolved oxygen (DO) and contributes to hypoxic conditions, when the DO decreases to a level that can no longer support living aquatic organisms. A DO concentration of less than 2 mg/L is considered hypoxic. Figure 1 is a diagram depicting the sequences resulting in eutrophic waters.

Eutrophication causes numerous issues for ecosystems and industries around the world. Eutrophic water bodies result in a loss of revenue for fish industries and recreational sites, a possible loss of fish and animal livestock due to toxic algal blooms, and human fatalities due to seafood harvested from a region that had a toxic algal bloom. Additional treatment costs are necessary for drinking water sources that have become eutrophic or contaminated with toxins. Moreover, there is a decreased conservation value and lowered aesthetic value to eutrophic water bodies. Figure 2 shows a world map of coastal waters that are experiencing eutrophic and hypoxic conditions.

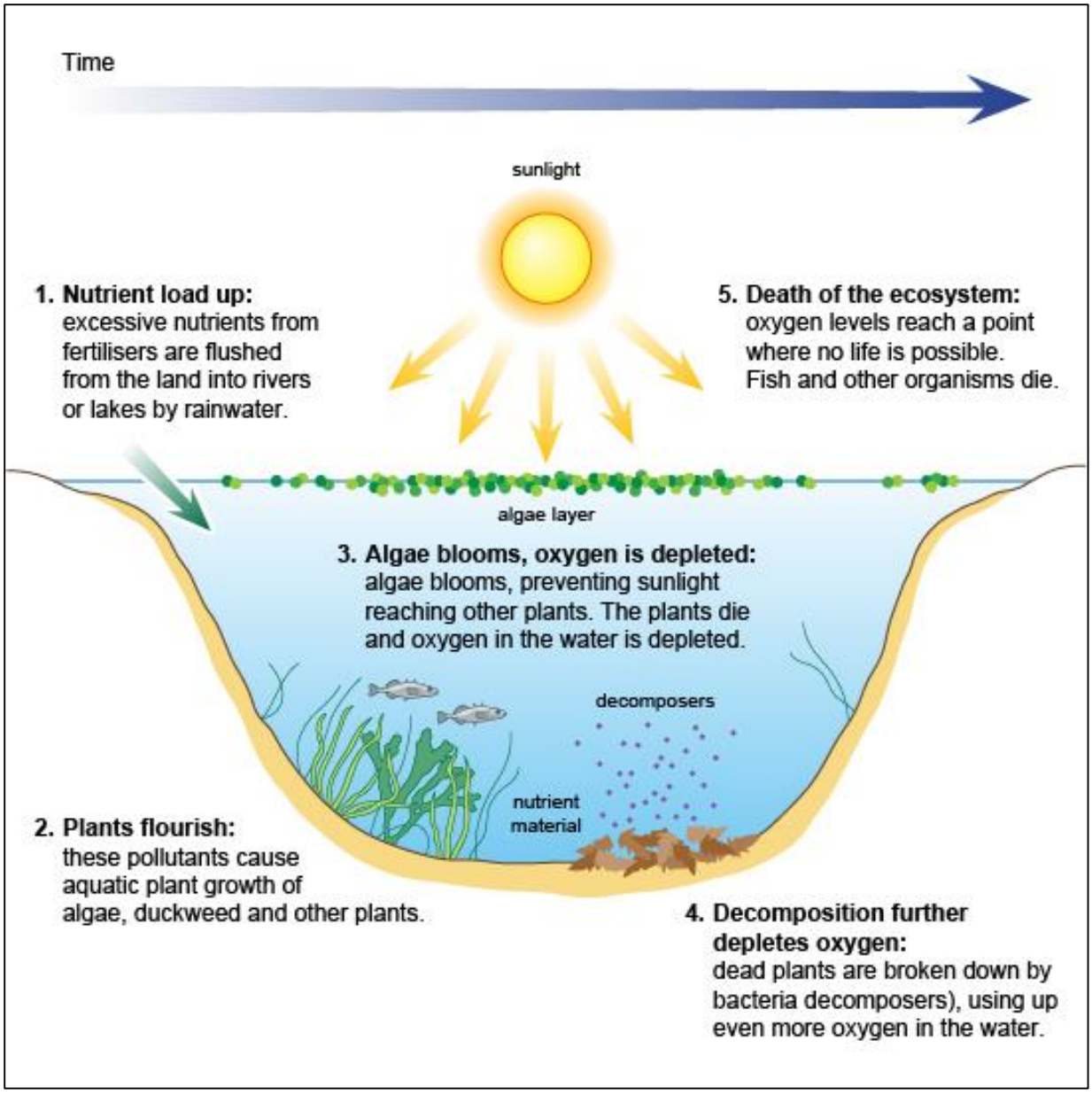

Figure 1. The eutrophication process. (BBC, 2014).

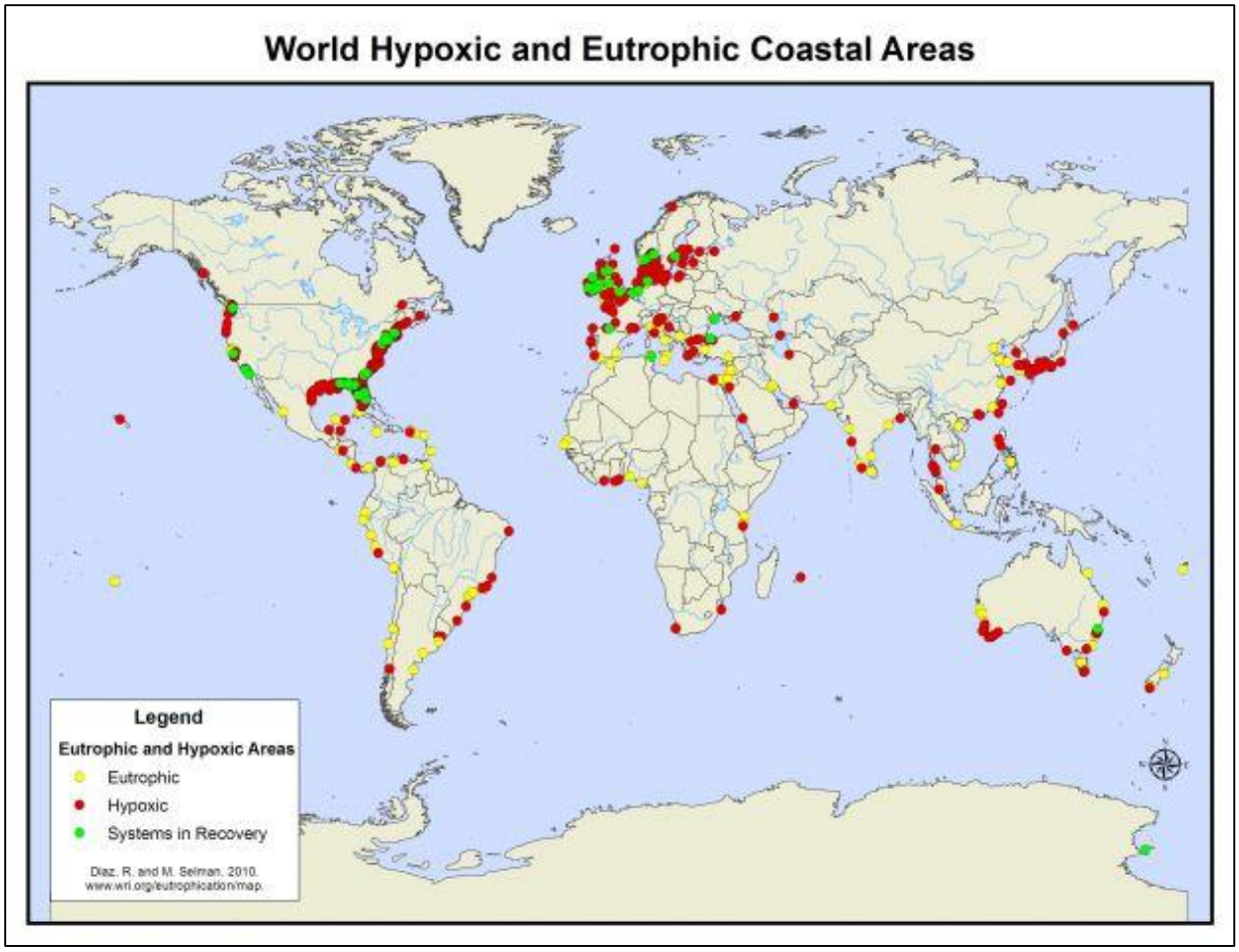

Figure 2. World map of coastal waters experiencing hypoxic and eutrophic condition. (WRI, 2008).

Lake Winnipeg and the Gulf of Mexico are two examples of major concern in North America. These waters receive excess loading of nutrients from rivers, surface runoff, and point-sources that flow into these water bodies. The Red River of the North flows to Lake Winnipeg and the Missouri River joins the Mississippi River, which then flows to the Gulf of Mexico. Both these rivers originate or pass through North Dakota and contribute to the eutrophication problem in these water bodies. The North Dakota 2012 Integrated Section 305(b) Water Quality Assessment Report indicates that 45% of the assessed lakes and reservoirs in the state are eutrophic as well.

Lake Winnipeg has experienced a steady increase of blue-green algae growth over the past 30 years. These blooms affect the 5.5 million people that rely on the health of the lake. It also affects the tourist and fishing industries of the lake that combined account for $125 million per year (Environment Canada, 2011). These algal blooms can destroy fresh water lake ecosystems and be toxic to humans and other species. Public beaches, tourist attractions, lakeside businesses, and fishing have all been closed due to these algal blooms over the past decades. The Lake Winnipeg algal blooms are considered the worst algae problem of any large freshwater lakes in the world and the Global Nature Fund reported it as the most threatened lake in the world for 2013.

The Gulf of Mexico has been experiencing a hypoxic zone, where low oxygen levels can no longer support the life of higher organisms, since the 1950's. The Gulf of Mexico tourist and fishing industries are far larger than those of Lake Winnipeg. The shores, beaches, and wildlife support a $20 billion tourist industry and marine animals support the Gulf States in a $1 billion fishing industry (EPA GMPO, 2014). These industries are affected by the large hypoxic zone caused by the elevated concentrations of nutrients discharging from the Mississippi River (which is contributed to by the Missouri River) along with other contributing rivers, streams, and coastal runoffs.

In both examples described above, as well with other lakes and reservoirs across the world, algal blooms grow from excess nutrient loading that is contributed by human activities. These increased loads are largely due to two factors. First there has been an increase in livestock production and the use of synthetic fertilizers in the Midwest, a region known for its crop and livestock production, along with wastewater contributions from cities along these rivers. Secondly the increased frequency and intensity of spring flooding in the watersheds, which has

enhanced the transfer of nutrients from the landscape to the rivers and lakes. These contributions result in the accumulation of nutrients in lakes, reservoirs, and the Gulf of Mexico reaching elevated levels that cause the algae to thrive and results in depleted oxygen levels necessary to support other life forms (Schoumans et al., 2013).

Both N and P management practices have been considered; however, managing P in water bodies is considered more effective since certain phytoplankton are capable of fixing N from the atmosphere into water bodies (Schindler et al., 2008). A limitation of P management is that waters and wastewaters are typically in N limiting conditions. This is a limitation because P is needed by organisms in smaller amounts than N is needed (Geider and La Roche, 2002) and achieving increasingly lower P concentrations becomes increasingly difficult and costly. Algal blooms tend to occur if concentrations of inorganic N and TP exceed 0.3 mg/L and 0.01 mg/L, respectively (Metcalf and Eddy, 2014).

2.2. Phosphorus-Speciation

To understand P uptake by algae, it is helpful to know different forms of P. P-speciation can be done either by grouping P containing molecules that are similar (also known as by P type) or through a chemical analysis. Another way to differentiate P is by bioavailability, to distinguish BAP and non-BAP.

2.2.1. By P Type

P compounds can be grouped into orthophosphates, poly- or condensed phosphates, and organic phosphates (OP). Orthophosphates are phosphates attached to or lacking hydrogen atoms (H_3PO_4, $H_2PO_4^-$, HPO_4^{2-}, and PO_4^{3-}). Polyphosphates and condensed phosphates are two or more phosphates attached to other groups and include pyrophosphates, tripolyphosphates, and metaphosphates. OP include nucleic acids, phospholipids, inositol phosphates, phosphoamides,

phosphoproteins, sugar phosphates, amino phosphoric acid, and organic condensed P species. These different types of P exist in both soluble and particulate forms. Particulate inorganic P includes phosphates attached to metals (Fe, Al, and Ca) and mineral phosphates (struvite and apatite). P attached to metals and struvite are classified as orthophosphates while apatite is classified as a polyphosphate.

2.2.2. By Chemical Analysis

P compounds can be grouped through chemical analysis as seen in Figure 3. Filtration separates particulate P (PP) from dissolved or soluble P, which consists of SRP, SAHP, and DOP (Figure 3). SAHP and DOP are non-reactive P (NRP). Without any chemical pretreatment, the ascorbic acid method measures SRP. With H_2SO_4 pretreatment, the ascorbic acid method measures SRP and SAHP together. Total dissolved or soluble P is measured through persulfate digestion pretreatment followed by the ascorbic acid method. SAHP and DOP are determined based on the differences in the results provided by different pretreatment conditions (including no pretreatment) according to the descriptions in Figure 3.

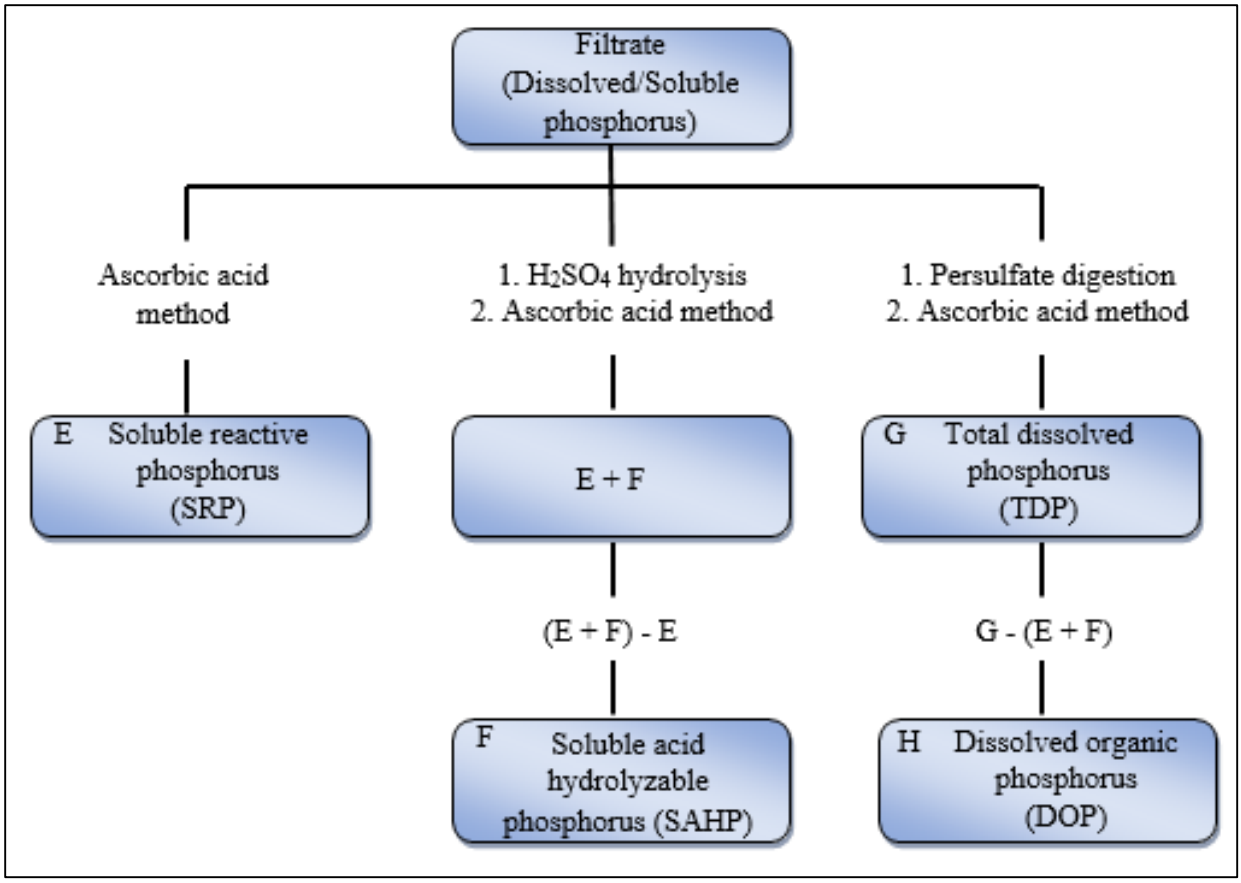

Figure 3. Dissolved phosphorus speciation by chemical analysis. Modified from Standard Methods Manual 21st Edition, pg. 4-147.

SRP represents orthophosphates; however, due to the acidic nature of the ascorbic acid method, which is used to measure SRP, it is possible that SAHP may hydrolyze during the test and be measured as SRP (Neethling et al., 2007; Majed et al. 2012). This indicates that the ascorbic acid method may not be able to accurately distinguish P-species. SAHP represents poly- and condensed-phosphates. Due to the acidic nature of the pretreatment with acid hydrolysis along with the ascorbic acid method, it is possible that some DOP compounds may be measured as SAHP, again indicating a possible flaw in P-species analysis with the pretreatment and ascorbic acid method. Table 1 summarizes the methods of categorizing P-species, how the different P-species categories relate to one another, and examples of P compounds for each P-species category.

Table 1. P-species related by type and chemical analysis categorization methods, and examples of each category.

By Type	By Chemical Analysis	Examples
Orthophosphates	SRP	H_3PO_4, $H_2PO_4^-$, HPO_4^{2-}, and PO_4^{3-}
Poly- and condensed phosphates	SAHP	Pyro-, meta-, poly-phosphates
Organic phosphates	DOP	Nucleic acids and phospholipids

2.2.3. By Bioavailability

BAP is the P plants, algae, and other phytoplankton use to grow, maintain, and reproduce themselves. Another way to describe BAP is as the sum of IAP and PAP. IAP is the portion that can be taken up by algae within a few hours, while PAP is the portion transformed, after longer durations, to IAP forms by physical (e.g. desorption), chemical (e.g. dissolution), and/or biological (e.g. enzymatic degradation) processes (Schoumans et al. 2013; Cade-Menun and Paytan, 2010; Jansson, 1993).

Both IAP and PAP are difficult to define further. Research has shown that apatite and humic-Al/Fe-bound P, which are classified as reactive P (RP), have low bioavailability, and would therefore not be classified as IAP, but possibly as PAP (Li and Brett, 2013). This research also shows that several OP and inorganic-P forms, which are classified as NRP, have high bioavailability, but does not discuss their associations with IAP and PAP. The research concludes that the classical assumption that BAP is approximated by SRP is unreliable.

It is likely that orthophosphates are considered IAP as orthophosphates are inorganic minerals that are readily available to algae for growth and reproductive purposes. The relation of polyphosphates and organic P to IAP and PAP has not been established. Similarly, it is not clear

how SRP, SAHP, and DOP are related to IAP and PAP. However, it is likely that each of these P categories have P-compounds that are PAP; because poly-/condensed-phosphates and organophosphates are larger P-containing molecules that require physical, chemical, or biological removal of the phosphate group and some orthophosphates require such separation as with metal bound phosphates.

2.3. Bioavailable Phosphorus

BAP has been quantified in sediments, agricultural runoffs, municipal wastewaters, rivers, and soils since the 1980's and early 1990's. Studies have quantified BAP through bioassays, bioluminescent sensor strains, iron oxide impregnated paper strips, and isotope uptake experiments (Ekholm et al., 2009; Muñoz-Martín et al. 2011; Dollard and Billard, 2003; Saavedra and Delgado, 2006; Muñoz-Martín et al., 2014; Robinson et al., 1994; Li and Brett, 2011; Bjorkman and Karl, 1994). BAP was originally thought to be SRP or orthophosphates as these are phosphates at the mineralized level, which algae use as primary P sources. Studies eventually determined that BAP is often greater than SRP (Ekholm and Krogerus, 1998; Li and Brett, 2013). Since then, studies have considered BAP to be the sum of SRP, SAHP, and bioavailable DOP; the sum of plant available P in soils (Olsen-P), readily desorbable P, and total dissolved P (TDP); or as the sum of TDP and bioavailable PP; (Thien and Myers, 1992; Sharpley, 1993; Oladeji et al., 2008; Nausch and Nausch, 2011).

