Utilizing Anaerobically Digested Dairy Manure for the Cultivation of Duckweed for Biomass Production, Nutrient Assimilation, and Sugar Production

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Presented in Partial Fulfillment of the Requirements for the

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by

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Authorization to Submit Thesis

This thesis of Kevin C. Kruger, submitted for the degree of Master of Science with a Major in Biological and Agricultural Engineering and titled "Utilizing Anaerobically Digested Dairy Manure for the Cultivation of Duckweed for Biomass Production, Nutrient Assimilation, and Sugar Production," has been reviewed in its final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

Nutrient management methods are needed to provide sustainable operation to livestock production that balance the costs of operation and maintenance. Cultivating duckweed on dairy wastes is considered an effective way of nutrient uptake and cycling. Duckweed cultivation has been implemented on nutrient management systems, such as constructed wetlands and waste stabilization ponds that use both domestic and swine wastewater. The objectives of this study were to (1) identify a nutrient concentration and duckweed strain that rapidly produces biomass, (2) removes nutrient content from anaerobically digested dairy manure, and (3) produces starch from nutrient starvation.

To complete these objectives, this study targeted estimating growth and nutrient rate constants as well as starch yield of duckweed under different cultivation conditions. The strains of duckweed, *Landoltia punctata 0128, Lemna gibba 7589*, and *Lemna minuta 9517* were identified as the promising candidates for their high levels of nutrient uptake, starch accumulation, and biomass production. The growth rate of the duckweed strain was assessed based on the effects of temperature, pH, dissolved oxygen, light intensity, nutrient concentration, and biomass accumulation. The nutrient uptake through duckweed cultivation on the anaerobically digested (AD) dairy manure, characterized by the changes of total nitrogen (TN), total Kjeldahl nitrogen (TKN), total phosphorus (TP), and ortho-phosphate-phosphorus (o-PO_4-P), was assessed in four nutrient dilution ratios 1:5, 1:13, 1:18, and 1:27 v/v at two light intensities of 10,000 and 3,000 lux to model seasonal variation.

The duckweed strain that exhibited the best biomass production, nutrient removal and starch accumulation was *Landoltia punctata 0128* at a dilution ratio of 1:27 at a light intensity of 10,000 lux. The growth rate constant established from zero order kinetics for *Landoltia punctata 0128* was 13.3 $gm^{-2}d^{-1}$. The rate constants for nutrient recovery were 0.122 $d^{-1}$of TN, 0.136 d^{-1} of TKN, 0.145 d^{-1} of TP, and 0.173d^{-1} of o-PO_4-P. The batch efficiency of cultivation for *Landoltia punctata 0128* on dilution ratio 1:27, in terms of nutrient uptake was 38% m/m in relation to the total nitrogen removed. The starch yield was measured at 30% w/w for *Landoltia punctata 0128* after the nutrient starvation process. Due to its ability to reduce nutrients from AD dairy manure, accumulate biomass at a rapid growth rate, and accumulate a high yield of starch, *Landoltia punctata 0128* has great potential to become a preferred choice for nutrient recovery and biomass and bioethanol production.

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Chapter 1 : Introduction

Excessive land applications of anaerobically digested (AD) dairy manure from agricultural operations often results in excess nutrient run-off into surface and ground water, causing eutrophication (Adhikari *et al.,* 2014; Zhao *et al.,* 2014). Due to the ratio of nitrogen (N) to phosphorus (P) present in AD dairy manure, which rarely matches the specific crop needs, an imbalance of nutrient content results (Adhikari *et al.,* 2014). To improve environmental sustainability of animal production, appropriate technologies are necessary to reduce nutrients from AD dairy manure while providing a feedstock for bioresource application (Sooknah *et al.,* 2004). Nutrient reduction and biomass production from the flowering plant duckweed is a technology that can reduce N and P from AD dairy manure and provide a feedstock for bioethanol production (Adhikari *et al.,* 2014; Sooknah *et al.,* 2004; Zhao *et al.,* 2012; Zhao *et al.,* 2014).

New nutrient management methods are needed to provide sustainable operation of livestock production when trying to balance the costs of operation, maintenance, and production. Such methods must be in line with legislation passed by the federal and state governments which require a nutrient management plan enforced by the United States Environmental Protection Agency (EPA). Low cost and low maintenance agricultural wastewater systems are desired by farmers in small agricultural communities, where livestock farming is a way of life. The cost of operating and maintaining a conventional wastewater system, such as an activated sludge system, for small farm use is simply impractical. However, larger livestock farms use anaerobic digestion to process their stock manure into a fertilizer.

To maintain sustainability, large dairies will anaerobically digest their livestock manure to profit from the fertilizer and the biogas produced. Anaerobic digestion is used to remove organic matter from livestock manure to produce methane (CH_4) and carbon dioxide (CO_2) which can be sold to electricity companies for a profit (Sooknah *et al.*, 2004). The process transforms ammonia nitrogen (NH_3-N) to ammonium nitrogen (NH_4-N) which is readily available to plants. The problem is that large scale anaerobic digestion is quite expensive and requires a high initial capital investment. Many smaller farms cannot afford to implement such a process so they use waste stabilization lagoons to treat their agricultural waste.

Current studies have indicated that constructed wetlands and waste stabilization lagoons are the main nutrient management practices designed for nutrient reduction and biomass accumulation, from agricultural waste, utilizing the aquatic angiosperm duckweed (Adhikari *et al.*, 2014; Al-Nozaily, 2001; Mohedano *et al.*, 2012). The advantages of operating constructed wetlands and waste stabilization lagoons is that they require minimal operation staff to function. The effluent from these systems can be suitable for water re-use systems in irrigation, due to their higher nutrient content compared to conventional wastewater systems, at the end of the process.

The disadvantage of using constructed wetlands and waste stabilization lagoons for nutrient management is that they can have longer detention times when reducing nutrients from wastewater. They are also less efficient in colder climates and may require more land in which to operate efficiently. One more disadvantage is that in waste stabilization lagoons, filamentous algae blooms can dominate, which can cause an odor during the spring thaws and inhibit duckweed growth, if left unchecked.

The cultivation of duckweed, on the surface of nutrient management systems, can reduce the growth of filamentous algae as well as improve N and P reduction and increase detention times. Constructed wetlands and waste stabilization lagoons designs have been utilized in several duckweed studies to remove N and P from swine manure (Adhikari *et al.*, 2014, Soda *et al.*, 2013; Mohedano *et al.,* 2012; Xu *et al.,* 2010). In this process, N is removed from constructed wetlands and waste stabilization lagoons by the means of ammonia volatilization, nitrification, denitrification, microbial uptake, and sedimentation (Adhikari *et al.,* 2014), which is in contrast to the removal of P by lagoon systems consisting of mainly microbial uptake and sedimentation. Without duckweed treatment, the removal of P from waste stabilization lagoons is generally poor (Al-Nozaily, 2001). The biomass accumulated after cultivation has been used as a fertilizer for soil enhancement, livestock feed, and bioethanol production.

The main problem observed with utilizing constructed wetlands for the removal of N and P from livestock manures is the loss of the solid manure from the environment which cannot easily be removed from the system and used as a fertilizer. When the manure is introduced to the constructed wetlands, a large portion of the manure settles out of solution and is lost. To remedy this problem a further controlled system can be implemented, such as a waste stabilization lagoon, which can be designed to implement the cultivation parameters of duckweed to remove N and P from agricultural wastewater.

Waste stabilization lagoons come in many forms based on how they handle oxygen in their environments. Aerobic lagoons can be aerated to remove biochemical oxygen demand (BOD) and ammonia (NH_3), from the system, which in turn would have to enter an anoxic or anaerobic lagoon, to remove nitrates (NO_3-N) from the system. The most commonly used

wastewater treatment system found in rural communities are facultative lagoons in conjunction with other storage or stabilization ponds attached in series or parallel (Quality, 2014). Facultative lagoons generally have a depth of 3 m, where sections of that depth are zones of aerobic, anaerobic, and facultative microbial activity (Al-Nozaily, 2001). Bacteria that require free oxygen to grow, are termed aerobic. Anaerobic bacteria can thrive in environments where no free oxygen is present. Facultative bacteria can grow in environments that can utilize free or combined oxygen sources.

Aerobic, anaerobic, and facultative environments can be found in facultative lagoon systems. The aerobic zone, at the surface of the lagoon, where algae and duckweed would grow provides oxygen to the, lagoon for microbial growth, improving nitrification within the system. Surface aeration provides 6 pounds of oxygen per acre per day whereas algae and duckweed using photosynthesis can provide 100 pounds of oxygen per acre per day (Quality, 2014). The dissolved oxygen content of the lagoon will be in the range of 2.0 to 8.0 mg L^{-1} depending on temperature (Quality, 2014). A symbiotic relationship between algae and bacteria exists within facultative lagoons. Algae provide the oxygen from photosynthesis and bacteria provide the CO_2 for algal growth.

Duckweed has been implemented in waste stabilization lagoons in several countries, among them Israel and Bangladesh, for the removal of nutrients from domestic wastewater. Rural farming communities are the best option for the implementation of duckweed for nutrient removal and biomass production. Waste stabilization lagoons could be designed to meet the specifications needed for the cultivation of duckweed. The AD dairy manure could be harvested from the lagoons as a product for fertilization of cropland while the reclaimed water could be

used for irrigation. Another sustainable product that duckweed can produce through nutrient starvation is starch for bioethanol production.

Bioethanol is a renewable energy source that is produced from biomasses that are high in carbohydrates. Bioethanol is produced from sugar or starch materials such as sugarcane, corn, or potatoes. These are enzymatically broken down into monomeric sugars and then fermented into bioethanol by the means of *Saccharomyces cerevisiae*. The production of bioethanol from agricultural products has shown great success as a renewable energy fuel, however, problems come from using agricultural products as feedstocks for bioethanol production. The area of land that is required to meet the energy demands for a growing nation is too great to supply. The wise use of agricultural land also supports the argument of using agricultural crops for food instead of fuel. To solve these problems, a feedstock is needed that will not use agricultural land and will not be an agricultural crop used for human consumption. Duckweed, a flowering aquatic plant, could be considered a third-generation biofuel for starch production.

Third generation biofuels generally come from microalgae and macroalgae. Microalgae contain lipids that can be utilized for biodiesel production and green macroalgae contain starch or amylopectin which can be used in the direct conversion of starch into bioethanol. The main feedstock for bioethanol production, in the United States, is corn. However, studies have shown that duckweed would be able to produce 10 times the amount of biomass as compared to corn based on duckweed's rapid growth rate (Yu *et al.,* 2014). Studies have also shown that duckweed can produce a starch content of 5-70% m/m with the conversion efficiency being favorable for bioethanol production (Yu *et al.,* 2014). The growth of duckweed would be superior to corn in that duckweed covers the entire surface area it inhabits and corn does not.

The highest recorded yield of corn was 192 bushels per acre in 2016 which in hindsight is not 100% efficient in relation to corn generated per surface area utilized (USDA, 2016).

This study evaluates the performance of the flowering plant duckweed, when cultivated on anaerobically digested (AD) dairy manure. Duckweed have been shown, throughout studies to rapidly accumulate biomass through vegetative growth, reduce N and P from domestic and swine wastewater, and to readily accumulate starch, when starved of nutrient content. These attributes are made possible by understanding the specific cultivation parameters used to efficiently cultivate duckweed. The main parameters that have been investigated, throughout studies have been medium temperature, pH, dissolved oxygen (DO), electric conductivity (EC), nutrient concentration, light intensity, photoperiod, and harvesting management (Chen *et al.,* 2015; Milledge *et al.,* 2014).

The objectives of this study were to identify a nutrient concentration and duckweed strain that:

1. Rapidly accumulates biomass when cultivated on anaerobically digested (AD) dairy manure.
2. Reduce nitrogen (N) and phosphorus (P) from AD dairy manure.
3. Accumulate starch from the cultivation of biomass on AD dairy manure.

It is expected, from this study, that cultivation parameters will be identified to find a duckweed strain that will rapidly produce biomass, remove N and P from AD dairy manure, and accumulate starch for bioethanol production.

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Chapter 2 : Literature Review

Abstract

The search for sustainable methods of reducing nutrients from agricultural wastewaters has led to the cultivation of duckweed on livestock manures in various studies. When analyzing these studies, it has become apparent that there are common cultivation parameters apply to each species of duckweed. The most common cultivation parameters, indicate, that duckweed need a medium temperature of 20 to 30°C, a light intensity of 3,000 to 7,500 lux, a pH of 6.5 to 7.5, dissolved oxygen (DO) of 2 to 8 mg L^{-1}, and an electric conductivity (EC) in the range of 600 and 1400 µS cm^{-1}, to attain active growth. Nutrient concentrations of livestock manure can vary with duckweed species and the nutrient management system applied.

To incorporate duckweed into current technologies, duckweed based cultivation lagoons (DCL) should be modeled based on relative growth rates, nutrient recovery rates, and harvesting intervals based on the cultivation parameters applied. Value-added products must also come out of the process, to create sustainable technology. Duckweed can accumulate starch, within the tissues of its biomass, through their natural processes. Starch content varies greatly in duckweed species, with a range of 5 and 70% m/m. Methods to stress duckweed have concluded that nutrient starvation is the best method for starch accumulation, in which the nutrients nitrogen (N) and phosphorus (P) are low, within the system. This review will analyze the parameters of cultivation, harvesting, and starch production methods for the utilization of DCL for the current technologies of nutrient removal from livestock manure and the advancement of bioethanol production.

2.1 Introduction

A desperate need exists, in the United States and abroad, for agricultural nutrient management systems that are cost-effective and provide a product that can balance operation costs, for the sustainable production of livestock (Adhikari *et al.,* 2014). The waste product of livestock production is manure accumulation. Livestock manure has the potential to pollute water systems containing high concentrations of nitrogen (N) and phosphorus (P) causing ground and surface water contamination (Mohedano *et al.,* 2012). Strict legislation in the United States, enforced by the United States Environmental Protection Agency (EPA) requires a nutrient management plan, to dispose of livestock manure using systems such as anaerobic digesters, waste stabilization lagoons and constructed wetlands (EPA, 2017). The water recovered from these systems can be used for the irrigation of cropland and the manure can be re-used as a fertilizer. Duckweed-based cultivation lagoons (DCL), for the removal of N and P from livestock manures have the potential to become a feasible technology that can be useful as a nutrient management system that provides a feedstock for bioethanol production, soil enhancement, or a feed for livestock.

Many studies have utilized duckweed as an aquatic plant used in constructed wetlands, wastewater stabilization lagoons, and simple systems of anaerobic digestion, and duckweed storage ponds (Adhikari *et al.*, 2014; Soda *et al.*, 2013; Mohedano *et al.*, 2012; El-Shafai *et al.*, 2006; Al-Nozaily, 2001). Duckweed is the standard name given to the aquatic plants that represent a small and simple flowering plant, which belong to the *Lemnoideae* family and can be found worldwide (Adhikari *et al.,* 2014; Wendeou *et al.*, 2013; Xu *et al.*, 2010). Within the family of *Lemnoideae,* duckweed belong to five different genera including *Lemna, Spirodela, Wolfia*, *Wolffiella* and *Landoltia* with 37 different species (Ziegler *et al.,* 2014).

Duckweed can be identified by their size with *Wolffia* having a frond of 2 mm or less across, *Lemna* being 6-8 mm across, and *Spirodela* being the largest at 20 mm (Hasan *et al.,* 2009). Duckweed can rapidly accumulate biomass, through vegetative growth, assimilating N and P from domestic and agricultural wastewater, making them ideal for water re-use systems such as irrigation, and starch accumulation for bioethanol production (Adhikari *et al.,* 2014; Zhao *et al.,* 2014).

Duckweed can be easily cultivated and harvested on unproductive land, providing they have a nutrient source, active sunlight, and space to grow (Konda *et al.,* 2015). Duckweed have an average photosynthetic efficiency of 6–8%, which is much higher than that of terrestrial biomass at 1.8–2.2% (Chen *et al.,* 2015). In a study conducted by Mohedano *et al.* (2012), duckweed ponds maintained a productivity of 68 ton/ha/year of dry *Landoltia punctata* biomass. Since 1993, Agriquatics Mirzapur in Bangladesh in South Asia have utilized the cultivation of duckweed for domestic wastewater treatment in lagoons systems to produce cod fish (Skillicorn 2008).

Here in the United States, Lemna Corporation out of Vadnais Heights, Minnesota has developed a commercial process approved by the EPA for applications in municipal wastewater treatment, utilizing duckweed in a sophisticated system of interlocking floating booms and hydraulically-driven mechanical harvesters, to enable the growth and harvesting of duckweed on large open ponds (Skillicorn 2008). In 1990, Lemna Corporation designed a duckweed lagoon system in Devils Lake, North Dakota, which to this day is still in operation and removes P from domestic wastewater at 91% of total phosphorus (TP) removed. The objectives of this review are to analyze the parameters of cultivation, harvest, and starch production methods for

the utilization of DCL, for the removal of N and P from livestock manure and for the advancement of bioethanol production.

2.2 Processes of Duckweed Cultivation

Duckweed have been utilized to actively remove N, P, and heavy metals form agricultural wastewater, both in the United States and abroad, to be used as a feedstock for livestock feed, soil enhancement, and bioethanol production (Yin *et al.,* 2015). Most studies have revolved around reducing the nutrient content in swine manure (Cheng *et al.,* 2001; Xu *et al.,* 2010; Mohedano *et al.,* 2012), however, few have used anaerobically digested (AD) dairy manure as a nutrient source (Sooknah *et al.,* 2004; Adhikari *et al.,* 2014). The common management practices, designed to target nutrient reduction from livestock manure include oxidative ponds, facultative lagoons, constructed wetlands, storage ponds, composting, and aerobic and anaerobic digestion facilities (Sooknah *et al.,* 2004; Adhikari *et al.,* 2014). Nutrient reduction from aquatic plants is a technology that can reduce N and P from AD dairy manure (Adhikari *et al.,* 2014; Sooknah *et al.,* 2004; Zhao *et al.,* 2012; Zhao *et al.,* 2014).

Several studies have utilized duckweed for the removal of nutrients, incorporating constructed wetlands and waste stabilization lagoons as nutrient management systems (Adhikari *et al.,* 2014; Mohedano *et al.,* 2012; Al-Nozaily, 2001). Yu *et al.* (2012) contends that the dominant N and P groups in AD livestock manure are ammonium nitrogen (NH_4–N) and ortho-phosphate-phosphorus (o-PO_4–P). Adhikari *et al.* (2014) maintains that duckweed preferentially absorbs NH_4–N over nitrate nitrogen (NO_3-N) which may sustain its growth in livestock lagoon wastewaters, where NH_4–N is the dominant form of N. In a study conducted by Wang *et al.* (2014), the NH_4–N concentration in livestock manure is too high to administer

to duckweed, which means that the manure must be diluted in a continuous or batch system. Other nutrients in livestock manure from which duckweed can benefit from include potassium, calcium, magnesium, sulfur, manganese, copper, chlorine, zinc, boron, iron, and molybdenum.

2.2.1 Nitrogen and phosphorus

Nitrogen is the limiting reactant, in cultivating duckweed on AD livestock manures (Soda *et al.* 2013). The current processes, to remove N from lagoon based systems, are ammonia volatilization, nitrification, denitrification, microbial uptake, and sedimentation (Adhikari *et al.*, 2014), which in contrast to the removal of P by lagoon systems consists of mainly microbial uptake and sorption into the soil via sedimentation. Further design specifications are needed, in the forms of growth and nutrient recovery rates to develop system parameters that will recover nutrients in the form of N and P from agricultural wastewater. Lagoon designs, combined with aquatic plants, would have to account for the natural processes that take place when agricultural wastewater stagnates.

Commonly, ammonia volatilization is the pathway for the removal of N batch systems, removing approximately 355 to 1534 mg m^{-2} of NH_3-N, increasing with temperature and pH (Middlebrooks *et al.*, 1999). Ammonia volatilization is mostly negligible in a DCL, if the surface area is completely covered with duckweed in at least a single layer (Xu *et al.*, 2010). Mohedano *et al.* (2012) stated that a pH of 7 and temperature of 20°C only accounts for 0.4% of ammonia in its volatile form, however lagoon systems can see a pH upwards of 9.0. The mat-structures stabilize the N in solution, while maintaining the nutrient concentration constant due to minimal evaporation of water, however, when an aerobic lagoon system is used without the aid of duckweed, large quantities of the NH_3 can dissipate into the atmosphere, as the pH of the system starts to increase (>9.0) with the temperature (>23°C) (Metcalf and Eddy, 2014).

Nitrification is the biological oxidation of ammonia nitrogen (NH_4-N), to nitrite nitrogen (NO_2-N), followed by the oxidation of NO_2-N to nitrate-nitrogen (NO_3-N), using microbes such as ammonia oxidizing bacteria (AOBs) and nitrite oxidizing bacteria (NOBs) (McLean *et al.,* 2000). NOBs and AOBs are aerobic chemoautotrophs, because they use CO_2 for their carbon (C) source and require dissolved oxygen to oxidize inorganic compounds, such as NH_4-N and NO_2-N to obtain cell energy, in order to reproduce (Metcalf and Eddy, 2014). Nitrification has several parameters to which it can be identified within a batch system.

To identify whether nitrification is taking place, the researcher should look for an increase in the concentration of NO_3-N, which naturally occurs during the process (Ndegwa *et al.,* 2007). The second indication that nitrification is taking place is an increase in the pH content and a decrease in dissolved oxygen (DO) content (Metcalf and Eddy, 2014; Ndegwa *et al.*, 2007). When oxygen is consumed within the system, via nitrification, the buffering capacity of the solution known as alkalinity begins to weaken. When the alkalinity of a solution decreases, this allows for the pH of the solution to increase, and become basic (pH>7). The third and final indication of nitrification is the consumption of oxygen. If the dissolved oxygen content is low within the system and becomes anaerobic or anoxic, with NO_3-N as the electron acceptor, this is a good indication of nitrification (Metcalf and Eddy, 2014). If a NPDES permit requires NH_3 to be removed from the system, then nitrification is the first step in the process.

Denitrification within a batch system, occurs with the reduction of NO_3-N and NO_2-N to nitrogen gas (N_2). This completes the N cycle in a wastewater treatment process, however, denitrification requires very specific conditions to transform NO_3-N to N_2. In an activated sludge system, NO_3-N must be transferred to an anoxic basin, where no is oxygen present, so that the facultative aerobic organisms only feed on the NO_3-N, the electron acceptor, to

transform it to N_2. Mohedano *et al.* (2012) claimed that nitrification and denitrification was occurring in their DSL system. The only place in a lagoon system for denitrification to occur would be in the anoxic zone at the bottom of the lagoon. At high concentrations of nutrients, the nitrification/denitrification process consumed more N and the biomass consumed less, however, when the concentration of nutrients was low, in their secondary basin, the nitrification/denitrification process was lower and more nutrients went into the duckweed biomass. It has also been found that in batch systems that *Landoltia punctata* will metabolize NO_3-N with NH_4-N present (Cedergreen *et al.*, 2002; Fang *et al.*, 2007).

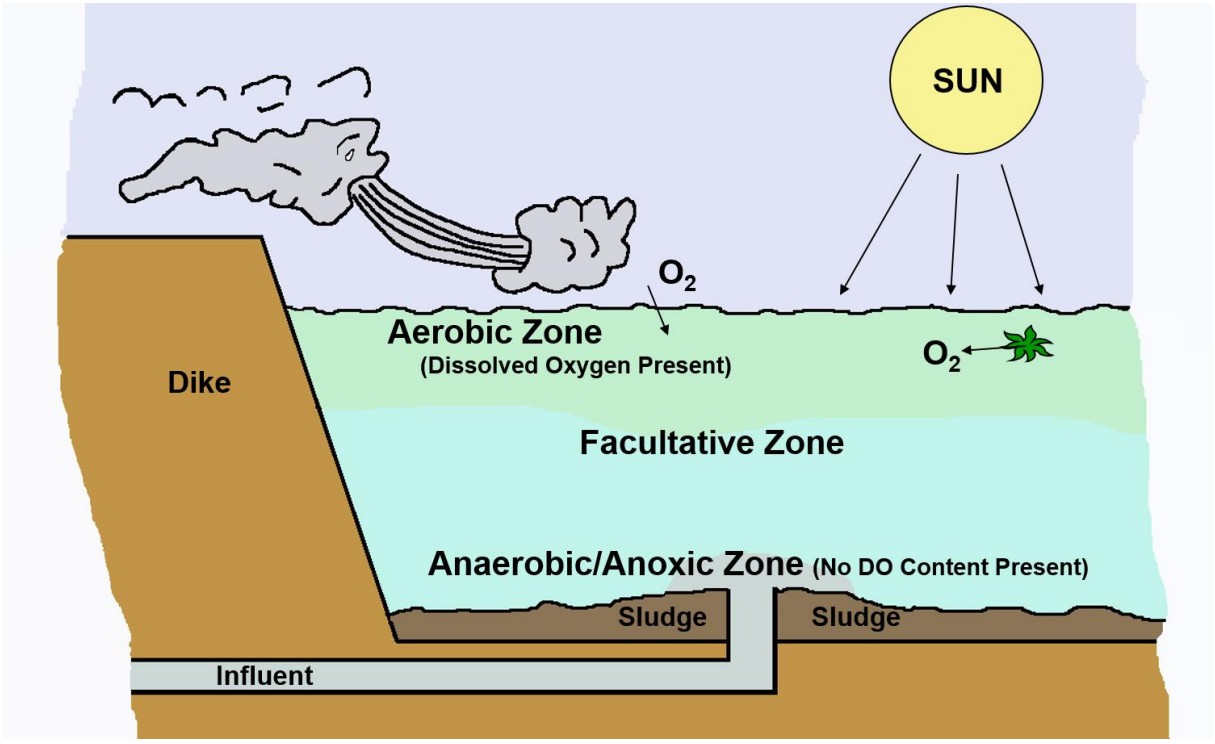

Figure 2-1 Zonal relationships in a lagoon system (Quality, 2014)

Figure 2-1 shows the zonal relationships in a lagoon system. N uptake, within the lagoon system, will take place, due to duckweed and algal vegetative growth, nitrification/denitrification and sedimentation (Mohedano *et al.*, 2012). Duckweed growth will

occur along with minimal algae growth at the top of the lagoon in the aerobic zone. Nitrification will also occur in the aerobic zone as NOB and AOB require oxygen to thrive. Denitrification occurs in the anaerobic/anoxic zones where no oxygen is present. Solid sedimentation will settle out of solution, at the bottom of the batch system. If the depth of the sedimentation of the batch system is shallow, the nutrients will actively travel between full suspension and settling through the process of diffusion. When the batch system is deep, the solids, which contain most of the N will settle to the bottom and not be transported to the top unless agitated, which present the option of their re-use on agricultural land for fertilization. Since the root systems of duckweed are very short, the shallow placement option is more feasible for active growth.

2.3 Cultivation of Duckweed

Duckweed generally have a cycling period of 30 days where they will follow the phases of growth (Farrell, 2012). Figure 2-2 shows the major phases of growth which can be broken down into the lag phase, exponential or log phase, stationary phase, and not shown, the death or decay phase (Kumar *et al.,* 2016). In the lag phase the duckweed start to adapt to the new environmental conditions. There is theoretically no growth occurring within the system during the lag phase. The exponential phase is where the most active growth in the system occurs.

During the exponential phase duckweed is rapidly producing more vegetative growth through budding. The stationary phase occurs when the duckweed start running out of critical nutrients for metabolism to produce energy for vegetative growth. The growth rate slows and eventually becomes constant. The death phase of duckweed is not shown in Figure 2-2 as there is no loss in biomass during the process. The ideal parameters for duckweed cultivation are a controlled medium temperature, pH, dissolved oxygen (DO), electric conductivity (EC), nutrient concentration and light source, photoperiod, and harvesting management plan for

continuous growth (Chen *et al.*, 2015; Milledge *et al.*, 2014). Most studies will compare the individual parameters to the relative growth rate (RGR), nutrient reduction rate or starch accumulation of the duckweed species.

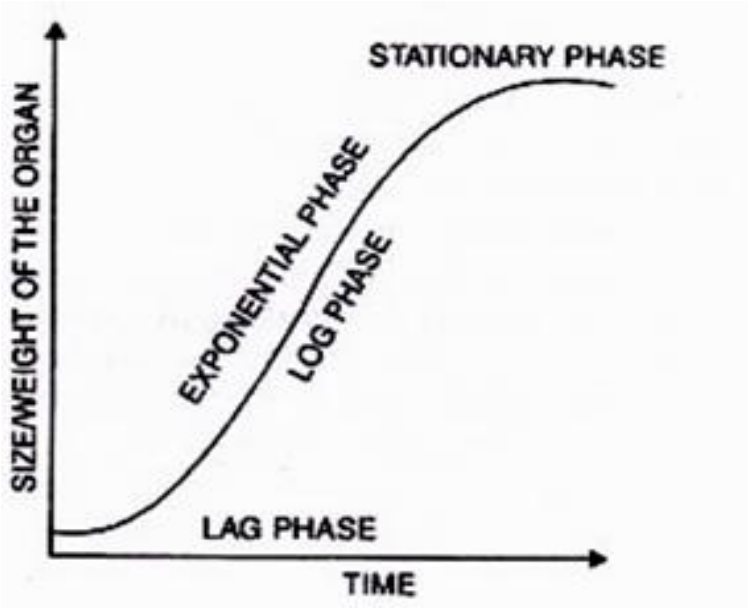

Figure 2-2 The phases of duckweed growth (Kumar et al., 2016)

2.3.1 Temperature and photoperiods

Duckweed thrive in a variety of climates and they adapt well to new environments. Xiao *et al.* (2013) states the maximum growth rate observed for duckweed was at an optimal temperature and photoperiod of 26°C and 12-13 hours of light, respectively. The lowest temperature observed for constant growth was 15°C. Figure 2-3 shows a study conducted by Zhao *et al.* (2014) contrasting two duckweed species *Lemna minor* and *Landolita punctata* when cultivated on Hoagland E-Medium at temperatures 20, 25 and 30°C. The relative growth rates (RGR) were recorded for each temperature. At 25°C, *Landoltia punctata* had the highest

RGR at 4.2 g m^{-2} d^{-1} on a dry basis. At 20°C the duckweed species had a lower RGR at approximately 3.6 g m^{-2} d^{-1} on a dry basis.

The growth rate of duckweed can be strongly inhibited at temperature ranges below 8°C or above 35°C (Lasfar *et al.*, 2007). Zhao *et al.* (2014) states that the range of optimal duckweed growth is between 20 to 30°C. Optimal temperatures can range depending on the species of duckweed. Most of the time it is determined experimentally but in some geographic locations the air temperature varies with the weather conditions of the given month.

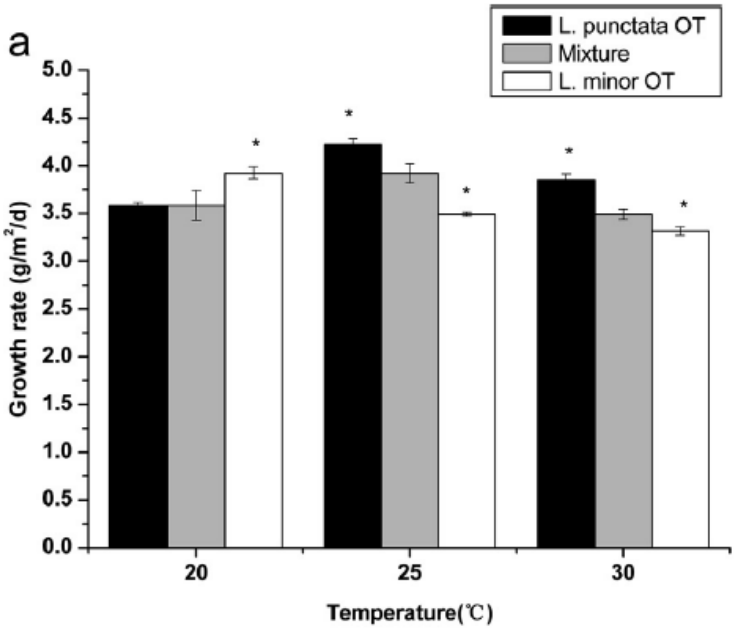

Figure 2-3 Relative growth rates of Landoltia punctata, Lemna minor, and a mixture of both as related to air temperature (Zhao et al., 2014)

For year around growth of duckweed the design is going to have to be in a geographic area where the air temperature does not go below 20°C. Duckweed do not grow well when in extreme heat or cold environmental conditions. During the winter months, the duckweed will float to the bottom of a lagoon and bury themselves in the sedimentation until the spring months

where the water will warm up and the duckweed will return to the surface of the stagnated water system.

