Luxury Uptake of Phosphorus by Microalgae in New Zealand Waste Stabilisation Ponds

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Thesis Abstract

The discharge of phosphorus to waterways within wastewater effluent causes significant environmental damage through microalgal blooms and eutrophication. This is a particular problem for wastewater treatment plants that rely on waste stabilisation ponds (WSPs) for the bulk of their treatment. While having simple designs and low running costs, WSPs are mostly ineffective at phosphorus removal, with only 15 – 50% removal achieved on average according to some studies.

The luxury uptake phenomenon within microalgae has been identified as one mechanism that could improve WSP phosphorus removal. This occurs when microalgae store phosphorus beyond what is required for their metabolism as polyphosphate, leading to phosphorus contents above the standard 1 %P/g VSS for microalgae. However, studies on this subject in full scale WSPs to date have been limited to just two different ponds. To improve knowledge on this mechanism, this study aimed to assess the impact of environmental conditions, climatic region and pond type on microalgal luxury uptake, as well as determine which specific microalgal and cyanobacterial genera were best able to perform this mechanism. To achieve these objectives, a yearlong study was conducted on 13 different WSPs from 7 sites within various climatological regions within New Zealand, as well as two pilot scale High Rate Algal Ponds (HRAPs).

From this study, it was found that luxury uptake was found to occur in 56% of the WSP, with a peak phosphorus content of 3.8 %P/g VSS. Conversely, only one sample taken from the HRAPs was found to exhibit luxury uptake. Total dissolved phosphorus (TDP) concentration and rainfall were found to have a significant effect on biomass phosphorus content at a 95% confidence level, while the WSP climate was found not have an influence. There were no significant differences between the biomass phosphorus contents in primary and secondary ponds, with averages of 1.31 %P/g VSS and 1.21 %P/g VSS respectively, while HRAPs (0.71 %P/g VSS) were significantly lower due to the low TDP concentrations experienced by these ponds.

22 of the 23 identified microalgal and cyanobacterial genera were found to perform luxury uptake, at varying frequencies. The cyanobacterium *Planktothrix* was most effective, storing polyphosphate as granules in 84% of the samples it was identified in. *Scenedesmus*, *Pediastrum*, and *Schroederia* were also effective, at frequencies of 73%, 82% and 79% respectively. There was some correlation between storage of phosphorus as polyphosphate and enhanced phosphorus contents in the biomass, with nearly all samples containing no visually identifiable polyphosphate granules exhibiting phosphorus contents below 1 %P/g VSS. However, there were only limited correlations between the amount of polyphosphate and the levels of the significant variables identified previously.

This research provides valuable insight into the phosphorus uptake behaviour of microalgae and cyanobacteria, and shows that there is some potential for development of a new engineered process targeting improved phosphorus removal. If the phosphorus content of

biomass in this new process could consistently attain a level of 3 %P/g VSS, phosphorus removal from wastewater for an average community of 500 people could be increased from 31% to 93%, thus greatly reducing the impact of wastewater discharge on the receiving environment.

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This thesis is dedicated to my Grandmother

Helen Enid Crimp

1932 - 2015

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1 Introduction

Phosphorus discharge to waterways from wastewater treatment systems has received increased attention in recent literature and research as a serious threat to the health of water bodies. The high concentrations of phosphorus compounds within wastewater can lead to eutrophication in receiving waters if not removed (Blackall, et al. 2002; Craggs 2005a; Ruiz, et al. 2014). Eutrophication is the extraordinary growth of microalgae as a result of excess nutrients in water bodies, such as lakes, rivers and seas (de-Bashan and Bashan 2004; Chislock, et al. 2013). Such growth can limit light penetration into water bodies, reducing growth and causing death of aquatic plants. In addition, the high rate of photosynthesis associated with eutrophication can deplete dissolved inorganic carbon and raise pH to extreme levels (Chislock, et al. 2013). Such microalgal growth can include toxic cyanobacteria (blue-green microalgae), which release toxins harmful to all aquatic life (Blackall, et al. 2002), as well as creating a public health risk. In 2007, a blue-green microalgal bloom within Lake Taihu in China significantly impacted the fresh water supply for 6.1 million people within the nearby Wuxi City (Ye, et al. 2011).

Even more significant effects on waterways occur when the microalgae grown through eutrophication eventually die out, through complete consumption of the nutrients in the waterway. The death of these microalgae promotes aerobic bacterial growth, which consume oxygen in order to metabolise the dead microalgae. This in turn depletes the waterways of oxygen, creating an anoxic dead zone which kills off all aquatic life (Chislock, et al. 2013).

The consequences of nutrient discharge are extremely widespread. A survey conducted by the International Lake Environment Committee found that 48% of lakes in North America are eutrophic, with 53% in Europe, 54% in Asia and the Pacific, 41% in South America, and 28% in Africa (Cai, et al. 2013). The estimated cost of damage mediated by eutrophication in the U.S. alone is approximately $2.2 billion annually (Dodds, et al. 2009). Recognition of these risks to waterways has led to the creation of extensive projects aimed at reducing phosphorus loading in receiving waterways. Examples of such projects have occurred in the Baltic Sea and the Great Lakes of North America (Harremoes 1994; Vallentyne 1994). However, eutrophication through high phosphorus release to waterways remains a problem. One significant source of phosphorus discharge is wastewater treatment plants, which release thousands of tonnes of phosphorus to waterways every year (Cai, et al. 2013). The bulk of this discharge is due to the inability of the employed wastewater treatment technology to remove phosphorus from the raw influent, leading to significant amounts of phosphorus passing straight through the system within the wastewater stream. Of particular concern is the phosphorus removal performance of treatment plants employing Waste Stabilisation Ponds (WSPs). WSPs are employed by thousands of communities around the world, and yet currently only remove 15 – 50% of influent phosphorus on average (Garcia, et al. 2000), leading to large masses of phosphorus being released into waterways. Improving the phosphorus removal ability of WSPs has been the subject of significant amounts of research; however there are still significant gaps in the knowledge in this area. Currently, very little is known about the internal phosphorus removal mechanisms within the WSPs, particularly the

luxury uptake mechanism which has been found to occur within microalgae. This mechanism has been known to result in enhanced phosphorus uptake by microalgae (Powell, et al. 2008), but has only been studied in two full scale WSP systems (Powell, et al. 2011). To improve knowledge of microalgal luxury uptake in WSPs, the following objectives were set for this project:

1. Confirm whether luxury uptake occurs in WSPs from a wide range of climates.
2. Determine the environmental effects that influence microalgal luxury uptake within WSPs.
3. Determine how different pond types influence the performance of luxury uptake.
4. Determine which microalgal species perform luxury uptake within WSPs.

2 Literature Review

2.1 Waste Stabilisation Ponds

The use of WSPs for treatment of wastewater is extremely widespread around the globe. Despite their simplicity to design and operate WSPs still provide good levels of treatment in terms of organic carbon and pathogen removal, making them ideal for smaller communities (Kayombo, et al. 2004; Shilton and Walmsley 2005).

The standard pond system utilises of a series of different types of ponds. This usually consists of a primary facultative pond, followed by a series of maturation ponds. Occasionally, an anaerobic pond is placed at the start of the pond system to remove the bulk of the organic load, allowing for reduced size in the following ponds (Kayombo, et al. 2004; Shilton and Walmsley 2005).

2.1.1 Types of Ponds

2.1.1.1 Anaerobic Ponds

Anaerobic ponds are typically 2 – 5 metres deep (Kayombo, et al. 2004; Shilton and Walmsley 2005), and are used to provide bulk removal of organic solids. As sunlight can only penetrate the upper layers of the pond, there is only limited microalgal growth within the pond due to the lack of sunlight available for microalgal photosynthesis. Due to the lack of significant photosynthesis, there is also a lack of dissolved oxygen production within the pond, creating an anaerobic environment favouring the growth of anaerobic bacteria. This in turn limits the amount of nutrient removal achieved by the pond, with the only nutrient removal achieved through assimilation into the bacterial biomass. Influent wastewater is typically kept in the pond for just a few days.

2.1.1.2 Facultative Ponds

Facultative ponds operate with both aerobic and anaerobic zones, as demonstrated in Figure 1.

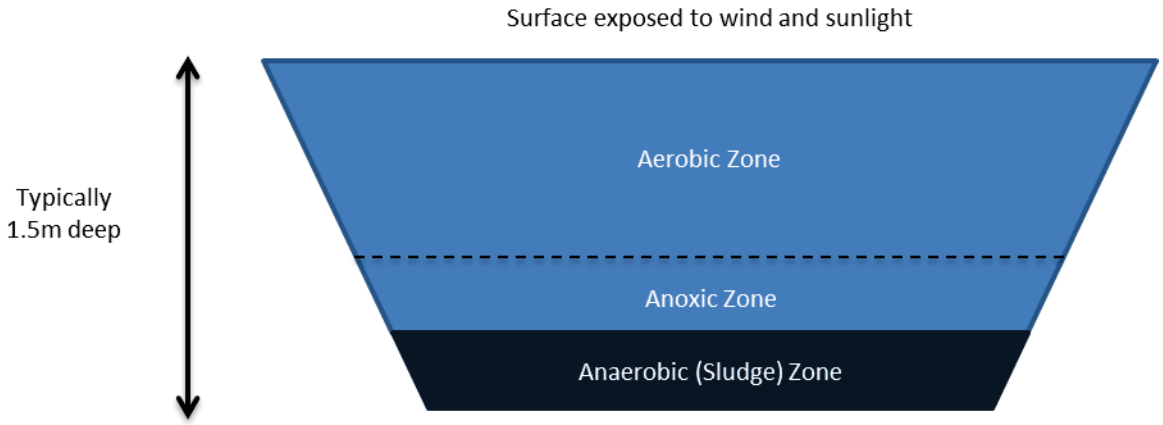


Figure 1: Outline of the different zones found within facultative ponds

As described in Shilton and Walmsley (2005), the top of the pond is typically the most irradiated due to its proximity to the surface. This leads to high levels of microalgal photosynthesis, leading to increased microalgal growth and oxygen production, creating an aerobic zone. With increased depth of the pond, there is less sunlight available for photosynthesis and in turn less dissolved oxygen production. Eventually, the dissolved oxygen is depleted, leading to the creation of an anoxic zone. Any dead microalgae and other solids settle to the bottom of the pond, forming an anaerobic sludge layer where anaerobic bacteria digest the sludge.

To support increased microalgal growth, facultative ponds are a lot shallower than anaerobic ponds, at around 1.5m in depth. They also have a longer retention time than anaerobic ponds, with retention times of a few weeks fairly common (US EPA 1983; Kayombo, et al. 2004; Shilton and Walmsley 2005). This pond is the most important in terms of ensuring good effluent quality, as it is typically the largest pond in the system and therefore relied upon to provide the bulk of the treatment for solids, nutrients and pathogens.

2.1.1.3 Maturation Ponds

Maturation ponds are designed to remove residual pathogens from the wastewater. They are typically much smaller than facultative ponds, at around 1m in depth and with a much lower surface area. They also are more highly oxygenated, due to the lower organic loading present (Kayombo, et al. 2004; Shilton and Walmsley 2005). Typically, a series of smaller maturation ponds are used rather than a single large pond for better hydraulic efficiency (Shilton and Walmsley 2005).

2.1.1.4 High Rate Algal Ponds

High rate algal ponds (HRAPs) are an alternative pond design to standard WSPs. While aiming to treat the same wastewater constituents, they are typically a lot shallower than facultative ponds at 0.2 – 0.8 metres in depth (Shilton and Walmsley 2005). They also have a much shorter hydraulic retention time than typical WSPs at around a week or less. A key feature is the paddlewheel, which drives water around the "raceway" configuration of the pond. The shallow depth and high degree of mixing promote the growth of large amounts of microalgae, which provide the bulk of the treatment of solids and nutrients (Craggs, 2005b). Elevated rates of disinfection also occur due to the high level of light penetration, pH and DO within the pond (Craggs, 2005b).

2.1.2 Pond Biology

Waste stabilisation ponds predominantly contain two different types of microorganisms: microalgae and bacteria.

2.1.2.1 Microalgae

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that can grow rapidly and live in harsh conditions due to their unicellular or simple multicellular structure (Mata, et al. 2010). Examples of different genera of microalgae are shown in Figure 2.

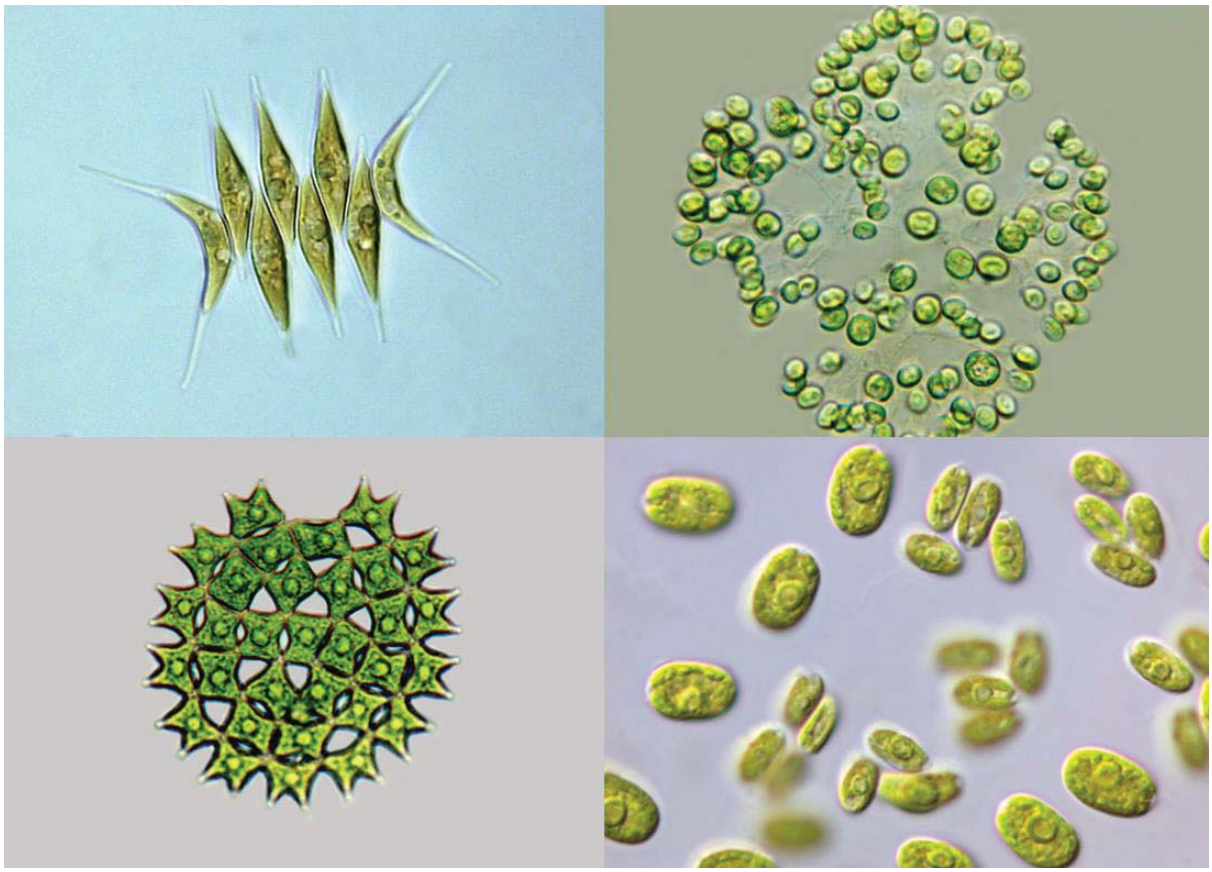


Figure 2: Examples of microalgae, taken from Landcare Research. Clockwise from top left: *Scenedesmus*, *Dictyosphaerium*, *Chlamydomonas* and *Pediastrum*.

Microalgae are present in all existing earth ecosystems. It is estimated that more than 50,000 species exist, but only around 30,000 have been studied and analysed (Richmond 2004). The unique environment present within WSPs leads to the growth of particular genera of microalgae that are not commonly found in other environments (Pearson 2005).

2.1.2.2 Bacteria

Bacteria in waste stabilisation ponds are typically either aerobic (need oxygen to survive) or anaerobic (survive in an absence of oxygen) (Shilton and Walmsley 2005). In the aerobic layer of the pond, bacteria form a symbiotic relationship with microalgae. This is demonstrated in Figure 3.

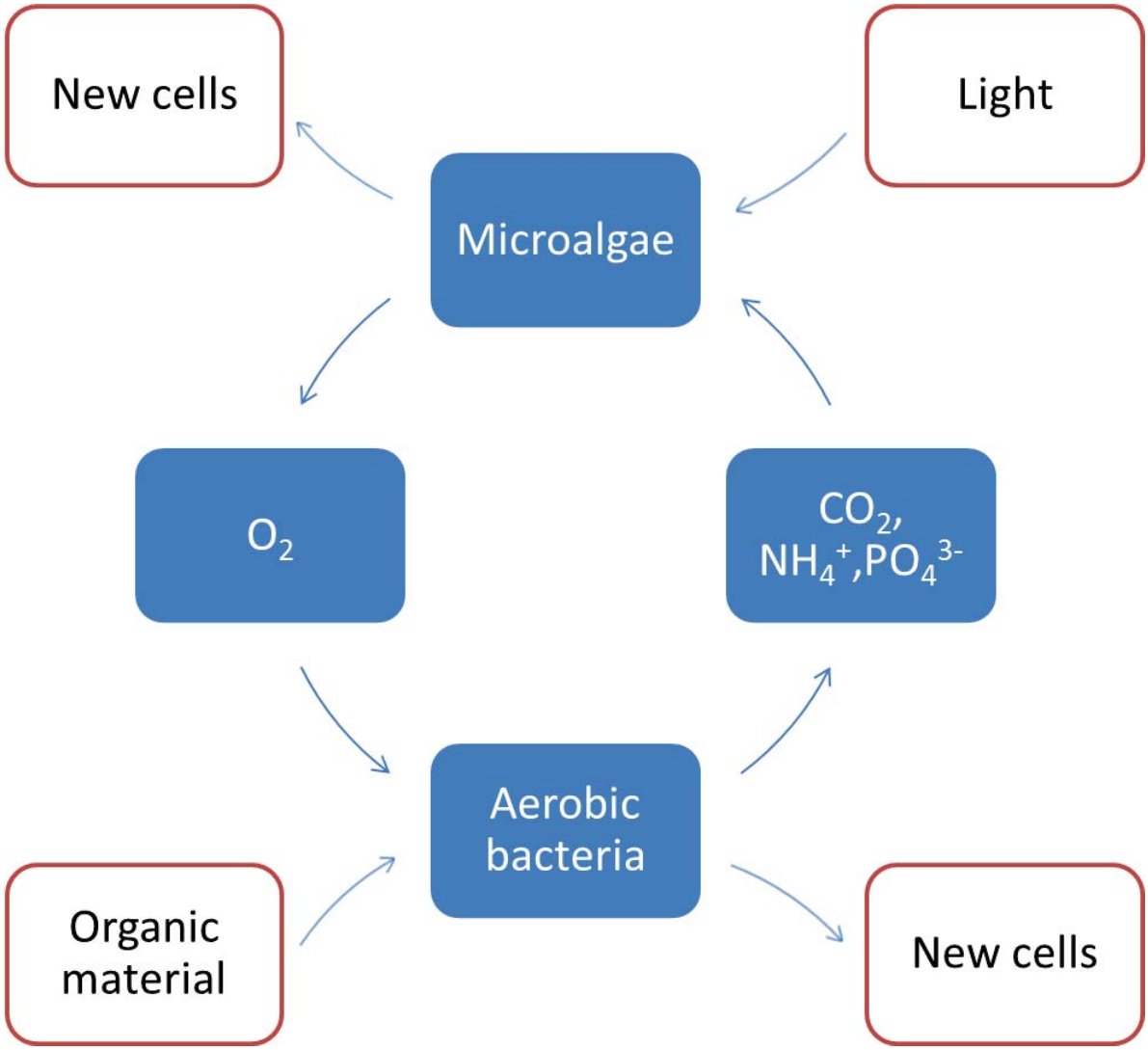


Figure 3: Diagram of the symbiotic relationship between microalgae and aerobic bacteria

Aerobic bacteria consume organic matter as part of their metabolism, producing CO_2 as a by-product. This CO_2 is then consumed by microalgae along with other nutrients within the facultative pond as part of photosynthesis, producing oxygen as a by-product. This oxygen is then used in bacterial metabolism, completing the symbiotic cycle. In this way, the organic carbon is fixed within the biomass, which then settles and can be removed when each pond is desludged (Walmsley and Shilton 2005).

One phylum of bacteria that is often present in WSPs are cyanobacteria (occasionally known as blue-green algae). These are bacteria that, like microalgae, obtain their energy through

photosynthesis (Durall and Lindblad 2015). While not common in WSPs, these bacteria have been known to appear during periods of high water temperature (Gloyna 1971).

2.1.3 Pond Treatment Mechanisms and Performance

2.1.3.1 Solids and Organic Carbon Removal

In pond systems featuring an anaerobic pond, the bulk of solids removal (40 – 70%) is carried out by this initial stage. The remaining solids removal is then performed by the facultative pond and, to a lesser extent, the maturation pond, with up to 95% removal rates achievable ((US EPA 1983; Shilton and Walmsley 2005). For pond systems that utilise facultative ponds as their primary treatment option, removal of between 75 – 85% of organic loading can be achieved (Walmsley and Shilton 2005). However, this requires a much larger pond size and much longer retention time than if a combination of an anaerobic pond and an aerobic pond was used. Solids removal within the ponds is achieved through two main mechanisms: sedimentation of larger solids and assimilation of organic carbon.

Sedimentation

Upon entering a wastewater stabilisation pond, settleable and colloidal solids settle on the bottom of the pond. This forms a sludge layer on the bottom of the pond where anaerobic decay occurs. Once the organic solids have been broken down to organic carbon molecules, anaerobic bacteria assimilate the organic carbon as part of its metabolism. Some release of organic carbon and nutrients back into the water also occurs as a result of this process. This occurs in both the anaerobic and facultative ponds (Kayombo, et al. 2004; Shilton and Walmsley 2005).

Assimilation

The remaining organic carbon not removed through sedimentation and anaerobic decay is consumed through assimilation into the bacterial biomass and in turn the microalgal biomass as part of the symbiotic relationship outlined in Figure 3.