A study by Li and Brett (2013) examined the influence of dissolved P form on bioavailability and found that bioavailable forms did not necessarily correspond to the classical assumption that SRP approximates BAP. In their study, they analyzed numerous organic-P compounds that were nearly entirely bioavailable, poly- or condensed-P compounds that had high bioavailability, and they analyzed compounds (apatite and humic-(Al/Fe)-P) that are

classified as SRP, but were barely bioavailable. They concluded that a portion of SRP in surface waters is likely non-BAP due to P bound to humic-metal compounds that are not released by phosphatase enzymes and that these compounds are too large to cross cell membranes.

Studies have found that BAP is often greater than SRP and therefore PP, DOP, and/or inorganic P (other than orthophosphates) must be contributing to BAP as well (Ekholm and Krogerus, 1998; Li and Brett, 2013). Li and Brett (2013) conducted studies of organic and inorganic compounds containing P that proved DOP and inorganic P (other than orthophosphates) as partially bioavailable. Bioassays are considered the best approach for determining BAP, yet underestimate it due to the release of P over the incubation period (Ekholm et al., 2009). While some studies starve the algae of P prior to the bioassay (in order to increase the uptake rate of P), it has been shown that this is not necessary as there is no significant difference in BAP between P starved algae and algae that were not starved (Moorleghem et al., 2013; Powell et al., 2008).

2.4. Algal Uptake and Usage of Phosphorus

2.4.1. Uptake Pathways

Ions can enter cells by one of four pathways: adsorption, passive transport, facilitated diffusion, and active transport. Adsorption ions enter the cell by moving across the boundary layer of water surrounding the cell, then it passively travels through the cell wall and plasmalemma into the cytoplasm. In passive transport, nonelectrolytes diffuse through the membrane at a rate proportional to their lipid solubility and inversely proportional to their molecular size. For ions the driving force is the difference between two electrochemical potential points along the path. Facilitated diffusion is similar to passive diffusion, but occurs at a faster rate. Carriers or enzymes bind to the ion at the outer membrane surface and escort it to

the inner membrane surface where it is released. Active transport involves the transportation of ions against an electrochemical gradient and is therefore termed "uphill" transport. In active transport, energy is expended to release the molecule or ion from the carrier. Active transport can be used to transfer solutes into or out of cells. Nutrient uptake usually occurs via passive diffusion, facilitated diffusion, and active transport. Inorganic nutrients are generally taken up in ionic form, such as phosphate, via active transport (Vymazal, 1995; Schachtman et al., 1998).

Uptake rates of ions in relation to their external concentration are generally described by a rectangular hyperbola, similar to the Michaelis-Menten equation for enzyme kinetics (Vymazal, 1995). Phytoplankton produces extracellular enzymes to mineralize organic matter and release nutrients into forms that can be assimilated. Production of extracellular enzymes increases when simple nutrients are scarce and complex nutrients are in abundance (Nedoma et al., 2003; Allison and Vitousek, 2005).

2.4.2. Phosphorus Uptake and Algal Bloom

P uptake and the portion of P that becomes bioavailable in water bodies can vary depending on the dominating species of algae present. This is evident from the reason that algal blooms occurred at different P levels and also differ in the type of algal species present. Algal blooms have been shown to occur at different P concentrations. For example blooms occurred at P levels as low as 10 µg/L in Australia, but tend to occur at relatively much higher levels in European water bodies (Shaw et al., 2001). Algal blooms also differed by the dominance of specific types of algal species. Red tides (e.g. *Alexandrium catenella*) are common algal blooms along the coasts of the USA and in the Gulf of Mexico, while green tides in many coastal regions are dominated by *Enteromorpha spp.* and *Codium isthmocladum* where they outcompete seagrass and coral reefs. Brown tides dominated by pelagophytes are commonly seen in the

northeast and mid-Atlantic USA estuaries, while blue-green algal blooms of Cyanobacteria are a major issue in fresh water lakes such as Lake Winnipeg and Lake Erie (Environment Canada, 2011).

2.4.3. Algal Usage of Phosphorus

P is a necessary element for all life as it is a key component in RNA, DNA, membranes, and the energy cycle for all organisms. RNA and DNA, the nucleic acids, are composed of 5-carbon sugar, a phosphate group, and a nitrogenous base. Membranes are composed of phospholipids, where the hydrophilic end contains a phosphate group. Inositol phosphates have diverse cellular functions including cell growth, apoptosis, cell migration, endocytosis, and cell differentiation. Sugar phosphates are used in biological systems to store and transfer energy. While both N and P are necessary nutrients for all organisms, P is required in smaller amounts.

2.5. Phosphorus Sources and Cycle

2.5.1. Sources

Sources of P to water bodies come from non-point sources or point sources. Non-point sources include runoff from pastures and croplands, urban runoff, non-agricultural rural runoff, seepage from individual sewage treatment systems, and stream bank erosion. Runoff from pastures and croplands is a major source of P in waterbodies, especially in rural farming areas, and are primarily due to the use of fertilizers, herbicides, and insecticides (MPCA, 2007). Studies have shown that P in forest and field runoff is typically about 20 to 40% bioavailable (Ekholm and Krogerus, 2003; Reynolds and Davies, 2001; Edwards and Withers, 2007). P also occurs naturally in minerals containing phosphate, such as struvite and apatite, which can erode into streams and rivers. Point sources include municipal and industrial WWTPs. Municipal point sources receive P from human excrement and from detergents containing P, although P

content in detergents has been reduced over the years. Based on bioassay methods, the bioavailability of P in tertiary wastewater effluents can range from 10 to 75% with most being 40 to 50% while that in secondary wastewater effluents is typically 80 to 90% (Ekholm and Krogerus, 1998; Li and Brett, 2011; Li and Brett, 2013; Qin et al, 2015).

Non-point sources, such as agricultural and urban runoff, contribute up to 80% of P loading to water bodies (WDNR, 1992), but they contain a lower bioavailable content compared with point sources (Edwards and Withers, 2007). Nutrient limits are more easily imposed on point sources since the wastewater is collected and treated. More stringent nutrient limits are expected in the coming years, but WWTPs in environmentally sensitive areas are already facing limit implementations (Clark et al., 2010). It is expected the future effluent P limit may be as low as < 0.01 to < 0.05 mg/L (Ragsdale, 2007). This would stress the capabilities of current treatment systems and require that all WWTPs incorporate tertiary nutrient removing processes.

Table 2 summarizes the percent contributions of TP to receiving waters by different sources as discussed above. Using the information on TP bioavailability (BAP/TP in %) which is also summarized in Table 2, the percent contributions of BAP to receiving waters by different sources were calculated and are presented in Table 2. Non-point sources contribute between 47 and 94% of all BAP in receiving waters, while point sources are responsible for 6 and 53% of BAP in receiving waters. It should be noted that the value for percent contribution of BAP to receiving waters by secondary treatment point sources in Table 2 is based on the assumption that all the point source discharges are from secondary treatment only. The values for tertiary treatment point sources are based on similar assumptions. These values demonstrated that point sources are important contributors of BAP to receiving waters. An explanation of how these

values were calculated and sample calculations can be found at the beginning of the appendix (Equations 1 and 2).

Table 2. Contributions of P and BAP to receiving waters by different sources.

Source		**Percent Contribution of TP to Waters (%)**	**Range of %(BAP/TP) of Effluent or Runoff (%)**		**Range of %BAP Contribution to Waters (%)**	
Non-point		80	20	40	47	94
Point	Secondary treatment	20	80	90	33	53
	Tertiary treatment (typical range)		40	50	20	38
	Tertiary treatment (full range)		10	75	6	48

2.5.2. Cycle

The major environmental reservoir of P is in rocks that are mined for human resources or otherwise eroded into water bodies. P leaching and erosion into water bodies leads to dissolved forms of P that are taken up by plants and phytoplankton allowing P to enter the food chain. P then remains in the food chain or is returned to sediments, soils, and rocks through excretion or death and decay. Humans mine P from rocks for fertilizers, detergents, and pesticides. From there, P is applied to crops which allow P to enter the food chain or return the P to soils via

adsorption. In the case of detergents and human excretion, P is eventually sent to WWTPs and discharged into rivers or separated in sludge processes that are used as fertilizers or sent to landfills (Ophardt, 2003).

2.6. Wastewater Treatment and Phosphorus

2.6.1. Phosphorus Treatment

P is removed from wastewater by chemical, biological, or physical means. Chemical treatment includes precipitation and physical-chemical adsorption, biological treatment includes assimilation and enhanced biological P removal (EBPR), and physical treatment includes filtration and membrane technologies. Chemical precipitation (typically with aluminum or iron slats) was the primary method of P removal from wastewaters, but as biological methods were developed they have gained more appeal due to the elimination or reduced use of chemicals and reduction of chemical sludge volume. Biological P removal is achieved under controlled environmental conditions beginning with an anaerobic zone, followed by an aerobic zone, for P accumulating organisms. While the anaerobic stage results in the release of some P due to starvation, this increases their P uptake above normal levels during the aerobic stage. Physical treatment processes include granular and membrane filtration that removes P mainly through size exclusion (PP for granular filtration, and PP + some soluble P molecules for membrane filtration).

Conventional chemical and biological P removal processes are capable of achieving 70 to 90% P reduction of the influent amount, with effluents concentrations reliably reaching 1 mg/L and even 0.5 mg/L (Neethling et al., 2008). In order to reliably and consistently achieve low P effluents tertiary processes such as EBPR, sedimentation, and filtration processes are necessary (Neethling et al., 2008). Ultrafiltration and reverse osmosis are also effective at achieving low P

effluents, but are primarily used to reduce dissolved inorganic solids (Viessman et al., 1993). WWTPs are also capable of recovering P, through settling processes, for use as fertilizer (Metcalf and Eddy, 2014).

Major P removal mechanisms in natural systems (such as ponds and constructed wetlands) are chemical precipitation and adsorption, although plants take up some P as well (Viessman et al., 1993). Ortho-P is adsorbed by clay minerals and organic soil. Biological P removal processes are an effective and relatively low-cost treatment option compared to chemical P removal and have the benefit of reduced chemical sludge production. While biological methods are reliable, they are sensitive and require sustained environmental conditions to work properly.

Gu et al. (2011) reported biological nutrient removing (BNR) processes, processes that remove both N and P, are efficient at removing SRP and PP (> 93% removal), had low efficiency at removing DOP (78%), and was not effective at removing SAHP. They also reported that chemical P removal is effective at removing SRP, SAHP, and POP; but was not effective at removing PAHP or DOP. For both biological and chemical processes, TDP and PP are effectively reduced, but DOP becomes a greater portion in the effluent wastewater. A study by Li et al. (2013) on advanced BNR systems concluded that chemical P removal in addition to BNR systems resulted in an effective removal of P-species that promote algal growth. The conclusion was based on observing a difference in %(BAP/TP) of effluents between processes with and without chemical addition.

Membrane technologies have been shown to remove the P in total suspended solids and dissolved P as well. Membrane bioreactors (MBRs), tertiary membrane filtration, and reverse osmosis (RO) treatment processes have been used in full-scale WWTPs reporting effluents of

<0.1 mg/L TP. It is possible to achieve reliable P levels of 0.04 mg/L for MBRs and tertiary membrane filtration and 0.008 mg/L for RO (Strom, 2006), which are below the future expected P limit of 0.1 mg P/L. The most stringent limit of 0.01 mg P/L can be met by using RO.

2.6.2. Phosphorus Levels in WWTPs and Water Bodies

Influent domestic wastewater typically has a P concentration of 11 mg/L (Qasim et al., 1998). Effluent P of secondary treatment systems is between 1 and 6 mg P/L, depending on the process (Qasim et al., 1998). While secondary effluents of WWTPs with P removal processes have <1 mg P/L (Neethling et al., 2008). Advanced tertiary WWTPs are capable of achieving very low levels of P in effluents (0.2 - 0.3 mg P/ L) (Neethling et al., 2008) and some are capable of achieving <0.05 mg P/L (Li and Brett, 2013). In tertiary effluents, DOP represents a greater portion of the effluent TDP (Liu et al. 2011; Liu et al., 2012; Li and Brett, 2012; Gu et al., 2014) and has been found to be approximately 75% bioavailable (Qin et al., 2015).

The P levels in various waterbodies have been reported, but vary from location to location. Lakes concentrations have been reported between 0.025 to 0.2 mg/L. Rivers and streams are known to have concentrations between 0.01 and 0.1 mg/L. Urban and agricultural runoff can have concentrations from 0.2 to 2.5 mg/L. These are approximate concentrations that have been found among several sources, but by no means represents all water bodies and runoff concentrations (Daverede et al., 2003; Osmond et al., 1995; Howell and Nakamoto, 2009; EEA, 2001; Lory, 1999).

2.6.3. Phosphorus Limits

Often being a major source of P to surface water through their effluent discharges. WWTPs may employ various types of P removal processes, but there is no current national regulation to limit P in effluents. However, present and potential future regulations on effluent P

concentrations (requiring as low as < 10 μg/L) would stress existing treatment technologies and require all WWTPs to incorporate tertiary nutrient removing processes. In the interest of reducing the economic and technological burden on the treatment systems, recent studies have focused on investigating the bioavailability of TP and have shown that only a portion of TP is bioavailable to support algal growth (Gu et al., 2011; Li and Brett, 2012; Li and Brett, 2013; Bjorkman and Karl, 1994; Qin et al., 2015).

WWTPs in major cities in North Dakota, such as Fargo and Bismarck have average and maximum effluent TP concentrations of approximately 3.5 and 6 mg/L, respectively as reported by plant operators. Currently there is no limit on P in the state of North Dakota, likewise there is no P limit for most states in the USA (EPA, 2015), but nationwide regulations are being proposed to limit effluent TP. Li and Brett (2011) suggested that TP management is better suited for non-nutrient removing secondary effluents due to their higher P concentrations with high bioavailability and BAP managements practice are better suited for nutrient removing treatment processes due to their lower P concentrations with lower bioavailability.

Table 3 shows the relation between P concentration, secchi depth in meters, and trophic class (Carlson and Simpson, 1996) as used by the limnological community to classify and rank lakes. This table indicates that water bodies can become eutrophic with P concentrations as low as 0.02 mg/L. Conventional WWTP secondary effluents typically contain between 1 and 6 mg P/L, these effluents are diluted by discharging to rivers, streams, lakes, and ditches. P concentration of the receiving waterbodies may then increase downstream to eutrophic levels. Moreover, the P concentrations may already be at eutrophic levels upstream of WWTPs due to other WWTP effluents and runoff events. Studies indicate that P-levels rise downstream of WWTPs (Douglas et al., 2006; Millier and Hooda, 2011; Yin et al., 2013; Ekka et al., 2006).

This rise in river and stream P concentrations is often an order of magnitude larger downstream and is a major source of eutrophication.

Table 3. Trophic class related to phosphorus concentrations (μg/L) and secchi depth (m).

Trophic State	**Phosphorus (μg/L)**	**Secchi Depth (m)**
Oligotrophic	0 - 12	4 - 8+
Mesotrophic	12 - 24	2 - 4
Eutrophic	24 - 96	0.5 - 2
Hypereutrophic	96 - 384+	<0.25 - 0.5

There is no national limit on the amount of P that may be discharged to waterbodies. However, some states (for example, Florida, New Jersey, Minnesota, and Wisconsin) have point source limits and provisions to reduce runoff from farms, construction sites, and urban areas. (WDNR, 1992). The adoption of numeric limits for N and P will help protect waters by assisting states in identifying and listing impaired waters, developing total maximum daily loads, and writing the National Pollutant Discharge Elimination System permits for facilities discharging N and P. Numeric criteria for N and P from point sources can further improve water quality by complementing best management practice implementation for non-point sources to limit N and P in runoff and infiltration (EPA, 2011).

3. SENSITIVITY OF PHOSPHORUS BIOAVAILABILITY TEST TO ALGAL SPECIES

3.1. Introduction

Eutrophication is a process where excess nutrients, such as N and P, enter water bodies. These nutrients are consumed by phytoplankton, such as algae, stimulating their growth which can result in large floating mats of algae called algal blooms. The algae eventually die and sink to the bottom of the water body, where they are consumed by primary consumers and other organisms. This consumption requires the oxidation of organic matter, which depletes DO levels in the water body, contributing to hypoxic condition, known as dead zones, for fish and other aerobic organisms (Schindler et al., 2008). Eutrophic water bodies results in revenue losses for fishing industries and recreational sites. Some algae are known to produce toxins that are dangerous to humans, livestock, and other animals resulting fatalities. Additionally, algal blooms can increase the treatment costs of drinking water (Smith, 2003; Anderson et al., 2002; Steffen et al., 2014). While some communities and industries already have N and P effluent limits, this is leading to the establishment of nationwide effluent limits on WWTPs in the near future.

WWTPs in environmentally sensitive areas are already facing effluent limit implementations (Clark et al., 2010). As more stringent nutrient limits are expected in the coming years, it is expected the future P limit may be as low as 0.009 to 0.05 mg/L (Ragsdale, 2007). These low limits are meant to minimize the amount of P available for algae and other phytoplankton in order to minimize human contributions to eutrophication. These low limits will stress existing treatment technologies and will require WWTPs to incorporate tertiary nutrient removing processes.

BAP is the P that algae use to metabolize, maintain, and reproduce themselves (Geider and La Roche, 2002). Another way to describe BAP is as the sum of IAP and PAP. IAP is the portion that can be taken up by algae within a few hours, while PAP is the portion transformed, after longer durations, to IAP by physical (e.g. desorption), chemical (e.g. dissolution), and/or biological (e.g. enzymatic degradation) processes (Schoumans et al., 2013; Cade-Menun and Paytan, 2010; Jansson, 1993). The enzymatic activity is known to vary among algal species (Kwon et al., 2011) and it is therefore possible that BAP as determined by different algal species may vary. It has been found that algal blooms occur at different P levels and different species dominate different locations around the world (Shaw et al., 2001; Environment Canada, 2011), further suggesting that BAP may vary among algal species. This brings into question whether the current algal species used to determine BAP provides a higher BAP estimate than other species, thereby providing a more conservative BAP value for monitoring and regulative purposes.

BAP has been measured through bioassays (Ekholm et al., 2009; Muñoz-Martín et al., 2011; Li and Brett, 2011; Bjorkman and Karl, 1994), isotope uptake rates (Moorleghem et al., 2013), soils strips (Robinson et al., 1994; Saavedra and Delgado, 2006), and bio-reporters (Dollard and Billard, 2003; Muñoz-Martín et al., 2011). In addition, it has been estimated based on concentrations of other P-species (Oladeji et al., 2008; Nausch and Nausch, 2011). While there is no set standard to determine BAP, bioassays are considered to provide the best approximation (Ekholm et al., 2009; Li and Brett, 2011).

Nutrient limits are more easily imposed on point sources such as WWTPs, despite non-point sources, such as agricultural and urban runoff, contributing up to 80% of TP loads to water bodies (WDNR, 1992);. However, studies show that the bioavailability of P (BAP fraction) in

agricultural and urban runoff is 20 to 40% (Ekholm and Krogerus, 2003; Reynolds and Davies, 2001) while secondary WWTP effluents, without nutrient removing processes, have bioavailability contents of 80 to 90% (Ekholm and Krogerus, 1998; Li and Brett, 2011). In tertiary effluents, with nutrient removing processes, the bioavailability content ranges from 10 to 75% with many being approximately 40% bioavailable (Qin et al., 2015; Ekholm and Krogerus, 1998; Li and Brett, 2011; Li and Brett, 2013). DOP has been found to represent a greater portion of the TP effluent in tertiary effluents (Liu et al., 2011; Liu et al., 2012; Li and Brett, 2012; Gu et al., 2014). This shows that DOP is a recalcitrant form of P in WWTP effluents and represents a greater portion of the effluent TP and potentially the effluent BAP as well.

The objective of this study is to determine if the current standard algal species of the bioavailability test, *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*), provides a higher or lower BAP estimate than other algal species tested in order to determine which provides a more conservative BAP value. This is accomplished by comparing the %(BAP/TDP), of secondary effluents without nutrient removing processes, measured via multiple algal species as individual species and a mixed culture. The value of %(BAP/TDP) was used instead of a BAP concentration to normalize results for different TDP concentrations among the samples tested. To accomplish this objective, the Printz Algal Assay Bottle Test (Miller et al., 1978) was modified for higher nutrient concentrations and high algal density measuring techniques were used in conjunction. TP and total N were analyzed with the ascorbic acid method and ultraviolet spectrophotometric method respectively, both following persulfate oxidation pretreatment (APHA et al., 2005). BAP was determined via standard growth curves (Miller et al., 1978; Li and Brett, 2011) and through TDP analysis prior to and after the incubation period. These two

BAP determination methods were used in order to determine which one is the more reliable method and to examine if a correlation between the two methods could be made.