2.3.2 pH

The conditions that have the potential to be toxic to duckweed and therefore inhibit growth are high EC, nutrient, and pH contents. The pH of the medium is a contributing factor that can result in the inhibition of duckweed growth. Duckweed actively grow when the pH is between 6.5 and 7.5 (Skillicorn, 2008; Xu *et al.*, 2012). Figure 2-4 shows the pH baseline of approximately 8.0 and the spikes of approximately 11.0 during the months of July and August because of the extra algae blooms within the duckweed (Xu *et al.*, 2012). The increase in pH was due to the blooms of filamentous algae and the consumption of CO_2, bicarbonate (HCO_3^-), and carbonate alkalinity (Farrell, 2012). When the pH increases to the range of 9 to 11 that indicates excess hydroxide ions (OH^-) within the medium. When the concentration of CO_2 is low within the medium from algae synthesis, the algae will begin to consume carbonate in the system lowering the alkalinity of the system.

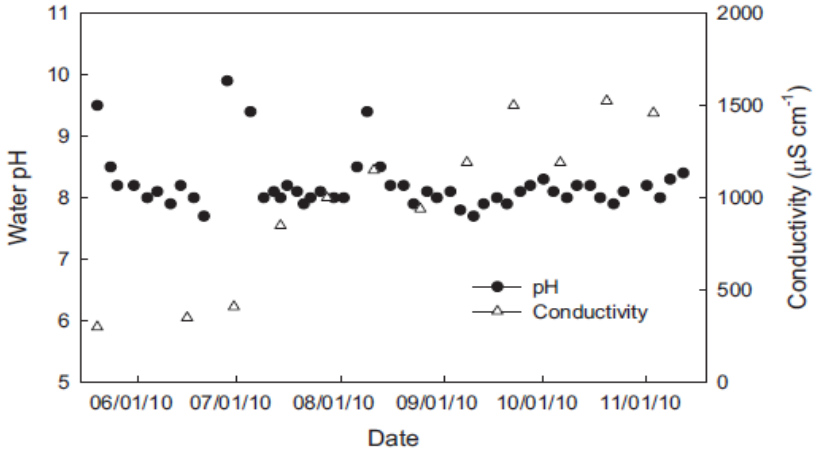

Figure 2-4 Changes in pH and Electric Conductivity within a Duckweed Pond (Xu et al., 2012)

2.3.3 Electric conductivity

In a study conducted by Sooknah *et al.* (2004), the electric conductivity (EC) within the anaerobically digested dairy manure and water mixture was 1,500 µS cm^{-1}. It was observed that the aquatic plant water hyacinth effectively reduced the EC within the system to 250 µS cm^{-1}. Sooknah *et al.* (2004) concluded that EC could be a feasible way to measure nutrient content within a system. In a study conducted by Wendeou *et al.* (2013), they concluded that the EC should be between 600 and 1,400 µS cm^{-1} for AD domestic wastewater when growing duckweed. Figure 2-5 shows that as the EC within the system increased the RGR of *Spirodela polyrrhiza* decreased. EC measures salinity within a nutrient source. When the concentration of nutrients is high within a system the salinity will also be high. The concentration of the nutrient source plays a role in the EC of the solution. The higher the concentration of nutrients are the higher the EC is going to be in solution. The lower the concentration of nutrients the lower the EC in solution.

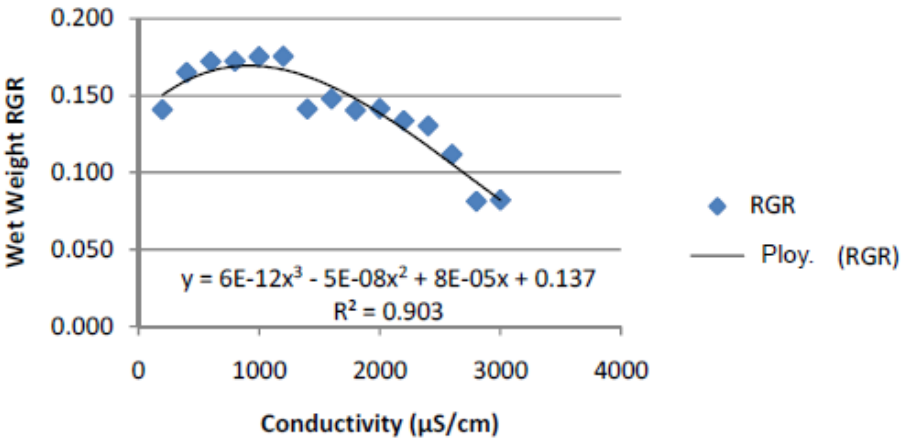

Figure 2-5 Spirodela polyrrhiza wet weight relative growth rate as a function of electric conductivity (Wendeou et al., 2013)

2.3.4 Dissolved oxygen

Duckweed provide oxygen to the to the environment through photosynthesis to produce energy for further biomass production and nutrient recovery. Sooknah *et al.* (2004) found an increase in the dissolved oxygen content within their system which comes from algae and duckweed. Oxygen is a byproduct of plant growth and can inhibit duckweed growth. With duckweed and algae producing oxygen within the system the top surface of the medium turns aerobic which is favorable for NOB and AOB for nitrification. A higher dissolved oxygen content is favorable for bacteria within the system but it is unfavorable to duckweed. For nutrient reduction in a waste stabilization lagoon it is recemented that the dissolved oxygen content is 2 to 8 mg L^{-1} to effective promote nitrification/denitrification within the system to remove ammonia.

2.3.5 Light intensity

Duckweed need UV light to fix carbon dioxide (CO_2) through photosynthesis. The main source of light intensity is the UV radiation of the sun. The brightest sunlight measured in illuminance lux one lumen per square meter, is averaged at 120,000 lux (Schlyter, 2006). During a typical day when there is shade illuminated by the entire clear blue sky the illuminance is averaged at 20,000 lux on the earth's surface (Schlyter, 2006). During an overcast day, the illuminance is averaged at 1,000 to 3,000 lux (Schlyter, 2006). In controlled duckweed studies, light intensity is often maintained using artificial light.

Figure 2-6 shows a study conducted by Zhao *et al.* (2014), the light source used was fluorescent lighting with a photoperiod of 16 hours of light and 6 hours of dark with the light intensities 10,000, 5,000 and 2,000 lux. The RGRs observed for 10,000 and 5000 lux were very close in the study at 3.25 g m^{-2} d^{-1} and 3.0 g m^{-2} d^{-1}, respectably, for *Landoltia punctata*. Since

the 10,000 and 5,000 lux are close in growth rates the optimal light intensity for duckweed could be around 7,500 lux. Yin *et al.* (2015) confirms the 7,500 lux statement by concluding that 7,500 lux is the optimal light intensity for starch accumulation and growth of the duckweed.

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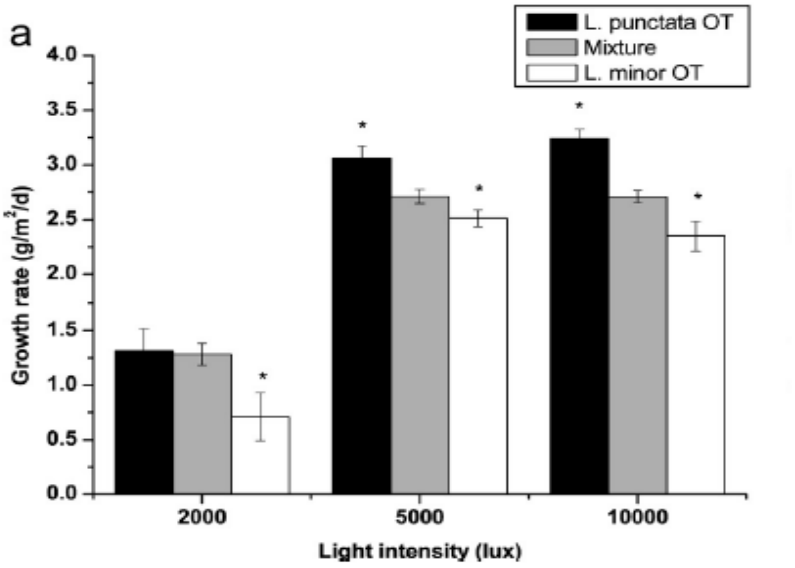

Figure 2-6 Growth rate based on light intensity (Zhao et al., 2014)

2.3.6 Biomass density

A study conducted by Driever *et al.* (2004) ran experiments to determine the limitations of *Lemna minor* growth in high plant densities. They concluded that the growth efficiency of *Lemna minor* decreases with increasing biomass density. Figure 2-7 shows the non-linear relationship of decreasing growth rate with increasing density of *Lemna minor*. Surface area limitations will play an active role in the cultivation of duckweed. In a study conducted by Farrell (2012) the duckweed biomass was 7 cm in thickness in a duckweed pond.

High densities of duckweed can also effect the efficiency of the transfer of light to the system. When multiple layers of duckweed grow in a system, light cannot effectively cover all the duckweed in the system. The main way to control duckweed population densities is to harvest the duckweed frequently throughout their cultivation cycle. Knowing when to harvest

the duckweed from the system and the quantity to harvest it is critical for biomass production and nutrient recovery.

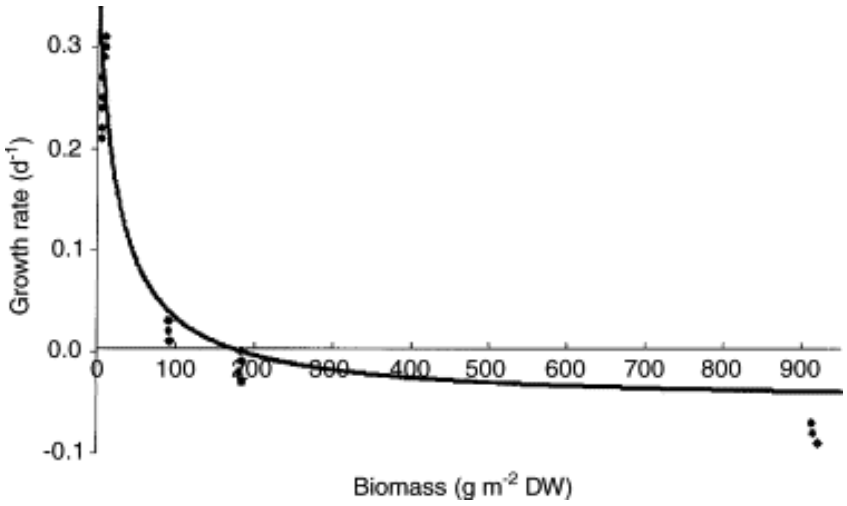

Figure 2-7 Growth rate as a function of the initial biomass of *L. minor (Driever et al., 2004)*

2.3.7 Harvesting

The frequency of harvesting is also a very important factor in maintaining an active growth rate and nutrient recovery rate for duckweed because it physically removes the N and P from the environment (Kesaano, 2011; Farrell, 2012). Xu *et al.* (2010) stated that harvesting twice every 14 days increased growth rate and nutrient reduction of duckweed. Willet (2005) stated that shorter harvesting intervals correlated to an increase in biomass production and nutrient recovery within their system. The study did indicate that the duckweed had a doubling time of between 5 and 7 days but gave no other evidence of the interval of harvesting. Edwards *et al.* (1992) also indicated that harvesting intervals may also depend on seasonal variation between dry and cool weather offering that the harvesting intervals between 2 and 15 days depending on the environmental conditions of the area.

If harvesting does not occur the duckweed biomass would settle to the bottom of the pond or lagoon and release the nutrients back into the system through the process of endogenous decay. The frequency of harvesting and the amount of biomass removed does vary from study to study. In a study conducted by Farrell (2012), duckweed must be harvested at least 20 days after initial seeding to prevent the duckweed from releasing N and P back into the water system. Researchers from Agriquatics Mirzapur in Bangladesh harvest their duckweed every 2-3 days at an average rate of 4.5 g dry m^{-2} d^{-1} to obtain 74-77% TP removal and 90-95% of o-PO_4-P (Cheng *et al*., 2001; Farrell, 2012). Continuous removal of duckweed improves biomass production, nutrient removal, prevents overcrowding, endogenous decay, and release of N and P back into the water system (Farrell, 2012). Figure 2-8 shows an example of a mechanical harvester called a skimmer (Smith, 2003).

Figure 2-8 Harvesting duckweed by skimming (Smith, 2003)

2.3.8 Nutrient concentration

The nutrient concentration for the cultivation of duckweed varies with the nutrient management system, cultivation conditions and duckweed species. Duckweed grow better under the nutrient concentrations of secondary and tertiary systems where the concentration of N and P are low. Figure 2-9 shows a study conducted by Wang *et al.* (2014), 840 mg L^{-1} of NH_4–N in standard solution was too high for duckweed to actively grow which indicates that diluted concentrations are needed in batch systems. Adhikari *et al.* (2014) stated that the ideal growth for *Lemna minor* is 32 mg L^{-1} of TN while *Spirodela polyrrhiza* can handle 98 mg L^{-1} of TKN in a batch system (Yu *et al.*, 2012). Soda *et al.* 2013 found that a concentration of 15 mg L^{-1} of TN was optimal for *Wolffia arrhiza* a smaller duckweed strain.

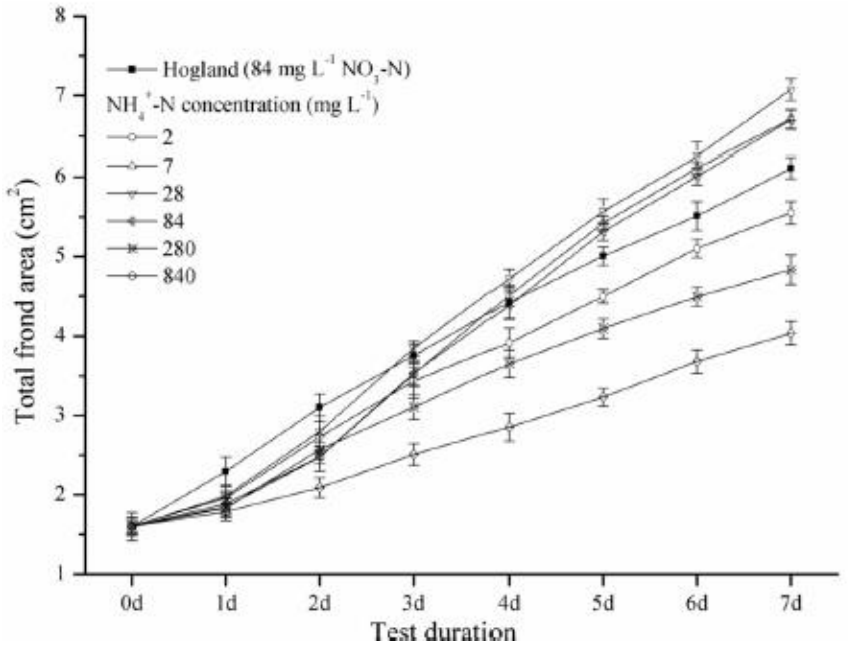

Figure 2-9 Growth curves of Lemna minor under varying concentrations of ammonium nitrogen (Wang et al., 2014)

2.4 Modeling Relative Growth Rate

Growth rates and nutrient reduction for duckweed vary per species. These variants include the beforementioned parameters of medium temperature, pH, and dissolved oxygen content, nutrient and light source, photoperiod, frequency of harvesting and surface area for growth. The estimation of duckweed biomass production is termed relative growth rate (RGR) which is the unit mass per unit area per day (Mohedano *et al.*, 2012). The typical method used to acquire RGR comes from the difference of the final and initial duckweed mass (g) divided by the area (m^2) and number of days of the study. Figure 2-10 shows an example of using zero-order kinetics to establish the RGR of *Lemna minor* (Cheng *et al.,* 2001). The slope of the line from the exponential growth rate determines the RGR.

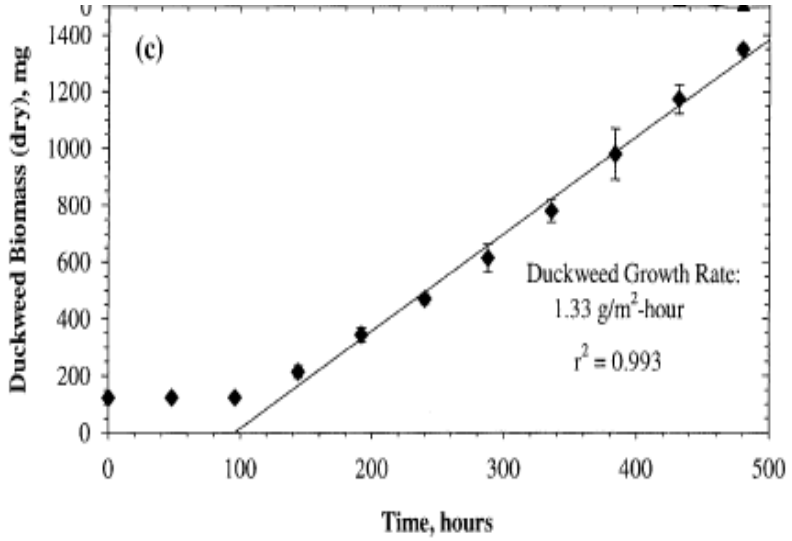

Figure 2-10 Zero-order kinetics model to obtain relative growth rate (Cheng et al.,2001)

The previously described equation for RGR was used to fine the growth rate of *Lemna minor* grown on swine lagoon wastewater and Schenk & Hildebrandt medium with a RGR of 3.5 and 14.1 g m^{-2} d^{-1} on a dry basis using white fluorescent lighting (Ge *et al.*, 2012). In a study conducted by Mohedano *et al.* (2012), duckweed ponds produced 16.90 g m^{-2} d^{-1} of dry

Landoltia punctata biomass. Other methods can look at the exponential growth using regression equations through zero-order or first order kinetics to acquire growth rate constants for duckweed growth.

2.5 Modeling Nutrient Reduction Rate

Researchers have quantified reduction of nutrients in different ways including using a percent reduction in the specific system being used. They have reported that duckweed-based treatment ponds could remove approximately 60% of N and 56% of P from pretreated dairy wastewater (Adhikari *et al.,* 2014). When using swine wastewater Mohedano *et al.* (2012) removed 98.0% of total Kjeldahl nitrogen (TKN) and 98.8% of TP on average using *Landoltia punctata.* Researchers have also used zero order kinetics to quantify nutrient reduction. The observed N removal rate for Mohedano *et al.* (2012) was 4.4 g m^{-2} d^{-1} of TKN. Adhikari *et al.* (2014) stated that the average N and P recovered by harvesting duckweed was 22.4 g N m^{-2} y^{-1} and 7.4 g P m^{-2} y^{-1}.

To model duckweed systems, Cheng *et al.* (2001) used simple linear models to characterize *Spirodela punctata* biomass, o-PO_4-P, and NH_4-N from synthetic swine manure. Figure 2-11 shows a zero-order kinetics model to obtain relative growth rate, ortho-phosphate-phosphorus, and ammonium nitrogen, to obtain the nutrient rate constants, the curves are split up into three linear sections or phases that can be compared to the *Spirodela punctata* growth rate curves. When looking at the duckweed growth curves, there is a lag phase at the beginning that lasts about 100 hours where the rate of nutrient uptake is slow at 0.483 mg L^{-1} h^{-1} of NH_4-N and 0.038 mg L^{-1} h^{-1} of o-PO_4-P

At roughly 130 hours the exponential phase of growth starts as indicated by the duckweed growth curve where the most growth and nutrient uptake occurs in the system. The nutrient uptake rates were 0.955 mg L^{-1} h^{-1} of NH_4-N and 0.129 mg L^{-1} h^{-1} of o-PO_4-P. The death phase of the system was not measured fully and there was not a stationary phase in the growth curve. However, the *Spirodela punctata* growth rate was 1.33 g m^2 h^{-1}. This model was based on zero-order kinetics to attain the duckweed growth rate constant, o-PO_4-P and NH_4-N nutrient reduction rate constants.

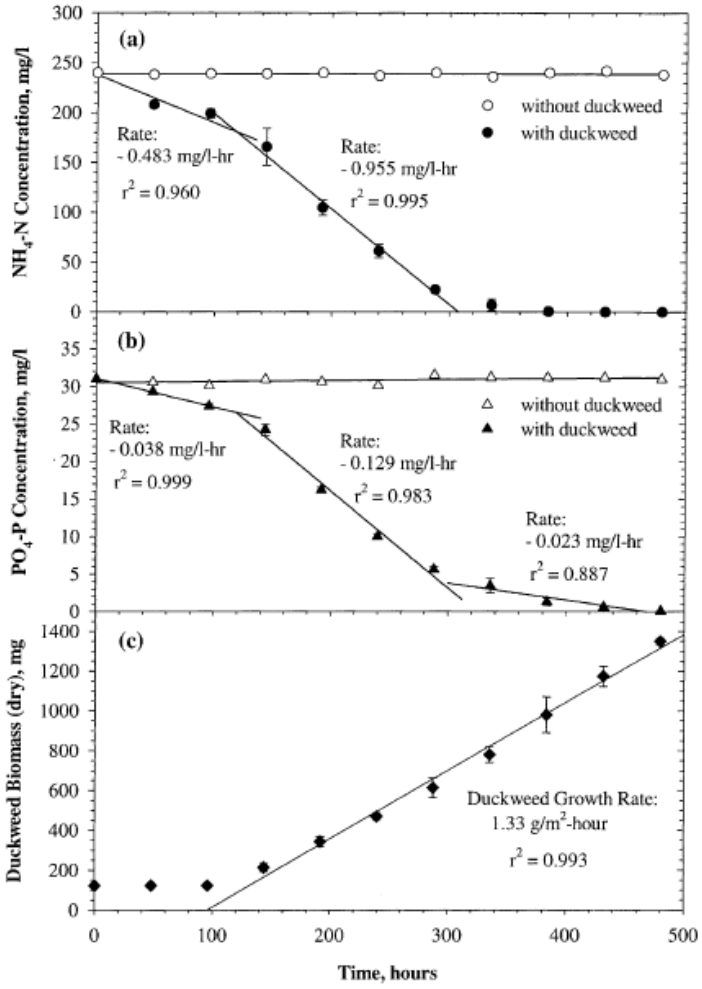

Figure 2-11 Zero-order kinetics model to obtain relative growth rate, ortho-phosphate-phosphorus, and ammonium nitrogen nutrient rate constants (Cheng et al.,2001)

2.6 Bioethanol Production

In the United States the dominate feedstock for bioethanol production is corn at a starch content of 65 to 73% m/m. Figure 2-12 shows the total corn production in the United States and the corn used for ethanol fuel production. In 2016, corn accounted for 36% of bioethanol production (USDA, 2016). The other 64% of corn went into human and livestock consumption. Over the last eight years (2009-2016) the use of corn for bioethanol production has held constant at an average of 5 billion bushels used for corn ethanol out of the 14.5 billion bushels produced (USDA, 2016).

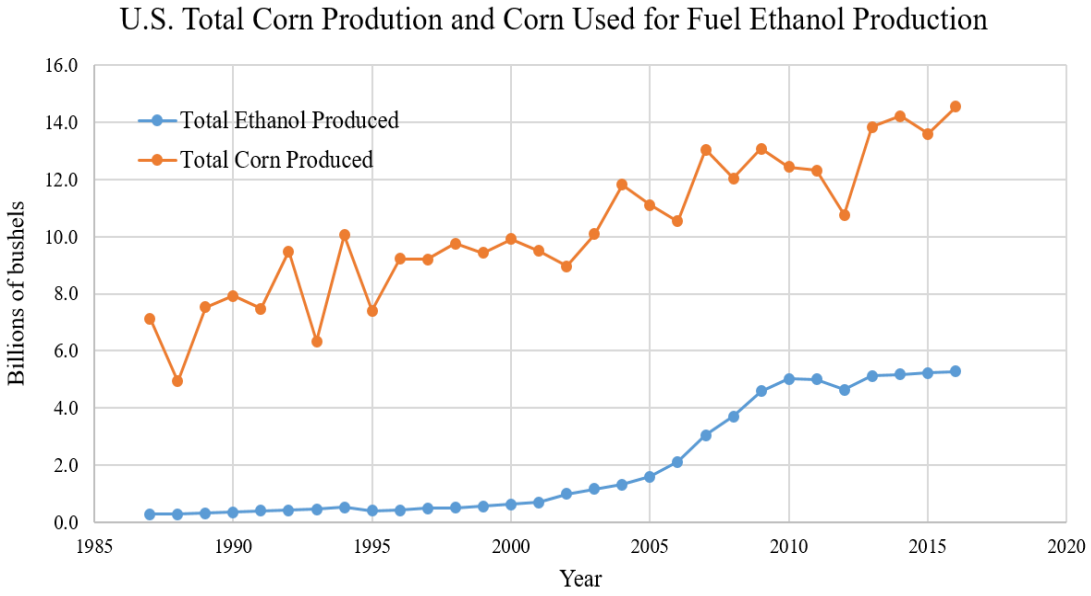

Figure 2-12 Total corn production in the United States vs. corn used for ethanol fuel production (USDA, 2016)

However, over that period the rate of corn production has stayed constant. This could indicate that an increase in the production of bioethanol from corn may not rise in the future due to land restraints, drought from climate change, or a shift in political favor. Alternative feedstocks are required to meet the demand of an increasing population, to reduce the consumption of fossil fuels and environmental pollution, and to secure the future of the world

on the security of energy production. The future will lie in our ability to develop third generation biofuels which utilize the chemical composition of macroalgae. Duckweed is considered a third-generation biofuel in that it is a macroalgae that can generate starch from its biomass.

2.6.1 Methods of starch production in duckweed

2.6.1.1 Nutrient starvation

Studies have indicated that duckweed can accumulate starch onto the surface of their fronds creating turions at a starch content of 5-70% on a dry weight basis (Chen *et al.,* 2012; Yu *et al.* 2014). Yu *et al.* (2014) stated duckweed grows about 10 times faster than terrestrial corn does making it a potential feedstock for bioethanol production and that many studies attribute starch accumulation to growth conditions such as nutrient levels, temperature, pH, and photoperiod. Among the studies observed the common method used to stress duckweed into producing starch is nutrient starvation where there is a low concentration of N and P within the system (Xiao *et al.,* 2013; Yu *et al.,* 2014).

The parameters used to cultivate duckweed can also be used to stress duckweed into producing starch. In nature when nutrients become scarce in winter the duckweed will form a white crystallized starch structure on the inner surface of the plant. To control this phenomenon, we must mimic the effects of winter by studying the effects of nutrient starvation, low air temperatures, longer periods of darkness, extreme pH increases or decreases, and high population densities to get the maximized starch content from the duckweed (Yu *et al.,* 2014; Yin *et al.,* 2015). According to a study conducted by Xiao *et al.* (2013), when duckweed is completely reliant on the nutrient content of a water body and those nutrients are sufficient for active growth (>25 mg L^{-1} of TN) the starch content will be low at 15.6% m/m. However, when

the nutrient content is relatively low (<15 mg L^{-1} of TN) the duckweed will begin to accumulate starch.

In a study conducted by Ge *et al.* (2012), duckweed without any prior treatment contains glucan (20.3±0.3%, m/m). Roughly half of the glucan is in the form of starch (amylopectin) (10.3±0.8%, m/m) and half is cellulose (9.4±0.5%, m/m). As nutrient starvation begins to occur the starch content in the duckweed will start to increase. According to Yu *et al.* (2014) the nutrient starvation technique accumulated a total starch content of approximately 40% m/m for the duckweed species *Lemna aequinoctialis* which can be seen in Figure 2-13.

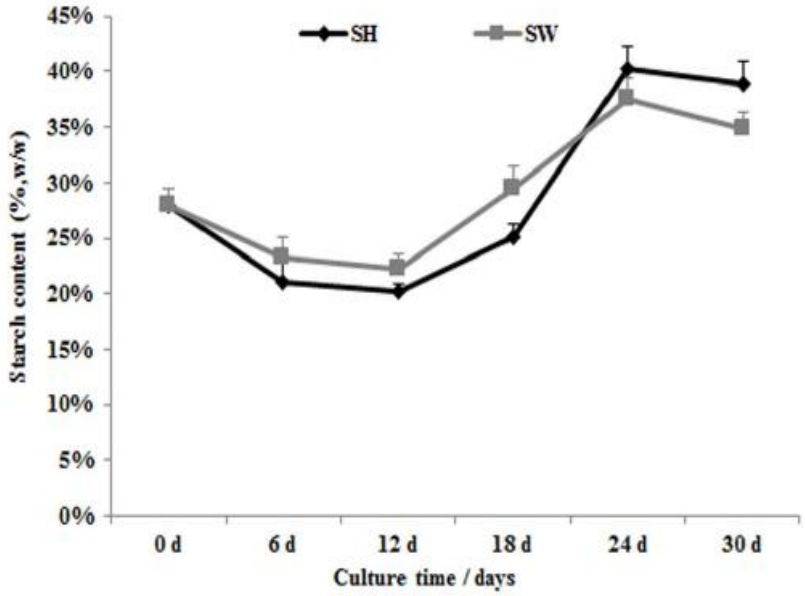

Figure 2-13 Starch Accumulation of Lemna aequinoctialis on sewage water and a control of standard solution

Nutrient starvation is the most common parameter that is used when stressing duckweed to accumulate starch. Figure 2-13 above demonstrates over time how starch begins to accumulate on the plant. Duckweed already contains approximately 24-28% m/m starch initially. When duckweed is stressed it forces the plant to pull from its own starch reserves. From Figure 2-13 above one can see that it isn't until day eighteen that duckweed will start to

metabolize starch in preparation for winter. According to Yu *et al.* (2014) the nutrient starvation technique accumulated a total starch content of approximately 40% m/m. To confirm these results, in another study Ge *et al.* (2012) reported that under stressed conditions they were able to produce a starch content of 10-36% m/m. Although in a recent article by Yin *et al.*, (2015), a maximum starch content of 62.24% was seen with *Lemna aequinoctialis* which is comparable to the starch content of corn which is approximately 70-72% on a dry basis. Xiao *et al.* (2013) also reported a starch content of 52.9% m/m using the duckweed species *Landoltia punctata*.

Nutrient starvation has been the used in conjunction with high and low light intensities to accumulate starch. In a study conducted by Zhao *et al.* (2014), a fluorescent light with a photoperiod of 16 hours of light and 8 hours of dark with the light intensities ranging from 10,000, 5,000 and 2,000 lux was used to cultivate duckweed. It was concluded in that study that a light intensity of 7,500 lux would be optimal for duckweed growth. Yin *et al.* (2015) confirmed that 7,500 lux is an optimal light intensity for duckweed cultivation and starch accumulation when using the nutrient starvation method. However, the researchers did see an increase in starch accumulation when they raised the light intensity to 24,000 lux and a photoperiod of 24 hours a day. However, practically the energy requirements on an industrial scale would be too great to maintain a light intensity of 24,000 lux for a period of 24 hours a day.

2.6.1.2 Uptake of nitrogen and carbon by duckweed

The mechanisms to which duckweed generates starch is not clearly known. In a natural setting, duckweed will often experience nutrient limitations such as low concentrations of N and P within a system. Nitrogen, phosphorus and carbon dioxide uptake are critical components of photosynthesis to produce energy for growth. Photosynthesis is dependent on N uptake and assimilation of carbon from either CO_2 within the air or a carbon source within the growth medium. If N and P are limited within the system duckweed may uptake compensate by removing carbon from the medium.

Yu *et al.* (2014) incorporated 10 g L^{-1} sucrose within the mixture of the standard solution and got a higher starch content than the sewage water produced. Vidakovi-Cifrek *et al.* (2013) used sucrose to generate growth from *Lemna minor* and observed that higher growth rates were simulated at low light intensities at sucrose concentrations of 7.5 and 10 g L^{-1}. Amy-Sagers *et al.* (2017) observed that sucralose a chlorinated artificial sweetener was found to have a positive influence on growth of *Lemna minor*.

2.6.2 Starch conversion to ethanol

The conversion efficiency of sugar-rich feedstocks into ethanol has been a problem for second generation biofuels such as lignocellulosic ethanol where the process to convert the woody feedstock into ethanol is energy intensive (Bayrakci *et al.,* 2013). Starch is a polymer of glucose that is composed of a ratio of amylose and amylopectin based on the genetic variation of the species of duckweed (Yu *et al.*, 2014). According to Yu *et al.* (2014), amylose (MW=160,000 dal) consists of 500-20,000 glucose units joined by α-1,4 glycosidic bonds. As the amylose content increases the energy conversion efficiency decreases.

