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Determination of the Effect of Constructed Wetlands on the Bioavailability of Phosphorus using Algal Bioassays

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July 15, 2016

ABSTRACT: *Phosphorus is the limiting nutrient in most freshwater aquatic environments. When a large amount of phosphorus enters a freshwater environment, such as a lake, this causes a rapid increases in primary production which can lead to anoxic conditions. Anoxic conditions can be detrimental to the local ecosystem as well as ecosystems downstream of this water source. Constructed wetlands have been used worldwide to remediate pollutants, such as phosphorus, from water entering the environment. Wetlands are able to do this through biological, physical and chemical process such as sedimentation, uptake, sorption and precipitation. There is some debate on whether or not this efficiency decreases as the wetland ages and growth rates slow. Studies have concluded that, chemically, constructed wetlands can remove phosphorus. This study is interested in whether or not the wetlands changes the bioavailability of the phosphorus through Leikvollbekken, a mature constructed wetland on the south western coast of Norway which is subject to inflows of nutrient rich run-off from agricultural lands. Experiments were performed over several months to assess the bioavailability at the inlet and the outlet of the wetland. Chemical analysis determined the phosphorus fractions and algal bioassays were used to quantify the amount of bioavailable phosphorus at the inlet and outlet of the constructed wetland. Samples were taken, sterilized, diluted and then split into 5 replicates. Each replicate was inoculated with Raphidocelis subcapitata and the algal growth potential was determined. The results of the algal assays were compared to chemical analysis and demonstrated no removal trend of bioavailable phosphorus from the inlet of the wetland to the outlet. These results were further studied by evaluating the fractionation of the total phosphorus and the bioavailable phosphorus in the inlet and the outlet and concluded that the fractionation of bioavailable phosphorus does change through the wetland. More studies should be done on this wetland using different algal bioassay methods to further determine the speciation of the bioavailable fractions. The wetland should also be studied to evaluate its performance and determine if there is maintenance that can be done to increase its efficiency.*

KEYWORDS: bioavailable, algal bioassay, constructed wetland, *Raphidocelis subcapitata*, phosphorus

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1. INTRODUCTION

The aquatic ecosystem within a lake is naturally at dynamic equilibrium, varying only with the season as changes in temperature and light affect photosynthesis and respiration. In most freshwater systems phosphorus (P) is the limiting nutrient and most of these systems, such as lakes, have a buffer capacity for an increased input of phosphorus. If that phosphorus buffer capacity is exceeded, the steady-state is shifted and there is a significant increase in primary production. Eutrophication, excessive nutrient input, leads to changes at all trophic levels via an increase in phytoplanktonic biomass. Especially in ecosystems where there is restricted water circulation this can lead to toxic algae blooms which transform the area into a highly anoxic environment.

Artificial wetlands are designed to use multiple mechanisms to remove excess phosphorus from the groundwater before it enters the natural water system in order to mitigate the eutrophication of lakes and prevent algae blooms. First it traps the phosphorus in the sediments through low flow which allows for sedimentation and long contacts times between the water and the sediments which allows for infiltration of the water and nutrients into the soil. Second, the plants and micro-organisms uptake phosphorus directly from the water preventing it from leeching into the lake. These mechanisms and their efficiency are highly dependent on the design of the constructed wetland and the local conditions.

The wetland

The wetland of interest in this study, Leikvollbekken, is located on the edge of Stokkavatnet, about 3.8 km west of Stavanger on the south-western coast of Norway. The wetland was built in 1994 and consists of 2

Figure 1: Wetland location in Stavanger, Norway (photo: Google Maps)

ponds in series which filter the input water. Diagrams and details on the construction of the wetland are given in Appendix A.

The watershed

In 2015, it was estimated that the watershed treated by this wetland is approximately 0.19 km^2 (Hill, 2015). The watershed area is hard to estimate as the original plans for the wetland are not available. As seen in Figure 1, the area surrounding the wetland is mostly used for agricultural production. Locals say that a series of underground channels and pipes were installed in order to divert groundwater from local farmlands into the wetlands, including waste lines from nearby greenhouses. This series of channels and pipes means the watershed feeding into the wetland can be significantly greater than estimated. For the purpose of this experiment, no soil testing was done, but a map of soil types was used to get a general soil characterization. From the soil map, Appendix A, the soil consists of podozols which are described as acidic with a distinct bleached eluviation horizon over an accumulation horizon of brownish soil showing evidence of organic matter containing aluminum or iron (Hartemink, 2006). The other descriptions of the soil indicate high clay content and high amounts of surface rocks and visible waterlogging of the soil. Visibly, shale (fine grained rock made up of compacted silt and mud) deposits can be seen in and around the wetland.

Previous Studies and Purpose

Experiments have been conducted to chemically measure and compare the phosphorus in the inlet and outlet of the wetlands and have proven that constructed wetlands can retain phosphorus (Dunne et al., 2015). Some experiments have concluded that, especially in the winter, the wetland has negative phosphorus retention meaning it is inputting phosphorus into the lake ecosystem (Hijosa-Valsero, Sidrach-Cardona, & Bécares, 2012). It has been suggested that this phosphorus is not reactive, i.e. not available for algae blooms, and therefore the wetland is still fulfilling its purpose.

The purpose of this study is to determine if the constructed wetland has an effect on the bioavailability of the phosphorus entering the lake. This will be done by performing algal bioassays on samples taken from the Leikvollbekken wetland inlet and outlet in order to determine the bioavailability of the phosphorus. Multiple tests will be performed at different times of year in order to get an idea of the general performance of the wetland.

2. THEORY

The purpose of this chapter is to present the general and specific factors affecting the functioning and efficiency of the wetland studied. General sources and fates of phosphorus in the aquatic ecosystem are presented and the current definition of the bioavailability of phosphorus is reviewed. The transformations of phosphorus by plants is examined as well as the purpose and structure of the constructed wetland.

2.1 Phosphorus Sources

This section will review the natural inputs of phosphorus to a lake and the human impacts to these inputs.

Natural Sources

There are two ways for phosphorus to enter a lake naturally; the atmosphere, and drainage input. Allochthonous input of phosphorus comes from sources outside of the lake system such as leaves, twigs, and soil (Fenoglio, 2014). Autochthonous input of phosphorus includes phosphorus being cycled within the lake through processes such as decomposition of materials originating in the lake. The atmosphere does not play a significant role in the phosphorus cycle as there are very few gaseous forms of phosphorus such as phosphine (PH_3) but you can also get some minor additions of particle bound phosphorus from dust deposition by wind currents (Wetzel, 2001). Drainage includes both surface and subsurface flows such as river water and ground water. The phosphorus that is released from drainage is primarily in 2 forms; dissolved organic phosphorus and particle bound phosphorus (Kalff, 2002). Due to the rapid uptake of orthophosphates by plants and microbes, they are not usually found in high concentration in natural runoff. River inputs during base flow are relatively constant, but during storm events, significant erosion can occur causing an increased load of particulate bound phosphorus in the river water (Mainstone & Parr, 2002).

Anthropogenic Sources

There are many anthropogenic inputs of phosphorus to the environment including wastewater, urban runoff, and agricultural runoff (manure, fertilizer, and pesticides) (Yuan, Shi, Wu, Zhang, & Bi, 2011). Most anthropogenic sources of phosphorus are point sources of highly reactive phosphorus (Mainstone & Parr, 2002). Many chemicals used in industrial and agriculture processes break down into phosphoric acid (phosphate) (Hoffman, 1991). This means lakes closer to urban areas and lakes with inputs of industrial, or agricultural discharge will see a higher than natural input of phosphorus and possible eutrophication.

The impact of these sources of phosphorus on a lake is dependent on the quantity of the phosphorus input as well as the reactivity and bioavailability of the phosphorus input when it enters the phosphorus cycle within the lake.

2.2 Aquatic Phosphorus Cycle

The phosphorus cycle in a lake can be very intricate, as demonstrated in Figure 2. Because of copious and sometimes rapid transformations, the speciation of phosphorus in aquatic systems can be hard to estimate. Some of these transformations are chemical processes, and some are catalyzed by biological processes. The phosphorus cycle involves all trophic levels thus changes in available phosphorus will have effects at all trophic levels.

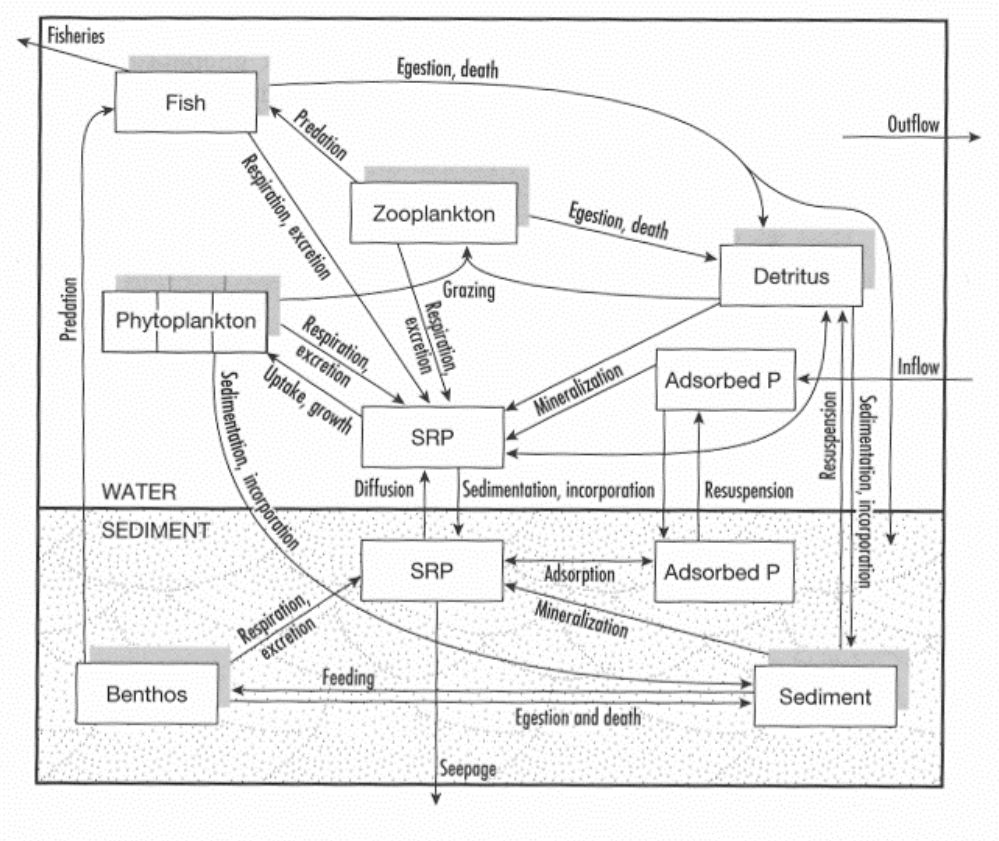

Figure 2: PCLOOS model of phosphorus and carbon flow in aquatic systems (Kalff, 2002).

The more basic transfer cycle of phosphorus in the aquatic system, Figure 3, simplifies Figure 2 into general compartments; particulate, soluble, organic and inorganic phosphorus.

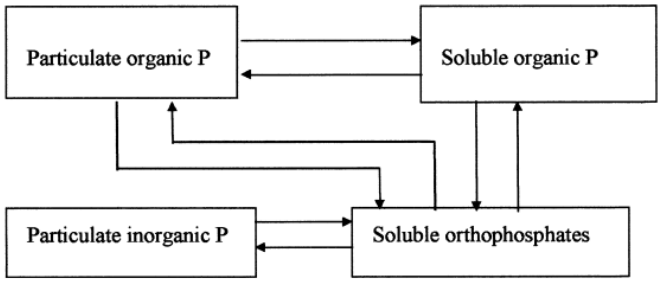

Figure 3: Transfer cycle of phosphorus in aquatic systems (B. Ya. Spivakov, 1999)

Particulate organic phosphorus (POP) includes organisms, and dead organisms (detritus) and other phosphorus bound to organic particles whereas soluble organic phosphorus (dissolved organic phosphorus, DOP) is phosphate bound to soluble organic molecules produced by organisms. Soluble organic phosphorus also includes some adsorbed polyphosphates which can act as colloids in water, staying in suspension (Hoffman, 1991).

Particulate inorganic phosphorus (PIP) includes phosphorus bound to minerals. It is important to note in Figure 3 that the only conversion of particulate inorganic phosphorus is to soluble orthophosphates (dissolved inorganic phosphorus, DIP), and there is no direct link to particulate organic phosphorus (organisms). This means organisms cannot directly uptake inorganically bound phosphorus; it must be released into soluble orthophosphates before organisms can uptake it.

In biological systems, phosphorus is used for intracellular molecular synthesis and transport as well as for the formation of nucleic acids (Madigan, Martinko, Stahl, Clark, & Brock, 2012). Phosphorus is biologically important and thus biotic transformations make up the bulk of its cycle in the environment as organisms desire to obtain it. In the environment, phosphorus is almost always found in the (+5) oxidation state, as phosphates (Hoffman, 1991).

2.3 Phosphorus Speciation

Total phosphorus (TP) in water quality testing is the total amount of detectable phosphorus in the sample and is determined by digesting the sample in a strong acid to solubilize all of the phosphorus (APHA). This can be split into two sub-categories; inorganic phosphorus and organic phosphorus as show in Figure 4 below. Interaction with carbon differentiates between inorganic phosphorus (IP) and organic phosphorus (OP). OP will be attached to a group containing carbon while IP will not be associated with any carbon.

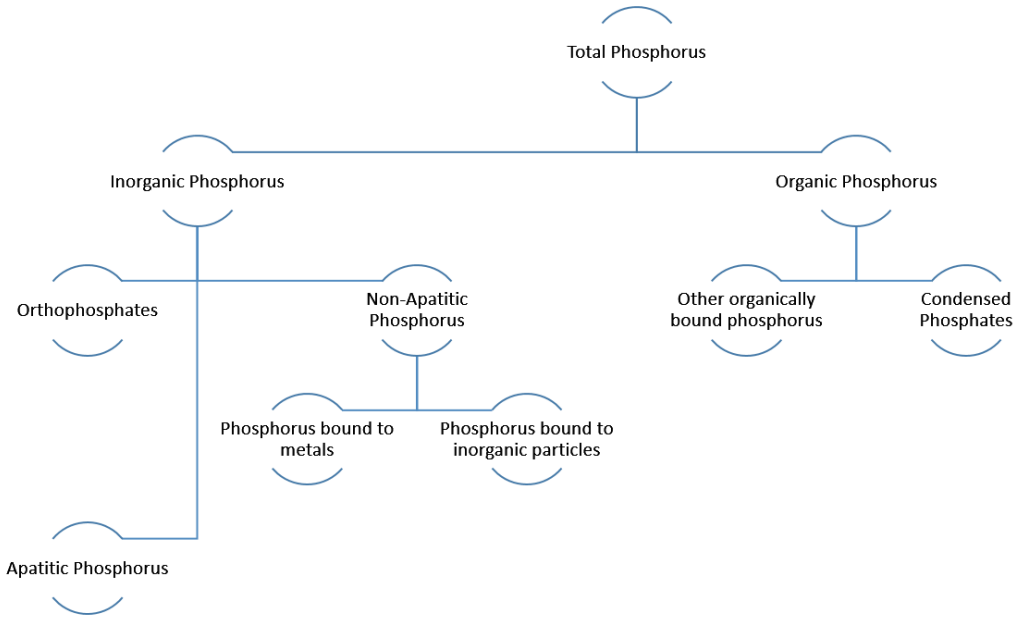

Figure 4: P Speciation based on Standard Method 4500-P

The IP is then separated, as it was in the basic speciation in Figure 3, into soluble orthophosphates (SP) and phosphorus bound to inorganic particles; apatitic (AP) and non-apatitic (NaIP) phosphorus. In general, apatitic phosphorus (AP) is phosphorus bound to calcium and non-apatitic phosphorus (NaIP) is phosphorus bound to non-calcium particles such as iron (Reynolds & Davies, 2001).

OP is divided into condensed phosphates and other organically bound phosphorus. The condensed phosphates category includes pyro-, meta-, and other polyphosphates (Hoffman, 1991). Other organically bound phosphorus can include organisms, detritus or chemicals such as pesticides and their degradation products. Detritus will release phosphorus in the form of common biological material such as nucleotides, inositol phosphates, phytin, and phosphorus monoesters.

Dissolved and particulate fractions of phosphorus are distinguished by filtration with a 0.45 µm membrane which separates algae, bacteria, and minerals but fails to exclude colloidal particles from the filtrate which is used to determine the concentration of dissolved P (APHA).

The colorimetric technique used to measure soluble reactive phosphorus (SRP) digests the sample and hydrolyzes all of the labile, or easily broken down, organic phosphorus (APHA). This loss of resolution of different phosphorus species is the reason the result of this test is no longer reported as orthophosphate but is instead called SRP (Kalff, 2002). Many articles use the term soluble reactive phosphorus (SRP)

interchangeably with soluble phosphorus (SP), orthophosphate, molybdate-reactive phosphorus (MRP), or dissolved phosphorus. SRP is the major form of bioavailable phosphorus (BAP) which is why it plays such a large role in the phosphorus cycle shown in Figure 2. Because different sources use different definitions and nomenclature related to SRP, it is important to define how it is used in this paper; the SRP is the unbound orthophosphates and the readily available inorganic phosphates associated with colloids, but does not include all dissolved phosphorus.

While chemical testing can interpret general phosphorus speciation from samples, phosphorus in the environment is very dynamic and undergoes many transformations within the ecosystem.

2.4 Phosphorus Transformations in the Aquatic Environment

Water Column

There are many factors affecting the speciation of the phosphorus in the water column including the presence of micro-organisms, trivalent metals, and colloids, as well as the oxygen content, and the pH. Particle bound phosphorus content in the water column is dependent on a lot of factors such as turbulence, and wind which prevent sedimentation and/or instigate resuspension of particles. Characteristics of the particles that phosphorus is sorbed or bound to will also affect if the phosphorus stays in the water column or has a tendency to sediment through increased aggregation or flocculation potential (Hoffman, 1991). Environmental conditions have a pronounced effect on the transformations and movement of phosphorus in the environment.

Sediments

One study found that 93% of the phosphorus entering a lake was immobilized into the sediments (Doremus & Clesceri, 1982). This makes the speciation of the phosphorus in the sediments particularly important to the bioavailability of phosphorus and the eutrophication potential within a lake.

There doesn't seem to be consensus on the distinction between sediment and soil. Some say that sediment is allochthonous material eroded from its parent material and redeposited while soil is developed in place with autochthonous materials such as plant remains that build up over time (Julien, 2010). Other sources ignore the difference completely, interchangeably using the terms soil and sediments (Aber, Pavri, & Aber, 2012). In this wetland in particular, the segregated definition presented above becomes unclear as the sediments that migrate into the wetland can get buried in the wetland with the plant detritus and thus the soils in the wetland can be considered a mixture of sediment and soil unique to each site and possibly even unique to each season as materials get washed out of and washed

in to the wetland. For the purpose of this paper, wetland soil will be used interchangeably with wetland sediment and defined as a mixture of allochthonous and autochthonous materials.

As depicted In Figure 5, smaller particles interact in the soil to form larger soil particles leaving pore space for air, and water movement. These associations can sometimes trap nutrients inside of the soil particle such that it is inaccessible for removal by water, or microbial interactions. The particle bound forms of phosphorus thus vary greatly on the geology of the rocks/particles in the sediment, and will vary greatly from lake to lake. Phosphorus in sediment can be found both soluble and insoluble forms, the soluble being found in the pore water.

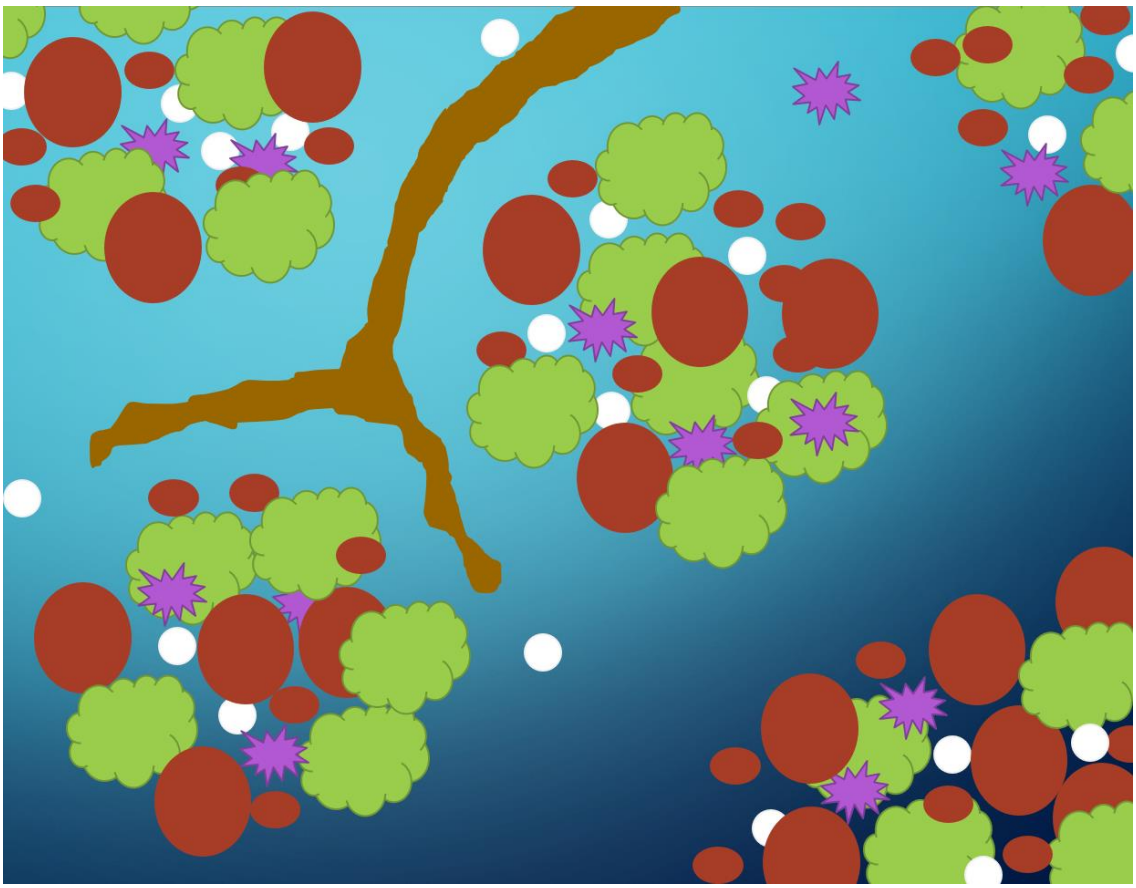

Figure 5: Diagram showing soil particles and water saturated pore space. The brown area represents a plant root. The green represents organic matter, red represents minerals such as clay, white represents air and purple represents nutrients. Figure adapted from (Wedlock, 2015).

Adsorption capacity is affected by the chemical and physical properties of the particles that make up the soil. The most common materials that interact with phosphorus in the sediments are humus, clay, and calcium (Ca), iron (Fe), or aluminium (Al) salts (Reynolds & Davies, 2001). Humus is the organic matter in

the soil that forms from the decay of plant and animal matter (partially decayed matter is non-humic) and it has a significant effect on soil moisture and nutrient retention (Barker & Pilbeam, 2007). Clay minerals primarily consist of a stacked-layered structure of crystalline aluminum or magnesium silicates (Stumm & Morgan, 1981). Aluminium, calcium and iron salts can be found in many environments usually in the form of variscite, calcite, and strengite rocks, respectively. Many rocks are a mixture of these minerals, such as shale , which is typically a combination of quartz and calcite layered with clay (Drizo, Frost, Grace, & Smith, 1999). Shale has a high clay content, and is easily broken into thin sheets giving it a larger surface area which allows it to have a high phosphorus adsorption capacity (Drizo et al., 1999).

There are many physio-chemical factors affecting the behavior of the phosphorus in sediments especially at the particle-pore water and sediment-water interface. These factors include adsorption/desorption, precipitation/dissolution, and advection/diffusion. Redox, pH, temperature, location are just some of the conditions which drive these interactions.