3.2. Materials and Methods

3.2.1. Glassware and Standard Chemicals

All glassware except assay bottles were washed with phosphorus-free Liquinox soap, rinsed with tap water and placed in a 10% v/v hydrochloric acid (HCl) acid bath overnight, then rinsed with deionized (DI) water. Assay bottles were sterilized by placing them in a 10% bleach solution for 10 minutes to break up the biomass and then were rinsed with tap water before placing them in an acid bath overnight. After that, they were rinsed with DI water and placed in an oven at 105°C. Potassium phosphate was used as a standard for P analysis and purchased from VWR International Co., PA, USA. Nutrients for algal media were purchased from VWR International Co., PA, USA. Sulfuric acid, antimony potassium tartrate, ammonium molybdate, ascorbic acid, boric acid, and sodium hydroxide were purchased from VWR International Co., PA, USA.

3.2.2. Wastewater Samples

Secondary effluent wastewater samples were collected from the City of Fargo WWTP (Fargo, ND, USA). The Fargo WWTP has a peak flow capacity of 29 MGD and an average of 15 MGD. This facility is not subject to total N or total P limits, but is subject to ammonia limits. The Fargo WWTP treats wastewater for biochemical oxygen demand (BOD) and ammonia through a two-stage trickling filter process. Eleven grab samples of secondary effluent were collected from the Fargo WWTP between June 2014 and May 2015. Grab samples were collected prior to the chlorination process.

3.2.3. Phosphorus Analysis

Grab samples were filtered through a 0.45 μm pore size filter (Advantec sterile mixed-cellulose-ester membrane filters Tokyo, Japan). Since the effluent TDP varied between 3.5 to 6.0 mg P/ L, a series of dilutions was used to bring the P levels into the reliable measuring range of the ascorbic acid method (for determining the TDP concentration of each grab sample). This method measures P between 0.02 and 2.60 mg P/L. These diluted samples were then subject to persulfate digestion and autoclaving pretreatment steps followed by the ascorbic acid method as described in the Standard Methods (APHA et al., 2005). This provides the TDP concentration. Standard calibration curves, of known potassium phosphate concentrations versus absorbance, were successfully created in order to determine the wastewater samples TDP concentration (R^2 = 0.9893). Standard calibration curve experiment was conducted in triplicate and repeated on separate occasions to ensure accuracy and reliability.

3.2.4. Nitrogen Analysis

A portion of each grab samples was also analyzed for total dissolved N (TDN) content after filtration through the 0.45 μm pore size filter. The TDN concentration of the secondary effluents samples ranged from 20 to 45 mg N/ L; therefore, a series of dilutions were used to bring the N levels into the reliable measuring range (0.0 and 3.0 mg N/L). Persulfate digestion pretreatment and the ultraviolet spectrophotometric screening method were used to determine the TDN concentration (APHA et al., 2005).

3.2.5. Stock Algae

The following algal strains were purchased from the UTEX Culture Collection of Algae, TX, USA: *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*) UTEX 1237, *Chlorella vulgaris* UTEX 2714, and *Chlamydomonas reinhardtii* UTEX 89. Stock algae were

maintained biweekly with the media solution of the Printz Algal Assay Bottle Test (Miller et al., 1978).

3.2.6. Algal Assay Bottle Test

The Printz Algal Assay Bottle Test, developed by the EPA (Miller et al., 1978), is a procedure used to test the effect of nutrients on algal growth using the algal species *R. subcapitata*. The procedure can be used to determine which nutrient is limiting algal growth (N, P, trace-elements, or co-limiting conditions). It can also be used to investigate heavy metal toxicity. The bioassay was used to measure BAP as the samples were placed in P-limiting conditions. It was conducted with the other two algal species, *C. vulgaris* and *C. reinhardtii*, and a mixed algal culture (*R. subcapitata, C. vulgaris*, and *C. reinhardtii* together) as well in this study. *C. vulgaris* and *C. reinhardtii* are common freshwater-green algal species. They were included in the study to represent alternate green algal species. A broader range of algal species was not used in order to minimize different characteristics and parameters among the algal species tested. The mixed culture was tested to represent a natural ecosystem, in which pure cultures are usually not found. The Algal Assay Bottle Test requires specific environmental conditions for the algae to grow as described in the following subsections.

3.2.6.1. Nutrients

In order to ensure P-limiting conditions during the bioassay test, P-free media was used. This P-free media was based on the media from the Algal Assay Bottle Test. The Redfield ratio relates C, N, and P content of algae, by weight, to each other and is estimated to be 106:16:1 (C:N:P). However, Geider and La Roche (2002) stated that the ratio of N to P varies between algal species from 9:1 to 23:1. Therefore, in this research a ratio of 26:1 was used to better ensure that P-limiting conditions are obtained and to add a safety factor. The ratio of C:N from

the Redfield ratio was maintained resulting in C:N:P ratios of 173:26:1 in this research. The macro- and micro-nutrients of the media from the Algal Assay Bottle Test were scaled up by the same factor the N was scaled up to achieve P-limiting conditions. Potassium phosphate was replaced with potassium chloride to maintain the potassium content, while removing undesired P sources.

Media was autoclaved in order to sterilize them. Carbonate would precipitate out of the media after autoclaving, but become soluble again after adjusting the pH to 7.5. However, if carbonate concentrations were too high, not all of the carbonate would become soluble after adjusting the pH. Therefore, wastewater sample bioassays were conducted at 1 mg P/L and 26 mg N/L, where carbonate could become soluble again.

3.2.6.2. Algal Inocula

Stock algae were centrifuged at 1,000 rpm for 5 minutes. The solution was decanted in order to remove the nutrient media, containing P, from the centrifuged stock algae. The tubes were then rinsed with DI water, centrifuged, and decanted three times in order to wash the stock algae of all nutrients. The rinsed stock algae were then suspended in DI water and counted through an Accuri C6 flow cytometer to determine the cell concentration for each species. The rinsed stock algae were then placed in volumetric flasks, as individual species and the mixed culture, and diluted so that these algal inocula would result in an initial cell concentration of 10,000 cells/L in the bioassay test bottles. The mixed culture had the same total initial concentration of algae as the pure cultures, but an equal number of each species. This was accomplished by taking one-third of the required volume for the individual algal inocula, mixing them together, and diluting so the mixed algal inocula would result in an initial cell concentration of 10,00 cells/L.

3.2.6.3. Incubation Setup and Conditions

Bioassay bottles containing wastewater sample, DI water, algal inocula, and P-free media supplement were placed on an orbital shaker. DI was added to dilute the samples to 1 mg P/L and the P-free media supplement was used to ensure P-limiting conditions. The mixture in the bottle was incubated at 24 ± 2°C and 110 rpm with lamps continuously providing 4300 ± 430 lumens adjacent to the sample surface. The bottles were sealed air tight to prevent the escape and/or addition of carbon as carbon dioxide. The incubation period was 14 days to achieve the maximum standing crop (Miller et al., 1978; Li and Brett, 2011).

3.2.7. Algal Growth/Biomass

There are several ways to measure biomass. The recommended methods described in the Algal Assay Bottle test (Miller et al., 1978) lists them in order of preference: particle counter, gravimetric method, chlorophyll-a, hemacytometer, and absorbance. The gravimetric method is better suited for high algal densities while the chlorophyll-a method works better for lower algal densities. The absorbance method, also more appropriate for lower algal densities, was recommended as a last resort. To accurately seed the samples and standards with an accurate number of cells, a flow cytometer was used for the algal inocula as a particle counter. However, since this research investigates relatively high TDP concentrations, resulting in high algal densities, the gravimetric method was utilized to quantify the biomass after the incubation period.

3.2.7.1. Particle Counter

A BD Accuri C6 Flow Cytometer (BD Biosciences, Franklin Lakes, NJ) was utilized to count cells of centrifuged and rinsed stock algal solution. Cell counts were done in triplicates

and the average cell count was used to dilute the cell densities to the desired concentration for the algal inocula.

3.2.7.2. Gravimetric Method

The gravimetric method described in the Algal Assay Bottle test (Miller et al., 1978) and the Standard Methods (APHA et al., 2005) were used to determine dry algal densities of bioassay samples and controls, after the incubation period. Pre-weighed 0.45 μm pore size membrane filters were used on each bioassay to separate the algal biomass and remaining media. After the filtration, each filter was placed on an aluminum dish. Four replicates of bioassay samples, for each individual algal species and the mixed culture, were used due to the variability involved in the bioassay procedure. The aluminum dish, the filter, and biomass were then dried overnight at 70°C. Control weights of standard metal weights weighing 1.0 and 2.0 g were used to monitor variations between weighing the filters prior to and after the filtration and drying process. If these standard metal weights varied in weight between the weighing periods, this difference was factored into the biomass weight to account for the error. The dry algal biomass was determined as the difference between the combined dried dish, filter, and biomass weight and the combined dish and filter weight. The filtered volume of the bioassay sample was recorded along with the recorded weights corresponding to each bioassay. The algal density was then determined by dividing the dry weight of the algal biomass by the volume filtered.

3.2.8. Bioavailable Phosphorus Estimation

3.2.8.1. Standard Growth Curve Method

One method of determining BAP is to create standard growth curves (Miller et al., 1978; Li and Brett, 2011), for each algal species individually and as a mixed culture. These standard growth curves compare dry algal density produced from known concentrations of potassium

phosphate and P-free media. The potassium phosphate, a SRP form, was assumed to be entirely bioavailable. Curves were created with known P concentrations of 0.0, 0.1, 0.5, 1.0, 1.5, 2.0, and 5.0 mg P/L for each individual algal species and the mixed culture. The BAP of wastewater samples was then determined by comparing the sample algal density produced and comparing it to the algal density of the standard growth curves for each respective algal species.

3.2.8.2. TDP Difference Method

Another method to determine BAP concentrations is via chemical analysis of the TDP prior to and after the incubation period. Here BAP is the difference of TDP before and after the incubation period (the corresponding equation used can also be found near the beginning of the appendix, Equation 3). A potential issue with this method is that as algae decompose during the incubation period, organic-P compounds are released. Due to the number of replicates compounded by the number algal species utilized and since the bioassay bottles were sealed during the incubation period nothing could be done to prevent this possible issue.

3.2.9. Inverted Microscope

Images of the standards and samples were taken after the incubation period to visually check for contamination and to see which algal species are present in the mixed culture. A Zeiss AxioObserver Z1 inverted microscope (Carl Zeiss, Thornwood, NY) mounted with a camera (Zeiss AxioCam HRc) using 20X 0.4 LD Plan-Neofluar objective was utilized. In order to localize and count algae on a Gridded Sedgewick Rafter 1 mm^2 (Wildlife Supply Company, Yulee, FL), a differential interference contrast (DIC) technique combined with bright field imaging was used. Images were processed with Zeiss AxioVision Rev. 4.8.1 image capture and analysis software (Carl Zeiss, Thornwood, NY).

3.2.10. Statistical Analysis

A one-way Analysis of Variance (ANOVA) test was done using MiniTab software (Version 17.1.0, 2013) to compare the %(BAP/TDP) among the individual algal species and the mixed culture. The Generalized Extreme Studentized Deviate Test, an extension of Grubb's test, was used to determine outliers that exhibited low P uptake by algal species, compared to the average for each algal species. The simpler interquartile range method was used to determine outliers that exhibited high or low algal densities, compared to the average for each algal species.

3.3. Results and Discussion

3.3.1. Standard Synthetic Media Study

Standard synthetic media solutions, for each individual algal species and the mixed culture, at different BAP concentrations of 0.0, 0.1, 0.5, 1.0, 1.5, 2.0, and 5.0 mg P/ L were created for use with the growth curve method. Potassium phosphate was used as the sole source of P in these standard solutions and was assumed to be entirely bioavailable. TP analysis of standard bioassays after the incubation period showed that 99% of the P was taken up by all algal species. Standard growth curves, for each algal species and the mixed culture, were created on three separate dates in order to achieve triplicates. In order to calculate BAP via the growth curve method, trendline equations were taken from the set of curves with the highest R^2 values. Figure 4 displays the trendlines of the standard curves with the highest R^2 along with the slope, y-intercept, and R^2 values for each individual algal species and the mixed culture. The trendline of the mixed culture appears similar to that of *C. reinhardtii*, suggesting that *C. reinhardtii* is the dominant species of the mixed culture. The appendix contains Figure A1 and Table A1 showing the average and standard deviation among each set of standard growth curves produced during this research.

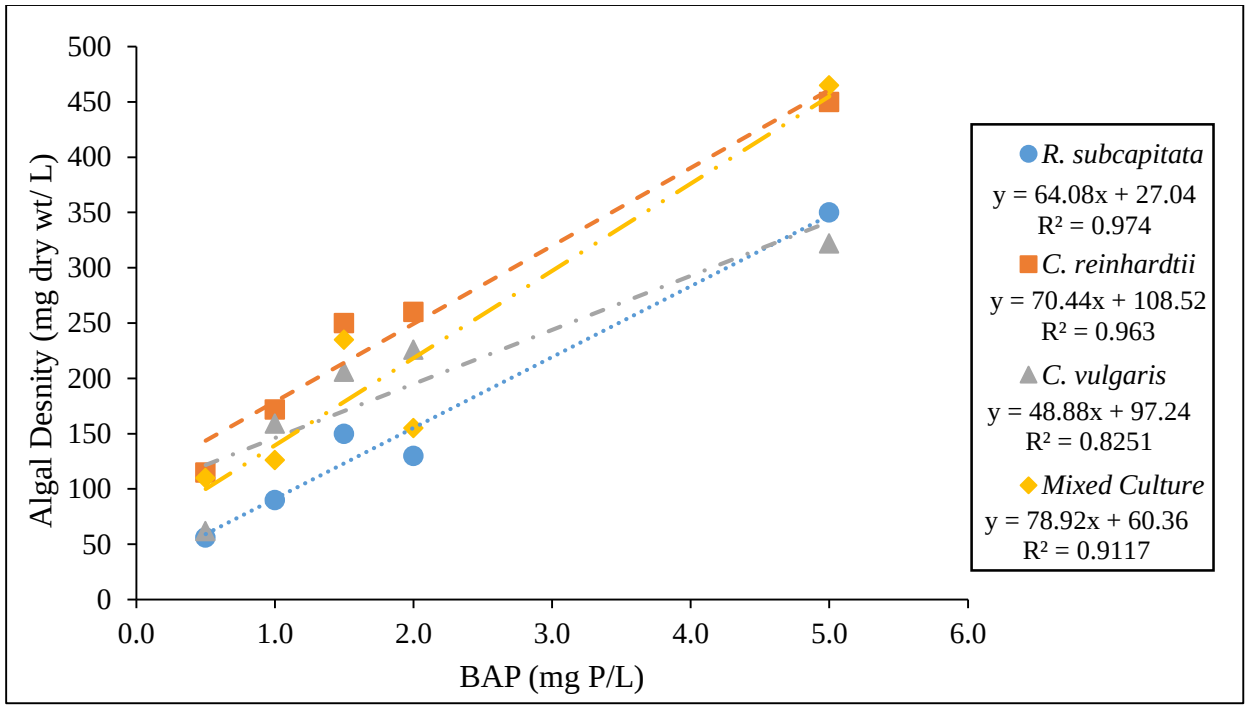

Figure 4. Standard bioassay algal densities, for each individual algal species and the mixed culture, and linear trendlines from 0.5 to 5.0 mg P/L BAP concentrations.

Figure 5 is an image of a mixed culture standard solution, at 1 mg P/ L, taken with an inverted microscope. Images of the mixed culture bioassays, in standards and samples, were used to determine which algal species was dominant. Images show that *C. reinhardtii* is the dominant species, with *R. subcapitata* and *C. vulgaris* making few appearances. This agrees with the standard curves, as seen above, because the mixed culture trendline is most similar to the trendline of *C. reinhardtii*. The standard curves also indicate that *C. reinhardtii* produces the highest algal densities at nearly all substrate concentrations. As seen in Figure 4 and Figure A2, found in the appendix, are instances where the mixed culture, dominated by *C. reinhardtii*, produced higher algal densities than *C. reinhardtii* as an individual algal species, further confirming that *C. reinhardtii* is the dominant species in the mixed culture.

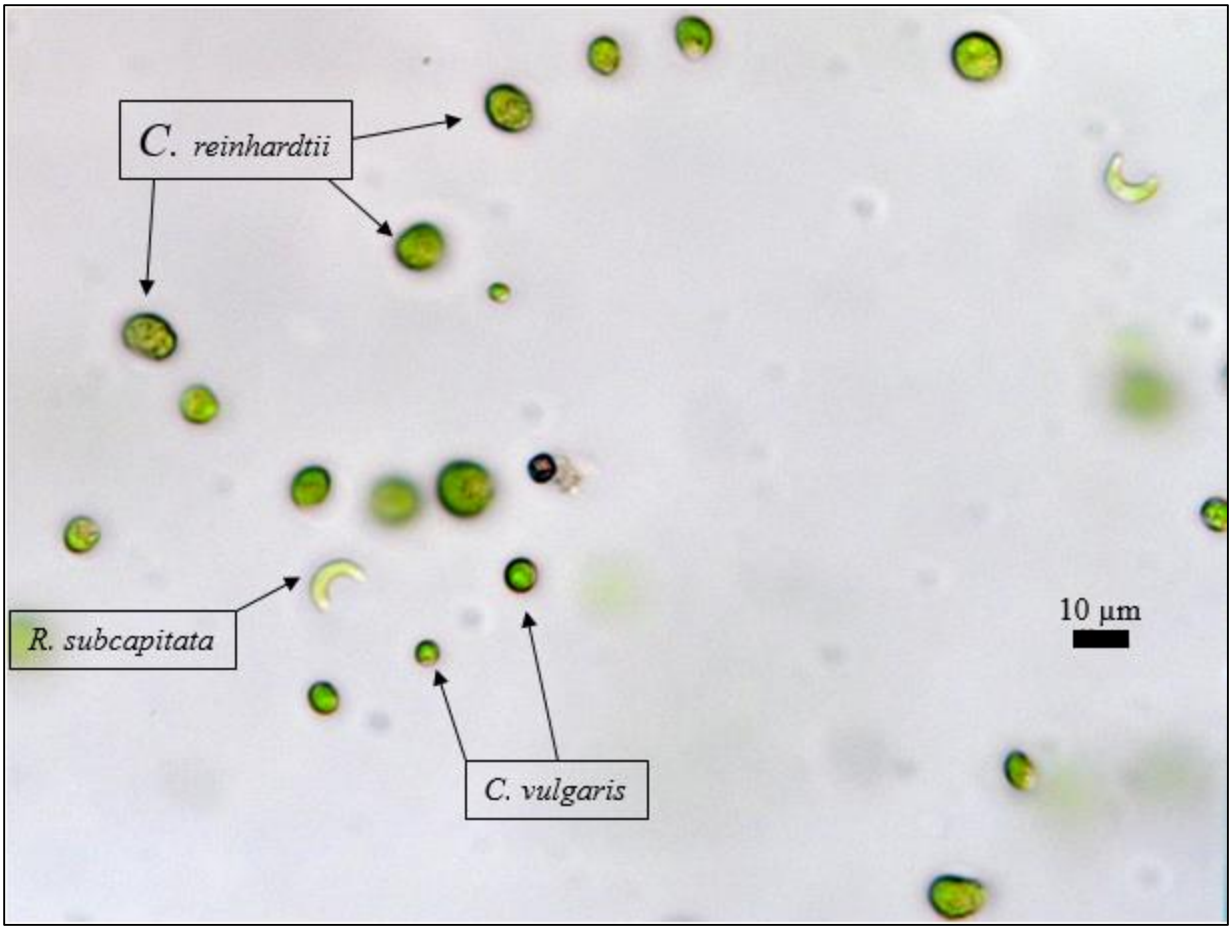

Figure 5. Microscopic image of algae from a standard bioassay of the mixed culture.