Figure 2-14 shows the conversion of amylopectin to D-glucose through enzymatic hydrolysis. Amylopectin (MW=32,400,000 dal) is a branched glucose polymer that has α-1,4 glycosidic bonds and side chains connected by α-1,6 glycosidic bonds. The conversion of starch to ethanol is less energy intensive and requires either enzymatic hydrolysis or acid hydrolysis to convert the starch into monomeric glucose units. The most common method for the conversion of starch to ethanol is enzymatic hydrolysis which uses α-amylase to hydrolyze starch into soluble branched and unbranched maltodextrins. Amyloglucosidase hydrolyses the maltodextrins to D-glucose which in turn can be fermented into ethanol using *Saccharomyces cerevisiae* (yeast).

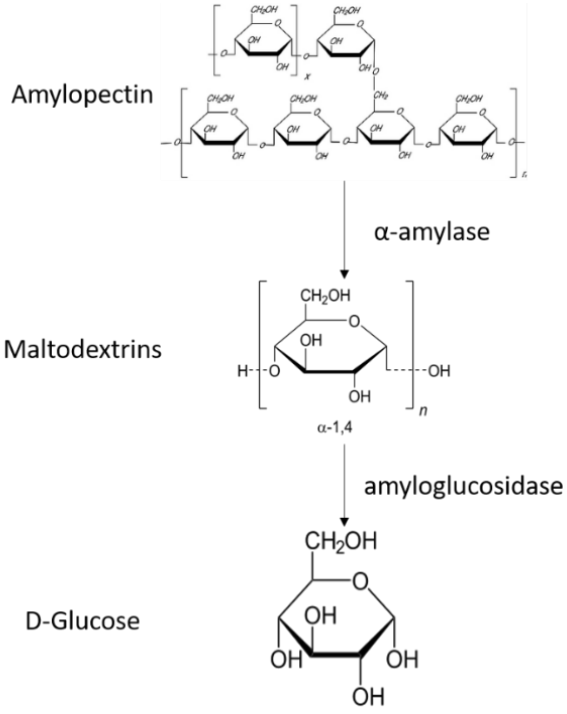

Figure 2-14 Conversion of Amylopectin to D-Glucose through enzymatic hydrolysis

2.7 Conclusion

The utilization of duckweed for sustainable operations of producing biomass, reducing nutrients from agricultural waste waters, and accumulating starch is a feasible technology. Duckweed application can be used for the re-use of water for irrigation, manure for cropland fertilization, and starch for bioethanol production. The common parameters for cultivating duckweed are a medium temperature of 20 to 30°C, a light intensity of 3,000 to 7,500 lux, a pH of 6.5 to 7.5, dissolved oxygen (DO) of 2 to 8 mg L^{-1}, and an electric conductivity (EC) in the range of 600 and 1,400 µS cm^{-1} for active growth.

The frequency of harvesting is one of the most important parameter in duckweed cultivation and yet is have not been fully optimized for biomass production and nutrient recovery. Studies indicate a variable of intervals ranging from 2 to 14 days of cultivation before harvesting takes place. Seasonal variation may play a role in harvesting times and should be considered throughout the process.

Nutrient concentrations must from livestock manure sources must still be found experimentally as they vary based on duckweed species, cultivation conditions and nutrient management system implemented. To design duckweed based cultivation systems, more data is needed based on relative growth rates, nutrient recovery rates, and harvesting periods based on the cultivation parameters applied.

To make the process more sustainable to a market place, value-added products must come from the process. Duckweed can accumulate starch within the tissues of its biomass through its natural processes when stressed. The starch content has varied by species producing a range of 5 and 70% m/m. Studies have conduced that nutrient starvation stresses duckweed when N and P are low within the system. However, the mechanism behind that process is

unknown. But what is known through studies is that the cultivation parameters directly affect the outcome of duckweed growth, nutrient recovery and starch accumulation.

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Chapter 3 : Growth and Nutrient Rates of Cultivating Duckweed on Flushed Anaerobically Digested Dairy Manure for Biomass Production

Abstract

Nutrient cycling from dairy manure to cropland is a national research priority for sustainable dairy operations and environmental protection. Cultivating aquatic plants on dairy wastes is considered as an effective way of nutrient uptake and cycling. This study investigated the growth rates of duckweed under different cultivation conditions for nutrient uptake and biomass production. Five strains of duckweed, i.e., *Landoltia punctata 0128, Lemna minor 9533, Lemna gibba 7589, Lemna minuta 9517,* and *Lemna valdiviana 8831,* were cultivated on anaerobically digested (AD) dairy manure as the nutrient source over a duration of 28 days. The growth rate of duckweed was assessed on the effects of temperature, pH, dissolved oxygen, light intensity, nutrient concentration and biomass accumulation.

Three strains, namely *Landoltia punctata 0128, Lemna gibba 7589* and *Lemna minuta 9517*, were identified as the most promising candidates for their high levels of nutrient uptake and biomass production. The cultivation temperature and light intensity were maintained in an environmental chamber at 25°C and 10,000 lux, respectively. After two days of growth the pH of the cultivation medium was adjusted to 6.7 using 5% v/v acetic acid. The dissolved oxygen content, which ranged from 2 to 5 mg L^{-1}, was monitored using a dissolved oxygen probe. The nutrient uptake through duckweed cultivation on the AD dairy manure, characterized by the changes of total Kjeldahl nitrogen (TN) and ortho-phosphate-phosphorus (o-PO_4-P) in the digested dairy manure, was assessed in three manure dilution ratios of 1:13, 1:18, and 1:27 v/v (AD) dairy manure: deionized (DI) water).

Experimental results revealed that nutrient recovery from dairy wastewater to duckweed followed first-order rate kinetics. The highest rates of nutrient recovery that produced the highest biomass production were found in the manure dilution ratio of 1:27 which accounts for a TKN of 54 mg L^{-1} and an o-PO_4-P of 6 mg L^{-1}. The growth rate constants came from the dilution ratio of 1:27 at 13.3 g m^{-2} d^{-1}, 19.0 g m^{-2} d^{-1}, and 4.8 g m^{-2} d^{-1} for *Landoltia punctata 0128, Lemna gibba 7589,* and *Lemna minuta 9517,* respectively. The rate constants for nutrient recovery were 0.136 $d^{-1}$of TKN and 0.173 d^{-1} of o-PO_4-P for *Landoltia punctata,* 0.112 $d^{-1}$of TKN and 0.166 d^{-1} of o-PO_4-P for *Lemna gibba,* and 0.118 $d^{-1}$of TKN and 0.172 d^{-1} of o-PO_4-P for *Lemna minuta*. Due to its ability to reduce nutrients from anaerobically digested (AD) dairy manure to accumulate biomass with a rapid growth rate, *Landoltia punctata* has a great potential to become the choice for nutrient recovery and algal biomass production.

3.1 Introduction

The utilization of aquatic plants for biomass production and nutrient reduction is a technology that can improve the environmental sustainability of animal production (Adhikari *et al.,* 2014; Sooknah *et al.,* 2004; Zhao *et al.,* 2012; Zhao *et al.,* 2014). The common management practices designed to target nutrient reduction from livestock manure include the use of oxidative ponds, facultative lagoons, constructed wetlands, storage ponds, composting, and anaerobic digestion facilities (Sooknah *et al.,* 2004; Adhikari *et al.,* 2014). These methods give the option of treating the livestock manure, before land application takes place by reducing the nutrient concentration.

The main nutrient management system being designed, for aquatic plants, has been constructed wetlands due to the associated low-costs of construction and management

(Adhikari *et al*., 2014). While preferred, many countries have invested in aquaculture, because they want to be able to control the parameters in which the aquatic plants grow naturally, where they are less productive than in controlled environments (Zhao *et al*., 2014). An oxidative or facultative lagoon, which includes an aquatic plant species, could have the potential to remove N and P from the anaerobically digested manure under controlled parameters.

The current processes, employed to remove N from lagoon based systems are ammonia volatilization, nitrification, denitrification, microbial uptake, and sedimentation (Adhikari *et al.,* 2014). Which in contrast the removal of P by lagoon systems consists of only microbial uptake and sedimentation. Design specifications are needed in the form of growth and nutrient recovery rates to develop system parameters that will recover nutrients in the form of N and P from agricultural wastewater. Lagoon designs, combined with aquatic plants, would have to account for the natural processes that take place when agricultural wastewater stagnates.

To create such a system, a species of aquatic plant must first be selected. Aquatic plants can be divided into three distinctive groups: microalgae, macroalgae, and flowering plants or angiosperms (Milledge *et al.,* 2014). The selected aquatic plant should reduce nutrients from animal-based agricultural waste, have a high biomass productivity rate, and be cost effective and easy to harvest. Of the three aquatic plants previously mentioned, angiosperms need further study, for nutrient reduction and biomass production capabilities (Ge *et al.,* 2012; Xiao *et al.,* 2013; Yu *et al.,* 2014). Cultivation parameters must be identified for each species of plant and the mechanisms behind nutrient reduction should be understood. Among the contender aquatic plants, is the flowering plant duckweed that grows actively on stagnant water systems. Duckweed is a potential candidate for agricultural wastewater treatment for nutrient recovery and biomass production.

Duckweed is the standard name given to the aquatic plants that represent a small and simple flowering plant which belong to the *Lemnoideae* family and can be found worldwide. Within the family of *Lemnoideae,* duckweed belong to five different genera including *Lemna, Spirodela, Wolfia, Wolffiella* and *Landoltia* with 37 different species (Ziegler *et al.,* 2014). The interest in duckweed has stemmed from the fact that they grow rapidly doubling their biomass in 2-5 days, depending on the species and environmental conditions. The natural growth of duckweed is developed in an ecosystem consisting of an angiosperm that intertwines itself with filamentous algae, microalgae, and bacteria to form mat-like colonies on the surface of brackish water systems (Adhikari *et al.,* 2014). They also actively reduce N, P, and heavy metals from wastewater sources, including domestic and agricultural waste streams to produce biomass that can be used as a feedstock for livestock, soil enhancement, or bioethanol production (Yin *et al.,* 2015).

Growth rates and nutrient reduction for duckweed vary per species. These variants include: medium temperature, pH, and dissolved oxygen content, nutrient and light source, photoperiod, frequency of harvesting, and surface area for growth. The estimation of duckweed biomass production growth rate per area or relative growth rate (RGR) (Mohedano *et al.*, 2012). The typical method used to acquire RGR, comes from the difference of the final and initial duckweed mass (g) divided by the area (m^2) and number of days of the study. The previously described equation for RGR was used to find the growth rate of *Lemna minor*, grown on swine lagoon wastewater and Schenk & Hildebrandt medium with a RGR of 3.5 and 14.1 g m^{-2} d^{-1}, on a dry basis using white fluorescent lighting (Ge *et al.,* 2012). In a study conducted by Mohedano *et al.* (2012), duckweed ponds produced 16.90 g m^{-2} d^{-1} of dry *Landoltia punctata* biomass. Other methods can be used to look at the exponential growth of the duckweed, using

regression equations through zero-order or first order kinetics, to acquire growth rate constants (k-values) for duckweed growth.

To model duckweed systems, Cheng *et al.* (2001) used simple linear models to characterize *Spirodela punctata* biomass, o-PO_4-P, and NH_4-N from synthetic swine manure which can be found in Figure 2-11. When observing the nutrient recovery graphs, the curves were split up into three linear sections or phases that can be compared to the *Spirodela punctata* growth rate curves. When looking at the duckweed growth curves, there was a lag phase at the beginning that lasted about 100 hours, where the rate of nutrient uptake was slow at 0.483 mg L^{-1} h^{-1} of NH_4-N and 0.038 mg L^{-1} h^{-1} of o-PO_4-P. At roughly 130 hours the exponential phase of growth started as indicated by the duckweed growth curve, where the most growth and nutrient uptake occurred in the system. The nutrient uptake rates were 0.955 mg L^{-1} h^{-1} of NH_4-N and 0.129 mg L^{-1} h^{-1} of o-PO_4-P. The death phase of the system was not measured fully and there was not a stationary phase in the growth curve. However, the *Spirodela punctata* growth rate was 1.33 g m^2 h^{-1}. This model was based on zero-order kinetics, to attain the duckweed growth rate constant, o-PO_4-P and NH_4-N nutrient reduction rate constants.

In a study conducted by Xiao *et al.* (2013), the maximum growth rate observed came from a medium temperature of 26°C. Temperatures lower than 15°C resulted in lower productivity rates. The growth rate of duckweed can be strongly inhibited at temperature ranges below 8°C or above 35°C (Lasfar *et al.*, 2007). The typical photoperiod used for many studies has been 16 hours of daylight and 8 hours of darkness (Yin *et al.*, 2015). The pH of the medium is also a contributing factor that can result in the inhibition of duckweed growth. Duckweed actively grow when the pH is between 6.5 and 7.5. Figure 2-4 shows a study by Mohedano *et al.* (2012), the duckweed ponds exhibited a pH baseline of approximately 8.0 and spikes of

approximately 11.0 during the months of July and August because of the extra algae blooms within the duckweed.

The increase pH was due to the blooms of filamentous algae and the consumption of CO_2, bicarbonate (HCO_3^-), and carbonate alkalinity (Farrell, 2012). When the pH increases to the range of 9 to 11 that indicates the presence of excess hydroxide ions (OH^-) within the medium. When the concentration of CO_2 is low within the medium from algae synthesis, the algae will begin to consume carbonate in the system lowering the alkalinity of the system. The dissolved oxygen content (DO) should stay in the region of 1.5 to 2.0 mg L^{-1}, to inhibit the growth of filamentous algae.

In an agricultural wastewater treatment system, the main source of light intensity is the UV radiation of the sun. The brightest sunlight measured in illuminance lux, which is one lumen per square meter, is averaged at 120,000 lux (Schlyter, 2006). During a typical day when there is shade illuminated by the entire clear blue sky the illuminance averages 20,000 lux on the earth's surface (Schlyter, 2006). During an overcast day, the illuminance averages 1,000 to 3,000 lux (Schlyter, 2006). In duckweed studies, light intensity is often controlled using artificial lights. Figure 2-6 shows a study conducted by Zhao *et al.* (2014), the light source used was fluorescent lighting, with a photoperiod of 16 hours of light and 6 hours of dark and the light intensities 10,000, 5,000 and 2,000 lux. The growth rates observed for 10,000 lux and 5,000 lux were very close in the study at 3.25 g m^{-2} d^{-1} and 3.0 g m^{-2} d^{-1}, respectably for *Landoltia punctata*.

To cultivate duckweed on agricultural wastewater treatment systems, an understanding of the mechanisms behind the nutrient source and the specific characteristics that enable duckweed to ideally grow is required. Yu *et al.* (2012) states that the dominant N and P groups

in anaerobically digested livestock manure are NH_4–N and ortho-phosphate-phosphorus (o-PO_4–P). Adhikari *et al.* (2014) stated that duckweed preferentially uptakes NH_4–N over nitrate nitrogen (NO_3-N) which may sustain its growth in dairy lagoon wastewaters where NH_4–N is the dominant form of N. However, duckweed will uptake NO_3-N if the NH_4–N concentration is low. In a study conducted by Wang *et al.* (2014), the NH_4–N concentration in pure livestock manures were too high to administer to duckweed which means that the manure must be diluted in a continuous or batch system. Adhikari *et al.* (2014) stated that the ideal growth for *Lemna minor* is 32 mg L^{-1} of TN, while *Spirodela polyrrhiza* can handle 98 mg L^{-1} of TKN in a batch system (Yu *et al.*, 2012). The TKN of a system is equivalent to the NH_4-N concentration of the manure. Other nutrients in livestock manure that duckweed can benefit from include potassium, calcium, magnesium, sulfur, manganese, copper, chlorine, zinc, boron, iron, and molybdenum.

The frequency of harvesting is also a very important factor in maintaining an active growth rate for duckweed, because it physically removes the N and P from the environment (Kesaano, 2011; Farrell, 2012). Xu *et al.* (2010) also stated that harvesting twice every two weeks increased growth rate and nutrient reduction of duckweed. If harvesting does not occur the duckweed biomass would settle to the bottom of the pond or lagoon and release the nutrients back into the system through the process of endogenous decay. The frequency of harvesting and the amount of biomass removed does vary from study to study. In a study conducted by Farrell (2012), duckweed must be harvested at least 20 days after initial seeding to prevent the duckweed from releasing N and P back into the water system. Researchers from Agriquatics Mirzapur in Bangladesh harvest their duckweed every 2-3 days at an average rate of 4.5 g dry m^{-2} d^{-1} to obtain 74-77% TP removal and 90-95% of o-PO_4-P (Cheng *et al.*, 2002; Farrell, 2012). Continuous removal of duckweed improves biomass production, nutrient removal, prevents

overcrowding, endogenous decay, and release of N and P back into the water system (Farrell, 2012). Continuous removal of duckweed improved biomass production, nutrient removal, prevents overcrowding, endogenous decay, and release of N and P back into the water system (Farrell, 2012).

Researchers have quantified reduction of nutrients in different ways including using a percent reduction in the specific system being used. They have reported that duckweed-based treatment ponds could remove approximately 60% of N and 56% of P from pretreated dairy wastewater (Adhikari *et al.*, 2014). Although, when using swine wastewater Mohedano *et al.* (2012) removed 98.0% of TKN and 98.8% of TP on average using *Landoltia punctata.* Researchers also use the rate of nutrient reduction which is commonly measured in unit mass per unit area per day. The observed nitrogen removal rate for Mohedano *et al.* (2012) was 4.4 g m^{-2} d^{-1} of TKN. Adhikari *et al.* (2014) stated that the average N and P recovered by harvesting duckweed was 22.4 g N m^{-2} y^{-1} and 7.4 g P m^{-2} y^{-1}.

The present study was designed to compare the potential of three duckweed strains along with a standard solution of Hoagland E-Medium and algal controls, in reducing the nutrient and organic content of effluent from an anaerobic digester receiving flushed manure from a dairy farm. These experiments established and compared the biomass yield, growth rate, nutrient removal rate, dissolved oxygen content, pH and electric conductivity within the cultured media when subjected to different nutrient concentrations for the specified duckweed strains. The specific objectives were to (1) identify a nutrient concentration and duckweed strain that significantly accumulates biomass while establishing relative growth rates that double the duckweeds mass every 2-5 days; (2) establish nutrient reduction rate constants for total nitrogen (TN), total Kjeldahl nitrogen (TKN), total phosphorus (TP), and ortho-phosphate-phosphorus

(o-PO_4-P); and (3) determine a minimum and maximum harvesting rate based on relative growth rates and nutrient reduction rates of the batch systems.

3.2 Materials and Methods

3.2.1 Pre-culturing of duckweed strains

Collected from Rutgers Duckweed Stock Cooperative, five strains of duckweed i.e., *Landoltia punctata 0128, Lemna minor 9533, Lemna gibba 7589, Lemna minuta 9517,* and *Lemna valdiviana 8831* were considered for this study. The duckweed strains were initially pre-cultured in 114 × 86 × 102 mm PET containers for seven days to allow for initial seeding. The duckweed strains started out at an initial mass of 0.25 g. The mass of the duckweed strains after initial seeding was 10 g. After the initial seeding was observed, the duckweed strains were transferred to larger rectangular PET containers (0.2670 m^2) for 30 days on a standard solution of Hoagland E-Medium at 200 g L^{-1}.

Figure 3-1 shows the rectangular PET container set-up used to cultivate the duckweed strains. The duckweeds were cultivated at a laboratory temperature of 24°C. A light intensity of 3,000 lux was maintained using conical fluorescent lighting at a photoperiod of 16 hours of light and 8 hours of darkness set on a timer to allow for some adaptation of the duckweed and to provide samples for future batch testing. The deionized water had to be replenished every two days to keep the concentration of the nutrients constant. The pH was initially set at 6.5 using a buffer solution of 5% v/v of acetic acid.

Figure 3-1 Preliminary initial lab set-up of duckweed strains: Left to right Landoltia punctata 0128, Lemna valdiviana 8831, Lemna gibba 7589, Lemna minuta 9517, and Lemna minor 9533

3.2.2 Selection of duckweed strains

Three strains of duckweed were selected for this study based on biomass productivity after 30 days of growth. The strains selected for further experimentation were *Landoltia punctata 0128, Lemna gibba 7589,* and *Lemna minuta 9517.* Table 3-1 shows the initial and final biomass (g wet wt. m^{-2}), biomass yield (g wet wt. m^{-2}), biomass productivities (g wet wt. m^{-2} d^{-1}), relative growth rates, and doubling time (days) of the three strains of duckweed. Initially, 10 g of biomass was inoculated into the larger PET containers at a nutrient concentration of 200 g L^{-1} of Hoagland E-Medium. Preliminary investigation found that it is imperative to inoculate the PET containers at 25% of the surface area to keep the growth of filamentous algae low while at the same time actively reducing nutrients and accumulating biomass.

The biomass productivities (BP) can be estimated using Equation (3-1),

$$BP = \frac{DW_f - DW_i}{t} \qquad \text{(Equation 3-1)}$$

where the final and initial biomass yields (g wet. m^{-2}) are DW_f and DW_i, respectively, at the start and the end of the experimental period and t is the variable for number of days. The relative growth rates were measured by initially measuring the mass (g) of the duckweed samples and after the 30-day period measuring the final mass of the duckweed samples. The fresh weight was measured by removing the excess water from the duckweed strains by blotting the fronds dry with absorbent paper tissues and then weighing the duckweed immediately afterwards. The duckweed's relative growth rate (RGR) could then be calculated after the initial and final biomass measurements were made. Equation (3-2) is used for calculating RGR,

$$RGR = \frac{\left(\ln\left(\frac{m_f}{m_i}\right)\right)}{t} \qquad \text{(Equation 3-2)}$$

where m_i and m_f are the initial and final duckweed mass (g), respectively at the start and end of the experiment period and t is the number of days. Using the RGR, the biomass doubling time (DT) can be estimated by Equation (3-3):

$$DT = \frac{(\ln(2))}{RGR} \qquad \text{(Equation 3-3)}$$

Figure 3-2 shows the initial and final surface area growth of the three duckweed strains after the 30-day duration period. The biomass productivity was calculated from Equation (3-1), the RGR was calculated from Equation (3-2), and the doubling time was calculated from Equation (3-3). The initial cultivation parameters have not provided the doubling rate of 2-5 days of growth on Hoagland E-Medium at 200 g L^{-1}, a light intensity of 3,000 lux and a temperature of

24°C. While not ideal, this experiment provides a good start at establishing the optimum cultivation conditions of the duckweed strains.

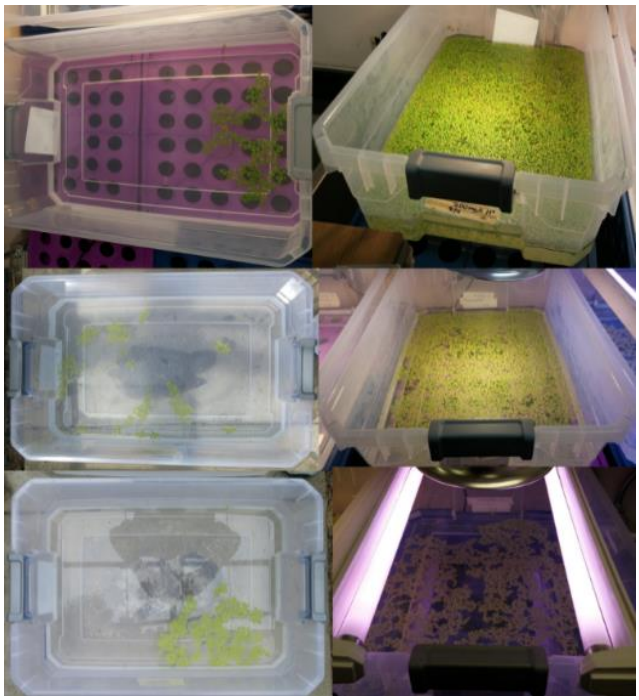

Figure 3-2 Initial and final surface area growth of duckweed strains selected for batch studies after 30 days: Top: Landoltia punctata 0128, Middle: Lemna gibba 7589, and Bottom: Lemna minuta 9517

Table 3-1 Relative growth rates of duckweed strains after 30 days of batch growth in 200g L^{-1} of Hoagland E-Medium

Treatment	**Initial Biomass**	**Final Biomass**	**Biomass Yield**	**Productivity**	**RGR**	**DT**
Units	**g wet wt. m^{-2}**			**g wet wt. m^{-2} d^{-1}**	**d^{-1}**	**d**
L. punctata	37.5	250.8	213.3	7.11	0.063	11
L. gibba	37.5	125.4	87.9	2.93	0.040	17
L. minuta	37.5	100.0	62.5	2.08	0.033	21

3.2.3 Batch tests

3.2.3.1 Developing nutrient basis

Anaerobically digested dairy manure was collected from a local dairy in the Southern Idaho region. A nutrient and physical analysis of the anaerobically digested dairy manure was performed to provide a basis for measurement. The nutrient parameters were analyzed using a spectrophotometer (DR5000, Hach, USA). The Hach methods used to obtain the data were Method 10242, Method 10214, and Method 10127 for chemical oxygen demand (COD), TN, TKN, nitrates and nitrites (NO_3-N+NO_2-N), o-PO_4-P, and TP, respectively. Suspended solids (SS) was analyzed using Hach Method 8006. Total and volatile solids were analyzed using standard methods (APHA, 2015). A pH, dissolved oxygen (DO) and electric conductivity (EC) analyses were conducted using a Sper Scientific 850049 water meter kit. Table 3-2 shows the characteristics of the anaerobically digested dairy manure. The results from Table 3-2 were compared to an analysis done by Northwest Laboratories LLC in Southern Idaho which can be found Appendix B.

Table 3-2 Characteristics of flushed anaerobically digested dairy manure tested seven-days after collection

Parameter	Units	Mean Value	S.D.
TN	mg L^{-1}	1600.4	±245.1
TKN	mg L^{-1}	1500.3	±243.2
NO_3-N+NO_2-N	mg L^{-1}	100.1	±3.2
TP	mg L^{-1}	188.0	±4.0
o-PO_4-P	mg L^{-1}	108.8	±27.4
COD	mg L^{-1}	7679.4	±81.0
TS	%	1.2	±0.03
VS	%	53.9	±2.25
pH		7.97	±0.15
SS	mg L^{-1}	11,714	±1283
EC	mS cm^{-1}	12.6	±0.08
DO	mg L^{-1}	2.6	±0.4

S.D., standard deviation of triplicate samples

3.2.3.2 Hoagland's No. 2 Basal Salt Mixture

The standard solution (thereafter referred to as Hoagland E-Medium throughout this study) used in these experiments was Hoagland's No. 2 Basal Salt Mixture (HOP01-50LT). Hoagland E-Medium is a mixture of micronutrients and macronutrients which contain the specific components: ammonium phosphate, monobasic ($NH_4H_2PO_4$), boric acid (H_3BO_3), calcium nitrate, tetrahydrate ($Ca(NO_3)_2$-$4H_2O$), cupric sulfate, pentahydrate ($CuSO_4$-$5H_2O$), ferric tartrate ($C_{12}Fe_2H_{12}O_8$), magnesium sulfate, anhydrous ($MgSO_4$), manganese chloride, tetrahydrate (MnC_{l2}-$4H_2O$), molybdenum trioxide (MoO_3), potassium nitrate (KNO_3), and, zinc nitrate, hexahydrate ($Zn(NO_3)_2$-$6H_2O$). The analysis of Hoagland E-Medium was carried out using the same methods to analyze the anaerobically digested dairy manure.

Table 3-3 Characteristics of the standard solution 1.6 g L^{-1} Hoagland E-Medium for batch growth of duckweed

Parameter	Units	Mean Value	S.D. *
TN	mg L^{-1}	258.6	±38.8
TKN	mg L^{-1}	69.9	±11.3
NO_3-N+NO_2-N	mg L^{-1}	188.5	±5.6
TP	mg L^{-1}	101	±2
o-PO_4-P	mg L^{-1}	95.0	±23.7
COD	mg L^{-1}	389.5	±4.1
pH		7.01	±0.15
EC	µS/cm	2029	±0.08
DO	mg L^{-1}	4.7	±0.4

* S.D. = standard deviation of triplicate samples

3.2.3.3 Batch test preparation

Dilution ratios were determined by mixing anaerobically digested dairy manure and deionized water to establish the nutrient content from Table 3-2. It was determined through a series of preliminary tests that the three-specific duckweed, *Landoltia punctata 0128*, *Lemna gibba 7589* and, *Lemna minuta 9517*, would be able to uptake a nutrient basis concentration of

TN at 114, 84, and 57 mg L^{-1}, respectively. It was also determined that the pH of the medium would increase while inside the environmental growth chamber. To keep an ideal growth environment, the pH was adjusted to 6.5 every 2 days with acetic acid at 5% v/v and 10M NaOH solution when the medium was over adjusted.

Each concentration of anaerobically digested dairy manure was initially prepared in a 4L container. Each 4-L container held a different dilution ratio of a mixture of AD dairy manure and deionized water at 1:13, 1:18, and 1:27. The initial pH values of the mixtures were 8.11, 8.12, and 8.19, respectively. These pH values were then adjusted to 6.5 adding the volumes of 24, 20, and 15 mL, respectively, using 5% v/v acetic acid. With the adjusted pH values for the dilution ratios of 1:13, 1:18 and 1:27, the initial electric conductivity, dissolved oxygen, and medium temperature were measured at 1,660 µS cm^{-1}, 4.7 mg L^{-1}, and 21.4°C, 1,331 µS cm^{-1}, 3.8 mg L^{-1}, and 23.4°C, and 1066 µS cm^{-1}, 4.5 mg L^{-1}, and 23.6°C, respectively. The dilutions were then transferred to PET containers with the dimensions of 114 × 86 × 102 mm with a surface area of 0.0116 m^2 and a total volume of 300 mL.

Batch tests were conducted inside an environmental growth chamber at a light intensity of 10,000 lux and a photoperiod of 16:8 (light: dark). The experiment consisted of a total of 45 PET containers with a total volume of 200 mL of deionized water and anaerobically digested dairy manure. Triplicate dilution ratios of 1:13, 1:18, and 1:27 were conducted on each strain of duckweed along with control/algae per dilution ratio and a standard solution of Hoagland E-Medium. For each dilution ratio, there were three strains of duckweed in triplicate followed by a triplicate set of control/algae and Hoagland E-Medium at 1.6 g L^{-1} utilizing 15 containers per dilution ratio.

3.3 Sampling and Analysis

Every 72 hours, random triplicate grab samples would be taken from different locations inside the PET containers containing the treated and untreated anaerobically digested dairy manure for nutrient analysis. The whole period of experimentation lasted 28 days with there being 8 days of testing on the following days: 0, 4, 8, 12, 16, 20, 24, and 28. Over this period, medium samples were analyzed for medium temperature, COD, TN, TKN, NO_3-N+NO_2-N, TP, o-PO_4-P, pH, DO, and EC. The nutrient parameters were analyzed using a spectrophotometer (DR5000, Hach, USA). Hach methods used to obtain the data were Method 10242, Method 10214, and Method 10127 for TN, TKN, NO_3-N+NO_2-N, o-PO_4-P, and TP. The wet and dry weights of the duckweed in each sample were measured immediately after sampling with a Mettler AE 260 Delta Range balance. Free water on the duckweed was removed with cheese cloth before measurement. At the end of the 28th day the wet duckweed was dried in an oven at 26.7°C for 24 hours to obtain a dry weight.

3.3.1 Statistical analysis

The performances of the control/algal systems were compared to those of the duckweed cultures by performing a Students t-test to determine if the differences between the rate constants were statistically significant, using the method of independent samples and unequal variances, employing a two-tailed p-value. All statements were based on a statistical significance of $P<0.05$. Relative growth rates (RGR) are established from zero-order kinetics, which in turn establish the doubling times. Simple linearized regression equations were calculated, to obtain rate constants and standard errors. Relative growth rates were estimated from linear regressions using Equation (3-4):

$$C_t = k \cdot t + C_o \qquad \text{(Equation 3-4)}$$

where C_o is the initial duckweed yield (g wet. m^{-2}) in the duckweed culture, C_t (g wet. m^{-2}) is the biomass yield at time t (days), and k is the relative growth rate constant (g wet. m^{-2} d^{-1}). Nutrient parameters are analyzed using first-order rate kinetics to establish nutrient rate constants. Equation (3-5) was used to estimate the first order rate equations for nutrient reduction:

$$A = A_o \cdot e^{-z \cdot t} \qquad \text{(Equation 3-5)}$$

where A_o is the initial nutrient concentration at time zero in the duckweed culture, A is the nutrient concentration (mg L^{-1}) at time t (days), and z is the nutrient reduction rate constant (d^{-1}).