Redox potential

Aerobic sediments are considered a sink for phosphorus while anaerobic sediments, such as those found in eutrophic lakes, are considered a source of phosphorus for the water column. This is due to microbial activity and the redox potential of the sediment. The redox potential, Eh, is the potential of chemical species to participate in electron exchange reactions known as oxidation-reduction reactions (Langmuir, 1997). When Eh is positive than the system is oxidizing; and if the Eh is negative than the system is a reducing system.

As seen in Figure 6 below, oxygen is the most energetically favorable electron donor; it has a very positive redox potential. That means if oxygen is present, as in aerobic systems, then aerobic respiration will be dominant. After oxygen is depleted, there follows a succession of organisms capable of reducing NO_3^-, FeOOH, SO_4^{2-} and CO_2, with each oxidant yielding successively less energy for the organism mediating the reaction. Following the redox couples on the left of Figure 6, when nitrate is depleted, the reduction of nitrate stops and the reduction of ferric oxide (pH 7) begins. A wide range of anaerobic bacteria are able to conserve energy through the reduction of iron from Fe^{3+} to Fe^{2+}.

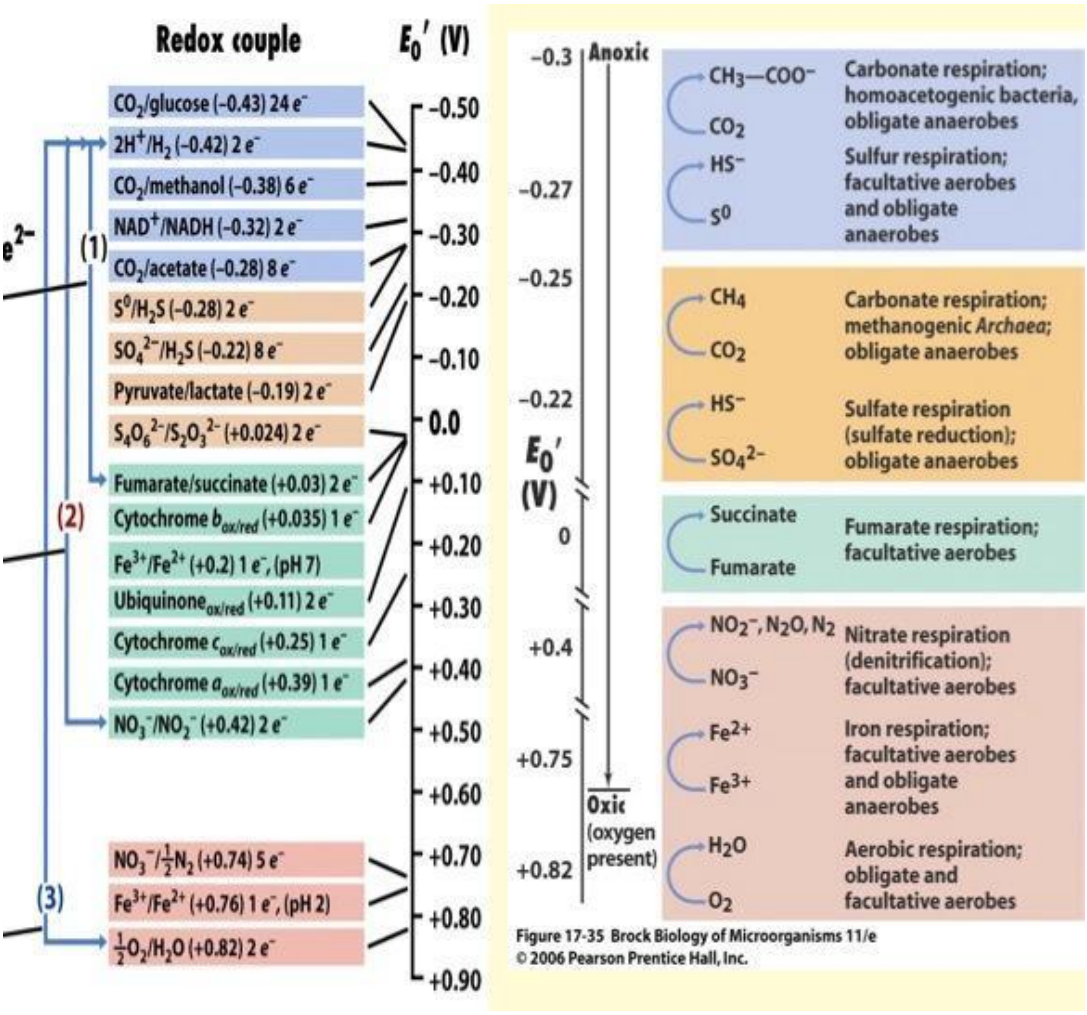

Figure 6: Redox tower showing microbial populations which thrive at different conditions (Madigan et al., 2012)

In wetland soil systems, these transitions from one electron acceptor to a less favorable electron acceptor tend to correspond to depth in the soil (Figure 7). Oxygen is introduced into the soil via diffusion from the atmosphere and as you get deeper in the soil, there is less free oxygen available because it has been used already. Aerobic conditions can be identified by the availability of dissolved oxygen (E_h >300mV) while anaerobic conditions are identified by the absence of dissolved oxygen (E_h <-100mV) (Kadlec et al. 1996).

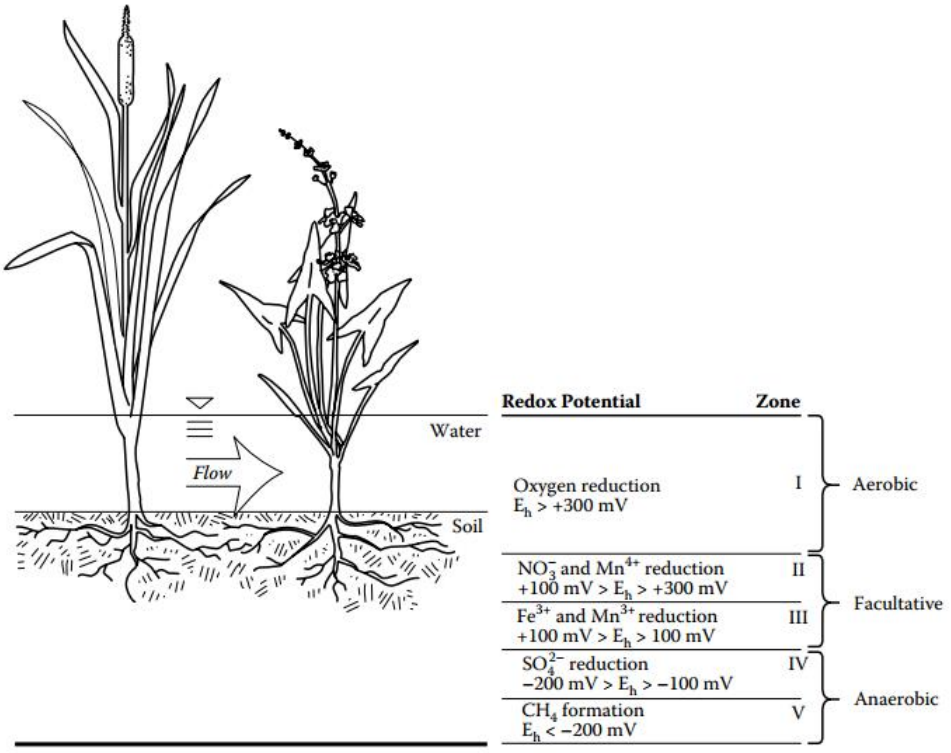

Figure 7: Redox potential in water, sediments, and soil in a wetland system (Kadlec & Wallace, 2008)

The facultative zone is where the redox potential is still positive, usually where dead plant debris has piled up and organic sediments have started to form but free oxygen is not always available. This is called the facultative zone because the organisms in this zone will use oxygen in aerobic respiration if it is available but can switch to fermentation or anaerobic respiration and reduce other substances if oxygen isn't available (Madigan et al., 2012). If an organism cannot switch its metabolic process it is called an obligate aerobe or an obligate anaerobe (Madigan et al., 2012). One the left side of Figure 6, some of the reduction are shown along with what kind of organisms can perform these processes. The anaerobic zone is deeper, consisting mostly of organic sediments and mineral soils and has a negative redox potential.

The availability of oxygen in the wetland is dependent on the flow of the water and how well mixed it is, as well as the adaptation of plants in the wetland. In water systems with no flow, the availability of oxygen in the water would also have a direct relationship with the distance to the surface. If good mixing is occurring in the water then more oxygen will be available at depths. Ultimately, wetland environments tend to be shallow but can be very biologically active and thus this mixing is important to keep the sediments oxidized.

Redox potential does not directly affect the inorganic phosphorus speciation as the oxidation state of phosphate ions (H_3PO_4, $H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}) is +5 and it is not directly involved in the transfer of electrons (Langmuir, 1997). Phosphorus is indirectly affected by changes in redox potential.

Both the oxygen and nitrate content of the sediments are important with respect to iron. In oxidized soils, inorganic phosphorus is present in the form of ferric phosphate; the mineral form is FePO4, strengite. Once the oxygen and nitrate have been reduced, microbes will begin to reduce iron. Under anaerobic conditions, the iron works as an electron acceptor and is reduced to ferrous iron releasing the phosphate as shown below (Stumm & Morgan, 1981).

$$FeNH_4PO_4(s) = Fe^{+2} + NH_4+ + PO_4^{-3}$$

$$Fe_3(PO_4)_2(s) = 3Fe^{+2} + 2PO_4^{-3}$$

Figure 8 shows increased P with depth in soils/sediments and decreased sulfate which implies anaerobic conditions and a negative redox potential. The phosphorus concentration increasing with depth then makes sense as iron is reduced releasing sorbed phosphorus. This phosphate is still not readily available because there would still be a reverse gradient of phosphate back into the environment and as the phosphate diffuses toward more P limited environments it will get consumed or resorbed. If there is a steady flow of water filtering through the soil, this phosphate could also continue to travel through the soil with the water.

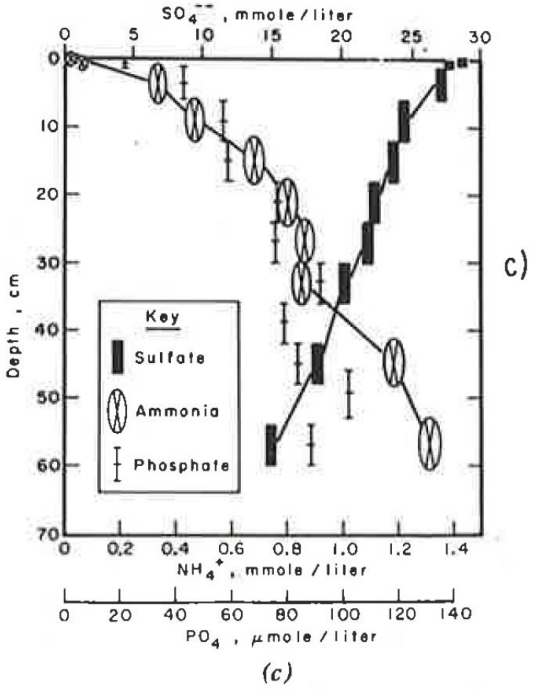

Figure 8: Change in sulfate, ammonia and phosphate concentration with depth in soil (Stumm & Morgan, 1981).

pH

Soil that contains clay and organic material tends to have a higher cation exchange capacity and therefore are able to resist changes in pH (Sparks, 2003). Humus and clay are both negatively charged and thus will have an area of low pH surrounding their particles due to the attraction of H^+ ions. Because they are negatively charged they will also attract and retain metals such as iron and calcium.

As previously discussed, iron and other metals play a very large role in the immobilization of phosphorus in the sediments. Phosphoric acid (SRP) is strongly attracted to iron in the form of Fe (III) and can be precipitated as $FePO_4$ under aerobic conditions (Peretyazhko & Sposito, 2005). When compounds are introduced that have a stronger affinity for iron, such as sulfur, this results in a release of soluble phosphorus as the iron binds to the other compound (Kalff, 2002).

Figure 9 shows the solubility of different metal phosphates at different pH. Around natural pH, some phosphate will be fixed by aluminum, iron and calcium if they are present in the soil. As the pH changes it will affect the fixation of this phosphorus. Increasing pH from 5.5 will cause the formation FeOOH-OH and will desorb the phosphorus from the iron (Wetzel, 2001). Increases in pH will also result in decreased Al fixation but increased calcium fixation of phosphorus. In alkaline, calcite sediments, the calcium ion is the dominant cation and as the alkalinity increases the phosphor species become less soluble and less bioavailable. Phosphorus release into sediments can cause rapid nucleation of amorphous calcium fluorapatite in the sediments which eventually stabilizes to Apatite (most stable phosphatic phase) (Konhauser, 2007). Decreases in pH will result in decreased calcium fixation but increased iron and aluminium fixation to a point, once a certain acidity is reached, seen in Figure 9 as the inverse peak of the curves, the Al and Fe fixation of phosphorus will decrease as well. The solubility of these metal salts is also dependent on interaction with other chemicals available in the environment. For example, aluminum phosphate can form very insoluble double salts with potassium and nitrogen called Taranakite which are often found in fertilized areas (Lindsay, 1979). Phosphate is most available (soluble) under neutral to slightly acidic conditions.

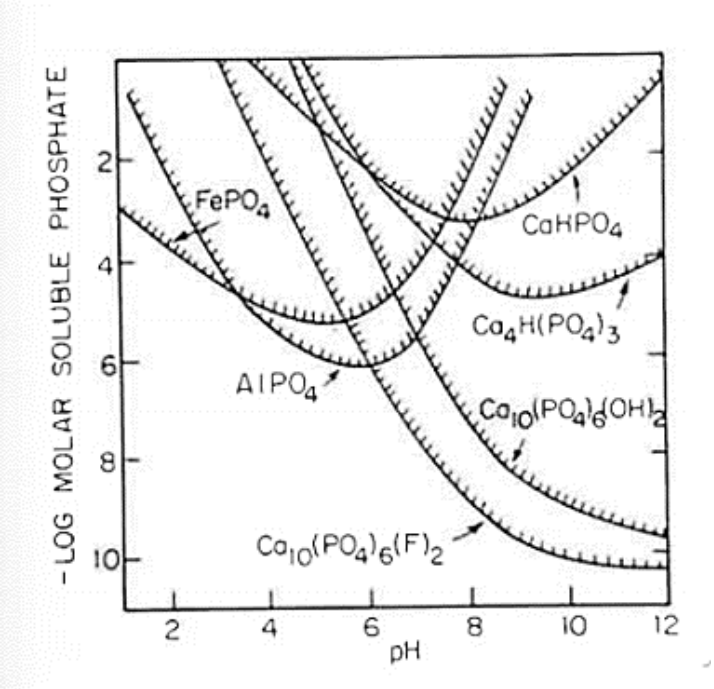

Figure 9: Solubility of metal phosphates in aquatic environments (Stumm & Morgan, 1981)

While redox did not directly affect phosphate, pH has a very noticeable effect. Figure 10, below, shows the phosphoric acid speciation based on pH. Phosphoric acid is a weak acid that is soluble in water. As seen in Figure 10 under acidic conditions, the dominant species of phosphoric acid is H_3PO_4 and under natural conditions, pH 7, the dominant phosphate species are $H_2PO_4^-$ and HPO_4^{2-}.

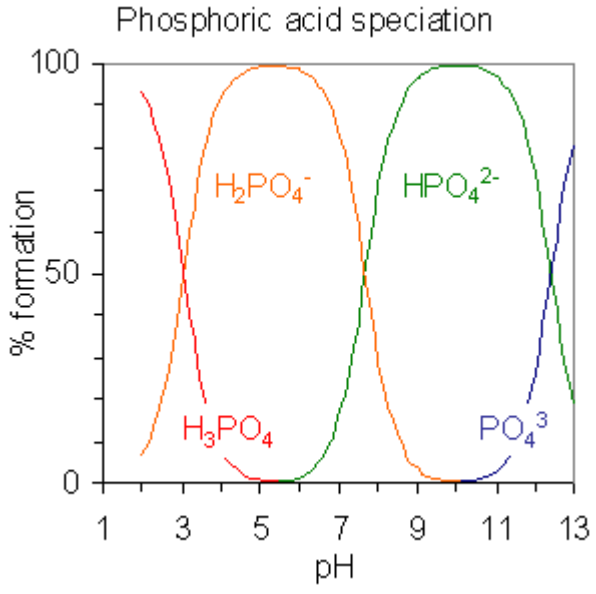

Figure 10: Percent formation of species of phosphoric acid based on changes in pH (Open Source)

There are some organisms that like pH higher than neutral conditions and some that prefer the pH to be lower. In general, the wetland environment is going to be at a neutral pH. Any significant increases or decrease in pH for a long period of time would stress and most likely kill the microbial population as well as plants in the wetland.

pH changes also affects organically bound phosphorus by causing the breakdown of poly- and meta-phosphates. Both poly- and meta- phosphates are stable at neutral pH but if the pH increases or decreases significantly, they break down rapidly into monophosphates (Hoffman, 1991).

Overall, the pH can have a significant effect on the speciation and availability of phosphorus.

Temperature

Higher temperature increases the speed of chemical reactions as molecules get excited and have more interactions with each other (Sparks, 2003). Research has shown that the adsorption of phosphorus to sediments actually increases with higher temperatures (Sugiyama & Hama, 2013). As the temperature increases, the poly- and meta- phosphates breakdown rapidly into monophosphates (Hoffman, 1991) which can be due to both chemical and biological reactions. Increased water temperature also results in increased decomposition. Other microbially-mediated activities such as extracellular enzyme hydrolysis and catabolic activities will also be affected by temperature.

As seen in Figure 11, within the range of natural temperature increase, biological reactions also increase with increased temperature (Banerjee, 2012). Higher temperature and increased microbial activity can increase the weathering of primary minerals which results in soluble phosphorus in the form of orthophosphates being released into the water (Peretyazhko & Sposito, 2005). Mineralization is the release of inorganic phosphorus from organic phosphorus, caused by micro-organisms in a process known as heterotrophic degradation (Konhauser, 2007). This process occurs more in warm, aerated soils because this is where decomposition is occurring. The phosphorus released can interact three ways: it can be stored as energy by micro-organisms, it can be adsorbed onto sediment particles, or it can diffuse into the water column (Konhauser, 2007). In order to break down these phosphorus containing materials, microorganisms release enzymes such as phosphatases into the environment where they remove phosphate groups from substrates (Madigan et al., 2012). The higher the biological diversity in the area, the greater the diversity of phosphatase enzymes will be and the greater number of phosphorus containing materials will be broken down. One study even suggests that the substrate is not the limiting factor, the concentration of enzymes is the limiting fraction (Van Moorleghem, De Schutter, Smolders, &

Merckx, 2013). Ecosystems at neutral pH, such as wetland environments, will have the greatest biodiversity because the organisms do not need special adaptations to live there (Madigan et al., 2012).

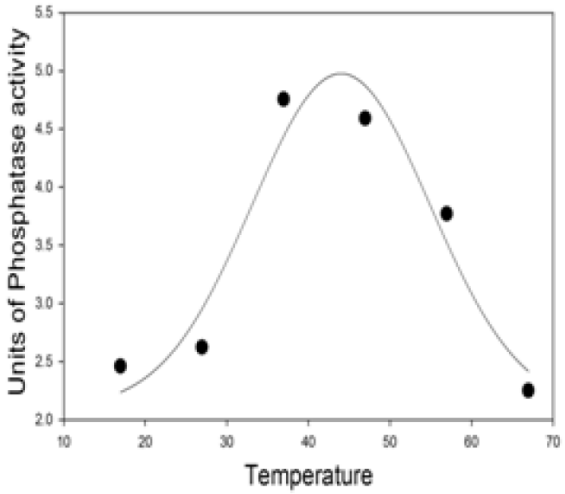

Figure 11: Effect of temperature in Celsius on phosphatase activity in soil (Banerjee 2012)

Location

Physical location of the phosphorus is also very important to the speciation. In the water column there is less interaction because of the dilution effect, while in the sediments there can be a variety of chemicals and interactions occurring. Shallower water tends to have more circulation, more oxygenation and more light infiltration than the deep zones in the lake (Wetzel, 2001). More light penetration means more primary production and more assimilation of phosphorus by organisms. Shallow, well-lit water also tends to be closer to atmospheric temperature and thus will be warmer in the summer and colder in the winter. Another aspect of physical location is depth, phosphorus containing sediments can get buried beneath other sediments effectively making that phosphorus unavailable to interact with the water column, and possibly containing the phosphorus in an anaerobic environment. Physical degradation processes can also have an effect on the phosphorus content and speciation in the sediments such as weathering, wind-induced water circulation, re-suspension and mixing of sediments and bioturbation (Wetzel, 2001). The reactions occurring in the sediments and the resulting diversity of phosphorus compounds has a significant effect on the bioavailability of the phosphorus.

2.5 Bioavailability of Phosphorus

In earlier sections the phosphorus cycle and the phosphorus speciation in aquatic environments was reviewed. This section will look at those forms and analyze their bioavailability.

Bioavailable Phosphorus (BAP) is phosphorus that can be utilized by plants and bacteria. It includes orthophosphate as well as a fraction of TDP and the fraction of PP that is readily usable therefore none of the commonly measured P fractions are accurate as a measure of total BAP. In general, all forms of P are more bioavailable in a p-limited environment because the number of enzymes released increases as the organisms become more desperate for phosphorus.

Readily bioavailable phosphorus is orthophosphate, free or adsorbed, which can be take up directly without conversion.

Biogenic phosphorus is dissolved organic phosphorus and particulate phosphorus originating from organisms, including organic P such as phosphonates, orthophosphate diesters and monoesters as well as condensed (pyro- and poly-) P (Jørgensen, Inglett, Jensen, Reitzel, & Reddy, 2015).

The phosphorus speciation diagrams presented in Figure 4 can be simplified as shown in Figure 12 below to emphasize the bioavailability of different fractions.

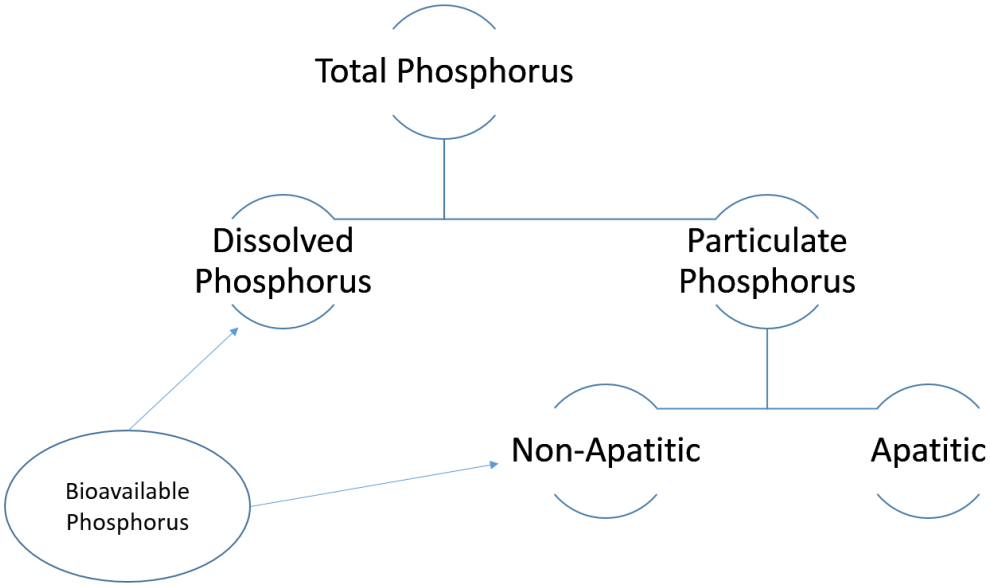

Figure 12: Simplified P-Speciation diagram

Bioavailability of Dissolved Phosphorus

In the aquatic environment, phosphorus is only present in low concentrations and is a limiting nutrient for many species. Because of this, aquatic plants have evolved very efficient phosphorus uptake mechanisms (Reynolds & Davies, 2001). Phosphorus is a macronutrient and it is taken up by plants as orthophosphate; $H_2PO_4^-$ and HPO_4^{2-} (Ridge, 1991). This means phosphate is easily incorporated into organisms via simple uptake mechanisms and therefore is bioavailable. But orthophosphates are also very reactive in general so they have a tendency to quickly adsorb to particles, especially metals, decreasing their bioavailability. Orthophosphates are both biologically available and chemically available hence many studies refer to them as part of soluble reactive phosphorus (SRP).