3.3.2. Bioavailable Phosphorus Estimates via Different Methods

BAP was estimated via two methods 1) standard growth curves, shown in Figure 4, and 2) P-analysis by taking the difference in TDP prior to and after the incubation period. The first method, via standard growth curves, has great variability in algal densities for each algal species and the mixed culture. This method nearly always reported BAP concentrations greater than the initial TDP of the incubating samples (which is not possible), sometimes the method provided %(BAP/TDP) values below 50% due to low algal densities of samples, and rarely produced result with %(BAP/TDP) values that seem reasonable (> 80%) (Ekholm and Krogerus, 1998; Li and Brett, 2011) for secondary effluents without nutrient removing processes. The inaccuracy of

this method is due to the variability of algal densities produced among and between sample and standard bioassays. The second method, BAP determined via P-analysis, provided more realistic and consistent BAP estimates that were below the initial TDP concentration. Following these results, BAP estimates taken via the P-analysis were determined to be the more accurate and reliable representation of the true BAP concentrations.

It should be noted that the growth curve method is reliable for solutions with low TP concentrations. Li and Brett used this method from 0 to 50 µP/L (Li and Brett, 2011). The reason the growth curve method is appropriate for low TP concentrations is because the minimum detection limit of P (0.01 mg P/L) is approached and the relative error is approximately the same value or more (APHA et al., 2005). Therefore, P-analysis is not sensitive enough at these low P levels while the biomass produced can be measured more reliably.

The algal densities of standard bioassays, at a concentration of 1 mg P/L BAP, and wastewater sample bioassays are compared below in Figure 6. The wastewater sample bioassays nearly always produced a higher algal density, for the respective algal species, than the standard bioassays. On rare occasions, the wastewater sample bioassays produced abnormally low algal densities and thereby increased the variation seen in sample bioassays. These unusually low algal densities in wastewater sample bioassays may have resulted from inhibitory compounds present in the wastewater effluent. It was expected that the standard synthetic media bioassays would produce higher algal densities, because wastewater can contain compounds that inhibit the growth of microorganisms. However, this research resulted in the wastewater sample bioassays producing larger amounts of algal biomass than the standard synthetic media bioassays. A

correlation plot of %(BAP/TDP) via each method is shown in Figure 7. No correlation was found between the results provided by the two methods having a R^2 value of 0.08.

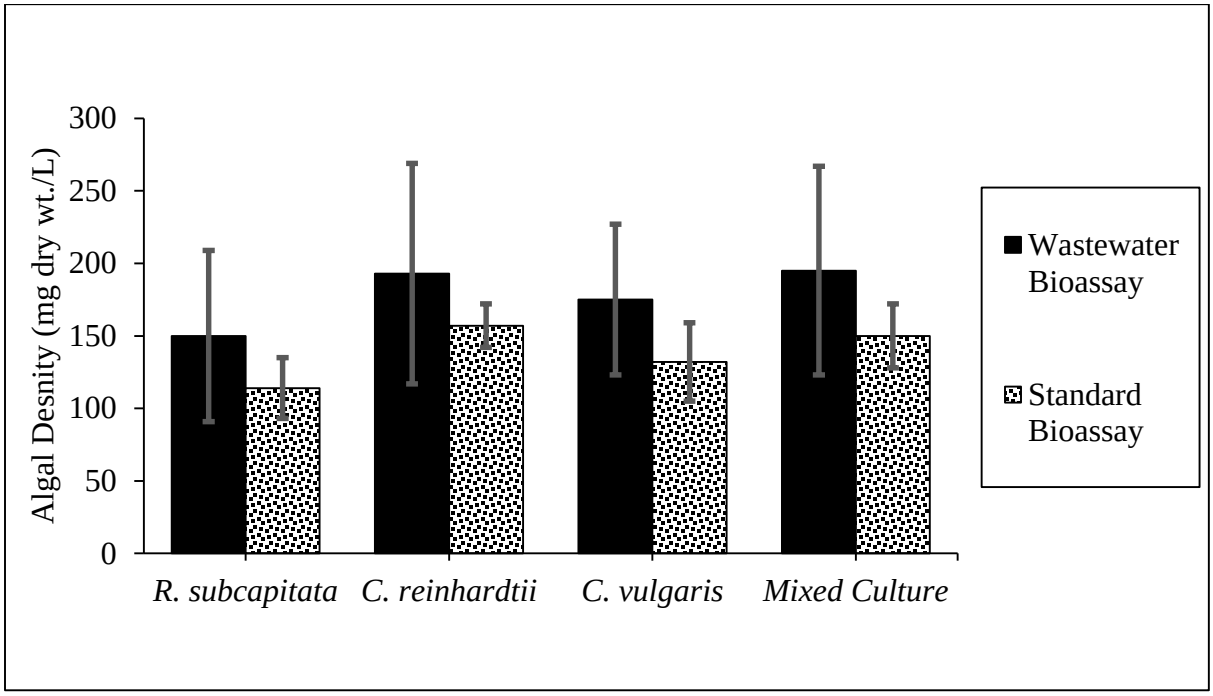

Figure 6. Average algal densities and standard deviations for standard and wastewater sample bioassays of solutions containing TDP of 1 mg P/L.

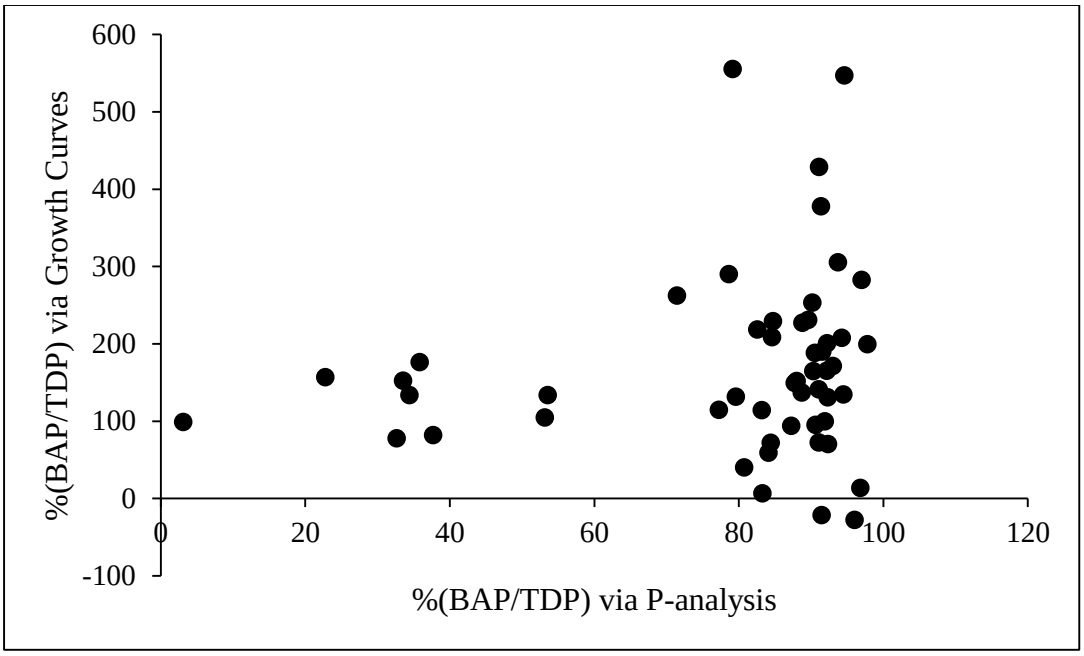

Figure 7. A correlation plot of wastewater bioassay %(BAP/TDP) as calculated via both BAP determination methods.

Nutrient concentrations in the standard and wastewater sample bioassays are compared to determine a possible reason for the wastewater samples producing higher algal densities. Below in Table 4 is a list of major elemental composition of the secondary effluent from the City of Fargo WWTP. The secondary effluent concentrations are displayed in the first column. The second column displays those effluent concentrations as found in a wastewater sample bioassay. The third column represents the concentration of nutrients in a standard solution bioassay. While the concentrations of boron and sodium were similar, the concentrations of calcium, magnesium, potassium, iron, and sulfur were greater in the sample than the standard bioassays. Because the wastewater bioassays typically had larger algal densities, this suggests that there was a micronutrient in the wastewater that was promoting algal growth. This micronutrient was either not present in the P-free media or had a smaller concentration in the standard bioassay than the wastewater bioassay. Following N and P, the next most common limiting nutrients are potassium and sulfur, which had a 76% and 250% higher concentration respectively in the wastewater

bioassays than the standard bioassays. Either of these nutrients could have further stimulated algal growth in the wastewater bioassays by being co-limiting with P.

Table 4. Concentrations of common elements in the Fargo wastewater secondary effluent, that effluent as if it were a bioassay wastewater sample (diluted and mixed with P-free media), and a standard synthetic media bioassay at 1 mg P/L.

Nutrient (mg/L)	**Secondary Effluent**	**Wastewater Bioassay (Secondary Effluent + P-free Media)**	**Standard Bioassay (at 1 mg P/L)**
Boron	0.33	0.23	0.20
Calcium	93.5	25.6	7.4
Magnesium	53	26	18
Sodium	221	288	297
Potassium	24.6	9.5	5.4
Iron	1.12	0.40	0.20
Manganese	0.03	0.588	0.714
Sulfur	153	42	12
Nitrogen	22.9	26.0	26.0
Phosphorus	4.8	1.0	1.0

C. reinhardtii by itself and in the mixed culture were prone to enter a palmelloid stage, where these cells formed colonies surrounded by a mucus layer and produced spores that would await more favorable environmental conditions for reproduction (Ratcliff et al., 2013). This occurred to *C. reinhardtii* as they reacted to stressful conditions such as organic acids, calcium deficiencies, chelating agents, or high phosphate concentrations (Ratcliff et al., 2013). An image

of a mixed culture bioassay that entered a palmelloid stage can be seen in the appendix in Figure A3.

Some of the wastewater samples and some of the standards with *C. reinhardtii* and the mixed culture experienced palmelloid stages; however, the standards were affected to a lesser degree as observed by images from the inverted microscope and by visual observation noting that the standard bioassays did not turn as pale in color as wastewater sample bioassays. The palmelloid stage was not observed in the standard samples with 5 mg P/L (the highest concentration studied) proving that high phosphate concentrations are not the stressor. Calcium to P ratios were maintained in all bioassays (in those that experienced a palmelloid stage and those that did not) and therefore calcium deficiency is not the cause of the stressor either. Also, the P-free media supplies an excess of all nutrients except P.

Ethylenediaminetetraacetic acid (EDTA), as a chelating agent, and organic acids are the other suggested stressors of *C. reinhardtii*. The EDTA concentration in the standard bioassays, at 1 mg P/L, is 938 µg/L. EDTA concentrations in the wastewater sample bioassays are not accurately known as EDTA was added in the wastewater sample and wastewater is known to contain EDTA. However, research shows that EDTA in municipal wastewater is often not removed and its concentration varies from 30 to 640 µg/ L (Alder et al., 1990; Xie et al., 2010; European Commission, 2004). Based on this concentration range the EDTA concentration in the wastewater bioassays in this research, after dilution and adding the P-free nutrient media supplement, could be as high as 944 to 1071 µg/L, which are not substantially different than those in the standard bioassays. While some studies suggest that concentrations above 1 mg/L of EDTA can interfere with biological processes (Christison et al., 2011) placing the wastewater bioassays around the limit for biological interference, while the standard bioassays at 5 mg P/L

contained EDTA concentrations approximately five times this level and did not experience a palmelloid stage. Therefore, EDTA is not the stressor either. This leaves organic acids as the possible stressor.

The secondary effluent might contribute organic acids to the wastewater bioassays. The amount of organic acids in the wastewater bioassays is not known. Furthermore, organic acids were not added to the standard bioassays; therefore, organic acids were not the cause of the palmelloid stage in the standard bioassays. Further research is needed to determine what is causing the palmelloid stage to occur in the bioassays with *C. reinhardtii*.

The palmelloid stage resulted in some *C. reinhardtii* and mixed culture bioassays taking up low amounts of P, suggesting that the bioassay failed due to stressful conditions. These samples of low P uptake were subject to the Generalized Extreme Studentized Deviate Test to determine whether these samples should be evaluated with the other data points that had a more consistent, typical %(BAP/TDP) of 80% or more. Results show that the low P uptake bioassays are considered outliers for *C. reinhardtii* and the mixed culture data sets and were not considered for further analysis. The Generalized Extreme Studentized Deviate Test results can be seen in the appendix in Tables A2 and A3 for *C. reinhardtii* and the mixed culture bioassays, respectively.

3.3.3. Bioavailable Phosphorus via Different Algal Species

BAP estimates for the wastewater sample bioassays were calculated via the two methods and reported as %(BAP/TDP) are listed below in Table 5. The %(BAP/TDP) was chosen over BAP concentration in order to normalize BAP between bioassay sets of different dates, as not all bioassays were diluted to exactly 1 mg P/L. Wastewater bioassays were diluted to 1 mg P/L due to limitations of the P-free media concentration and volume, where higher P concentrations in the wastewater sample bioassays would require proportionately higher P-free media

concentrations or volumes. However, if the concentrations of bicarbonate in the composition of the P-free media became too high, the bicarbonate would not completely re-dissolve after the sterilization process (prior to mixing with the bioassays), thereby limiting the concentration of the P-free media. This limitation limited the P concentration in the wastewater sample bioassays.

As previously stated, the estimates via the standard curves are not reliable due to the wastewater sample bioassays typically producing a higher algal density than the standard bioassays. This results in BAP estimates greater than the initial TDP of the bioassays which is not possible. However, the estimates via the P-analysis method prove to be more reliable since they were less than the initial TDP of the bioassays and were roughly 80 to 90% as expected of secondary effluents without nutrient removal processes.

Table 5. Average and standard deviation of wastewater bioassay %(BAP/TDP) as calculated via the P-analysis and growth curve BAP determination methods.

Algal Species	**%(BAP/TDP) via P-analysis**	**%(BAP/TDP) via Growth curves**
R. subcapitata	91 ±4	194 ±91
C. reinhardtii	83 ±8	110 ±105
C. vulgaris	90 ±5	146 ±111
Mixed Culture	87 ±7	157 ± 89

In this study, BAP via the P-analysis was considered to be the accurate representation of the BAP. The average %(BAP/TDP) and the standard deviation for each individual algal species and the mixed culture are presented in Figure A4 in the appendix. The average of %(BAP/TDP) of *R. subcapitata* and *C. vulgaris* are nearly identical, while that of the mixed culture is slightly lower and that of *C. reinhardtii* is slightly lower still. The lower %(BAP/TDP) values of *C. reinhardtii* and the mixed culture are likely due to a stressor, leading to the palmelloid stage, and therefore preventing *C. reinhardtii* from uptaking as much P as the other species. However, when considering the standard deviation, all algal species fall with in a similar range of %(BAP/TDP). A one-way ANOVA test indicates that there is no significant difference between %(BAP/TDP) among different algal species [$F(3, 41) = 2.12$, $P = 0.113$] at a 95% confidence interval. The (3, 41) represents 3 degrees of freedom between groups (algal species), 41 degrees of freedom within groups (wastewater sample bioassays). The F value of 2.12 indicates that there is a slight variation in %(BAP/TDP) among the algal species tested, as an F value of 1 represents that the averages among groups are the same. However, the P value of 0.113 indicates that there was no significant difference in %(BAP/TDP) among the different algal species tested.

These findings suggest that any of the algal species tested in this study may be used with the bioassay method to achieve relatively the same BAP concentration from a wastewater sample. However, it is suggested that *R. subcapitata* remain the standard algal species for the bioassay method, because this algal species provides the highest BAP concentration and would therefore provide the most conservative BAP estimate for monitoring and regulative purposes. In addition, the species has been used as in numerous studies for several decades and most of the existing studies also use this species.

A normalized BAP (mg P/g dry wt) ratio is calculated by dividing the BAP concentration by the respective algal density, to compare the amount of BAP used per mass of algae. This represents the amount of P (mg P) needed to produce 1 g, of dry weight, for each algal species. By taking the inverse of this value multiplied by 1000 results in the biomass (g) produced per g of BAP. This may be used to predict the amount of algae that would be produced from a water or wastewater solution with a known BAP concentration. This could then be used to roughly predict the extent of a green algal species bloom in a given water body. These normalized values are presented in Table 6. An additional column is added to this table that excludes outliers in order to represent a more consistent value that was observed. Outliers were samples that exhibited abnormally high or low algal densities during this study and were determined through the Interquartile Range Method.

Table 6. Average and standard deviations for normalized BAP ratio (mg P/g dry wt.), from wastewater bioassays, for each algal species and the mixed culture.

Algal Species	**Normalized BAP (mg P/g dry wt)**	
	All Samples	**Select Samples (omitting outliers)**
R. subcapitata	7.34 ± 3.44	5.53 ± 0.79
C. reinhardtii	5.45 ± 2.94	4.01 ± 0.47
C. vulgaris	5.76 ± 2.02	5.50 ± 0.88
Mixed Culture	5.71 ± 3.67	4.73 ± 1.00

An algal growth test was conducted to ensure P-limiting conditions were met, since the wastewater bioassays produced higher algal densities than the standard bioassays and the comparison of nutrients in Table 4 suggests that other nutrients may have been co-limiting with P. If P-limiting conditions exist, than increasing the P content should result in an increase in the algal density. For this experiment, bioassays were created that had P-free media concentrations previously used with the 0.1 mg P/L standard bioassay, while the P concentration was increased to 1 mg P/L. Table 7 below shows that the algal growth bioassays had higher algal densities which were above the standard deviation of the standard bioassays (from the growth curves) for each respective algal species, therefore showing that P-limiting conditions did exist. Interestingly the algal densities produced by the algal growth test bioassay are similar to the values predicted by the normalized BAP ratio for a bioassay with a TP concentration of 0.1 mg P/L. The normalized BAP ratio predicts an algal density of 18.1, 24.9, and 18.2 mg dry wt/L for *R. subcapitata*, *C. reinhardtii*, and *C. vulgaris*, respectively; by taking the inverse of the

normalized BAP ratio and multiplying by the standard bioassay concentration of 0.1 mg P/L. For example, dividing the standard bioassay P concentration of 0.1 mg P/L by the normalized BAP value for *R. subcapitata* of 5.53 mg P/g dry wt and multiplying by 1000 mg/g yields 18.1 mg dry wt/L. This seems to indicate that the 0.1 mg P/L standard bioassay did not achieve the maximum standing crop. Also it is the 0.1 mg P/L standard bioassay that is not linear (compared to the bioassays from 0.5 mg P/L to 5.0 mg P/L) with the other standard bioassays as seen in the appendix in Figure A1. This supports that the maximum standing crop may not have been achieved for the 0.1 mg P/L bioassay as the others are linear. These results show that P-limiting conditions did exist in the bioassays as an increase in algal densities was observed by increasing the P content relative to the other nutrient concentrations.

Table 7. Algal densities for standard bioassays and algal growth test bioassays.

Bioassay	**N Content (mg/L)**	**P Content (mg/L)**	**Algal Density (mg dry wt/L)**		
			R. subcapitata	***C. reinhardtii***	***C. vulgaris***
Standard Bioassay	2.6	0.1	3.6 ±11	4.6 ±13	3.3 ±3
Algal Growth Test Bioassay	2.6	1.0	18.0	24.0	20.6

3.4. Summary

With the implementation of future P effluent limits on WWTPs, TP and BAP will be important management tools throughout the treatment process, from the influent to the effluent. This study shows that TDP is a more reliable parameter than algal density (via growth curve) for determining BAP in secondary effluents without nutrient removing processes. The study further

shows that there is no significant difference in %(BAP/TDP) when measured from different green algal species, as individual species or a mixed culture. BAP may have been similar between these species because the 14 day incubation period provides sufficient time for all species to uptake the maximum or near maximum amount of P possible. Other green algal species may be utilized to determine BAP concentrations; however, use of *C. reinhardtii* runs the risk of entering a palmelloid stage that may affect its ability to uptake P. Also, it is recommended that *R. subcapitata* remains the standard species as it provides the highest BAP concentration and would therefore provide the most conservative value for monitoring and regulative purposes. While %(BAP/TDP) over the incubation period is not significantly different, the amount of P needed per gram of algal biomass for each species differs, as suggested by the growth curves and the normalized BAP ratio. This may affect eutrophication and the size of the floating algal-mats in water bodies depending on the dominant algal species and P levels that are present. The normalized BAP ratio may be used to predict the amount of green algae that would be produced from secondary effluent with a known BAP concentration and in turn potential contribution to eutrophication in receiving water bodies by the effluent.