3.4 Results

3.4.1 Experimental conditions

The air temperature within the environmental growth chamber was set at 25°C and the average medium temperature was 23.0°C. The maximum and minimum medium temperatures observed were 24.0°C and 19.7°C, respectively. As time progressed for all strains of duckweed the medium temperature was lowest at the end of the experiment at an average of 19.0°C. Upon measuring the outside light intensity in Southern Idaho during the summer months it was found that a light intensity of 10,000 lux was the average light intensity. The average light intensity of 10,000 lux was set inside the environmental growth chamber with an Extech LT300 light meter. Studies did indicate that 7,500 lux was the optimal light intensity for duckweed growth

but the light intensity of the geographic area of Southern Idaho is going to be used for the analysis. The PET containers were randomly placed on the racks inside the environmental growth chamber so that each sample saw the same average light intensity.

3.4.2 Biomass production

Biomass accumulation was modeled using zero order kinetics to establish RGRs of the duckweed strains. These measurements generated the growth rate which allows engineers to calculate the area required to grow a targeted amount of duckweed each day at the specific cultivation conditions presented. To quantify duckweed production, growth curves were produced to estimate RGR. To begin with, growth curves were established to measure duckweed biomass production (g wet. m^{-2} d^{-1}). Figure 3-3 shows the curves of each duckweed strain and dilution ratio of AD dairy manure. Each point is a triplicate mean value with a standard deviation. The lag phase of the curve was not established due to the duckweed samples being pre-cultured on Hoagland E-Medium solution. The curves show the exponential and stationary phases for duckweed growth. The biomass growth curves will reflect that of duckweed and algal biomass within the batch systems.

Figure 3-3 shows that the growth rate of *Landoltia punctata 0128* and *Lemna gibba 7589* were very similar for dilution ratio 1:18 of anaerobically digested dairy manure, while the growth rate of *Lemna minuta 9517* was slower. When observing dilution ratio 1:27, it appeared that *Lemna gibba 7589* grew the fastest followed by *Landoltia punctata 0128* and *Lemna minuta 9517*. When observing the growth on Hoagland E-Medium, *Landoltia punctata 0128* grew the fastest and *Lemna gibba 7589* and *Lemna minuta 9517* followed in second and third place.

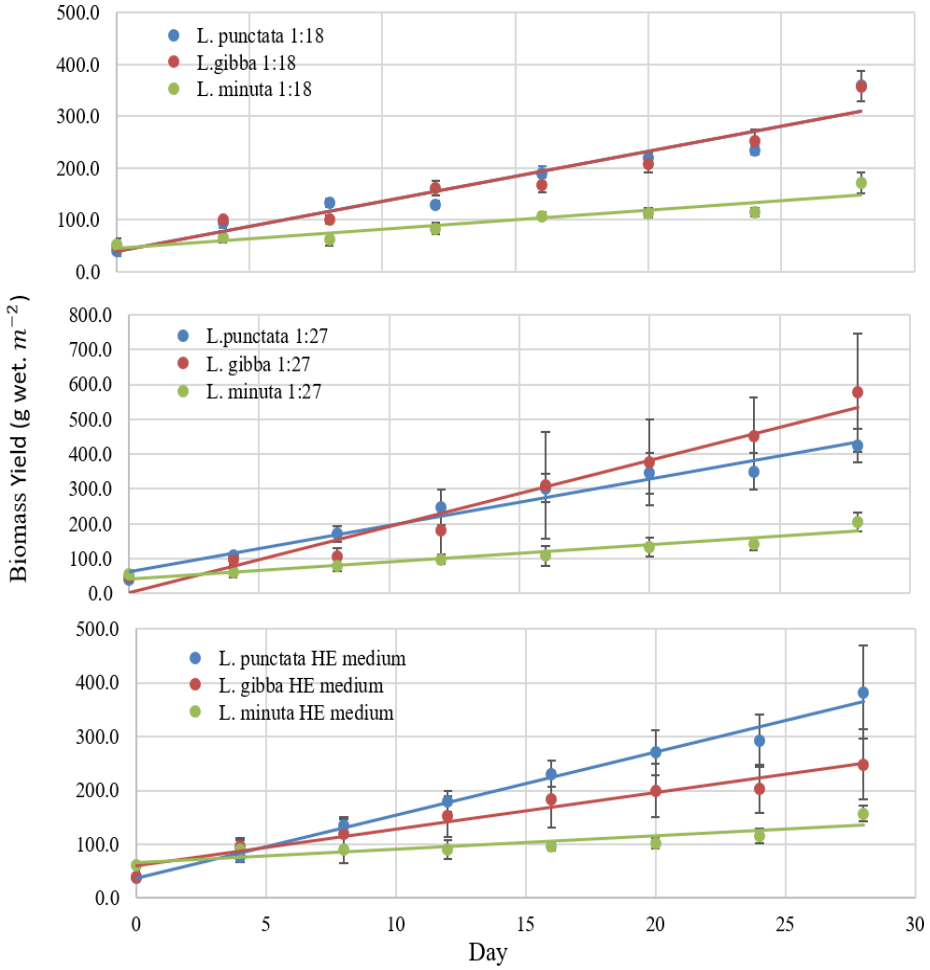

Figure 3-3 Biomass yields of L. punctata, L. gibba, and L. minuta at dilution ratio 1:18, 1:27 on anaerobically digested dairy manure and 1.6 g L^{-1} Hoagland E-Medium for a 28-day batch growth

When observing the duckweed curves, one can see an exponential and stationary phase in the dilution ratio 1:27, where the nutrients may run out faster than dilution ratio 1:18. The duckweed strains at both dilution ratios and the standard solution Hoagland E-Medium appear to be entering the exponential phase immediately at day 0 and end at day 16. From the 16th day to the 24th day, the duckweed strains appear to be in the stationary phase. From day 24 to 28, there seems to be a second exponential phase starting, possibility due to the duckweed strains adapting to the new nutrient concentration which could indicate that a lower concentration of

nutrients required. However, it could also indicate that the duckweed stains have come to the end of their life cycle to get ready for harvesting.

Growth rate constants were estimated from zero order linear regression equations established through Equation (3-4). The performances of the duckweed strains grown in the standard solution Hoagland E-Medium are compared to the duckweed strains grown in the dilution ratios of anaerobically digested dairy manure. Those equations can be seen in Table 3-4 along with the regressions.

Table 3-4 Regression between biomass yield and test duration at 28-days at different dilution ratios of anaerobically digested dairy manure

Treatment	**Dilution**	**Regression equation**	**R^2**
L. punctata	1.6g/L HE	y=11.765x+35.948	0.9888
L. gibba	1.6g/L HE	y=6.7402x+60.972	0.9596
L. minuta	1.6g/L HE	y=2.4942x+65.268	0.7794
L. punctata	1:18	y=9.6786x+39.309	0.9253
L. gibba	1:18	y=9.6601x+39.430	0.9275
L. minuta	1:18	y=3.7077x+44.896	0.8860
L. punctata	1:27	y=13.340x+61.967	0.9736
L. gibba	1:27	y=19.059x+1.7459	0.9633
L. minuta	1:27	y=4.8711x+41.545	0.9252

Table 3-5 provides a summary for biomass yields of the three duckweed strains and the three dilution ratios observed. The table also provides the initial and final biomass yields (g wet. m^{-2}), total biomass yields (g wet. m^{-2}), and relative growth rate constants (g wet. m^{-2} d^{-1}) that were derived from simple linear regression equations. All the duckweed strains growing in the dilution ratio 1:18 of anaerobically digested dairy manure were found to be insignificant when compared to the growth in the Hoagland E-Medium. The RGR of dilution ratio 1:27 were found to be significant for duckweed strains *Landoltia punctata 0128* and *Lemna gibba 7589*

at 13.0 and 19.0 g wet. m^{-2} d^{-1}, respectively. Doubling time was calculated using the growth rate constant as the RGR in Equation (3-4). When observing the doubling times in Table 3-5 it is found that *Landoltia punctata 0128* and *Lemna gibba 7589* at dilution ratio 1:27 had the lowest number of days needed to double the mass of the duckweed at 4.5 and 3.1 days, respectively.

Table 3-5 Biomass yield of duckweed strains after 28 days of batch growth in anaerobically digested dairy manure [a]

Treatment	**Dilution**	**Initial Biomass**	**Final Biomass**	**Biomass Yield**	**RGR [b]**	**DT**
Units		**g wet. m^{-2}**			**g wet. m^{-2} d^{-1}**	**d**
L. punctata	1.6 g L^{-1} HE	36.9	382.3	345.4	11.8 (±1.6)	5.1
L. gibba	1.6 g L^{-1} HE	39.4	248.2	208.8	6.70 (±1.3)	8.9
L. minuta	1.6 g L^{-1} HE	60.4	157.1	96.7	2.5 (±0.2)	23.9
L. punctata	1:18	40.2	358.6	318.4	9.7_{nsc} (±0.04)	6.2
L. gibba	1:18	49.4	357.9	308.5	9.7_{nsc} (±0.9)	6.2
L. minuta	1:18	53.3	171.4	118.1	3.7_{nsc} (±0.40)	16.1
L. punctata	1:27	40.2	425.6	385.4	13.3 (±0.8)	4.5
L. gibba	1:27	49.0	576.8	527.8	19.0 (±3.5)	3.1
L. minuta	1:27	53.6	204.4	150.8	4.9_{nsc} (±0.5)	12.2

[a] A student t-test was performed to compare k values of the control/algae and Hoagland E-Medium with those of duckweed cultures. All k values were significant (α=0.05), except those bearing the subscript: nsc (not significantly significant)

[b] Values in parentheses are standard errors of rate constants

3.4.3 Nutrient recovery rate

To model nutrient recovery from the batch systems treated by duckweed, nutrient reduction curves were produced to estimate nutrient reduction rate constants. Next, nutrient recovery curves were established from measuring the liquid medium from the samples. The reductions for TN, TKN, TP, and o-PO_4-P were compared for each of the dilution ratios and controls to identify significance. The period in which the greatest nutrient removal was

observed was from day 0 to day 16. The period most clearly represented first-order kinetics. First-order rate constants were modeled using equation (3-5).

3.4.4.1 Total nitrogen

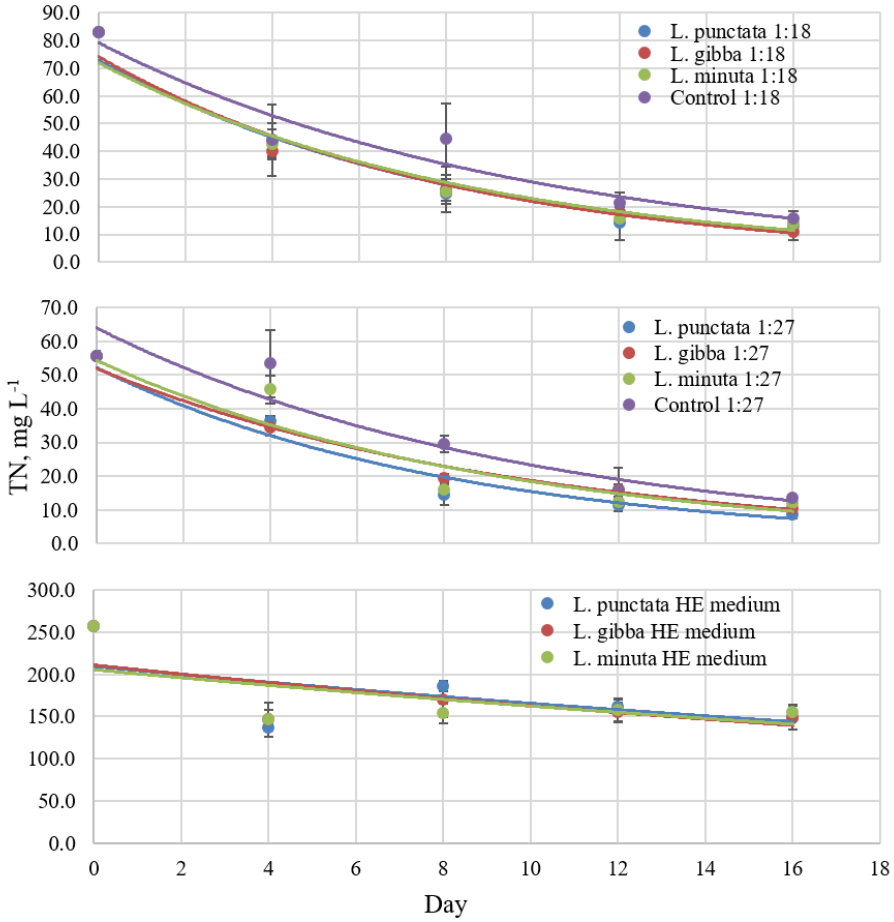

Figure 3-4 TN Reduction of L. punctata, L. gibba, and L. minuta at dilution ratio 1:18 and 1:27 on anaerobically digested dairy manure and 1.6 g L^{-1} Hoagland E-Medium for a 16-day batch growth

TN is the sum of TKN and NO_3-N+NO_2-N. TKN is made up of total organic N and NH_3-N. The development of TN, for the three duckweed strains, is shown in Figure 3-4. Exponential equations were established from Figure 3-4 above to estimate TN reduction rate constants. Those equations are illustrated in Table 3-6, along with the regressions. The

exponential equations modeling TN were found to fit the data well with regressions for the AD dairy manure medium having R^2 values in the upper 0.80s and 0.90s. However, the regressions for the Hoagland E-Medium model equations were low, at R^2 equal to 0.30 to 0.48. The regression equations for modeling Hoagland E-Medium might not be very reliable for reducing TN, but the regression equations for the dilution ratios of AD dairy manure will be quite reliable for modeling TN.

Table 3-6 Regression between TN reduction and test duration at 16-days at different dilution ratios and treatments of anaerobically digested dairy manure and 1.6 g L^{-1} Hoagland E-Medium

Treatment	Dilution	Regression equation	R^2
Control	1:18	$y = 79.201e^{-0.101x}$	0.9432
Control	1:27	$y = 63.957e^{-0.101x}$	0.9371
L. punctata	1.6g/L HE	$y = 209.44e^{-0.023x}$	0.3571
L. gibba	1.6g/L HE	$y = 210.97e^{-0.026x}$	0.4872
L. minuta	1.6g/L HE	$y = 205.43e^{-0.023x}$	0.4069
L. punctata	1:18	$y = 72.888e^{-0.12x}$	0.9524
L. gibba	1:18	$y = 74.185e^{-0.122x}$	0.9853
L. minuta	1:18	$y = 71.982e^{-0.115x}$	0.9581
L. punctata	1:27	$y = 52.191e^{-0.122x}$	0.9452
L. gibba	1:27	$y = 51.977e^{-0.102x}$	0.9781
L. minuta	1:27	$y = 54.355e^{-0.108x}$	0.8701

3.4.4.2 Total Kjeldahl nitrogen

The development of TKN for the three duckweed strains can be seen in Figure 3-5. Exponential regression equations were established from the Figures 3-5, to estimate TKN reduction rate constants. Those equations are expressed in Table 3-7 along with the regressions. The TKN rate constants are very similar between the duckweed strains but not necessarily within the controls. The exponential equations, modeling TKN, were found to fit the data, well,

with regressions for the AD dairy manure medium having R^2 values in the upper 0.80s and 0.90s. However, the R^2 values of the regression equations for the Hoagland E-Medium ranged from 0.30 to 0.48. The exponential regression equations for modeling Hoagland E-Medium might not be very reliable for modeling TKN.

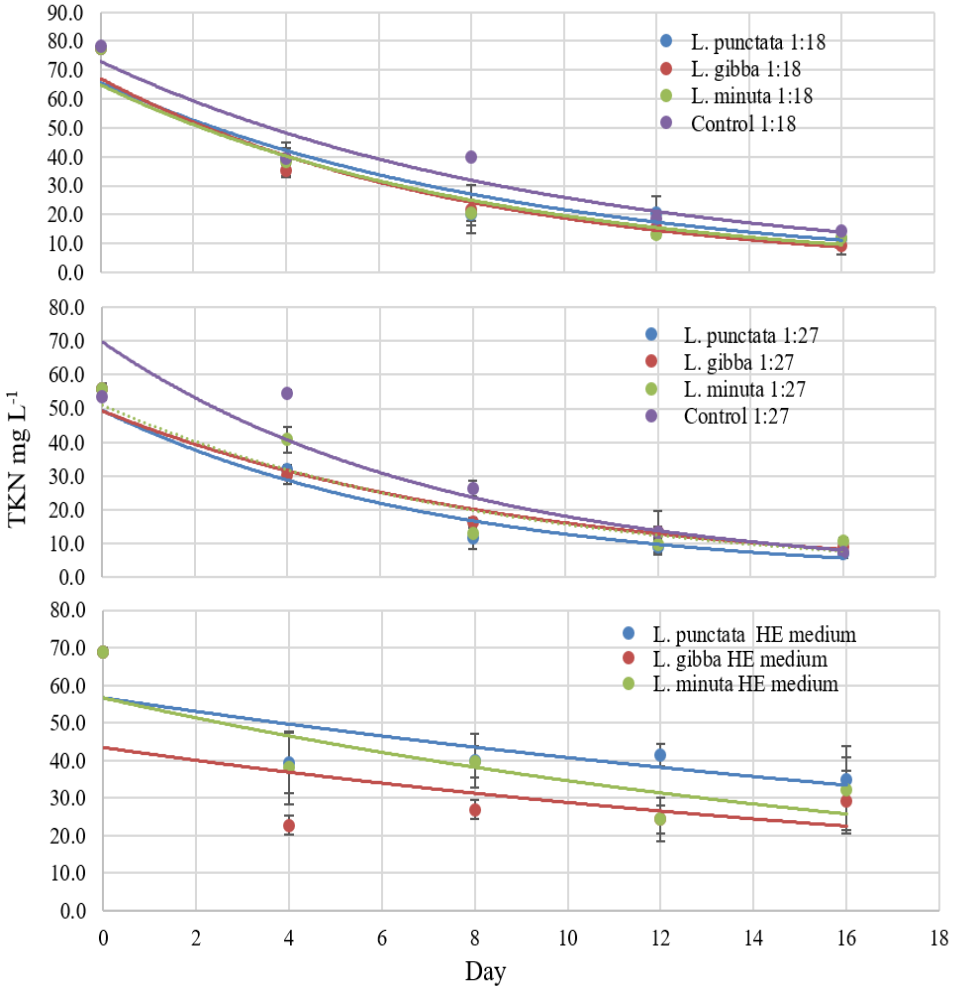

Figure 3-5 TKN Reduction of L. punctata, L. gibba, and L. minuta at dilution ratio 1:18 and 1:27 on anaerobically digested dairy manure and 1.6 g L^{-1} Hoagland E- Medium for a 16-day batch growth

Table 3-7 Regression between TKN reduction and test duration at 16-days at different dilution ratios of anaerobically digested dairy manure and 1.6 g L^{-1} Hoagland E-Medium

Treatment	Dilution	Regression equation	R^2
Control	1:18	$y = 72.927e^{-0.103x}$	0.9370
Control	1:27	$y = 69.776e^{-0.135x}$	0.9423
L. punctata	1.6g/L HE	$y = 56.65e^{-0.033x}$	0.6158
L. gibba	1.6g/L HE	$y = 43.429e^{-0.041x}$	0.3298
L. minuta	1.6g/L HE	$y = 56.617e^{-0.049x}$	0.6658
L. punctata	1:18	$y = 65.671e^{-0.111x}$	0.9291
L. gibba	1:18	$y = 67.014e^{-0.127x}$	0.9789
L. minuta	1:18	$y = 64.851e^{-0.119x}$	0.9379
L. punctata	1:27	$y = 49.472e^{-0.136x}$	0.9345
L. gibba	1:27	$y = 49.341e^{-0.112x}$	0.9677
L. minuta	1:27	$y = 51.04e^{-0.118x}$	0.8386

3.4.4.3 Total phosphorus

TP, in AD dairy manure consists mainly of o-PO_4-P; however, particulate P is present within the sediment that diffuses into the upper level of the solution. TP accounts for both sedimentary P and o-PO_4-P portions of the dairy medium. The development of TP, for the three duckweed strains, is visualized Figure 3-6. Exponential equations were established from Figure 3-6, to estimate TP reduction rate constants. Those equations can be seen in Table 3-8 along with the regressions. The exponential equations modeling TP were found to fit the dairy manure data, well, with R^2 values in the upper 0.80s and 0.90s. However, the regression equations for the Hoagland E-Medium had R^2 values, ranging from 0.30 to 0.48 for *Lemna gibba 7589* and *Lemna minuta 9517* but above 0.80 for *Landoltia punctata 0128*. The regression equations for modeling Hoagland E-Medium might not be very reliable for predicting TP reducing. However, the regression equations for the AD dairy manure with the two dilution ratios could be used for modeling TP given that their R^2 values were greater than 0.8.

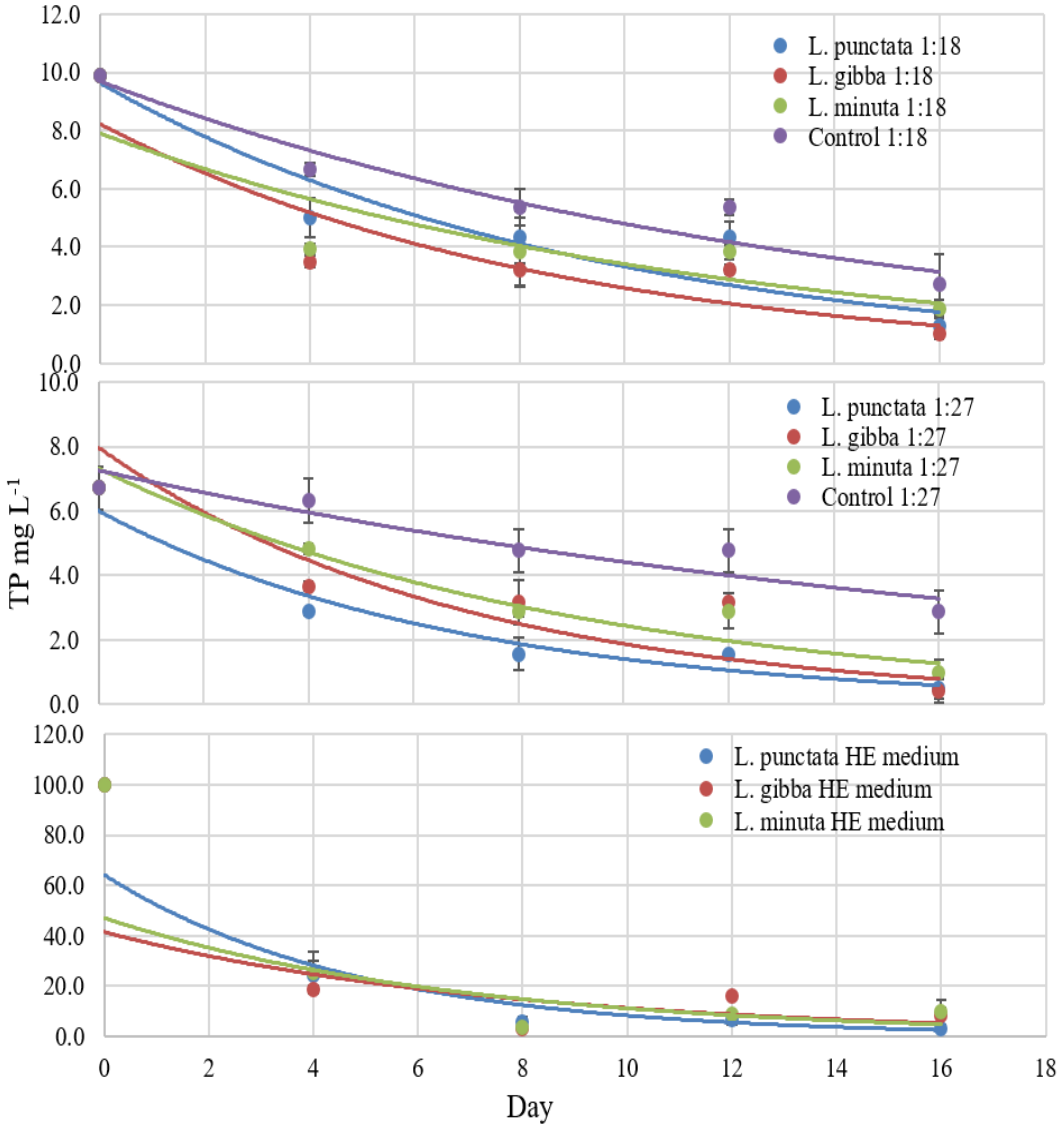

Figure 3-6 TP Reduction of L. punctata, L. gibba, and L. minuta at dilution ratio 1:18 and 1:27 on anaerobically digested dairy manure and 1.6 g L^{-1} Hoagland E- Medium for a 16-day batch growth

Table 3-8 Regression between TP reduction and test duration at 16-days at different dilution ratios of anaerobically digested dairy manure and 1.6 g L^{-1} Hoagland E-Medium

Treatment	Dilution	Regression equation	R^2
Control	1:18	$y = 9.6906e^{-0.07x}$	0.8907
Control	1:27	$y = 7.2586e^{-0.05x}$	0.8689
L. punctata	1.6g/L HE	$y = 64.124e^{-0.206x}$	0.8763
L. gibba	1.6g/L HE	$y = 41.373e^{-0.131x}$	0.3981
L. minuta	1.6g/L HE	$y = 46.955e^{-0.145x}$	0.5076
L. punctata	1:18	$y = 9.628e^{-0.106x}$	0.8221
L. gibba	1:18	$y = 8.227e^{-0.116x}$	0.826
L. minuta	1:18	$y = 7.8981e^{-0.084x}$	0.8044
L. punctata	1:27	$y = 6.0005e^{-0.145x}$	0.9292
L. gibba	1:27	$y = 7.9699e^{-0.145x}$	0.7292
L. minuta	1:27	$y = 7.3208e^{-0.11x}$	0.8908

3.4.4.4 Ortho-phosphate-phosphorus

O-phosphate-phosphorus is the active state to which plants readably uptake P. The development of o-PO_4-P for the three duckweed strains can be seen in Figure 3-7. Exponential equations were established from Figure 3-7 to estimate o-PO_4-P reduction rate constants. Those equations can be seen in Table 3-9 along with R^2 values. The exponential equations modeling o-PO_4-P were found to fit the dairy manure data well with R^2 values in the upper 0.90s. The regressions for the Hoagland E-Medium also had R^2 values equal to 0.95 and above. These R^2 values indicated that the regression equations could be used to predict o-PO_4-P reduction for both the Hoagland E-Medium and anaerobically digested dairy manure with the two dilution ratios.

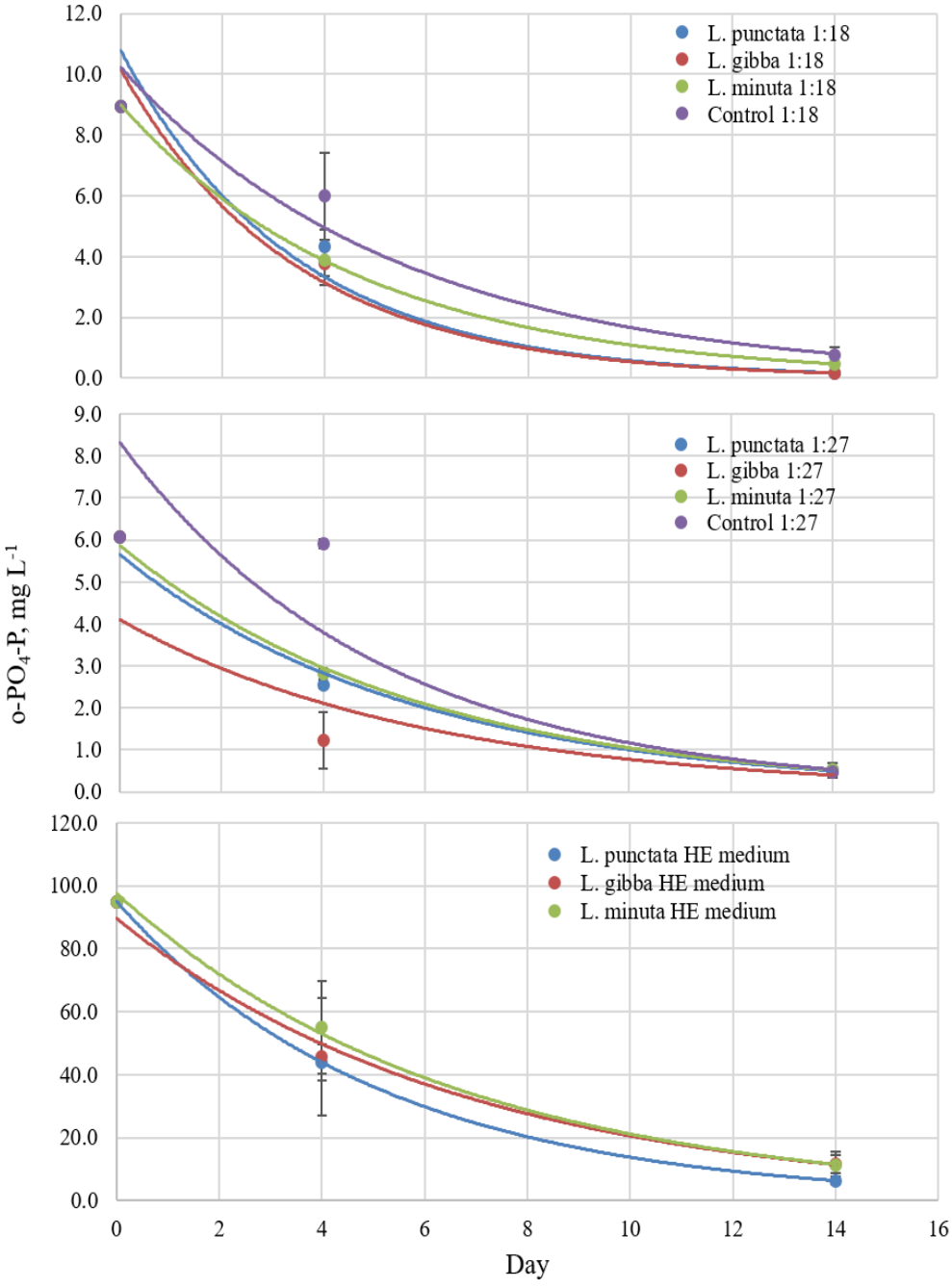

Figure 3-7 o-PO_4-P Reduction of L. punctata, L. gibba, and L. minuta at dilution ratio 1:18 and 1:27 on anaerobically digested dairy manure and 1.6 g L^{-1} Hoagland E- Medium for a 16-day batch growth

Table 3-9 Regression between o-PO_4-P reduction and test duration at 16-days at different dilution ratios of anaerobically digested dairy manure and 1.6 g L^{-1} Hoagland E-Medium

Treatment	Dilution	Regression equation	R^2
Control	1:18	$y = 10.236e^{-0.182x}$	0.9836
Control	1:27	$y = 8.3217e^{-0.196x}$	0.9281
L. punctata	1.6g/L HE	$y = 95.007e^{-0.193x}$	1.0000
L. gibba	1.6g/L HE	$y = 89.582e^{-0.147x}$	0.9953
L. minuta	1.6g/L HE	$y = 97.59e^{-0.153x}$	0.9991
L. punctata	1:18	$y = 10.785e^{-0.294x}$	0.9879
L. gibba	1:18	$y = 10.17e^{-0.294x}$	0.9943
L. minuta	1:18	$y = 8.9964e^{-0.211x}$	1.0000
L. punctata	1:27	$y = 5.6427e^{-0.173x}$	0.9947
L. gibba	1:27	$y = 4.1024e^{-0.166x}$	0.8575
L. minuta	1:27	$y = 5.8786e^{-0.172x}$	0.9990

3.4.4 Comparison nutrient reduction rate constants

Total Nitrogen (TN), total kjeldahl nitrogen (TKN), ortho-phosphate-phosphorus (o-PO_4-P), and total phosphorus (TP) followed a first order decay model and all the rate constants were significant (α=0.05) using a Student's t- test when compared to the standard Hoagland E-Medium which can be found in Table 3-10, along with the dilution control treatments and standard errors. However, when comparing to the controls of anaerobically digested dairy manure it was found that significance varied among dilution ratios and parameters. For the reduction of TN, the dilution ratio 1:27 was determined to be significant for the duckweed strain *Landoltia punctata 0128.* For the reduction of TKN, it was found that the dilution ratio 1:27 was significant for the duckweed strain *Lemna gibba 7589.* For the reduction of o-PO_4-P, it was established that both dilution ratios of 1:18 and 1:27 were significant for the duckweed strains *Landoltia punctata 0128* and *Lemna gibba 7589.* For the reduction of TP, it was found that the dilution ratio 1:18 and 1:27 was significant for *Lemna gibba 7589* and *Landoltia punctata 0128,*

respectively. The *Landoltia punctata 0128* culture exhibited the highest reduction rates for all the parameters, except o-PO_4-P in dilution ratio 1:27 when compared to all other duckweed cultures.