The other component of dissolved phosphorus in the form of colloids. These are particulate bound phosphorus that are so small they remain suspended in the water column and thus interact more. This increased interaction makes them more biologically and chemically reactive than particles in the sediments, but they aren't necessarily bioavailable. One study found that the bioavailability of phosphorus in the dissolved (soluble) fraction varied from 0-55% (Van Moorleghem et al., 2013). From the same study, these dissolved forms include nucleic acids (readily available), monoesters (variable availability), and humic acid and phytic acid complexes (not available),

Bioavailability of Particulate Phosphorus

As discussed in the previous section, orthophosphates are readily bioavailable and thus the particulate phosphorus has a much more significant impact on the variation in bioavailability. A fraction of SRP is particulate phosphorus but is readily soluble meaning it is ready to diffuse into solution and thus under the right conditions it is also highly bioavailable. Table 1, below, shows the bioavailability of different fractions of phosphorus within the sediments.

Table 1: Phosphorus fractions in sediments and their significance (B. Ya. Spivakov, 1999)

Phosphorus Fraction	Bioavailability
Adsorbed phosphorus	Easily available fraction
Non-apatitic phosphorus	Available in absence of oxygen
Apatitic phosphorus	Not available (stable and inert)
Organic phosphorus	Slowly but continuously available

As discussed previously, the speciation and thus the bioavailability of particulate phosphorus is variable based on many factors including the pH, oxygen availability, temperature, light penetration. pH not only affects the speciation of phosphorus but also affects the micro-organisms present which will affect the amount of phosphorus that can be consumed. 10-75% of sediment phosphorus is held within the cells of sediment microbes so microorganisms can play an important role in the retention of bioavailable phosphorus in the sediments (Kalff, 2002). By maintaining a high sediment redox potential through elevated concentrations of nitrate and sulfate, microbes help keep soluble phosphorus in the sediments (Kalff, 2002). Iron also helps to keep phosphorus in the sediments by creating a similar redox barrier between the sediments and the water column (Reynolds & Davies, 2001). Fluctuations from anoxic to oxic conditions have been shown to not slow the degradation of biomass (and subsequent release of soluble phosphorus) but the anoxic conditions cause the reduction of Fe(III) to Fe(II) releasing the sorbed phosphorus into the readily bioavailable fraction (Peretyazhko & Sposito, 2005). Bacteria release soluble reactive phosphorus during cell lysis under aerobic conditions and during anaerobic conditions they release solubilized polyphosphate granules (Kalff, 2002). Because of the anoxic conditions, organisms that can take up this released phosphorus may or may not be available to utilize this phosphorus released from organisms and the sediment.

Organic forms of phosphorus such as those found in detritus need to be converted to inorganic phosphorus in order to be bioavailable (Reddy 2008). Organic P forms can be generally grouped into easily decomposable and slowly decomposable organic P. The slowly decomposable group consists of biological materials such as nucleic acids, phospholipids and sugar phosphates while the slowly decomposable contains inositol phosphates (phytin) (Reddy 2008). Inositol phosphates are complex cycle compounds, containing a lot of oxygen and as much as one third of the inositol P will be complexed with humic and fulvic acids, thereby further reducing bioavailability of this organic P (Reddy 2008). Other organic forms of phosphorus include pesticides and herbicides which would be included in the particulate fraction because pesticides in general are not made to be water soluble because it would decrease their effectiveness (Alexander, 1999). The bioavailability of these compounds is variable as they may be toxic to some species and easily broken down by others.

pH is particularly significant when it comes to adsorption and precipitation of phosphorus. Figure 13 shows the correlation between pH (x-axis) and different forms of sorbed and precipitated phosphorus (left axis) and compares this to bioavailability (right axis).

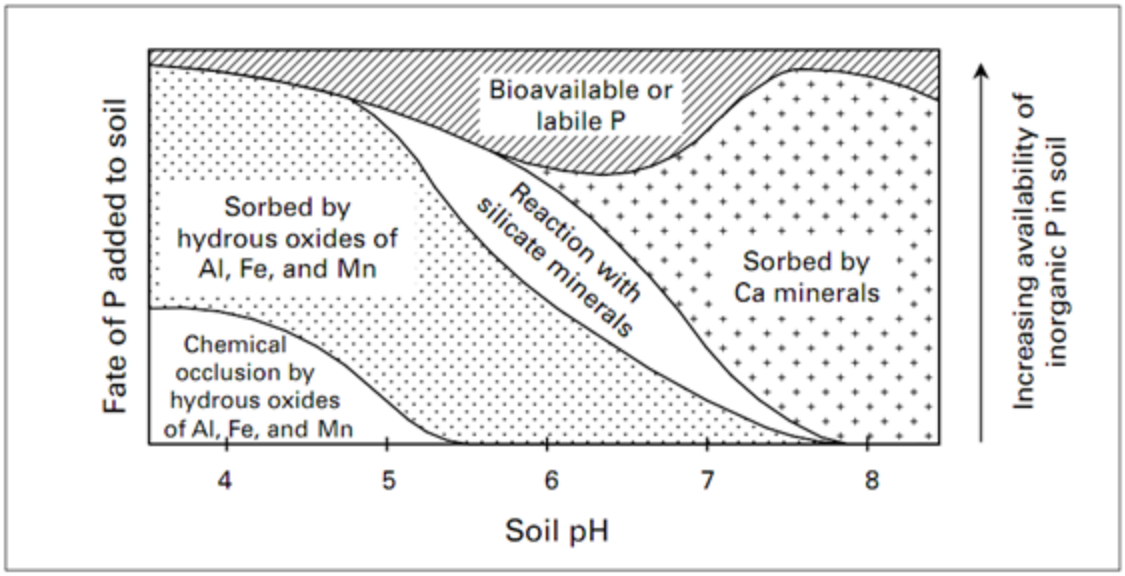

Figure 13: Bioavailability of soil phosphorus compound in relation to pH (Ebrary)

Sediments are of important when it comes to the eutrophication potential of phosphorus within a lake system and the conditions within the lake control the speciation and thus the bioavailability of the phosphorus contained within the sediments.

2.6 Plants, Organisms, and Phosphorus

The role of phosphorus in the environment is defined by how it is used. Phosphorus is used in the most basic building blocks of our cells and thus it is very important for growth. This relationship in plants and organisms is defined by the Redfield ratio, photosynthesis, and Liebig's law of minimum. Some wetland plants have adapted special to wetland environments in order to increase their success at obtaining essential nutrients such as phosphorus.

Redfield ratio

In 1934, British marine biologist Alfred Redfield found that the elemental composition of organic matter in the ocean is remarkably uniform (Falkowski & Raven, 2013). This ratio of elements has become known as the Redfield Ratio and, in the field of biology, it is typically used to define the ratio of carbon to nitrogen to phosphorus in organisms. Originally he defined it as 106 C:16 N:1 P, and that is a good rule of thumb, but over time that ratio has been refined for particular environments and organisms as seen in Table 2 (Falkowski & Raven, 2013). The EPA algal bioassay method defines the ratio as 11 N:1 P for this algae in particular (Agency, 1978).

Table 2: Molar C:N:P ratios for selected groups of organisms. Adapted from (Singer, 2016)

	Relative molar abundance		
Group	C	N	P
Bacteria (soil)	46	7	1
Fungi	165	11	1
Trees (foliage)	1212	28	1
Marine animals	64	12	1
Marine Plants	265	18	1
Humans	84	6.3	1

Photosynthesis

In the wetland, both plants and microorganisms use photosynthesis as a way to get energy from the sunlight in order to drive their metabolic cycle. Photosynthetic reactions are light dependent; photosynthetic organisms consume O2 in the dark via respiratory processes and produce O2 in the light (Madigan et al., 2012). Light intensity is important to photosynthesis and the rate of fixation. Figure 14 shows the relationship between light intensity and the rate of photosynthesis.

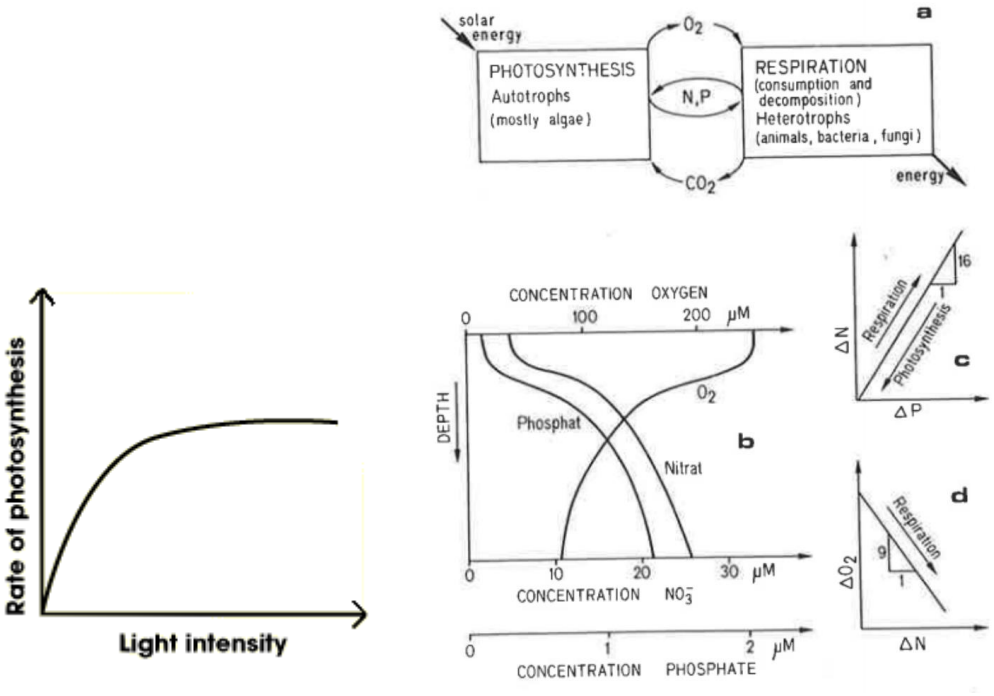

Figure 14: Effect of light intensity on the rate of photosynthesis (left) and the dynamics between photosynthesis, respiration and nutrients (right). (Stumm & Morgan, 1981)

The rate of photosynthesis in these organisms drives growth. Many components in the cell contain phosphorus including phospholipids, to nucleic acids, to coenzymes. Plants can also store phosphorus, such as when they are making seeds, it is stored as phytic acid (Barker & Pilbeam, 2007). It can also be stored in vacuoles and the cytosol as inorganic orthophosphate and polyphosphate (Falkowski & Raven, 2013). On the right of the figure you can see that photosynthesis results in a net uptake of phosphorus while respiration processes such a degradation result in a net release of phosphorus.

Algae Growth Kinetics and Liebig's Law of Minimum

Growth is defined as an increase in the number of cells in a population and dynamic growth includes 4 phases; lag phase, exponential growth phase, stationary phase and death (decay) phase (Madigan et al., 2012). The lag phase is the period of low to no growth before the exponential phase where organisms introduced into a new begin producing the enzymes and cell components necessary to survive and grow. Exponential growth phase is the most rapid growth phase where cell division is occurring, but this growth is limited. Exponential growth in a batch culture can be limited by nutrients, or it can be limited by the increase in toxic byproducts of the organism's growth. Stationary phase happens when the limit of exponential growth phase is reached and the population stabilizes with growth and decay balancing each other. The death (decay) phase occurs when the population begins to decline.

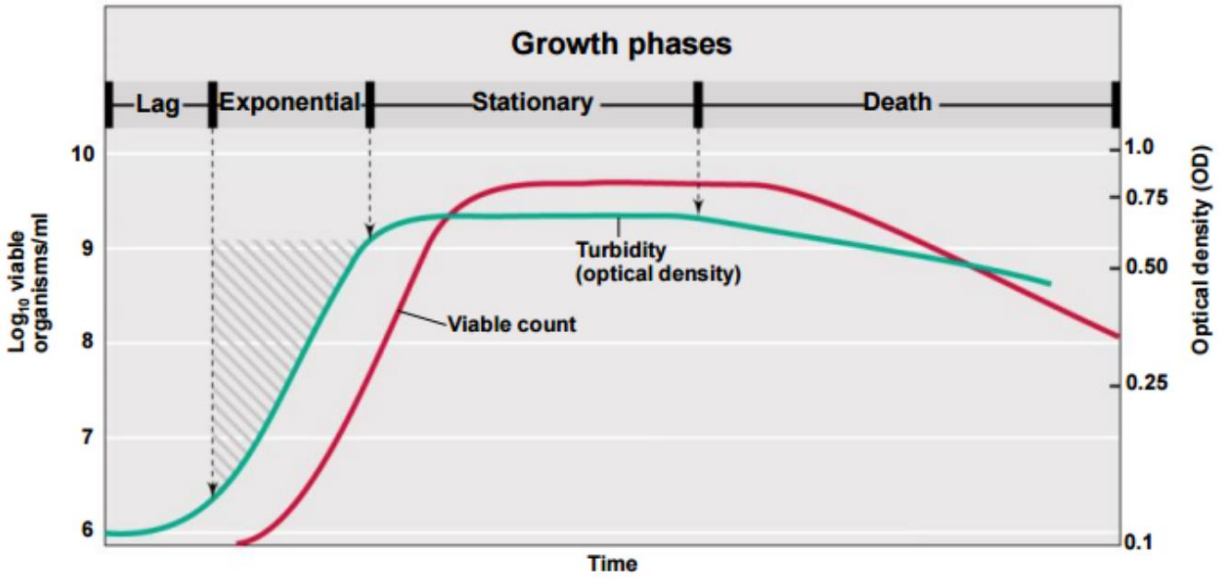

Figure 15: Graphical depiction of the growth phases of microorganisms (Madigan et al., 2012)

The maximum yield of biomass is proportional to the concentration of nutrients that are present and biologically available in sufficient quantity for the growth requirements of the organisms (Agency, 1978). As discussion previously, the ratio of elements C:N:P varies per organism and is important in regulating the maximum yield. In Figure 15, the yield can be expressed as the initial organisms per ml subtracted from the organisms per ml at stationary phase. Generally this is then multiplied by an average organism size in order to produce the amount of biomass created. Through the C:N:P ratio, this can be correlated to the amount of phosphorus that was consumed.

Liebig's law of minimum defines that this growth will be limited not by the total amount of nutrients available but by the scarcest nutrient (Barker & Pilbeam, 2007). The scarcest nutrient is not always the one in least concentration as it still needs to hold to the C:N:P ratio. For example if you have 4 g/l of N and 1 g/l of P, you have a smaller concentration of P but you are limited by N because the Redfield ratio defines the relationship of nitrogen to phosphorus by weight to be 5:1.

Adaptations of Wetland Plants

Aquatic and wetland plants are knowns as hydrophytes. Hydrophytes must cope with many stressful conditions including a lack of oxygen, flooding, drying, a lack of nutrients, and low pH.

The most important adaption for plants living in aquatic environments is a mechanism for transporting oxygen to the roots. The most common is an extremely porous tissue designed to move oxygen from the leaves above the water to the roots called aerenchyma (Aber et al., 2012). The aerenchyma also enables reverse transport of methane from the anaerobic zone to the atmosphere (Pezeshki & DeLaune, 2012). Some other plants have root segments that grow upwards from the roots allowing for oxygen diffusion, while wetland trees tend to have large pores in their trunks (Aber et al., 2012). Many of these structures serve a double function for wetland plants as the water logged soil is soft and does not provide a lot of support these air filled pockets help keep the plant from sinking into the sediments (Aber et al., 2012). This area around the roots is called the rhizosphere, and because of the infiltration of oxygen, a gradient of soil available oxygen forms (Figure 16). This creates an oxidizing environment around the roots leading to microbial activity and oxidation of iron.

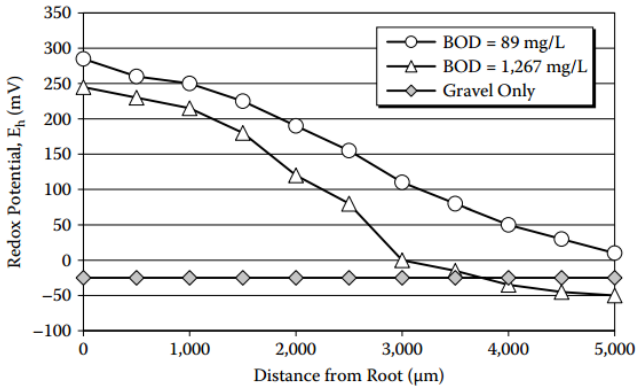

Figure 16: Change in redox potential with increased distance from roots (Kadlec & Wallace, 2008)

Phosphorus uptake through the roots in catalyzed by the presence of microbes and fungi in the soil. The release of oxygen from the roots is part of a symbiotic relationship between the plant and these microbes. Some of the processes involved in this relationship are shown in Figure 17.

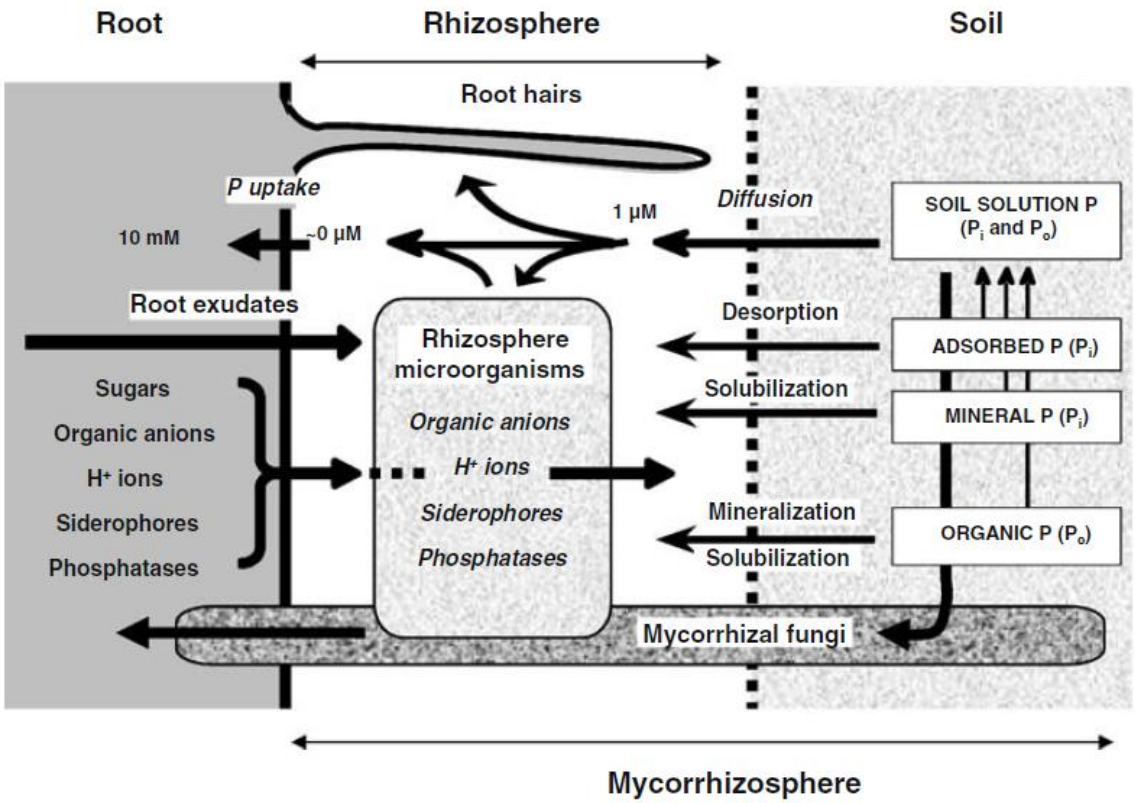

Figure 17: Visualization of the processes occurring in the rhizosphere (Richardson, Barea, McNeill, & Prigent-Combaret, 2009)

The rape plant is known to mine alkaline soils for phosphorus by producing an acid around its roots that solubilizes the phosphorus in a process called “Rhizosphere acidification” (Ridge, 1991). This emphasizes

that biological activity such as acidification, respiration and enzyme release can have a significant effect on the uptake of phosphorus and ultimately on the speciation of phosphorus in this environment.

2.7 Constructed Wetlands

Constructed wetlands are designed and built with a specific purpose in mind whether it be removal of a specific contaminant or general treatment of storm water or wastewater before it is released into the receiving waters. The purpose of the wetland will affect the size and layout of the wetland in order to accomplish the treatment goals. In general, all wetlands will be composed of several zones to facilitate the necessary mechanisms of increased hydraulic retention time and pollutant retention. These zones include the inlet zone, the bypass for high flow zone, macrophyte zone, outlet zone, and open water zone (Kadlec & Wallace, 2008). All of these zones, except the bypass can be found at the Leikvollbekken wetland, Figure 18. The inlet zone begins as water exits the drainpipe and encounters a rock barrier intended to slow down the water and disperse it over the larger area of the wetland. Then there is an open water zone to allow for sedimentation after which the water goes through a macrophyte zone where nutrients are removed. A pipe is used to convey water between pond 1 and pond 2 so permeable barriers made up of rocks are used to slow down and filter water between the 2 ponds. Pond 2 begins with a macrophyte zone and ends in an open water zone for final sedimentation before the water exits the wetland via a rocky stream and V-notch weir. As far as is documented, this wetland was built without a bypass.

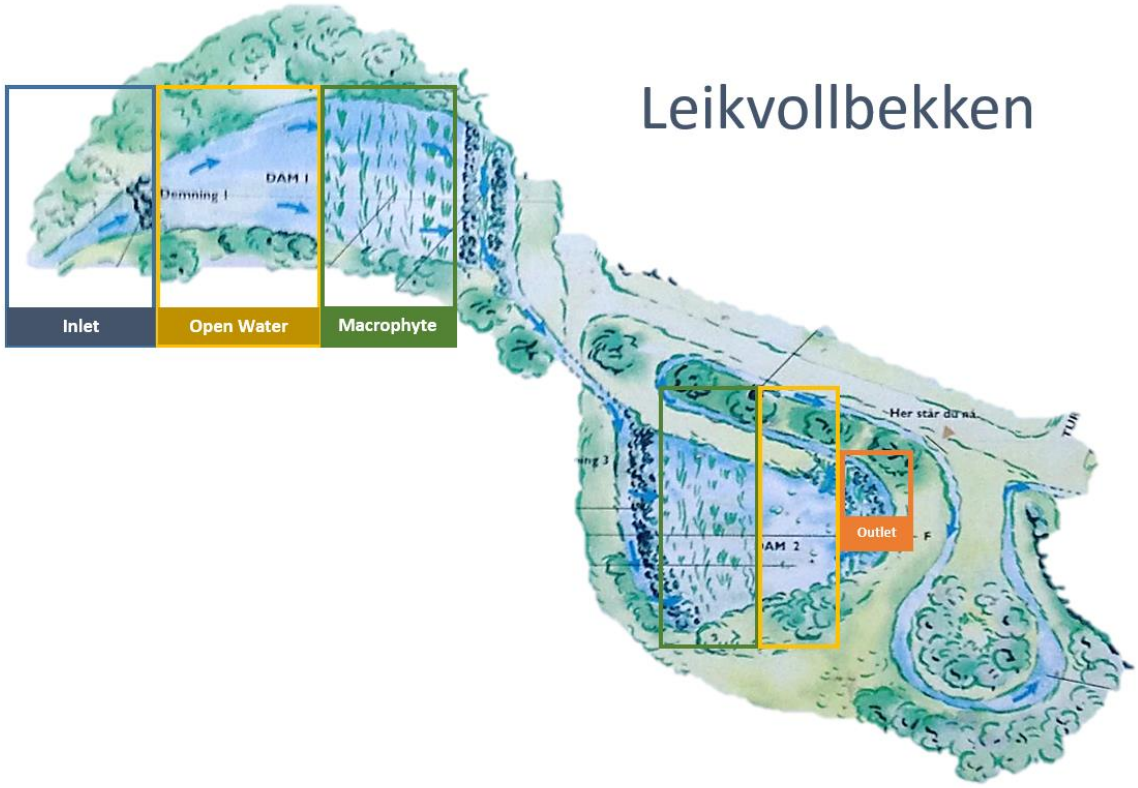

Figure 18: Diagram of Leikvollbekken with emphasis on zones. The inlet is shown outlined in dark blue, the open water zone is outlined in yellow, the macrophyte zone is outlined in green, and the outlet zone is outlined in orange. Adapted from the sign posted at the wetland

Leikvollbekken was built in 1994 and it is unsure how much maintenance has been done on the wetland. It is possible that the open water zones meant for sedimentation now contain over 20 years of sediment and thus have converted to macrophyte zones as the sediment layer approached the surface and plants began growing there. Analysis of the state of the wetland was not done for the purpose of this thesis but it can visibly be seen that plants are growing in the areas marked as open water zones. It is also possible that these zones have been maintained by natural wash outs of sediment materials during high flow periods. When sediment samples were attempted in the wetland, the majority of the sample was root materials which supports the idea that washouts can and have occurred and the plants are not sitting directly on sediment but instead of supported by a network of their own roots. This allows for filtering of the through flow and possibly sedimentation. A lack of maintenance can also affect the ability of the wetland to remove phosphorus from the ecosystem because without biomass removal, the plants which have retained the excess phosphorus will die in the winter and decay on site. This detritus is then susceptible to wash out into the lake during heavy rainfall. It is made more susceptible by the lack of a

bypass in this wetland. During heavy rain events, the excess water is not flowing through a separate channel, but instead flowing rapidly through the wetland which will re-suspend and wash out sediments. During especially heavy events water flow may creep outward from the defined wetland and wash out soil from places around the wetland which are usually not saturated. Sediment samples were successfully taken at the outlet of the wetland near the V-notch weir. Anne described the sediment as a floc, an unconsolidated layer of saturated material that is loose (Haws, 2016). This indicates that the sediments making it through the wetland and out of the outlet are fine particles that are resistant to sedimentation.