4. BIOAVAILABILITY OF PHOSPHORUS SPECIES IN SECONDARY EFFLUENTS

4.1. Introduction

Eutrophication is the accumulation of nutrients, particularly N and P, in water bodies that promote algal blooms. Nationwide P and N effluent limits for WWTPs are expected to be implemented in the near future. Managing P in effluents and water bodies is a more effective practice because there are phytoplankton that are capable of fixing N from the atmosphere (Schindler et al., 2008) and would circumvent N management practices. While current WWTP regulations are based on TP, studies have shown that only a portion of TP is bioavailable to support the growth of algae (Gu et al., 2011).

There are a few P-species that compose TP. P-speciation can be defined via reactivity and through filtration. Filtration separates particulate P from soluble P or TDP and reactivity distinguishes reactive P from non-reactive P; defining P as SRP and SNRP for soluble P, and particulate reactive P and particulate non-reactive P for particulate P. Studies often overlook a test that can further distinguish SNRP into SAHP and DOP via acid hydrolysis and persulfate digestion steps (APHA et al., 2005). This study focuses on soluble forms of P since particulate P is effectively removed from wastewaters by current treatment technologies.

According to Neethling et al. (2007), it is possible that due to the acidic nature of the ascorbic acid method, some SAHP may hydrolyze during the ascorbic acid test and alias as SRP. Likewise, it is possible that DOP may hydrolyze during the acid hydrolysis pretreatment and alias as SAHP during the analysis. This may result in erroneous P-analysis, but is unavoidable due to the acidic nature of the tests.

In the past BAP was believed to be approximated by SRP or orthophosphates. This is because algae, as primary consumers, were thought to use inorganic minerals only and SRP is the simple inorganic mineral form of P. Studies eventually demonstrated that BAP is often greater than SRP (Ekholm and Krogerus, 1998; Li and Brett, 2013) proving that other P-species were contributing to the BAP concentration. Other studies have since considered BAP as the sum of various P-species. Some studies describe BAP as the sum of SRP, SAHP, and bioavailable DOP; others as the sum of plant available P in soils (Olsen-P), readily desorbable P, and TDP; and others as the sum of TDP and bioavailable PP; (Thien and Myers, 1992; Sharpley, 1993; Oladeji et al., 2008; Nausch and Nausch, 2011).

Li and Brett (2013) investigated how dissolved P form influenced bioavailability. They used various P containing compounds of different P-species categorizes as the sole source of P in their bioassays. They found that P-species did not necessarily correspond to the classical assumption that SRP approximates BAP. They analyzed organic-P compounds that were nearly entirely bioavailable, poly- or condensed-P compounds that had high bioavailability, and compounds (apatite and humic-(Al/Fe)-P) that are classified as SRP, but were barely bioavailable. Their results show that some DOP and SAHP can contribute to BAP, while some SRP forms do not contribute to BAP. They concluded that a portion of SRP in surface waters is likely non-BAP due to P bound to humic-metal compounds that are not released by phosphatase enzymes and that these compounds are too large to cross cell membranes.

Currently there is limited research on BAP and P-speciation in comparison to N bioavailability and N-speciation, especially in wastewater effluents. This may be due to the fact that P is found in smaller concentrations than N and achieving increasing low concentrations through treatment is difficult and costly, thereby increasing research focus on N.

The work presented in this chapter investigates the contributions of P-species to BAP and how P-speciation is altered by algae. This will identify which P-species in wastewater effluents are contributing to BAP and thereby indicate which P-forms to focus on removing from WWTP effluents in the future. Studies often overlook distinguishing SAHP and DOP when examining wastewaters (Gao et al., 2014; Oladeji et al., 2008; Nausch and Nausch, 2011; Li and Brett 2011, 2012, 2013; Ekholm et al., 1998, 2003, 2009; Saavedra and Delgado, 2006) and this distinction may be an important parameter to use in the estimate of BAP through chemical P-analysis. Acid hydrolysis and persulfate oxidation pretreatments with the ascorbic acid method were used for P-speciation and the algal assay bottle test was used to determine BAP.

4.2. Materials and Methods

4.2.1. Glassware and Standard Chemicals

All glassware was purchased from VWR International Co., PA, USA. Glassware was washed with phosphorus-free Liquinox soap, rinsed with tap water placed in 10% v/v hydrochloric acid (HCl) acid baths overnight, then rinsed with DI water. Potassium phosphate, sodium hexametaphosphate, and adenosine monophosphate (AMP) were standards for P analysis and were purchased from VWR International Co., PA, USA. Nutrients for algal media were purchased from VWR International Co., PA, USA. Sulfuric acid, antimony potassium tartrate, ammonium molybdate, and ascorbic acid were purchased from VWR International Co., PA, USA.

4.2.2. Wastewater Samples

Secondary effluent wastewater samples were collected from the City of Fargo WWTP (Fargo, ND, USA) and the City of Moorhead WWTP (Moorhead, MN, USA). The Fargo WWTP has a peak flow capacity of 29 MGD and an average of 15 MGD. The Moorhead WWTP has a

peak flow capacity of 18 MGD and an average of 6 MGD. These facilities are not subject to total N or total P limits, but they are subject to ammonia limits. The Fargo WWTP treats wastewater for BOD and ammonia through a two-stage trickling filter process. The Moorhead WWTP treats wastewater for BOD and ammonia through high purity oxygen activated sludge (HPOAS) and moving bed biofilm reactors (MBBR).

Seven grab samples were collected from the Fargo WWTP between February and May 2015, while three grab samples were collected from the Moorhead WWTP between August 2014 and July 2015. All samples were collected from points prior to chlorination. All Fargo samples were collected after the two-stage trickling processes. Two of the Moorhead samples were collected from the activated sludge effluent and one from the MBBR effluent.

4.2.3. Phosphorus Analysis

P analysis was conducted prior to and after the incubation period in order to determine the P composition in the bioassays at the beginning and end of the incubation period. The wastewater samples were filtered through a 0.45 μm pore size filter (Sterile MCE Membrane Filter, Advantec MFS) to separate particulate matter from the substrate. The filtrates were then analyzed to categorize their P concentrations into SRP, SAHP, and DOP categories. Since P levels are greater than the detection limit of the ascorbic acid method, a series of dilutions were used to determine the P concentration of each P-species for all grab samples. A branch diagram of the P-species categorization methods can be found in Section 2.2 in Figure 3.

4.2.3.1. Soluble Reactive Phosphorus

The sample was analyzed via the ascorbic acid method (APHA et al., 2005) with a 10 mm light path to determine the SRP concentration of the sample. This method measures P between 0.01 and 1.30 mg P/L. Standard calibration curves, of known potassium phosphate

concentrations versus absorbance (R^2 = 0.9885), were successfully created in order to determine the sample SRP concentration. The calibration curve experiment was conducted in triplicate and repeated on separate occasions to ensure accuracy and reliability.

4.2.3.2. Soluble Acid-Hydrolysable Phosphorus

A preliminary acid hydrolysis and autoclaving step was used prior to the ascorbic acid method as described in the Standard Methods (APHA et al., 2005) to determine the combined SRP and SAHP concentration. This measures P between 0.01 and 1.30 mg P/L. The SAHP concentration is then determined by subtracting the SRP concentration (see Equation 4 in the appendix). Sodium hexametaphosphate was used to create the combined SRP and SAHP standard calibration curves (R^2 = 0.9871). The calibration curve experiment was performed in triplicate and repeated similar to the SRP calibration curves.

4.2.3.3. Dissolved Organic Phosphorus

The sample was subject to a preliminary persulfate digestion and autoclaving step prior to the ascorbic acid method as described in the Standard Methods (APHA et al., 2005). This procedure provides the TDP concentration and measures P between 0.02 and 2.60 mg P/L. DOP is then determined by subtracting the combined SRP and SAHP concentration from the TDP concentration (see equation 5 in the appendix). TDP standard calibration curves were created from known concentrations of AMP (R^2 = 0.9893). The calibration curve experiment was conducted in triplicate and repeated on separate occasions to ensure accuracy and reliability.

4.2.4. Nitrogen Analysis

Nitrogen analysis was conducted in the same manner as found in Section 3.2.4.

4.2.5. Stock Algae

This study used the standard algal strain *R. subcapitata* UTEX 1237 which was purchased from UTEX Culture Collection of Algae, TX, USA. Stock algae was maintained biweekly with the media solution of the Printz Algal Assay Bottle Test (Miller et al., 1978).

4.2.6. Algal Assay Bottle Test

The Printz Algal Assay Bottle Test, developed by the EPA (Miller et al., 1978; Li and Brett, 2011), is a procedure to test the effect of nutrients on algal growth using the algal species *R. subcapitata*. This test is used to measure BAP as the samples were placed in P-limiting conditions. The specific environmental conditions for the algae to grow, in this procedure, are described below.

4.2.6.1. Nutrients

The nutrient media was the same as that described in Section 3.2.6.1.

4.2.6.2. Algal Inocula

The algal inocula were prepared following the same procedure found in Section 3.2.6.2.

4.2.6.3. Incubation Setup and Conditions

The environmental conditions were the same as those described in Section 3.2.6.3.

4.2.7. Initial Algal Concentration via Particle Counter

The initial algal count to seed the bioassays was conducted in the same manner as found in Section 3.2.7.1.

4.2.8. Bioavailable Phosphorus Estimation

BAP is determined via chemical analysis of the TDP measured prior to and after the incubation period. This was determined to be the most reliable method for the P concentrations

used in these studies as described in Section 3.3.2. BAP is the difference between the TDP before and after the incubation period.

4.2.9. Sterilization

The same sterilization procedures as found in Section 3.2.10 were used.

4.2.10. Statistical Analysis

Regression analysis was conducted using MiniTab software (Version 17.1.0, 2013) in order to determine if a relation between any of the P-species or combination thereof could be used to predict the BAP concentration of samples. One-way ANOVA tests was conducted using MiniTab software (Version 17.1.0, 2013) to compare the respective P-species concentrations before and after the incubation period as well as to compare the P-species between the samples from the two WWTPs.

4.3. Results and Discussion

4.3.1. Phosphorus Speciation of Wastewater Samples

Seven (7) and three (3) grab samples were collected from the Fargo WWTP and the Moorhead WWTP, respectively, between June 2014 and June 2015. These samples were filtered and analyzed for P-speciation via the ascorbic acid method and pretreatment with acid hydrolysis and persulfate digestion in order to determine the SRP, SAHP plus SRP, and TDP concentrations respectively. By taking the difference among these values the SAHP and DOP concentrations were determined. In order to determine these P concentrations, the grab samples were subject to series of dilutions: 40, 50, 60, 70, and 80% for SRP; 50, 60, 70, 80, and 90% for SAHP plus SRP; and 70, 75, 80, 85, and 90% for TDP. These dilutions were to ensure that the P concentrations of the samples were brought into the detection limit of their respective P analysis methods.

P-speciation results show that SRP was the predominant form of the TDP effluent, with all but one sample being greater than 85% SRP/TPD. The concentration ranges among both WWTPs were 1.89 to 5.79 mg P/L for TDP, 1.86 to 4.45 mg P/L for SRP, 0.03 to 0.46 mg P/L for SAHP, and <0.01 to 0.49 mg P/L for DOP. These results corroborate with previous studies (Ekholm et al., 2009; Li and Brett, 2013) that conventional secondary effluents are primarily composed of SRP. Table A5 in the appendix displays the concentrations of each P-species and the TDP concentration for all wastewater samples analyzed for this research. Table A6 in the appendix shows each P-species as a percentage of TDP.

A blank and two controls were used during the P-speciation experiments to verify the reliability of the tests. The blank represents a P concentration of zero, was used to zero the spectrophotometer, and was used to verify that P contamination did not occur during the analysis procedures. Additionally two controls of known concentration were used for SRP, SAHP, and DOP analyses each. P-species controls were composed of known concentrations of potassium phosphate, sodium hexametaphosphate, and AMP as the P sources for SRP, SAHP, and DOP respectively. Results show that R^2 value and normalized root mean square error (RMSE) for P-species controls calculated from all experiment sets and using multiple concentrations, were 0.761 and 0.19 for SRP, 0.839 and 0.15 for SAHP+SRP, and 0.607 and 0.22 for TDP. Sodium nitrate was used as a N standard control. TDN controls had a R^2 value of 0.920 and a normalized RMSE of 0.14. These R^2 and RMSE values indicate that the P-analysis and N-analysis of controls and therefore the wastewater samples are acceptable, but potentially subject to an error of approximately 0.15 to 0.22 mg/L for P and 0.14 mg/L for N.

The average and standard deviation of the effluent TDP for the Fargo WWTP (two-stage trickling filter process) and Moorhead WWTP (HPOAS and MBBR processes,) was 4.34 ±0.90

and 2.85 ±0.94 mg/L, respectively. The portion each P-species that made up the effluent TDP is shown in Figure 8. The SRP fraction was higher in the Moorhead WWTP, although one sample from the Fargo WWTP had low %(SRP/TDP) of roughly 63% bringing the average down from 92% to 88.3%. Both WWTPs had approximately a comparable fraction of SAHP in the effluent. The DOP fraction constitutes a larger fraction of the Fargo effluent compared to the Moorhead effluent. This is due to the Fargo effluent %(DOP/TDP) having two samples greater than 10%, one of which was 36%, which increased the average and standard deviation for the Fargo samples. One-way ANOVA tests showed there was no significant difference in P-speciation between the samples from the two WWTPs (ANOVA results are in the appendix in Table A6.

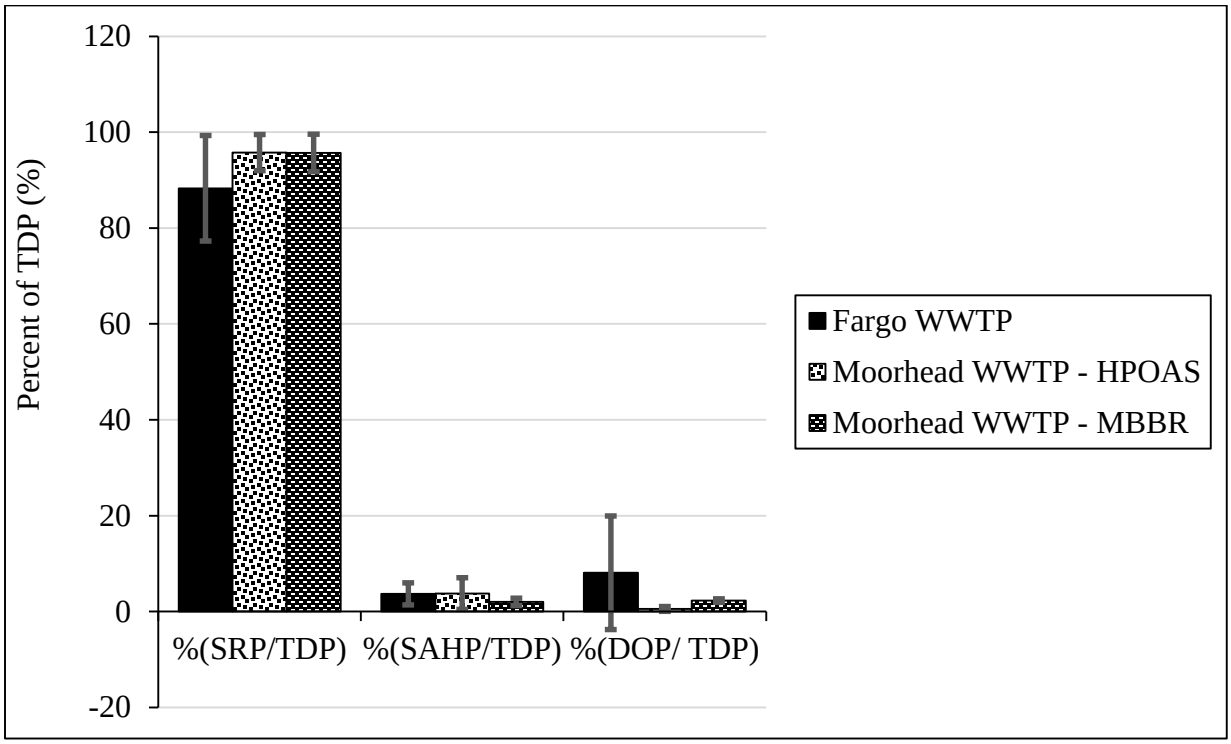

Figure 8. P-species as a percentage of TDP for the secondary effluent samples (before the incubation period for the bioassays).

For both WWTPs, SRP was the greatest portion of effluent TDP; this is due to the nature of the biological treatment in these WWTPs that breaks down organic matter and larger compounds into smaller compounds, thereby breaking down organic P and polyphosphate

compounds to orthophosphate compounds (Metcalf and Eddy, and AECOM, 2014). Based on the effluent P-composition of these WWTPs and current P removal treatment options, chemical, biological, or a combined chemical and biological P removal process could be added to remove much of the remaining SRP, and therefore TDP, in both WWTPs to achieve lower TP and BAP effluent concentrations.

The Fargo two-stage trickling filter and the Moorhead HPOAS processes utilize some P-compounds for microbial growth and metabolism. The Moorhead WWTP has an MBBR system following the HPOAS process that focuses on nitrifying ammonia. The Fargo two-stage trickling filter also incorporates a nitrifying trickling filter. These nitrifying processes do not oxidize P, as they do N, since the P in the wastewater is mostly if not entirely found in a phosphate form; therefore these processes do not affect P-compounds other than by breaking down larger P-compounds into smaller P-compounds (down to orthophosphates) through biodegradation or by uptaking readily available orthophosphates. However during these processes some of the organisms die, decompose, and release DOP. This DOP may be broken down during the process or potentially be washed out with the process effluent.

4.3.2. Phosphorus Speciation Changes during Incubation

During the incubation period, the algae uptake P and the change in concentration for each P-species is shown in Figure 9. P-analysis for all P-species was done in triplicate before and after the incubation period. Results are similar among the WWTP processes examined. SRP, which was the predominant form before incubation, is taken up by algae and composed most of the change in TDP concentrations. Each P-species is shown to decrease over the incubation period, except for some instances where DOP increased, this shows that each P-species is partially bioavailable. On three of the sampling dates for the Fargo WWTP and all three of the

sampling dates for the Moorhead WWTP, the DOP concentration increased during the incubation period, suggesting that algal death and decay released DOP compounds into the bioassay solutions.

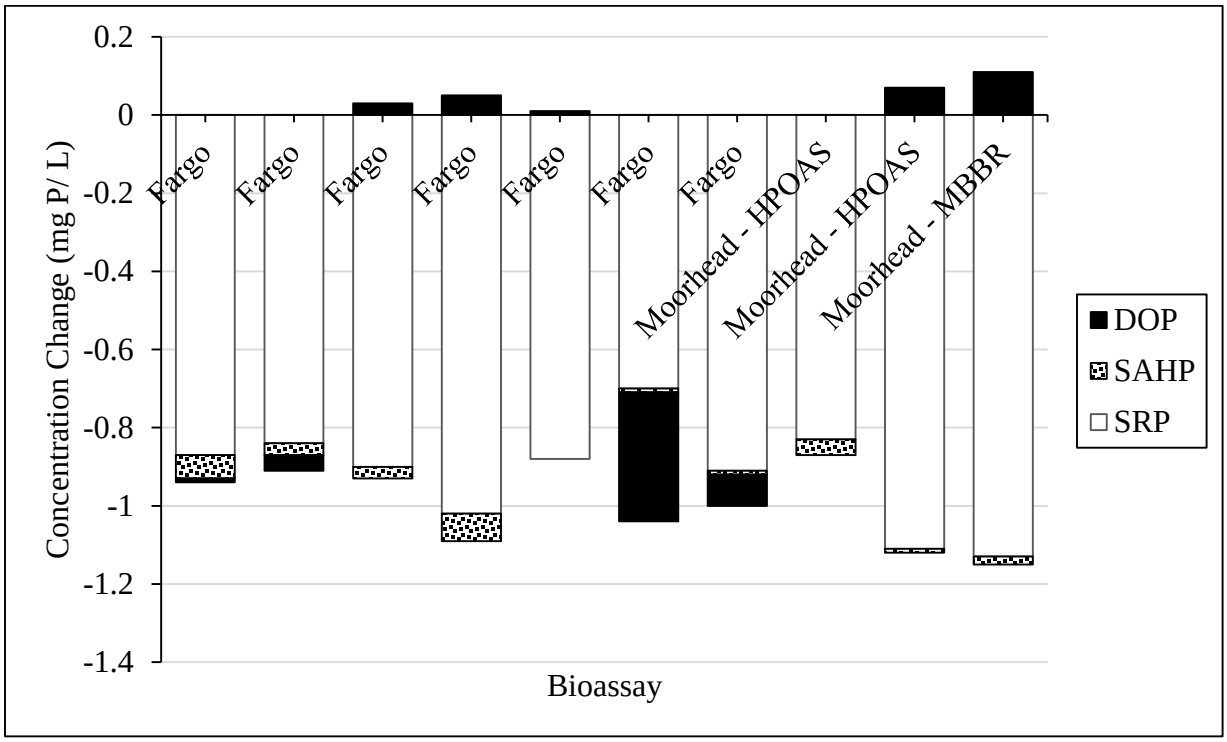

Figure 9. The change in P-species concentrations over the BAP incubation period for the bioassays.