Table 3-10 First-order rate constants of nutrients in anaerobically digested dairy manure during a 16-day growth cycle of the duckweed strains L. punctata, L. gibba, and L. minuta [a, b]

Treatment	Dilution	k_{TN}	k_{TKN}	$k_{o\text{-}PO4\text{-}P}$	k_{TP}
Control	1:18	0.101 (±0.007)	0.103 (±0.008)	0.182 (±0.01)	0.07 (±0.01)
Control	1:27	0.101 (±0.009)	0.135 (±0.005)	0.196 (±0.001)	0.05 (±0.01)
L. punctata	1.6g/L HE	0.023 (±0.004)	0.033 (±0.005)	0.193 (±0.005)	0.206 (±0.01)
L. gibba	1.6g/L HE	0.026 (±0.001)	0.041 (±0.01)	0.147 (±0.005)	0.131 (±0.01)
L. minuta	1.6g/L HE	0.023 (±0.002)	0.049 (±0.01)	0.153 (±0.02)	0.145 (±0.02)
L. punctata	1:18	0.120 (±0.002)	0.111 (±0.001)	0.294 (±0.001)	0.106 (±0.005)
L. gibba	1:18	0.122 (±0.009)	0.127 (±0.01)	0.294 (±0.03)	0.116 (±0.006)
L. minuta	1:18	0.115 (±0.002)	0.119 (±0.001)	0.211 (±0.001)	0.084 (±0.004)
L. punctata	1:27	0.122 (±0.002)	0.136 (±0.002)	0.173 (±0.001)	0.145 (±0.03)
L. gibba	1:27	0.102 (±0.003)	0.112 (±0.006)	0.166 (±0.003)	0.145 (±0.03)
L. minuta	1:27	0.108 (±0.001)	0.118 (±0.002)	0.172 (±0.007)	0.11 (±0.01)

[a] Values in parentheses are standard errors of rate constants

[b] A student t-test was performed to compare k values of the control/algae and Hoagland E-Medium with those of duckweed cultures. All k values were significant (α=0.05) for reduction of Hoagland E-Medium

3.5 Discussion

3.5.1 Visual observations

Initially, the PET containers were inoculated at 25% of the surface area to promote biomass accumulation and nutrient reduction. All the duckweed strains started out in their natural frond pigmentation states with *Landoltia punctata 0128* being a dark green color and *Lemna gibba 7589* and *Lemna minuta 9517* exhibiting more of a light green color, indicating ideal cultivation conditions. The medium colors for the dilution ratios are a dark brown for 1:13,

a lighter shade of brown for 1:18, and the lightest shade of brown for 1:27. The Hoagland E-Medium solution began as a clear liquid.

At 4 days of growth, the duckweed strains noticeably doubled their biomass for dilution ratio 1:18, 1:27, and Hoagland E-Medium. Dilution ratios 1:18 and 1:27 kept their original pigmentations. However, for dilution ratio 1:13 the duckweed strains began to turn a very light shade of yellow while their fronds curled up on themselves. The was no noticeable color change or growth in with the controls with no duckweed treatment. The medium color for the Hoagland E-Medium did turn a light shade of green indicating algal growth.

After 8 days of growth, the duckweed strains *Landoltia punctata 0128* and *Lemna gibba 7589* appear that they have doubled their biomass taking up ¾ of the surface area in the PET containers for both dilution ratios. In dilution ratio 1:18, bubbles formed on the surface of the PET containers holding *Lemna gibba 7589* and *Lemna minuta 9517* in place. It is possible that nitrogen (N_2) or oxygen (O_2) gas are dissipating from the samples. Dilution ratio 1:27 had a thick film of filamentous green algae growing among the duckweed. Both the dilution ratios had a light green color mixed in with the solution. The duckweed strains pigmentation had turned white for dilution ratio 1:13 and died. Both control dilution ratios without duckweed, began to exhibit a thin film of filamentous algae growing on the surface of the medium. The color of the medium for Hoagland E-Medium became fully green with algal activity.

After 12 days of growth, a clear difference between dilution ratios and duckweed strains became self-evident. Dilution ratio 1:27 produced the most biomass of the three duckweed strains. *Landoltia punctata 0128* is superior to the other two taking up the most surface area in the PET containers. At dilution ratio 1:18, about ¾ of the surface area was occupied and on dilution ratio 1:27 all the surface area was taken up. Dilution ratio 1:27 has indicated a healthier

vegetative growth than has ratio 1:18. The fronds, for dilution ratio 1:18, became diminished, almost shrinking in size in comparison to dilution ratio 1:27. The controls, without duckweed treatment, turned green with algal growth. The Hoagland E-Medium treatments grew slower than the AD dairy manure treatments, but *Landoltia punctata 0128* grew the fastest followed by *Lemna gibba 7589* and *Lemna minuta 9517.*

After 16 days of growth, *Lemna gibba 7589* and *Lemna minuta 9517* were observed to increase in biomass density, for dilution ratio 1:27, but stayed the same for dilution ratio 1:18. They were also observed to have a white pigmentation on some of the fronds. *Landoltia punctata 0128* increased in surface area, on dilution ratio 1:18, but stayed the same for dilution ratio 1:27. *Landoltia punctata 0128* started to stack on top of itself, forming a second layer of duckweed growth for dilution ratio 1:27. The controls, without duckweed treatments, were observed to have more algal growth with a darker green pigmentation in the medium. In the Hoagland E-Medium treatments, *Landoltia punctata 0128* completely covered the surface area of the PET containers and remained dark green and the other two duckweed strains grew poorly.

After 20 days of growth, *Landoltia punctata 0128* for both dilution ratios began to show white colored fronds throughout the samples. For dilution ratio 1:18, *Lemna gibba 7589* and *Lemna minuta 9517* started to turn a light shade of brown with a white tint going through the samples. It appeared as if growth had ceased for both dilution ratios except for the duckweed strain *Lemna minuta 9517* that appeared to be still growing on dilution ratio 1:27. The controls with no duckweed treatment are starting to turn a brown color again with a visible growth of algae at the bottom of the containers. On the Hoagland E-Medium, there is still growth occurring for *Landoltia punctata 0128*, however, the visible growth for the other duckweed

strains ceased. At days 24 through 28 no noticeable different were observed. Pictures of the duckweed cultivation samples can be found in Appendix B.

3.5.2 Biomass growth

When observing Figure 3-3, the biomass growth among the three dilution ratios were contrasting. The most duckweed growth occurred in the first 16 days of activity for all duckweed strains. The scale for biomass growth for 1:27 was approximately two times larger than dilution ratio 1:18. The duckweeds grown on the Hoagland E-Medium followed the scale of dilution ratio 1:18 in terms of biomass yield. Comparing the duckweed growth on dilution ratios 1:18 and 1:27 to the Hoagland E-Medium treatments, dilution ratio 1:18 was found insignificant and 1:27 was found to be significant. It is apparent that nutrient concentration inhibited the growth of duckweed cultivated on AD dairy manure within the dilution ratio 1:18.

On observation of the standard deviation between samples, dilution ratio 1:18 had very small deviation between the treatments. The standard deviation between the duckweed strains *Landoltia punctata 0128* and *Lemna minuta 9517* grown on AD dairy manure with the dilution ratio 1:27 were small. The standard deviation between the treatments of *Lemna gibba 7589* were questionably high on days 16 to 28. A potential reason for that could be in the residual water that was left behind, on the duckweed, which was quite different from case in each *Lemna gibba 7589* sample. It could be associated with the mortality rate for *Lemna gibba 7589,* as over time *Lemna gibba 7589* perished faster, which could contribute to the mass of the duckweed, since duckweed naturally gain weight for the winter months. This hypothesis appears valid, as after the 16-day mark, *Lemna gibba 7589* started to turn brown and die on the surface of the medium. Underneath the surface, new growth emerged, but grew slowly due to low light intensity.

It was observed that *Landoltia punctata 0128, Lemna gibba 7589,* and *Lemna minuta 9517* growing on the dilution ratio 1:27 produced the most biomass. The productivity of *Landoltia punctata 0128, Lemna gibba 7589* and *Lemna minuta 9517* were 13.3, 19.0, and 4.9 g m^{-2} d^{-1}, respectively. By contrast, the productivity for *Landoltia punctata 0128, Lemna gibba 7589* and *Lemna minuta 9517* for dilution ratio 1:18 was 9.7, 9.7 and 3.7 g m^{-2} d^{-1}, respectively. All duckweed strain rates were observed in a fresh wet environment.

3.5.3 Nutrient reduction

When measuring TN (Figure 3-4) there was a TKN and NO_3-N and NO_2-N component that must be taken into account. The reduction of TKN (Figure 3-5) in the duckweed cultures would mainly be due to the uptake of NH_4-N, volatilization of NH_3, nitrification/denitrification, and sedimentation. An increase in NO_3-N and NO_2-N within the batch systems indicated that nitrification was taking place within the cultures. Nitrification occurs when ammonia is oxidized to NO_3-N by nitrifying bacteria (NOB). A plethora of microorganisms can form a symbiotic relationship with the root system of the duckweed which provides a large surface area for microbial attachment. A major factor for the reduction of nutrients is the relationship between the microorganisms and the duckweed.

In the study conducted by Sooknah *et al.* (2004), they suggested that the support of the root system of the duckweed strains provided more favorable conditions for the NOBs by providing them with the oxygen they need to reduce N, in contrast to the control/algal systems. For this study, it was found that NO_3-N and NO_2-N were detected within the medium, at the beginning of the study; however, it was found that there was no increase in NO_3-N or NO_2-N, throughout the study, indicating that no nitrification took place. A decrease in NO_3-N and NO_2-N, was observed, meaning that duckweed likely reduced the NO_3-N and NO_2-N. Reduction of

N, within the controls/algae system likely came from ammonia volatilization and not the nitrifying bacteria within the batch system.

Filamentous algae would have contributed to the reduction of NH_4-N, but the main mechanism would have been ammonia volatilization. As the pH and the medium temperature increased, N transformations would occur within the anaerobically digested dairy manure. In this study, there was an attempt to control pH by adjusting to 6.5, with a buffer every two days; however, the pH did steadily increase during the two-day period, after each adjustment, where ammonia gas would have had the chance to form and dissipate out of the PET containers. When the pH of a batch system reached 9.6 there would have been a 1:1 ratio of ammonia (NH_3) to ammonium (NH_4^+) forms within the medium. When the pH went beyond 9.6 there was a higher concentration of NH_3 in the medium than NH_4^+ which results in ammonia volatilization when the temperature increases above 23°C.

Ammonia volatilization is of great concern because it is a gas that inherently alters the chemistry of our atmosphere causing air quality issues. With the mat structure that duckweed formed ammonia volatilization decreases forcing N to remain in solution. However, there was no mat structure for the controls which would allow ammonia to readily dissipate from solution. NH_4-N reduction from the batch system, for the dilution ratios 1:18 and 1:27, likely was attributed to duckweed uptake, since no NO_3-N was produced for nitrification, and possibly due to sedimentation. It was likely that most of the nutrients were diffused back up into solution. Control/Algae reduction of NH_4-N was likely attributed to filamentous algae uptake and ammonia volatilization.

The main component in TP that duckweed actively uptake is o-PO_4-P which can be seen in Figure 3-7. All the duckweed strains actively took up o-PO_4-P at both dilution ratios. The

control/algal however did not uptake P at the same rate as the duckweed strains. The assumption can be made that the algal activity in the controls reduced o-PO_4-P within the medium and due to duckweed uptake in the plant cultures. The reduction of TP (Figure 3-6) would have been mainly due to the uptake of o-PO_4-P, filtration of particulate matter through the root system, and sedimentation. Reduction of TP and o-PO_4-P occurred rapidly within first 16 days of growth, which indicates that harvesting would have reduced nutrients within the system if a portion of the duckweed were harvested at 16 days. To improve o-PO_4-P removal and biomass accumulation a step feed system could be implemented to give the duckweed a continuous supply of nutrients which would result in a continuous supply of biomass after a hydraulic retention time (HRT) of 16 days. Algal type systems show good performance in reducing nutrients from agricultural waste streams. However, duckweed based systems have a more practical approach when it comes to biomass growth, nutrient reduction, and ease of harvesting,

3.5.4 Modeling nutrient reduction

Two different methods of modeling nutrient reduction were considered for this study: zero order and first order kinetics. When applying zero order kinetics it was found that distinguishing between duckweed strains and controls was almost impossible because the slopes were so close together that a meaningful conclusion could not be developed. Another problem with using a zero-order kinetic model was that the scale between dilution ratio 1:18 and 1:27 was too large indicating that dilution ratio 1:18 acquired more nutrients when it wasn't observed that nitrification took place, to help remove N and no higher growth rate from dilution ratio 1:18 was observed. The benefits of being able to use zero order kinetics would be the units taken from the rate constants in concentration per unit time. If the researcher knows how much nutrient content needs to be reduced, each day, then a reactor based on the volume dimension

(L) of the rate constant could be designed. The model however was only statistically significant with TP removal.

First-order kinetics proved to be a better approach for predicting nutrient reduction. When implementing first order kinetics it was determined that the model fit the data better. The first order model matched visual observations throughout the study. It was found that the *Landoltia punctata 0128* culture exhibited the highest reduction rates for all the parameters, except for o-PO_4-P in dilution ratio 1:27 when compared to all other duckweed cultures. Further work is needed, to establish a kinetics model, to identify parameters such as volume, to identify dimensions for a system. However, the first step in establishing design specifications is establish rate constants for nutrient parameters.

3.5.5 Harvesting frequency

The harvesting of duckweed is a critical part of managing a duckweed based agricultural waste water recovery system. The harvest process physically removes the nutrients or contaminates from the agricultural waste stream. Before harvesting duckweed from a system, at least two layers of duckweed should completely cover the surface area of the system to reduce the growth of filamentous algae. The doubling rates are based on the RGRs of the duckweed strains and they represent the minimum amount of days in which the duckweed should be harvested. The doubling rates for dilution ratio 1:27 for the duckweed strains *Landoltia punctata 0128, Lemna gibba 7589,* and *Lemna minuta 9517* were 4.5, 3.1, and 12.2 d, respectively. The nutrient rate analysis conducted within this study established that the maximum harvesting frequency is 16 days of growth. After 16 days, the duckweed started to decay and transfer the nutrients back into the system.

3.6 Conclusion

This study evaluated the potential of five duckweed strains i.e. *Landoltia punctata 0128, Lemna minor 9533, Lemna gibba 7589, Lemna minuta 9517,* and *Lemna valdiviana 8831* in improving the water quality of diluted anaerobically digested (AD) dairy manure. Three strains of duckweed were selected, *Landoltia punctata 0128, Lemna gibba 7589,* and *Lemna minuta 9517* based on biomass yield for further evaluation. The treatments of duckweed strains were compared to the controls without duckweed treatment cultivated on the media of Hoagland E-Medium and AD dairy manure diluted by deionized water at three dilution ratios. Both physical and chemical tests were carried out to evaluate the growth media and duckweed strains at specified time points. Batch tests were evaluated based on observations of RGR and DT, and nutrient removal rates.

The RGR of this study were modelled after zero order kinetics to establish significance between treated and untreated batch samples. RGRs were used to identify a duckweed strain that actively produced biomass when cultivated on a concentration of AD dairy manure. The duckweed strain that actively accumulated the most biomass as compared to the standard solution was *Landoltia punctata 0128.* The RGR of *Landoltia punctata 0128* was 13.0 g m^{-2} d^{-1} with a DT of 4.5 days for a light intensity at 10,000 lux, air temperature of 25°C, an EC of 1,066 µS cm^{-1}, a DO of 4.7 mg L^{-1}, and a pH of 6.5.

Nutrient rate constants were established from first-order kinetics to establish rate constants for TN, TKN, TP and o-PO_4-P to establish significance between the treated and untreated batch samples. The treatment of *Landoltia punctata 0128* on dilution ratio 1:27 significantly reduced N and P within the batch system at 57 mg L^{-1} of TN and 7 mg L^{-1} of TP. The nutrient rate constants for *Landoltia punctata 0128* established from this study are 0.122 d^{-}

^{1} for TN, 0.136 d^{-1} for TKN, 0.145 d^{-1} for TP, and 0.173 d^{-1} for o-PO_4-P at 16 days of cultivation. The RGR and the nutrient rate constants was used to establish a minimum and maximum harvesting period for *Landoltia punctata 0128*.

Knowing the minimum and maximum days of cultivation before harvesting *Landoltia punctata 0128* is a critical step in the cultivation cycle. It removes N and P from the system and establishes growth during the harvesting period that can be carried on throughout the cultivation period. The maximum amount of days is required for the initial cultivation. After the initial cultivation period is established the minimum amount of days can be used to harvest *Landoltia punctata 0128*.

This study analyzed the minimum and maximum cultivation days required before harvesting takes place. The nutrient rate constants are based on a cultivation period of 16 days due to *Landoltia punctata 0128* entering the stationary phase of growth at that point. The maximum length of time that *Landoltia punctata 0128* should be cultivated on AD dairy manure is 16 days. The DT was established from the RGR of *Landoltia punctata 0128* to establish a minimum cultivation time. The period of 4.5 days should be given before early harvesting takes place. At day 16 there was only 5 mg L^{-1} of TKN and 0.6 mg L^{-1} of o-PO_4-P left in the batch system which indicates that the nutrients must be replenished while harvesting takes place.

The treatment of *Landoltia punctata 0128* on 1.6 g L^{-1} Hoagland E-medium was successful. The use of this control provided an extreme environment to observe how the duckweed strains would react. *Landoltia punctata 0128* managed to handle the high concentration of nutrients favorably. However, the other two duckweed strains could not handle the recommended concentration of 1.6 g L^{-1} of Hoagland E-Medium.

The untreated controls, in which filamentous algae grew, exhibited the nutrient rate constants that were lower than the treated duckweed samples. Algal reduction would have been a major contributor of N and P removal in the control batch systems due to the high light intensity and low concentration of nutrients. The duckweeds mat structure in the batch samples would have prevented much of the evaporation and volatilization of ammonia. Ammonia volatilization is present in agricultural lagoon systems. The use of a floating macroalgae such as *Landoltia punctata 0128* would be a practical implementation to improve lagoon systems in the reduction of ammonia volatilization to the environment.

To control the inhibition of duckweed growth, EC, pH, DO, and N content would have to be controlled using wastewater monitoring equipment such as a probe or sensor. The EC depends on how much nutrients are introduced into the system. If the nutrients meet a TN content of 57 mg L^{-1}, the EC should be approximately 1,066 µS cm^{-1}. If the whole surface area of the confinement area is covered with duckweed the DO should stay in the range of 1.5 to 2.0 mg L^{-1}, reducing the filamentous growth in the system. The pH must stay in the range of 6.5 to 7.5. Buffered solutions such as volatile fatty acids (VFA), acetic acid may have to be introduced to the system to bring the pH down.

The overall performance of the *Landoltia punctata 0128* treatment indicates that it can thrive in a system that incorporates AD dairy manure as a nutrient source for dairy wastewater treatment. Visual observations indicated that *Landoltia punctata 0128* covers surface areas quite efficiently with its larger fronds size and quickly adapts to the environment to which it is placed. To assess the potential of such as system for year-round performance, further modeling and investigation are needed.

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Chapter 4 : The Cultivation of *Landoltia punctata* on Anaerobically Digested Dairy Manure for Nitrogen Uptake and Biomass Production

Abstract

The duckweed strain, *Landoltia punctata 0128* has been identified as a potential candidate for agricultural, lagoon-based wastewater treatment, supplied with anaerobically digested dairy manure for sustainable nutrient management, due to its high level of nutrient uptake and biomass production. This study was targeted at investigating batch system efficiency, which involved harvesting periods for *Landoltia punctata 0128,* based on relative growth rates (RGR) and nitrogen reduction rates of anaerobically digested (AD) dairy manure. A mass balance on the nitrogen reduction pathways of ammonia volatilization, nitrification/denitrification, sedimentation, and *Landoltia punctata 0128* uptake of nitrogen (N).

The RGR of *Landoltia punctata 0128* was assessed, based on the effects of light intensity, nutrient concentration, temperature and pH. Two light intensities were maintained in an environmental chamber at 3,000 and 1,000 lux. An air temperature was maintained 25°C, and a medium pH buffered at 7.0 using 5% v/v acetic acid. Relative growth rates were modeled by means of zero-order kinetics. The highest relative growth rate of *Landoltia punctata 0128* was 22.7 $gm^{-2}d^{-1}$ at 8 days of growth with a doubling time of 2.6 days.

The most practical relative growth rates were observed from dilution ratio 1:13 at 17.2 (±0.70) g m^{-2} d^{-1}, 16.4 (±0.65) g $m^{-2}d^{-1}$, and 14.6 (±0.60) g m^{-2} d^{-1} for *Landoltia punctata 0128* after 8, 16 and 24 days of growth respectively. These relative growth rates also produced doubling times of 3.5, 3.6, and 4.0 days. The nutrient uptake was characterized by the changes

of total nitrogen (TN) and total Kjeldahl nitrogen (TKN) in the digested dairy manure. This was assessed in three nutrient dilution ratios of 1:5, 1:13, and 1:27 v/v. The highest rates of nutrient recovery that produced the highest biomass production were found from the nutrient dilution ratio of 1:13 which accounts for TN and TKN of 114 mg L^{-1} and 107 mg L^{-1}. The rate constants for TN for dilution ratio of 1:13 were 0.223 (±0.001) d^{-1}, 0.104 (±0.002) d^{-1}, 0.084 (±0.003) d^{-1} at 8, 16, and 24 days. The rate constants for TKN for dilution ratio of 1:13 were 0.304 (±0.001) d^{-1}, 0.148 (±0.002) d^{-1} and 0.084 (±0.003) d^{-1} for 8, 16, and 24 days of growth.

4.1 Introduction

The United States is the world's largest producer of beef cattle, at 92 million head, as of 2016, up by 3% from 89.1 million head in 2015 (USDA, 2016). One of the major consequences of growing cattle for milk production or meat products is the accumulation of manure which contains urea (CH_4N_2O) and phosphorus (P) components (Horn *et al.,* 1994). The USDA estimates that 335 million tons of dry matter waste is produced from farms in the United States every year (Agriculture, 2012). Large quantities of nitrogen (N) and phosphorus (P) are entering water systems through the means of run-off from land applications and buildup of manure near waterways. This causes eutrophication in lakes and streams and contamination of groundwater (Adhikari *et al.,* 2014; Zhao *et al.,* 2014). The EPA provides some guidelines on what a nutrient management plan should entail, given the type of operation undertaken. For any livestock operation in which the operator wishes to discharge waste into a water system, a National Pollutant Discharge Elimination System (NPDES) is required for processing (EPA, 2017).

As part of a nutrient management plan, it is in the best interest of dairy farmers to re-use their recovery wastewater from agricultural waste for irrigation, in order to cut costs of fertilization and water use (Scott *et al.,* 2004). The first step in the nutrient management process is anaerobic digestion, where the product produced can be sold to crop farmers as a fertilizer. Anaerobic digestion is a nutrient management process that transforms inactive forms of nutrients into reactive groups, which plants can absorb (Sooknah *et al.,* 2004). The inactive forms of N and P are ammonia nitrogen (NH_3-N) and phosphorus pentoxide (P_2O_5). When raw dairy manure is processed by the means of anaerobic digestion in which the two inactive groups NH_3-N and P_2O_5 become ammonium nitrogen (NH_4-N) and ortho-phosphate-phosphorus (o-PO_4-P) or soluble reactive phosphorus (SRP) (Adhikari *et al.,* 2014). The processes behind anaerobic digestion are hydrolysis, acidogenesis, acetogenesis, and methenogenesis which occur in controlled environments (Metcalf and Eddy, 2014). The organic matter within the system is metabolized into carbon dioxide (CO_2) and methane (CH_4), which if flared must be reported to the EPA if more than 125,000 metric tons of CO_2 is released in a year (EPA, 2017). In a majority of cases, CH_4 and CO_2 are burned to generate electricity and sold to electricity companies for a profit.

To discharge the wastewater back into a water system, the concentration of N and P must be generally low. The problem is that anaerobic digestion does not remove the N and P from the system (Mohedano *et al.,* 2012). It only removes carbon (C) from the system, resulting in a low C:N ratio. Each stream or river has a limit on how much N and P can be discharged per year and that quantity is dictated by the findings in ecological studies which are enforced and regulated by the state to which the facility is producing manure (EPA, 2017).

The result of the anaerobic digestion process for dairy manure is high concentrations of N and P, which are easily metabolized by plants at diluted concentrations (Wang *et al.*, 2014). In AD dairy manure the concentration of N is typically higher than that of P, which results in an uneven balance for growing crops (Adhikari *et al*., 2014). A potential cost-effective operation for nutrient management for livestock waste reduction could involve a duckweed-based cultivation system to improve the assimilation of N and P from agricultural wastewater. These new systems would be termed duckweed stabilization lagoons (DSL) (Sooknah *et al.*, 2004; Adhikari *et al.*, 2014).

Within the family of *Lemnoideae,* duckweed belong to five different genera including *Lemna, Spirodela, Wolfia, Wolffiella* and *Landoltia* with 37 different species (Ziegler *et al.*, 2014). *Landoltia punctata* is a small (2-5mm), tropical, free-floating plant that actively reduces N and P from wastewater streams, grows rapidly due to its relatively high vegetative growth, and has the potential to be an aquatic species used for agricultural wastewater technologies such as DSLs (Soda *et al.*, 2013; Mohedano *et al.*, 2012). The implementation of a *Landoltia punctata*-based cultivation system (LCS) for agricultural wastewater treatment is a cost-effective method for attaining sustainability of livestock production.

The successful implementation of such a system would require a thorough knowledge of the cultivation parameters in *Landoltia punctata* and the growth and nutrient reduction kinetics behind the specific process undertaken. Kinetic models have been studied on DSL systems, which look at simply reducing nutrients, completely from the system, instead of generating large quantities of duckweed biomass, by determining the endpoint at which duckweed should be harvested (Mohedano *et al.*, 2012; Soda *et al.*, 2013). Many studies lack a breakdown of kinetic information needed to analyze harvesting periods, to stimulate plant

productivity, based on specific cultivation conditions that enable *Landoltia punctata* to grow efficiently. They likewise lack information based on the quantity of N reduced by each pathway of the batch system always only looking at the end result.

Nitrogen is the limiting reactant, when cultivating duckweed on AD livestock manures (Soda *et al.,* 2013). This is the case as N has multiple transport pathways which can result in a skewed view of the overall process. Nitrogen can take five reduction pathways, in a batch system: ammonia volatilization, nitrification, denitrification, microbial uptake, and sedimentation (Adhikari *et al.,* 2014). LCS systems have a great advantage for the problem of ammonia volatilization, since the mat-like structures of the duckweed keep the system mostly closed to the atmosphere (Mohedano *et al.,* 2012). Soda *et al.* (2013) modeled ammonia volatilization as evapotranspiration, which is the escape of water and ammonia vapor from the system, to the environment.

Commonly ammonia volatilization is the pathway for the removal of N batch systems removing approximately 355 to 1534 mg m^{-2} of NH_3-N increasing with temperature and pH (Middlebrooks *et al.,* 1999). Ammonia volatilization is mostly negligible in a LCS if the surface area is completely covered with duckweed in at least a single layer (Xu *et al.,* 2010). Mohedano *et al.* (2012) states that a pH of 7 and temperature of 20°C only accounts for 0.4% of ammonia in its volatile form. The mat-structures stabilize the N in solution while keeping the nutrient concentration constant due to minimal evaporation of water. However, when an aerobic lagoon system is used without the aid of duckweed large quantiles of the NH_3 can dissipate into the atmosphere as the pH of the system starts to increase (>9.0) with the temperature (>23°C) (Metcalf and Eddy, 2014).

Nitrification is the biological oxidation of ammonia nitrogen (NH_4-N) to nitrite nitrogen (NO_2-N), followed by the oxidation of NO_2-N to nitrate-nitrogen (NO_3-N), using microbes such as ammonia oxidizing bacteria (AOBs) and nitrite oxidizing bacteria (NOBs) (McLean *et al.*, 2000). NOBs and AOBs are aerobic chemoautotrophs, because they use CO_2 for their C source and require dissolved oxygen to oxidize inorganic compounds such as NH_4-N and NO_2-N, to obtain cell energy, to reproduce (Metcalf and Eddy, 2014). Nitrification has several parameters to which it can be identified, within a batch system. When identifying whether nitrification is taking place, the first indication is an increase in the concentration of NO_3-N, which naturally occurs during the process (Ndegwa *et al.,* 2007). The second indication that nitrification is taking place is an increase in the pH content and a decrease in dissolved oxygen (DO) content (Metcalf and Eddy 2014; Ndegwa *et al.,* 2007). When oxygen is consumed within the system, via nitrification the buffering capacity of the solution known as alkalinity, begins to weaken. When the alkalinity of a solution decreases, it is easy for the pH of the solution to increase, and become basic (pH>7). The third and final indication of nitrification is the consumption of oxygen. If the dissolved oxygen content is low within the system and becomes anaerobic or anoxic, with NO_3-N as the electron acceptor, which is a good indication of nitrification (Metcalf and Eddy 2014). If a NPDES permit requires NH_3 to be removed from the system, then nitrification is the first step in the process.

Denitrification, within a batch system, occurs with the reduction of NO_3-N and NO_2-N to nitrogen gas (N_2). This completes the N cycle in a wastewater treatment process, however, denitrification requires very specific conditions in order to transform NO_3-N to N_2. In an activated sludge system, NO_3-N must be transferred to an anoxic basin, where there is no oxygen present, so that the facultative aerobic organisms only feed on the NO_3-N, the electron

acceptor, to transform it to N_2. Mohedano *et al.* (2012) claimed that nitrification and denitrification occurred in their DSL system. The only place in a lagoon system for denitrification to occur would be in the anoxic zone at the bottom of the lagoon which is depicted in Figure 2-1. At high concentrations of nutrients, the nitrification/denitrification process consumed more N and the biomass consumed less. However, when the concentration of nutrients was low, in their secondary basin, the nitrification/denitrification rate was lower and more nutrients transferred to the duckweed biomass. It has also been found, that in batch systems, that *Landoltia punctata* will metabolize NO_3-N with NH_4-N present (Cedergreen *et al.*, 2002; Fang *et al.,* 2007).

N uptake within the batch system will be due to duckweed and algal vegetative growth, nitrification/denitrification and sedimentation (Mohedano *et al.,* 2012). Duckweed and filamentous green algae will compete for nutrients within the system. If the concentration of filamentous algae is too high, they will inhibit the growth of duckweed. Solid sedimentation will settle out of solution at the bottom of the batch system. If the depth of the sedimentation of the batch system is shallow the nutrients will actively travel between full suspension and settling through the process of diffusion. When the batch system is deep the solids which contain most of the N will settle to the bottom and not be transported to the top unless agitated. Which have the option of re-use on agricultural land for fertilization. With the root systems of duckweed being very short the shallow option is more feasible for active growth.

In a study conducted by Mohedano *et al.* (2012), the researchers looked at the nutrient reduction efficiency of their DSL systems and produced a mass balance equation for the pathways of N reduction. They came up with a mass balance on N which accounts for the five pathways that N can be removed from a lagoon system. They found that

nitrification/denitrification and biomass uptake attributed to 72% and 28%, respectively at 47.3 (±22.6) mg L^{-1} of TKN. In the second pond with the lower concentration of TKN at 14.1 (±10.6), it was observed that duckweed up took most of the TKN at 96% and 4% for nitrification/denitrification. It is important to understand that when operating a DSL system that the N is assimilated by the duckweed to actively produce biomass.

In the study conducted by Zhao *et al.* (2014), the light source used was fluorescent lighting with a photoperiod of 16 hours of light and 8 hours of dark with the light intensities 10,000, 5,000 lux and 2,000 lux. The RGRs observed for 10,000 lux and 5,000 lux were very close in the study at 3.25 g m^{-2} d^{-1} and 3.0 g m^{-2} d^{-1}, respectably for *Landoltia punctata*. Which indicates that a light intensity of 7,500 lux could be the optimal light intensity for duckweed growth. Soda *et al.* (2013) confirms the use of a light intensity of 7,500 lux at 16 hours of daylight and 8 hours of darkness resulting in a RGR of 86-160 g-wet $m^{-2}d^{-1}$ of *Wolffia arrhiza*.