2.1.3.2 Pathogen Removal

Sunlight has been deemed to be the single most important factor causing disinfection in WSPs (Davies-Colley 2005). This occurs primarily in facultative and maturation ponds, where the high surface area and low depth allows for large amounts of light penetration. Removal of pathogens occurs through UV light damaging the DNA of the pathogenic bacteria, preventing replication, as well as other photo-oxidative damage to external cell structures (Davies-Colley 2005). Increased dissolved oxygen and pH are known to increase disinfection levels, due to the effect these quantities have on the photo-oxidative reactions (Davies-Colley 2005).

2.1.3.3 Nitrogen Removal

Nitrogen removal in WSPs is achieved through a variety of different mechanisms.

Ammonia Volatilisation

Nitrogen as ammonia may be lost through the surface of WSPs through volatilisation of ammonia gas. This process is dependent on the concentration of free ammonia within the pond, as well as pond temperature, pH, and mixing conditions (Craggs, 2005a).

Nitrification/Denitrification

Under aerobic conditions, ammoniacal-N may be oxidised by nitrifying bacteria to nitrite and then nitrate, in a process known as nitrification. Anaerobic bacteria then perform denitrification by reducing nitrate to nitrous oxide (N_2O) and then nitrogen gas (N_2), which is released to the atmosphere (Craggs, 2005a).

In most WSPs, nitrification/denitrification occurs intermittently due to the changing dissolved oxygen, temperature and pH within the pond, all of which affect the process (Craggs, 2005a). As a result, nitrification/denitrification is not a major removal mechanism for nitrogen in WSPs (Ferrara and Avci 1982).

Assimilation and Sedimentation

Organic nitrogen is often removed from the incoming wastewater through simple sedimentation (Craggs, 2005a). This occurs in two different ways – either sedimentation of the nitrogen residing within the wastewater solids, or more frequently, sedimentation of microorganisms that have assimilated nitrogen as a basic requirement for growth (Pedersen and Borum 1996). Nitrogen is the second most important nutrient after carbon, and may comprise more than 10% of the cells biomass (Becker 1994). Nitrogen is primarily assimilated as ammonium [NH_4^+] and nitrate [NO_3^-], but can also be assimilated as urea [$(NH_2)_2CO$] and nitrite [NO_2^-] (Larsdotter 2006). Many authors have concluded that assimilation of nitrogen into microorganisms followed by sedimentation is the main process of nitrogen removal, with Ferrara and Avci (1982) estimating that 96% of total nitrogen was removed from WSPs through this process. These mechanisms have also been found to be significant for phosphorus removal (Wrigley and Toerien 1990). However, if the sludge formed by the sedimentation of this biomass is not removed, its eventual breakdown results in the release of nitrogen and phosphorus back into the water (Reed, et al. 1995).

Adsorption to Cations

Inorganic phosphates and ammoniacal nitrogen may be removed by adsorption to pond sludge, or, at high pH, to ferric oxyhydroxide [Fe(OOH)], aluminium hydroxide [$Al(OH)_3$] and calcium carbonate [$CaCO_3$] crystals (Craggs, 2005a). However, such removal requires an uncommonly high concentration of cations, usually requiring chemical dosing.

2.1.3.4 Phosphorus Removal

In wastewater treatment, phosphorus is removed by converting the phosphorus ions in wastewater into a solid fraction (Hoffman 1998; de-Bashan and Bashan 2004). There are a number of different mechanisms within WSPs that perform this conversion.

Precipitation

During growth, microalgae consume inorganic carbon, usually in the form of CO_2. If this consumption exceeds the rate at which carbon is absorbed from the atmosphere and from bacterial oxidation of organic waste, then the pH increases. An elevated pH can cause precipitation of phosphorus as phosphate (PO_4^{3-}) through complexation with metal ions such as calcium and magnesium in the water (Goldman, et al. 1982; Powell, et al. 2008).

While pH is the primary trigger for phosphorus precipitation, Goldman, et al. (1982) found that precipitation is also dependent on temperature, phosphate concentration and cation concentration. For every rise of one pH unit above a pH of 8.2, the concentration of phosphate remaining in the pond water decreases by a factor of ten (Craggs, 2005a). At pH >8.2, precipitation can account for up to 80% of phosphorus removal in WSPs (Ellis, 1983). However, to achieve the high cation concentration (50 – 100 mg/L) required for complete phosphorus precipitation, chemical dosing is usually required. At low cation concentrations (<50 g/m^3), there is only significant phosphate concentration at pH > 10 (Diaz, et al. 1994). In addition, phosphate precipitated at high pH during the day may be subsequently released at night when pH declines to <8 (Diaz, et al. 1994).

Adsorption

Adsorption to the surface of the cells of microalgae has been recognised as being a significant mechanism in the removal of phosphorus from wastewater. A study by Xu, et al. (2014) using *Chlorella emersonii* in membrane bioreactors found that extracellular phosphorus accounted for more than 90% of the total phosphorus in the microalgal biomass. Average extracellular phosphorus content ranged from 6.4% to 8.4% of dry weight, however this was likely due to the presence of calcium deposits that were found on the surface of the microalgae. Gomez, et al. (2000) also found that the presence of cations had a significant impact on phosphorus adsorption, with adsorption to pond sludge found to be a significant mechanism when the sludge contained high concentrations of iron and aluminium. A study by Martinez, et al. (2000) also found evidence of adsorption being a prevalent mechanism. The authors noted that there was a sharp drop in the residual phosphorus content of wastewater solution within the first 3 hours of inoculation with *Scenedesmus obliquus*. As this time period is too short to allow for significant microalgal growth and corresponding assimilation of phosphorus to occur, this phosphorus removal was attributed to adsorption to the surface of the microalgal cells. The drops in phosphorus concentration implied elimination of phosphorus of up to 51% within the first 3 hours. The magnitude of this phosphorus removal did not appear to depend on temperature or stirring, but on the concentration of phosphorus and available surface area for adsorption. However, another study by Lu, et al. (2014) found that environmental conditions did have a significant effect on adsorption of phosphorus. The authors found that the temperature and light intensity experienced by periphyton in phosphorus solution significantly affected the phosphorus removal achieved by adsorption. The removal rate for phosphorus increased from 22% to 96% with an increase in temperature from 5°C to 25°C, while decreasing the light intensity from 12,000 to 4800 Lux resulted in phosphorus removal decreasing from 98% to 10%. It was also found that adsorption could not occur in absence of light. Despite the significant influence of environmental variables, all of these studies indicate

that adsorption may be a highly prevalent mechanism in the removal of phosphorus from aquatic environments.

Assimilation

Phosphorus is an essential element needed for cellular constituents such as phospholipids, nucleotides and nucleic acids, and thus requires microalgae and bacteria to absorb it through assimilation (Cai, et al. 2013). According to Larsdotter (2006) and Cai, et al. (2013), assimilation in both bacteria and microalgae occurs when phosphorus is absorbed from solution as orthophosphate (either $H_2PO_4^-$ or HPO_4^{2-}), or removed from organic substrates by phosphatase enzymes found on the surface of the cell which then convert the phosphorus to orthophosphates. The orthophosphates are then transported across the plasma membrane through energized transport, which begins with coupling of the orthophosphate cell with a cation, most likely H^+ in wastewater and freshwater environments (Taylor, et al. 2012). Creation of an electrochemical gradient across the plasma membrane leads to the cation along with the orthophosphate molecule being pulled into the microalgal cell (Taylor, et al. 2012). Once inside the cell, the orthophosphates are incorporated into organic compounds through phosphorylation, much of which involves the generation of adenosine triphosphate (ATP) from adenosine diphosphate (ADP), accompanied by a form of energy input (such as that from oxidation of substrates, electron transport from mitochondria, or through light from photosynthesis).

One factor influencing the effectiveness of this mechanism is the amount of nitrogen in the wastewater. According to Redfield (1934), the N:P atomic ratio for both microalgae and bacteria is around 15:1. As the N:P ratio within wastewater is typically about 4:1, it contains insufficient nitrogen to enable complete removal of phosphorus by assimilation (Craggs, 2005a). As a result, while significant phosphorus removal can be achieved through assimilation from wastewater (Wang, et al. 2009), complete removal is not possible without elevated levels of nitrogen.

It must be noted that while the values above have assumed that assimilation was the sole mechanism in operation, it is highly likely that some adsorption to the surface of the microalgae was also occurring. This may not have been considered by the authors, as the dry weight analysis method used by the authors above does not differentiate between phosphorus adsorbed to the surface of the microalgae and phosphorus assimilated within the microalgae. While the degree of adsorption may not have been significant, it could still have influenced the results.

Luxury Uptake

Another mechanism of phosphorus removal within WSPs is the luxury uptake phenomenon in microalgae. While microalgae typically contain approximately 1% phosphorus by dry weight (Xu, et al. 2014), microalgae undergoing luxury uptake within WSPs have been known to contain up to 3.85% phosphorus through storage of polyphosphate (Powell, et al. 2011). In periods of high growth, this polyphosphate is utilised by cells for production of cellular constituents such as phospholipids, nucleic acids and nucleotides (Wu, et al. 2015).

This makes it rare to simultaneously have high levels of microalgal biomass and luxury uptake (Powell, et al. 2011).

However, the presence of polyphosphate does not necessarily mean that luxury uptake is occurring. Powell, et al. (2008) found that despite the phosphorus content of studied microalgae being below 1%, some polyphosphate was still present in the cell.

There are two types of polyphosphate present in microalgal cells. Acid-soluble polyphosphate is used for synthesis of cellular constituents, such as DNA or phosphoproteins (Powell, et al. 2008). Acid-insoluble polyphosphate is stored in microalgal biomass for use when the external phosphate level becomes limiting (Powell, et al. 2008). Luxury uptake of phosphorus has also been known to occur within cyanobacteria (Craggs, 2005a).

2.1.4 Possible Upgrade Options

One possibility for improving phosphorus removal from WSPs is to upgrade the plants with technologies that have been proved to be effective at removing phosphorus. Some of these technologies are described below.

2.1.4.1 Chemical dosing

Chemical dosing is the main process for removing phosphorus from wastewater effluents (de-Bashan and Bashan 2004). This approach involves the addition of soluble salts (such as aluminium sulphate, ferric salts or lime) to wastewater. As mentioned in 2.1.3.4, the dissolved cations from these salts precipitate with phosphate as an insoluble sludge (Szabo, et al. 2008). There are several different types of dosing agents currently used, each with their own different chemical mechanisms.

Alum

In wastewater treatment, Alum is initially present as $Al_2(SO_4)_3.nH_20$. Once this has entered the wastewater, it quickly dissociates and reforms as Al hydroxo complexes. These complexes then react with negatively charged contaminants in the water, such as phosphates and natural organic matter, forming stable complexes that can then be settled out (Edzwald and Kaminski 2008). This is shown in Figure 4.

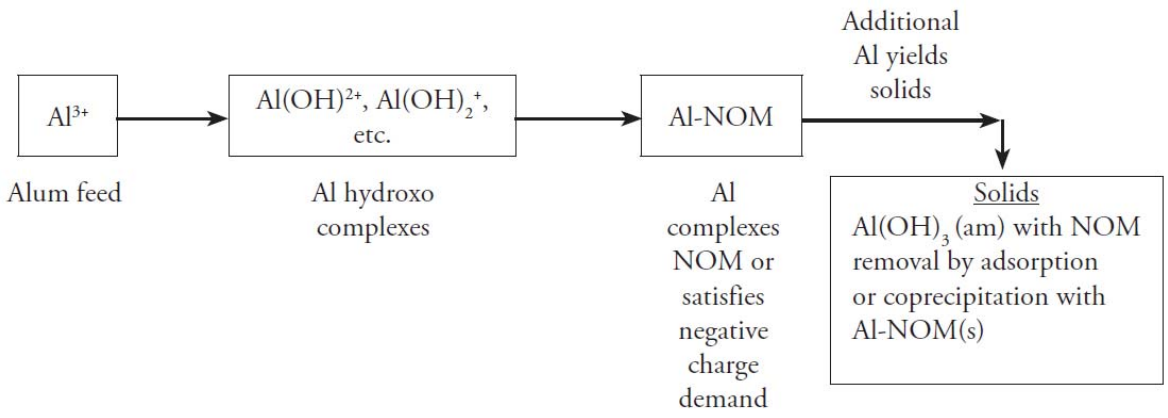


Figure 4: Chemical reaction pathway for alum in wastewater, from Edzwald and Kaminski (2008). Note that NOM = Natural Organic Matter

One downside of the use of alum is that it requires a very specific range of pH to be totally effective – between 6 and 7 (depending on temperature) according to Edzwald and Kaminski (2008). Given that the pH within wastewater can range from below 5 to as high as 11 in some cases, pH adjustment may be necessary prior to dosing with alum (Craggs, 2005a; Edzwald & Kaminski, 2008).

Another complication with the use of alum is the difficulty in calculating the amount of alum required for dosing, as a number of competing chemical reactions can occur alongside the phosphate precipitation reaction. In this case, the correct alum concentration must be determined in the laboratory for each addition to the wastewater stream (Tchobanoglous, et al. 1991).

There is also a significant cost associated with alum. According to Nind (2012), it costs approximately $7000 (NZ) per year to provide sufficient alum to treat wastewater for a community of around 500 people, not including the price of pumping equipment and facilities. This equates to a price of $56 per household per year (assuming four people per household in the community). Additionally, the large quantities of alum precipitate produced can be difficult to dispose of (Powell, et al. 2008), often requiring landfilling at significant cost.

Despite all of these downsides, alum has been proven to be highly effective at removing phosphorus from wastewater over an extended period of time, with phosphorus removal of 95% achievable using this technology (Tchobanoglous, et al. 1991). The reliability of alum use makes it extremely popular in larger scale treatment plants, which have the resources available to overcome any associated downsides.

Lime

Lime (CaO) is commonly used to remove water hardness and particulate matter from water (Drapcho and Brune 2000). However, phosphorus can also be removed through direct precipitation of calcium phosphate, $Ca_5(PO_4)_3OH$, using calcite (the most stable form of calcium carbonate, $CaCO_3$) as a seeding material. Calcium-phosphorus precipitation is a common method of phosphorus removal, mainly because of low cost and ease of handling (de-Bashan and Bashan 2004). However, a downside to this process is that large amounts of sludge are produced, which require disposal. Up to 50% more sludge is produced through lime dosing than if no dosing agent was used (US EPA 2000). Varying levels of alkalinity in wastewater also make it difficult to calculate how much lime is required (US EPA 2000), as the degree of alkalinity in the wastewater has a large effect on the removal ability of lime for phosphorus. Lastly, the addition of lime can result in the formation of calcium-phosphate scale on the inside of outlet pipes, as the calcium-phosphates precipitate on the surface of the pipes rather than within the wastewater (Pitman, et al. 1991).

Ferric Salts

Ferric salts used in wastewater treatment include ferric chloride ($FeCl_3$) and ferric sulphate ($FeSO_4$) (US EPA 2000). Phosphorus reduction using ferric salts is achieved through the formation and sedimentation of ferric orthophosphate ($FePO_4$). They can also act as coagulants or flocculants, and can act as a sludge thickening, conditioning and dewatering agent (Chemtrade 2014).

The downside of both these chemicals is that they are both highly corrosive, particularly ferric chloride (Chemtrade 2014). As with alum, competing chemical reactions also make it difficult to determine the correct amount of the ferric salt required for phosphorus removal, requiring more laboratory experimentation (Tchobanoglous, et al. 1991). Similarly to other dosing agents, the large sludge volumes produced through dosing with ferric salts, particularly with ferric sulfate, greatly increase the running costs of wastewater treatment plants due to transport and disposal requirements (US EPA 2000).

2.1.4.2 Enhanced Biological Phosphorus Removal

Enhanced Biological Phosphorus Removal (EBPR) involves the selective enrichment of microorganisms (known as Polyphosphate-Accumulating Organisms – PAOs) that perform, luxury uptake and accumulate inorganic polyphosphate as a part of their metabolism, (Blackall, et al. 2002; de-Bashan and Bashan 2004). In contrast to WSPs, the microorganisms within EBPR processes are almost entirely made up of bacteria. An outline of the EBPR process is shown in Figure 5.

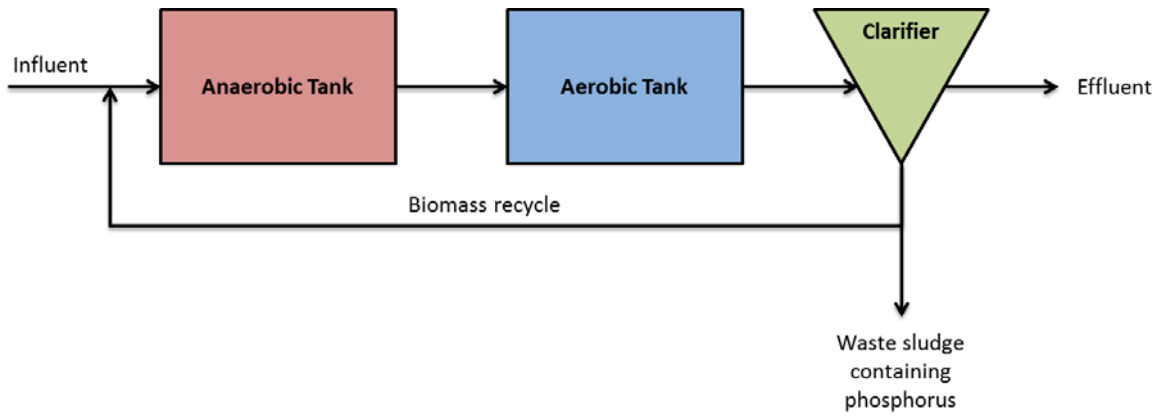


Figure 5: Outline of the EBPR process

As shown in Figure 5, EBPR involves mixing the influent wastewater with returned sludge (biomass), which then passes through an anaerobic stage followed by an aerobic stage (Blackall, et al. 2002). This reactor configuration gives PAOs a competitive advantage over other bacteria, ensuring that PAOs are the dominant microorganism within the system (Tchobanoglous, et al. 1991). Within the anaerobic tank, fermentation of organic substrates results in the production of acetate. Using already stored polyphosphates for energy, the PAOs assimilate the acetate, producing intracellular polyhydroxybutyrate (PHB) storage products. This results in a net release of phosphorus from biomass in the anaerobic stage as a result of the use of polyphosphates for energy. In the aerobic stage, the stored PHB is

metabolized, providing energy and carbon for new cell growth. The energy released from PHB oxidation is used to form polyphosphate bonds in cell storage, leading to the uptake of soluble phosphorus from solution, and the formation of new polyphosphates within the bacterial cell. In addition, cell growth occurs due to the PHB utilisation, with the additional cells produced also absorbing phosphorus to be stored as polyphosphate. This additional cell growth and phosphorus uptake leads to a net phosphorus removal from solution over the course of the EBPR process. The recycle stream recycles some of this biomass to the anaerobic tank, where it is mixed with the influent to restart the process, while excess sludge (along with the stored phosphorus) is removed for disposal. The sludge produced from this process has a phosphorus content of up to 4-5% of dry weight (Blackall, et al. 2002). This large amount of phosphorus removal has allowed EBPR processes to produce effluent with phosphorus concentrations of less than 0.1 mg P/L. All these factors combine to make EBPR a very effective technology for phosphorus removal, one that is used extensively in treatment plants around the world (Tchobanoglous, et al. 1991).

Despite it's success, there are significant downsides to the EBPR system. The complex nature of the system as a whole, as well as the significant power requirement for aeration make it a very expensive plant to install and operate. While treatment plants in larger settlements can afford such costs, plants serving small communities typically cannot afford the expense of an EBPR process, often making it unsuitable for smaller wastewater treatment plants such as those employing WSPs (Powell, et al. 2008).

2.1.5 Summary

While there are some options available for improving phosphorus removal from WSPs, the significant costs associated with installation and operation of the required technologies can make these options untenable for some smaller communites. One potential avenue for improved phosphorus removal from WSPs lies in improvement of the phosphorus removal processes found within the ponds, particularly those found in microalgae. Through mechanisms such as adsorption, assimilation and luxury uptake, microalgae have shown the potential to remove significant amounts of phosphorus from wastewater. Because of these mechanisms, microalgae make up the largest organic phosphorus pool in the water column of pond systems (Pearson 2005). To better understand how microalgal nutrient uptake could affect phosphorus removal from WSPs, the focus of the rest of this literature review will be on microalgae and the factors which influence both their growth and their ability to remove phosphorus.

2.2 Factors Affecting Growth and Phosphorus Removal by Microalgae

Microalgae have been shown to be very efficient at removing phosphorus from municipal wastewater, either in a free-swimming suspension or in an immobilized form (Hoffman 1998; Pittman, et al. 2011). Many studies have confirmed the ability of microalgae to remove significant amounts of phosphorus. Colak and Kaya (1988) found that *Chlorella vulgaris* could remove 85.7% of phosphorus from industrial wastewater, and 97.8% of phosphorus from domestic wastewater in laboratory scale reactors. Ji, et al. (2014) found that *Desmodesmus* sp. could remove 100% PO_4-P and TP from a sample of anaerobic digestion

wastewater after 14 days. In both these cases, assimilation for growth was the primary mechanism.

Despite the advantages of microalgal treatment, there are still some difficulties surrounding removal of the microalgae from suspension once phosphorus has been assimilated. This often requires installation of solids separation units such as clarifiers, which can carry significant cost.

In addition, there are a multitude of variables which affect microalgal growth. The key process in microalgal metabolism is known as photosynthesis, an energy producing reaction undertaken by microalgae. This involves the absorption of CO_2 (as a carbon source) and light (as an energy source), producing oxygen as a by-product (Pearson 2005). While the underlying reaction is simple, there are a large number of factors that influence the reaction. As a result, microalgal growth is inherently complex, predominantly showing nonlinear responses to various environmental parameters such as temperature, light and several nutrients (Ye, et al. 2011). An accurate portrayal of microalgal growth is one of the most difficult areas in water quality monitoring (Sterner and Grover 1998; Ye, et al. 2011). This was found in a detailed study by Sterner and Grover (1998) of microalgal growth in two water reservoirs in Texas, which despite the large scale of experimentation produced a model that could only explain 25% of the variation in microalgal community growth. The authors found that site specificity and the diverse microalgal communities found within natural environments made modelling microalgal growth in natural systems extremely problematic.

As the environmental conditions greatly affect microalgal metabolism, they also have corresponding effects on the ability of microalgae to remove phosphorus. A large number of studies have aimed to quantify the effect of various environmental variables on not only the growth of microalgae, but also the phosphorus uptake behaviour of the microalgae as detailed further below.