An increase in DOP can only happen through algae decay and release of organic compounds in the bioassays. However, this disrupts subsequent data calculations and therefore for the remainder of this chapter for bioassays where the DOP concentration increased, the final DOP concentration is set to the initial DOP concentration (before the incubation period). Otherwise, negative DOP bioavailability would result from the calculations.

After the incubation period, P-analysis was conducted again in triplicate. Each P-species is displayed in Figure 10 as a percentage of the TDP after the incubation. SRP still composes a large portion of the remaining TDP after the incubation, but to a lesser extent than before the incubation period. After the incubation period, DOP composes the majority of the TDP. The

concentrations of each P-species and TDP after the incubation period can be found in the appendix in Table A5 and the P-species as a percentage of TDP can be found in Table A6. As shown in Figure 10, the average P-speciation of the Fargo WWTP and the Moorhead HPOAS bioassays are nearly identical, while the Moorhead MBBR bioassay has a low %SRP/TDP and high %DOP/TDP after the incubation period. The standard deviations are high due to the low concentrations remaining after the incubation period. It should also be noted that only one sample was collected from the Moorhead MBBR system. While P-speciation after the incubation period appear different between samples from the MBBR system and those from the other processes, one way ANOVA tests indicate that there was no significant difference in P-speciation among samples from all the processes tested, these results can be found in the appendix in Table A7. This increase in proportion of DOP suggests that DOP is potentially recalcitrant to algal uptake. Even in samples where the DOP concentration decreased over the incubation period, the %DOP/TDP for these bioassays after incubation is 55.8%. In samples where the DOP increased the %DOP/TDP is 73.0% on average.

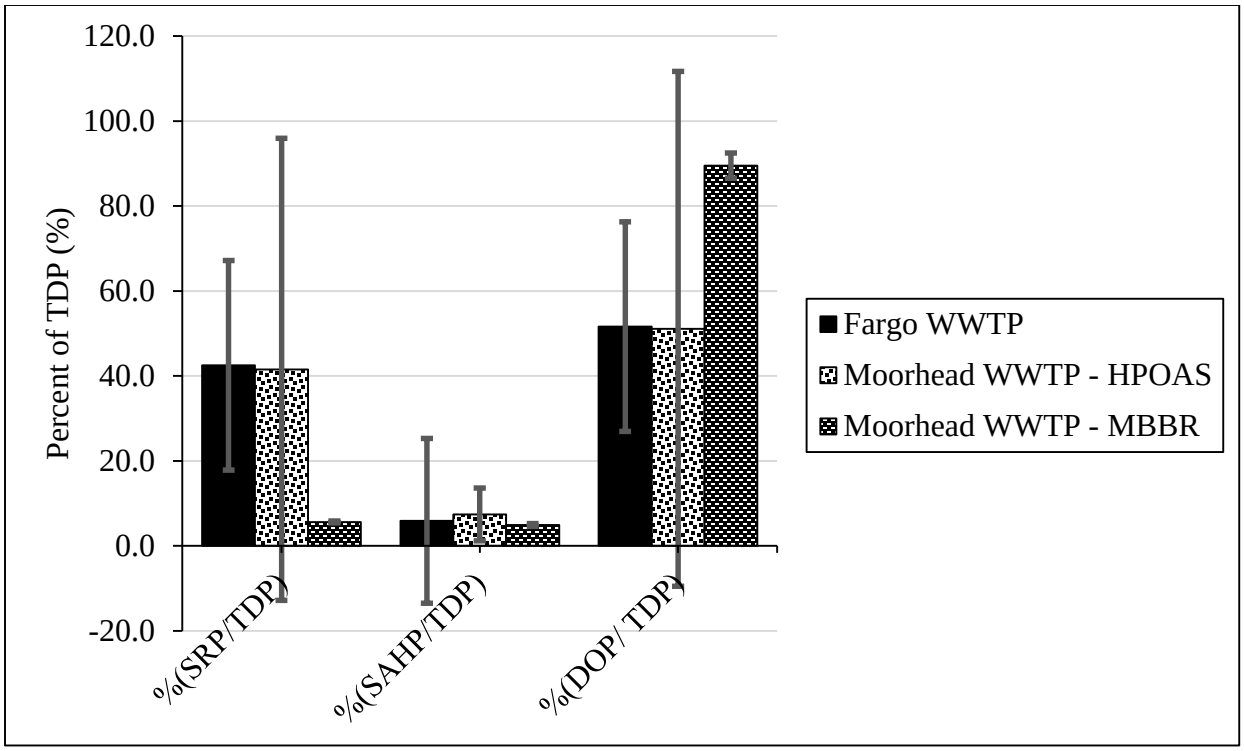

Figure 10. P-species as a percentage of TDP after the incubation period for the bioassays.

As previously stated DOP increased in over half of the bioassays suggesting that algal decay occurs during the incubation period. This helps explain why DOP composes a larger fraction of the TDP after the incubation in some bioassays; algae decay released organic compounds into the bioassay solution and thereby increasing the DOP concentration. Even though the effect of this on the data was minimized, by limiting the post-incubation DOP concentrations to their pre-incubation concentrations, it is possible that some of the initial DOP bioassay concentration was taken up by the algae and replaced by DOP that was later released by the algae. Since the DOP concentration before incubation is such a small portion of the TDP, its effect on BAP is minimal. Furthermore, it is possible that DOP is composed of recalcitrant forms of P that are not taken up by algae or hydrolyzed during the incubation period, as another potential explanation to why DOP composes the largest fraction of TDP after the incubation period. However, as SRP still represented a large portion of the TDP after the incubation period,

this suggests that not all SRP is bioavailable. This could be due to P bound humic-metal-complexes that do not release P to phosphatase enzymes, but do hydrolyze during SRP chemical P-analysis (Li and Brett, 2013).

These results corroborate with previous studies (Gu et al., 2011; Li and Brett, 2011) on tertiary effluents, where an increase in the %DOP/TDP is seen between the WWTP influent and effluent, while the TP concentration decreases. This is because the bioassay procedure mimics P assimilation treatment processes, where P would be taken up by microorganisms and subsequently settled out. Results also corroborate with studies (Li and Brett, 2013; Gu et al., 2011; Ekholm et al., 2009) indicating that for secondary effluents BAP is primarily composed of SRP, but that other P-species are bioavailable as well. This study shows that each P-species is at least partially bioavailable and that some DOP is potentially recalcitrant to algal uptake or its uptake is masked by the release of organic P from the algae.

4.3.3. Bioavailability of P and Contribution of P-species to BAP

The %(BAP/TDP) of the Fargo WWTP and Moorhead WWTP samples were 93.7 ±1.9% and 94.3 ±6.2%, respectively. This demonstrates that there was little difference in %(BAP/TDP) provided by the two treatment facilities. Likewise there was little difference in %(BAP/TDP) between the samples from the two process at the Moorhead WWTPs (HPOAS and MBBR), which were 93.4 ±8.5% and 96.3 ±2.8%, respectively. Table A8 in the appendix displays the average %(BAP/TDP) for each wastewater sample collected. A one way ANOVA test shows that there was no significant difference in %BAP/TDP among the WWTP processes tested, with [$F(2, 7) = 0.249$, $P = 0.786$] at a 95% confidence interval. This is due the similarity in the type of treatment among the processes tested, as each is a biological treatment process that is not

designed to remove P, both are even equipped with a nitrification process. It also shows that these WWTPs are discharging effluents composed of readily available P for algal uptake.

On the 8/27/14, 2/4/15, 2/26/15, and 5/15/15 sampling dates, the difference in TDP before and after the incubation period (which is also the BAP concentration) was greater than the initial SRP concentration indicating that more than SRP must be bioavailable; although they were not statistically different, except for the 2/26/15 sample. All P-species were shown to contribute to BAP as each P-species decreased in most of the bioassays as seen in Figure 9 above.

The proportion of each P-species in relation to BAP is displayed below in Figure 11. These proportions were found by taking the difference in each P-species before and after the incubation period then dividing the difference by the BAP concentration (see equation 6 in the appendix). This shows the composition of BAP. In the case where DOP increased over the incubation period, the difference (before and after the incubation period) was set to zero, otherwise a negative %(DOP/BAP) would be given. These results indicate that SRP, SAHP, and DOP accounted for 90.5%, 3.1%, and 6.5% of the BAP concentration for the samples from the Fargo WWTP, respectively. For the Moorhead HPOAS samples, SRP, SAHP, and DOP made up 96.9%, 3.1%, and 0.0% of the BAP concentration, respectively. For the Moorhead MBBR samples, BAP consisted of 98.6% of SRP, 1.4% of SAHP, and 0.0% of DOP. DOP accounted for 0.0% in the Moorhead samples (because all Moorhead bioassay resulted in an increased DOP concentration after the incubation period) suggesting that association of DOP with BAP in the samples from the Moorhead WWTP is inconclusive. Omitting the Fargo bioassays that saw an increase in the DOP concentration over the incubation period, the %DOP/BAP is 11.3%, this value may more accurately represent the DOP portion in BAP, as this value is the average of

bioassays that had DOP uptake. However the real %DOP/BAP may be even higher if DOP was also released in the bioassays where DOP uptake was recorded, but the algae uptake is greater than the release of DOP.

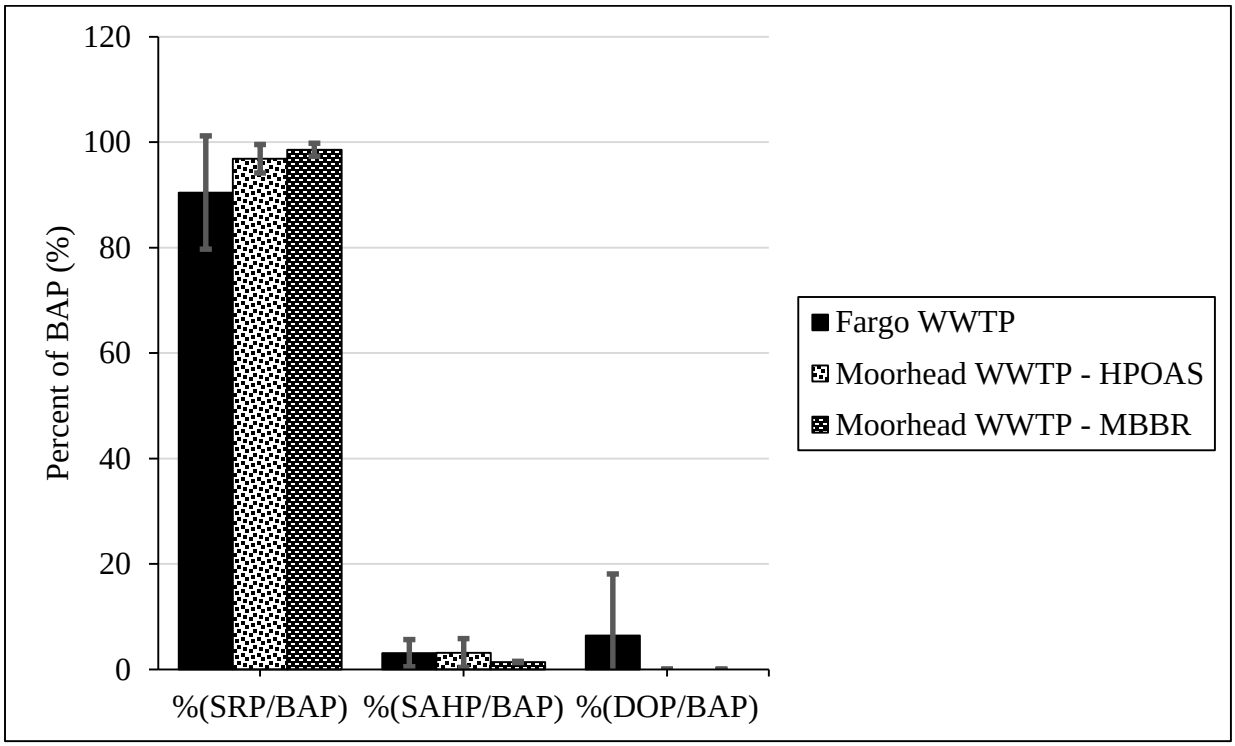

Figure 11. The portion each P-species composed of BAP in the wastewater bioassays.

The bioavailability of each P-species was determined as the difference in the concentrations of each P-species (before and after the incubation period) divided by the initial concentration of each respective P-species (see equation 7 in the appendix). These bioavailability values are shown in Figure 12. For the Fargo WWTP, Moorhead HPOAS, and Moorhead MBBR processes, SRP was almost entirely bioavailable at 96.9%, 93.5%, and 99.2%, SAHP was mostly bioavailable at 75.6%, 78.3%, and 67.5% respectively, and DOP was 33.1%, 0.0%, and 0.0% bioavailable. For the Fargo samples, excluding the bioassays that saw an increase in the DOP concentration, DOP bioavailability was 58.0%. The bioavailability of the SRP and SAHP are nearly the same among the treatment processes and while the Fargo samples

have higher bioavailability in DOP than the Moorhead samples. One way ANOVA tests indicate that there is no significant difference in P-species bioavailability among the treatment processes examined (see detailed results in Table A9 in the appendix). These results indicate that WWTPs should focus on removing SRP and SAHP as they had high bioavailability. While DOP appears to be recalcitrant and non-BAP based on the results, it is still possible that DOP has moderate to high bioavailability that is masked by the uptake and release of DOP by the algae during the bioassay.

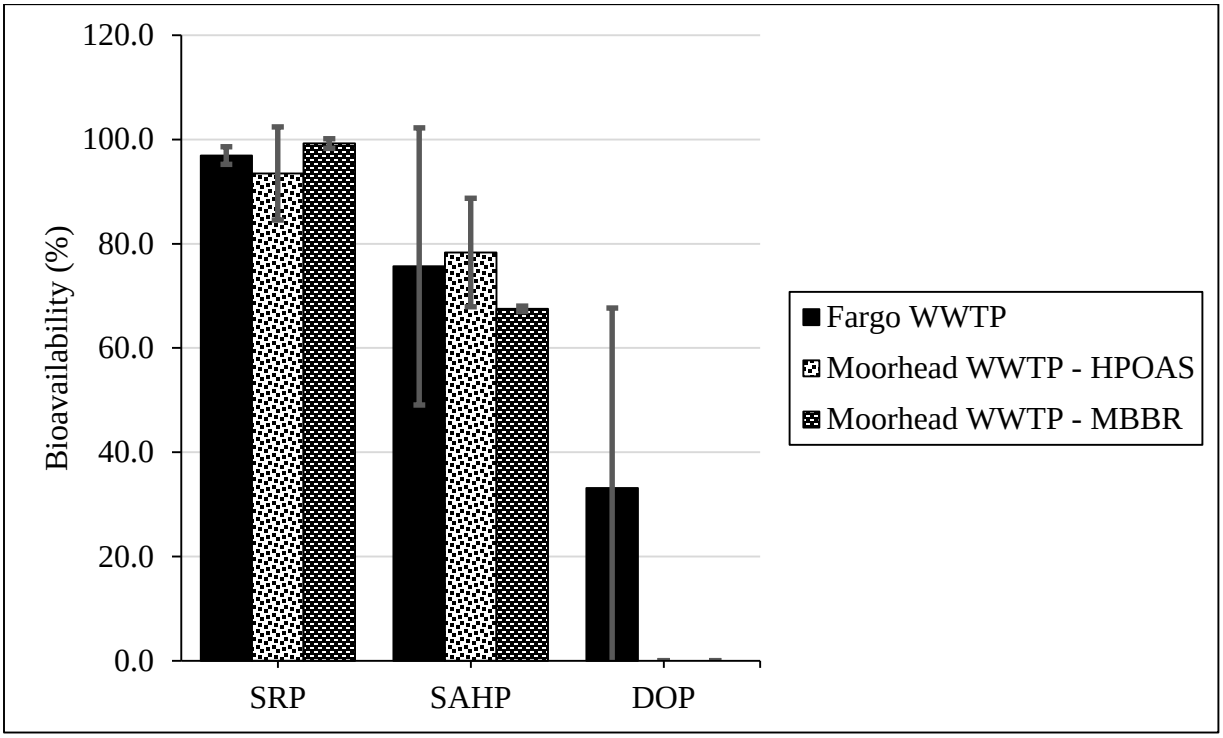

Figure 12. The bioavailability of each P-species.

P-species were also compared to BAP in order to determine if a relation could be made between the chemically analyzed P-species and the BAP concentration. The regression analysis results indicate that no significant relation could be formed between any combinations of P-species concentrations to the BAP concentration, which could have been used as a predictor of BAP concentrations in WWTPs. The regression analysis results can be found in appendix.

While no relation could be formed it is recommended that for WWTP of similar capabilities as those tested in this research, SRP be used as a low BAP estimate and TDP (or even TP) as a high BAP estimate. These are recommended as relatively fast and inexpensive BAP estimates compared to the bioassay procedure.

4.4. Summary

With the future implementation of P-limits on WWTPs, it is important to know which P-species of effluents contribute to BAP and which processes are more effective at reducing BAP in effluents. This research shows that there was no significant difference in P-speciation of the effluent from the Fargo and Moorhead WWTP processes and that their effluent is primarily composed of SRP. This research also shows that all P-species are at least partially bioavailable, since all P-species showed a decrease in concentration over the incubation period, except for some instances with DOP. DOP composed a greater portion of the TDP concentration after the incubation period, indicating that DOP is released by algae over the incubation period (as observed in some bioassays) and possibly that DOP is partially composed of recalcitrant forms of P that are not available to algae and do not hydrolyze during the incubation period. There was no significant different in the %(BAP/TDP) between the treatment processes tested. The bioavailability of each P-species is approximately 95% and 75% for SRP and SAHP for the samples from both WWTP bioassays. Bioavailability of DOP was 33% and 0% for the Fargo and Moorhead WWTP samples, respectively. The accuracy of the bioavailability of DOP is more difficult to determine as DOP is released by algae during the incubation period, thereby underestimating its true bioavailability. Additionally, an AONVA test indicates that no relation could be made between the P-species and BAP concertation.

While a relation between P-species and BAP could not be made, it is still possible to use SRP as the low estimate of BAP in effluents and TDP or TP as the maximum estimate of BAP. This allows for a faster and more cost effective method of estimating BAP in effluents compared to bioassays that have long incubation periods and are more costly. The TDP effluent from these WWTPs is determined to be readily bioavailable to algae for uptake. Since the bioavailability of SRP and SAHP were found to be higher, WWTPs should focus on removing these P-species. In the case of the Fargo and Moorhead WWTPs, this could be accomplished by the use of chemical, biological, or a combined chemical and biological P removal process. This would remove much of the remaining SRP, which composes a majority of the TDP in the effluent.

5. CONCLUSIONS AND FUTURE WORK RECOMMENDATIONS

5.1. Conclusions

With future nationwide N and P limits for WWTPs expected to be implemented, due to issues resulting from eutrophication, there is a need to better understand the relationship between P and algae. Bioassays are an effective method for determining BAP of wastewater samples. However, it is not known whether the standard algal species, *Raphidocelis subcapitata* (formerly known as *Selenastrum capricornutum*), provides a reliable BAP estimate. This research investigates whether *R. subcapitata* is a reliable species for determining BAP. Also, it is useful to know what P-species are contributing to algal growth. Research has been done concerning this, but often overlook a test that can further distinguish SNRP into SAHP and DOP. Furthermore, there is currently limited research investigating BAP of wastewater.

The Printz algal assay bottle test was used to determine BAP, for three algal species (*Raphidocelis subcapitata, Chlorella vulgaris,* and *Chlamydomonas reinhardtii*) as individual species and a mixed culture, of wastewater samples under P-limiting conditions. BAP was determined through algal growth curves and via P-analysis of TDP prior to and after the incubation period. It was the latter method that proved to provide reliable results as the algal growth curve method tended to overestimate the BAP above the initial TDP concentration of the bioassay solutions. Acid hydrolysis and persulfate oxidation along with the ascorbic acid method were used to determine P-species (SRP, SAHP, and DOP) concentrations prior to and after the incubation period of the bioassay test, these concentrations were then used to determine the bioavailability of each P-species and the contribution of each P-species to BAP.