The soil types local to Stavanger and this wetland are considered poor quality for farming because they are eluviated, i.e. the minerals and clays have migrated to deeper in the soil (Hartemink, 2006). The area around the wetland is farmland, so it is likely that each year truckloads of top soil are brought in to supplement and improve the natural soil. Because of this, we can expect high sediment loads to the wetland of nutrient rich soil that has run off of the top of the farmland. It has been defined as having a low cation exchange capacity meaning it has a low ability to hold on to cations and other nutrients (Hartemink, 2006).

Chemical Transformations and Mechanisms for Phosphorus Removal in the Wetland

Figure 19 depicts the transformations and mechanisms for phosphorus removal in the wetland which includes:

- direct uptake of phosphorus by plants
- direct uptake by bacteria and other organisms in the ecosystem
- degradation of bound phosphorus by enzymes from micro-organisms on the plant roots
- sedimentation of solids due to the decreasing velocity of flow through the wetland
- filtering of large particles by the rock barriers and the roots of the plants
- adsorption of nutrients by particles and sediments
- death and sedimentation of microbes and plants containing phosphorus

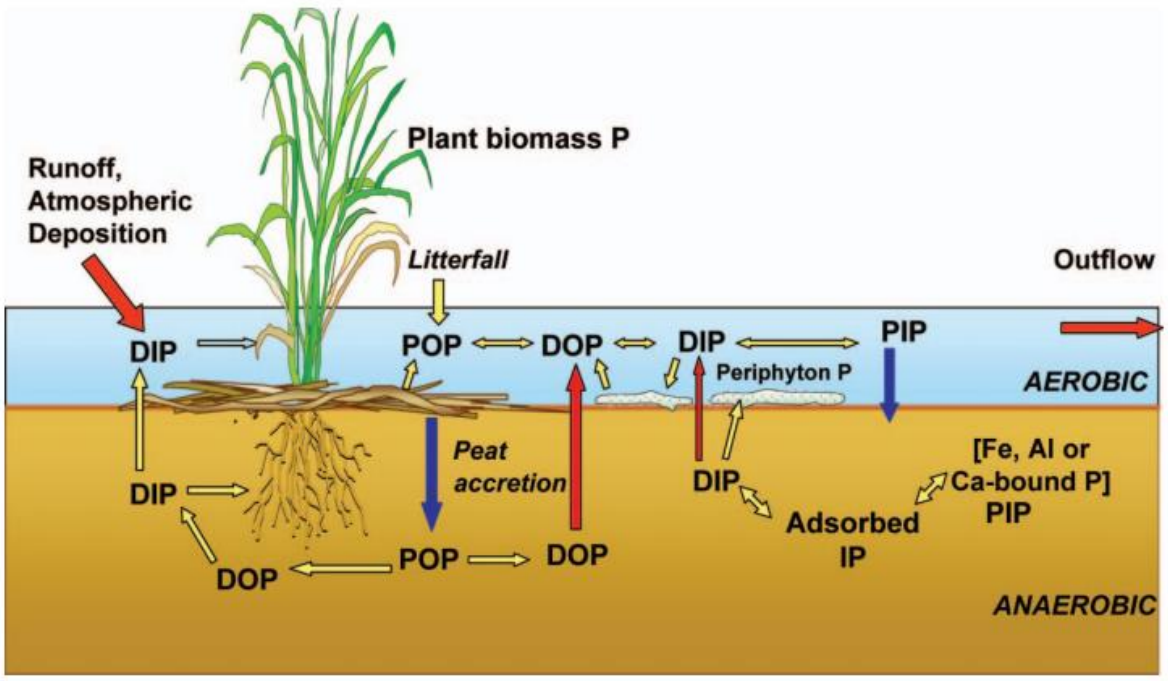

Figure 19: Phosphorus cycle in wetlands (Reddy & DeLaune, 2008). Dissolved inorganic phosphorus (DIP); dissolved organic phosphorus (DOP); particulate organic phosphorus (POP); particulate inorganic phosphorus (PIP); inorganic phosphorus (IP).

Some studies have shown that the efficiency of these phosphorus removal mechanisms decreases with age. In one study, suspended solids removal and total phosphorus removal declined over time as the gravel was clogged with solids and the sorption capacity for phosphorus was reached (Tanner, Sukias, & Upsdell, 1998).

Flow rate through the wetland and retention time of water in the wetland both have a significant effect on the efficiency of these mechanisms. At higher flows, particles will not settle and will possibly get resuspended from the wetland (Figure 20). As water rapid flows through soil pores (Figure 5), some soil particles will get resuspended making the soil pores larger. Eventually the soil will become too heavy for these larger soil pores and the soil particles will collapse, compacting itself and reducing the soil pore space. In this way the soil in the wetland is constantly restructuring itself and the flow of water through it will change over time. If something, such as plant roots, give this soil more restructure than the flow of water can be slowed by the roots and have less effect on the soil. If the retention time of the water is not high enough, the microorganisms will not have had enough time in exponential growth phase to consume the incoming nutrients.

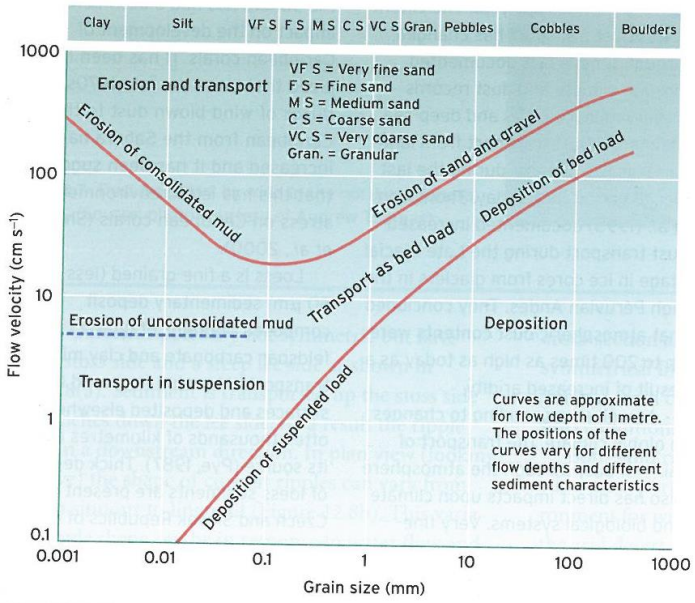

Figure 20: Erosion and deposition limits based on particle grain size and flow velocity (Wetzel, 2001).

There are many factors associated with the efficient uptake and removal of phosphorus by the constructed wetland. In this way it is important that the wetland is designed for the location taking into account soil type and temperatures and is designed for its input in order to get the desired effluent water quality.

2.8 Objective

The objective of this study is to determine if the constructed wetland has an effect on the bioavailability of the phosphorus. The hypothesis of this study is that the wetland decreases the bioavailability of the phosphorus through passive and active processes such as sedimentation, uptake of the soluble reactive phosphorus and release of organically bound particulate phosphorus. This will be analyzed by performing algal bioassays on samples taken from the wetland at different times of year in order to determine the bioavailability of the phosphorus. Both the inlet and outlet will be tested separately in order to get an idea of the performance of the wetland.

3. METHODS

These methods are based on methods provided by Åge Molversmyr titled "Algal Growth Procedures" supplemented with information from the EPA Algal Assay Bottle Test (Agency, 1978).

3.1 Materials

Culture Flasks: Duran rounded flasks

Scale for solution preparation:

Ohaus PA4102 (0.01 g readability)

Scale for filling flasks with sample:

Ohaus CS2000 (1g readability)

Coulter Counter: Beckman Coulter Multisizer 4 Serial Number: AV11021 Software: Particle Characterization Version 4.01 (2008)

Shaker Table: Edmund Bühler SM-30

Lightbulbs: Sylvania Fluoro Bulbs (high blue and red light emittance for plant growth)

Flow Cytometer: Accuri C6 Serial Number: 3073 Software: CFlow version 1.0.227.4 (2008)

Spectrometer: Spectroquant Pharo300 Serial Number 104321401 Software: 1.40-Merck-1.7 (2010)

3.2 Wetland Sampling

For the purpose of this experiment, wetland samples were collected weekly from both the inlet and the outlet of the wetland. Wetland samples were composite samples comprised of a subset of smaller samples taken over the course of a week. The inlet auto-sampler broke in March 2016 and was not longer able to take samples proportional to the flow of the water. So inlet samples were taken based on time and the outlet samples were taken proportional to flow. On site, composite samples were collected, untreated, in two large plastic bottles, one for the inlet sample and one for the outlet sample. Wetland samples were collected from the site in 2L glass bottles, brought to campus and stored in a refrigerator overnight. Chemical analysis of samples include iron content, ortho-P, TP and filtered TP. Wetland samples were monitored, collected, and chemically analysed by Anne-Marie Haws (Haws, 2016). The day after chemical analysis, samples were prepared and used for the algal bioassay.

Occasionally grab samples were collected due to freezing conditions or equipment failure. These samples were taken at approximately the same location as the composite sample intake locations.

3.3 Sample Preparation

Wetland samples were transferred from the 2L bottles into 200 mL bottles; one for the inlet sample and one for outlet sample. Before use, the 200 mL bottles were rinsed in tap water, rinsed in 0.5 N H2SO4, rinsed in tap water 6 times, then rinsed in DI water 2 times. The wetland sample was sterilized in the 200 mL bottles by placing them in a heating cabinet at 105 degrees C for approximately 40 minutes until the sample reached 60 degrees C.

3.4 Solution Preparation

Solutions were prepared according to the following instructions:

Sodium Carbonate Solution: 20 grams of Na_2CO_3 was added to 1 liter of de-ionized water

Hydrochloric Acid Solution: 10 mL of 38% HCl was diluted to 1 liter with de-ionized water

10% Z8 Solution: The growth medium Z8 is a standard growth medium for green algae in Norway (Skullberg OM, 1990), but it is important to note that it differs in its concentration of elements from the EPA nutrient solution for Algal Assays (Agency, 1978). The 10 % Z8 used for growing stock algae was made from stock solutions purchased from NIVA. Growth medium Z8 was prepared at a 10% concentration by adding the 1 mL of each stock solution (I, II, and III) plus 0.1 mL of stock solution IV in 1 liter of de-ionized water.

10% Z8-P Solution: The growth medium 10 % Z8-P was made from stock solutions purchased from NIVA. 4 different stock solutions were used, and growth medium was prepared by adding the 1 mL of each stock solution (I, IIa, and III) plus 0.1 mL of stock solution IV in 1 liter of de-ionized water.

Starvation Medium: The starvation medium was prepared from stock solutions purchased from NIVA. 1 mL of each of 4 stock solutions was added to 1 liter of de-ionized water.

Table 3: Comparison of content of 10% Z8, 10%Z8-P and the starvation medium

`	**10% Z8 (mg/l)**	**10% Z8-P**	**Starvation Medium**
I	5.9 mg/l $Ca(NO_3)_2{\cdot}4H_2O$	5.9 mg/l $Ca(NO_3)_2{\cdot}4H_2O$	35 g/L $NaHCO_3$
	2.5 mg/l $MgSO_4{\cdot}7H_2O$	2.5 mg/l $MgSO_4{\cdot}7H_2O$	84 mg/L K_2HPO_4
II	46.7 mg/l $NaNO_3$	46.7 mg/l $NaNO_3$	1.8 g/L $NaNO_3$
	2.1 mg/l Na_2CO_3	2.1 mg/l Na_2CO_3	10.3 g/L $MgSO_4{\cdot}7H_2O$
	3.1 mg/l K_2HPO_4		

III	0.37 mg/l EDTA 0.28 mg/l $FeCl_3 \cdot 6H_2O$	0.37 mg/l EDTA 0.28 mg/l $FeCl_3 \cdot 6H_2O$	3.8 g/L KCl 29.8 g/L $CaCl_2 \cdot 2H_2O$
IV	22.3 µg/l $MnSO_4 \cdot 4H_2O$ 31 µg/l H_3BO_3 1.2 µg/l KBr 0.83 µg/l KJ 2.87 µg/l $ZnSO_4 \cdot 7H_2O$ 1.54 µg/l $Cd(NO_3)_2 \cdot 4H_2O$ 0.88 µg/l $(NH_4)_6M_7O_{24} \cdot 4H_2O$ 1.25 µg/l $CuSO_4 \cdot 5H_2O$ 1.46 µg/l $Co(NO_3)_2 \cdot 6H_2O$ 1.98 µg/l $NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$ 4.74 µg/l $AL_2(SO_4)_3K_2SO_4 \cdot 24H_2O$ 0.33 µg/l $Na_2WO_4 \cdot 2H_2O$ 0.41 µg/l $Cr(NO_3)_3 \cdot 9H_2O$ 0.09 µg/l V_2O_5	22.3 µg/l $MnSO_4 \cdot 4H_2O$ 31 µg/l H_3BO_3 1.2 µg/l KBr 0.83 µg/l KJ 2.87 µg/l $ZnSO_4 \cdot 7H_2O$ 1.54 µg/l $Cd(NO_3)_2 \cdot 4H_2O$ 0.88 µg/l $(NH_4)_6M_7O_{24} \cdot 4H_2O$ 1.25 µg/l $CuSO_4 \cdot 5H_2O$ 1.46 µg/l $Co(NO_3)_2 \cdot 6H_2O$ 1.98 µg/l $NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$ 4.74 µg/l $AL_2(SO_4)_3K_2SO_4 \cdot 24H_2O$ 0.33 µg/l $Na_2WO_4 \cdot 2H_2O$ 0.41 µg/l $Cr(NO_3)_3 \cdot 9H_2O$ 0.09 µg/l V_2O_5	75.6 mg/L $FeCl_3 \cdot 6H_2O$ 100 mg/L EDTA

Standard Ortho-Phosphate Solution (1µg/mL): Using standard method 4500-P, 54.875 mg of KH_2PO_4 was added to 250 mL of DI water to give a solution with 50 µg/ml. Five mL of this 50 µg/mL solution was diluted to 250 mL to create a standard orthophosphate solution with a concentration of 1 µg/mL (APHA). A sample of this solution was diluted 10x and tested for accuracy. On February 1st, 2016, the standard solution was tested using a Spectroquant® phosphate cell test kit (1.14543.001) which determined the phosphate concentration to be 1.04 mg/L (*µg/mL).*

3.5 Nitrogen Testing

Cell test kits were used to determine the nitrogen concentration and speciation within a few of the wetlands samples. The kits used were as follows:

Table 4: Spectroquant® test kits used in nitrogen analysis

	Spectroquant® Number
Nitrogen (Total)	1.00613.0001
Nitrate	1.14563.0001
Ammonium	1.14739.0001

3.6 Washing culture flasks

Culture flasks were rinsed well with tap water after use and left in tap water until washing. A toothbrush was used to clean the sides of the glass if necessary. Culture flasks were emptied of tap water, and filled to the top with hot Na_2CO_3 -solution. Culture flasks were left in Na_2CO_3 –solution for at least 5 minutes. Na_2CO_3 –solution was poured back into the bottle for re-use (maximum 3 times). The culture flasks were rinsed with tap water, then filled to the top with HCl solution. They were left in HCl solution for at least 5 minutes. The HCl solution was poured from flasks back into the bottle for re-use (maximum 3 times). Culture flask were rinsed with water at least 6 times then rinsed 3 times with distilled water. Flasks were dried upside down on bench paper until use.

3.7 Preparing Algae

Stock culture

The test algae *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum)* was grown in 50 mL of 10 % Z8 growth medium at 25 Celsius under continuous light (approx. 70 $\mu E \cdot m^{-2} \cdot s^{-1}$ as PAR). 1.5 mL of algae was transferred weekly to 50 ml new 10 % Z8 solution.

P-limited inoculum culture

Algae to be used in AGP tests was transferred to starvation medium 7-10 days prior to start of a test. 1.5 mL of algae into 50 mL of starvation medium. 1 flask of starved algae was prepared for the approximately 50 test flasks. The inoculum culture was incubated in the same conditions as the stock culture. Starved algae was considered ready for use in the test when the cell count changed less than 10% over a 24 hour period.

3.8 Algal Bioassay Test Method

Set-up of a test

The speciation of phosphorus (TP, Ortho-P, and dissolved TP) in the wetland sample was determined using chemical analysis by Anne Marie Haws (Haws, 2016). 100 mL flasks were used, so test water was limited to 25 mL (Agency, 1978). The test was set up as follows (with 5 replicates):

I. Distilled water, ortho-P, algae (negative algae control)
II. Test water, no algae (negative w.s. control)
III. Test water, algae (test)
IV. Test water, ortho-P, algae (positive control)

Flasks in Group I contained added algae and ortho-P to a concentration of 20 µg P/l in dDI water. This group is not repeated for inlet and outlet since no wetland sample is used for this group. This group serves as a negative control for this experiment where no growth is expected from the algae when P is not the limiting nutrient because no other nutrients have been provided. This will be referred to as the "Negative Algae Control."

Flasks in Group II contained wetland sample diluted so that the total-P concentration was 20 µg P/l. No alga was added, and this test water served as a blank for possible particles in the test water since wetland samples were unfiltered. This group contains a separate set of flasks for inlet (group 2) and outlet (group 2.2). This group serves as a negative control for this experiment as no growth is expected at all from sterilized test water with no algae added. These will be referred to as the "Negative W.S. Control In" and "Negative W.S. Control Out," the W.S. standing for wetland sample.

Flasks in Group III contained algae, and wetland sample diluted so the concentration of total-P was 20 µg P/l. This group contains a separate set of flasks for inlet (group 3) and outlet (group 3.2). These will be referred to as "Test In" and "Test Out."

Flasks in Group IV contained algae, wetland sample diluted to a total-P concentration of 20 µg P/l, and additional ortho-P to a concentration of 10 µg P/l (total-P concentration is thus 30 µg P/l). This group contains a separate set of flasks for inlet (group 4) and outlet (group 5.2). This group serves as a positive control to check for inhibitory effects as it should have a higher growth when compared to Group 3. These will be referred to as "Positive Control In" and "Positive Control Out."

The flasks were labelled with numbers 1-45 so the same flasks were always used for Group 1, 2, 3 and 4 respectively, and always had the same placement on the shaker table (Appendix C). All flasks were covered with a plastic petri dishes overturned to sit on top of the flask and prevent contamination while still allowing air exchange and light penetration.

Wetland samples were diluted with 10%Z8-P to 20 µg P/L in 500 mL batches before putting 25 mL into each flask so the dilution of the sample would result in equivalent test water in each flask.

Cultures were incubated on a shaker table operating at 110 rpm (confirmed using a tachometer), at room temperature under continuous light (70 $\mu E \cdot m^{-2} \cdot s^{-1}$ as PAR). On August 26th, the lighting for the test shaker table was set up. It consisted of 3 light banks hanging above the shaker table with 2 long bulb in each. The light intensity was measured at multiple locations on the shaker table and although the 3 light banks were hanging at the same level, several different intensities were measured across the table. Aluminum foil was

applied over the light bulb in several locations until the light intensity was 70 $\mu E \cdot M^{-2} \cdot S^{-1}$ ± 10% at all locations.

Sampling

October to December:

A 250 µL sample of each flask was taken initially and stored in lugol.

250 µL samples were taken, at least one from each group, on day 6 and stored in lugol.

250 µL samples were taken, at least one from each group, on day 7. Lugol was added and they were run through coulter counter to determine if stationary phase had been reached based on Log Concentration of cells vs. time growth curve.

250 µL samples were taken daily, and fixed in lugol from day 7 in order to determine if in stationary phase.

Once stationary phase was been reached 250 µL samples were taken from all flasks and fixed with lugol for final cell count.

January-February: 1 mL samples were taken every day, fixed with lugol and stored until analysis could be done due to equipment malfunction.

February-June: 0.5 mL samples were taken and analysed immediately on the flow cytometer.

Standard Curve

The relation between bio-available phosphate concentration and concentration of cells at stationary phase was established using a standard orthophosphate series. A series of test concentrations were made using 10 % Z8 – P solution adding the standard orthophosphate solution to final concentrations of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 and 30 µg P/l. One or more test concentrations (groups 5, 6 and 7) were run with each sampling depending on space available on the shaker table. Triplicates of each concentration were prepared, and were inoculated with the same test algae as samples. Flasks were incubated under the same conditions as the samples.

Sample Storage

Samples taken during the AGP test were not always analysed the same day. All samples taken and not analysed were preserved with Lugol's solution and stored in the refrigerator until analysis could be done.

3.9 Analysis

Growth rate cannot be used as a growth parameter since this test is a batch culture which is why cell count or MSC is usually used to measure the change in algae in Algal Bioassays (Agency, 1978).

Coulter Counter

The coulter counter was used to analyse samples from October to December 2015.

20 mL samples were required for the accuvette container on the coulter counter so the 250 µL samples fixed in lugol were diluted to 20 mL with electrolyte solution (0.1% NaCl solution). Algae was separated from background particles by subtracting the initial particle count from the final particle count.

In January 2015 the coulter counter was intermittently available due to inaccurate calibrations and high background counts. A calibration of both flow cytometer and coulter counter was done in March 2016. 3.2 µm beads were used, 40 drops of 2 sets of beads containing $5x10^6$ beads per ml were added to 15 ml of dDI water. There is approximately 25-26 drops in 1 ml so 80 drops is between 3.2 and 3.4 ml. There means there should be approximately 902,000 beads per ml in the solution. The results flow cytometer measured 923,010 beads per ml while the coulter counter measured: 5,280,000 beads per ml.

From this calibration test, the decision was made in April to begin using the Flow Cytometer instead. Many samples that had been stored for analysis on the coulter counter were then analysed using the flow cytometer instead.

Flow Cytometer

It took nearly a month to get the flow cytometer operational. All the tubing had to be removed and cleaned due to growth throughout. It was determined ready for use when the blanks showed no particles within the fluorescent range of interest.

Live Samples

Samples were taken from test flasks into Eppendorf tubes and analyzed on the flow cytometer directly from these tubes after being re-suspended using a vortex at 150 rpm.

The figures below demonstrate how the gating was used to discern between live algae and background particles in the live samples, Figure 21, and the fixed samples, Figure 22. Fluorescence was used to separate the live algae from background in live samples.

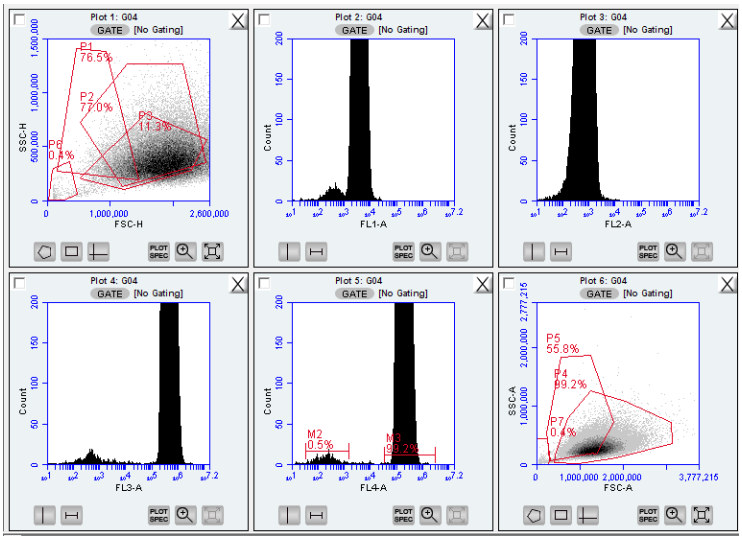

Figure 21: Typical result for live algae samples displayed on the Accuri C6 software. The most distinct and separate peaks are seen in Plot 5 using the FL4 laser, therefore the shown M3 gating was used to select for live algae when running samples.