2.2.1 Bacteria

As mentioned in 2.1.2.2, the symbiotic relationship that is present between bacteria and microalgae in WSPs means that bacteria have a significant impact on microalgal growth. Without the presence of bacteria in the pond, microalgae would consume all of the available CO_2 within the pond, leading to a rise in pH (often greater than 10) and the eventual death of the microalgae from either the high pH or lack of a carbon source. However, some species of bacteria, such as *Azospirillum brasilense*, are known to enhance microalgal growth through means beyond the symbiotic relationship, such as through nutrient transport, control of potentially harmful diseases, and the enhancement of mineral uptake (Bashan 1998).

Bacteria have also been known to affect microalgal phosphorus removal at laboratory scale, through co-immobilisation - the fixing of microalgae with bacteria in polysaccharide gel beads (de-Bashan, et al. 2002). Hernandez, et al. (2006) found that co-immobilization of microalgal cultures in alginate beads with the microalgal growth-promoting bacterium *A. brasilense* increased the phosphorus absorption of both *Chlorella sorokiniana* and *Chlorella*

vulgaris significantly, both in terms of peak phosphorus removal and in terms of per cell removal. The study also found that there was a direct correlation between the initial load of phosphorus in the domestic wastewater and the efficiency level of removal. The use of a control reactor featuring just alginate beads proved that the co-immobilised microalgae and bacteria were responsible for the phosphorus removal, as no removal was achieved by the alginate beads. Liang, et al. (2013) also found that a combined bacteria and microalgae system involving *C. vulgaris* and *Bacillus lichenformis* resulted in increased phosphorus removal by the combined system when compared to bacteria-only and microalgae-only systems. However, in some cases co-immobilisation was found to not result in beneficial phosphorus removal. In a project by de-Bashan, et al. (2002) the microalga *C. vulgaris* was co-immobilized in alginate with *A. brasilense* in synthetic wastewater culture. While significantly more phosphorus was removed by the co-immobilized culture in the first wastewater cycle, the co-immobilized culture actually produced phosphorus in the second cycle, with removal rates remaining similar to that of immobilized *C. vulgaris* alone for the remaining 4 cycles. This shows that co-immobilization does not necessarily result in improved phosphorus removal. In addition, close attention needs to be paid in future studies on whether any nutrients removed by the immobilised cultures aren't immediately re-released by the microorganisms soon after.

2.2.2 Light

Microalgae require an energy source for growth. This can be either an organic compound for heterotrophic growth or, more commonly, light energy for photoautotrophic growth (Grobbelaar 2009). Photoautotrophic microalgae convert light energy to chemical energy such as ATP and nicotinamide adenine dinucleotide phosphate (NADP) (Kim, et al. 2013). Generally, microalgae use light of wavelengths from 400 to 700 nm for photosynthesis (Kim, et al. 2013). While this process may seem straightforward, there are many variables which influence how light affects both microalgal growth and phosphorus uptake.

2.2.2.1 Light Intensity

Many studies have been performed on the effect of light intensity on microalgal growth. The light intensity received by the microalgae is dependent not only on the intensity of the incident light entering the pond but also on the position of the microalgae within the pond. Cultures near the surface receive the most light, with light intensity decreasing exponentially with depth in the pond in accordance with Beer's Law for light travelling through a medium (Torzillo, et al. 2003; Powell, et al. 2008; Zhang, et al. 2008). This is due to the shading effect created by the growths of microalgae near the surface, which reduce the light intensity experienced by microalgae at greater depths. To best explain the influence of light intensity on photosynthesis, a photosynthesis versus irradiance response curve can be used, as shown in Figure 6.

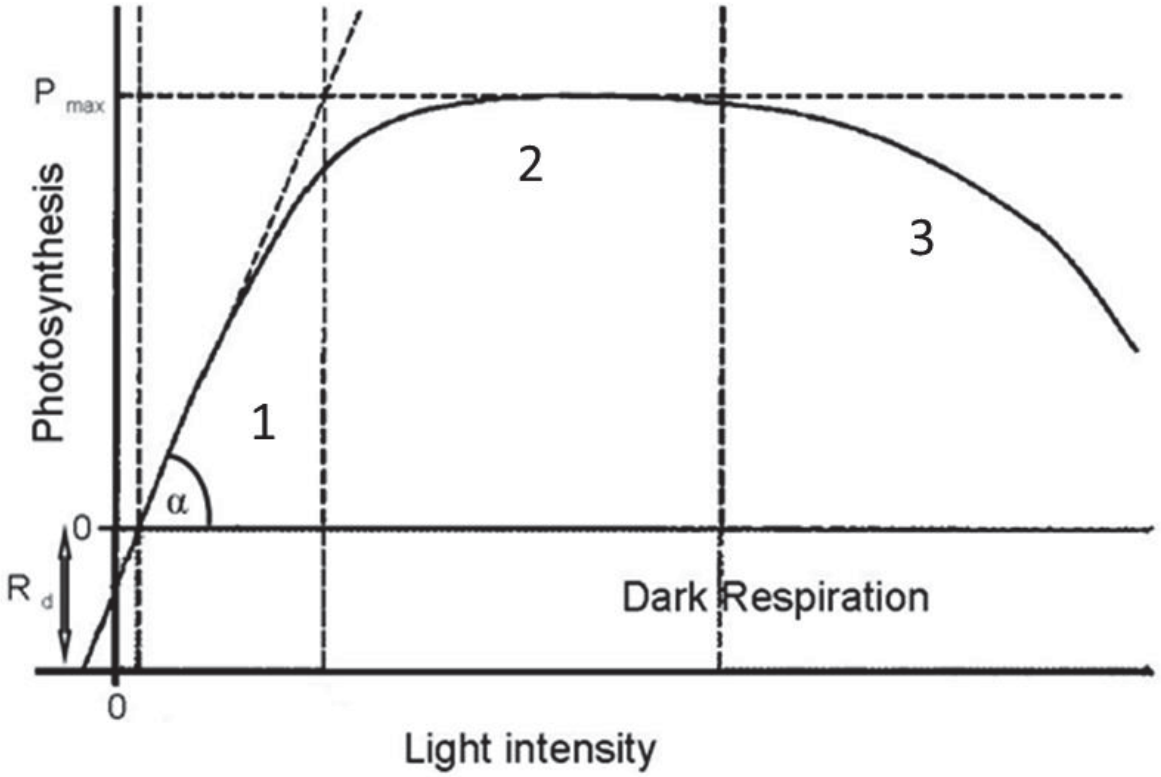


Figure 6: Photosynthesis vs. irradiance response curve for microalgae. Note that Pmax = light saturated rate of photosynthesis, and Rd = dark respiration. Taken from Grobbelaar (2013).

From this plot, three distinct regions (labelled 1, 2 and 3) can be seen:

1. An initial light-limited region at low light intensities where photosynthetic rates increase with increasing irradiance. The rate of photon (light) absorption by microalgae is correlated with the rate of electron transport from water to CO_2, with the liberation of O_2.
2. A light saturated region where photosynthetic rates are independent of irradiance. At this point, photosynthetic rates reach a maximum for the microalgae. The transition from the light-limited to the light-saturated phase can be gradual or abrupt, implying a non-linearity between absorbed light and photosynthetic rates.
3. A region of photoinhibition in which photosynthetic rates decrease with an increase in irradiance.

This curve explains the significant variation in microalgal growth with depth. Upper layers of the pond are exposed to more light, with the microalgae present undergoing more photosynthesis (Drapcho and Brune, 2000; Paterson and Curtis, 2005). Deeper cultures are exposed to less light, and so have lower growth rates (Paterson and Curtis 2005). As a result of these conditions, biomass concentration in WSPs is typically significantly higher near the surface of the pond than in lower layers. However, there have been some studies which had conflicting results with the information given above. One such study was performed by Sterner and Grover (1998). The authors found that mean light intensity in a mixed culture of microalgae had little influence on microalgal community growth, as a result of study on

microalgal samples subject to a wide variety of light intensities. However, this was likely due to the fact that other limiting factors were present in the sample.

Several studies have also been completed on the influence of light intensity on phosphorus removal by microalgae, specifically on the phosphorus content of the microalgal biomass. A study by Powell, et al. (2008) found that light intensity actually had a negative effect on the phosphorus content in the biomass. This was unexpected because phosphorus uptake is known to require energy, most commonly through photosynthesis which is enhanced by increased light levels. However, this effect was consistent with other reports (Hessen, et al. 2002). Further investigation by Powell, et al. (2008) found that the reduction in phosphorus content with increased light was due to consumption of internal phosphorus stores to facilitate increased growth at higher light intensities

In another surprising result, a study by Ruiz, et al. (2014) found that the phosphorus content of *Scenedesmus obliquus* grown in reactors of secondary treated wastewater increased in the absence of light. Mechanistically, this could have been due to the absence of light preventing growth in the microalgae. With no growth occurring, phosphorus stores that would normally be consumed to support growth would remain unused, even as the microalgae continued to uptake phosphorus. The low biomass concentration present in the sample may also have had an effect, as there could have been a larger amount of phosphorus available to each individual cell than if more biomass was present.

2.2.2.2 Light and Dark Variations

According to Grobbelaar (2009), another factor influencing the effect of light on microalgal growth is light and dark (L/D) variations. These are variations caused by artificially changing the period in which microalgae are exposed to (and conversely deprived of) light. Other studies have shown that photosynthesis can be enhanced through the "flashing light" effect – alternating between light and darkness at high frequency. Grobbelaar, et al. (1996) showed that this only becomes important with L/D cycles of less than 1 Hz. He also found that:

- Photosynthetic rates increases exponentially with increasing L/D frequencies.
- A longer dark period in relation to the light period can further increase photosynthetic efficiencies, but not vice versa.
- Microalgae do not acclimate to specific L/D frequencies, but they become low light-acclimated at long L/D frequencies and high light-acclimated at short L/D frequencies.

This effect has also been studied for effects on phosphorus uptake in a limited fashion by Lee and Lee (2001). The authors found that the removal efficiency of organic carbon and phosphorus with *Chlorella kessleri* was greater under a 12 h light/12 h dark lighting scheme, than under continuous lighting. This was despite significantly lowered cell counts in the culture experiencing the light/dark cycle compared to the culture experiencing continuous lighting. However, this only represented an increase in phosphorus removal from 8% to 20%. The authors were unable to explain this effect mechanistically, with more research into the component mechanisms being required.

2.2.2.3 Effect of Wavelength of Light

Kim, et al. (2013) provided a detailed study on the effects of wavelength of light on both microalgal growth and phosphorus uptake by the microalgae. The study found that white light providing both 450 – 475 nm and 630 – 675 nm wavelengths of light promoted the highest growth of microalgae. However, as the wavelength of sunlight is typically made up of only 43% visible light (400 – 700 nm) (American Society for Testing and Materials 2013), these ranges of wavelength only represent a small amount of the incident light present in most microalgal environments. In terms of phosphorus uptake, blue light (400 – 500 nm) was found to promote the highest phosphorus removal rate in a batch study, with removal of 1.8 mg P/L/d, compared with removal of 1 mg P/L/d for white light (Kim, et al. 2013). This occurred despite the decreased level of microalgal growth for this culture compared to the white light culture. This effect could be explained by observations made by Ruyters (1984) who found that blue light was involved in enzyme activation, regulation of gene transcription and energy-derivation of microalgae, all of which could contribute to a microalgal cell's phosphorus uptake behaviour.

Kim, et al. (2013) also found that a mixture of red (630 – 675 nm) and blue light at a ratio of 5:5 or higher (in terms of the blue light portion) resulted in further increases in phosphorus removal – 2.3 times higher than under white light conditions. However, this increase in phosphorus removal also corresponded with an increase in microalgal growth within the culture, which may have contributed to this increased removal.

2.2.3 Temperature

Temperature is seen as one of the most important factors affecting microalgal growth, particularly in WSPs, as it dramatically affects the rate of biological processes (Paterson and Curtis 2005). When nutrients are not limited, increased temperatures will greatly accelerate microalgal growth (Sterner & Grover, 1998; Ye, et al. 2011). There have been many studies demonstrating the effect of temperature on microalgal growth, in many different environments. For example, a study by Sterner and Grover (1998) found that maximal growth of microalgae within two Texas reservoirs increased as a function of temperature. This finding was backed up by a study by Ye, et al. (2011) on microalgae within Lake Taihu in China, which found that the rate of photosynthesis of microalgae within the lake increased exponentially with increased temperature (less than 35°C). Ye, et al. (2011) also found that the air temperature at Lake Taihu had a great effect on microalgal growth. This is probably due to the fact that photosynthesis mainly occurs near the water surface, where the air temperature has the greatest influence over the temperature of the water. In addition, increased temperatures generally coincide with increased sunlight levels, which also increase growth through photosynthesis. However, just as temperature can lead to increased growth in microalgae, it can also inhibit growth when low. A combination of low temperature and high irradiance is one of the more common environmental stresses that negatively affect biomass growth. In the morning hours, while increases in light intensity occur in a range of 1 – 2 hours, the increase of temperature is much slower, taking 4 – 5 hours. This disconnect can cause photo-inhibition at relatively low light intensity, due to the sub-optimal temperatures

(Torzillo, et al. 2003; Walker 2009) This effect is less severe near the surface of water bodies, as shallower cultures experience a greater temperature rise throughout the day than deep cultures according to Drapcho and Brune (2000).

Temperature is known to affect cell composition as well as the rate of biological reactions, and so should be expected to affect phosphorus uptake in some way. Temperature had a positive effect on the percentage of phosphorus in the biomass according to Powell, et al. (2008). However, high temperatures can also have an inhibitive effect. Consecutive studies by Martinez, et al. (1999) and Martinez, et al. (2000) found that the growth rate and phosphorus removal of *Scenedesmus obliquus* in wastewater decreased beyond temperatures of 30°C. Further analysis revealed that at the higher temperatures the cells began to lyse and break apart, thus leading to a release of phosphorus.

2.2.4 pH

Most microalgae have a specific tolerance range for pH (Grobbelaar 2009). Xin, et al. (2010) and Li, et al. (2010) found that microalgae did not grow well in acidic pH environments below 6. Increasing pH from this point yielded far greater values for the microalgal biomass. Another study by Martinez, et al. (2000) found that cell rupture within microalgal cultures occurred when pH concentrations reached their highest point, at around 11 for *Scenedesmus obliquus*. The optimum pH for microalgal growth according to a study by Goldman, et al. (1982) is around 8. However, microalgal nutrient assimilation and photosynthesis significantly influence the pH of pond water, making it difficult to maintain a particular pH within WSPs. Photosynthesis causes an increase in pH through a shift in the equilibrium reactions shown in equation 1 and 2.

$$2HCO_3^- \leftrightarrow 2CO_3^{2-} + H_2O + CO_2 \text{ (Eq. 1)}$$
$$CO_3^{2-} + H_2O \leftrightarrow 2OH^- + CO_2 \text{ (Eq. 2)}$$

Equation 1 and Equation 2: Carbonate-bicarbonate equilibrium relationship as seen in WSPs. Equations taken from Pearson (2005).

As more CO_2 is removed by the microalgae in the system, the equilibrium shifts to produce more CO_2, with hydroxyl (OH^-) ions being produced as a byproduct. This leads to an increase in the pH of the system, raising the pH to as high as 10 or more (Pearson 2005; Abdel-Raouf, et al. 2012). Nitrate assimilation by microalgae also raises the pH (Oh-Hama and Miyachi 1988), while ammonia assimilation decreases pH due to the H^+ released as a result of the reduction of NH_4^+ to NH_3 by the microalgae (Azov and Goldman 1982). The result is that pH is highly variable within pond environments, and therefore has a varying effect on microalgal growth.

These fluctuations in pH can have significant effects on the presence of phosphorus in the wastewater. As mentioned previously in 2.1.3.4, a rise in pH above 8.2, combined with a high dissolved oxygen concentration and high cation concentration can cause precipitation of phosphorus (Cai, et al. 2013; Ji, et al. 2014). The elevated pH also promotes phosphorus adsorption onto the surface of microalgae, as noted by Xu, et al. (2014). However, changes in pH do not necessarily lead to changes in phosphorus removal by the microalgae. A combined

system of *C. vulgaris* and *B. lichenformis* grown in solution was found to have similar removal for total phosphorus with the pH controlled at 7 compared to the pH left uncontrolled. This is despite the uncontrolled pH getting as low as 3.5 (Liang, et al. 2013).

2.2.5 Availability of Carbon

Microalgae also require a carbon source for growth, in the form of either dissolved CO_2 or HCO_3^- (Grobbelaar 2009) or, less commonly, organic carbon (Pearson 2005). Carbon only affects growth when it becomes limiting (Drapcho and Brune 2000). This can occur when CO_2 is consumed by microalgae at a rate higher than it can be replaced by either bacteria or the air-water interface. This results in a shift in the carbonate-bicarbonate equilibrium shown in Equation 1 and Equation 2 to produce more CO_2 and OH^-, leading to a rise in pH (Pearson 2005). This often occurs in WSPs, particularly in secondary ponds where CO_2 production via bacterial respiration is less than in primary ponds (Pearson 2005).

2.2.6 Dissolved Oxygen Concentration

Dissolved oxygen (DO) concentration is an important factor in the symbiotic relationship between bacteria and microalgae (Shilton and Walmsley 2005). Low oxygen content (<1.5 mg/L) in the water inhibits bacterial respiration, thus removing a source of CO_2 for microalgal metabolism (Tchobanoglous, et al. 1991). In contrast, high oxygen concentration can also limit growth of microalgae. High dissolved oxygen levels in water can lead to creation of potentially damaging radical oxygen species, disrupting the electron transport pathway for photosynthesis, in a process known as photorespiration (Hartig, et al. 1988; Torzillo, et al. 2003). According to Torzillo, et al. (2003), photorespiration occurs once the DO concentration in the water reaches 70 – 80 mg/L. However, Su, et al. (2012) stated that photorespiration can occur at DO levels as low as 35 mg/L.

There is also significant diurnal variation in the dissolved oxygen concentration of ponds, as noted by Paterson and Curtis (2005). At night microalgae continue to respire by consuming oxygen, leading to a reduction in the dissolved oxygen level of the pond. This leads to the creation of an oxypause – a level in the pond below which the dissolved oxygen concentration is zero. Low dissolved oxygen concentrations lead to a higher oxypause during the night, which results in the death of many aerobic bacteria. Once the sun rises, photosynthesis can begin again and the dissolved oxygen concentration gradually rises. As a result, ensuring that there is a high (but not too high) DO level within the microalgal growth environment is integral to ensuring the sustained symbiotic relationship between bacteria and microalgae.

Very little research has been performed on the influence of DO levels on the phosphorus content of microalgae in any environment. As DO is a key variable in the operation of WSPs, more research should be completed on this topic to identify any potential effects.

2.2.7 Nutrients

From literature, the two key nutrients that most significantly affect microalgal growth are nitrogen and phosphorus, both of which have been found to be growth limiting (Craggs,

2005a). As a result, total phosphorus (TP) and total nitrogen (TN) at low levels control microalgal blooms in lakes and oceans (Ye, et al. 2011). However, at the levels found in WSPs, nutrients very rarely become limiting. Typical concentrations of total nitrogen and phosphorus within WSPs range between 15 – 60 mg N/L and 4 – 15 mg P/L respectively (Craggs, 2005a).

While there is very little research on the effect of nitrogen concentration on phosphorus uptake, a large amount of research has been put into identifying the effect of phosphorus concentration on phosphorus uptake by microalgae, with differing conclusions. A number of studies have found that the uptake rate of phosphorus is proportional to the initial nutrient concentration (Cai, et al. 2013). Ji, et al. (2014) found that higher initial phosphorus concentration resulted in higher rates of phosphorus removal via assimilation into *Desmodesmus* sp. This effect was also found in a study of *Scenedesmus obliquus* by Martinez, et al. (1999). However, this also corresponded with an increase in the production of biomass in both cases. The increased removal of phosphorus is therefore most likely due to the additional phosphorus required to sustain this microalgal growth, and thus does not represent elevated phosphorus uptake by the microalgae.

Another study by Powell, et al. (2009) found that initial phosphorus concentration had a significant effect on microalgae grown in batch reactors with synthetic wastewater. However, when continuous reactors were used with synthetic wastewater, it was found that the initial nutrient concentration did not affect the phosphorus uptake of wastewater microalgae in the range of phosphorus concentrations studied (5 – 15 mg P/L) (Powell, et al. 2008). The authors found that the positive effect of nutrient uptake found by past studies on continuous reactors was likely only applicable to cells that have been starved of phosphorus prior to introduction to (comparatively) high phosphorus concentrations. The result obtained by Powell, et al. (2008) was backed up by Wang, et al. (2013) in a study comparing growth and nutrient removal of *C. vulgaris* grown in different dilutions of influent and effluent wastewater. In the study, more than 90% of TP in influent wastewater and 60% of TP in effluent wastewater were removed by *C. vulgaris* over a period of 24 days, regardless of the initial wastewater concentration. It appeared that *C. vulgaris* could use phosphorus at extremely low concentrations, possibly due to pre-stored polyphosphate. This is indicated by the fact that the PO_4-P was nearly exhausted in the 50% influent and effluent wastewater concentrations after 14 days of culture, after which the microalgae continued to multiply. These studies suggest that at the levels experienced within WSPs, the initial concentration of phosphorus is unlikely to have an effect on the phosphorus uptake of the biomass.

Another factor that could have an effect on phosphorus uptake is the bioavailability of the phosphorus. Wastewater effluent contains various forms of phosphorus, all of which may have different availabilities to microalgae and other primary producers (Ekholm and Krogerus 1998). A study by Ekholm and Krogerus (1998) of wastewater effluent from treatment plants in Finland found that only 36% of the total phosphorus was biologically utilized by microalgae. This may have been due to the more available forms of phosphorus having already been removed by prior processing. However, this study did not account for the

effect of microalgal concentration on the wastewater, as there may have been insufficient microalgae in the sample to remove larger amounts of phosphorus than that which was taken up.

Another interesting effect of nutrient concentration occurs when a nutrient "pulse" occurs. Microalgae have been known to maintain higher than expected growth when nutrients are low if they have recently experienced a "pulse" of high nitrogen and phosphorus content solution, which could explain the unexpected variation in microalgal growth sometimes seen in microalgal systems upon nutrient addition (Sterner and Grover 1998). This could be due to luxury uptake by microalgae occurring upon reception of the pulse, with the phosphorus stores maintained until external phosphorus is depleted, as was found to occur in a study by Aitchison and Butt (1973).

2.2.8 Concentration of Microalgae in the Pond

Biomass concentration can have a large impact on nutrient removal due to nutrients adsorbed to the surface of cells (in the right conditions), as well as assimilated into biomass (Boyd and Musig 1981; Xu, et al. 2014). A study by Lau, et al. (1995) was performed to assess the effect of varying the starting microalgal inoculum density on phosphorus uptake by microalgae in primary wastewater. This study found that the higher the initial microalgal density, the higher the speed and amount of phosphorus removal. This would seem to indicate a direct correlation between microalgal concentration and phosphorus removal by the microalgae.