Results show that the bioassays of wastewater samples consistently produced higher algal densities than standard synthetic solution bioassays. This is likely due to potassium or sulfur

concentrations that were higher in the wastewater bioassays than the standard solutions bioassays and resulted in co-limiting conditions with P, since P was proven to be limiting. *C. reinhardtii* was proven to be the dominant species in the mixed culture by comparing the standard growth curves and normalized BAP concentrations of the mixed culture to each individual species, and by observing the mixed culture after the incubation period through an inverted microscope. The bioassays containing the species *C. reinhardtii,* including the mixed culture, were also prone to enter a palmelloid stage that produced spores due to stressful conditions of the bioassays. Possible stressors, including calcium deficiencies, high phosphate concentrations, chelating agents, and organic acids were ruled out as the cause of the palmelloid stage, yet further research is needed to determine the cause for certain.

No significant difference of %(BAP/TDP) was found among the algal species as individual species or a mixed culture. Therefore, any of the algal species tested could be used to measure BAP and produce comparable results. A normalized BAP concentration was also determined for each algal species and could be used to predict the amount of green algae that would be produced from samples or waters with a known BAP concentration. This could be used to predict the extent of an algal bloom that would be created in the water or wastewater tested. While any of the algal species tested could be used in the bioassay method to produce comparable results, it is recommended that *R. subcapitata* remain the standard algal species because this species provided slightly higher BAP concentrations and would therefore provide more conservative BAP estimates than the other algal species.

TDP in the effluent samples from both WWTPs is composed primarily of SRP (>85%). Both WWTPs have similar %(BAP/TDP) values of approximately 94%. After the incubation, the P-composition was composed of DOP having the largest fraction (at 50% for the Fargo

bioassays and the Moorhead HPOAS bioassays and approximately 90% for the Moorhead MBBR bioassay), while SRP still composed a large portion for the Fargo bioassays and the Moorhead HPOAS bioassays at 40% each. Over half of the bioassays observed a rise in the DOP concentration over the incubation period. This indicates that DOP is released by algae and possibly that DOP contains recalcitrant forms of P that are non-BAP and do not hydrolyze during the incubation period. All P-species were shown to be partially bioavailable due to the decrease seen in all bioassays for SRP and SAHP and in some bioassays for DOP over the incubation period. SRP contributed to the majority of the BAP concentration, comprising 91% and 97% of BAP in the Fargo and Moorhead WWTP samples, respectively. These results indicate that both WWTPs are discharging effluents with readily bioavailable P.

The bioavailability of each P-species was also determined. SRP and SAHP were approximately 95% and 75% bioavailable for the samples from both WWTPs. The bioavailability of DOP differed, being 33% and 0% bioavailable for the samples from the Fargo and Moorhead WWTPs, respectively. However the DOP bioavailability of the Fargo WWTP samples was 58% when neglecting the bioassays that observed an increase in the DOP concentration over the incubation period. The accuracy of the bioavailability of DOP is difficult to determine because DOP may have been released by the algae during the incubation period in all bioassays. This leads to a potentially false conclusion that portions of DOP are non-BAP because the DOP that is taken up is masked by a larger DOP concentration that is released; however, portions of DOP may actually be non-BAP and further testing would be needed to verify this.

A relation between P-species and BAP could not be made according to a regression analysis. However, it is possible to use SRP as a low BAP estimate and TDP or TP as a high

BAP estimate. This allows for a quicker and more cost effective method of estimating BAP compared to bioassays. Results suggest that the WWTPs studied should focus on removing SRP and SAHP in order to reduce BAP, as their bioavailabilities were high. This could be accomplished by incorporating biological P removal or chemical P precipitation and clarification after the secondary biological processes, in order to remove most of the remaining SRP from the effluent and thereby reducing the BAP concentration.

5.2. Future Work Recommendations

This thesis research was an initial step in understanding the relation between P and algae. Results show that algal species tested do not affect the amount of P that is bioavailable and that all P-species are partially bioavailable. However, further research could be conducted to further distinguish P-species, examine BAP across a broader range of algal species, and to optimize the P-limiting conditions to exclude co-limiting conditions. Some further research ideas are summarized below.

- Use a broader range of algal species (such as green, red, and brown algae) to determine whether %(BAP/TDP) varies among a broader range of algal species.
- If more P-speciation tests could be developed to distinguish phosphite, phosphines, and phosphides; perhaps a relation of P-species to BAP may be developed.
- Investigate the %(BAP/TDP) of tertiary plant effluents along with P-speciation studies, to determine which P-species are not being removed from the wastewater and determine the fraction of the effluent that is bioavailable.

- Determine the precise N:P level needed to achieve P-limiting conditions in *R. subcapitata* in order to minimize the amount of nutrients required for the P-free media.
- In order to achieve P-limiting conditions, wastewater samples had to be diluted due to the amount of carbonate that was required (to ensure P-limiting conditions). If a method was developed that circumvented this dilution requirement, the bioassays would be able to analyze samples with higher P concentrations and the examination of P-species after the incubation period would benefit from larger initial concentrations.
- P forms can be transformed after sufficient exposure to ultraviolet light or sunlight. Tests can be conducted to determine whether pretreatment with UV light affects BAP.
- Determine the bioavailability of DOP by filtering bioassays and replenishing their algal supply during the incubation period.
- Conduct kinetic studies to determine P uptake rates that can be applied to both wastewater treatment and receiving water quality models.

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APPENDIX

A.1. Equations

1) "BAP Contribution" = "Percent Contribution of TP to Waters (%)" * "Range of %(BAP/TP) of Effluent or Runoff (%)"
2) "Range of %BAP Contribution to Waters (%)" equals a Point Source "BAP Contribution" divided by the sum of the same Point Source "BAP Contribution" and a Non-point Source "BAP Contribution" value. The lowest and highest "Range of %BAP Contribution to Waters (%)" values, for each level of point source treatment, were then used to populate Table 2. Sample calculations for Equations 1 and 2:
    - Non-point "Percent Contribution of TP to Waters (%)" = 80% and "Range of %(BAP/TP) of Effluent or Runoff (%) = 20 to 40%. Non-point "BAP Contribution" = 80*20/100 = 16 and 80*40/100 = 32.
    - Conventional Secondary "Percent Contribution of TP to Waters (%)" = 20 and "Range of %(BAP/TP) of Effluent or Runoff (%) = 80 to 90. Conventional Secondary "BAP Contribution" = 20*80/100 = 16 and 20*90/100 = 18.
    - The low range of the non-point "Range of %BAP Contribution" (as calculated with the conventional secondary point source) is determined by dividing the lowest non-point "BAP Contribution" by the sum of the lowest non-point "BAP Contribution" and the highest Conventional Secondary treatment "BAP Contribution". This results in the low "Range of %BAP Contribution" for non-point sources, 16*100/(16+18) = 47%
3) BAP = Initial TP – Final TP
4) SAHP = (SAHP+SRP) – SRP

5) DOP = TDP – (SAHP+SRP)

6) $\%(\text{P-species/BAP}) = \frac{(\Delta\text{P-species})*100}{\text{BAP}}$

7) $\text{P-species Bioavailability} = \frac{(\Delta\text{P-species})*100}{(\text{P-species})_{\text{initial}}}$

A.2. Tables and Figures

Table A1. Average and standard deviations from each set of standard growth curve algal densities for each algal species and the mixed culture.

BAP (mg P/L)	Average Algal Densities (mg dry wt./L)			
	R. subcapitata	***C. reinhardtii***	***C. vulgaris***	**Mixed Culture**
0.0	-4 ±10	-12 ±12	-5 ±4	-3 ±8
0.1	4 ±11	5 ±13	3 ±3	6 ±7
0.5	88 ±47	115 ±15	106 ±50	114 ±40
1.0	114 ±21	157 ±15	132 ±27	150 ±22
1.5	165 ±16	223 ±36	191 ±17	233 ±28
2.0	172 ±37	265 ±23	223 ±25	199 ±40
5.0	378 ±40	435 ±21	364 ±59	447 ±25

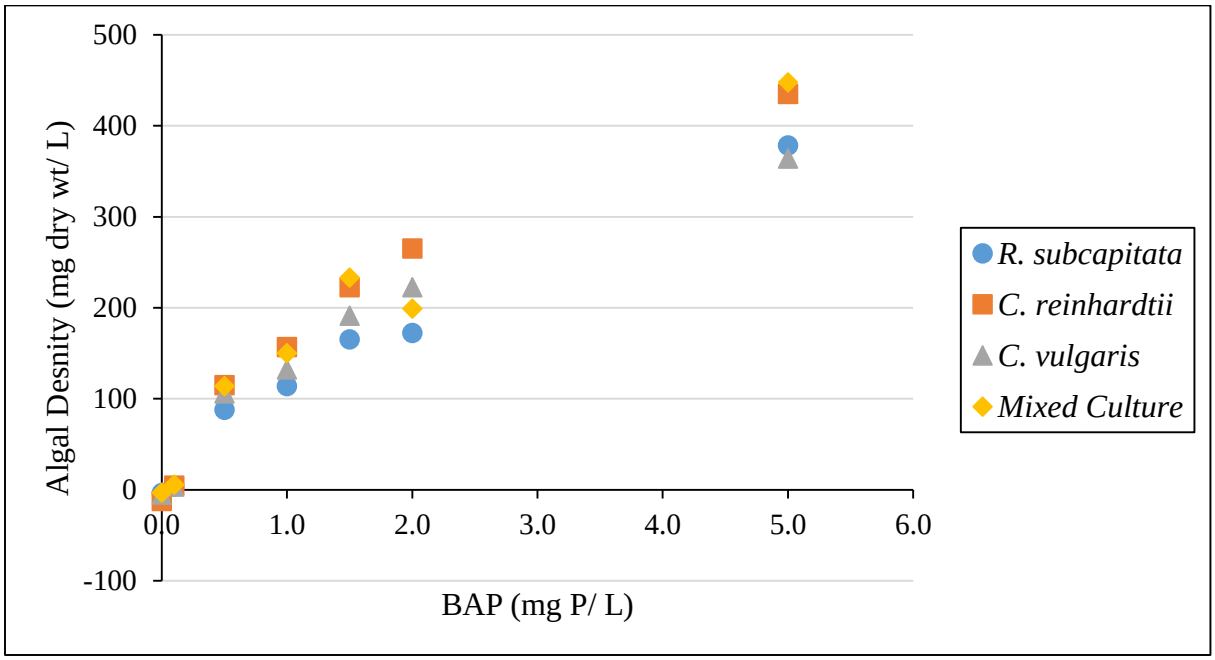
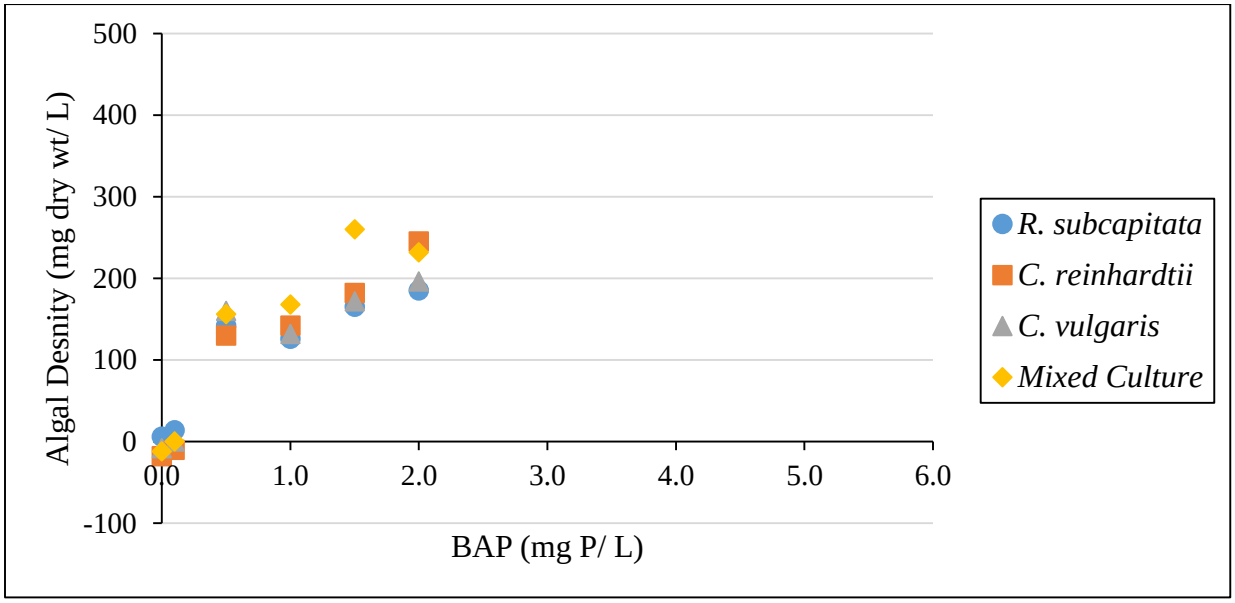


Figure A1. Plot of the average standard growth curve algal densities, from each set of growth curves, for each algal species and the mixed culture.

a)



b)

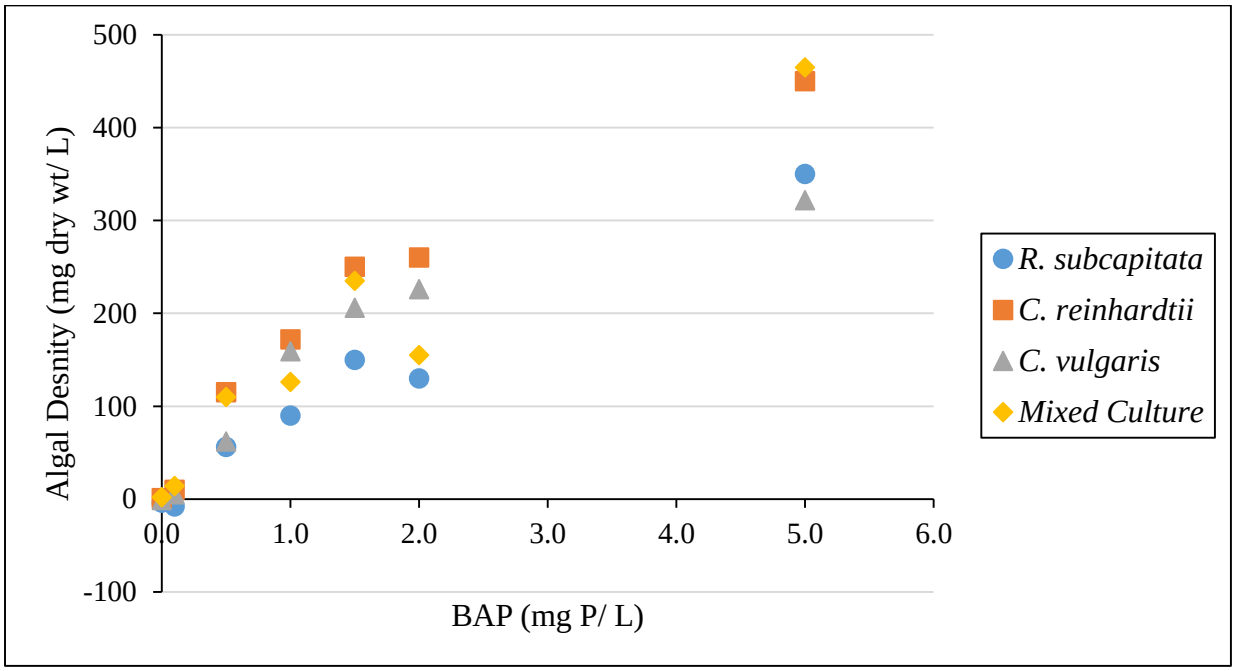


Figure A2. Standard growth curves from a) 11/2014 b) 1/2015 and c) 3/2015.

c)

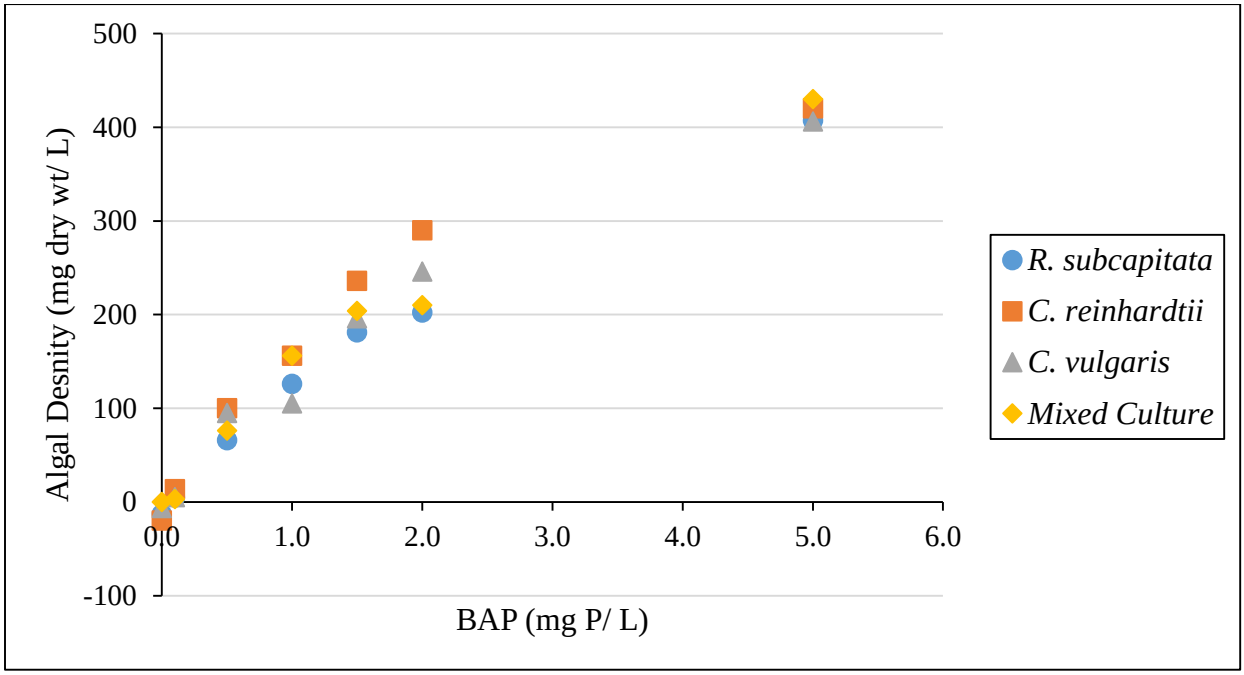


Figure A2. Standard growth curves from a) 11/2014 b) 1/2015 and c) 3/2015 (continued).