Fixed Samples

500 µl of dDI water was added to the original sample of test water which was fixed with lugol and stored in a test tube. This mixture was vortexed for at least 15 seconds at 150 rpm before being analyzed on the flow cytometer directly from the original test tube.

The fixed samples did not fluoresce and as can be seen in the upper right hand corner of Figure 22, the size and shape of the algae was so altered by the storage in lugol that it was not adequate to separate the algae from background. Instead, the two most discernable peaks on FL3 and FL4 were compared to separate the algae from the background as shown in the lower left hand corner of Figure 22.

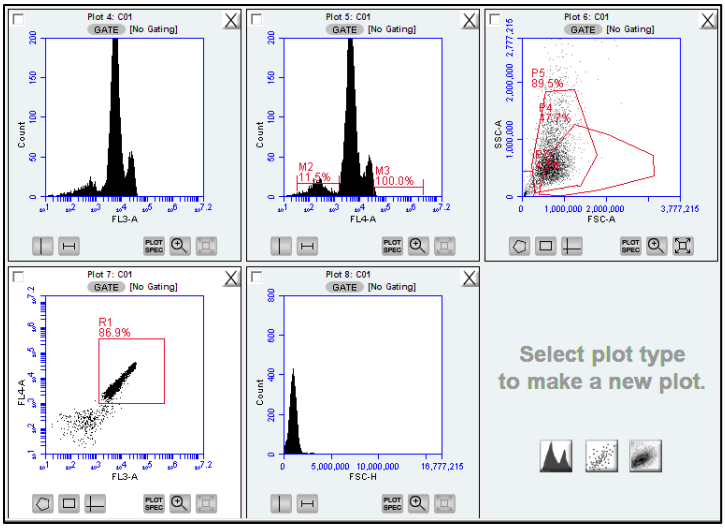

Figure 22: Typical result for fixed samples as displayed on the Accuri C6 software. The peaks are not distinct enough to use to select for algae, so the comparison of the FL3 and FL4 lasers were used to select for algae as shown on Plot 7 using R1.

3.10 Error Analysis

Error between replicates was calculated using a simple standard deviation for a sample (Equation 1)

$$s = \sqrt{\frac{\sum(X-\bar{X})^2}{n-1}} \qquad \textbf{Equation 1}$$

where, s is the standard deviation of the sample, $\bar{X}$ is the sample mean and n is the number of data points in the sample.

Error of estimations using the linear regression were calculated using the simple standard error (Equation 2) of the data used to make the linear regression.

$$s_{est} = \sqrt{\frac{\sum(Y-Y')^2}{N-2}} \qquad \textbf{Equation 2}$$

Exclusion of data

Some test water, due to various errors, returned samples that were inconsistent with other results. Outlier analysis was performed on these using Dixon's Q test which uses the equation

$$Q = \frac{gap}{range} \qquad \textbf{Equation 3}$$

paired with a table of acceptable values for Q based on certainty (Appendix B)

4. RESULTS

This section first presents the results of the pertinent wetland analysis completed by Anne-Marie Haws (Haws, 2016). Next the chemical analysis of nitrogen is reviewed. Algal assay results are then presented in 2 sections; fixed sampling and live sampling. Next the precision of the methodology is demonstrated. The calculation of the bioavailable phosphorus is presented last.

4.1 Wetland Analysis

The results of Haws' analysis showed that average phosphorus retention over a year was 6.9 kg/yr with 2.6 of that being dissolved and 1.8 of that being phosphate (Appendix D). Looking at monthly data it can be seen that this spring shows a net negative retention meaning the wetland was actually inputting phosphorus into the lake. The summer and fall show positive retention (Appendix D).

Haws completed 2 retention time tests on the wetland, Leikvollbekken. The first was on April 20th and the demonstrated retention time was 0.5 hours. The second was on June 14th and the retention time was 9 hours. It can be inferred from this data that the retention time in this wetland is highly variable. Retention time is based on flow into and out of the wetland. The flow out of the wetland was all that was measured so it is assumed that the flow in and out are equivalent. The average flow through the wetland during the week that composite samples were collected is presented in Table 5.

Table 5: Average flow in Leikvollbekken the week of the composite sampling

Analysis Date	Average flow (l/s)
November 18	5.8
December 5	23.7
January 28	12.3
April 6	3.1
May 25	2.4
June 2	8.2
June 18	2.4
June 25	3.9

4.2 Nitrogen Analysis

Several wetland samples were analyzed using standard cell test kits to determine nitrogen concentration in the form of total nitrogen, nitrate and ammonium (Table 6). The uncertainty presented is the stated accuracy of the measurement value for each test method by Spectroquant®.

Table 6: Measured total nitrogen, nitrate and ammonium values in 11 wetland samples, and comparison of phosphorus to nitrogen ratios.

	Total Nitrogen (mg N/l)	Nitrate (mg N/l)	Ammonium (mg N/l)	Total Phosphorus according to chemical analysis (µg P/l)	Test Water Nitrate:TP ratio (g:g)
Feb 04					
Inlet*	9.2±0.5	8.3±0.5	0.15±0.19	242	34:1
Mid*	4.5±0.5	4.0±0.5	ND**		
Outlet*	6.5±0.5	5.6±0.5	0.06±0.19	61	92:1
Feb 09					
Inlet	5.9±0.5	5.0±0.5	0.03±0.19	84	60:1
Outlet	5.0±0.5	4.3±0.5	0.04±0.19	78	55:1
Mar 29					
Inlet	11.2±0.5	5.3±0.5	0.014±0.19	63	84:1
Outlet	9.9±0.5	5.0±0.5	0.054±0.19	113	44:1
Apr 5					
Inlet	9.8±0.5	7.9±0.5	0.06±0.19	177	45:1
Outlet	9.3±0.5	7.2±0.5	0.032±0.19	111	65:1
June 22					
Inlet	19±5	17.5±0.5	0.51±0.19	843	21:1
Outlet	23±5	16.2±0.5	3.43±0.19	1507	11:1

*these samples were grab samples, not composite

**ND = Non-Detect, or below detection limit

The first 3 column shows the results of the nitrogen analysis. The fourth column shows the total phosphorus concentration as determined by chemical analysis (Haws, 2016). The last column is the nitrate to total phosphorus ratio by weight. Earlier it was presented that the molar element ratio for this algae is 11 nitrogen to 1 phosphorus which is 5g N to 1g P by weight. The test water had a nutrient ratios strongly indicate P limiting conditions. The samples analyzed for nitrogen show that nitrogen was not the limiting

nutrient in the samples and thus should not affect the ability of the algal assay to evaluate the bioavailability of phosphorus based on growth potential. For these calculations is was assumed that only the nitrate is available for growth to show that even if all the phosphorus were bioavailable it would still be a limiting nutrient in these samples. Additionally, the 10% Z8-P used to dilute samples for analysis contains 8.4 mg N/l which further decreases the possibility of nitrogen limited conditions. From the results of this analysis, it was assumed that phosphorus was the limiting nutrient in all the algal assays performed.

4.3 Algal Assay

This section is divided into fixed sampling and live sampling, and then further divided into specific test results in order by date, with the most recent tests coming last.

Fixed Sampling

Test 1

Test 1 took place starting November 18th with wetland sample taken from the auto-sampler on November 17th. The standard concentrations run during this test were 0, 5 and 15 µg P/l. The results of the chemical analysis originally concluded 90 µg P/l as total phosphorus in both the inlet and outlet wetland samples. In order to dilute to 20 µg P/l based on total phosphorus, 111.32 g of inlet sample was diluted to 500.04 g using 10% Z8-P and 111.26 g of outlet sample was diluted to 500.59 g using 10% Z8-P. After the test was completed, the chemical analysis was amended to 96 µg P/l as total phosphorus in the inlet and 100 µg P/l as total phosphorus in the outlet. The P-limited inoculum culture contained approximately 440 particles/µl as measured on the coulter counter so each flask was inoculated with 10^6 cells using 2.3 ml of this culture. Samples from this test were fixed with lugol, stored at 4 degrees C and analyzed over the course of several months using first the coulter counter and then the flow cytometer. The results of this analysis are shown in Figure 23.

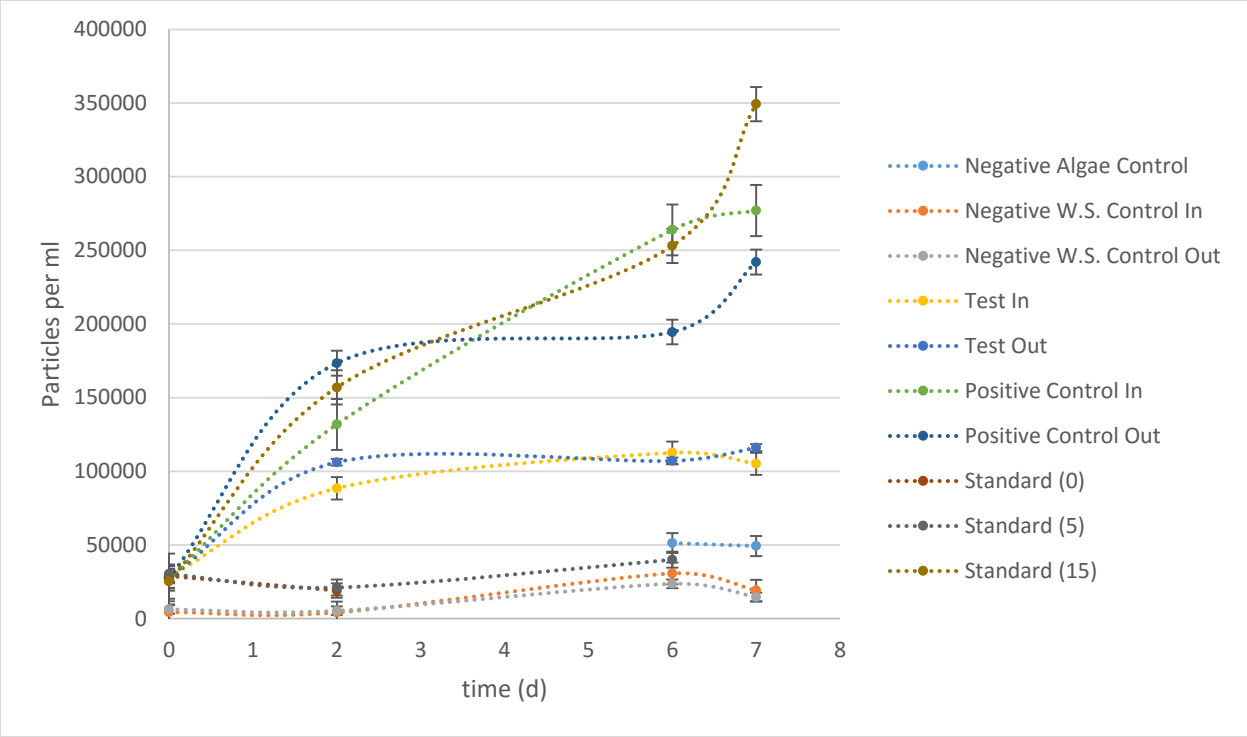

Figure 23: Number of particles per ml in sample groups over 7 days from November 18th to November 25th with error bars showing the standard error.

The difference in chemically analyzed total phosphorus between the inlet and the outlet was small, and the results in Figure 23 show the concentration of particles in inlet and outlet test water to be nearly identical. Stationary phase was assumed to be reached at the time, but both standard (15) and positive control out appear to possible be growing still.

Test 2

Test 2 took place starting December 4th with wetland sample taken from the on December 3rd. The standard concentrations run during this test were 4, and 6 µg P/l. The results of the chemical analysis originally concluded 108 µg P/l as total phosphorus in the inlet and 92 µg P/l as total phosphorus in the outlet wetland samples. In order to dilute to 20 µg P/l based on total phosphorus, 92.75 g of inlet sample was diluted to 500.05 g using 10% Z8-P and 108.75 g of outlet sample was diluted to 500.08 g using 10% Z8-P. After the test was completed, the chemical analysis was amended to 99 µg p/l as total phosphorus in the inlet and 95 µg P/l as total phosphorus in the outlet. The P-limited inoculum culture contained approximately 864 particles/µl so each flask was inoculated with 10^6 cells using 1.2 ml of this culture. Samples from this test were fixed with lugol, stored at 4 degrees C and analyzed over the course of several months using both the coulter counter and then the cytometer. The results of this analysis are shown in Figure 24.

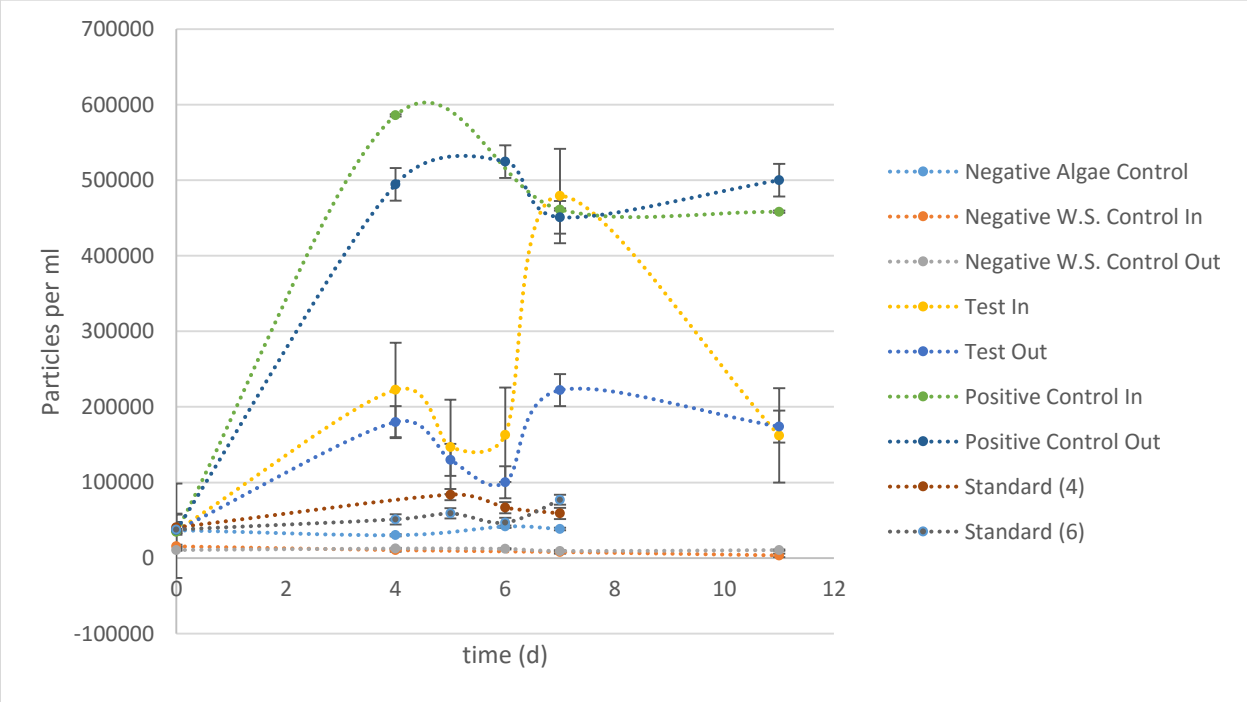

Figure 24: Number of particles per ml in sample groups over 11 days from December 4th to December 15th with error bars showing the standard error.

The difference in chemically analyzed total phosphorus between the inlet and the outlet was small, and the results in Figure 24 show the concentration of particles in inlet and outlet test water to be close except for on day 7. Outlier analysis was performed and determined that point to be an outlier so it was excluded when the stationary phase average particles per ml was calculated.

Test 3

Test 3 took place starting January 28th with wetland sample (grab sample) taken on January 27th. The standard concentrations run during this test were 25, and 30 µg P/l. The results of the chemical analysis concluded 242 µg P/l as total phosphorus in the inlet and 74 µg P/l as total phosphorus in the outlet wetland samples. In order to dilute to 20 µg P/l based on total phosphorus, 41.33 g of inlet sample was diluted to 500.16 g using 10% Z8-P and 135.18 g of outlet sample was diluted to 500.34 g using 10% Z8-P. The P-limited inoculum culture contained approximately 125 particles/µl so each flask was inoculated with 10^6 cells/l using 21 µl of this culture. Samples from this test were fixed with lugol, stored at 4 degrees C and analyzed over the course of several months using both the coulter counter and then the flow cytometer. The results of this analysis are shown in Figure 25.

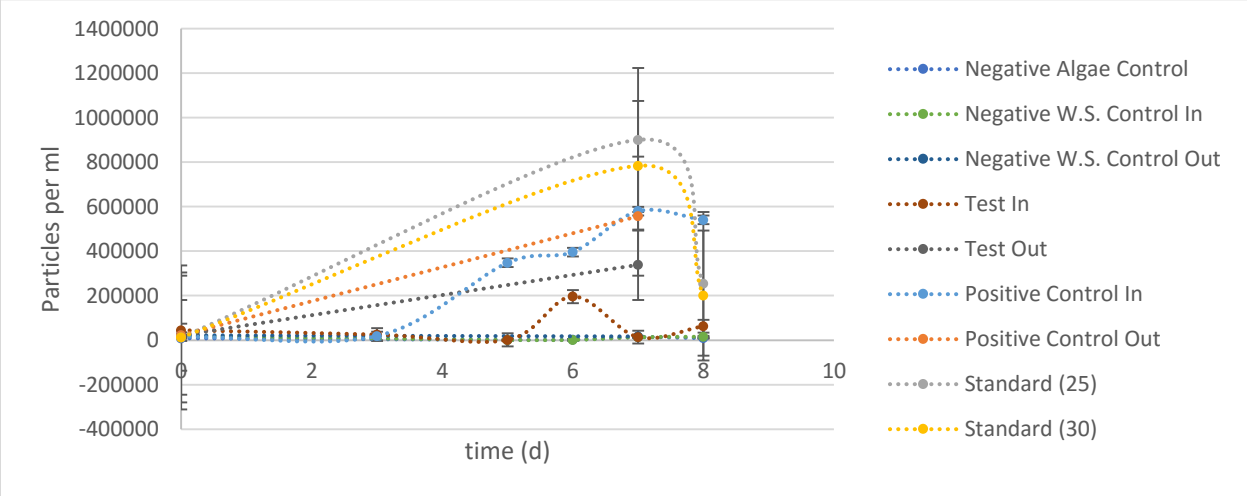

Figure 25: Number of particles per ml in sample groups over 8 days from January 28th to February 5th with error bars showing the standard error.

The difference in chemically analyzed total phosphorus between the inlet and the outlet was large, and the results in Figure 25 show the concentration of particles in inlet and outlet test water to be significantly different. Good resolution was not obtained for this analysis due to malfunctioning equipment. There is a lot of error which cannot be quantified due to a lack of enough data points. The peak shown could be the stationary phase of the algae but without further data points these are not certain and the assumption that this point is the stationary phase is weak.

Live Sampling

Test 4

Test 4 took place starting April 6th with wetland sample taken on April 5th. The standard concentrations run during this experiment were 2 µg P/l and 7 µg P/l. The results of the chemical analysis concluded 177 µg P/l as total phosphorus in the inlet and 111 µg P/l as total phosphorus in the outlet. In order to dilute to 20 µg P/l based on total phosphorus, 58.09 g of inlet sample was diluted to 501.14 g and 90.7 g of outlet sample was diluted to 500.17 g using 10% Z8-P solution. The concentration of algae in the P-limited inoculum culture was approximately 220 cells/µl so each flask was inoculated with 10^6 cells/L using 113.4 µl of this culture. The results of this analysis are shown in Figure 26.

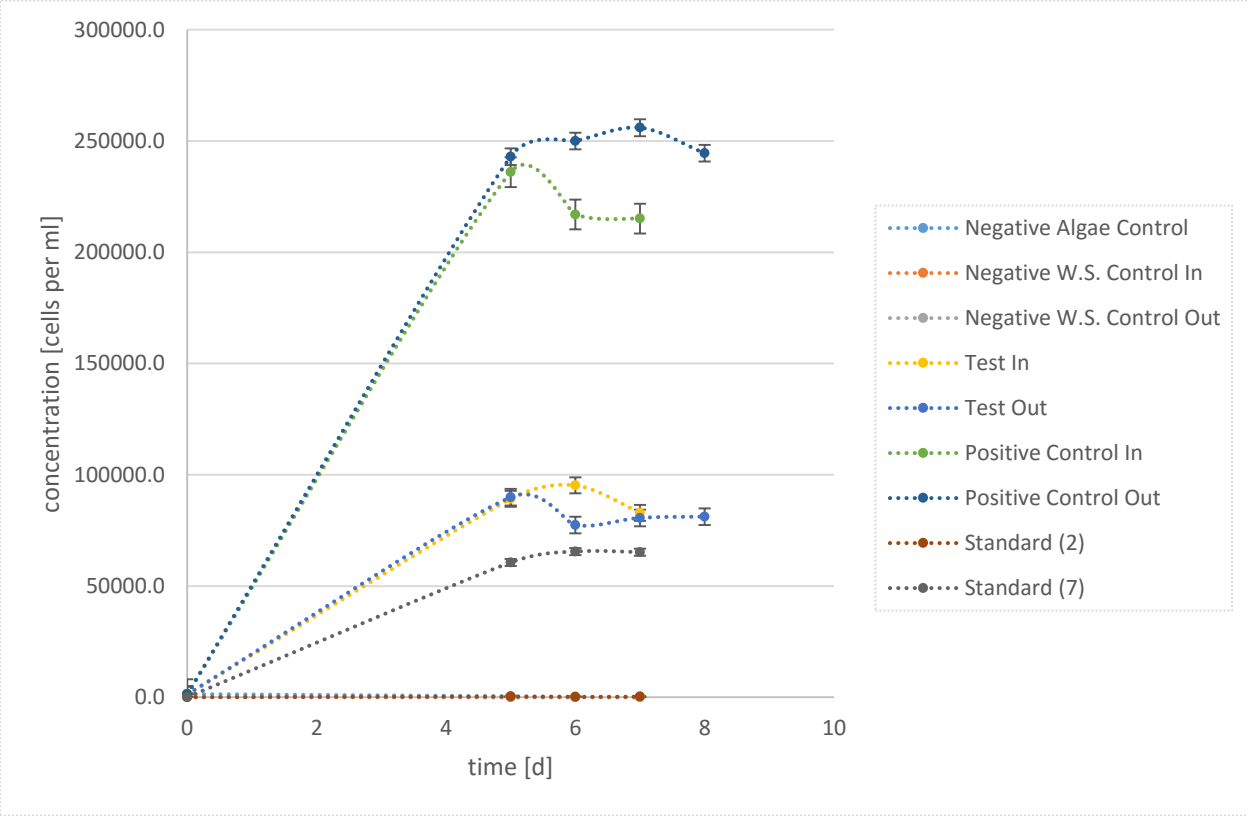

Figure 26: Concentration of algae in cells per ml of groups 1-6 over 8 days from April 6th to April 14th with error bars showing the standard error per test group.

While the difference in chemically analyzed total phosphorus between the inlet and the outlet was large, the results in Figure 26 show the concentration of algae in inlet and outlet test water to be close with the inlet peaking just above the outlet on day 6.

Test 5

Test 5 took place starting May 25th with wetland sample taken on May 24th. The standard concentrations run during this experiment were 8 µg P/l and 20 µg P/l. The results of the chemical analysis concluded 250 µg P/l as total phosphorus in the inlet and 482 µg P/ as total phosphorus in the outlet. In order to dilute to 20 µg P/l based on total phosphorus, 40.31 g of inlet sample was diluted to 500.08 g and 21.17 g of outlet sample was diluted to 500.19 g using 10% Z8-P solution. The concentration of algae in the P-limited inoculum culture was approximately 300 cells/µl so each flask was inoculated with 10^7 cells/L using 800 µl of this culture. The results of this analysis are shown in Figure 27.