Contrasting results were found in a study by Powell, et al. (2011) in a study on biomass samples taken from four different WSPs. This study found that, while it was possible to have high biomass phosphorus contents at low biomass concentrations, a combination of high biomass levels and high biomass phosphorus contents were not often found within WSPs. This indicates the presence of an inverse relationship between biomass concentration and phosphorus uptake within the studied WSPs.

Mechanistically these effects can be understood when considering the perspective of one microalgal cell. In the study by Lau, et al. (1995) the increased microalgal concentration combined with low phosphorus concentration (4 mg P/L) would have led to greater competition for the available phosphorus amongst the available microalgal cells, leading to the increased speed of uptake. In contrast, the study by Powell, et al. (2011) considered microalgae growing in conditions where phosphorus was far more abundant, with total phosphorus concentrations in the samples often above 10 mg P/L. With phosphorus being significantly more abundant, there would be more phosphorus available to each individual microalgal cell, increasing the likelihood that the cell will be able to absorb phosphorus for storage as polyphosphate as well as for basic metabolism.

2.2.9 Species of Microalgae Present

Many studies have found that different genus of microalgae have different affinities for phosphorus uptake. *Chlorella* and *Scenedesmus* are usually the predominant microalgal communities in WSPs (Masseret, et al. 2000) and in high rate microalgal ponds (Canovas, et

al. 1996), and are therefore the most commonly studied for nutrient removal (Wang, et al. 2013). A study of free-grown cultures of *S. obliquus* and *C. vulgaris* carried out by Ruiz-Marin, et al. (2010) showed that *S. obliquus* took up significantly more phosphorus in both artificial and urban wastewater. However, this was due to the increased growth of *S. obliquus*, as both species had similar uptake rates per unit cell concentration.

Some studies considered a wider range of microalgal species for phosphorus uptake. A study of *Phormidium* sp., *Chlorella reinhardtii*, *C. vulgaris* and *Scenedesmus rubescens* by Su, et al. (2012) showed different rates of phosphorus uptake for the different species when placed in wastewater. This was measured by determining the time for 98% removal of phosphorus. The results of this study are displayed in Table 1.

	Time for 98% Removal (d)	**Phosphorus Removal Capacity (mg P/L/d)**
C. reinhardtii	2	0.89
C. vulgaris	3	0.76
S. rubescens	4	0.6
***Phormidium* sp.**	4	0.56

Table 1: Phosphorus uptake of different species/genera of microalgae. Results taken from Su, et al. (2012).

The results showed that *C. reinhardtii* had the fastest removal rate for phosphorus and the highest removal capacity as a result. All of the microalgal species had similar growth rates (averaging 6.3 g/m^{2}d), excluding *Phormidium*, which was significantly lower at 2.71 g/m^{2}d. This may explain why it had the lowest phosphorus removal capacity. The phosphorus uptake performed by the microalgae was attributed to basic assimilation, with the differences in times of removal attributed to the speed of assimilation achieved rather than the presence of an additional phosphorus removal mechanism.

While not mentioning the presence of mechanisms of phosphorus removal outside of basic assimilation, the results from the studies of Ruiz-Marin, et al. (2010) and Su, et al. (2012) show that the species of microalgae present has a significant effect on the rate of phosphorus removal, and therefore should be monitored in future studies on phosphorus removal in WSPs.

2.2.10 Preparation of Microalgae

Many different studies have focused on applying specific preparations to microalgae cultures, to see whether these have any effect on the behaviour of microalgae in terms of nutrient removal. Such treatments include immobilisation and starvation.

2.2.10.1 Immobilisation

Immobilisation involves the fixing of cultures of microalgae within a gel media, such as calcium alginate gel. According to Tam, et al. (1994) and Zhang, et al. (2008), the major advantages of this are:

- Low light energy consumption for cells
- High and rapid nutrient uptake and short retention time
- A small area requirement

- The ease of recovery of the excess biomass
- The screens used to hold the fixed microalgae can be reused, which is less costly and more convenient for continuous flow reactors
- Increase in cell retention time
- Higher metabolic activity

Studies have indicated that efficient and rapid removal of nitrogen and phosphorus from wastewater can be achieved by immobilized microalgae (Zhang, et al. 2008). However, there are several important factors that influence the nutrient removal achieved by the immobilized microalgae. Excessively low cell densities within gels result in reduced nutrient removal efficiency, while excessively high cell densities and gel thickness results in reduced amounts of light penetrating through the bioreactor, enhancing the self-shading effects and limiting the growth and activities of the microalgal cells. High gel cell densities are also believed to lead to difficulties with diffusion of substrate to the microalgae within the gel (Vichez and Vega 1994). Both of these factors were examined in a study by Zhang, et al. (2008), who found that for the same gel thickness, increasing the cell density also increased the phosphorus removal efficiency up to a density of 6×10^7 cells/mL. Beyond this point, nutrient removal efficiency decreased due to self-shading. From further analysis, it was proved that get thickness was not the key factor in removal efficiency and that cell density in the gel is most important in nutrient removal. In addition, the high levels of nutrient removal with increased cell density proved that resistance to diffusion of substrate was not a significant factor. This finding was supported by Lau, et al. (1997), who supported the idea that simple inorganic nutrient ions such as nitrate, ammonium and phosphate would be as freely available to the immobilized microalgae as to their free counterparts, because nutrients must diffuse through the alginate pores to reach the microalgal cells.

2.2.10.2 Starvation

It is well known that the nutrient removal efficiency of immobilized microalgae not only depends on cell density, but also on whether the cultures used have been starved of nutrients prior to inoculation (Hernandez, et al. 2006). There is a large amount of evidence which suggests that pre-starved microalgae absorb much higher amounts of phosphorus when inoculated than if the microalgae had not been starved (Aitchison and Butt 1973). This applies to both immobilised microalgae and microalgae grown in suspension.

An example of this was found in a study by Hernandez, et al. (2006), who found that phosphorus starvation of *Chlorella sorokiniana* over 72 hours resulted in greatly increased phosphorus removal when introduced to wastewater, in terms of both peak removal and removal per cell. However, phosphorus removal decreased when the period of starvation was applied to a population *C. vulgaris*, which was also introduced to a sample of wastewater. This was an interesting result, as the phosphorus content of a phosphorus starved culture of *C. vulgaris* was found to increase significantly upon addition to phosphorus solution in a study by Aitchison and Butt (1973). This could have been due to the more complex environment present in wastewater when compared to the phosphorus solution, which could have provoked a differing response in the microalgae.

Another example of the effect of starvation occurred in a study of 9 consecutive starvation-treatment cycles of *Scenedesmus* sp. immobilized in alginate beads conducted by Zhang, et al. (2008). In this study, it was found that the nutrient removal efficiency was maintained at high levels after 8 full cycles, before declining after the 9th cycle due to the excessive growth of microalgae, which limited light and substrate diffusion.

While starvation may have some effect on phosphorus uptake by microalgae, this is unlikely to occur in ponds due to the significant amounts of phosphorus present in wastewater, and so is typically only seen in lakes and river environments.

2.2.11 Other Factors

2.2.11.1 Wind Speed and Mixing

Wind speed and direction are known to affect the horizontal distribution of microalgae. This effect was observed in a long term study of Lake Taihu in China, carried out by Chen, et al. (2003). They found that while microalgal concentrations were low in the centre of the lake, the concentration was significantly higher in one of the bays within the lake which received high winds coming from across the lake, with some microalgal blooms visible. This also resulted in a horizontal distribution of genera of microalgae, with different genera found in the centre of the lake compared to the bay.

Several studies have shown that mixing has a significant effect on microalgal growth. Ugwu, et al. (2002) found that microalgal growth increased by 40% within a *Chlorella* culture when mixing was introduced. Drapcho and Brune (2000) have also suggested that increasing the degree of mixing increases microalgal productivity by increasing the exposure of the microalgal cells to sunlight, decreasing the effects of photoinhibition. Grobbelaar (1994) also mentioned the influence of turbulence of the water body on nutrient and oxygen transport by microalgae. The higher the turbulence, the thinner the boundary layer around microalgae, the more readily nutrients are taken up and the faster metabolites (e.g. oxygen) are taken away from the cell, thus increasing growth rates. However, this analysis was performed in a batch system within a laboratory, and so did not consider the multitude of variables present in a WSP.

In terms of phosphorus removal, a study by Martinez, et al. (2000) found that stirring of a solution of partially treated wastewater increased the speed at which phosphorus was taken up by a culture of *S. obliquus*. However, this also corresponded with an increase in growth of the culture, with the biomass phosphorus content remaining constant. In addition there were some limitations to this study. The study only considered complete mixing versus no mixing, thus did not consider any variation in degree of mixing. In addition, the completely mixed condition is not typically seen in WSPs, and would take significant upgrades to achieve. However, further study in this area could yield more relevant results to WSPs.

2.2.11.2 Hydraulic Retention Time

Drapcho and Brune (2000) found that microalgal productivity increased within an aquaculture pond with an increase in the retention time of the pond from 1.2 to 2.5 days. This is to be expected, as the longer length of time in the pond gives microalgae more exposure to sunlight for photosynthesis, as well as more time to absorb nutrients for growth. However, there is currently very little knowledge on the influence of hydraulic retention time on phosphorus uptake by biomass, a significant knowledge gap given the importance of hydraulic retention time on pond treatment performance.

2.3 Phosphorus Removal by Microalgae in Natural Environments

Natural systems (such as lakes and rivers) present a very different environment for microalgae compared to WSPs, with phosphorus concentrations in that are typically lower than those found in WSPs. Peak yearly average TP in the eutrophic Lake Taihu in China over a 30 year study was only 0.205 mg P/L (Ye, et al. 2011), as compared to 4 -15 mg P/L commonly found in wastewater (Craggs, 2005a). The species of microalgae present within natural environments also differs to WSPs. Lakes with TP concentrations below 1 mg P/L typically favour growth of cyanobacteria, as observed in Lake Taihu in China where the cyanobacteria *Microcystis* was dominant (Chen, et al. 2003). However, once the TP concentration increases above 1 mg P/L, the cyanobacteria population within lakes are often replaced by green microalgae (such as *Scenedesmus* and *Pediastrum*), as observed in a study of Danish lakes by Jensen, et al. (1994). These microalgae are more typical of the type of microalgae seen within WSPs. However, the microalgal species found within Lake Taihu did not demonstrate particularly high phosphorus uptake. Using data from Chen, et al. (2003), the average phosphorus uptake by the biomass within the lake was only 0.307 %P/g SS for areas near the shore and 0.109 %P/g SS for the middle of the lake. While this figure does not represent the true phosphorus uptake of the biomass (as the solids data is based on suspended solids and not volatile solids), it does provide an indicator of the levels of phosphorus uptake by the biomass.

Unfortunately, there were only few studies available on phosphorus uptake by microalgae in natural environments, making it difficult to make any conclusions regarding the behaviour of microalgae in lakes and rivers.

2.4 Conclusions from Literature Review

From the studies examined, microalgae display significant potential for phosphorus removal, through simple assimilation of phosphorus into the biomass. However, some studies have highlighted the potential of microalgae in wastewater environments to remove higher than expected amounts of phosphorus, through mechanisms such as adsorption, precipitation and luxury uptake. Through these mechanisms, it is possible to have a phosphorus content in the microalgal biomass of greater than the 1% standard in microalgae, and thus remove greater than expected levels of phosphorus.

However, while there have been some studies of adsorption of phosphorus to microalgae, and on precipitation of phosphorus within WSPs, there has been very little research performed on

the ability of microalgae to perform luxury uptake, and what causes this phenomenon to occur. Powell, et al. (2008) and Powell, et al. (2009) both demonstrated that luxury uptake was possible in microalgae taken from WSP and placed in lab scale reactors, with phosphorus contents of up to 3.16% P/g VSS observed, over three times the standard value required for microalgal metabolism. Further study by Powell, et al. (2011) revealed that luxury uptake could also occur within WSPs, with phosphorus contents of up to 3.85% P/g VSS observed.

While this is a significant result in terms of better understanding luxury uptake by microalgae, the study by Powell, et al. (2011) was limited by the fact that only four ponds were studied. In addition, while this study did look into the effect of pH and time of year on the biomass phosphorus content within WSPs, it did not consider a number of other environmental variables, such as dissolved oxygen (DO), temperature, phosphorus concentration, the effect of weather-related variables such as wind speed and light intensity, or the influence of geographical (climatic) location. While some experiments on the effect of different environmental variables on phosphorus uptake by microalgae have been performed by Shilton, et al. (2006) and Powell, et al. (2008), these studies were performed on laboratory scale reactors rather than full scale WSPs. The added complexity of WSPs may result in new effects becoming apparent that were not present at laboratory scale. The study also did not assess whether facultative (primary) or maturation (secondary) ponds contained biomass of higher phosphorus content, or whether biomass within other types of ponds would be more effective at taking up phosphorus.

A last factor that could have had a significant effect on the phosphorus uptake of the biomass is the genera of microalgae present. Currently, it is unknown which genera of microalgae are capable of exhibiting luxury uptake, and whether some genera are more likely to store polyphosphate than others. This could have a significant effect on the phosphorus removal performance of WSPs, and could explain some of the variation in phosphorus removal present in WSPs.

To improve the knowledge in this area, a number of objectives were set for this project, as shown in the introduction. Through fulfilment of these objectives, a better understanding of the ability of microalgae to uptake phosphorus in WSPs will be established..

3 Methodology

3.1 Site Selection

A large number of sites were required for the study, in order to provide stronger conclusions and better account for any random between site variations. To ensure that there was some comparability between the different sites, the following conditions were set:

- The study would focus on two-pond systems wherever possible. These were the most common type of pond system found in New Zealand, and so could be easily found and compared.
- The study would only consider municipal WSPs.
- Pilot scale HRAPs would be used to assess the influence of pond types, given the absence of a functional full scale municipal HRAP in New Zealand.

3.1.1 Manawatu Sites

It was decided to sample from four sites within the Manawatu region. This sample size would account for any random between site variations, while also making the results gathered from these sites independent of climatic conditions, due to the relatively close proximity of the sites to one another. The sites selected were Halcombe, Sanson, Foxton Beach and Rongotea. Weather data for Halcombe, Sanson and Rongotea was taken from the NIWA weather station in Palmerston North, while weather data for Foxton Beach was taken from the NIWA weather station located in Levin.

3.1.1.1 Halcombe

The Halcombe wastewater treatment plant is a small two pond system serving the nearby town of Halcombe, a town of 534 people according to Statistics New Zealand 2013. An aerial photo of Halcombe is shown in Figure 7.

Figure 7: Aerial photo of Halcombe WSPs. Note that sampling locations are indicated by the white crosses, while the black arrows indicate the direction of flow of wastewater through the system.

3.1.1.2 Sanson

Sanson Wastewater Treatment Plant utilises a two pond system to treat the wastewater of the nearby town of Sanson, a town of 537 people according to Statistics New Zealand 2013. An aerial photograph of the plant is shown in Figure 8.

Figure 8: Aerial photo of Sanson WSPs. Note that sampling locations are indicated by the white crosses, while the black arrows indicate the direction of flow of wastewater through the system.

3.1.1.3 Foxton Beach

Foxton Beach Wastewater Treatment plant is a two pond system found between the townships of Foxton and Foxton Beach. This plant serves Foxton Beach, a township with a permanent population of 1641 according to the Statistics New Zealand 2013. However, this plant has significant fluctuations in influent volumes during summer due to the large number of visitors to the area over this time period.

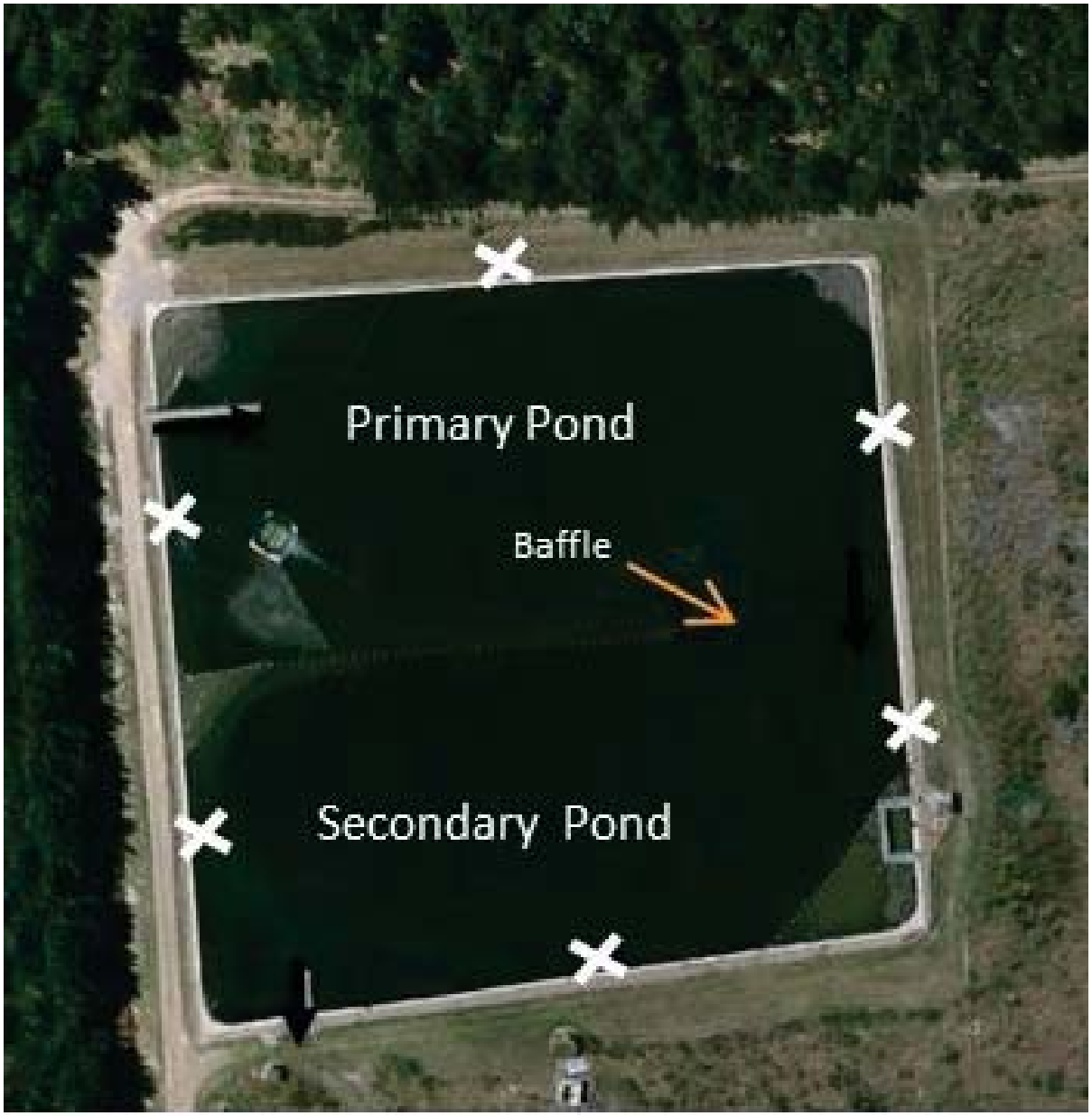


Figure 9: Aerial photo of Foxton Beach WSPs. Note that sampling locations are indicated by the white crosses, while the black arrows indicate the direction of flow of wastewater through the system.

3.1.1.4 Rongotea

The Rongotea Wastewater Treatment Plant is a two pond system found on the outskirts of Rongotea, a town with a population of 594 according to the Statistics New Zealand 2013. In addition to municipal waste, the Rongotea ponds receive some effluent from the nearby stockyards, as well as from septic tank disposal. A third pond is present in Rongotea, but is too small to provide significant treatment. An aerial photo of the site can be found in Figure 10.

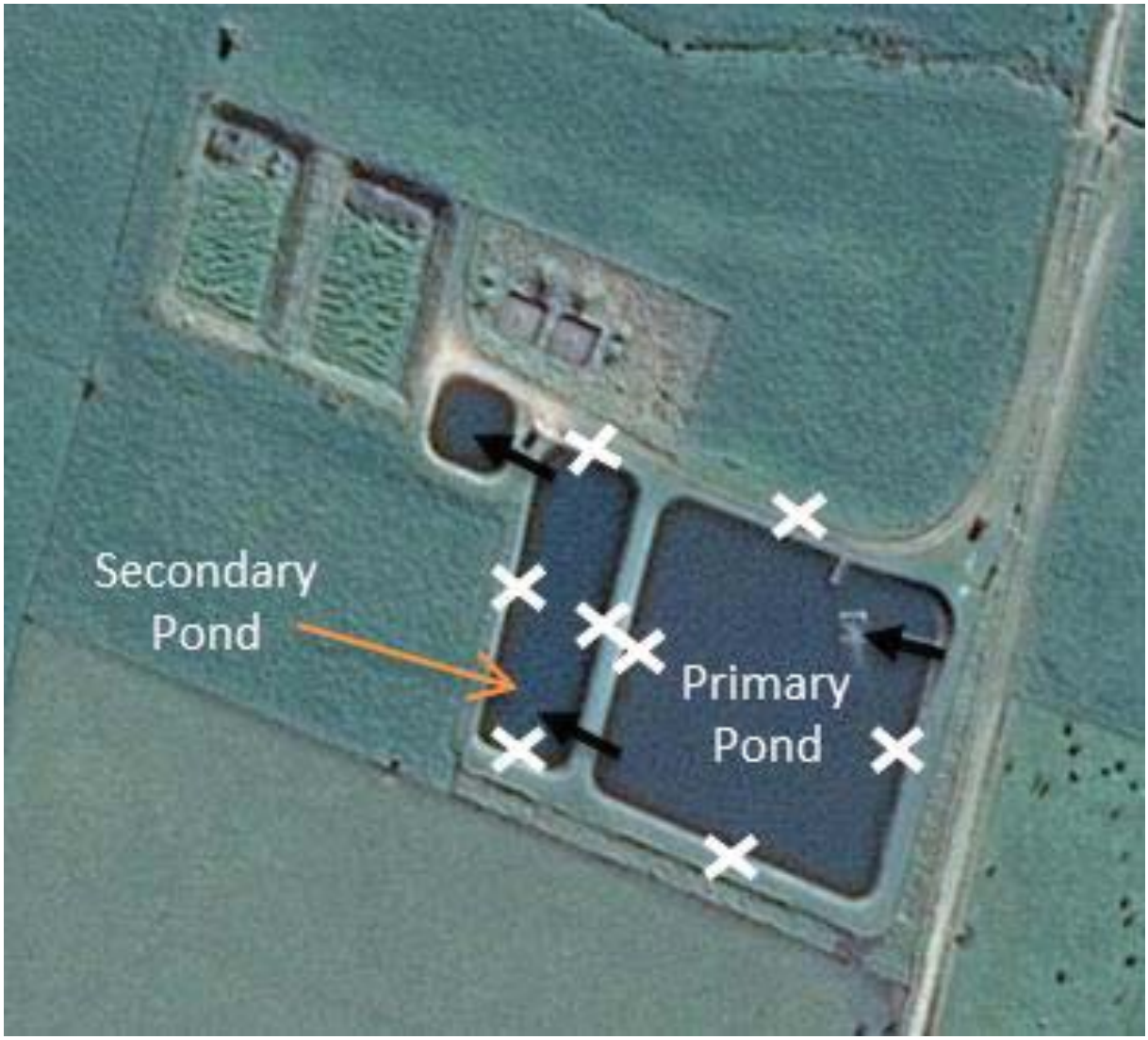


Figure 10: Aerial photo of Rongotea WSPs. Note that sampling locations are indicated by the white crosses, while the black arrows indicate the direction of flow of wastewater through the system.