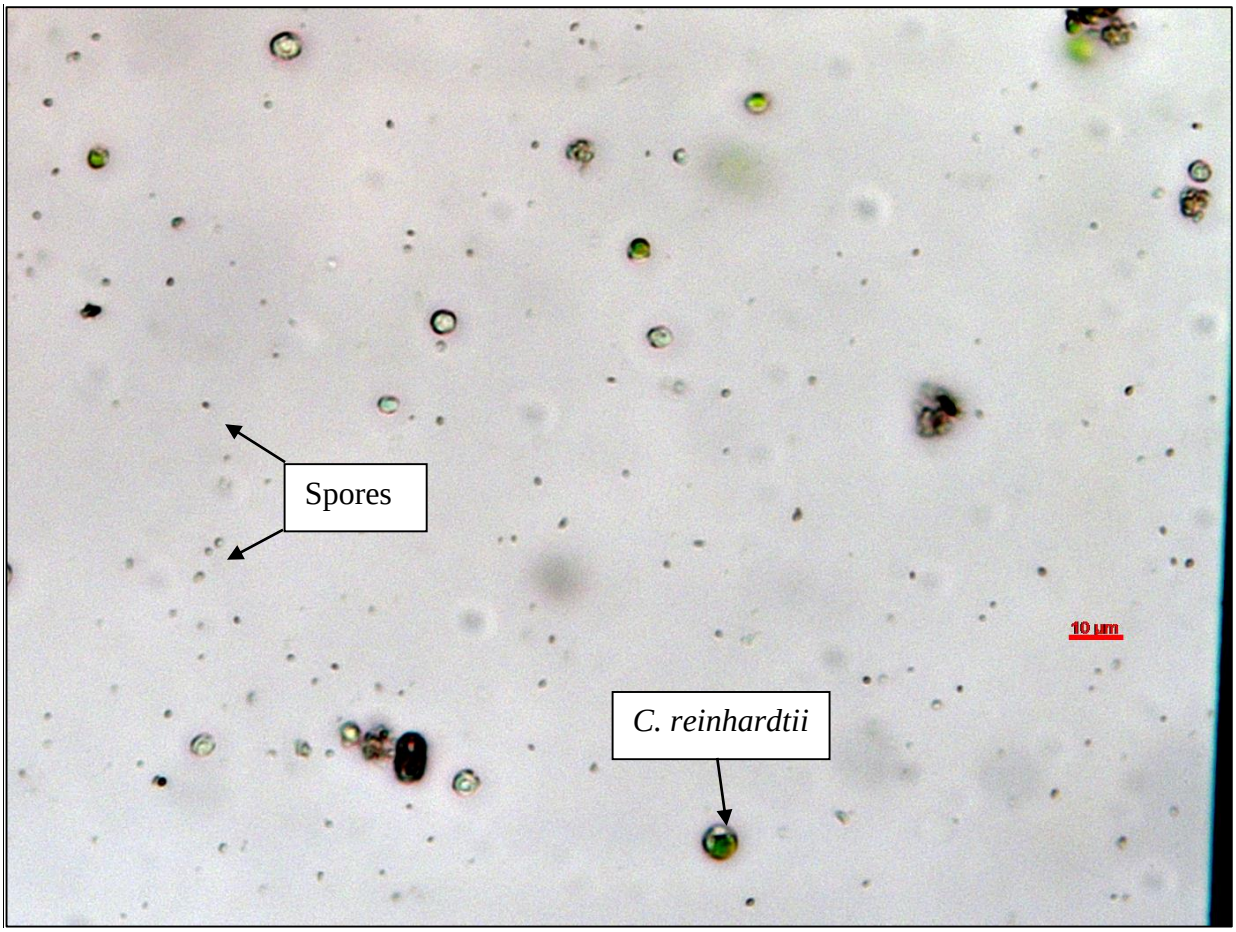


Figure A3. Microscopic image of a mixed culture wastewater bioassay where *C. reinhardtii* has entered a palmelloid stage and released spores.

Table A2. The Generalized Extreme Studentized Deviate Test for *C. reinhardtii*. The R_i value greater than the critical value indicates the number of outliers.

r	p	DOF	$t_{p,v}$	R_i	Critical Value
1	0.9979	11	3.776	1.913	2.435
2	0.9975	10	3.778	1.306	2.361
3	0.9969	9	3.741	1.474	2.270
4	0.9958	8	3.594	1.765	2.147
5	0.9938	7	3.097	2.288	1.941
6	0.9875	6	0.893	1.581	0.841

Table A3. The Generalized Extreme Studentized Deviate Test for the mixed culture. The R_i value greater than the critical value indicates the number of outliers.

r	p	DOF	$t_{p,v}$	R_i	Critical Value
1	0.9979	11	3.776	2.002	2.435
2	0.9975	10	3.778	2.034	2.361
3	0.9969	9	3.741	1.770	2.270
4	0.9958	8	3.594	2.335	2.147
5	0.9938	7	3.097	1.355	1.941

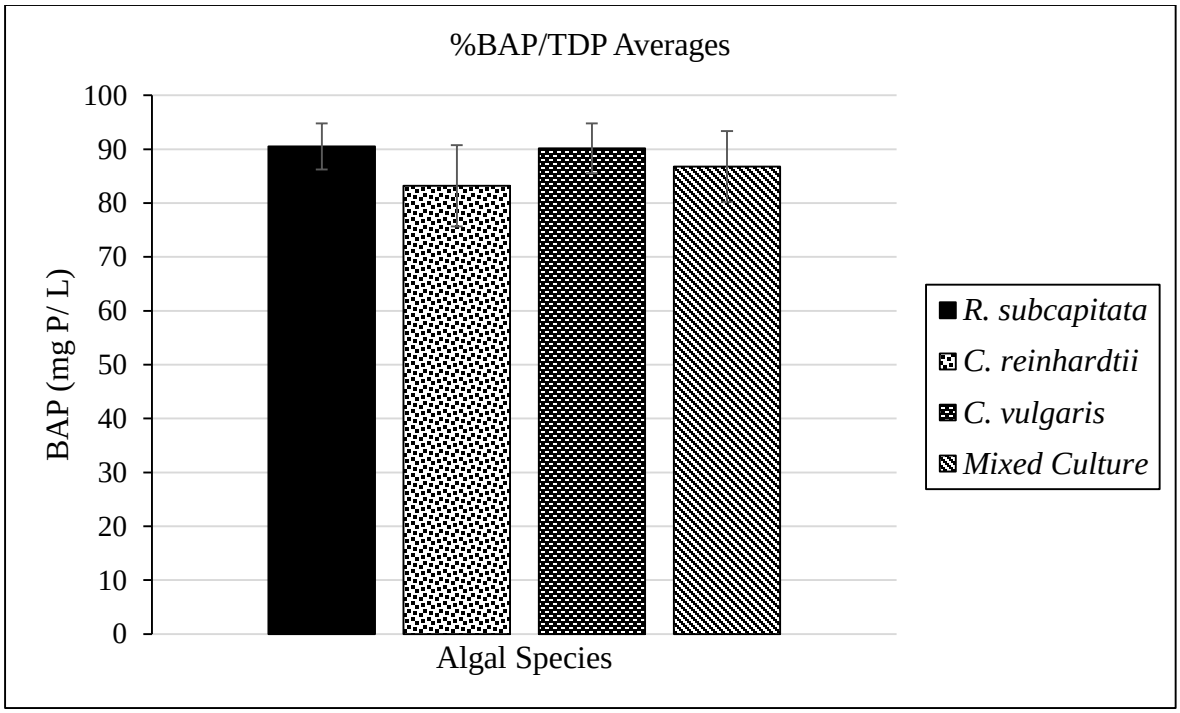


Figure A4. A bar graph of the %BAP/TDP averages with standard deviation indicators for each algal species and the mixed culture.

Table A4. P-speciation, representing each individual P-species, measured prior to and after the incubation period.

Date	P-Species	Concentration Before Incubation (mg P/ L)	Concentration After Incubation (mg P/ L)	Difference (mg P/ L)
8/27/2014	SRP	0.95 ±0.00	0.12 ±0.00	0.83 ±0.00
	SAHP	0.06 ±0.00	0.02 ±0.01	0.04 ±0.01
	DOP	0.01 ±0.04	0.01 ±0.00	0.00 ±0.04
	TDP	1.02 ±0.04	0.15 ±0.00	0.87 ±0.04
2/4/2015	SRP	0.91 ±0.08	0.03 ±0.01	0.87 ±0.09
	SAHP	0.06 ±0.08	0.00 ±0.00	0.06 ±0.08
	DOP	0.03 ±0.01	0.02 ±0.01	0.01 ±0.02
	TDP	1.00 ±0.01	0.06 ±0.01	0.94 ±0.02
2/26/2015	SRP	0.87 ±0.09	0.04 ±0.02	0.84 ±0.11
	SAHP	0.06 ±0.11	0.03 ±0.01	0.03 ±0.12
	DOP	0.07 ±0.01	0.03 ±0.03	0.04 ±0.04
	TDP	1.00 ±0.01	0.10 ±0.03	0.90 ±0.04
2/27/2015	SRP	0.93 ±0.03	0.03 ±0.01	0.90 ±0.04
	SAHP	0.04 ±0.00	0.01 ±0.01	0.03 ±0.01
	DOP	0.02 ±0.00	0.06 ±0.02	-0.03 ±0.02
	TDP	1.00 ±0.00	0.09 ±0.02	0.91 ±0.02

Table A4. P-speciation, representing each individual P-species, measured prior to and after the incubation period (continued).

Date	P-Species	Concentration Before Incubation (mg P/ L)	Concentration After Incubation (mg P/ L)	Difference (mg P/ L)
4/20/2015	SRP	1.08 ±0.06	0.06 ±0.02	1.02 ±0.08
	SAHP	0.04 ±0.03	-0.03 ±0.01	0.07 ±0.04
	DOP	0.02 ±0.00	0.07 ±0.01	-0.05 ±0.01
	TDP	1.14 ±0.00	0.10 ±0.01	1.04 ±0.01
5/1/2015	SRP	0.91 ±0.02	0.03 ±0.00	0.88 ±0.02
	SAHP	0.00 ±0.03	0.00 ±0.01	0.00 ±0.04
	DOP	0.02 ±0.02	0.03 ±0.00	-0.01 ±0.02
	TDP	0.93 ±0.02	0.05 ±0.00	0.88 ±0.02
5/15/2015	SRP	0.71 ±0.06	0.01 ±0.00	0.70 ±0.06
	SAHP	0.01 ±0.00	0.00 ±0.00	0.01 ±0.00
	DOP	0.41 ±0.01	0.07 ±0.00	0.33 ±0.01
	TDP	1.13 ±0.01	0.09 ±0.00	1.04 ±0.01
5/26/2015	SRP	0.92 ±0.00	0.01 ±0.00	0.91 ±0.00
	SAHP	0.02 ±0.01	0.01 ±0.00	0.01 ±0.01
	DOP	0.13 ±0.01	0.05 ±0.01	0.08 ±0.02
	TDP	1.06 ±0.01	0.07 ±0.01	1.00 ±0.02

Table A4. P-speciation, representing each individual P-species, measured prior to and after the incubation period (continued).

Date	P-Species	Concentration Before Incubation (mg P/ L)	Concentration After Incubation (mg P/ L)	Difference (mg P/ L)
7/6/2015	SRP	1.11 ±0.03	0.00 ±0.00	1.11 ±0.03
	SAHP	0.02 ±0.01	0.00 ±0.00	0.01 ±0.01
	DOP	0.00 ±0.00	0.07 ±0.01	-0.07 ±0.01
	TDP	1.13 ±0.00	0.07 ±0.01	1.05 ±0.01
7/6/2015 (After MBBR)	SRP	1.14 ±0.04	0.01 ±0.00	1.13 ±0.04
	SAHP	0.02 ±0.01	0.01 ±0.00	0.02 ±0.01
	DOP	0.03 ±0.00	0.14 ±0.03	-0.11 ±0.03
	TDP	1.19 ±0.00	0.16 ±0.03	1.03 ±0.03

Table A5. Proportion of each individual P-species in relation to TDP.

Date	Pre-Incubation			Post-Incubation		
	%(SRP/ TDP)	%(SAHP/ TDP)	%(DOP/ TDP)	%(SRP/ TDP)	%(SAHP/ TDP)	%(DOP/ TDP)
8/27/2014	93.1 ±0.5	6.1 ±0.1	0.9 ±3.8	80.0 ±1.5	11.8 ±6.6	8.2 ±1.6
2/4/2015	90.8 ±8.4	5.9 ±8.2	3.3 ±1.2	58.6 ±15.4	0.4 ±4.8	41.1 ±12.9
2/26/2015	87.4 ±8.8	6.0 ±10.9	6.5 ±0.6	37.0 ±19.1	33.9 ±9.3	29.1 ±34.9
2/27/2015	93.3 ±2.7	4.3 ±0.2	2.4 ±0.0	46.7 ±5.3	15.1 ±12.7	38.2 ±21.1
4/20/2015	94.8 ±0.8	3.4 ±2.0	1.7 ±0.3	127.5 ±23.7	-67.2 ±9.9	39.7 ±9.9
5/1/2015	98.0 ±2.2	0.2 ±2.8	1.8 ±2.5	61.2 ±4.7	-1.5 ±10.1	40.2 ±4.6
5/15/2015	62.7 ±5.3	1.3 ±0.0	36.0 ±1.0	11.3 ±2.2	5.0 ±2.0	83.7 ±5.7
5/26/2015	86.2 ±0.3	2.0 ±0.7	11.9 ±0.9	10.5 ±0.0	20.1 ±3.6	69.4 ±9.4
7/6/2015	98.4 ±3.0	1.4 ±1.1	0.2 ±0.3	32.2 ±0.6	31.4 ±1.4	36.3 ±19.9
7/6/2015 (After MBBR)	95.7 ±3.3	2.0 ±0.6	2.3 ±0.3	20.0 ±1.4	17.5 ±2.3	62.4 ±18.8

Table A6. One-way ANOVA test results for P-speciation before the incubation period; a) for %SRP/TDP, b) for %SAHP/TDP, and c) for %DOP/TDP.

a)

Anova: Single Factor	**%SRP/TDP**					
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Fargo WWTP	7	613.2706	87.61009	137.5936		
Moorhead WWTP - HPOAS	2	191.4258	95.7129	14.08954		
Moorhead WWTP - MBBR	1	95.66459	95.66459	#DIV/0!		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	137.3307	2	68.66536	0.572449	0.588496	4.737414
Within Groups	839.6512	7	119.9502			
Total	976.9819	9				

Table A6. One-way ANOVA test results for P-speciation before the incubation period; a) for %SRP/TDP, b) for %SAHP/TDP, and c) for %DOP/TDP (continued).

b)

Anova: Single Factor	**%SAHP/TDP**					
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Fargo WWTP	7	23.12048	3.302925	5.117348		
Moorhead WWTP - HPOAS	2	7.480482	3.740241	10.94521		
Moorhead WWTP - MBBR	1	2.011469	2.011469	#DIV/0!		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.032975	2	1.016487	0.170841	0.846367	4.737414
Within Groups	41.64929	7	5.949899			
Total	43.68227	9				

Table A6. One-way ANOVA test results for P-speciation before the incubation period; a) for %SRP/TDP, b) for %SAHP/TDP, and c) for %DOP/TDP (continued).

c)

Anova: Single Factor	**%DOP/TDP**					
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Fargo WWTP	7	63.6089	9.086985	154.1398		
Moorhead WWTP - HPOAS	2	1.093715	0.546857	0.198247		
Moorhead WWTP - MBBR	1	2.323944	2.323944	#DIV/0!		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	134.756	2	67.37801	0.509867	0.621274	4.737414
Within Groups	925.037	7	132.1481			
Total	1059.793	9				

Table A7. One-way ANOVA test results for P-speciation after the incubation period; a) for %SRP/TDP, b) for %SAHP/TDP, and c) for %DOP/TDP.

a)

Anova: Single Factor	**%SRP/TDP**					
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Fargo WWTP	7	259.8809	37.12584	443.9671		
Moorhead WWTP - HPOAS	2	83.04632	41.52316	2953.396		
Moorhead WWTP - MBBR	1	5.605111	5.605111	#DIV/0!		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	980.5816	2	490.2908	0.610987	0.569412	4.737414
Within Groups	5617.199	7	802.457			
Total	6597.781	9				

Table A7. One-way ANOVA test results for P-speciation after the incubation period; a) for %SRP/TDP, b) for %SAHP/TDP, and c) for %DOP/TDP (continued).

b)

Anova: Single Factor	**%SAHP/TDP**					
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Fargo WWTP	7	35.33306	5.04758	430.2399		
Moorhead WWTP - HPOAS	2	14.83564	7.417819	38.77023		
Moorhead WWTP - MBBR	1	4.90819	4.90819	#DIV/0!		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9.138492	2	4.569246	0.012207	0.987888	4.737414
Within Groups	2620.21	7	374.3157			
Total	2629.348	9				

Table A7. One-way ANOVA test results for P-speciation after the incubation period; a) for %SRP/TDP, b) for %SAHP/TDP, and c) for %DOP/TDP (continued).

c)

Anova: Single Factor	**%DOP/TDP**					
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Fargo WWTP	7	404.7861	57.82658	352.1649		
Moorhead WWTP - HPOAS	2	102.118	51.05902	3668.935		
Moorhead WWTP - MBBR	1	89.4867	89.4867	#DIV/0!		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1061.111	2	530.5556	0.642328	0.554476	4.737414
Within Groups	5781.924	7	825.9892			
Total	6843.035	9				

Table A8. Bioavailability of P in secondary effluent wastewater samples.

Date	%(BAP/TDP)	WWTP
8/27/2014	87.4 ±1.6	Moorhead
2/4/2015	94.3 ±0.7	Fargo
2/26/2015	90.3 ±3.4	Fargo
2/27/2015	93.8 ±2.0	Fargo
4/20/2015	95.6 ±0.9	Fargo
5/1/2015	95.6 ±0.2	Fargo
5/15/2015	92.3 ±0.5	Fargo
5/26/2015	93.7 ±0.6	Fargo
7/6/2015	99.4 ±1.5	Moorhead
7/6/2015 (After MBBR)	96.3 ±3.0	Moorhead

Table A9. One-way ANOVA test results on P-species bioavailability among the WWTPs sampled; for a) SRP, b) SAHP, and c) DOP.

a)

Anova: Single Factor	**SRP Bioavailability**					
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Fargo WWTP	7	678.3255	96.90364	2.910928		
Moorhead WWTP - HPOAS	2	186.9612	93.48059	79.63512		
Moorhead WWTP - MBBR	1	99.21996	99.21996	#DIV/0!		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	26.74799	2	13.37399	0.964133	0.426735	4.737414
Within Groups	97.10069	7	13.87153			
Total	123.8487	9				

Table A9. One-way ANOVA test results on P-species bioavailability among the WWTPs sampled; for a) SRP, b) SAHP, and c) DOP (continued).

b)

Anova: Single Factor	**SAHP Bioavailability**					
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Fargo WWTP	7	529.5308	75.64725	707.9099		
Moorhead WWTP - HPOAS	2	156.6511	78.32554	108.3335		
Moorhead WWTP - MBBR	1	67.51429	67.51429	#DIV/0!		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	79.72074	2	39.86037	0.064058	0.938494	4.737414
Within Groups	4355.793	7	622.2561			
Total	4435.514	9				

Table A9. One-way ANOVA test results on P-species bioavailability among the WWTPs sampled; for a) SRP, b) SAHP, and c) DOP (continued).

c)

Anova: Single Factor	**DOP Bioavailability**					
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Fargo WWTP	7	231.8513	33.12161	1194.148		
Moorhead WWTP - HPOAS	2	0	0	0		
Moorhead WWTP - MBBR	1	0	0	#DIV/0!		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2303.786	2	1151.893	1.125384	0.376895	4.737414
Within Groups	7164.887	7	1023.555			
Total	9468.673	9				

A.3. Regression Analysis: BAP (mg P/ L) versus SRP (mg P/ L), SAHP (mg P/ L), and DOP (mg P/ L)

The following terms cannot be estimated and were removed: DOP (mg P/ L)

Stepwise Selection of Terms

α to enter = 0.15, α to remove = 0.15

Beginning with step 1, the model may not be hierarchical because some required terms are impossible to estimate.

At your request, the stepwise procedure included these terms in every model: SRP (mg P/ L), SAHP (mg P/ L), SRP (mg P/ L)*SAHP (mg P/ L), SRP (mg P/ L)*DOP (mg P/ L), SAHP (mg P/L)*DOP (mg P/ L), SRP (mg P/ L)*SAHP (mg P/ L)*DOP (mg P/ L)

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	6	0.010932	0.001822	0.75	0.25
Error	6	0.014634	0.002439		
Total	12	0.025566			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0493863	42.76%	0.00%	0.00%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	-433	310	-1.40	0.211	
SRP (mg P/ L)	432	308	1.40	0.211	275130.27
SAHP (mg P/ L)	108	110	0.99	0.361	25205.35
SRP (mg P/ L)*SAHP (mg P/ L)	375	253	1.48	0.189	103442.00
SRP (mg P/ L)*DOP (mg P/ L)	493	352	1.40	0.211	110409.23
SAHP (mg P/ L)*DOP (mg P/ L)	7439	5465	1.36	0.222	211626.75
SRP (mg P/ L)*SAHP (mg P/ L) *DOP (mg P/ L)	-8060	5957	-1.35	0.225	186263.01

Regression Equation

BAP (mg P/ L) = -433 + 432 SRP (mg P/ L) + 108 SAHP (mg P/ L) + 375 SRP (mg P/ L) *SAHP (mg P/ L) + 493 SRP (mg P/ L)*DOP (mg P/ L) + 7439 SAHP (mg P/ L) *DOP (mg P/ L) - 8060 SRP (mg P/ L)*SAHP (mg P/ L)*DOP (mg P/ L)

Fits and Diagnostics for Unusual Observations

Obs	BAP (mg P/ L)	Fit	Resid	Std Resid
4	0.8473	0.8176	0.0297	2.04 R
12	0.9101	0.9230	-0.0129	-2.15 R

R Large residual