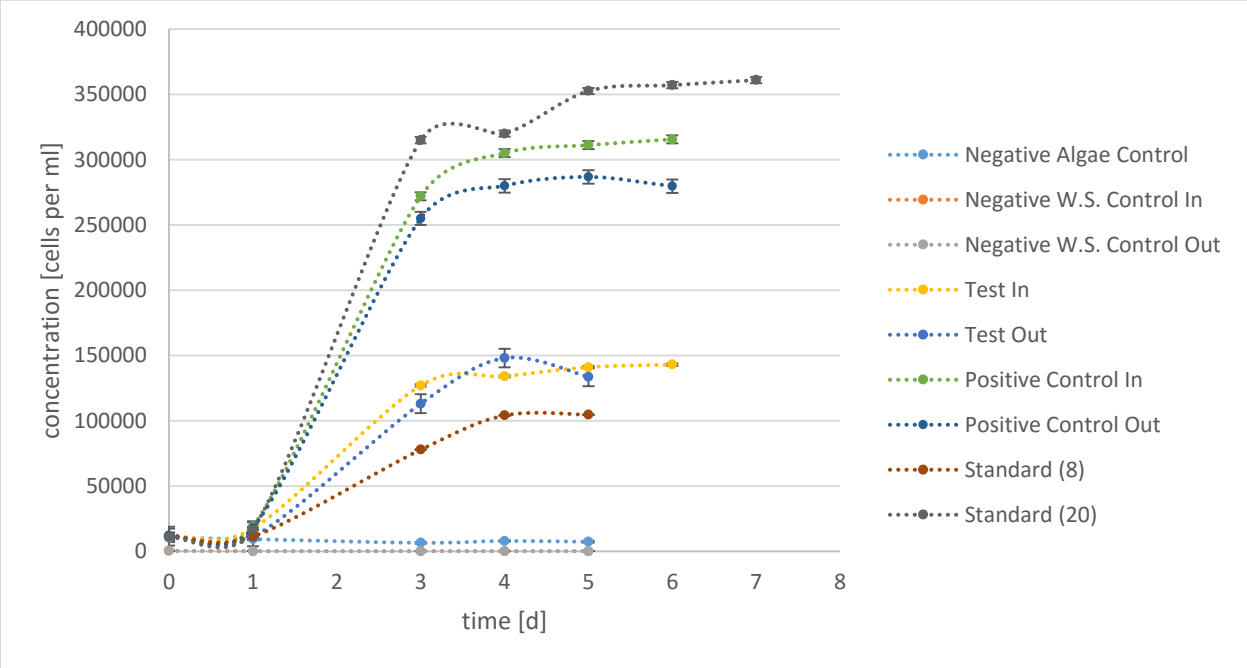

Figure 27: Concentration of algae in cells per ml of groups 1-6 over 7 days from May 25th to June 1st with error bars showing the standard error per test group.

While the difference in chemically analyzed total phosphorus between the inlet and the outlet was large, the results in Figure 27 show the concentration of algae in inlet and outlet test water to be close with the outlet peaking just above the inlet on day 4 and just below on day 5.

Test 6

Test 6 took place starting June 2nd with wetland sample taken on May 30th. The standard concentrations run during this experiment were 5 µg P/l and 10 µg P/l. The results of the chemical analysis initially concluded 255 µg P/l as total phosphorus in the inlet and 247 µg P/l as total phosphorus in the outlet. In order to dilute to 20 µg P/l based on total phosphorus, 39.67 g of inlet sample was diluted to 501.50 g and 40.99 g of outlet sample was diluted to 500.18 g using 10% Z8-P solution. The chemical analysis was later amended to 166 µg P/l as total phosphorus in the inlet sample and 153 µg P/l as total phosphorus in the outlet sample making this test over diluted compared to other tests. The concentration of algae in the P-limited inoculum culture was approximately 256 cells/µl so each flask was inoculated with 10^7 cells/L using 980 µl of this culture. This test water was sampled more frequently to get a better resolution of what was occurring during the test period. The results of this analysis are shown in Figure 28.

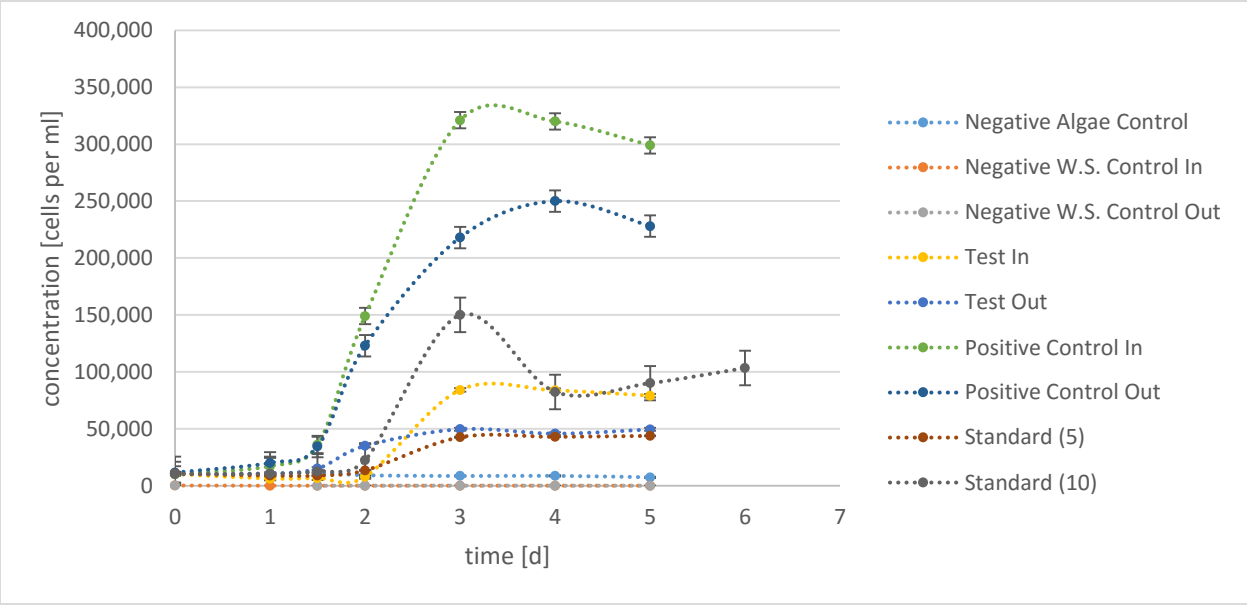

Figure 28: Concentration of algae in cells per ml of groups 1-6 over 6 days from June 2nd to June 8th with error bars showing the standard error.

The difference in chemically analyzed total phosphorus between the inlet and the outlet was relatively small, but the results in Figure 28 show the concentration of algae in inlet test water to be higher than the outlet test water, peaking at about double the outlet on day 3. This trend is also seen in the positive controls.

Test 7

Test 7 took place starting June 18th with wetland sample taken on June 14th. The standard concentrations run during this experiment were 15 µg P/l, 30 µg P/l, and 4 µg P/l. The results of the chemical analysis concluded 150 µg P/l as total phosphorus in the inlet and 91 µg P/l as total phosphorus in the outlet. In order to dilute to 20 µg P/l based on total phosphorus, 66.87 g of inlet sample was diluted to 501.03 g and 110.71 g of outlet sample was diluted to 500.49 g using 10% Z8-P solution. The concentration of algae in the P-limited inoculum culture was approximately 1,000 cells/µl so each flask was inoculated with 10^7 cells/L using 250 µl of this culture. This test water was sampled more frequently to get a better resolution of what was occurring during the test period. The results of this analysis are shown in Figure 29.

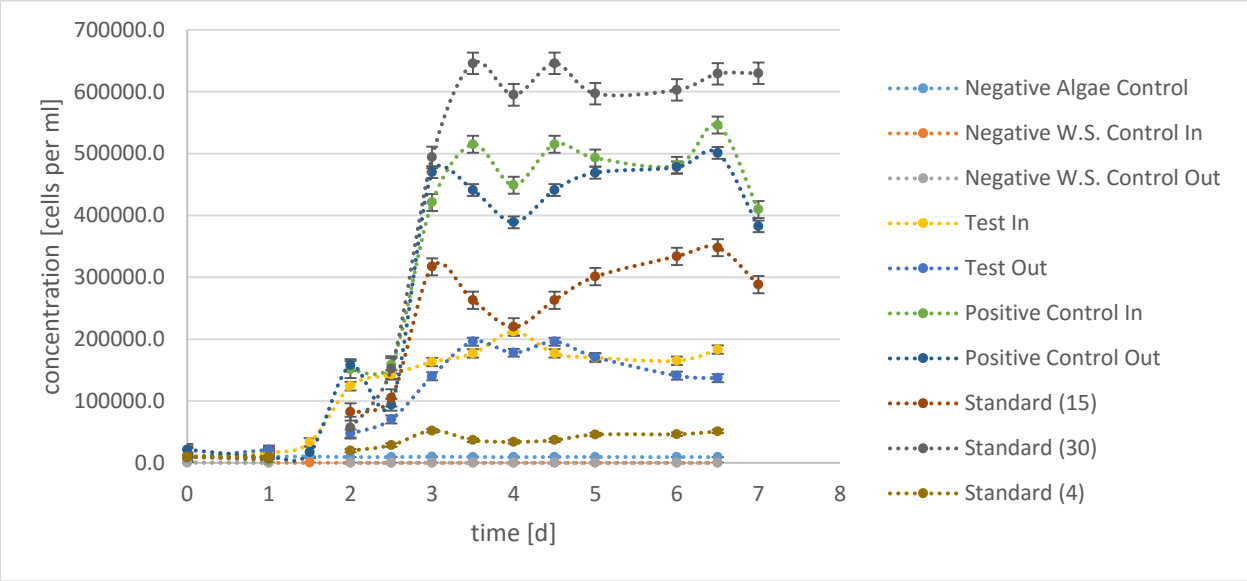

Figure 29: Concentration of algae in cells per ml of groups 1-7 over 7 days from June 18th to June 25th with error bars showing the standard error.

While the difference in chemically analyzed total phosphorus between the inlet and the outlet was large, the results in Figure 29 show the concentration of algae in Group 3 inlet and outlet to be close with the inlet peaking just above the outlet alternating peaks.

Flasks 39, 40 and 41 were replicates of the standard P concentration of 30 µg P/l. It can be seen in Figure 30 that samples taken from flask 40 were very different from samples out of flasks 39 and 41. Outlier analysis was performed for flask 40. Chauvenet's test was done using all data points from day 4 to 7 resulted a probability of 1.14 of getting a point that differs from the mean by 2.4 standard deviations. This value is higher than the 0.5 needed to reject the data so the data was not rejected using this test.

Chauvenet's test was done using data on from day 4 which resulted in a probability of 0.44 which is less than the 0.5 set by Chauvenet's criterion so the data can be rejected. A box and whisker plot was made showing -327783 cells per ml to be the lowest value of inclusion, which is also a failure to reject the data. The Q test was done which did result in this data being excluded. Given the inconsistency of the outlier analyses, and the visible difference in the samples, it was chosen to exclude flask 40 from the values presented as Standard (30) in the results in Figure 29. The most likely scenarios for why this test water wasn't presenting the same growth as the two other test waters in the same group is either the phosphorus wasn't added to flask 40 or the cleaning of the glassware of flask 40 wasn't adequate and some acid residuals sterilized the test water.

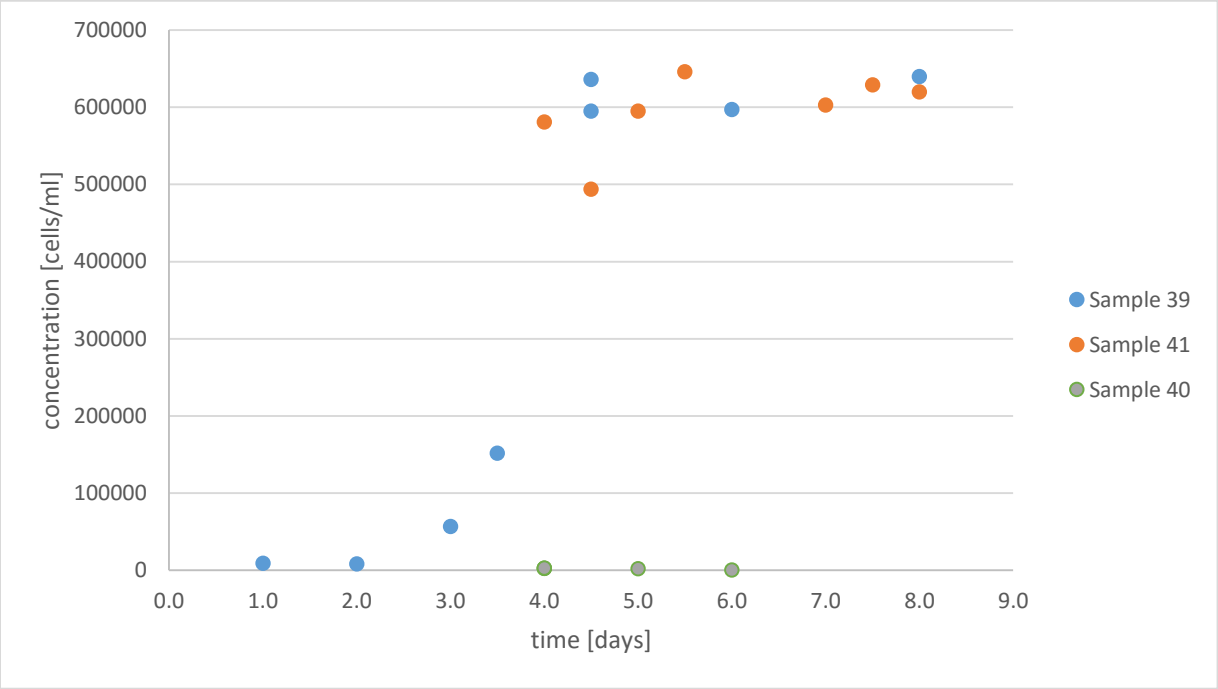

Figure 30: Plot of all measurements of Group 6 (Standard 30) samples during Test 7.

Test 8

Test 8 took place starting June 25th with wetland sample taken on June 21st. The standard concentrations run during this experiment were 6 µg P/l, 9 µg P/l, and 25 µg P/l. The results of the chemical analysis concluded 843 µg P/l as total phosphorus in the inlet and 1507 µg P/l as total phosphorus in the outlet. In order to dilute to 20 µg P/l based on total phosphorus, 12.01 g of inlet sample was diluted to 500.78 g and 6.76 g of outlet sample was diluted to 500.53 g using 10% Z8-P solution. The concentration of algae in the P-limited inoculum culture was approximately 700 cells/µl so each flask was inoculated with 10^7 cells/L using 360 µl of this culture. The results of this analysis are shown in Figure 31.

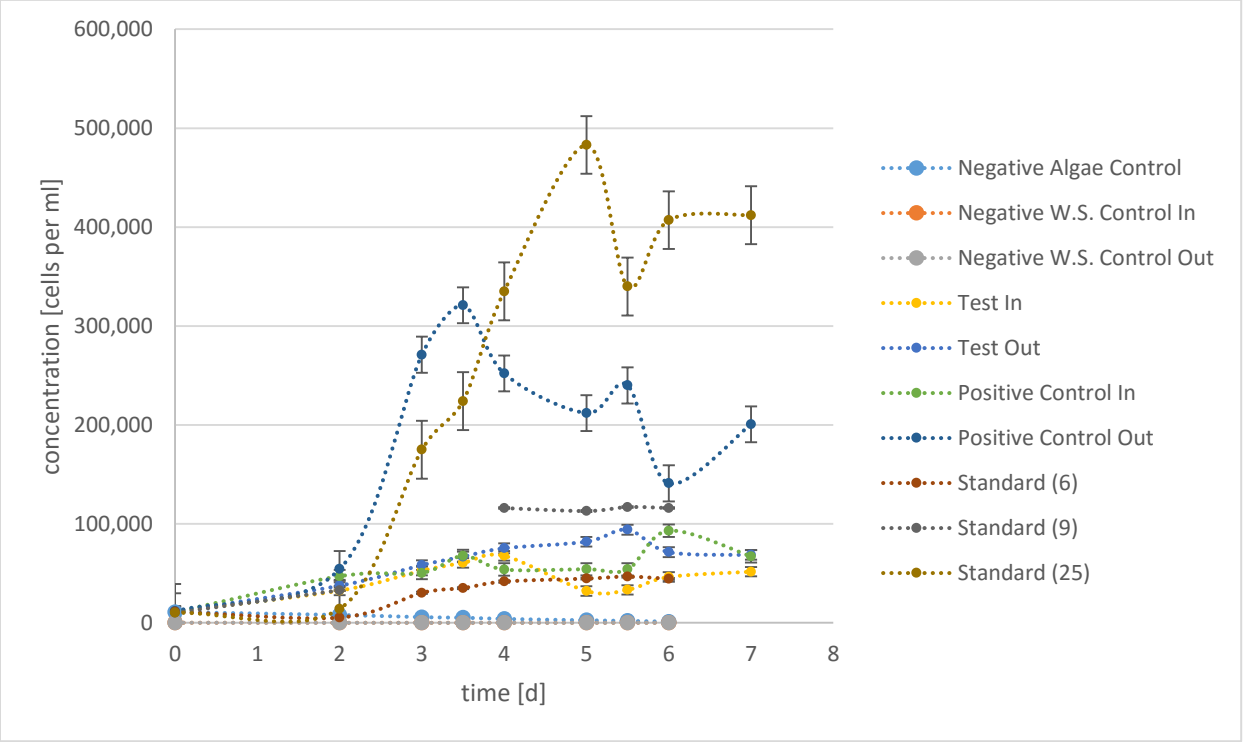

Figure 31: Concentration of algae in cells per ml of groups 1-7 over 7 days from June 25th to July 2nd with error bars showing the standard error.

Positive Control In doesn't appear to show expected results of significantly higher growth than Test In. The difference in chemically analyzed total phosphorus between the inlet and the outlet was very large, and the results in Figure 31 show the concentration of algae in Test out to peak at nearly twice the inlet on day 5.

4.4 Methodology

Standard concentrations from each test were plotted against their average measured change in concentration of algae from initial inoculum to stationary growth phase. A simple linear regression was fitted to the data with the method of least squares.

Inclusion or exclusion of the fixed analysis data

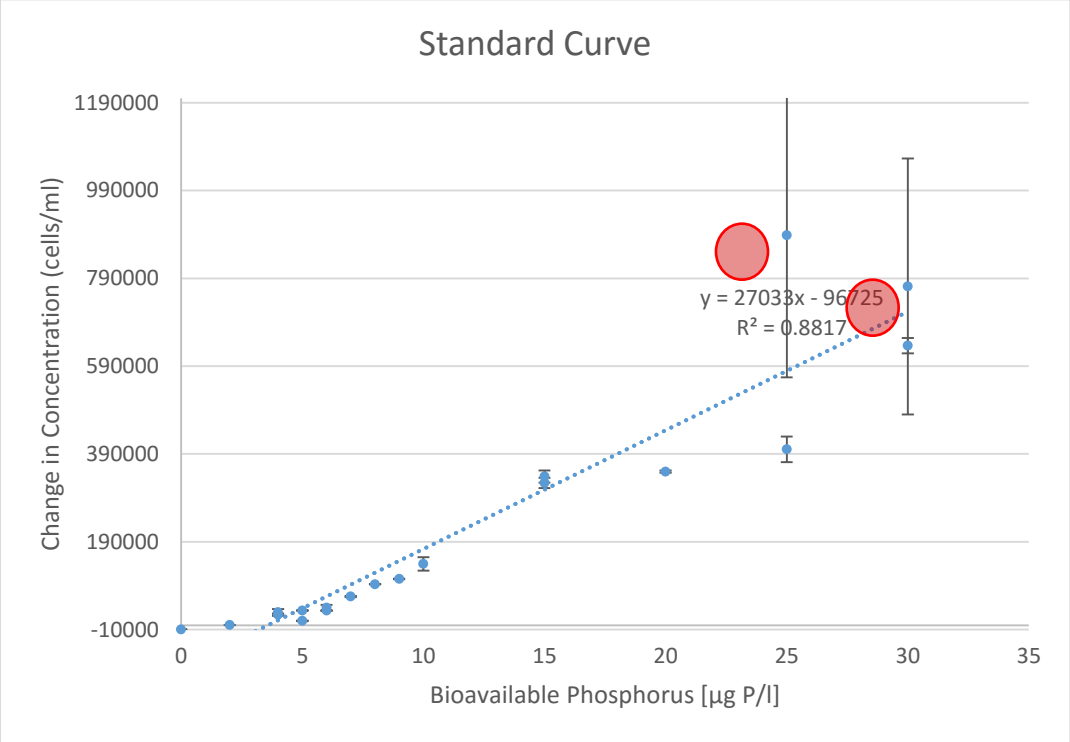

Figure 32: Standard concentrations and their respective change in cell concentration during testing including both fixed and live analysis data

The R^2 value (R^2= 0.8817) for this data indicates that the data does correlate, but it is not the precision desired for this experiment. The fixed analyses results using the coulter counter were less reliable than the live analysis using the flow cytometer. It cannot be determined if what was counted on the coulter counter was algae cells or other particles in the test water. Initial was subtracted from final concentrations which should cancel out the effect of background particles. Ultimately, this experiment was not set up in order to make a comparison between the two methods of counting and thus it was complicated to include both measurements in the final results. The last test completed using the coulter counter, Jan 27, highlighted in red in Figure 32, had significant error and deviation from the other results. For these reasons, the choice was made to exclude the fixed data from the standard curve.

Table 7: Analysis results from chemical analysis and algal bioassay for each test, sorted by sample group.

		Max Growth [cells per ml]	Anne's Measured P Concentrations (Haws, 2016) [µg P/l]			Experimental Ortho-P Conc. (diluted to 20 µg P/l based on Anne's TP) [µg P/L]	Experimental Dissolved TP Conc. (diluted to 20 µg P/l based on Anne's TP) [µg P/L]	Calculated Bio-P using trendline * [µg P/l]	Undiluted bio P [µg P/l]	Actual OP [µg P/l]	Standard Error [cells per ml]
			TP	Ortho P	Diss TP						
18-Nov	**Test In**	80404	96	26	61	5.8	13.6	6.6	31.5		
	Test Out	82125	100	26	54	5.8	12.0	6.6	33.1		
	Positive Control In	243458						12.6			
	Positive Control Out	190883						10.6			
	Standard (0)	-9849								0	777
	Standard (5)	9758								5	840
	Standard (15)	324047								15	11588
4-Dec	**Test In**	141430	99	35	59	6.5	10.9	8.8	43.6		
	Test Out	124715	95	33	55	7.2	12.0	8.2	38.9		
	Positive Control In	467168						20.9			
	Positive Control Out	455528						20.4			
	Standard (4)	29432								4	7343
	Standard (6)	39474								6	6714
28-Jan	**Test In**	18100	242	42	61	3.5	5.0	4.2	51.4		
	Test Out	316980	74	14	32	3.8	8.6	15.3	56.6		
	Positive Control In	544475						23.7			
	Positive Control Out	534590						23.4			
	Standard (25)	887750								25	323535
	Standard (30)	771260								30	291645

		Max Growth [cells per ml]	Anne's Measured P Concentrations (Haws, 2016) [µg P/l]			Experimental Ortho-P Conc. (diluted to 20 µg P/l based on Anne's TP) [µg P/L]	Experimental Dissolved TP Conc. (diluted to 20 µg P/l based on Anne's TP) [µg P/L]	Calculated Bio-P using trendline * [µg P/l]	Undiluted bio P [µg P/l]	Actual OP [µg P/l]	Standard Error [cells per ml]
			TP	Ortho P	Diss TP						
6-Apr	**Test In**	94047	177	12	52	1.4	5.9	7.5	66.6		3594
	Test Out	88543	111	18	59	3.2	10.6	7.3	40.4		3744
	Positive Control In	234538						14.0			6660
	Positive Control Out	254643						15.0			3756
	Standard (2)	237								2	36
	Standard (7)	65442								7	1593
26-May	**Test In**	131617	250	31.5	54	2.5	4.3	9.3	115.9		816
	Test Out	136420	482	29.5	74	1.2	3.1	9.5	228.7		7200
	Positive Control In	304233						17.3			3074
	Positive Control Out	254643						15.0			5098
	Standard (8)	93233								8	267
	Standard (20)	349833								20	2408
2-Jun	**Test In**	73800	166	20	33	1.6	2.6	6.6	83.4		1615
	Test Out	38700	153	18	27	1.5	2.2	5.0	60.6		1273
	Positive Control In	311020						17.6			7172
	Positive Control Out	238500						14.2			9452
	Standard (5)	33267								5	350
	Standard (10)	139700								10	15147
18-Jun	**Test In**	201500	150	23	34	3.1	4.5	12.5	93.8		6766
	Test Out	187660	91	14	28	3.1	6.2	11.9	54.0		6369

		Max Growth [cells per ml]	Anne's Measured P Concentrations (Haws, 2016) [µg P/l] TP	Ortho P	Diss TP	Experimental Ortho-P Conc. (diluted to 20 µg P/l based on Anne's TP) [µg P/L]	Experimental Dissolved TP Conc. (diluted to 20 µg P/l based on Anne's TP) [µg P/L]	Calculated Bio-P using trendline * [µg P/l]	Undiluted bio P [µg P/l]	Actual OP [µg P/l]	Standard Error [cells per ml]
	Positive Control In	535200						27.9			13634
	Positive Control Out	479735						25.4			9528
	Standard (15)	338360								15	13932
	Standard (30)	636620								30	17450
	Standard (4)	24071								4	2776
25-Jun	**Test In**	35,933	843	22	43	0.5	1.0	4.8	204.1		4795
	Test Out	54,200	1507	437	518	5.8	6.9	5.7	428.5		4949
	Positive Control In	69,250						6.4			6224
	Positive Control Out	247,500						14.6			18175
	Standard (6)	33300								6	1028
	Standard (9)	105400								9	866
	Standard (25)	400400								25	29213

*Fixed Analysis (November, December, and January) used trendline from Figure 32 while the Live Analysis used the trendline from Figure 33.