It is known that light intensity, nutrient concentration, pH, and an optimized harvesting period are the four main factors that must be controlled for *Landoltia punctata* to produce biomass that doubles every 2-5 days. Light intensity controls duckweed and algal growth within batch systems. As discussed in Chapter 3, low concentrations (< 57 mg L^{-1} TN and < 7 mg L^{-1} TP) of AD dairy manure at high light intensities (>7500 lux) resulted in rapid vegetative growth and low algal growth within a batch system. Preliminary studies in Chapter 3 also found that a pH of 6.5 was optimal for duckweed growth. In Chapter 3, an investigation into the minimum and maximum harvesting period reveled that *Landoltia punctata* initially can be harvested at a maximum cultivation period of 16 days at initial startup and a minimum of 4.5 days after the duckweed has had time to adapt itself to the environment and is having its nutrients replenished

or mixed after every harvest. Further investigation is needed to quantify nutrient reduction and biomass growth for the light intensity 3,000 lux which is modeled for the fall months.

The present study was designed to compare the potential of two light intensities and three dilution ratios in reducing N content of effluent from an anaerobic digester receiving flushed manure from a dairy farm using the duckweed strain *Landoltia punctata 0128.* These experiments will establish and compare the biomass yield, relative growth rates, N removal rates, and potential harvesting periods within the cultured media when subjected to different nutrient concentrations when treated with *Landoltia punctata 0128*. The specific objectives were to: (1) Establish relative growth rates (RGRs) and nutrient reduction rates for total nitrogen (TN) and total Kjeldahl nitrogen (TKN) for optimizing a harvesting period; (2) identify a batch system efficiency model of N when *Landoltia punctata 0128* treats AD dairy manure in batch samples.

4.2 Methods and Materials

As noted in Chapter 3, three strains of duckweed, *Landoltia punctata 0128, Lemna gibba 7589,* and *Lemna minuta 9517,* were collected from Rutgers Duckweed Stock Cooperative and cultivated on AD dairy manure at dilution ratios 1:18 and 1:27, to determine growth and nutrient reduction rates. *Landoltia punctata 0128* was found to significantly reduce N and P from AD dairy manure at a dilution ratio of 1:27, as characterized in Table 3-2. *Landoltia punctata 0128* was cultivated at a light intensity of 10,000 lux, an air temperature of 25°C, a pH of 6.5, and an EC of 1066 µS cm^{-1}. *Landoltia punctata 0128* was found to significantly reduce N and P from AD dairy manure and accumulate biomass over a period of 28 days at the beforementioned parameters. However, further experimentation is needed to

determine the parameters for *Landoltia punctata 0128* to be cultivated at lower light intensities, to model that of fall and winter months.

4.2.1 Batch tests

Batch tests were conducted within an environmental growth chamber, at light intensities 3,000 and 1,000 lux and at a photoperiod of 16:8 (light: dark). The experiment consisted of a total of 18 PET sample containers, with a total volume of 200 mL of deionized water and AD dairy manure. Triplicate dilution ratios of 1:5, 1:13, and 1:27 were conducted on *Landoltia punctata 0128,* to analyze how they react to each dilution ratio at the given light intensity. The dilution ratios consisted of a TN basis concentration of 266, 114, and 57 mg L^{-1}, respectively, which was calculated in Table 3-2 in Chapter 3.

Each concentration of AD dairy manure was initially prepared in a 4L container. Each 4L container held a different dilution ratio of a mixture of deionized water and AD dairy manure at 1:5, 1:13, and 1:27. The initial pH values of the mixtures were 8.11, 8.12, and 8.19, respectively. These pH values were then adjusted to 6.5, by adding the volumes 40, 35, and 25 mL, respectively, using 5% v/v acetic acid. With the adjusted pH values, for the dilution ratios 1:5, 1:13, and 1:27, the initial electric conductivity, dissolved oxygen, and medium temperature were measured at 1,660 µS cm^{-1}, 4.7 mg L^{-1}, and 21.4°C, 1331 µS cm^{-1}, 3.8 mg L^{-1}, and 23.4°C, and 1066 µS cm^{-1}, 4.5 mg L^{-1}, and 23.6°C, respectively. The dilutions were then transferred to PET containers with the dimensions of 114mm × 86mm × 102mm (L × H × W) with a surface area of 0.0116 m^2 and a total volume of 300 mL.

4.2.2 Sampling and analysis

Every 72 hours random grab samples were taken from different locations within the PET containers for nutrient analysis. The whole period of experimentation lasted 24 days, with

there being 6 days of testing on days 0, 4, 8, 16, 20, and 24. Over this period, medium samples were analyzed for medium temperature, TN, TKN, NO_3-N+NO_2-N, pH, DO, and EC. The nutrient parameters were analyzed using a spectrophotometer (DR5000, Hach, USA). The Hach method used to obtain the data was Method 10214 for TN, TKN, and NO_3-N+NO_2-N. A pH, dissolved oxygen (DO), and electric conductivity (EC), analysis was conducted using a Sper Scientific 850049 water meter kit. The wet and dry weights of the duckweed in each sample were measured immediately after sampling, with a Mettler AE 260 Delta Range balance. Free water on the duckweed was removed with cheese cloth, before measurement. At the end of the 24th day, the wet duckweed was dried in an oven at 80°C for 24 hours to obtain a dry weight.

To observe the batch efficiency of the removal of N, within the batch samples, a mass balance was performed, to account for N content among the different processes. A N mass balance was performed in accordance with Equation (4-1), where total nitrogen removed (TNR) was obtained from the sum of biomass removal (BMR), ammonia volatilization (NH_3), nitrogen sedimentation (NS), and the nitrification and denitrification process (N_2) (Mohedano *et al.*, 2012).

$$TNR = BMR + NH_3 + NS + N_2 \qquad \text{(Equation 4-1)}$$

The TNR of the batch samples was observed by measuring total nitrogen (TN) content of the batch samples, as N decreased over time. Biomass removal of N was analyzed at the University of Idaho's Analytical Science Laboratory. Ammonia volatilization was calculated by first estimating the ammonia dissociation constant (pKa), of the solution, by measuring the average medium temperature in the batch systems using Equation (4-2) (Metcalf and Eddy 2014). The resulting percent ammonia nitrogen (NH_3-N) content was estimated by measuring

the average pH of the medium solution in the batch systems in Equation (4-3) (Metcalf and Eddy 2014). However, Mohedano *et al.* (2012) states that a pH of 7 and temperature of 20°C only accounts for 0.4% of ammonia in its volatile form indicating that ammonia volatilization will be negligible under low pH and temperature. Nitrification and denitrification (N_2) is observed by the increase and decrease of NO_3-N and NO_2-N within the batch systems. Nutrient sedimentation (NS) is solved for, in Equation 4-1, to determine how much manure can be reused on agricultural land.

$$pKa = 0.09108 + \frac{2729.2}{273.2 + T} \qquad \text{(Equation 4-1)}$$

$$\%NH_3 = \frac{100}{1 + 10^{(pKa - pH)}} \qquad \text{(Equation 4-2)}$$

4.2.3 Statistical analysis

The performance of the duckweed cultures at 10,000 lux was used as a control to compare to the duckweed cultures at 3,000 and 1,000 lux by performing a Students t-test to determine if there were significant differences between the k values using the method of independent samples and unequal variances using a two-tailed p-value. All statements were based on a statistical significance of $P<0.05$.

Relative growth rates (RGR) are established from zero-order kinetics which in turn establish the doubling times (DT). Simple linearized regression equations were calculated to obtain rate constants and standard errors. Relative growth rates (RGR) were estimated from

linear regressions established in Chapter 3 using Equation (3-4). Equation (3-5) was used to estimate first order rate equations for nutrient reductions established in Chapter 3.

$$C_t = -k \cdot t + C_o \qquad \text{(Equation 3-4)}$$

$$A = A_o \cdot e^{-z \cdot t} \qquad \text{(Equation 3-5)}$$

4.3 Results and Discussion

4.3.1 Experimental conditions

The air temperature within the environmental growth chamber was maintained at 25°C which resulted in a 20.3°C of the average medium temperature. The maximum and minimum medium temperatures observed were 23.3°C and 18.0°C, respectively. The PET containers were randomly placed on racks of the environmental growth chamber so that each sample was subjected to the same averaged light intensity. During the experiment, the maximum pH observed for dilution ratios 1:5, 1:13, and 1:27 was 9.48, 9.95, and 10.01, respectively. The minimum pH that was observed from the dilution ratios was 6.5 which was the starting pH value. The average pH values for dilution ratios 1:5, 1:13, and 1:27 were 8.18, 8.30, and 8.41 which were more ideal than the average pH values discussed in Chapter 3. Relative growth rates were estimated from linear regressions using Equation (3-4). See Chapter 3 for definition of variables.

4.3.2 Biomass production

4.3.2.1 Effect of light intensity on RGR

In the previous chapter, it was found that *Landoltia punctata 0128,* cultivated on AD dairy manure at dilution ratio 1:27 (57 mg L^{-1} of TN) and a light intensity of 10,000 lux had a RGR of 13.3 g m^{-2} d^{-1} was discussed. Figure 4-1 shows the RGRs at the two dilution ratios 1:13 and 1:27 at the three light intensities 10,000, 3,000, and 1,000 lux. When comparing the control to dilution ratio 1:13 at a light intensity of 3,000 lux it was found that the two RGRs were not significantly different. When comparing the dilution ratio 1:27 at 3,000 lux to the control it was found that they were significantly different. When *Landoltia punctata 0128* was cultivated, at a light intensity of 1,000 lux and a dilution ratio of 1:27 it was found that there was no significant difference between the treatment and control. When comparing the dilution ratio 1:13 to the control it was found that they were significantly different.

In Chapter 3, we see that the dilution ratio 1:13 failed to grow at a light intensity of 10,000 lux. It is hypothesized that this was due to the high pH, EC, and DO content of the AD dairy manure. That hypothesis was confirmed, in this study, because the pH of the system was more controlled using more buffering solution. Algal activity was also controlled using an initial surface area of 50% coverage instead of 25%. The lower light intensity also decreased algal activity, within the batch systems evidenced by the lack of algal growth on the surface of the containers and on the surface of the medium. The EC within the system decreased slightly within the batch systems and DO content of the medium remained constant.

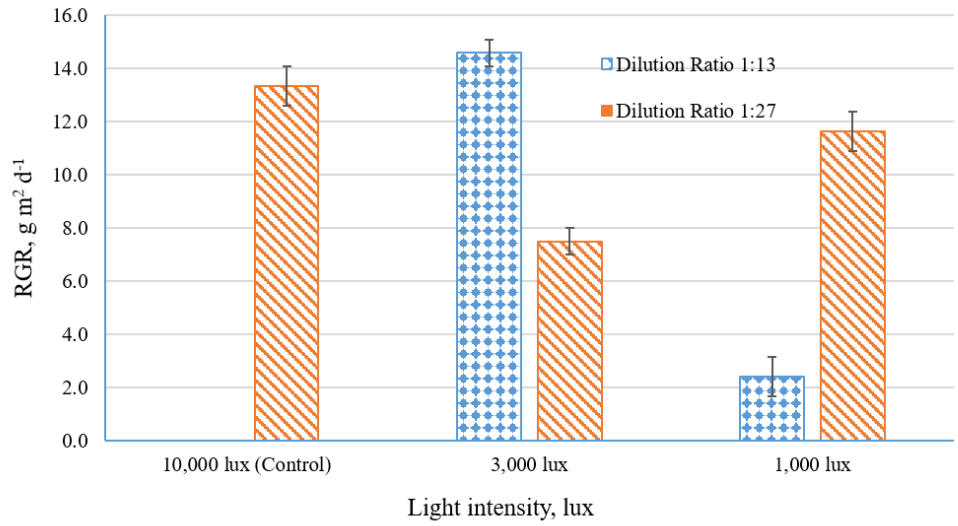

Figure 4-1 Relative growth rates of L. punctata at light intensities 10,000, 3,000 and 1,000 lux on dilution ratios 1:13 and 1:27 of AD dairy manure at period of 28 days (10,000 lux) and 24 days (3,000 lux and 1,000 lux).

Cultivation of *Landoltia punctata 0128,* at a lower light intensity of 3,000 lux and a dilution ratio 1:13 proved to match that of the control in RGR, which could mean that higher concentrations of nutrients are needed under lower light intensities for *Landoltia punctata 0128* to ideally grow. However, at a light intensity of 1,000 lux, the dilution ratio 1:13 was not favorable but the dilution ratio 1:27 was favorable. This could indicate that not enough energy has been inducted into the system to stimulate the cultivation of *Landoltia punctata 0128* at a dilution ratio of 1:13, however, a dilution ratio of 1:27 could generate growth at a light intensity of 1,000 lux.

Under light intensity 3,000 lux it is possible that *Landoltia punctata 0128* can not only fix C in the form of CO_2 as a result of photosynthesis, but it can also assimilate C from the AD dairy manure in the form of sugars which could indicate how the duckweed was able to match the growing capacity of the higher light intensity. Since a higher concentration of nutrients was

present in the system, that could indicate why dilution ratio 1:13 produced an increase in growth at 3,000 lux and dilution ratio 1:27 resulted in a decrease. However, with light intensity 1,000 lux, again not enough energy was again available, to push the duckweed into metabolizing the excess sugar content, in the lower dilution ratio, thereby giving a lower RGR. In the former case, enough energy must have been present, in photosynthesis to stimulate the metabolism of N and P assimilation which resulted in a higher RGR from dilution ratio 1:27, at the light intensity of 1,000 lux. Consequently, the light intensity 3,000 lux will be further investigated throughout this study starting with the visual observations of the batch samples.

4.3.2.2 Visual observations

Verma *et al.* (2015) stated that initially covering the surface area at 40%, reduced the most nutrients in a batch system. To keep the growth of filamentous algae at bay, for the benefit of the higher concentrations of AD dairy manure, the batch PET sample containers were initially inoculated at 50%, of the surface area, to promote biomass accumulation and nutrient reduction. *Landoltia punctata 0128* began with a healthy dark green pigmentation indicating a healthy duckweed strain on each of the dilution ratios. The medium colors, for the dilution ratios, were a dark brown for 1:5, a lighter shade of brown for 1:13, and the lightest shade of brown for 1:27.

After 4 days of growth, *Landoltia punctata 0128* noticeably doubled its biomass for dilution ratios 1:13 and 1:27, but it did not for dilution ratio 1:5. All the dilution ratios kept their original pigmentation of dark green. A thin film of filamentous algae was seen on the surface of the mediums. After 8 days of growth, *Landoltia punctata 0128* made up over ¾ of the surface area of the PET containers for dilution ratios 1:13 and 1:27. Dilution ratio 1:5 had only taken

up a little over half of the surface area. There were a few bubbles forming on the surface of the medium and all the dilution ratios had a light green color mixed in with the solution.

After 16 days of growth, there was a clear difference between the dilution ratios. Dilution ratios 1:13 and 1:27 dominated in biomass growth and dilution ratio 1:5 inhibited the growth of *Landoltia punctata 0128.* Both dilution ratio 1:13 and 1:27 produced the most biomass with the surface area all filled up for two of the three sample containers. Dilution ratio 1:5 had just started to produce biomass. Dilution ratios 1:13 and 1:27 were harvested during this time to promote a faster growth rate.

After 20 days of growth, dilution ratios 1:13 and 1:27 were actively growing after being harvested. Dilution ratio 1:5 still had problems but continued slowly growing and remained a dark shade of green pigmentation. At day 24, the first sign of white pigmentation appeared on dilution ratio 1:13 and 1:27 indicating that there are not enough nutrients for the plant to keep growing effectively. The duckweed biomass for dilution ratios 1:13 and 1:27 almost completely covered the surface of the PET containers for a second time after being harvested once.

4.3.2.3 Modeling biomass accumulation

Biomass accumulation was modeled using Equation (3-4) in Chapter 3, to establish the RGRs of *Landoltia punctata 0128,* at the dilution ratios 1:5, 1:13, and 1:27. Each point was a triplicate mean value, with a standard deviation. Growth curves were established to measure duckweed RGRs at three points in time 8, 16, and 24 days. Figure 4-2 shows the pathway of *Landoltia punctata 0128* biomass density over time.

In Figure 4-2, a lag phase was not observed in the batch samples 1:13 and 1:27 due to a pre-adaption period of *Landoltia punctata 0128*. However, a lag phase is observed for dilution ratio 1:5 at 0 to 8 days. Figure 4-2 identifies only two phases of growth the exponential and

stationary phases. *Landoltia punctata* 0128 was harvested before the death phase could start which was indicated by white pigmentation on the fronds at day 24. The exponential phase for dilution ratio 1:13 went from days 0 to 20 at a maximum biomass density of 275 g wet. m^{-2}. The exponential phase for dilution ratio 1:27 went from 0 to 8 days at a maximum biomass density of approximately 175 g wet. m^{-2}. The exponential phase for dilution ratio 1:5 started at day 8 and went to day 24 at a maximum biomass density of 175 g wet. m^{-2}. The cultivation points of 8,16 and 24 are based on the exponential and stationary phases.

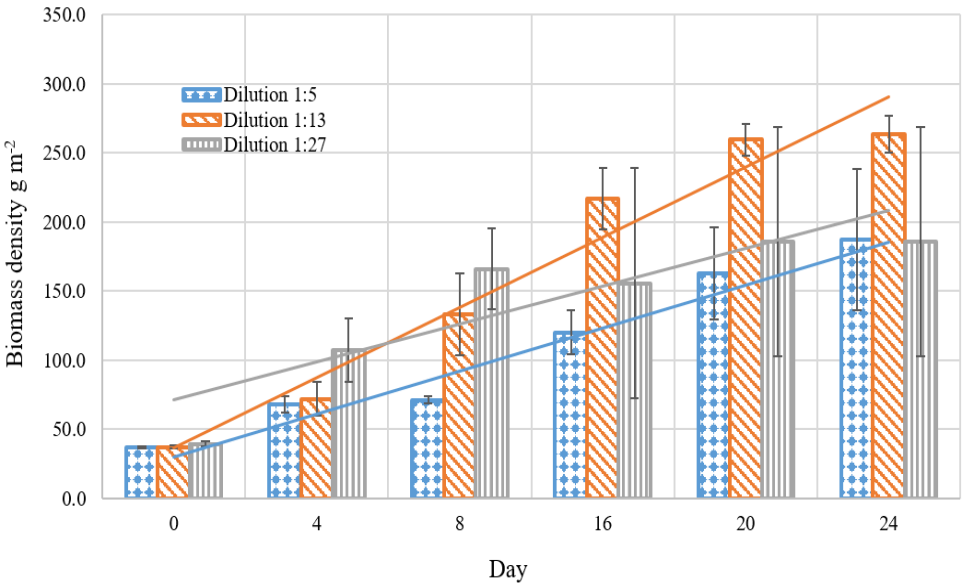

Figure 4-2 Biomass density of L. punctata at dilution ratio 1:5, 1:13, and 1:27 on anaerobically digested dairy manure modeled using zero-order kinetics for a 24-day batch growth period

Biomass density was also used to establish the regression equations on dilution ratios 1:5, 1:13, and 1:27 of AD dairy manure to predict the RGRs of each batch system. The zero-order regression equations established from Equation (3-4) in Chapter 3 can be found in Table 4-1 along with the R^2 values to establish the predictive capability of the model. After 8 days of

growth dilution ratio 1:27 established a regression equation with the highest slope value with a R^2 value at 0.9983. After 16 days of growth, dilution ratio 1:13 had the highest slope value and a R^2 value at 0.9983 and continued after 24 days of growth, with the highest slope value and R^2 value at 0.9773.

The slopes of the equations in Table 4-1 denoted the RGR value of the growth period. Table 4-2 provides a summary for RGR and doubling times (DT) of the three dilution ratios observed. At 8 days into growth, dilution ratio 1:5 was found not to be significant when compared to dilution ratio 1:27 and 10,000 lux of *Landoltia punctata 0128* in Chapter 3. The dilution ratios 1:13 and 1:27 after 8 days of growth were found to be significant at 17.2 and 22.7 g wet wt. m^{-2} d^{-1}, respectively. The DT values were derived using the RGRs established from Equation (3-4). When the RGRs were found, they were inserted into Equation (3-3) to solve for DT.

Table 4-1 Regression between biomass density and test duration at 8, 16, and 24-days at dilution ratios 1:5, 1:13, and 1:27 of AD dairy manure

Dilution	**Day**	**Regression equations**	**R^2**
1:5	8	y = 6.1408x + 59.244	0.8228
1:13	8	y = 17.207x + 46.653	0.9753
1:27	8	y = 22.695x + 58.396	0.9983
1:5	16	y = 7.0772x + 56.331	0.9595
1:13	16	y = 16.443x + 49.028	0.9931
1:27	16	y = 9.9192x + 98.144	0.6695
1:5	24	y = 8.8368x + 48.118	0.9741
1:13	24	y = 14.561x + 59.553	0.9773
1:27	24	y = 7.4915x + 110.47	0.7510

Table 4-2 Relative growth rates of L. punctata after 8, 16, and 24 days of batch growth in dilution ratios 1:5, 1:13 and 1:27 of AD dairy manure [a]

Dilution	Day	RGR [b] (g wet. m^{-2} d^{-1})	DT (Days)
1:5	8	6.1_{nsc} (±0.45)	9.8
1:13	8	17.2 (±0.70)	3.5
1:27	8	22.7 (±0.65)	2.6
1:5	16	7.07_{nsc} (±0.40)	8.5
1:13	16	16.4 (±0.65)	3.6
1:27	16	9.9_{nsc} (±0.75)	6.0
1:5	24	8.8_{nsc} (±0.40)	6.8
1:13	24	14.6 (±0.60)	4.0
1:27	24	7.5 (±0.50)	8.0

[a] A student t-test was performed to compare k values of *Landoltia punctata 0128* at 10,000 lux and dilution ratio 1:27 with those of 3,000 lux and dilution rations 1:5, 1:13, and 1:27. All k values were significant (α=0.05), except those bearing the subscript: nsc (not significantly significant)

[b] Values in parentheses are standard errors of rate constants

The DT values after 8 days of growth for dilution ratios 1:13 and 1:27 were 3.6 and 2.6 days. At 16 days of growth only dilution ratio 1:13 was significant at a RGR of 16.4 g wet. m^{-2} d^{-1}. The DT value at the period of 16 days of growth was 3.6 days. At 24 days' dilution ratio 1:13 was still significant at 14.6 g wet. $m^{-2}d^{-1}$ while the other two dilution ratio were not. The DT value at the period of 24 days of growth was 4.0 days. The recommended time to harvest *Landoltia punctata 0128* on dilution ratio 1:27 under the light intensity of 3000 lux was 8 days. If a higher concentration of 1:13 is used the best time to harvest the duckweed is going to be at 16 days as the dilution ratio 1:13 is approximately double that of 1:27 in terms of TN.

4.3.3 Modeling N recovery

To model N recovery, from the batch systems treated by *Landoltia punctata 0128*, N reduction curves were produced, to estimate TN and TKN reduction rate constants. To begin, N recovery curves were established, by measuring TN and TKN in the liquid medium. The reductions for TN and TKN were compared for each dilution ratio and control, to determine significance. N rate constants were evaluated from periods of 0 to 8 days and 0 to 24 days. The

reduction of NO_3-N+NO_2-N were also observed although it was not modeled by first-order kinetics but instead percent NO_3-N+NO_2-N reduction.

4.3.3.1 TN reduction

TN reduction was modeled using Equation (3-5), to establish TN rate constants, of the duckweed treatment *Landoltia punctata 0128* at the dilution ratios 1:5, 1:13, and 1:27. The TN rate constants were modeled at three points, 8, 16, 24 days, to establish possible harvesting periods to further simulate *Landoltia punctata 0128* growth. The rate constants, modeled from TN will be used to establish the total mass of N, reduced from the AD dairy manure during the batch system process or the TNR in Equation (4-1) at each period. Figure 4-3 looks specifically at days 0 to 16, where the TN content began to slow down and go constant. However, the three periods were analyzed to determine when *Landoltia punctata 0128* should be harvested based on the rate constants of TN.

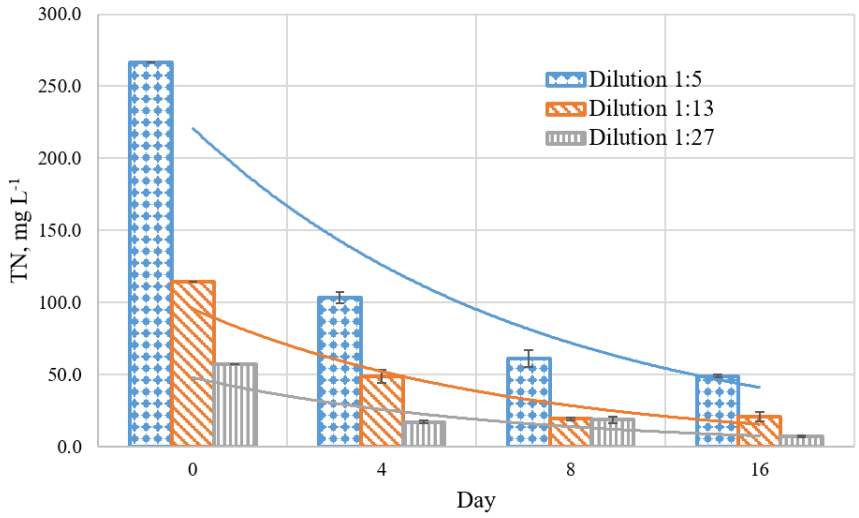

Figure 4-3 TN Reduction of L. punctata at dilution ratio 1:5, 1:13 and 1:27 on anaerobically digested dairy manure for 16-day batch growth

In Chapter 3, it was concluded that 16 days is the maximum amount of time the duckweed should be grown, on the dilution ratio 1:27, before it is harvested and that period is used as a baseline for this study. As time progresses, the nutrients can be released back into the water system, by the means of endogenous decay. If the *Landoltia punctata 0128* can be harvested earlier, in the process, it would be beneficial to do so, in order to extract the nutrients out of the system, reduce the duckweed density, thereby, providing more room for *Landoltia punctata 0128* to actively grow, generating more biomass, and allow for a more continuous system. An optimized harvesting period is directly related to the RGRs and the N recovery rates, when cultivating *Landoltia punctata 0128*. Figure 4-3 shows the exponential curves established to model TN reduction, from AD dairy manure at dilution ratios 1:5, 1:13, and 1:27.

Each point in Figure 4-3 is a triplicate mean value, with a standard deviation. The bar graphs in Figure 4-3 show the standard deviation at each point. Table 4-3 below shows the exponential regression equations used to model the reduction of TN, within the batch systems, at days 8, 16, and 24. Table 4-3 also shows the regression equations and R^2 values for the three specific periods. Dilution ratio 1:13 had the highest TN rate constant of the three dilution ratios, with a R^2 value of 0.9994 after 8 days of growth. After 16 days of growth, dilution ratio 1:27 had the highest rate content of the three dilution ratios with a R^2 value of 0.8636. After 24 days of growth, dilution ratio 1:5 had the highest rate constant of the three dilution ratios, with a R^2 value of 0.8944.

Table 4-3 Regression between TN reduction and test duration at 8, 16, and 24-days at dilution ratios 1:5, 1:13, and 1:27 of AD dairy manure

Dilution	Day	Regression equations	R^2
1:5	8	$y = 248.42e^{-0.184x}$	0.9732
1:13	8	$y = 115.69e^{-0.223x}$	0.9994
1:27	8	$y = 46.111e^{-0.14x}$	0.6959
1:5	16	$y = 190.64e^{-0.099x}$	0.8064
1:13	16	$y = 79.981e^{-0.104x}$	0.7226
1:27	16	$y = 42.789e^{-0.116x}$	0.8636
1:5	24	$y = 179.12e^{-0.088x}$	0.8944
1:13	24	$y = 71.889e^{-0.084x}$	0.8149
1:27	24	$y = 35.268e^{-0.078x}$	0.8047

4.3.3.2 TKN reduction

TKN reduction was also modeled using Equation (3-5), to establish TKN rate constants, of the duckweed treatment *Landoltia punctata 0128* at the dilution ratios 1:5, 1:13, and 1:27. The TKN rate constants were measured at three points: 8, 16, and 24 days. Figure 4-4 looks specifically at days 0 to 16, where the TKN content began to slow down and become constant. Each point was a triplicate mean value with a standard deviation. Table 4-4 shows the regression equations and R^2 values for the three specific periods.

TKN had the highest rate constant values as it measures NH_4-N, the N state in which plants actively uptake. After 8 days of growth, dilution ratio 1:13 had the highest rate constant of the three dilution ratios, with a R^2 value of 0.9696, at day 16 with a R^2 value of 0.7375. At 24 days of growth, dilution ratio 1:5 had the highest rate constant of the three dilution ratios, with a R^2 value of 0.8501. Through observation, after 8 days of active growth, both TN and TKN had the highest N rate constants. This creates the conclusion that after 8 days of growth

the nutrient content should be replenished, by a step feed system and *Landoltia punctata 0128* should be harvested with a built-in skimmer.

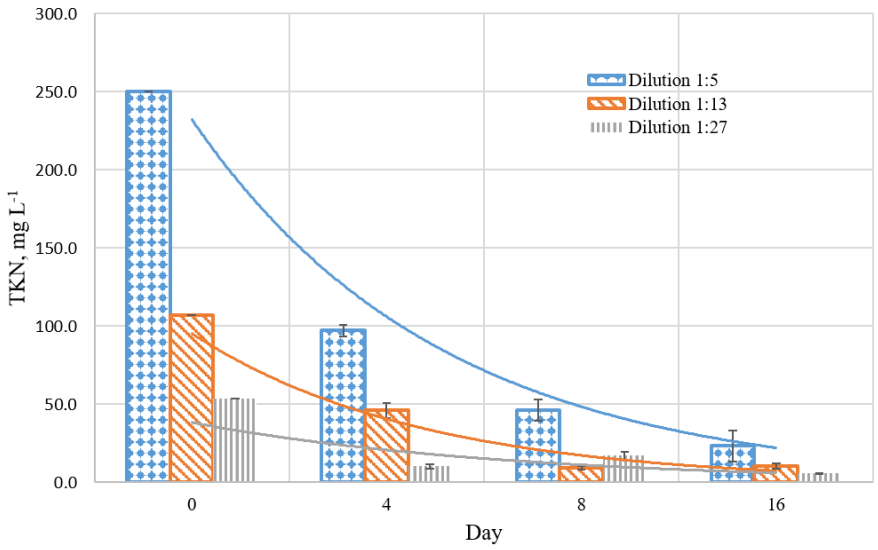

Figure 4-4 TKN Reduction of L. punctata at dilution ratio 1:5, 1:13 and 1:27 on anaerobically digested dairy manure for 16-day batch growth

Table 4-4 Regression between TKN reduction and test duration at 8, 16, and 24-days at dilution ratios 1:5, 1:13, and 1:27 of AD dairy manure

Dilution	**Day**	**Regression equations**	**R^2**
1:5	8	$y = 241.65e^{-0.211x}$	0.9951
1:13	8	$y = 121.32e^{-0.304x}$	0.9696
1:27	8	$y = 37.293e^{-0.143x}$	0.4534
1:5	16	$y = 196.41e^{-0.144x}$	0.9382
1:13	16	$y = 74.796e^{-0.148x}$	0.7375
1:27	16	$y = 34.218e^{-0.115x}$	0.6942
1:5	24	$y = 154.52e^{-0.095x}$	0.8501
1:13	24	$y = 55.604e^{-0.087x}$	0.6427
1:27	24	$y = 27.92e^{-0.075x}$	0.6729

4.3.3.3 Comparison of TN and TKN

TN and TKN followed a first order decay model and the significance between the dilution ratios and periods of measurement varied as compared to the N reduction of *Landoltia*

punctata 0128 on dilution ratio 1:27 at 10,000 lux, at a TN and TKN nutrient rate constant of 0.122 d^{-1} and 0.136 d^{-1}, respectively, as the control. A Student's t-test was used to assess significance between the treatments of 3,000 and 10,000 lux. Table 4-5 compares TN and TKN nutrient rate constants of L. punctata after 8, 16, and 24 days of batch growth in dilution ratios 1:5, 1:13 and 1:27 of AD dairy manure. For the reduction of TN, at a growth period of 8 days all dilution ratios were significant. At 16 and 24 days of growth, the dilution ratios were not significant. For the reduction of TKN at the growth periods of 8 and 16 days the dilution ratios were significant and the growth period of 24 days was not significant. The reduction of TKN will always be higher than TN because, TKN measures NH_4-N to which plants will actively uptake.