3.1.2 Nationwide sites

In contrast to the Manawatu sites, the aim of site selection for nationwide sites was to measure the effect of different climatic conditions on microalgal biomass within WSPs. To maximise the effect of the climatic conditions, and therefore make it more easily observed, it was decided to sample from two regions as far away as possible – Gore, near the bottom of the South Island, and Kaitaia near the top of the North Island. However, the distance of these regions from Massey University in the Manawatu (the location of the laboratory where analysis was to be carried out) meant that samples sent from these sites would not be received on the same day as sampling, creating a potential bias due to change in the biomass over this time. To avoid this, Lugol's iodine prepared using a recipe provided by the research unit at Cawthron Institute in Nelson was used to preserve the samples and prevent any change in the biomass. Lugol's iodine has been found to be an effective preservative for microalgae, according to Wetzel and Likens (2000). Once the samples had been gathered, 5 mL of Lugol's Iodine was added, enough to make the concentration within the sample to 0.5% as

per recommendation by Cawthron Institute. This allowed analysis to be carried out as normal the following day.

3.1.2.1 Gore

The wastewater treatment plant at Gore utilises a large two pond system which serves the entire Gore district, which has a population of 12033 people according to the Statistics New Zealand 2013. Weather data for Gore was taken from a weather station located within the town. An aerial photo of the Gore WSPs is shown in Figure 11 below.

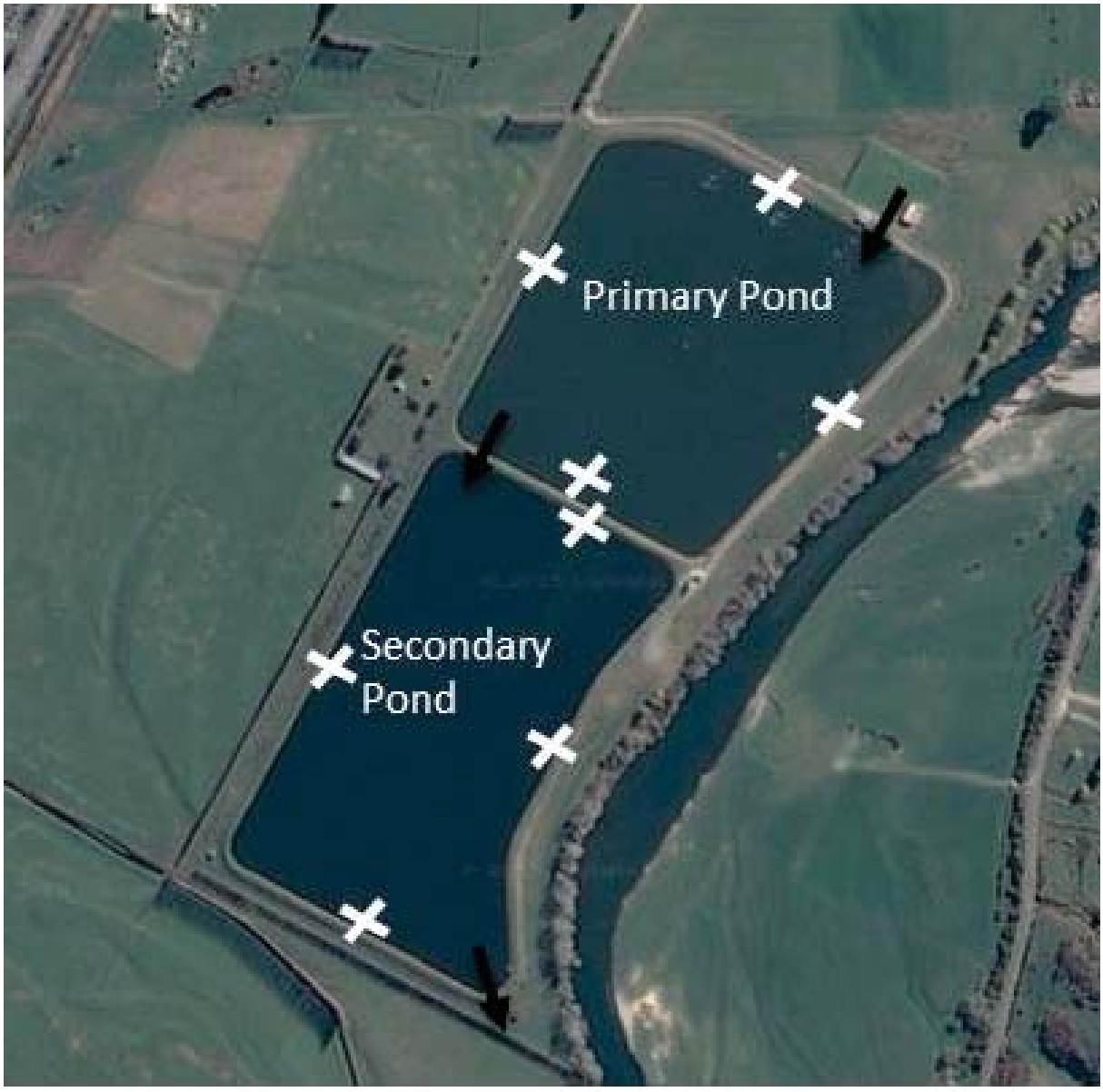


Figure 11: Aerial view of Gore WSPs. Note that sampling locations are indicated by the white crosses, while the black arrows indicate the direction of flow of wastewater through the system.

3.1.2.2 Kaitaia

Kaitaia Wastewater Treatment Plant is a large three-pond system that serves Kaitaia, a town with a population of 3093 people. This site was selected due to the lack of any suitable two-pond systems in the area. Samples were taken only from the first two ponds. Weather data

was taken from a weather station located in the town. An aerial photo of the Kaitaia plant is shown in Figure 12.

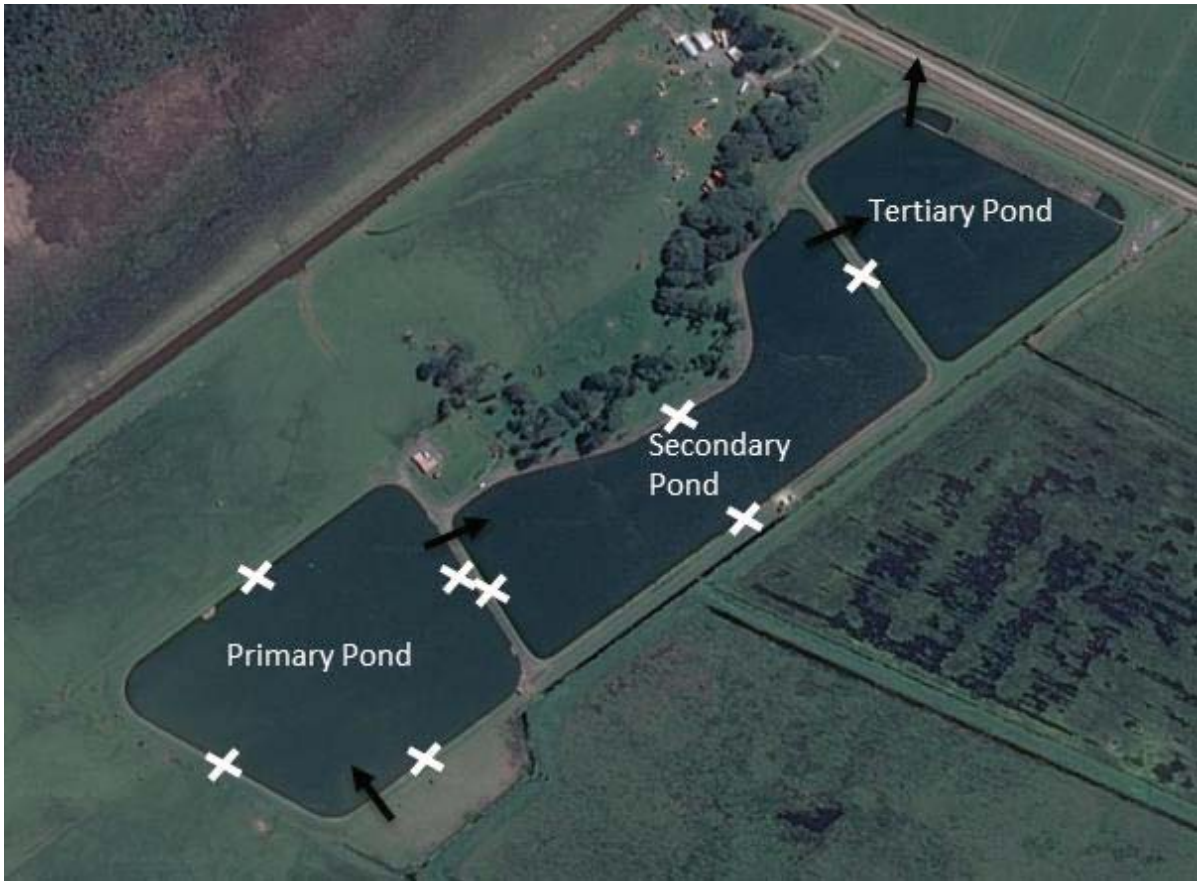


Figure 12: Aerial view of Kaitaia WSPs. Note that sampling locations are indicated by the white crosses, while the black arrows indicate the direction of flow of wastewater through the system.

3.1.3 Different Pond Types

To determine the effect of different pond types on phosphorus uptake by biomass required the assessment of both WSPs and non-WSPs. Firstly, the primary ponds of all the sites studied were compared to the secondary ponds, to see whether the different conditions found in each pond had an effect on the phosphorus content of the biomass. In addition, two pilot scale HRAPs located at NIWA in Hamilton were also studied. These ponds provide a significantly different environment for microalgal biomass due to their shallower depth and increased mixing. In addition, pH adjustment through CO_2 addition was employed by this pond to keep the pH below 8, further influencing the environmental conditions experienced by the microalgae. One obstacle to the study of the NIWA HRAPs was that the DO values for the NIWA site were expressed as a percentage DO rather than mg/L as used elsewhere. To solve this issue, a DO converter taken from the website Hamza's Reef was used to convert the values to mg/L. The salinity value used for the conversion was a typical value for wastewater taken from Paterson and Curtis (2005).

The NIWA HRAPs are shown in Figure 13.

Figure 13: Twin HRAPs located at NIWA. Sampling points were located at the ends of both ponds.

To ensure that climactic effects that may have affected the results from this site were accounted for, a nearby WSP in Ngaruawahia was also assessed. The Ngaruawahia WSP served the local population of Ngaruawahia, a town of 5127 people according to Statistics New Zealand 2013. This was the only WSP located near to the HRAPs at NIWA. Lugol's iodine was used as a preservative for both these sites due to their distance from the Manawatu. Weather data for both sites was obtained from a weather station located within Hamilton, due to its proximity to both sites.

An aerial photo of the Ngaruawahia WSP is shown in Figure 14 below.

Figure 14: WSP found at Ngaruawahia. Note that sampling locations are indicated by the white crosses, while the black arrows indicate the direction of flow of wastewater through the system. The black dotted lines indicate the position of baffles in the system.

3.2 Sampling Methodology

At each site used in the study, WSP samples were taken using a 500 mL Duran bottle attached to a pole submerged to 15 cm. This shallow depth was chosen as the proximity to the surface meant that photosynthetic activity would almost always be occurring at this depth, guaranteeing that microalgae would be present in the sample.

Using the sampling pole, 500 mL samples were taken from each of the four sides of each pond studied, and combined into one container. This combined 2L sample therefore represented an "average sample" of the pond. Samples were taken from the sides of the pond rather than the corners to ensure the point where the sample was taken did not contain any stagnant material. This sampling methodology only differed at the HRAPs at NIWA, where the arrangement of the ponds meant a different sampling protocol was required. 1 L of sample was taken from each end of the pond, and combined into one sample, to account for any variations across the pond. This process was repeated for both HRAPs present at NIWA.

3.3 Analytical Analysis

The determination of the phosphorus content of the biomass within the samples was the key measure in the study. As mentioned previously in section 2.1.3.4, a value for phosphorus content above 1% P/g VSS indicates that a phosphorus uptake mechanism beyond

assimilation is occurring within the biomass. However, this measure required determination of the volatile suspended solids concentration, total phosphorus concentration and dissolved phosphorus concentration before it could be calculated.

3.3.1 Volatile Suspended Solids

Volatile suspended solids (VSS) measurements were used as a measure of the biomass content of the wastewater. VSS concentrations of the samples were measured in triplicate using the method outlined by APHA, et al (1995). This analysis was performed on the same day as the sample was taken when possible.

3.3.2 Total Phosphorus and Total Dissolved Phosphorus

To gain a measure of the amount of phosphorus present both within the biomass and dissolved in the wastewater, total phosphorus (TP) and total dissolved phosphorus (TDP) measurements were required.

TP samples were first digested to breakdown the biomass using the sulphuric acid-nitric acid method outlined by APHA, et al. (1995). Analysis of phosphorus content was then performed using the ascorbic acid method also found in APHA, et al (1995). This provided a measure of the total amount of phosphorus present in the sample.

TDP samples were filtered through a 0.45 μm filter then digested and analysed using the same techniques as the TP process. As all of the phosphorus within the biomass was removed using the 0.45 μm filter, this analysis provided a measure of the amount of dissolved phosphorus present in the sample. The TDP samples were prepared on the same day as the sample was taken, before any significant changes in the biomass could occur. Both TP and TDP measurements were made in duplicate.

An analysis of the error in the TP analysis is presented in Appendix 1. To ensure that the Lugol's iodine used on the nationwide samples would not interact with the reagents used in the total phosphorus analysis, testing had to be performed. This testing compared the results for TP and TDP from pairs of identical samples – one with iodine and one without iodine. The results showed no significant differences between the iodised samples when compared with the non-iodised samples, indicating that the Lugol's iodine did not introduce bias into the total phosphorus analysis. The results of this analysis are shown in Appendix 2.

3.3.3 Percentage of Phosphorus in Biomass

To calculate the phosphorus content of the biomass as a percentage, Equation 3 was used:

$$\frac{\%P}{g\ VSS} = \frac{TP - TDP}{VSS} \times 100$$

Equation 3: Formula for calculating phosphorus content of the biomass, where: %P/g VSS = Biomass phosphorus percentage, TP = TP concentration, TDP = TDP concentration, VSS = VSS concentration.

3.4 Environmental Variables Measured

In addition to the variables measured in 3.3, the environmental variables that could affect the behaviour of microalgae, and in turn the phosphorus content of the biomass, needed to be measured. This study aimed to examine as many of these variables as possible, to best understand the environmental conditions that affect the phosphorus uptake behaviour of the biomass.

3.4.1 In Pond Variables

Primary variables measured within the pond were pH, DO and temperature. These were measured using a Thermo Scientific Orion Star A326 pH-DO meter, at the same points and depths as where the bulk samples were taken. The total dissolved phosphorus concentration of the wastewater was also considered to be an environmental variable, as this had been found to influence phosphorus uptake by microalgae in past studies (Powell, et al. 2009).

3.4.2 Weather Variables

To assess the effect of weather variables on the behaviour of microalgae in the pond, data from NIWA weather stations located near the different sites was used. The weather variables measured using these weather stations were air temperature, wind speed, rainfall and solar radiation.

One issue with the use of the NIWA weather stations was that it ignored the influence of local site characteristics (such as tree cover) on the weather conditions at the site. To assess whether these were significant, Aercus WS-3083 weather stations were installed at each of the Manawatu sites, and the data recorded compared to the data received from the NIWA weather stations.

3.5 Visual Analysis

The aim of the visual analysis was to identify the different genera of microalgae present in the sample, and identify which types stored phosphorus as polyphosphate granules. To do this, the slides had to first be stained to make the polyphosphate granules visible, before viewing under a photo microscope.

3.5.1 Sample Preparation

Microscope slides for visual analysis were prepared by taking a sample using a sawn off 1000 μL pipette tip. The tips were sawn off halfway up the slope of the tip to widen the inlet and thus prevent any filtration caused by the narrow inlet of the standard pipette tip. The pipettes were set to 500 μL and used to pipette fluid onto the slide, which was then placed in an oven at 100 °C to fix the biomass to the slide. Dried slides were stored in vacuum desiccators.

3.5.2 Staining Methodology

The staining methodology used followed the lead nitrate staining methodology used by Bolier, et al. (1992). This stained the polyphosphate granules within the cell black, making them easily identifiable under a microscope. However, slides from samples that used Lugol's

iodine as a preservative were found to react to the stain, with a yellow precipitate forming on the surface of the slide when first applied to the sample. The formation of this precipitate prevented the stain from reaching the cells of the sample, thus meaning that the polyphosphate within the cells remained invisible under microscopic examination. To avoid this, the slides were initially washed with the lead nitrate used in the first part of the staining to remove any of the excess iodine, and then stained as per the normal procedure. This wash method was tested by visual comparison of iodised samples stained under both the wash method and the standard method with uniodised samples stained under the standard method. This analysis found that the difference between the visibility of polyphosphate granules was negligible between the iodised sample stained under the wash method and the uniodised sample stained under the standard method. However, there were significant differences in visibility between the iodised sample stained under the wash method and the iodised sample stained under the standard method, indicating that the wash method was necessary in order to properly view the polyphosphate granules present.

3.5.3 Visual Analysis of Samples

Visual analysis of the samples was conducted using a Leica light microscope with camera attachment at the Manawatu Microscopy and Imaging Centre (MMIC) located on the Massey University campus.

The visual analysis was made up of two parts – visual identification of the microalgae, and the granule rating. The aim of visual identification was to determine the different genera of microalgae present on the slide. This was achieved by scanning the slide at 1000x magnification and noting the different genera of microalgae present. Samples from each pond were sent to Cawthron Institute once every three months for independent verification of the microalgal identification. This allowed for any microalgal genera that had been misidentified initially to be properly identified.

The granule rating was used to determine which genera were undergoing luxury uptake, and the relative amount of polyphosphate granules that were being stored. Granule ratings were based on the number of polyphosphate granules present in the cell, using a 0 – 5 scale. This is outlined in Table 2 below.

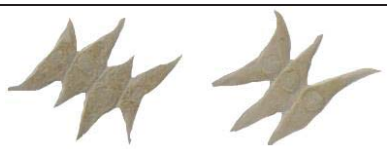
Score	Picture	Description
0		• *No polyphosphate present*
1		• *Feature only one granule per cell in group or less* • *Also includes any cells or colonies groups that have just one granule of polyphosphate in the group.*
2		• *Contain two granules per cell*
3		• *Usually feature 3 or more granules per cell* • *Can contain large granules*
4		• *Contain multiple large and small granules.*
5		• *Large portions of cell occupied entirely by polyphosphate.*

Table 2: Granule Rating allocation table, using cells of the genus *Scenedesmus* as examples

The overall granule rating for each genus was calculated by averaging the granule ratings for each of the cells photographed. A minimum of 10 colonies or single cells were used to calculate the granule score for each genus, unless less than 10 colonies or single cells of a particular genus were present.

While the granule score cannot quantify the amount of polyphosphate present in the cell, it does allow for comparison of different microalgal genera in terms of polyphosphate uptake.

3.6 Summary of Sampling Plan

To account for the effect of different seasons on microalgal behaviour, a full years sampling was planned. However, due to time and equipment constraints, it was not possible to sample every site every month. It was instead decided to sample each site twice every season, with Rongotea and Foxton being sampled every month to ensure there were no significant month-to-month variations. The sampling plan is demonstrated in Table 3.

	WINTER			SPRING			SUMMER			AUTUMN		
	June	July	August	September	October	November	December	January	February	March	April	May
Rongotea	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Foxton Beach	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Halcombe	✓	✓		✓	✓		✓	✓		✓	✓	
Sanson	✓	✓		✓	✓		✓	✓		✓	✓	
Gore		✓	✓		✓	✓		✓	✓		✓	✓
NIWA		✓	✓		✓	✓		✓	✓		✓	✓
Ngaruwahia		✓	✓		✓	✓		✓	✓		✓	✓
Kaitaia		✓	✓		✓	✓		✓	✓		✓	✓

Table 3: Sampling schedule for the project

In total, the phosphorus content of the WSP biomass was compared with the following variables:

- VSS
- TDP
- pH
- DO
- Pond temperature
- Air temperature
- Rainfall
- Wind speed
- Solar radiation
- Pond type
- Geographic location
- Genus of microalgae present

4 Results and Discussion

4.1 Phosphorus Content of Biomass

An overview of the phosphorus uptake performance of the WSPs and HRAPs used in this study is presented in Table 4.

	WSPs	HRAPs
Avg (%P/g VSS)	1.21%	0.71%
Std Dev	0.7%	0.2%
95% CI	0.1%	0.3%
Max	3.8%	1.3%
Min	0.1%	0.5%
>1%P/g VSS	56%	8%

Table 4: Summary table of phosphorus uptake performance by WSPs and HRAPs

Table 4 shows that luxury uptake occurred frequently within WSP biomass, with 56% of the samples examined exhibiting phosphorus uptake above 1 %P/g VSS for an average biomass phosphorus content of 1.21 %P/g VSS. In total, 12 of the 13 ponds studied exhibited elevated levels of phosphorus uptake at some point in the study. This is a much larger number of ponds than those examined at two sites by Powell, et al. (2011), and so proves that the elevated WSP biomass phosphorus content observed in their study was not an isolated case. The biomass within the HRAPs was not as effective at performing luxury uptake, as only one sample exhibited luxury uptake, with an average biomass phosphorus content of 0.71% P/g VSS.