Standard Curve

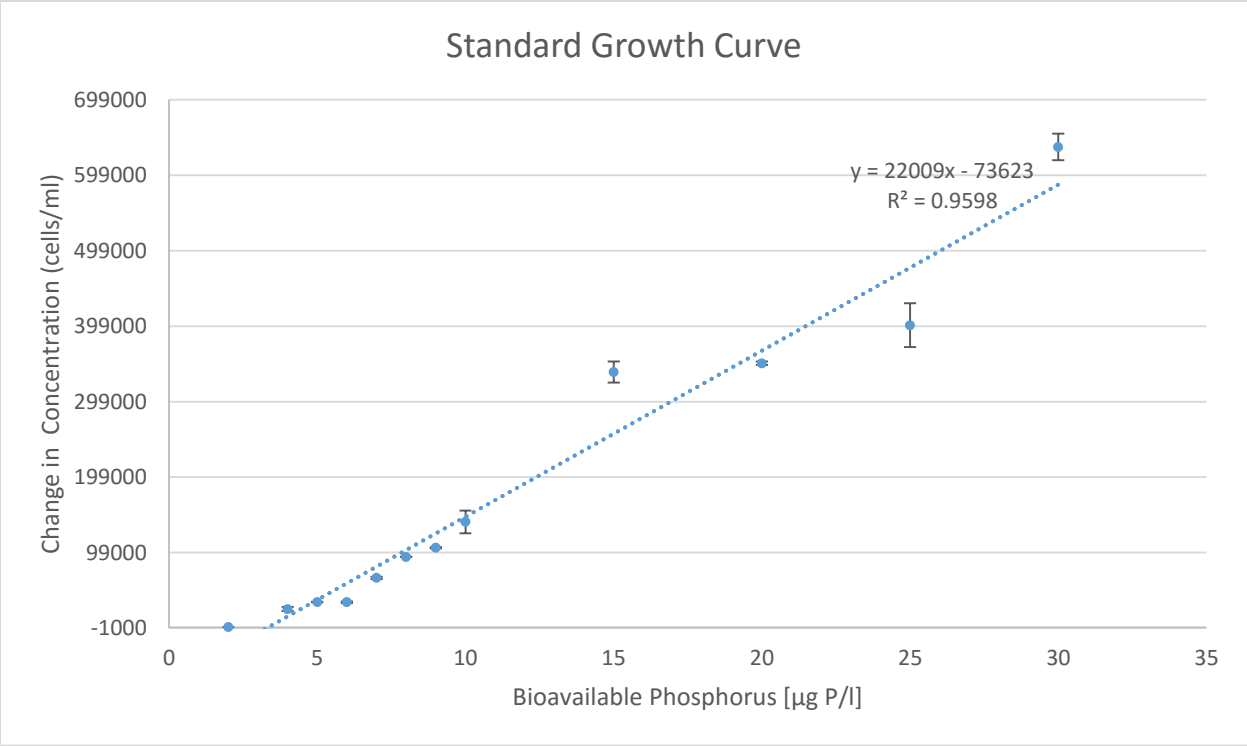

Figure 33: Standard phosphorus concentrations from each test and the associated change in concentration of algae from initial to stationary phase

The R^2 value of 0.9598 indicates that the regression line is a good fit to the data, and the data has good precision with respect to each other which validates the assumption that algal growth will correspond to phosphorus concentration. Results from the regression analysis can be found in Appendix B.

Comparison of Test and Controls

The negative controls functioned very well to demonstrate no growth in a sterilized sample as well as no growth in media lacking nutrition, but not lacking phosphorus.

The positive control did not produce the expected results. Data from Table 7 is presented below as the difference between the test samples and their respective positive controls. The positive control is designed to have 10 µg P/l more bioavailable phosphorus than the test water, thus it would be expected that the difference between the calculated bioavailable phosphorus in the positive control and the test water would be equal to 10 µg P/l. As shown, the difference varies from 20 to 1.5. The standard error of the estimate for phosphorus concentration, calculated in Appendix B, is 1.9, so for the difference observed

at “June 25, In” it can be inferred that an error was made and the additional phosphorus was not added. The largest discrepancy is seen in “January 28, In” which is an analysis which had significant error.

Table 8: The difference between test water and positive control for inlet and outlet samples

	In (µg P/l)	Out (µg P/l)
November 18	6.0	4.0
December 4	12.0	12.2
January 28	19.5	8.0
April 6	6.0	7.7
May 26	8.0	5.5
June 2	11.0	9.2
June 18	15.4	13.5
June 25	1.5	8.9

4.5 Estimation of Bioavailable Phosphorus

The bioavailable phosphorus in each wetland sample was calculated using the linear regression obtained from the standard curve (Figure 33) and presented in Table 7. The bioavailable phosphorus calculated from the trendline is significantly higher than the orthophosphate in the sample but still well below the total phosphorus concentration. This indicates that the biologically available phosphorus in the sample is not limited to the orthophosphate but also includes some of the dissolved phosphorus and particle bound phosphorus.

Table 9: Overview of the concentrations of types of phosphorus in µg P/l. The “BP-OP” column subtracts the orthophosphate concentration from the bioavailable phosphorus concentration to get the concentration of non-orthophosphate bioavailable phosphorus. Chemical TP-OP represents the amount of the total phosphorus that is not orthophosphate. The final column shows the fraction of the chemical TP-OP that is bioavailable based on BP-OP.

		Bioavailable Phosphorus (BP)	Chemical TP	Chemical OP	Chemical TP-OP	BP-OP	Fraction of (TP-OP) that is bio-available
6-Apr	In	67	177	12	165	55	0.33
	Out	40	111	18	93	22	0.24
25-May	In	116	250	31.5	218.5	84	0.39
	Out	229	482	29.5	452.5	199	0.44
2-Jun	In	83	166	20	146	63	0.43
	Out	61	153	18	135	43	0.32
18-Jun	In	94	150	23	127	71	0.56
	Out	54	91	14	77	40	0.52
25-Jun	In	204	843	22	821	182	0.22
	Out	481	1507	437	1070	44	0.04

The table above assumes that all of the orthophosphate is bioavailable in these tests which is a reasonable assumption. Given that assumption, the chemically measured amount of orthophosphate was subtracted from the bioavailable phosphorus in order to determine the amount of combined particulate and dissolved phosphorus that was bioavailable. The concentration of the non-orthophosphate fraction that is bioavailable changes from the inlet to the outlet of the wetland. This means that chemical changes on the speciation of phosphorus must occur in the wetlands.

Table 10: Comparison of the change in percent of non-orthophosphate bioavailable phosphorus (non-OP) to orthophosphate (OP) to bioavailable phosphorus (BP), all as fractions of TP. Negative change in percent from inlet to outlet indicates retention within the wetland and have been highlighted in green for easy recognition.

		% of non-OP available	Change in % non-OP from inlet to outlet	OP as % of TP	Change in % OP from inlet to outlet	BP as % of TP	Change in % BP from inlet to outlet
6-Apr	In	33%		7%		38%	
	Out	24%	-9%	16%	9%	36%	-1%
25-May	In	39%		13%		46%	
	Out	44%	5%	6%	-6%	47%	1%
2-Jun	In	43%		12%		50%	
	Out	32%	-12%	12%	0%	40%	-11%
18-Jun	In	56%		15%		63%	
	Out	52%	-4%	15%	0%	59%	-3%
25-Jun	In	22%		3%		24%	
	Out	4%	-18%	29%	26%	32%	8%

Table 10 shows retention of non-orthophosphate as a percent of total phosphorus and retention of bioavailable phosphorus as a percent of the chemically calculated total phosphorus. In comparison, the table also shows the orthophosphate fraction and retention represented as a percent of total phosphorus. June 2 and June 18th are particularly interesting because they show retention of orthophosphate when comparing chemical analysis concentrations and show retention of bioavailable phosphorus as a percent of total phosphorus but show no change in the percent of orthophosphate with respect to total phosphorus. On June 25th, the change in OP from inlet to outlet was 26% while the change in bioavailable phosphorus was only 8% which implies that the orthophosphate was not a significant fraction of the bioavailable phosphorus.

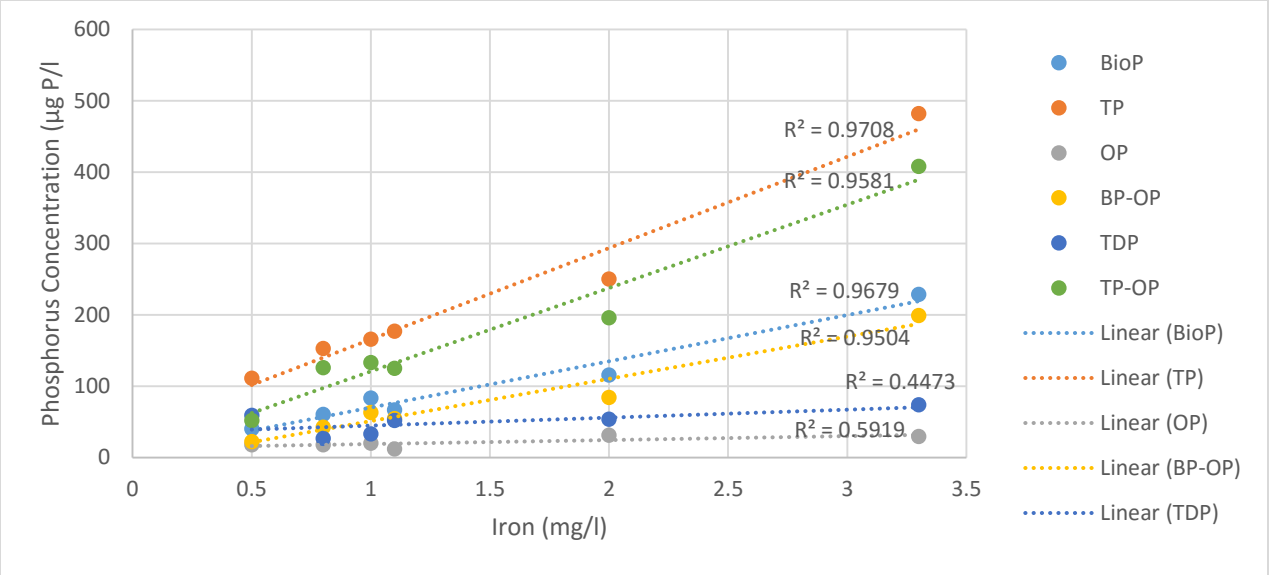

Figure 34: Correlation between the iron content of the samples and phosphorus

The graph in Figure 34 shows a good correlation between the iron content and the bioavailable phosphorus. It also shows a good correlation with total phosphorus and the non-orthophosphate fractions of both total phosphorus and bioavailable phosphorus. This implies that iron bound phosphorus is bioavailable. Iron content does not correlate well with the dissolved or orthophosphate fractions in the samples. A previous study on the bioavailability of phosphorus colloids determined that there is no correlation between the amount of iron and the amount of bioavailable phosphorus in the dissolved phosphorus fraction which is confirmed by these results (Van Moorleghem et al., 2013).

5. DISCUSSION

The methodology of this experiment relied on the conditions in the test flask being phosphorus limited conditions. The nitrogen analysis results validated this assumption. Chemical phosphorus analysis showed that spring was a period of net phosphorus output from the wetland while the fall and summer were periods of retention (Haws, 2016). It was expected that the spring would be the greatest period of retention due to it being a period of significant growth for the plant species. Spring is also the period when farmers would be fertilizing their crops, increasing the load of orthophosphates on the wetland which may be the reason we did not see significant changes in orthophosphate fraction from inlet to outlet. The lack of consistent decrease in orthophosphate from the inlet to the outlet of the wetland could indicates that the wetland isn't functioning properly as orthophosphate is the most reactive form of phosphorus and thus it should be rapidly consumed by organisms and plants. It is the main form of phosphorus that we would expect to decrease from the inlet to the outlet of the wetland. It could also indicate significant decay within the wetland as one of the byproducts of decay can be orthophosphate. The microbial degradation processes can decrease the pH and decrease the redox potential causing the release of phosphate from the sediments as well as from the degraded materials (Figure 14). If the new growth of plants doesn't exceed the microbial degradation processes in the soil this could explain the lack of uptake of phosphate within the wetland.

5.2 Bioavailability of Phosphorus

Over the course of the 5 live tests, the bioavailable phosphorus at the outlet was sometimes higher and sometimes lower than the inlet. From these results it cannot be concluded that the wetland reduces bioavailability of phosphorus in the water entering the lake.

Further comparison of chemical results and bioassay results is displayed graphically in Figure 35. The outlet for Test 4 and Test 8 are the only result that shows bioavailable phosphorus (BAP) to be less than the total dissolved phosphorus (TDP) but the error bars of those results include the total dissolved phosphorus. Not all of the TDP is bioavailable, many of the more complex organic colloids cannot be easily degraded (Van Moorleghem et al., 2013). This discrepancy between TDP and BAP could be due to a high content of degradation byproducts such as phytic acid, and phosphorus containing esters in the dissolved phosphorus fraction. Another explanation is lower amounts of particulate bound bioavailable P than normal. Another fraction of dissolved total phosphorus could be herbicides and pesticides and their degradation products. Depending on the chemical this could inhibit algae growth making the concentration of bioavailable phosphorus appear to be less.

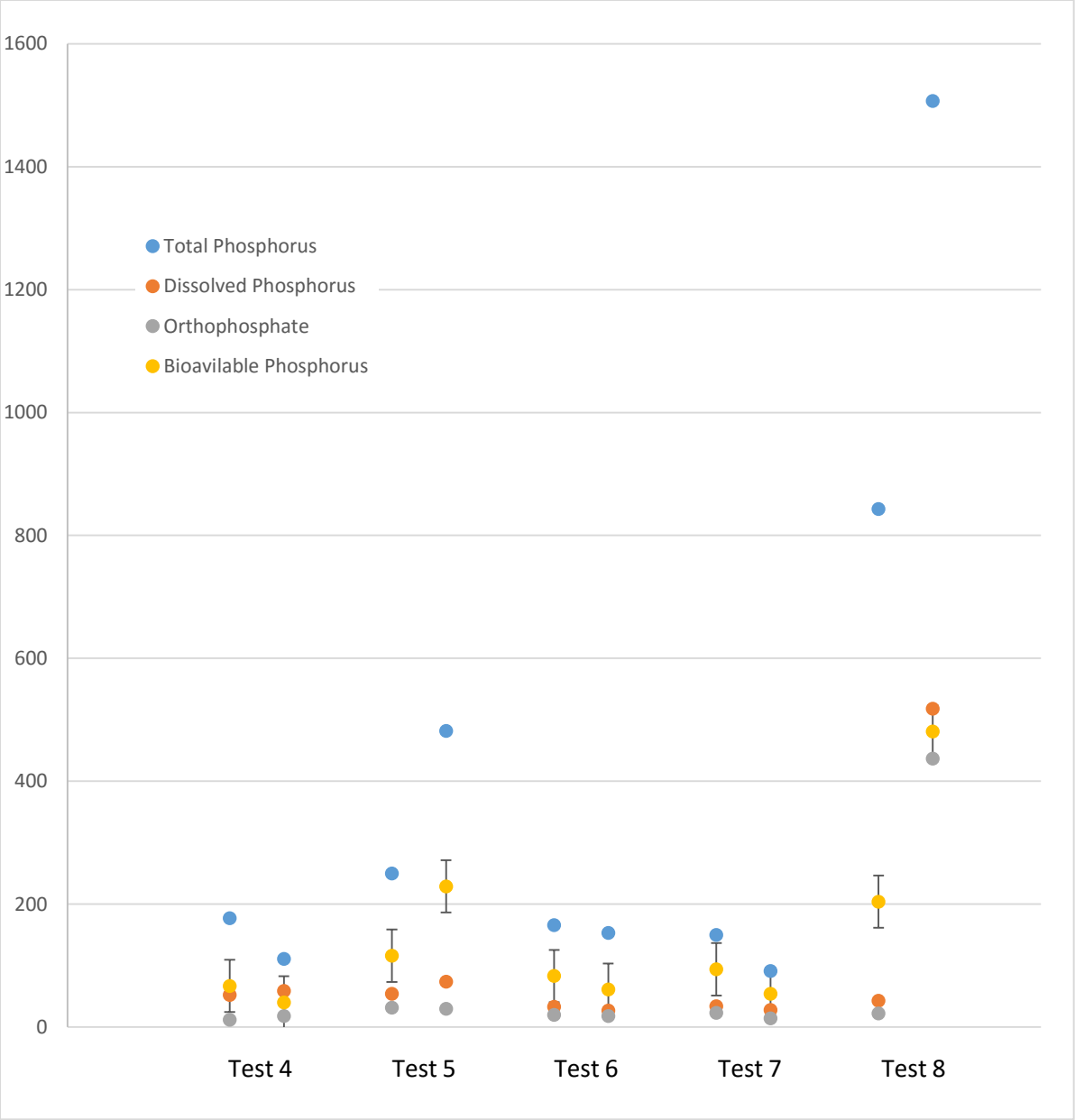

Figure 35: Comparison of chemically measured and biologically measured phosphorus in wetland sample with standard error bars on the bioavailable phosphorus.

The average flow data does not correlate with the change in retention as would be expected (Appendix B). This may indicate channeling within the wetland such that the water always travels the same path through a smaller area of the wetland. The average flow data does not give a good idea of storm event that may have occurred, increasing flow rate for a short period of time and when averaged over a week it appears as though it was low flow. While it is not the purpose of this thesis to estimate the optimal retention time for these concentrations of phosphorus, the variability in the retention times and the lack of correlation between the flow and phosphorus retention for this wetland indicates that it may not

working optimally. Chemical reactions such as sorption and biological processes such as nutrient uptake can be rapid, especially with respect to orthophosphate, but the diffusion or mixing required for enough contact to occur is much more time dependent. If the incoming water is only spending 30 minutes traveling through the wetland, it is not getting a lot of contact time with the plants and the sediments.

From Table 10, it appears the wetland has better retention of the non-orthophosphate fraction of total phosphorus. This could indicate that sedimentation is occurring in the wetland. This methodology might not be the best at determining the bioavailability of the samples in a lake because in this method particles are kept in suspension by the 100 rpm shaker table. This means that particulate bound P that might have been unavailable in a natural system is more readily available in this test method. Overall it can be seen that the fractionation of bioavailable phosphorus changes from inlet to outlet of the wetland which indicates that some processes are working on the water. More in depth testing will be needed to determine precisely how and why the fractionation is changing.

5.2 Error

One of the most prominent potential sources of error for this experiment comes from the initial sampling. The outlet sampling is based on flow while the inlet sampling is based on time. This would result in fewer measurements by the inlet during a storm (high flow) event which could result in a non-representative amount of sediment, especially when compared to the flow based outlet sampling which will take many more samples during a storm event. This affected all of the live algal bioassay samples and thus they could show less retention than was actually achieved.

The positive control used should have had 10 µg P/l more bioavailable phosphorus than the corresponding test group. The actual difference in bioavailable P between the two ranged from 1.5 to 20 µg P/l with a mean of 9.3 µg P/l and a standard deviation of 4.5 µg P/l. The concentration of the standard phosphate solution was checked and found to be fairly accurate, but perhaps adsorption of the phosphate to the glass increases over time or with temperature change. These discrepancies could also indicate that something in the test water was inhibiting growth. Given that only one test (June 2) was reasonably close to the expected difference, and some were above 10, inhibition is not likely the cause of the error in all the tests. Lastly, this error could be due to error in the experiment method. It is possible this method doesn't give an accurate enough estimation of bioavailable phosphorus to get a difference of precisely 10 µg P/l.

Error in positive control. Talk about why this could be. Mention the standard phosphate solution was stored in the fridge and perhaps this solution was at different temperatures for each experimental

addition. This could also explain variations in standards. Error in standard phosphate solution. Error in inhibition. Error in analysis. Discuss Group 4 and how it should have 10 µg P/l more bioavailable P by design and does it show that?

It was noticed during the experiment that black particles the size of ground pepper would appear in the flasks around day 2. These larger particles were determined to be aggregated particles present in the original sample. It was assumed that the centrifugal forces of the shaker table caused the sediment particles to aggregate in the flask. This could cause error in particle counts, and the initial-to-final comparisons of background particles, as well as error in the bioavailable phosphorus fraction as potential bioavailable phosphorus could get trapped in the inaccessible subsurface areas of the particle.

The coulter counter counts total particles and it was not possible to separate out the algae from background particles in the sample. It was observed during the experiment that the number of particles in the background change randomly through the sampling most likely due to aggregation and physical degradation of the particles.

The way the auto-sampler is set up, the samples are taken based on flow and stored in a container that is open to the atmosphere. When the samples got back to the lab for the algal bioassay the pH was always 7. Assuming the low pH in the wetland is due to highly biological activity, the on-site storage allowed for gas exchange which would equalize the CO_2 and O_2 in the sample before it got back to the lab. This will affect the amount of bioavailable phosphorus (Figure 13). In the algal bioassay, the water is fully mixed throughout the experiment thus would have had the same gas exchange anyways. Similarly, the lake water at the outlet of the wetland is shallow and oxygenated so this effect can be considered insignificant as the bioavailability of the phosphorus is important at the outlet of the wetland where it enters the lake at which point it will be exposed and diluted in water at a pH of 7.

The lighting on the experimental shaker table was known to be inconsistent across the area of the table. These slight changes in light availability per flask could account for some of the variation seen between replicates.

5.3 Methodology

In these tests, the dilutions of test water were made in one batch for better uniformity between flasks, and then each flask was inoculated individually but there was still deviation between the results of each flask in a sample group. In future experiments, this deviation might be reduced by making the test water

for each group completely in one container including phosphorus, algae and wetland sample, mixed, and then divided into the flasks. This will better ensure the same test water goes into each flask.

Over time, the amount of algae the test water was inoculated with changed due to error or purposeful adjustment. Consistent algae inoculum in each test could possibly reduce the error seen between tests, but with such high deviation between the individual groups it is hard to say if the initial algae concentration will have a significant difference on the final concentration as long as the inoculum is small enough to allow for initial exponential growth of the algae. The correlation of the bioavailable phosphorus regression was really good which demonstrates that the difference in initial algae did not have a statistically significant effect.

The results obtained with the coulter counter were comparable to the results obtained with the flow cytometer but this introduced error in comparing data with two different sets of systematic error from the equipment. Overall, the flow cytometer is much more accurate at detecting algae both in live and fixed samples so in further experiments one piece of equipment should be used to analyze samples and the flow cytometer is preferable.