Table 4-5 TN and TKN nutrient rate constants of L. punctata after 8, 16, and 24 days of batch growth in dilution ratios 1:5, 1:13 and 1:27 of AD dairy manure [a, b]

Dilution	Day	k_{TN}	k_{TKN}
1:5	8	0.184 (±0.001)	0.211 (±0.001)
1:13	8	0.223 (±0.001)	0.304 (±0.001)
1:27	8	0.140 (±0.001)	0.143 (±0.001)
1:5	16	0.099_{nsc} (±0.002)	0.144 (±0.002)
1:13	16	0.104_{nsc} (±0.002)	0.148 (±0.002)
1:27	16	0.116_{nsc} (±0.002)	0.115_{nsc} (±0.002)
1:5	24	0.088_{nsc} (±0.004)	0.095_{nsc} (±0.003)
1:13	24	0.084_{nsc} (±0.003)	0.087_{nsc} (±0.003)
1:27	24	0.078_{nsc} (±0.004)	0.075_{nsc} (±0.003)

[a] Values in parentheses are standard errors of rate constants

[b] A student t-test was performed to compare k values of *Landoltia punctata 0128* at 10,000 lux and dilution ratio 1:27 with those of 3,000 lux and dilution rations 1:5, 1:13, and 1:27. All k values were significant (α=0.05), except those bearing the subscript: nsc (not significantly significant)

4.3.3.4 Nitrates and nitrites

Over the development of nutrient reduction, TN can fluctuate due to the conversion of NH_4-N to NO_3-N+NO_2-N, which indicates nitrification within the batch systems. Figure 4-5 shows the increase of NO_3-N+NO_2-N within the batch samples. From day 0 to 4, NO_3-N+NO_2-N reduced within the batch samples in each dilution ratio. From day 4 to 16, dilution ratios 1:5 and 1:13 experienced nitrification, as the concentration of NO_3-N+NO_2-N increases to a peak value of 31 and 10 mg L^{-1} for dilution ratios 1:5 and 1:13, respectively. From days 0 to 24, the concentration of NO_3-N+NO_2-N for dilution ratio 1:27 decreases over time, and no nitrification occurs.

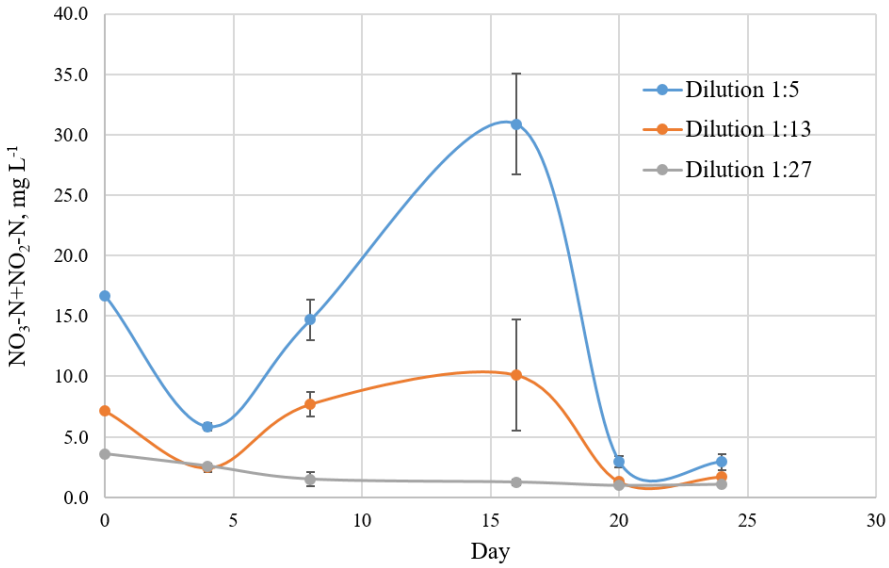

Figure 4-5 NO_3-N+NO_2-N reduction of L. punctata at dilution ratio 1:5, 1:13 and 1:27 on anaerobically digested dairy manure for 24-day batch growth

Figure 4-5 shows that nitrification occurred in the batch samples, for dilution ratios 1:5 and 1:13, with the increase in NO_3-N from days 4 to 16. Nitrification is a favorable process, when being used to reduce N in domestic wastewater systems, but it is not necessarily favorable when a DSL is being implemented due to the increase in NO_3-N within the system which is

unfavorable to the growth of duckweed. Duckweed and algae provide the oxygen required for nitrification to take place, because NOB and AOB require oxygen to grow. At lower dilution ratios, there were more nitrifying bacteria to populate in the batch samples, which resulted in a higher probability that nitrification took place. The concentration of NO_3-N within the batch systems was low, in comparison to the total nitrogen within the system and the concentration of NH_4-N was higher, which allowed for active growth during the period of 0 to 16 days.

From day 16 to 24 the NO_3-N+NO_2-N concentration of dilution ratios 1:5 and 1:13, decreased rapidly, indicating denitrification within the batch samples converting NO_3-N+NO_2-N into N_2. Visual observations showed bubbles in the solutions indicating either N_2 or O_2 or a combination of both. It is assumed that denitrification occurs within the batch systems in the facultative zone of the batch samples where facultative bacteria reduce NO_3-N+NO_2-N to N_2 depicted in Figure 2-1. It is possible that *Landoltia punctata 0128* and the filamentous algae also removed NO_3-N+NO_2-N from the batch samples after nitrification took place, but during that period (day 16 to 24) the duckweed were in the stationary phase of growth, so no biomass was being produced. At lower dilution ratios, there was a higher concentration of microbes within the batch samples which resulted in a higher probability of facultative bacteria being in the mix to reduce NO_3-N from the system. In this study the process of nitrification/denitrification was accounted for in Equation (4-1) as (N_2) and data was taken from Figure 4-5 for the analysis.

Table 4-6 shows the percent reduction of NO_3-N+NO_2-N within the batch systems. Dilution ratios 1:5, 1:13, and 1:27 showed reductions at 82.6%, 76.0%, and 72.0%, respectively. Table 4-6 also shows the initial and final concentrations of NO_3-N+NO_2-N. Initially, a high concentration was not present in the batch systems, until nitrification converted some of the

NH_4-N to NO_3-N+NO_2-N. To keep nitrification from occurring, it is recommended that a lower concentration of N is used to cultivate *Landoltia punctata 0128.* However, the concentrations of NO_3-N+NO_2-N after nitrification occurred in the batch systems, did not seem to affect the growth of *Landoltia punctata 0128,* since the RGRs at both light intensities observed were considered not significantly different, while no nitrification was observed, at a light intensity of 10,000 lux and a dilution ratio of 1:27.

Table 4-6 NO_3-N+NO_2-N percent reduction of L. punctata at dilution ratio 1:5, 1:13 and 1:27 on AD dairy manure for 24-day batch growth

Dilution	**Initial (mg L^{-1})**	**Final (mg L^{-1})**	**Total Reduction (mg L^{-1})**	**% NO_3-N+NO_2-N Reduction**
1:5	16.7	2.9	13.8	82.6 (±0.6)
1:13	7.1	1.7	5.4	76.0 (±0.2)
1:27	3.6	1.0	2.6	72.0 (±0.08)

4.3.3.5 N recovered from biomass

The N recovered by *Landoltia punctata 0128* was modeled using zero order kinetics, as it is modeled after the dried mass of *Landoltia punctata 0128.* The zero-order model was based on Equation (3-4) in Chapter 3. Zero-order kinetic rate constants were used to establish BMR in Equation (4-1). Figure 4-6 shows the TN of the *Landoltia punctata 0128* biomass, over the period of 24 days, using zero order kinetics. The bar graphs are used to show the standard deviation at each triplicate point and the lines indicate zero order kinetics. The rate constants in Table 4-7 are used to establish an N mass (mg) for BMR in Equation (4-1). For dilution ratio 1:13, Figure 4-6 shows that the maximum N that can be removed is 825 mg m^{-2} after 20 days of growth. At dilution ratio 1:5, the maximum N that can be removed at day 16 was

approximately 380 mg m^{-2} and dilution ratio 1:27 was 350 mg m^{-2} at day 8. Dilution ratio 1:13 removed the most N from the dairy medium after 20 days of growth. When observing Table 4-7, dilution ratio 1:13 has the highest slope at day 8, 16, and 24.

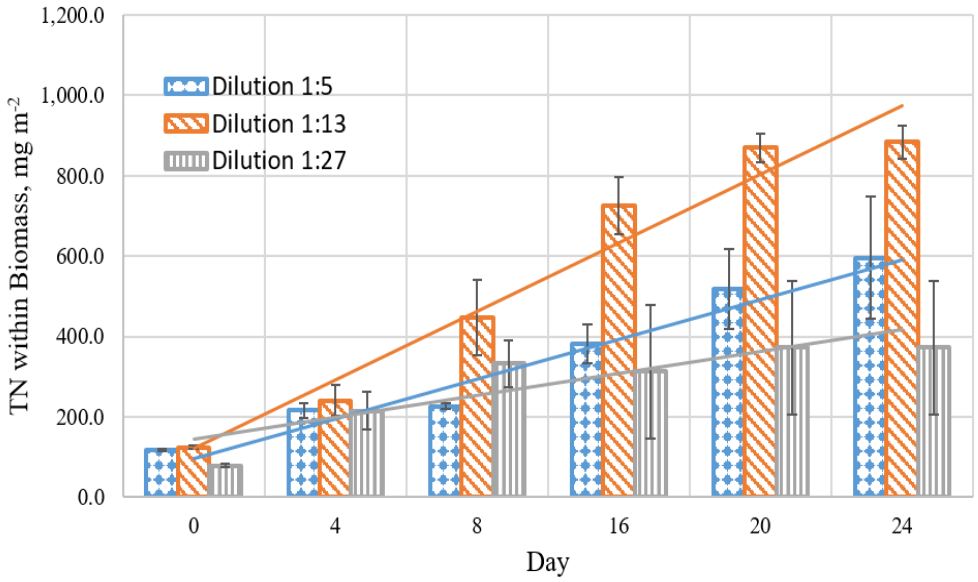

Figure 4-6 TN within L. punctata biomass at dilution ratio 1:5, 1:13, and 1:27 on anaerobically digested dairy manure for a 24-day batch growth

Table 4-7 Zero order regression between TN within L. punctata biomass and test duration at 8, 16, and 24-days at dilution ratios 1:5, 1:13, and 1:27 of AD dairy manure

Dilution	**Day**	**Zero order regression equations**	**R^2**
1:5	8	y = 13.633x + 131.52	0.8228
1:13	8	y = 40.263x + 109.17	0.9753
1:27	8	y = 31.774x + 81.754	0.9983
1:5	16	y = 15.711x + 125.05	0.9595
1:13	16	y = 38.477x + 114.72	0.9931
1:27	16	y = 13.887x + 137.40	0.6695
1:5	24	y = 19.618x + 106.82	0.9741
1:13	24	y = 34.072x + 139.35	0.9773
1:27	24	y = 10.488x + 154.66	0.7510

Studies have used zero order kinetics to establish nutrient productivities, from the duckweed species' (Cheng *et al.*, 2001). In Table 4-8, the most N recovered in the biomass was established at day 8 at a dilution ratio of 1:13 with a N recovery rate of 40.3 mg $m^{-2}d^{-1}$. As the days increase with dilution ratio 1:13, the N recovery rate decreases. However, at day 16 the N recovery rate was within the standard deviation range of day 8 at 38.5 mg m^{-2} d^{-1} (±0.65) indicating that the same N recovery rate was occurred at day 16 which means that there were enough nutrients in the system to keep *Landoltia punctata 0128* actively accumulating N from the system. Dilution ratio 1:27 saw a N recovery rate of 31.8 mg m^{-2} d^{-1} in the first 8 days of growth. However, the dilution ratio was not able to maintain the accumulation of biomass to day 16. A dramatic decrease in the uptake of N in the system occurred after 8 days of growth. Dilution ratio 1:5 did not begin to accumulate N until day 24 at an N recover rate of 19.6 m^{-2} d^{-1}.

Table 4-8 Zero order TN rate constants of (dried biomass) L. punctata after 8, 16, and 24 days of batch growth in dilution ratios 1:5, 1:13 and 1:27 of AD dairy manure

Dilution	**Day**	**Zero order (mg $m^{-2}d^{-1}$)**
1:5	8	13.6 (±0.45)
1:13	8	40.3 (±0.75)
1:27	8	31.8 (±0.65)
1:5	16	15.7 (±0.40)
1:13	16	38.5 (±0.65)
1:27	16	13.9 (±0.75)
1:5	24	19.6 (±0.40)
1:13	24	34.1 (±0.65)
1:27	24	10.5 (±0.55)

4.3.3.7 N sedimentation

In batch systems, sedimentation removes about 70% of the nutrients from the aerobic zone of the batch system. The remaining 30% of the nutrients can be assimilated by algae or duckweed, be released via ammonia volatilization, or undergo nitrification/denitrification from

bacteria within the system (Metcalf and Eddy, 2014). In a batch system, the solids from the AD dairy manure will settle out of solution and take N content with it. A portion of the N content will remain in solution and the duckweed plants will feed on the content in the aerobic zone. The sediment that is left behind can be used for land applications since the N content will be at a lower concentration than when it started.

Multiple runs, however, may be required to bring the N to the concentration required. A recirculation system may be needed to pump the solids from the bottom of the batch system to the top, to provide more access for duckweed to absorb the nutrients. There will be some diffusion that takes place between the bottom of the batch system and the top, although, if the tank is deep enough, not much diffusion will take place. Sedimentation is important, as it allows for the reuse of the dairy manure, for crop land. *Landoltia punctata 0128* can reduce the N concentration, in small increments, so that the N within the system can be decreased to match the concentration of P within the system to provide a balance between the nutrients.

Figure 4-7 shows the N content in the sedimentation, at each period of growth. Equation (4-1) was used to solve for the NS concentration. At each period, the medium was mixed and allowed to settle, allowing *Landoltia punctata 0128* to have an equal chance at removing N from the system each time. Table 4-9 shows the quantities of sedimentation, at the points of 8, 16, and 24 days of growth along with the standard deviations. The highest sedimentation concentration came from day 16, from dilution ratio 1:5 at 167.4 mg L^{-1} of TN. The lowest sedimentation came from dilution ratio 1:27, at day 24 at 19.3 mg L^{-1}. The concentration of N sedimentation increased with time for dilution ratio 1:13. However, with dilution ratio 1:27 the highest N sedimentation was at day 16 and the content was reduced further after that point.

Dilution ratio 1:5 saw a similar pattern, where at day 16, the N sedimentation content is higher than at day 24.

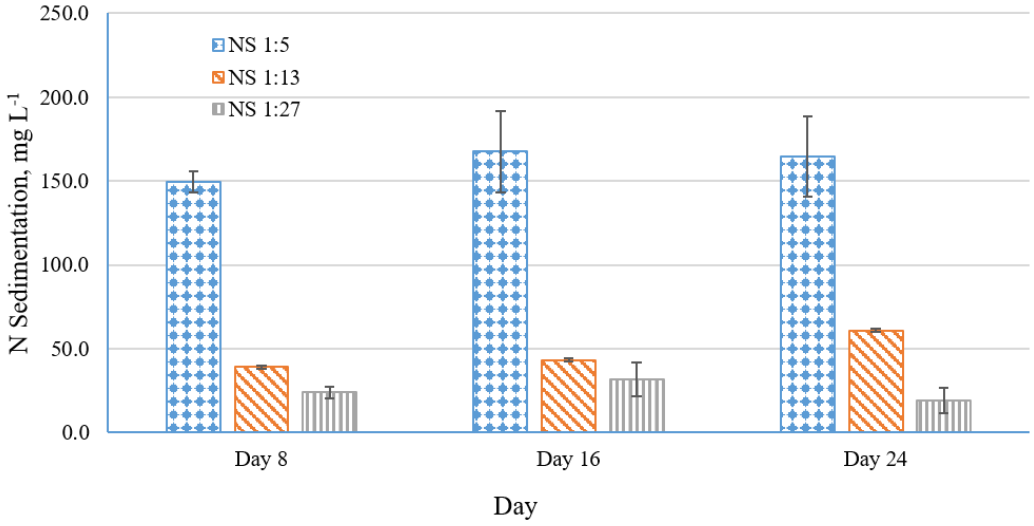

Figure 4-7 Sedimentation of N at 8, 16, and 24-days at dilution ratios 1:5, 1:13, and 1:27 of anaerobically digested dairy manure

Table 4-9 Sedimentation of N at 8, 16, and 24-days at dilution ratios 1:5, 1:13, and 1:27 of AD dairy manure

Dilution	**Day**	**N Sedimentation (mg L^{-1})**
1:5	8	149.7 (±6.3)
1:13	8	39.2 (±14.5)
1:27	8	24.0 (±3.35)
1:5	16	167.4 (±24.2)
1:13	16	43.1 (±5.0)
1:27	16	32.0 (±10.2)
1:5	24	164.4 (±23.8)
1:13	24	60.8 (±2.0)
1:27	24	19.3 (±7.5)

4.3.4 Batch system efficiency

Figure 4-8 shows the batch system efficiencies of dilution ratios 1:5, 1:13 and 1:27 on AD dairy manure at a light intensity of 3,000 lux and a cultivation period of 24 days. The batch systems efficiencies were calculated based on the TNR in Equation (4-1). BMR, N_2 and NS were found to be the major parameters for a LCS system. The three dilution ratios 1:5, 1:13 and 1:27 were evaluated at day 24 of the cultivation period. Dilution ratio 1:5 had the most N sedimentation among the dilution ratios at 73% m/m. It also had the highest nitrification/denitrification at 14% m/m. Dilution ratio 1:5 is too low for active *Landoltia punctata 0128* growth. It had the lowest N recovery at 13% m/m of the TNR and a nitrification/denitrification of 14% m/m of the TNR. Table 4-10 shows all the N reduction batch efficiency percentages with standard deviations.

Out of the three dilution ratios 1:13 performed the best in terms of N uptake of *Landoltia punctata 0128,* at and efficiency of 50% m/m and a N sedimentation content of 41% m/m. The nitrification/denitrification efficiency was low, at 9% m/m of the TNR. Dilution ratio 1:27 had the second highest BMR at 38% m/m of the total N removed from the system with no nitrification occurring in the system. To actively cultivate *Landoltia punctata 0128,* at a light intensity of 3,000 lux an increase in N is required. However, if the N content is too high, *Landoltia punctata 0128* will not actively grow and the nitrification/denitrification and sedimentation processes will remove most of the N from the system.

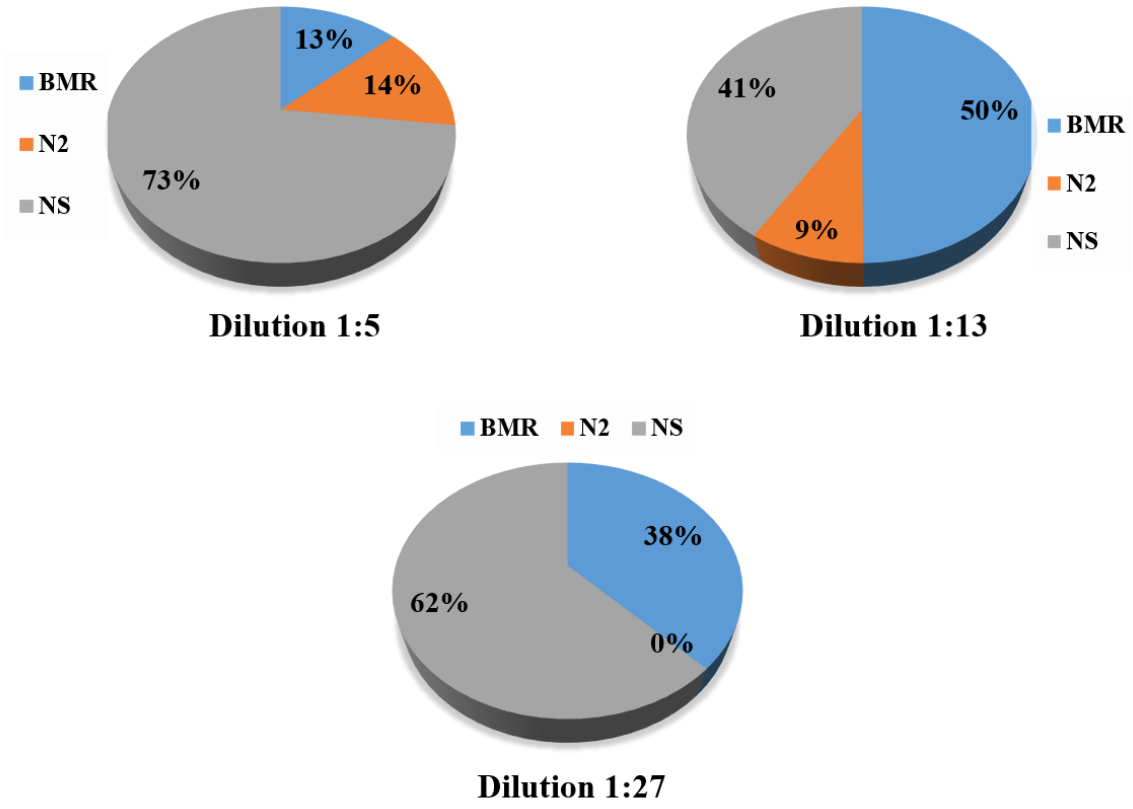

Figure 4-8 Batch system efficiencies of dilution ratios 1:5, 1:13 and 1:27 on anaerobically digested dairy manure at a light intensity of 3,000 lux and a cultivation period of 24 days

Table 4-10 Batch system efficiencies of dilution ratios 1:5, 1:13 and 1:27 on anaerobically digested dairy manure at a light intensity of 3,000 lux and a cultivation period of 24 days

Dilution	**Batch Efficiency (%)**		
	BMR	**N2**	**NS**
1:5	13 (±0.40)	14 (±0.6)	73 (±23)
1:13	50 (±0.65)	9 (±0.2)	41 (±2)
1:27	38 (±0.55)	0 (±0.08)	62 (±7)

4.4 Conclusion

This study evaluated the potential of *Landoltia punctata 0128* in improving the water quality of diluted AD dairy manure. The harvesting of *Landoltia punctata 0128* was evaluated based on biomass density and N reduction after 8, 16, and 24 days of growth. The treatments were compared to the study in Chapter 3 of *Landoltia punctata 0128* cultivated on dilution ratio 1:27 at a light intensity of 10,000 lux to determine significance. Batch tests were evaluated based on observations of RGR, DT, and nutrient removal rates to establish an effective harvesting period and batch system efficiency.

RGRs were established from zero-order kinetics to identify and model *Landoltia punctata 0128* growth at the three dilution ratios of 1:5, 1:13, and 1:27 at a light intensity of 3,000 lux at days 8, 16, and 24. Each of the RGRs was contrasted with *Landoltia punctata 0128* cultivated on dilution ratio 1:27 at a light intensity of 10,000 lux to determine significance. The dilution ratio that had the highest RGR was 1:27 at 8 days of growth. The RGR of *Landoltia punctata 0128* was 22.7 g m^{-2} d^{-1} with a DT of 2.6 days. At 8 days of cultivation, dilution ratio 1:13 followed closely with a RGR of 17.2 g m^{-2} d^{-1} and a DT of 3.5 days.

For further validation, the first 8 days of cultivation had the highest reduction of N based on the TN and TKN rate constants. Dilution ratio 1:13 has the highest rate constant of TN at 0.223 d^{-1} and TKN at 0.304 d^{-1}. The next highest TN and TKN reduction rate constants came from dilution ratio 1:27 at 0.140 d^{-1} and 0.143 d^{-1}, respectively. These values are much higher than that of the control signaling that a lower light intensity promotes a better N reduction and biomass growth at a shorter period. The optimal harvesting period for both dilution ratios based on RGRs and nutrient reduction constants is 8 days of cultivation at a light intensity of 3,000 lux.

When analyzing the overall batch efficiency of *Landoltia punctata 0128* it was found that a lower light intensity of 3,000 lux was easier to control the parameters of pH, DO, and filamentous algae growth than a light intensity at 10,000 lux in Chapter 3. The lower light intensity allowed *Landoltia punctata 0128* to grow at higher concentrations of AD dairy manure. One of the speculated reasons for this is the lack of filamentous algal influence. Less algae grew with the duckweed in the batch systems. *Landoltia punctata 0128* dominated the ecosystem with the batch samples resulting in a rapid growth rate from dilution ratios 1:13 and 1:27.

When observing the batch system efficiency, it was found that dilution ratio 1:13 assimilated more N content into the *Landoltia punctata 0128* biomass at 50% m/m of the TNR, had minimal nitrification/denitrification influence at 9% m/m of the TNR and 41% m/m of the N sedimentation of the TNR within the batch systems. It is recommended that higher concentrations of N (114 mg L^{-1}) should be utilized at lower light intensities (3,000 lux) for the cultivation of *Landoltia punctata 0128.*

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Chapter 5 : Starch Production from *Landoltia punctata* as a Feedstock for the Advancement of Bioethanol Production

Abstract

Biofuel production and utilization are, at present limited by the availability of feedstocks. An angiosperm called duckweed have presented themselves as a feasible feedstock, for bioethanol production due to their ability to readily accumulate starch through their natural cycles. Duckweeds can accumulate starch, when growing on anaerobically digested (AD) dairy manure under low concentrations of nitrogen (N) and phosphorus (P). The objective of this study was to maximize the starch accumulation in the macroalgal biomass of three strains of duckweed by optimizing the cultivation conditions.

Preliminary experimental results showed that the most influential factors in the starch accumulation, of duckweed, are nutrient starvation, controlled lighting, and growth applications. Nutrient starvation was conducted by controlled addition of nutrients, to the system, and the controlled growth time through the stationary phase in 12 days. Destructive batch sampling was used to assess the effects of nutrient starvation, which started at the nitrogen (N) and phosphorus (P) concentrations of $\leq$5 mg L^{-1} of TKN and $\leq$ 0.3 mg L^{-1} of o-PO_4-P. The controlled lighting applications were assessed at 10,000 lux to measure the accumulation of starch.

Once harvested, the duckweed biomass is dried at 27°C for 24 h, and then ball milled and sieved through a 0.5µm sieve. The total starch content of the dried duckweed was determined, using the Megazyme total starch assay kit. Cultivated at a nutrient dilution ratio of 1:27, the starch accumulation was measured at 30, 17, and 33% m/m for *Landoltia punctata*

0128, Lemna gibba 7589, and *Lemna minuta 9517,* respectively. It was observed that, the high yield of duckweed biomass will lead to high yields of starch, under optimized cultivation conditions, which in turn result in high yields of fermentable sugars.

5.1 Introduction

Rising concerns have elevated, both in the United States and abroad, over the future of energy production. The nature of current energy production has introduced issues of rising energy demand, due to increases in population and economic development, and environmental pollution, such as climate change which is caused by large-scale combustion of fossil fuels producing high volumes of carbon dioxide (CO_2) entering the atmosphere, resulting in global warming (Yu *et al.,* 2014). Solutions to counteract the effect of global warming and climate change, reside in the utilization of alternative energies such as biofuels, solar, wind, geothermal, and hydroelectric technologies which are carbon neutral. The development of biofuels has been the most successful in transforming our energy system from the use of fossil fuels in the means of transportation. The production of biodiesel and bioethanol has resulted in a technological revolution in the realm of energy production.

In the United States, Figure 2-12 shows, the dominate feedstock for bioethanol production is corn, which in 2016, accounted for 36% of bioethanol production (Xu *et al.,* 2011; USDA, 2016). The other 64% of corn was consumed by humans and livestock. Over the last eight years (2009-2016), the use of corn for bioethanol production has held constant, at an average of 5 billion bushels used for corn ethanol, out of the 14.5 billion bushels produced (USDA, 2016). However, over that period, the rate of corn production has remained constant. This trend could signal that an increase in the production of bioethanol, from corn, may not

occur in the future due to land use restraints, drought due to climate change, or a shift in political opinion. Alternative feedstocks are required to meet the demand of an increasing population, to reduce the consumption of fossil fuels and environmental pollution, and to secure the future of the world, on the security of energy production. A favorable future reality will manifest in our ability to develop third generation biofuels which utilize the chemical composition of aquatic plants.

Duckweed is a freshwater green angiosperm that floats on the surface of stagnate aquatic systems. Duckweed are studied for their rapid growth rate i.e. (2-5 days) and their ability to reduce N and P, from livestock manure, through phytoremediation for water purification (Xu *et al.,* 2011). Studies have also indicated that duckweed can accumulate starch onto the surface of their fronds, creating turions at a starch content of 5-70% on a dry weight basis (Chen *et al.,* 2012; Yu *et al.,* 2014). Yu *et al*. (2014) stated that duckweed grows about 10 times faster than terrestrial corn, making it a potential feedstock for bioethanol production. Many studies attribute starch accumulation to growth conditions, attributed to specific nutrient levels, temperature, pH, and photoperiod. Among the studies observed, the common method used to stress duckweed, into producing starch is nutrient starvation, where there is a low concentration of N and P are resident within the system (Xiao *et al.,* 2013; Yu *et al.,* 2014).

The parameters used to cultivate duckweed can also be used to stress duckweed into producing starch. In nature, when nutrients become scarce in winter, the duckweed will form a white crystallized starch structure on the inner surface of their fronds. To control this phenomenon, we must mimic the effects of winter by studying the effects of nutrient starvation, low air temperatures, longer periods of darkness, extreme pH increases or decreases, and high population densities to optimize the starch content, from the duckweed (Yu *et al.,* 2014; Yin *et*

al., 2015). According to a study conducted by Xiao *et al.* (2013), when duckweed is completely reliant on the nutrient content of a water body and those nutrients are sufficient for active growth (>25 mg L^{-1} of TN) the starch content will be low at 15.6% m/m, however, when the nutrient content is relatively low (<15 mg L^{-1} of TN) the duckweed will begin to accumulate starch.

In a study conducted by Ge *et al.* (2012), duckweed without any prior treatment contains glucan (20.3% ± 0.3, m/m). Roughly half of the glucan is in the form of starch (amylopectin) (10.3% ± 0.8, m/m) and half is cellulose (9.4% ± 0.5, m/m). As nutrient starvation begins to occur the starch content in the duckweed will start to increase. According to Yu *et al.* (2014), the nutrient starvation technique accrued a total starch content of approximately 40% m/m, for the duckweed species *Lemna aequinoctialis*. To confirm these results, in another study, Ge *et al.* (2012) reported that under nutrient starvation, they could produce a starch content of 10-36% m/m. Xiao *et al.* (2013) also reported a starch content of 52.9% m/m, via the duckweed species *Landoltia punctata*. The starch content of duckweed can vary greatly depending on the conditions of growth and decay.

Nutrient starvation has been the used in conjunction with the application of high and low light intensities to aid in the accumulation of starch. In a study conducted by Zhao *et al.* (2014), a fluorescent light, with a photoperiod of 16 hours of light and 6 hours of dark, with the light intensities ranging from 10,000, 5,000, and 2,000 lux, was used to cultivate duckweed. The study concluded that a light intensity of 7,500 lux would be optimal for duckweed growth. Yin *et al.* (2015) confirmed that 7,500 lux is an optimal light intensity for duckweed cultivation and starch accumulation, when using the nutrient starvation method, however, the researchers did see an increase in starch accumulation, when they raised the light intensity to 24,000 lux and the photoperiod of 24 hours a day. However, approximately the energy requirements of an

industrial scale operation would be necessary to maintain a light intensity of 24,000 lux for a period of 24 hours a day.

The conversion efficiency of sugar-rich of converting feedstocks into ethanol has been a problem, for second generation biofuels such as lignocellulosic ethanol, where the process to convert the woody feedstock into ethanol is energy intensive (Bayrakci *et al.,* 2013). Starch is a polymer of glucose that is composed of a ratio of amylose and amylopectin, based on the genetic variation of the species of duckweed (Yu *et al.,* 2014). According to Yu *et al.* (2014), amylose (MW=160,000 dal) consists of 500-20,000 glucose units joined by α-1,4 glycosidic bonds. As the amylose content increases the energy conversion efficiency decreases. Amylopectin (MW=32,400,000 dal) is a branched glucose polymer that has α-1,4 glycosidic bonds and side chains connected by α-1,6 glycosidic bonds. The conversion of starch to ethanol is less energy intensive and requires either enzymatic hydrolysis or acid hydrolysis to convert the starch into monomeric glucose units. The most common method for the conversion of starch to ethanol is enzymatic hydrolysis which uses α-amylase to hydrolyze starch into soluble branched and unbranched maltodextrins. Amyloglucosidase hydrolyses the maltodextrins to D-glucose which in turn can be fermented into ethanol using *Saccharomyces cerevisiae* (yeast).