Four samples taken from the WSPs exceeded 3 %P/g VSS, with a peak phosphorus uptake of 3.8 %P/g VSS, nearly four times the standard value for phosphorus content of the biomass. Interestingly, this peak value was very similar to the peak phosphorus content of 3.85 %P/g VSS measured by Powell, et al. (2011) in their study on WSPs. This result shows great potential for improvements in phosphorus removal from WSPs. Consistent replication of this elevated value for phosphorus uptake could nearly quadruple the amount of phosphorus removed from wastewater by microalgae, which combined with subsequent biomass removal, could result in greatly improved phosphorus removal. However, such a process would likely require the ability to cultivate microalgae under specific conditions to promote phosphorus uptake as well as subsequent removal of this biomass, a process which is not feasible under current WSP design.

Another interesting observation from Table 4 is the existence of biomass with extremely low phosphorus contents. In total 44% of the samples taken contained biomass at or below 1 %P/g VSS, with phosphorus contents as low as 0.1 %P/g VSS recorded on three occasions. This is an interesting result, as these low phosphorus contents would indicate that the microalgae are in a state of near-starvation of phosphorus, and yet are growing within a phosphorus rich environment. One possibility is that a combination of a spike in growth rate just prior to sampling and a lack of readily available dissolved phosphate within the pond could lead to consumption of internal phosphorus stores within the microalgae, as noted by

Wu, et al. (2015). However, it is highly unlikely that, at the levels available in WSPs, such a lack of available phosphorus would exist. As a result, more research is required on this phenomenon before any conclusions can be made.

While the elevated phosphorus contents measured in this study could have been due to the precipitation and adsorption mechanisms that exist within WSPs, this was deemed unlikely due to the sub-optimal conditions present for both mechanisms to occur. This will be covered further in section 4.2.

4.2 Environmental Effects on Phosphorus Content of Biomass

To identify the environmental variables that had significant effects on the phosphorus uptake of the biomass, the statistical software MINITAB was used to perform a stepwise linear regression analysis on the environmental data produced from this study, following the elimination method. This analysis was performed over multiple steps, with the least significant variable removed after each step until the only variables remaining were those significant at a 95% level of confidence. A summary of the environmental variables measured in this study can be found in Appendix 3, while details of the regression analysis are found in Appendix 4. Comparison of weather data from the NIWA weather stations with weather data from the local stations revealed no significant differences between the two station types, showing that localised site effects were not significant.

The results from the MINITAB analysis are shown in Table 5.

Variable	p-Value	Step Removed	Significant (95% Confidence Level)
VSS	0.881	2	NO
pH	0.382	5	NO
DO	0.406	6	NO
TDP	0.009	N/A	YES
Rainfall	0.024	N/A	YES
Wind speed	0.873	3	NO
Air Temperature	0.948	1	NO
Solar Radiation	0.654	4	NO

Table 5: Statistical significance values for variables measured in the study. Note that the p-Values given for each variable are for the final iteration before the variable was eliminated (unless found to be significant). A p-Value less than 0.05 indicates that the variable is significant at a 95% confidence level.

Table 5 shows that the only significant variables found using this analysis were rainfall and TDP concentration, both at a 95% confidence interval. Increasing rainfall was found to have a negative effect on the phosphorus content of the biomass while increases in TDP concentration were found to have a positive effect. To ensure that the model produced using these variables was statistically valid, residual plots were created. Residual plots are used to determine whether the residuals from a statistical model follow a normal distribution, or

whether there is a source of unexplained variation that is influencing the results in some way (Montgomery, et al. 2010). These plots are shown in Figure 15.

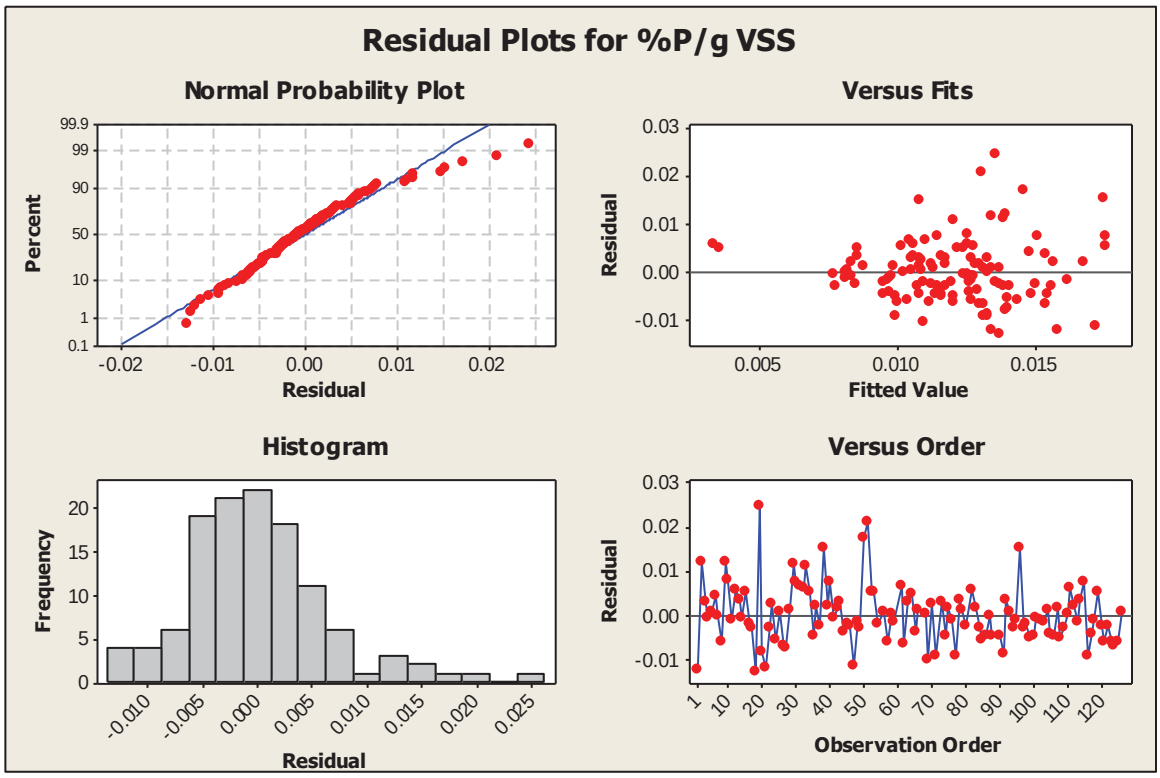


Figure 15: Residual plots for stepwise regression model

From these plots, it can be seen that the residuals very closely follow a normal distribution. The normal probability plot shows that the residuals follow very closely to the normal probability line, with a rough bell curve shape evident in the histogram. While there are two unusual residuals in the plot of residuals versus fits, the bulk of the residuals are random as following a normal distribution. This confirms that the results presented in Table 5 have not been influenced by unforeseen variation.

While the significant variables measured in this study were of great interest, the lack of influence of pH, sunlight and temperature provided some insight into the phosphorus removal mechanisms that occur within the WSPs. As mentioned in 2.1.3.4, significant precipitation of phosphorus requires a combination of high levels of cations and elevated pH above 8.2 (Craggs, 2005a). While the pH within the ponds did increase beyond 8.2 at times in the study, this only occurred in 31% of the samples, making it unable to explain the consistently elevated phosphorus contents that occurred within the WSP biomass. There was also no detectable relationship between biomass phosphorus uptake and pH measured in the MINITAB analysis. This combination of factors makes it unlikely that precipitation was a significant mechanism in the phosphorus uptake achieved by the ponds. Likewise, despite being found to have a significant positive effect on microalgal adsorption and in turn biomass phosphorus content by Lu, et al. (2014), both temperature and solar radiation were found to have no effect on the phosphorus uptake of the biomass in WSPs. As a result, it can be

concluded that the elevated phosphorus uptake achieved by the biomass present in the WSPs is most likely due to the luxury uptake mechanism.

Another interesting finding from this analysis is that the VSS concentration had no influence on the phosphorus content of the biomass. This is despite research by Powell, et al. (2011) and Wu, et al. (2015) that state that enhanced growth of microalgae leads to decreased biomass phosphorus contents. This could be due to the fact that phosphorus was not limiting within the WSPs studied, meaning that consumption of internal phosphorus stores was not required during growth. The timing of the sampling could have also had an effect. With microalgal phosphorus stores being consumed throughout the growth phase of the microalgae to facilitate growth, the time of sampling would directly impact the amount of phosphorus present within the microalgae. Sampling at the beginning of the growth phase would yield biomass with relatively high phosphorus contents, whereas sampling at the end of the growth phase could have yielded low phosphorus content biomass. While it was impossible to control the point in the growth phase where sampling occurred, it is a source of error that could have influenced the results.

4.2.1 TDP

To further examine the effects of the significant variables on the phosphorus content of biomass within WSPs, scatter plots of each of the variables were plotted. A plot of the TDP concentration against the biomass phosphorus content is shown in Figure 16.

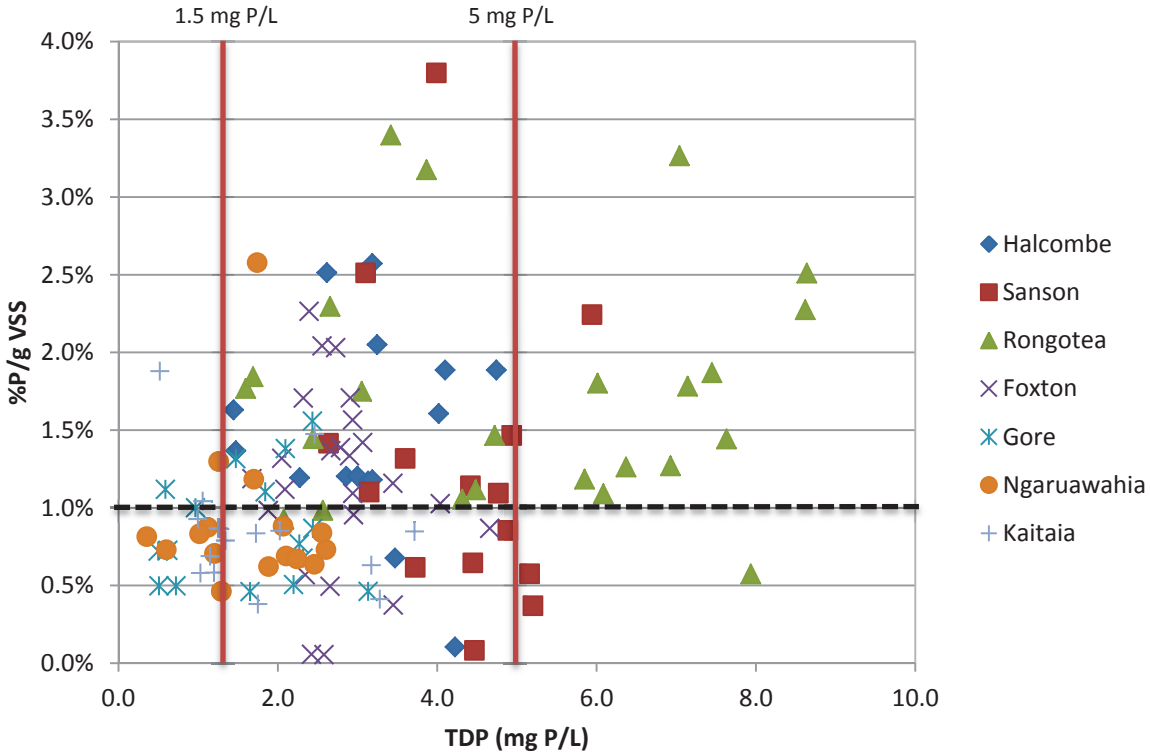


Figure 16: Plot of TDP against biomass phosphorus content

While initial observations from this plot make the influence of TDP unclear, clearer conclusions can be made when considering the intervals outlined by the vertical lines on

Figure 16. Below 1.5 mg P/L, only 32% of samples exhibit luxury uptake. Between 1.5 and 5 mg P/L, this value increases to 59%, while above 5 mg P/L, 80% of samples performed luxury uptake. This demonstrates that TDP does have a positive effect on the phosphorus content of the WSP biomass. Mechanistically, this effect may be due to the increased availability of phosphorus to the individual microalgae present in the WSP. The increased concentrations of TDP would increase the frequency at which an individual microalga comes into contact with phosphorus molecules, thus increasing the likelihood that these molecules will be absorbed. This in turn would increase the phosphorus content of the individual cell. This effect had been noted by Aitchison and Butt (1973), where it was observed that increasing the phosphorus concentration within the growth media for *C. vulgaris* resulted in increased polyphosphate content in the biomass. However, the *C. vulgaris* inoculum in this study was taken from a phosphorus-starved culture, a condition unlikely to be replicated in the phosphorus rich environments found within WSPs. As a result, more research is required to confirm the true mechanism behind the relationship between TDP and biomass phosphorus content.

4.2.2 Rainfall

A plot of the monthly average rainfall for each site against the phosphorus content of the biomass is shown in Figure 17.

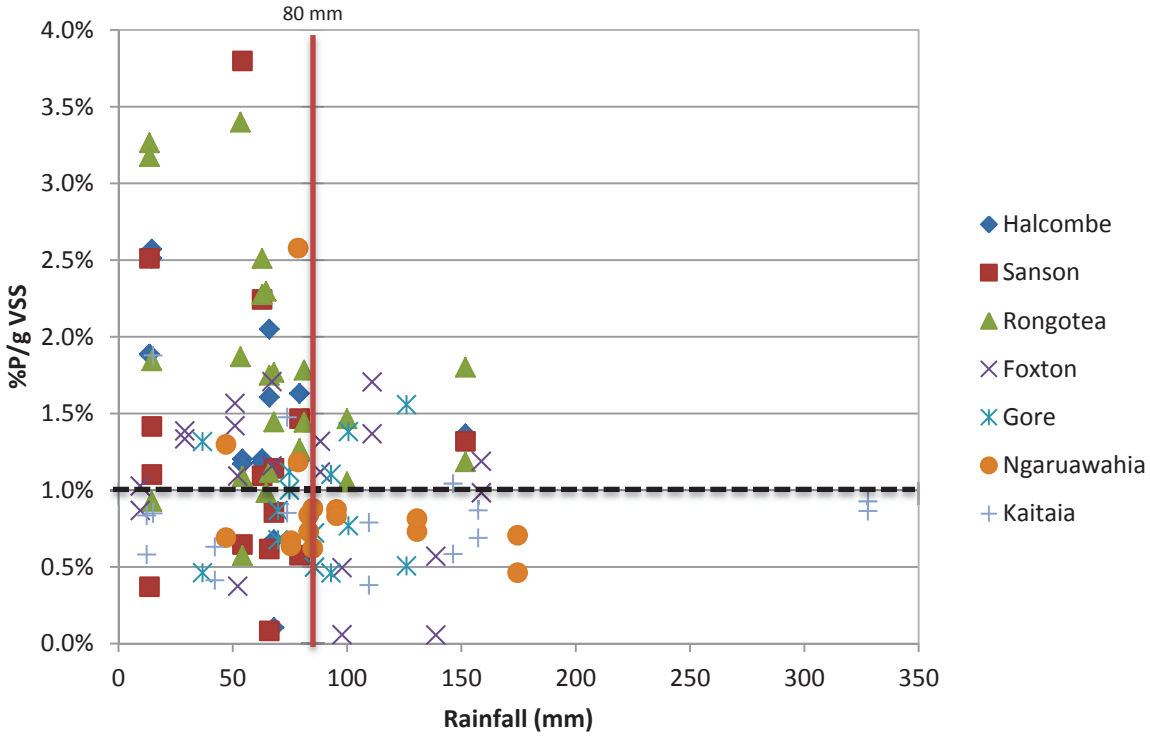


Figure 17: Monthly average rainfall versus phosphorus content of WSP biomass

Figure 17 demonstrates a clear negative trend between rainfall and phosphorus content. This trend is particularly clear around the value for rainfall of 80mm. Below 80mm of rainfall, 67% of all samples exhibited luxury uptake, while above 80 mm of rainfall this rate reduced to 39%. Most tellingly, of all the measured phosphorus contents of microalgae above 2 %P/g

VSS, none occurred above an average monthly rainfall of 80mm. Mechanistically, this effect is unlikely to be due to rain falling directly on the pond surface, but more likely due to increased flowrates through the WSPs as a result of stormwater infiltration into sewer lines. This increased flow could cause washout of microalgal cells from the WSPs, leading to extremely low biomass concentrations within the pond, as well as dilution of TDP within the pond. This could initiate a stress response in the microalgae, with growth rates rapidly increasing to replenish the microalgal population. Such an effect has been noted by Hartig, et al. (1988), who found that growth rates of the microalga *Scenedesmus obliquus* were highest when the biomass concentration was only 12 mg TSS/L, with growth rates decreasing beyond this value. Facilitation of this increased growth in a low TDP environment would require consumption of polyphosphate stores within the microalgae, as noted by Wu, et al. (2015), as well as use of all available energy from photosynthesis. Such energy supply could be limited by the lowered levels of solar radiation reaching the pond due to the overcast conditions that occur during rainfall. As a result, there would only be limited energy available for phosphorus uptake. The combination of these factors could explain the decreased phosphorus contents of the microalgal biomass at higher levels of rainfall; however more research is required on the component mechanisms before any firm conclusions can be made.

4.3 Climatic Effects on Biomass Phosphorus Content

To identify whether any climatic effects beyond the measured environmental variables had an influence on the phosphorus content of the biomass, the phosphorus uptake performance of biomass from the different geographical locations were compared with one another. This is displayed in Figure 18.

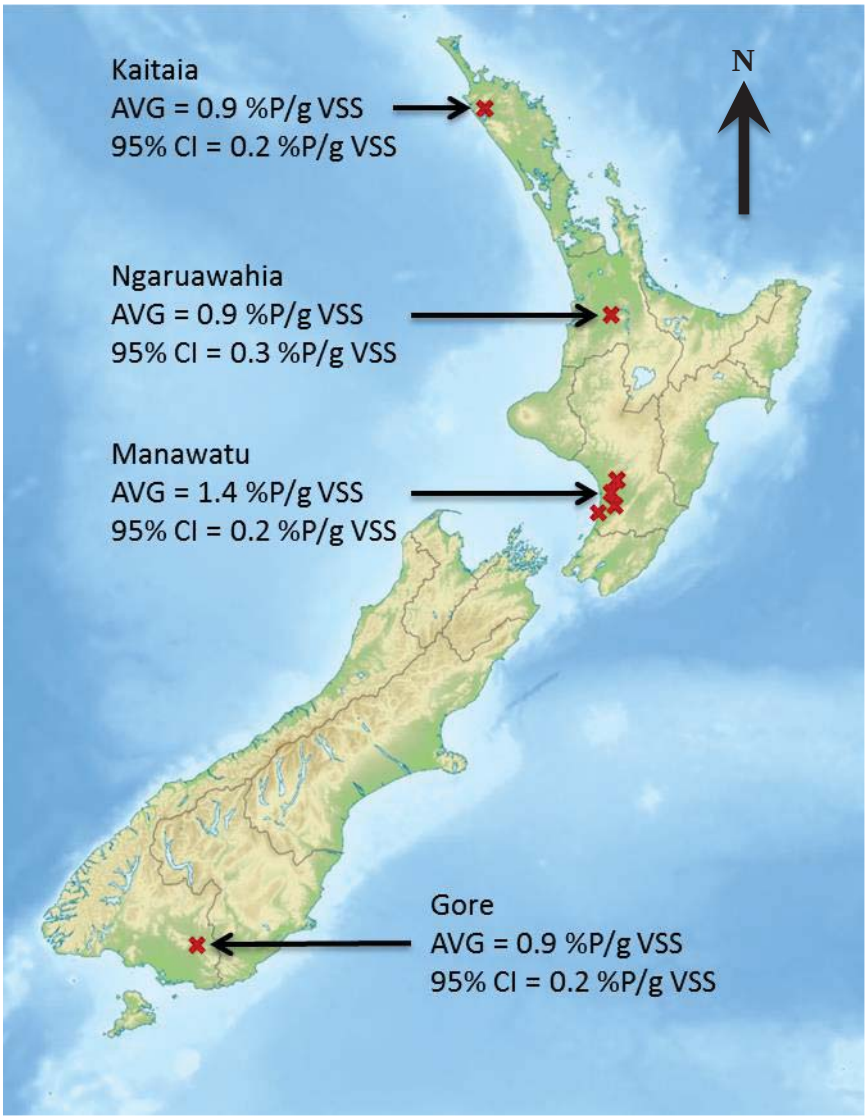


Figure 18: Average phosphorus content of WSP biomass with confidence interval at each geographical location in the study. Note that CI = Confidence Interval

As shown in Figure 18, the biomass phosphorus contents of WSP biomass within and around the Manawatu region was significantly higher than that of the any other region, with none of the 95% confidence intervals for the other regions overlapping with the confidence interval for the Manawatu. The average biomass phosphorus content was the same for all other regions, with the confidence intervals all overlapping. This occurred despite the significantly different climatological conditions present at each locale, with Gore, Ngaruawahia and Kaitaia existing within the cool temperate, warm temperate and sub-tropical regions of New Zealand respectively.

While the results of this analysis appear to suggest that the Manawatu region is the most ideal location for performance of luxury uptake, this effect may have been due to other environmental factors as covered previously. This was analysed in a two sample t-test between the rainfall and TDP concentrations experienced by the Manawatu WSPs as compared with those experienced by the WSPs in other regions. The results of these t-tests are presented in Table 6.

	N	Mean	p-Value
Manawatu TDP (mg P/L)	74	3.85	
Other Regions TDP (mg P/L)	46	1.67	0.000
Manawatu Rainfall (mm)	78	67	
Other Regions Rainfall (mm)	48	98	0.005

Table 6: Comparison of TDP concentration and rainfall experienced by Manawatu WSPs and WSPs from other regions

As shown by Table 6, the Manawatu WSPs experienced significantly higher TDP concentrations compared to WSPs in other regions, while also experiencing significantly lower rainfall levels. This combination of factors makes it more likely that the discrepancy in the biomass phosphorus contents between Manawatu WSPs and the WSPs in other regions is due to the significantly different environmental conditions than any climatic factor. As a result, it can be concluded that the geographic position of the WSPs does not have any effect on the phosphorus content of the biomass beyond that measured by the environmental variables.