The temperature of the standard phosphate solution was not considered important for this experiment methodology. After error analysis comparing the test group to the positive control showed deviation from expected results it was determined there might have been some inconsistencies in the phosphorus concentration added to the positive control. It might benefit the experiment to heat up the standard phosphate solution to minimize adsorption of phosphate to the glass container. Then dispense it into the Z8-P solution before the algae so the increased temperature doesn't negatively affect the algae inoculation. Another possible remedy is to make and store a more concentrated standard so the effects of adsorption are less significant. Then a dilution can be made for each test.

When analyzing the standard curve, NIVA used an exponential trendline for concentrations of phosphorus under 5 µg P/l and a linear trendline for higher concentrations with the expectation that the x intercept should be at 0. In Figure 33, the P-value for the intercept is 0.0119, which is lower than normal significance of 5% thus the intercept can be considered distinctly different from 0. Therefore if there is no phosphorus (x=0), it is expected there would be no growth, but instead decay which correlates with the y-intercept of the trendline at (-68714) cells per ml. The algae used in these tests is starved which means it is already in stationary phase. Theoretically you can say the growth will be 0, but experimentally if you introduce starved algae into an environment without P there will be measureable decay. The tests that were run in

this experiment only include 3 values of phosphorus that are 5 µg P/l or lower; 2, 4, 5 µg P/l. These three points correlated better with a linear trendline (Figure 36).

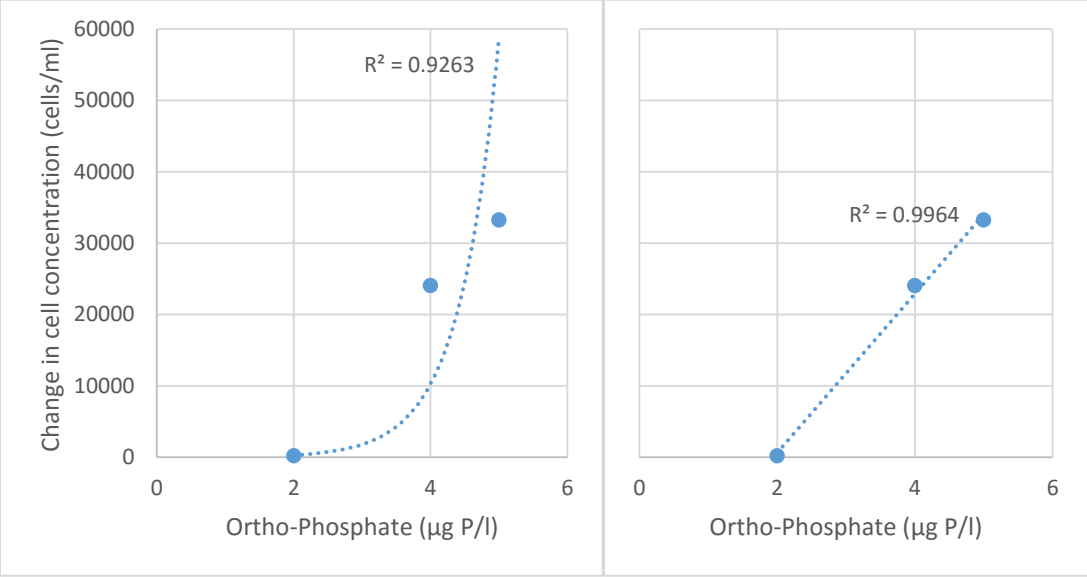

Figure 36: Correlation of standard orthophosphate concentrations under 5 µg P/l with an exponential trendline (left) and a linear trendline (right)

NIVA chose to use an exponential curve to correlate the data for concentrations under 5 µg P/l while in this experiment is was determined that a linear regression was a better fit. In future experiments further resolution for concentrations under 5 µg P/l should be obtained so the regression line can be fitted for better accuracy.

Error in this experiment was complicated to calculate. In future studies using this method, a statistician should be used to develop a good sampling regime with an adequate number of replicates for both the test water and sampling during the test. The statistician should also assist with the final error calculations.

This methodology did not mimic natural conditions at the wetland. In order to get results more accurate to the wetland of interest the method could be changed in several ways. Constant light and 25 degrees C might be ideal conditions but they are not natural conditions for this lake. It has been shown that the adsorption of phosphorus to sediments actually increases with higher temperatures and therefore under natural lower temperatures there may be more phosphorus bioavailable. Increased temperature increases biological activity, so enzymes may work faster to release bound phosphorus. Conducting this experiment under more natural conditions would give a more accurate representation of what will be bioavailable in this environment. This experiment could also be conducted under multiple temperatures to see how seasonal variations in water temperature might affect the bioavailability of the phosphorus.

Some studies using the algal bioassay method did not use the shaker table but instead mixed the test flasks once a day by hand. This method might give a more accurate analysis of the bioavailability of test water in a lake system as it will allow for settling. Another possibility is using a lower rpm which will allow some settling of particles. This experiment could be conducted with several mixing speeds to see how much the rpm of the shaker table affects the results of the experiment. Even with the outlined changes, the algal bioassay still only looks at what is available to algae while in the natural environment algae and bacteria co-exist which may result in an increase in variety of extracellular enzymes and increased bioavailability of the phosphorus compounds.

5.4 Further Research

The experiment presented in this paper should be run again over the course of a year to get a better idea of the annual changes in the bioavailability phosphorus. This experiment should also be performed on other wetlands in the area, preferably ones of different age, to compare performance between wetlands.

For further delineation of the bioavailable phosphorus it would be interesting to compare algal assays on filtered sample as compared to the unfiltered algal assays presented in this paper. This would allow for a more in depth discussion on the speciation of bioavailable phosphorus.

Further chemical speciation of the phosphorus may also help delineate the bioavailable phosphorus fractions. The sediments have been shown to be of particular importance when it comes to bioavailable phosphorus so a chemical sediment release test could be performed. Apply different conditions to the sterilized wetland sample such as a change in dissolved oxygen (redox potential) to see if labile P, orthophosphates, are released. A P-NMR test could be used to determine the organic fractions present and sort them into fast or slow degradation giving more clarity to the bioavailability potential.

A major point in this discussion was the proper functioning of the wetland. More analysis of the wetland should be completed to determine if it is functioning as intended. Given the lack of correlation between flow and retention for the samples analyzed in this paper, it would be interesting to set up multiple probes throughout the wetland and see to what extent the water penetrates the area of the wetland during different flow conditions using a tracer test. Another tracer test that could be done on the wetland would be to dose the input with radioactive phosphorus (P-32, P-33) and watch for it at the outlet. Outlet radioactive phosphorus in the inlet and outlet could then be compared to the chemically measured orthophosphate to see if orthophosphate has a source in the wetland.

6. CONCLUSION

The hypothesis of this study was that the wetland would decreases the bioavailability of the phosphorus. Based on the results of this study, the wetland does not affect the overall bioavailable phosphorus concentration from the inlet to the outlet. Testing did confirm the fractionation of the bioavailable phosphorus did change by the time the water exited the wetland but these changes varied between tests therefore they could not be attributed to any specific process.

The lack of consistent retention of total phosphorus, orthophosphate or bioavailable phosphorus indicates that the wetland is not performing as expected. Assuming the wetland is not working as it was designed to, some maintenance may be needed. This wetland is lacking a proper bypass which could help maintain a better hydraulic retention time and reduce wash outs of sediments from the wetland.

Weaknesses in the algal bioassay method used include weakness was insufficient sampling of test flasks. In initial tests only a few samples were taken but definitive determination of stationary phase couldn't be made. When sampling frequency was increased, an oscillating trend in algae concentrations was observed indicating that prior less frequent sampling may have misdiagnosed reduced cell counts as death phase. This experiment could be improved with more frequent sampling of the test water during the test. This test could be improved by better resolution of standard solutions between 0 and 5 µg P/l to clarify the trend of growth at low concentration of phosphorus. Furthermore a statistician should be employed for better quantification of error and possible remediate of error through different sampling methods.

A weakness that should be addressed before performing another algal bioassay on this wetland is the auto-sampler. The inconsistency of sampling frequency between the inlet and the outlet made them less comparable. For future studies the outlet should also be set to take samples on the same time increment as the inlet or the flow measurement sampling interface on the inlet should be fixed so it can take flow based samples again.

Further work should focus on understanding the bioavailability of the different fractions of phosphorus in and out of the wetland by further fractioning the sample using chemical analysis as well as separation of fractions to be used in the algal bioassay.

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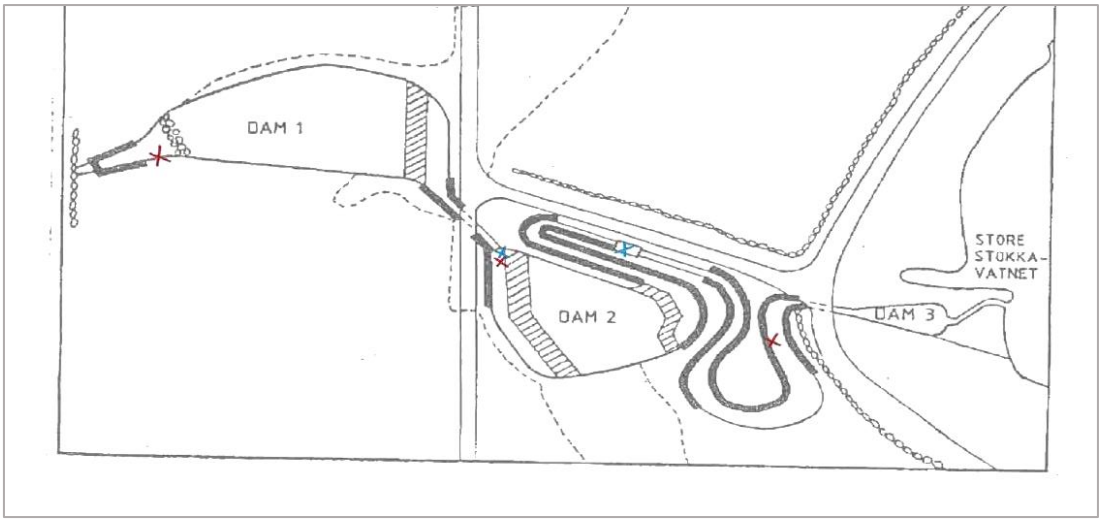
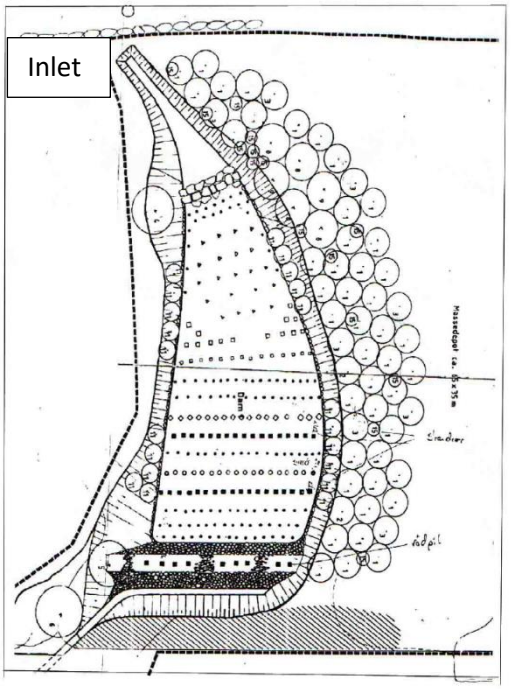
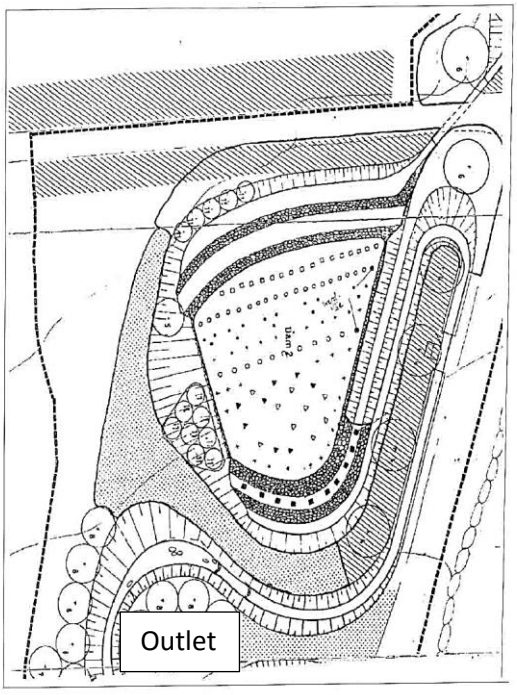
APPENDIX A

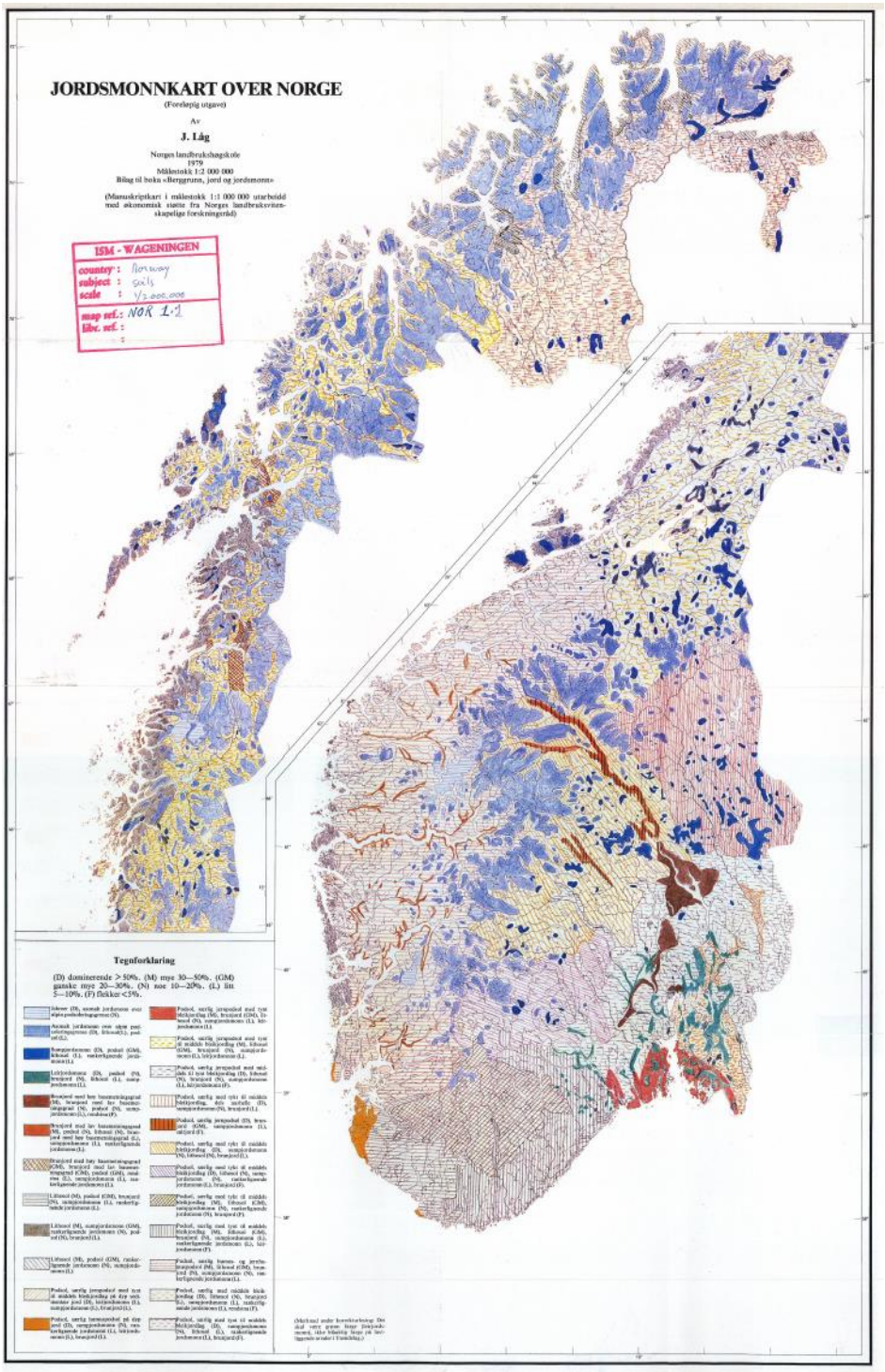
Wetland Maps:







Soil Map of Norway (Hartemink, 2006):



APPENDIX B

Microsoft Excel Chart

Raw Data from Live Analysis:

Microsoft Excel Chart

and Fixed Analysis:

Dixon's Q Test Table

Number of values:	3	4	5	6	7	8	9	10
$Q_{90\%}$:	0.941	0.765	0.642	0.560	0.507	0.468	0.437	0.412
$Q_{95\%}$:	0.970	0.829	0.710	0.625	0.568	0.526	0.493	0.466
$Q_{99\%}$:	0.994	0.926	0.821	0.740	0.680	0.634	0.598	0.568

Results from Regression Analysis on Live Data Only

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.97968159
R Square	0.95977602
Adjusted R Square	0.95575362
Standard Error	41984.5612
Observations	12

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	4.21E+11	4.21E+11	238.6079	2.64E-08
Residual	10	1.76E+10	1.76E+09		
Total	11	4.38E+11			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-73622.719	20668.42	-3.56209	0.005163	-119675	-27570.6	-119675	-27570.6
X Variable 1	22009.4737	1424.844	15.44694	2.64E-08	18834.72	25184.22	18834.72	25184.22

Standard Error of the Estimate for Phosphorus Concentrations

X Expected	X based on linear regression	$X_{LR} - X_a$	$(X_{LR} - X_a)^2$
2	3.355886	1.355886	1.838427
4	4.43884	0.43884	0.192581
5	4.856634	-0.14337	0.020554
6	4.858149	-1.14185	1.303824
7	6.318538	-0.68146	0.464391
8	7.581277	-0.41872	0.175329
9	8.134082	-0.86592	0.749815
10	9.692535	-0.30747	0.094535
15	18.71884	3.718842	13.82979
20	19.24014	-0.75986	0.577381
25	21.53769	-3.46231	11.9876
30	32.27057	2.270571	5.155493
		Sum	36.38971
		Standard Error	**1.9**

Regression Analysis of All Data (Fixed and Live)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.938988
R Square	0.881698
Adjusted R Square	0.874739
Standard Error	97962.22
Observations	19

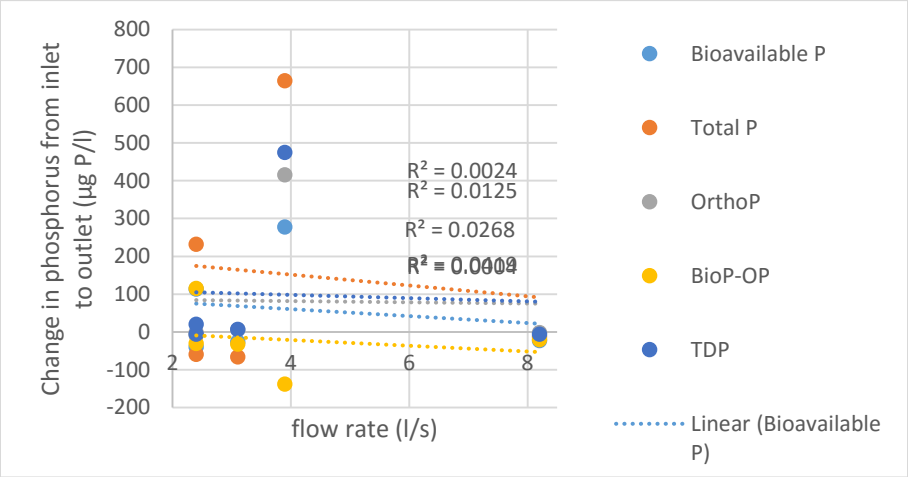
ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1.22E+12	1.22E+12	126.6999	2.66E-09
Residual	17	1.63E+11	9.6E+09		
Total	18	1.38E+12			

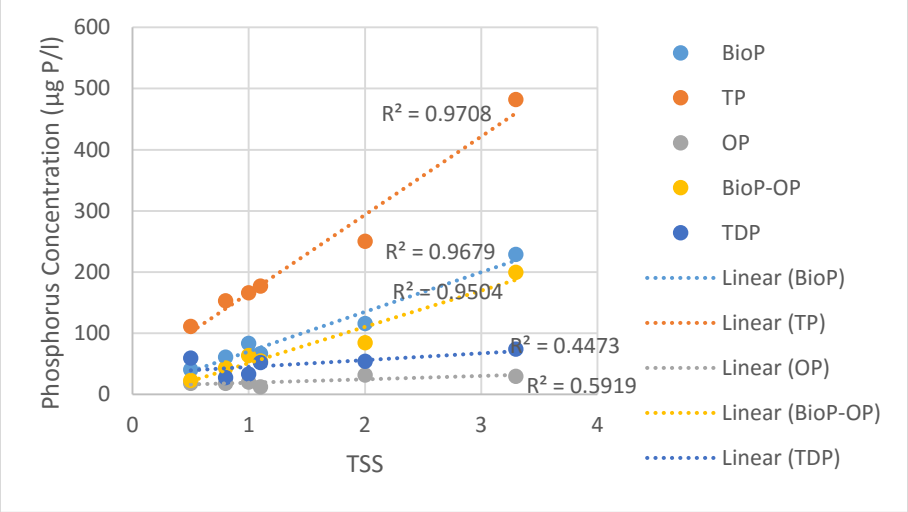
	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*

Intercept	-96724.7	36347.71	-2.66109	0.016457	-173412	-20037.7	-173412	-20037.7
X Variable 1	27033.21	2401.649	11.2561	2.66E-09	21966.17	32100.24	21966.17	32100.24

Attempt to determine a correlation between flow rate and phosphorus retention

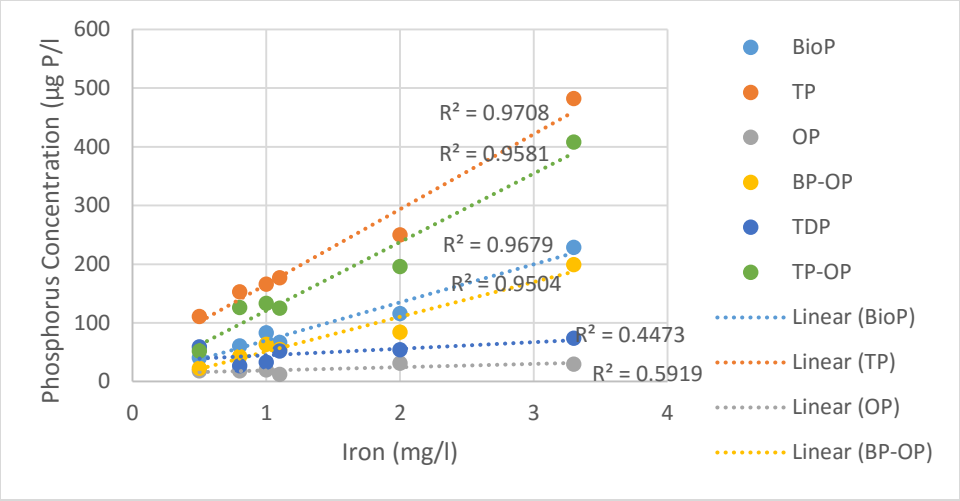


Correlation between TSS data and phosphorus



Correlation between iron content (mg/l) and phosphorus

Determination of the Effect of Constructed Wetlands on the Bioavailability of Phosphorus using Algal Bioassays



APPENDIX C

Placement of culture flasks on the shaker table consistently followed this set up when looking down at the top of the shaker table.

42	1	9	39	36	33	30	27	24	21	18	14	10	6	2
43	5	13	40	37	34	31	28	25	22	19	15	11	7	3
44		17	41	38	35	32	29	26	23	20	16	12	8	4

If lighting was consistent through the whole testing period, then each flask got the same intensity of light in each test.

APPENDIX D

Results from Anne-Marie Haws (Haws, 2016)

	TP	*TFP*	*PO_4*
Retention (kg/yr)	6.9	2.6	1.8
Retention (g/m^{2}/year)	9.0	3.5	2.3

Month	*Year*	*TP Retention g/wk*
June	2015	-77.3
July	2015	235.9
August	2015	426.6
September	2015	70.2
October	2015	69.6
November	2015	16.8
December	2015	-287.8
January	2016	1245.7
February	2016	-43.5
March	2016	-59.5
April	2016	128.8
May	2016	-130.5