4.4 Effect of Pond Type on Phosphorus Content of Biomass

To assess whether there were significant differences between biomass phosphorus content in different types of ponds, MINITAB was used to perform two sample t tests on the phosphorus contents of the samples from the different pond types. Using this method it was possible to determine which pond type was best for improving phosphorus content of biomass.

4.4.1 Primary vs. Secondary Ponds

The MINITAB output from the two sample t test comparing primary and secondary WSPs is shown in Table 7.

	N	Mean (%P/g VSS)	Std Dev	Std Error	p-Value
Primary	71	1.22	0.69	0.08	
Secondary	55	1.20	0.71	0.10	0.881

Table 7: MINITAB output for average phosphorus content of biomass for primary vs. secondary ponds.

This analysis shows that while the average phosphorus content of the biomass within primary ponds is higher than the average phosphorus content of the biomass within secondary ponds, at the 95% level of confidence this difference was determined not to be significant. This is an interesting result, as the different loading rates experienced by the primary and secondary ponds typically result in significantly different environmental conditions experienced by each pond. To identify the reason for this similarity, the significant variables that influence both ponds were compared. While rainfall was the same for both ponds in the study, the TDP concentrations were able to be compared with a two sample t-test, as shown in Table 8.

	N	Mean (mg P/L)	Std Dev	Std Error	p-Value
Primary TDP	71	2.87	1.81	0.22	
Secondary TDP	55	3.21	1.79	0.24	0.283

Table 8: Results from two sample t-test comparing TDP concentration in primary and secondary WSPs

While the secondary ponds TDP concentration was higher on average than that of the primary pond, Table 8 shows that this difference was not significant at any confidence level. This result explains the similarity in biomass phosphorus contents for primary and secondary ponds, as there were negligible differences in the levels of the environmental variables which influence the luxury uptake phenomenon. It also shows that the degree of organic loading experienced by the pond has no influence on the phosphorus uptake behaviour of the microalgae within the pond, as there was no difference in biomass phosphorus uptake across either pond despite the different levels of organic loading experienced by the two different ponds.

4.4.2 High Rate Algal Ponds

MINITAB analysis of the phosphorus content of HRAP biomass compared with both primary and secondary WSPs is shown in Table 9.

	N	Mean (%P/g VSS)	Std Dev	Std Error	p-Value
HRAP	13	0.71	0.21	0.06	
Primary	71	1.22	0.69	0.08	0.000
Secondary	55	1.20	0.71	0.10	0.000

Table 9: MINITAB comparison of biomass phosphorus contents between HRAPs, primary WSPs and secondary WSPs. Note that the p-values are for the two-sample t tests used to determine whether the differences between biomass phosphorus content in HRAPs and primary and secondary ponds were significant at a 95% confidence level.

Table 9 shows that the average biomass phosphorus content of HRAPs is significantly lower at a 95% confidence level than both primary and secondary WSPs. While this could be due to the differing design characteristics between HRAPs and WSPs, the different environmental conditions experienced by the site could also have had an effect. This is analysed in Table 10.

	N	Mean	Std Dev	Std Error	p-Value
WSP TDP (mg P/L)	126	3.02	1.80	0.16	
HRAP TDP (mg P/L)	14	2.31	0.58	0.15	0.002
WSP Rain (mm)	120	79	52	5	
HRAP Rain (mm)	13	85	31	9	0.535

Table 10: Comparison of TDP concentrations and rainfall levels for WSPs and HRAPs. Note that the p-Values are for the two sample t-test to determine whether HRAP pond quantities and WSP pond quantities are significantly different at a 95% level of confidence

As shown in Table 10, while the rainfall levels experienced by both WSPs and HRAPs in the study were statistically similar, HRAPs had significantly lower TDP concentrations than WSPs. This discrepancy may explain the decreased biomass phosphorus contents displayed in HRAPs, as with the difference in biomass phosphorus content between Manawatu WSPs and WSPs in other regions highlighted in Table 6. As a result it can be concluded that the

decreased biomass phosphorus contents exhibited by the biomass within HRAPs is more likely due to the differing environmental conditions than any particular design characteristic of the HRAPs.

4.5 Luxury Uptake of Different Microalgal and Cyanobacterial Genera

4.5.1 Commonality of Microalgal and Cyanobacterial Genera and Frequency of Luxury Uptake

The visual analysis identified 22 different microalgal and cyanobacterial genera that were present in the WSP samples. Comparison of the identified genera with Cawthron Institute results showed that all of the major genera identified by Cawthron Institute were also identified in this analysis. However, while there was some knowledge of the microalgal and cyanobacterial genera present in WSPs prior to this study, there was no information available on which genera were capable of performing luxury uptake within WSPs, and how frequently these genera performed this phenomenon. The visual analysis performed in this study was able to greatly increase the knowledge in this area, as illustrated in Table 11.

Genera	Luxury Uptake	%Samples ID	%Lux. Uptake
Actinastrum	✓	47%	46%
Chlamydomonas/Cryptomonas	✓	76%	68%
Closterium	✓	14%	65%
Crucigeniella	✓	17%	65%
Cyclotella	✓	5%	17%
Dictyosphaerium	×	3%	0%
Elakatothrix	✓	2%	50%
Euglena	✓	37%	73%
Kirchneriella	✓	21%	14%
Merismopedia	✓	24%	17%
Micractinium/Microcystis	✓	88%	50%
Monoraphidium	✓	71%	37%
Oocystis	✓	14%	41%
Pandorina	✓	2%	50%
Pediastrum	✓	14%	82%
Phacus	✓	34%	53%
Planktothrix	✓	32%	84%
Pseudanabanaceae	✓	29%	23%
Scenedesmus	✓	70%	73%
Schroederia	✓	32%	79%
Tetraedron	✓	8%	30%
Unknown Colonial	✓	2%	100%

Table 11: Frequency of appearance and frequency of luxury uptake of microalgal and cyanobacterial genera in WSPs. Genera marked with a * are cyanobacteria. Note that the categories for *Chlamydomonas* and *Cryptomonas* as well as *Micractinium* and *Microcystis* were combined due to the difficulty of differentiating the two genera after the staining process

The analysis presented in Table 11 shows that both microalgae and cyanobacteria within WSPs frequently store polyphosphate, showing that luxury uptake by these genera does occur across a wide range of ponds. Interestingly, nearly all of the genera identified in WSP samples were seen to perform luxury uptake. Of the 22 genera identified, 21 exhibited luxury uptake at some point in the study, demonstrating that luxury uptake is in fact widespread amongst microalgal and cyanobacterial genera present in WSPs. Only *Dictyosphaerium* was not found with granules in any samples. However, this may have been due to the low overall number of samples that each of this genus was identified in, thus reducing the odds of luxury uptake being found in the cells.

Table 11 also demonstrates that the most prevalent genera within the studied WSPs were *Chlamydomonas/Cryptomonas*, *Micractinium/Microcystis*, *Monoraphidium* and *Scenedesmus*, which were identified in 76%, 88%, 71% and 70% of the samples respectively. While appearing frequently in WSP samples, *Chlamydomonas/Cryptomonas*, *Micractinium/Microcystis* and *Scenedesmus* were also consistent performers of luxury

uptake, at frequencies of 68%, 50% and 73% respectively. Only *Monoraphidium* was a poor performer of luxury uptake, performing the phenomenon in only 37% of the samples it was identified in. The most consistent performers of luxury uptake were *Pediastrum, Planktothrix* and *Schroederia*, which contained stored polyphosphate in 82%, 84% and 79% of the samples they were identified respectively. However, these genera were only identified in 14%, 32% and 32% of the samples respectively. While *Pediastrum* and *Schroederia* were sporadically identified throughout the year, *Planktothrix* appeared to be heavily affected by the changing seasons, as it was identified frequently over the summer months (December – February) but declined in abundance outside of this time period, and was not identified at all during winter (June – August).

While these results suggest that luxury uptake is widespread amongst microalgal and cyanobacterial genera, the different frequencies of performance of this phenomenon suggest that some genera are more likely to perform luxury uptake than others. This may be linked to the findings highlighted in section 2.2.9 by Ruiz-Marin, et al. (2010) and Su, et al. (2012), where it was found that different microalgal species have different affinities for taking up phosphorus. This is a significant finding in terms of understanding the performance of this phenomenon in WSPs, and may explain the high degree of variation in phosphorus uptake present within WSPs.

4.5.2 Granule Scores for each Microalgal and Cyanobacterial Genus

While frequency of occurrence of luxury uptake within the samples by different microalgal genera is an important measure in assessing the ability of each individual microalgal and cyanobacterial genus to perform luxury uptake, the mass of polyphosphate stored within the cells is not considered by this method. This is an important consideration, as while a microalgal or cyanobacterial genus may perform luxury uptake at a low frequency, the mass of polyphosphate that it stores may be far greater than the total amount of polyphosphate stored by a genus that performs luxury uptake at a high frequency. While quantification was not possible, the granule score as defined in section 3.5.3 given to each microalgal genus in each sample provided a method to qualitatively compare the amount of polyphosphate stored. The granule scores for each genus could then be averaged over the number of samples in which the genus was identified in to assess which genus was best able to perform luxury uptake. The results from this analysis are given in Figure 19.

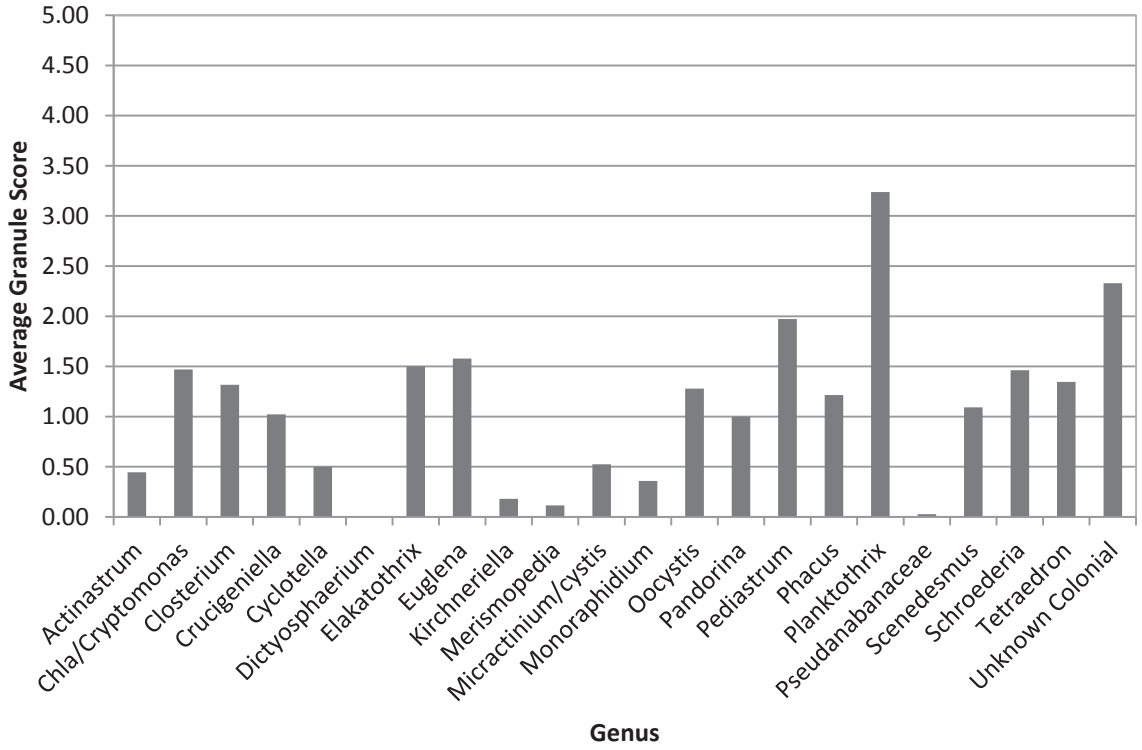


Figure 19: Plot of average polyphosphate granule scores for each genus of microalgae found in the study. Averages were taken across the number of times each genus was identified.

From this analysis, it can be seen that the genus with the highest granule score per sighting was the cyanobacterium *Planktothrix*, with an average score of 3.2. This makes *Planktothrix* not only the most frequent performer of luxury uptake, but also the genus that stores the largest amount of polyphosphate. This score was well ahead of the other genera, with the next best performers being *Pediastrum* (2.0), *Euglena* (1.6), *Chlamydomonas/Cryptomonas* (1.5) and *Schroederia* (1.46). *Elakatothrix* (1.5) and the unknown colonial microalgal genus (2.3) were also effective; however these genera were only identified in one sample each, making it difficult to make conclusions regarding their ability to store polyphosphate granules. Interestingly, the most common genera present in the sample had significant variations in the amounts of polyphosphate they stored. While *Chlamydomonas/Cryptomonas* stored a relatively high amount of polyphosphate, *Scenedesmus* had an average granule score of only 1.1, while *Micractinium/Microcystis* and *Monoraphidium* stored only small amounts of polyphosphate as granules with scores of 0.5 and 0.4 respectively.

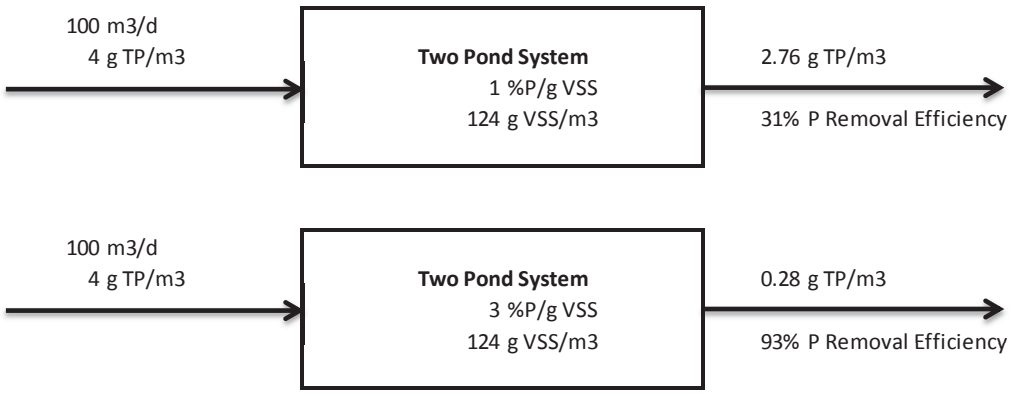
The results presented in Table 11 and Figure 19 have some important implications for the potential development of a new engineered process for improving phosphorus removal from WSPs. If microalgal or cyanobacterial genera that have a high likelihood of undergoing luxury uptake could be selectively favoured in WSPs, then the phosphorus uptake by these ponds could be greatly increased. Alternatively, a genus with a low frequency of luxury uptake but a high capacity for polyphosphate storage could be made to perform luxury uptake

through manipulation of certain conditions. Both these pathways could lead to the improvement in phosphorus removal from WSPs, as displayed in Box 1.

BOX 1

Comparison of phosphorus removal achieved in WSPs if normal microalgal assimilation occurred versus if enhanced phosphorus removal occurred.

Through development of a new engineered process targeting increased phosphorus removal, it may be possible to greatly increase phosphorus removal from WSP-type systems. Assuming that the phosphorus content of the biomass in this new process could be increased to 3% through luxury uptake, and if this biomass was subsequently removed, the improvements in phosphorus removal displayed below could be achieved. Note that the values for VSS concentration and TP concentration used are the averages taken from this study, while the flowrate is calculated based upon values for a standard township of 500 people from Tchobanoglous, et al. (1991).



This mass balance shows that by increasing the biomass phosphorus content, phosphorus removal could be improved from 31% for WSP microalgae undergoing standard assimilation (top mass balance), to 93% for microalgae undergoing luxury uptake in the new engineered process (bottom mass balance). However, more research is required into methods to consistently trigger the occurrence of this luxury uptake and be able to remove this biomass from suspension effectively before this new process can be implemented.

4.5.3 Granule Score and Biomass Phosphorus Content

In an attempt to provide a link between the elevated biomass phosphorus uptake measured in the WSP biomass and the luxury uptake visually confirmed to occur within the WSP samples, the average granule score for all luxury uptake performers for each sample was plotted against the biomass phosphorus content. The purpose of this graph was to see whether the samples within which luxury uptake was confirmed to occur also had biomass phosphorus contents above the typical 1 %P/g VSS. This plot is shown in Figure 20.

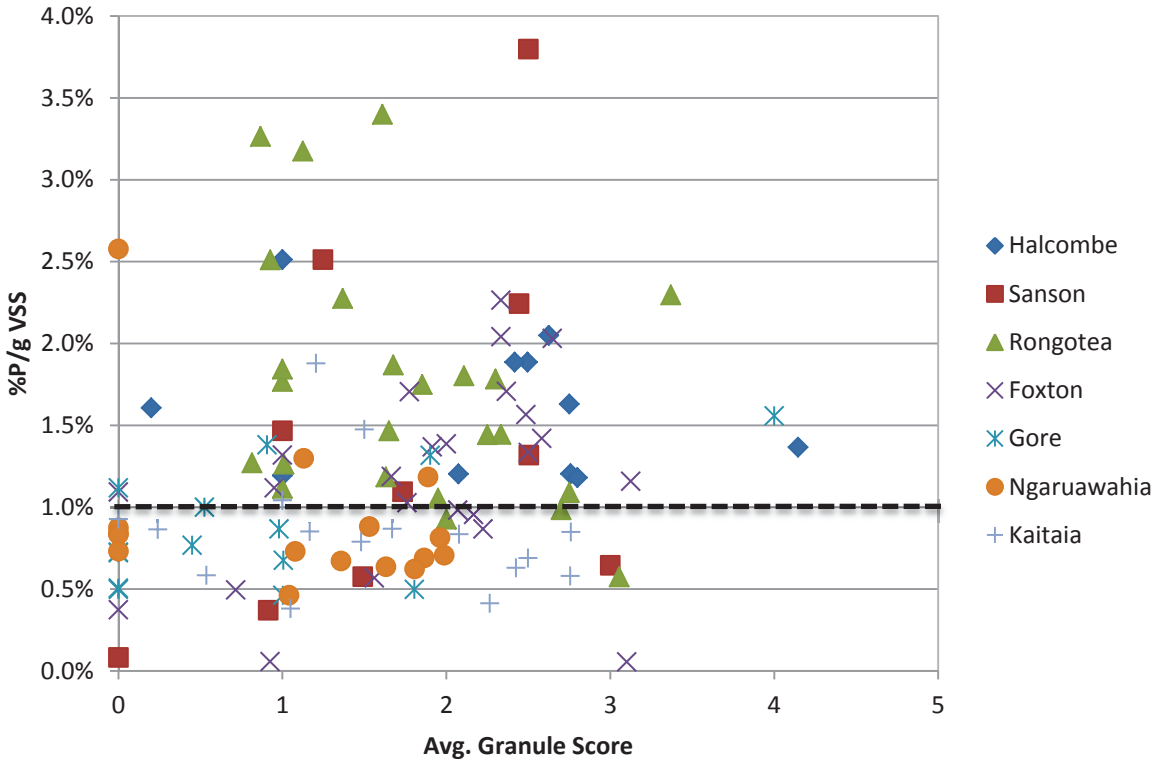


Figure 20: Phosphorus content of WSP biomass versus the average sample granule score

From this analysis, it can be seen that nearly all of the samples without any visually identified polyphosphate granules were also at or below the 1%P/g VSS threshold, as expected if no luxury uptake was present. The only exceptions were two points at 1.1%P/g VSS, a small enough amount above 1%P/g VSS to be considered negligible, and a point at 2.6 %P/g VSS. The point at 2.6% P/g VSS appears to be an inexplicable observation, as while luxury uptake did not occur, the low pH, temperature and solar radiation experienced by the Ngaruawahia pond that month also make the occurrence of precipitation or adsorption unlikely. Conversely, a number of samples containing microalgae or cyanobacteria undergoing luxury uptake had biomass phosphorus contents that were below the 1%P/g VSS threshold. These results indicate that while the lack of polyphosphate within the sample is a strong indicator that the biomass phosphorus content will be at or below 1%P/g VSS, the presence of polyphosphate in the sample does not necessarily mean that the biomass phosphorus content will be above 1%P/g VSS. This result is consistent with observations made in Powell, et al. (2008), and could be due to large fractions of biomass being made up of microalgae that did not perform luxury uptake, thus bringing the overall biomass phosphorus content down.

4.5.4 Influence of Environmental Variables on Granule Storage by *Scenedesmus* and *Chlamydomonas/Cryptomonas*

To further investigate the potential link between the quantitative measurements made in the study and the qualitative measurements made in the visual analysis, the impact of the significant variables identified in 4.2 on polyphosphate storage was plotted. The objective of these plots was to identify any possible influence of the environmental variables that were known to affect the phosphorus uptake on the amount of polyphosphate stored by selected

microalgal genera. If the granule score method used in this study were an accurate representation of the phosphorus uptake achieved by the microalgae, then the effect of the environmental variables on the granule score would mimic the effect the variables had on the phosphorus uptake. For this analysis, *Scenedesmus* and *Chlamydomonas/Cryptomonas* were chosen due to their high frequency of appearance and high frequency of luxury uptake, meaning that there was a significantly larger data set for these two genera than for other genera. The plots of *Scenedesmus* and *Chlamydomonas/Cryptomonas* granule scores against TDP concentration are shown in Figure 21.

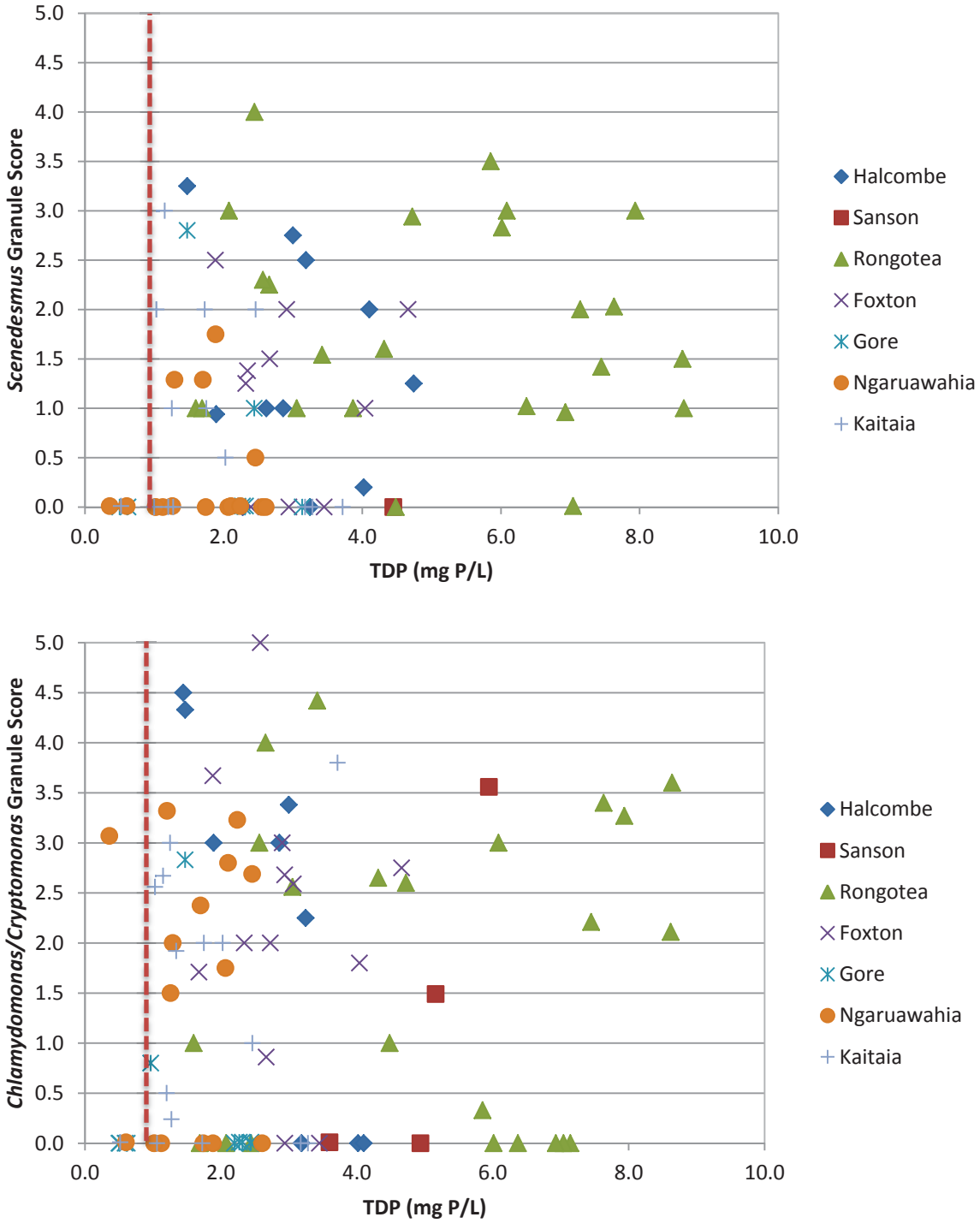
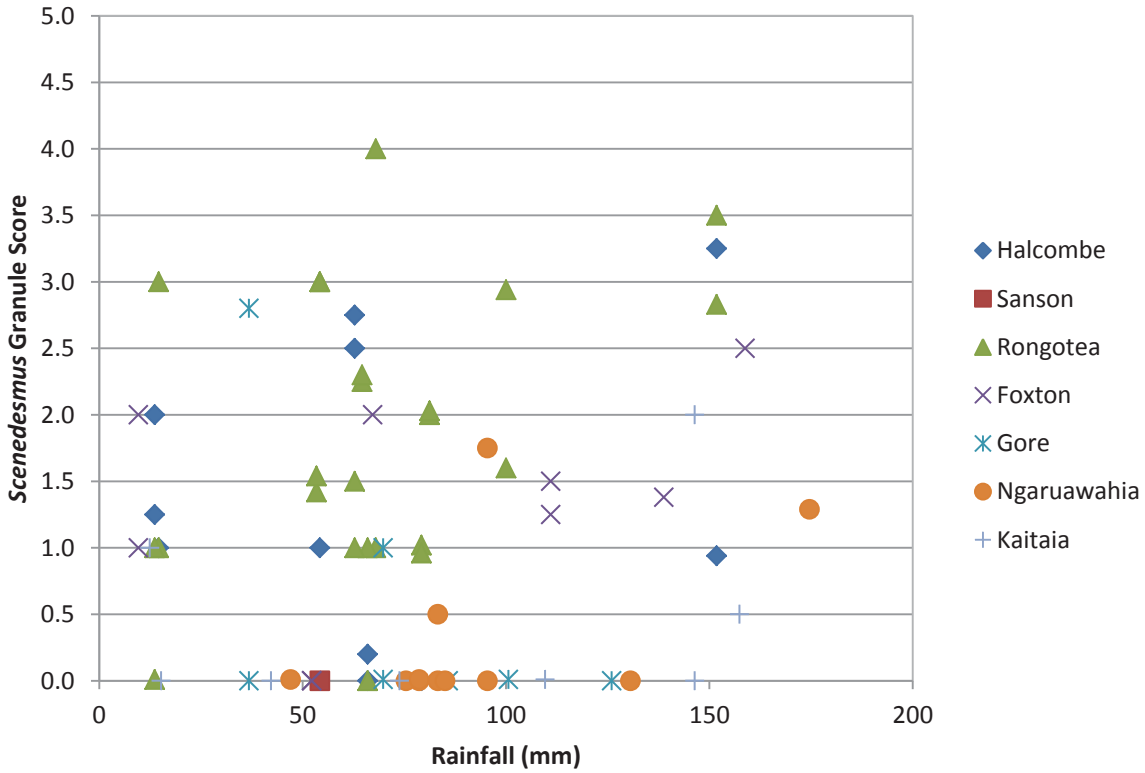


Figure 21: *Scenedesmus* (top graph) and *Chlamydomonas/Cryptomonas* (bottom graph) granule score against TDP

The plots shown in Figure 21 appear to show that there is a cutoff value at around 1 mg P/L below which no luxury uptake occurs for either genus, as indicated by the dotted line. This could indicate that there is a certain threshold value for TDP concentration below which there is insufficient phosphorus for luxury uptake to be performed by either genus, as following the mechanistic description presented in section 4.2.1. The only exception is one sample containing *Chlamydomonas/Cryptomonas* from Ngaruawahia, which has a granule score of 3.1 at a TDP concentration of only 0.36 mg P/L. This appears to be an outlier given that none of the other 5 samples within this range contained *Chlamydomonas/Cryptomonas* that stored polyphosphate granules. However, it must be noted that increasing TDP values above this threshold did not necessarily mean that luxury uptake occurred within these genera, as evidenced by the zero granule scores located throughout the range of TDP values measured. There also does not appear to be any correlation between TDP concentrations and granule scores in either plot. As a result, while the impact of lack of TDP in the wastewater can be seen using the plots in Figure 21, this analysis cannot provide any conclusions on the impact of increased TDP on the polyphosphate storage performance of *Scenedesmus* and *Chlamydomonas/Cryptomonas.*

Figure 26 shows the granule scores for *Scenedesmus* and *Chlamydomonas/Cryptomonas* plotted against the rainfall levels experienced by the samples.



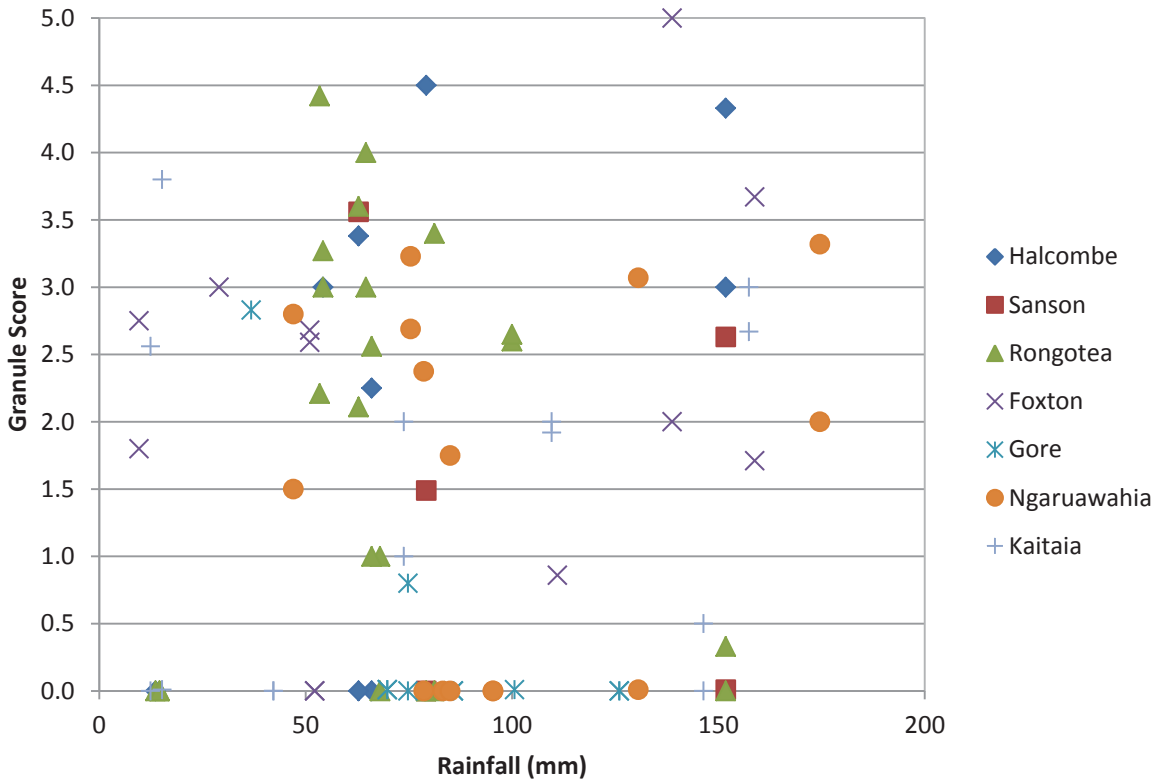


Figure 22: *Scenedesmus* (top) and *Chlamydomonas/Cryptomonas* (bottom) granule scores against rainfall levels

Unlike Figure 21, there does not appear to be a relationship between the rainfall and granule scores displayed in Figure 22, with high levels of variability in granule scores for both genera of microalgae across the range of rainfall measured. This is despite the clear relationship between rainfall and biomass phosphorus content displayed in Figure 17. This may indicate that the granule score measure is not adequate to fully capture the phosphorus uptake behaviour of the microalgae within the WSPs. It is also possible that the behaviour of either *Chlamydomonas/Cryptomonas* or *Scenedesmus* varied to that of other microalgae under certain rainfall conditions, thus leading to a discrepancy between the phosphorus uptake behaviour of these particular genera and the overall biomass phosphorus uptake achieved by the sample.

From Figure 21 and Figure 22, it can be concluded that while the granule score method can adequately measure the influence of low TDP concentrations on the sample, the trends in phosphorus uptake with changing levels of TDP and rainfall highlighted in section 4.2 are not visible using this measure. While the granule score is useful as an indicator of whether polyphosphate storage in a microalgae is occurring or not, it is not capable of measuring any trends in phosphorus uptake that occur with changing variables.

5 Conclusions

5.1 Phosphorus Content of WSP Biomass.

This study confirmed that luxury uptake in microalgae does occur across WSPs within a range of different climates, with biomass within 12 out of the 13 ponds studied containing phosphorus levels of significantly higher than 1% P/g VSS at some point in the study, and 56% of all samples performing luxury uptake. In comparison, only one sample from a HRAP exhibited luxury uptake. The peak biomass phosphorus content was found to be 3.8%P/g VSS, nearly four times the standard value for microalgal phosphorus content.

5.2 Environmental Effects on Phosphorus Uptake

From an elimination method stepwise regression performed using MINITAB, TDP and rainfall were found to be significant at a 95% confidence level. Increases in TDP were found to have a positive effect on the phosphorus content of the biomass, while increases in rainfall were found to have a negative effect. The positive effect of increased TDP concentrations was attributed to the increased availability of phosphorus to each individual microalga, thus increasing the amount of phosphorus that can be stored by the microalgae. The negative effect of rainfall was attributed to an increase in flow through the WSPs as a result of stormwater infiltration into sewer lines. It was theorised that the biomass washout as a result of this increased flowrate would trigger a spike in microalgal growth to replenish the population, resulting in consumption of internal stores of phosphorus and prioritisation of energy use for growth rather than phosphorus uptake. This would lead to a decrease in the phosphorus content of the biomass. However, more research is required on the component mechanisms for this effect to be confirmed.

The lack of influence of pH, temperature and sunlight on the biomass phosphorus content made it unlikely that precipitation or adsorption were significant phosphorus removal mechanisms within the WSPs studied, therefore meaning that the elevated phosphorus contents measured in this study were most likely due to the luxury uptake mechanism.

5.3 Climatic Effects on Phosphorus Uptake

It was found that the sites within the Manawatu had significantly higher biomass phosphorus contents than sites in other parts of the country. While this could have indicated the presence of climatic effects outside of the environmental variables measured previously, the increased levels of rainfall combined with lower TDP concentrations experienced by WSPs outside of the Manawatu was probably the main factor in this discrepancy.

5.4 Effect of Different Pond Types

From the study of the effect of different pond types, it was found that primary ponds had an average biomass concentration of 1.22% P/g VSS, compared with 1.20 %P/g VSS for secondary ponds. However, according to a MINITAB two sample t test, the differences between the two values could be considered negligible, indicating that there were no significant differences in biomass phosphorus content between the two ponds. The average

biomass phosphorus content of HRAPs was 0.71% P/g VSS, which was found from a two sample t test to be significantly lower than both primary and secondary WSPs. However, this difference was more likely due to the lowered TDP concentrations experienced at the NIWA HRAPs compared to the WSPs than any differences in design characteristics between the different pond types.

5.5 Luxury Uptake by Different Microalgal and Cyanobacterial Genera

From microscopic visual analysis of all of the WSP samples, it was found that 21 out of the 22 different genera identified within the WSP samples underwent luxury uptake at some point in the study, proving that luxury uptake does occur across a wide range of different microalgal and cyanobacterial genera within WSPs. However, the frequency of performance of luxury uptake varied significantly across the identified genera. The most commonly identified genera were *Chlamydomonas/Cryptomonas, Micractinium/Microcystis, Monoraphidium* and *Scenedesmus* which were identified in 76%, 88%, 71% and 70% of the samples respectively. However, of these genera, only *Chlamydomonas/Cryptomonas* and *Scenedesmus* were found to perform luxury uptake more than 50% of the time, at 68% and 73% respectively. The cyanobacterium *Planktothrix* was found to be the most consistent performer of luxury uptake, storing polyphosphate in 84% of the samples it was identified in. By this measure, *Pediastrum* (82%) and *Schroederia* (79%) were also found to be effective performers of luxury uptake. In addition to being the most consistent performer of luxury uptake, *Planktothrix* was also found to store the largest amount of polyphosphate using the granule score measure, with *Pediastrum* also found to be effective. In terms of the effect of luxury uptake on the phosphorus content of the biomass, it was found that nearly all of the samples that did not contain any polyphosphate were below the 1% P/g VSS threshold. However, a number of samples containing polyphosphate were also below the 1%P/g VSS threshold. This indicates that while the absence of luxury uptake is an indicator that the biomass phosphorus content will most likely be below 1%P/g VSS, the presence of polyphosphate within the microalgae or cyanobacteria does not necessarily mean that there will be an elevated biomass phosphorus content. Analysis of the TDP concentration against granule scores of different genera of microalgae appeared to indicate that there was a threshold point for TDP for both genera, below which luxury uptake was not performed. However, comparison of granule scores with rainfall yielded no relationship, demonstrating that the granule score measure could not accurately measure the relationship between the environmental variables and the phosphorus uptake.

5.6 Potential for Future Application of Findings

Development of a new engineered process which manipulates the conditions within WSPs to favour the growth of a microalgal or cyanobacterial genus that has a high propensity for storing phosphorus as polyphosphate could lead to improved phosphorus removal. Assuming the favouring of one of these species could increase the biomass phosphorus content achieved to 3 % P/g VSS, phosphorus removal efficiencies from WSPs could be increased from 31% to 93% at a biomass level of only 124 g VSS/m^3. This would greatly reduce impact of discharge of effluent from WSPs on the receiving environment, using WSP systems that are already in place around the world.

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Appendix 1 – Error Bounds in Total Phosphorus Analysis

To test the accuracy of the TP analysis, one sample was analysed 6 times on 6 different occasions. The statistics of this test from MINITAB are shown below:

```
Variable    Mean  SE Mean   StDev  Minimum  Maximum
Test 1     2.555    0.141   0.346    2.100    2.914
Test 2    2.7839   0.0428  0.1048   2.6781   2.9548
Test 3    2.9982   0.0402  0.0984   2.8978   3.0932
Test 4    2.6903   0.0672  0.1345   2.5560   2.8653
Test 5     2.719    0.105   0.257    2.491    3.207
Test 6    2.7622   0.0446  0.1093   2.5967   2.9223
```

From this analysis, the overall error on the TP analysis was found to be +/- 7.6%.

Appendix 2 – Effect of Lugol's Iodine on Total Phosphorus Analysis

Comparison of TP analysis on four separate samples both with and without Lugol's Iodine (LI):

	Without LI	With LI
N1	4.23	4.17
	4.05	4.17
N2	3.95	3.71
	4.03	3.77
W1	1.77	2.48
	2.12	2.54
W2	1.89	2.08
	1.83	2.54

Two sample t test comparing values produced from this analysis

```
Two-sample T for LI vs Normal

        N   Mean  StDev  SE Mean
LI      8  3.184  0.854     0.30
Normal  8   2.99   1.16     0.41


Difference = mu (LI) - mu (Normal)
Estimate for difference:  0.198
95% CI for difference:  (-0.913, 1.310)
T-Test of difference = 0 (vs not =): T-Value = 0.39  P-Value = 0.704  DF = 12
```

	N	Mean	Std Dev	Std Error
Without LI	8	3.18	0.85	0.30
With LI	8	2.99	1.16	0.41

P-value = 0.704

Difference in TP value between samples with Lugol's Iodine and without Lugol's Iodine found not to be significant.

Appendix 3 – Summary of Variables Measured in Study

	WSPs			HRAPs		
	Average	Max	Min	Average	Max	Min
VSS (mg/L)	124	1839	28	178	297	32
TDP (mg P/L)	3.02	8.64	0.36	2.37	3.26	1.29
pH	7.92	9.83	4.39	7.61	8.55	6.32
DO (mg/L)	9.29	20.30	0.22	4.83	9.03	2.04
Pond Temp (°C)	16.1	27.9	4.2	15.6	21.4	6.5
Air Temp (°C)	13.7	20.6	5.7	14.5	20.2	9.3
Wind Speed (km/h)	12.1	19.4	7.6	9.7	13.0	7.6
Rainfall (mm)	79	328	10	91	175	47
Solar Radiation (W/m^2)	163	299	51	180	299	93

Appendix 4 – Full Stepwise Regression Output

Part 1 – Correlation Analysis

	%P/g VSS	Rainfall (mm)	Air Temp (°C)
Rainfall (mm)	-0.273		
Air Temp (°C)	0.141	-0.168	
Radiation (W/m2	0.157	-0.347	0.780
Wind speed (km/h	-0.023	0.144	0.002
VSS (mg/L)	-0.011	0.055	0.203
TDP (mg P/L)	0.294	-0.281	0.328
pH	0.020	-0.116	0.256
DO (mg/L)	-0.094	0.068	0.083
Pond Temp (°C)	0.095	-0.172	**0.898**

	Radiation (W/m2	Wind speed (km/h	VSS (mg/L)
Wind speed (km/h	0.260		
VSS (mg/L)	0.068	-0.056	
TDP (mg P/L)	0.294	0.073	-0.051
pH	0.318	0.331	-0.030
DO (mg/L)	0.073	0.350	-0.046
Pond Temp (°C)	**0.898**	0.191	0.144

	TDP (mg P/L)	pH	DO (mg/L)
pH	0.061		
DO (mg/L)	-0.007	0.598	
Pond Temp (°C)	0.240	0.330	0.167

Cell Contents: Pearson correlation

A Pearson correlation coefficient greater than 0.8 indicates the presence of a strong correlation between variables. From this analysis, pond temperature was found to correlate with air temperature and radiation. As a result, pond temperature was excluded from the stepwise regression analysis.

Part 2 – Stepwise Regression

```
Backward elimination.  Alpha-to-Remove: 0.1


Response is %P/g VSS on 8 predictors, with N = 120
N(cases with missing observations) = 6 N(all cases) = 126
```

Step	1	2	3	4	5	6
Constant	0.005882	0.006001	0.006095	0.006202	0.005769	0.012341
Rainfall (mm)	-0.00002	-0.00002	-0.00002	-0.00002	-0.00002	-0.00003
T-Value	-1.75	-1.82	-1.83	-1.85	-2.04	-2.23
P-Value	0.084	0.071	0.071	0.067	0.044	0.028
Air Temp (°C)	0.00002					
T-Value	0.07					
P-Value	0.948					
Radiation (W/m2)	0.00000	0.00000	0.00000	0.00000		
T-Value	0.17	0.38	0.41	0.45		
P-Value	0.862	0.701	0.684	0.654		
Wind speed (km/h)	0.00005	0.00004	0.00004			
T-Value	0.18	0.17	0.16			
P-Value	0.857	0.867	0.873			
VSS (mg/L)	0.00000	0.00000				
T-Value	0.13	0.15				
P-Value	0.897	0.881				
TDP (mg P/L)	0.00085	0.00086	0.00085	0.00086	0.00090	0.00091
T-Value	2.28	2.38	2.38	2.43	2.61	2.65
P-Value	0.024	0.019	0.019	0.017	0.010	0.009
pH	0.0007	0.0007	0.0007	0.0008	0.0009	
T-Value	0.65	0.66	0.66	0.69	0.88	
P-Value	0.517	0.511	0.511	0.489	0.382	
DO (mg/L)	-0.00019	-0.00019	-0.00019	-0.00018	-0.00019	-0.00011
T-Value	-1.13	-1.13	-1.14	-1.14	-1.20	-0.83
P-Value	0.262	0.260	0.257	0.258	0.234	0.406
S	0.00666	0.00663	0.00660	0.00657	0.00655	0.00654
R-Sq	14.02	14.02	14.00	13.98	13.83	13.25
R-Sq(adj)	7.83	8.65	9.44	10.21	10.83	11.01
Mallows Cp	9.0	7.0	5.0	3.1	1.2	-0.0

Step	7
Constant	0.01140
Rainfall (mm)	-0.00003
T-Value	-2.29
P-Value	0.024
Air Temp (°C)	
T-Value	
P-Value	
Radiation (W/m2)	
T-Value	
P-Value	
Wind speed (km/h)	
T-Value	
P-Value	
VSS (mg/L)	
T-Value	
P-Value	
TDP (mg P/L)	0.00091
T-Value	2.65
P-Value	0.009

pH
T-Value
P-Value

DO (mg/L)
T-Value
P-Value

S	0.00653
R-Sq	12.73
R-Sq(adj)	11.24
Mallows Cp	-1.3