It was determined, through a series of tests, reported in Chapter 3 that *Landoltia punctata 0128, Lemna gibba 7589,* and *Lemna minuta 9517* can significantly uptake a total nitrogen content (TN) of 84 mg L^{-1} (1:18) and 57 mg L^{-1} (1:27) on anaerobically digested (AD) dairy manure followed by a standard solution control of 1.6 g L^{-1} Hoagland E-Medium. It was also observed that a light intensity of 10,000 lux, an air temperature of 25°C, and a pH of 6.5 promoted active duckweed growth. It was found that the first 16 days of growth were the most productive for nutrient reduction and biomass accumulation for the duckweed strains, however,

when the duckweed strains were grown an extra 12 days on AD dairy manure it was observed that growth and nutrient reduction had slowed. At the sixteenth day of growth a white pigmentation had started to form on the duckweed fronds indicating that the fronds had turned to turions which are rich in starch.

This study was designed to compare the potential of three duckweed strains in the accumulation of starch, using nutrient concentrations of effluent from an anaerobic digester receiving flushed manure from a dairy farm. These experiments will observe starch accumulation, when the duckweed strains are cultivated on two dilution ratios of AD dairy manure and a control of Hoagland E-Medium. The duckweed strains will be subjected to a light intensity of 10,000 lux, to examine starch content when subjected to different nutrient concentrations. The specific objectives were to: (1) Determine a duckweed strain that significantly accumulates starch when cultivated on AD dairy manure; (2) identify a nutrient concentration that maximizes starch content in the duckweed biomass; (3) validate the nutrient concentration of AD dairy manure by providing a process behind starch production from nutrient starvation of duckweed.

5.2 Methods and Materials

5.2.1 Batch testing

Batch tests were conducted inside an environmental growth chamber, at the light intensity 10,000 lux and a photoperiod of 16:8 (light: dark). The first set of tests consisted of 27 samples in PET containers (114mm × 86mm × 102mm), for the three strains of duckweed, *Landoltia punctata 0128, Lemna gibba 7589,* and *Lemna minuta 9517,* at dilution ratios 1:18 and 1:27 of AD dairy manure and DI water with a control of 1.6 g L^{-1} Hoagland E-Medium. For each dilution ratio, there were three strains of duckweed were cultivated, in triplicate,

followed by a triplicate set of 1.6 g L^{-1} Hoagland E-Medium, for each duckweed strain as the control. The second set of tests consisted of 63 samples, in triplicate, at the dilution ratios of 1:27 and 1:149, of AD dairy manure and DI water with a control of 355 mg L^{-1} Hoagland E-Medium.

5.2.2 Sampling and analysis

Destructive sampling was carried out throughout each experiment set. The first experiment lasted 28 days and was evaluated for starch accumulation, at the end of the period. During the second experiment, set the selected duckweed strain is tested for starch content, every 5 days. Random triplicate samples would be taken from different locations within the environmental growth chamber, for starch accumulation analysis. The whole period of experimentation lasted 30 days, with there being 7 days of testing, on the following days: 0, 5, 10, 15, 20, 25, and 30. The wet duckweed strains were dried in an oven at 80°C for 24 hours, to obtain a dry weight, using a Mettler AE 260 Delta Range balance. The samples were then ball milled to a powder that would pass through a 0.5-µm sieve. The total starch content of the dried duckweed strains was measured, using the Megazyme total starch assay kit (Megazyme International, Ireland), using modified AOAC Method 996.11. The assay is specific for α-glucans, including starch, glycogen, phytoglycogen, and non-resistant maltodextrins. Amylopectin (starch) is the targeted compound being measured in the duckweed samples due to its significance to bioethanol production.

The employed procedure followed that of the manufacturers' recommendations. The detailed procedure is as follows: 100 mg of the ball milled duckweed sample was weighted and transferred into a glass test tube (16 × 120 mm). The glass test tube was then tapped to ensure that all the plant material was at the bottom. To aid in dispersion, 0.2 mL of aqueous ethanol

(80% v/v) was added to the test tube. The mixture was then mixed vigorously on a vortex mixer. Immediately after mixing, 3 mL of thermostable α-amylase was added to the test tube. The test tubes were then transferred to a boiling water bath, for a total of 6 min. The tubes were stirred after 2, 4, and 6 min. The tubes were then placed in a bath of 50°C for 5 min. At the end of the period, 0.1 mL of amyloglucosidase (330 µl on starch) was added to the test tubes, stirred on a vortex mixer and incubated at 50°C for 30 min.

The contents of the test tubes were then transferred to a 100-mL volumetric flask, with a glass funnel. The test tubes were rinsed, using a wash bottle of DI water, to flush all the solid materials left behind. The volume of the 100 mL volumetric flask was then adjusted to dilute the starch concentration, for measurement. After mixing the volumetric flask, a sample of 10 mL was taken from the flask and transferred to a centrifuge vial. The samples were then centrifuged at 3,000 rpm for 10 min. to remove all the solids.

The clear fluid at the top of the centrifuge vial was used for the assay. Duplicate samples were taken from each centrifuge vial and transferred at 0.1 mL, to glass test tubes (16 × 100 mm). Each test tube received 3.0 mL of glucose oxidase/peroxidase (GOPOD) and were incubated at 50°C for 20 min. Each 0.1 mL D-glucose control received 3 mL of GOPOD reagent, for testing. Reagent blank solutions consisted of 0.1 mL of water and 3.0 mL of GOPOD reagent. The absorbance for each sample, and the D-glucose control were read at 510 nm against the reagent blank. The calculations for obtaining the percent starch content, was carried out using an excel spreadsheet that was obtained from the manufacturer. The calculations can be seen in Appendix C.

5.3 Statistical Analysis

The performances of the AD dairy manure systems were compared to those of the Hoagland E-Medium cultures, by performing a Student's t-test to determine if the differences between the values were statistically significant using the method of independent samples and unequal variances, using a two-tailed p-value. All statements were based on a statistical significance of $P<0.05$.

5.4 Results and Discussion

5.4.1 Biomass yield

It was previously reported in Chapters 3, that *Landoltia punctata 0128, Lemna gibba 7589,* and *Lemna minuta 9517* actively remove N and P from AD dairy manure from dilution ratios 1:27 and 1.6 g L^{-1} Hoagland E-Medium. Figure 5-1 shows the biomass yield of the three duckweed strains, at a dilution ratio of 1:27, on AD dairy manure, for the full period of 28 days. Over that period, it was observed that a white pigmentation began to form on the duckweed strains fronds, after 16 days of growth. *Landoltia punctata 0128* in Figure 5-1 shows the beginnings of the stationary phase, at approximately 350 g wet m^{-2} after 20 days of cultivation. This indicates a change in the duckweeds growth state. When a plant enters the stationary phase, that means that there are not enough nutrients are present or the conditions are not right to for continued metabolism. One can theorize that the duckweed start accumulating sugar from the AD dairy manure to fix carbon.

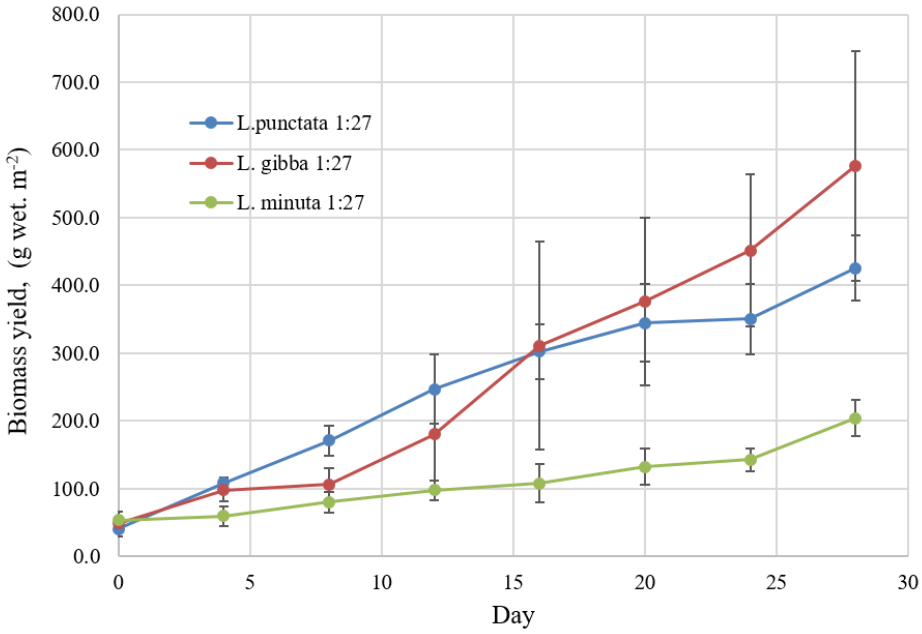

Figure 5-1 Growth curves of L. punctata, L. gibba, L. minuta at a light intensity of 10,000 lux, a dilution ration of 1:27 on AD dairy manure for a batch period of 28 days.

5.4.2 TKN and o-PO_4-P reduction

To confirm that the stationary phase occurs between days 16 and 28, Figure 5-2 shows the full removal of total Kjeldahl nitrogen (TKN) and o-phosphate-phosphorus (o-PO_4-P) for all the duckweed strains and control for dilution ratio 1:27. Upon observation, the most active depletion of TKN and o-PO_4-P occurred during the first 16 days of cultivation. The last 12 days were not mentioned in Chapter 3, because no active reduction took place during that period. In this study, researchers can hypothesize that starch accumulation starts in the stationary phase of growth, at approximately 5 mg L^{-1} of TKN and 0.3 mg L^{-1} of o-PO_4-P within the batch samples, which transpires at approximately day 20. No tests were completed on the AD dairy manure, for the reduction of sugars within the system, but tests were conducted on the duckweed strains to indicate the uptake of sugars within the tissues of the duckweed biomass, by measuring the starch content.

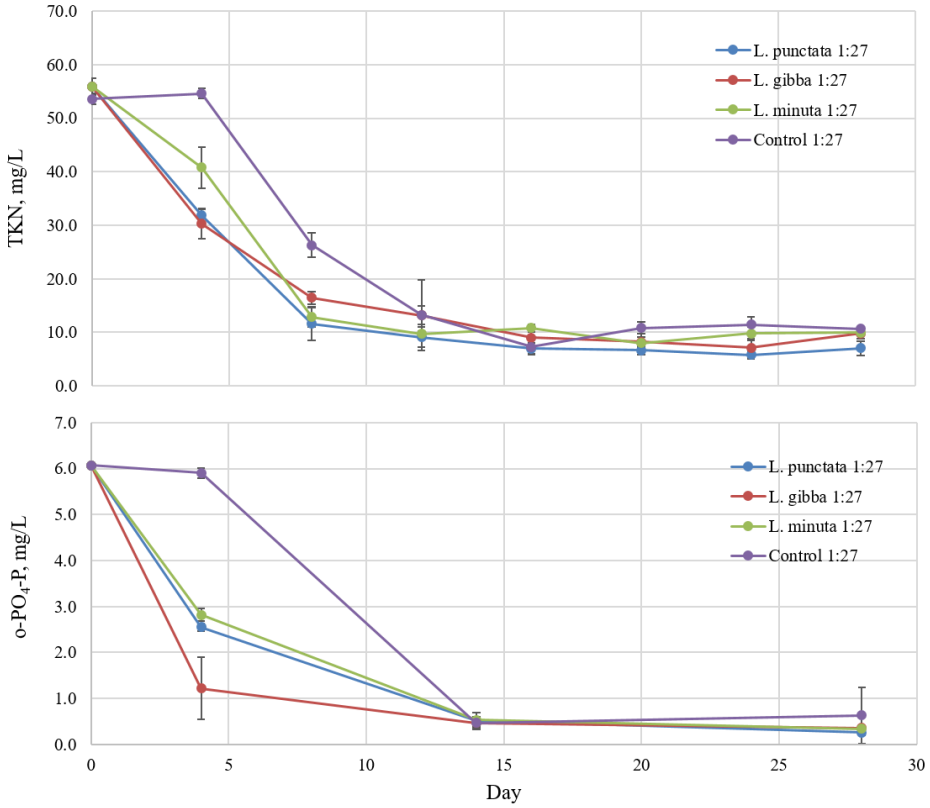

Figure 5-2 TKN and o-PO_4-P Reduction of L. punctata, L. gibba, and L. minuta at a light intensity of 10,000 lux, a dilution ration of 1:27 on AD dairy manure for a batch period of 28 days.

5.4.3 Identifying starch contents

The first experiment of this study sought to identify the duckweed strain that accumulated the most starch content, while being cultivated on AD dairy manure, at dilution ratios 1:18 and 1:27, with a control of 1.6 g L^{-1} Hoagland E- Medium, at a light intensity of 10,000 lux. Figure 5-3 shows the results of the duckweed strains after a 28-day period of growth. At dilution ratio 1:18, *Landoltia punctata 0128* produced the most starch content at 26% m/m. When observing dilution ratio 1:27, *Lemna minuta 9517* produced the most starch content at 33% m/m, followed closely by *Landoltia punctata 0128* at 30% m/m

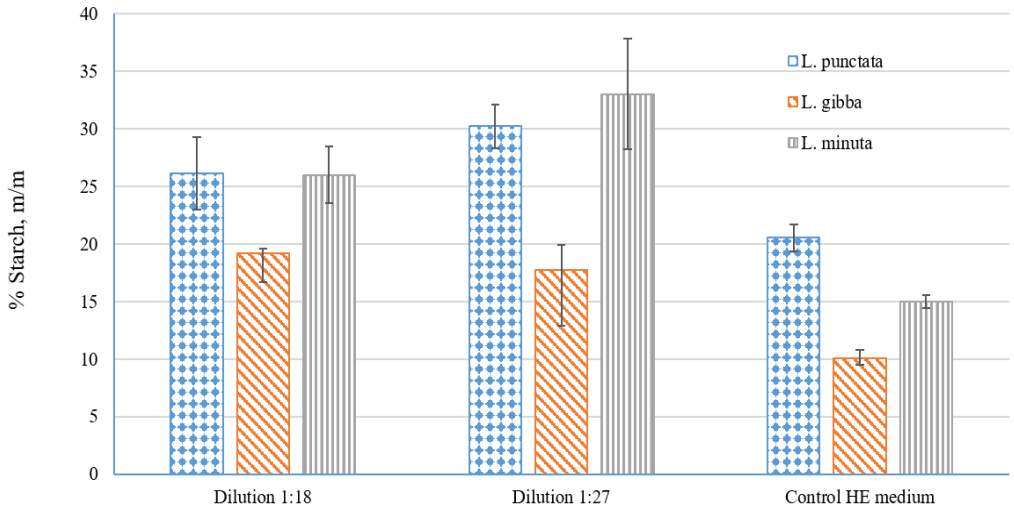

Figure 5-3 Starch accumulation of L. punctata, L. gibba, and L. minuta on dilution ratios 1:18 and 1:27 on AD dairy manure and 1.6 g L^{-1} Hoagland E-Medium for a period of 28-days

When the duckweed strains were cultivated on 1.6 g L^{-1} Hoagland E-Medium, *Landoltia punctata 0128* produced the highest starch content, at 20% m/m. All the dilution ratios had a significantly higher starch content than their controls. When comparing the starch percentage of *Landoltia punctata 0128* to the starch percentage of *Lemna minuta 9517,* no significance between the two duckweed strains was found. Overall, the duckweed strain that was chosen to proceed on to further batch testing was *Landoltia punctata 0128,* due to previously determined parameters, observed in Chapter 3, such as its rapid growth rate, its ability to reduce N and P from AD dairy manure and currently its ability to accumulate a high percentage of starch.

5.4.4 Starch accumulation of *Landoltia punctata 0128*

Dilution ratios 1:27 and 1:149 of AD dairy manure were used to gain a representation of how *Landoltia punctata 0128* accumulates starch throughout its biomass and nutrient cycle. The previous experiment indicates that starch accumulation occurs at the end of *Landoltia punctata 0128's* nutrient cycle. Figure 5-4 shows the lifecycle of *Landoltia punctata 0128*, at dilution ratios 1:27 and 1:149, with a control of 355 mg L^{-1} Hoagland E-Medium, when accumulating starch over a period of 30 days.

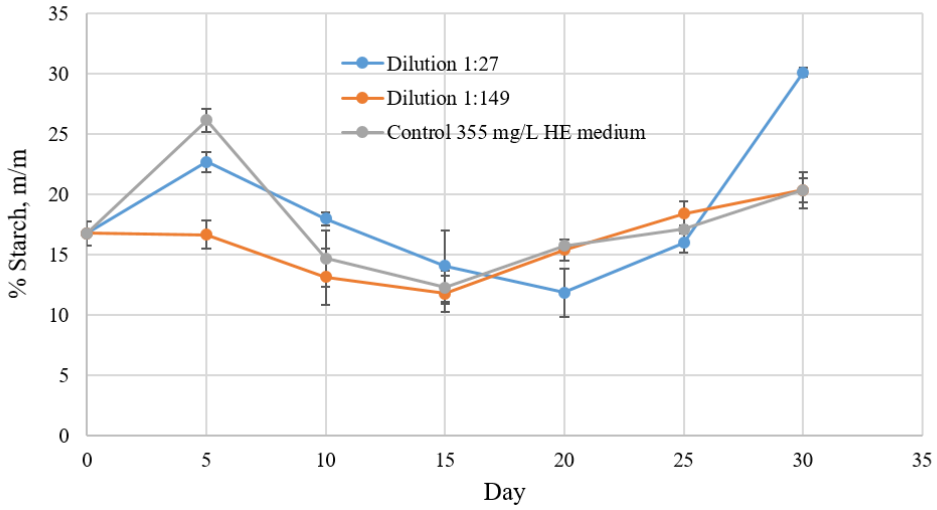

Figure 5-4 Starch accumulation of L. punctata, on dilution ratios 1:27 on AD dairy manure with a control of 355 mg L^{-1} Hoagland E-Medium for a period of 30-days

When observing Figure 5-4, it shows that dilution ratio 1:27, 1:149 and the control of 355 mg L^{-1} Hoagland E-Medium began at an initial starch content of 16% m/m. The initial starch content was measured, in triplicate, from pre-cultured *Landoltia punctata 0128*, growing on a dilution ratio of 1:27 of AD dairy manure at a light intensity of 3,000 lux. After 5 days of growth, one sees an increase in starch content, within the *Landoltia punctata 0128* cultures, for

dilution ratio 1:27, at approximately 23% m/m and the control at 26% m/m. The value for dilution ratio 1:149 is mostly constant up to day 5. That increase, within the first 5 days, could be a harvesting point for *Landoltia punctata 0128* biomass, at dilution ratio 1:27. This sharp increase could be due to the duckweed adjusting to the environment, at the higher light intensity.

The internal starch content or carbon, within the *Landoltia punctata 0128* biomass, is consumed from day 5 to 20, for dilution ratio 1:27 and from day 5 to day 15, for dilution ratio 1:149 and the control. When contrasting between Figure 5-1 and Figure 5-4, the graphs show that *Landoltia punctata 0128* is entering its exponential phase of growth, during the period of 0 to 16 days. It is possible that metabolism is consuming the internal carbon, along with N and P, to produce the energy required for *Landoltia punctata 0128* growth. The lowest percentage of starch, within dilution ratio 1:27, 1:149 and the control, was 13% m/m.

When N and P are used up or are low, within the system, *Landoltia punctata 0128* will begin to accumulate starch, within the system for growth. Figure 5-4 shows that at days 20 to 30, for dilution ratio 1:27, starch accumulation begins to occur. Dilution ratio 1:149 and the control start at day 15, for starch accumulation, where the N and P, within the system should be depleting. Figure 5-1 shows the stationary phase, for this period, where no growth exists, within the system. *Landoltia punctata 0128* is storing carbon, in the form of sugars, from the AD dairy manure. The total concentration of sugar (sucrose), in the AD dairy manure is 250 mg L^{-1}, according to Northwest Lab LLC, based in Southern Idaho and shown in Appendix B. At the dilution ratio 1:27 and 1:149, the AD dairy manure has approximately 9 and 2 mg L^{-1} of sugar concentration, respectively. No sugar is added to the system of the controls. It is possible that *Landoltia punctata 0128* uptakes the remaining sugar content, within the system when N and P are low in concentration. Figure 5-2 shows that the TKN and o-PO_4-P concentrations, within

the batch systems. The concentrations of TKN and o-PO_4-P start to become constant at day 16. At that point, the concentration of TKN and o-PO_4-P for *Landoltia punctata 0128,* within the batch system, is approximately 5 and 0.3 mg L^{-1}, respectively.

Landoltia punctata 0128 can only metabolize carbon, within two environmental systems. The first place is in the air in the form of carbon dioxide (CO_2) and the second is from the AD dairy manure. Lids were placed on the batch systems, preventing evaporation and ammonia volatilization, but the lids were not air tight. Once the initial CO_2 was exhausted in the batch system, *Landoltia punctata 0128* is required to uptake carbon from the AD dairy manure, however, as can be seen in Figure 5-4 that carbon is first taken from the internal storage of *Landoltia punctata 0128,* instead of from the AD dairy manure. The point at which *Landoltia punctata 0128* cannot take any more carbon, from internal storage, must be at approximately 12 to 13% m/m starch content. Instead of taking the carbon from the internal storage, *Landoltia punctata 0128* must take it from the AD dairy manure.

A study conducted by Yu *et al.* (2014) found that, when adding 10 g L^{-1} of sucrose to Schenk & Hildebrandt medium, the duckweed strain *Lemna aequinoctialis 6000* saw a maximum starch content of 39% m/m. This indicates that a higher sugar content, within the medium, produces a higher starch content within a duckweed species. That is confirmed in Figure 5-4, when we see that the dilution ratio 1:27 peaks at a starch content of 30% m/m. Dilution ratio 1:149 and the control peak at 20% m/m, which indicates that dilution ratio 1:27 is significantly different from the control, but dilution ratio 1:149 is not. The sugar concentration in dilution ratio 1:149 is 2 mg L and the control does not have any sugar added, which proves that the sugar component within the AD dairy manure is assimilated by *Landoltia punctata 0128,* at dilution ratio 1:27, producing a higher starch content than the control.

5.5 Conclusion

This study evaluated three duckweed strains, *Landoltia punctata 0128, Lemna gibba 7589,* and *Lemna minuta 9517* as potential feedstocks to produce starch for bioethanol production. Two experiments were completed, to first identify a duckweed strain, with the capability of accumulating starch. The second identified the life cycle of starch accumulation, based on nutrient concentration, to identify points of high starch accumulation. Duckweed strain, *Landoltia punctata 0128,* was identified as the best feedstock for starch production, producing a maximum starch content of 30% m/m, when cultivated on a dilution ratio of 1:27, utilizing AD dairy manure at the light intensity 10,000 lux.

Biomass yield and nutrient concentration can be used to identify the period of starch accumulation, for *Landoltia punctata 0128*. In both experiments, the highest starch accumulation occurred at the end of the 30 day cultivation cycle. When duckweed enters its exponential phase of growth, the starch content will be at its lowest due to the energy being used for metabolism. When *Landoltia punctata 0128* enters the stationary phase of growth, at approximately 350 g wet.m^{-2}, that is the beginning of starch accumulation at a light intensity of 10,000 lux, a dilution ratio of 1:27, and a cultivation period of 20 days. Biomass yield and nutrient concentration reduction are related, in that when biomass yield increases, nutrient concentration decreases due to the nutrient uptake of duckweed.

It was proven that *Landoltia punctata 0128* assimilates carbon, in the form of sugar within the batch systems of AD dairy manure, accumulating a starch content. At dilution ratio 1:27, *Landoltia punctata 0128* accumulated the highest starch content, as compared to dilution ratio 1:149 and the control, as there was a higher concentration of sugar within the batch system, compared to the lower concentration of AD dairy manure and the control with no sugar

concentration. That confirms that a higher concentration of AD dairy mature is beneficial to starch accumulation, however to accumulate the starch, the duckweed must go through their complete life cycle. That means that the nutrient concentration can be no higher than what is feasible for the duckweed to actively uptake. For this analysis, that concentration is based on a TKN and o-PO_4-P analysis at 56 and 6 mg L^{-1}.

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Chapter 6 : Summary and Recommendations for Future Research

6.1 Summary

Three strains of duckweed were selected, *Landoltia punctata 0128, Lemna gibba 7589,* and *Lemna minuta 9517* based on biomass yield of batch studies to evaluate the biomass production and starch accumulation based on nutrient concentrations of AD dairy manure. Seasonal variation of summer, fall, and winter were modeled based on light intensity's 10,000, 3000, and 1000 lux. Nutrient concentrations of anaerobically digested (AD) dairy manure was tested at dilution ratios 1:5, 1:13, 1:18, and 1:27. Both physical and chemical tests were carried out to evaluate the growth media and duckweed strains at specified time periods. Batch tests were evaluated based on observations of relative growth rate (RGR), doubling time (DT), nutrient removal rates and starch accumulation.

When modeling summer growth at 28 days of cultivation, RGRs were used to identify a duckweed strain that actively produced biomass when cultivated on dilution ratios 1:18 and 1:27 of AD dairy manure. The duckweed strain that actively accumulated the most biomass as compared to the standard solution was *Landoltia punctata 0128.* The RGR of *Landoltia punctata 0128* was 13.0 g m^{-2} d^{-1} with a DT of 4.5 days at a dilution ratio of 1:27. During the fall, at 8 days of cultivation, *Landoltia punctata 0128* had the highest RGR of 22.7 g m^{-2} d^{-1} with a DT of 2.6 days at dilution ratio 1:27. To keep the growth rate constant *Landoltia punctata 0128* access to at total nitrogen content (TN) of 57 mg L^{-1} and a total phosphorus (TP) content of 7 mg L during both summer and fall growing seasons.

During the summer cultivation period, nutrient rate constants were established from first-order kinetics for TN, TKN, TP and o-PO_4-P. The treatment of *Landoltia punctata 0128*

on dilution ratio 1:27 significantly reduced N and P within the batch system at 57 mg L^{-1} of TN and 7 mg L^{-1} of TP. The nutrient rate constants for *Landoltia punctata 0128* established from this study were 0.122 d^{-1} for TN, 0.136 d^{-1} for TKN, 0.145 d^{-1} for TP and 0.173 d^{-1} for o-PO_4-P at 16 days of cultivation.

During the fall cultivation cycle at 8 days of growth, dilution ratio 1:13 has the highest rate constant of TN at 0.223 d^{-1} and TKN at 0.304 d^{-1}. The next highest TN and TKN reduction rate constants came from dilution ratio 1:27 at 0.140 d^{-1} and 0.143 d^{-1}, respectively. These values are much higher than that of the summer cultivation cycle signaling that a lower light intensity promotes a better N reduction and biomass growth at a shorter period.

A harvesting model for both summer and fall were evaluated based on RGR, DT and nutrient recovery through the cultivation cycle. For both summer and fall the maximum harvesting interval was determined by nutrient recovery cycle to be 16 days. The minimum harvesting period was determined by the DT of the cultivation cycle for *Landoltia punctata 0128*. During the summer cultivation cycle the minimum harvesting interval at a dilution ratio of 1:27 is 4.5 days. During the fall, the minimum cultivation cycle harvesting interval at dilution ratio 1:27 is 2.6 days.

To find the optimal harvesting period the exponential and stationary phase of growth were observed. During the summer at a dilution ratio of 1:27, *Landoltia punctata 0128* enters the exponential phase from day 0 to 16. From 16 to 28 the duckweed is in the stationary phase. In the summer, optimal harvesting occurs at 16 days after cultivation. During the fall at a lower light intensity, *Landoltia punctata 0128* entered its exponential phase at dilution ratio 1:27 from day 0 to 8. At days 8 to 24, *Landoltia punctata 0128* remained in the stationary phase of growth indicating that the optimal harvesting interval during the fall is 8 days after cultivation.

During the fall season, overall batch efficiency of N reduction within the *Landoltia punctata 0128* batch systems at 24 days of cultivation was investigated. It was observed that *Landoltia punctata 0128* having access to an ideal concentration of nutrients at dilution ratio 1:27 resulted in higher biomass accumulation. It was found that *Landoltia punctata 0128* assimilated 38% m/m of N out of the TNR content into its biomass. It was also found that 62% m/m of the N in the batch system settle out of solution as sedimentation.

It was identified that the duckweed strain *Landoltia punctata 0128* would be the best feedstock for starch production producing a maximum starch content of 30% m/m when cultivated on a dilution ratio of 1:27 utilizing AD dairy manure at the light intensity 10,000 lux. It was identified that the stationary phase of growth promotes starch accumulation within *Landoltia punctata 0128.* It was also determined that sucrose content within the medium may play a role in starch accumulation in AD dairy manure in that dilution ratio 1:27 produced a higher starch content than dilution ratio 1:149 as there is a higher concentration of sucrose in the higher concentration of nutrients.

The overall performance of the *Landoltia punctata 0128* treatment indicates that it can thrive in a system that incorporates AD dairy manure as a nutrient source for dairy wastewater treatment. Visual observations indicated that *Landoltia punctata 0128* covers surface areas quite efficiently with its larger fronds size and quickly adapts to the environment to which it is placed. The lower light intensity allowed *Landoltia punctata 0128* to grow at higher concentrations of AD dairy manure. One of the speculated reasons for this is the lack of filamentous algal influence. Less algae grew with the duckweed in the batch systems. *Landoltia punctata 0128* dominated the ecosystem with the batch samples resulting in a rapid growth rate from dilution ratios 1:13 and 1:27 during fall season.

6.2 Recommendations for Future Research

The cultivation of *Landoltia punctata 0128* on anaerobically digested dairy manure at dilution ratio 1:27 and a light intensity of 10,000 lux for nutrient removal, biomass production, and starch accumulation. However, there are some important aspects that need to be investigated in order to asses further *Landoltia punctata 0128* cultivation. Therefore, I recommend the investigation of the following in future works:

1. Develop *Landoltia punctata* for large scale cultivation using design specifications from this study
2. Test harvesting intervals based on the models of this study.
3. Test *Landoltia punctata* response to varying concentrations of sucrose within AD dairy manure.
4. Identify the filamentous algae that grows with *Landoltia punctata* when cultivated on AD dairy manure and determine if that filamentous algae is high in lipid content for biodiesel production.
5. Identify a wavelength of light that *Landoltia punctata* will respond to through biomass production when cultivated on AD dairy manure
6. Model seasonal variation of the Northwest Region to identify feasibility of geographic area.
7. Conduct a life cycle analysis on the cultivation of *Landoltia punctata.*

Appendix A

Detailed Materials, Equipment, and Experimental Methods

A.1 Materials

In this study, flushed anaerobically digested (AD) dairy manure was collected from an anaerobic digester from a local dairy in the Southern Idaho region. Deionized water was used from a reverse osmosis resin unit to dilute the AD dairy manure. The standard solution used in these experiments was Hoagland's No. 2 Basal Salt Mixture (HOP01-50LT). Hoagland's No. 2 Basal Salt Mixture is a mixture of micronutrients and macronutrients which contain the specific components: ammonium phosphate, monobasic ($NH_4H_2PO_4$), boric acid (H_3BO_3), calcium nitrate, tetrahydrate ($Ca(NO_3)_2$-$4H_2O$), cupric sulfate, pentahydrate ($CuSO_4$-$5H_2O$), ferric tartrate ($C_{12}Fe_2H_{12}O_8$), magnesium sulfate, anhydrous ($MgSO_4$), manganese chloride, tetrahydrate (MnC_{l2}-$4H_2O$), molybdenum trioxide (MoO_3), potassium nitrate (KNO_3), and, zinc nitrate, hexahydrate ($Zn(NO_3)_2$-$6H_2O$). To keep an ideal growth environment, the pH was adjusted to 6.5 every 2 days with acetic acid at 5% v/v and 10M NaOH solution when the medium was over adjusted.

In this study batch experiments were conducted in 300 mL PET containers, inside an environmental growth chamber, at set light intensities of 10,000, 3,000, and 1,000 lux and an air temperature of 25°C. The environmental growth chamber was set up to hold a constant temperature in the system. It was also set on a timer to hold 16 hours of light and 8 hours of darkness.

A.2 Experimental Methods

A.2.1 Batch test preparation

Dilution ratios were determined by mixing AD dairy manure and deionized water to establish the nutrient content. Each concentration of AD dairy manure was initially prepared in a 4L container. Each 4-L container held a different dilution ratio of a mixture of AD dairy manure and deionized water at 1:5, 1:13, 1:18, 1:27 and 1:149. On a TN basis, these dilution ratios account for 266, 114, 84, 57, 10 mg L^{-1}. The dilutions were then transferred to PET containers with the dimensions of 114 × 86 × 102 mm with a surface area of 0.0116 m^2 and a volume of 200 mL.

A.2.2 Batch testing

A nutrient, physical, and starch analysis of the diluted AD dairy manure and duckweed biomass was performed to provide data for nitrogen (N) and phosphorus (P) reduction of AD dairy manure, starch accumulation and N and P uptake from duckweed biomass. The nutrient parameters were analyzed using a spectrophotometer (DR5000, Hach, USA). Hach methods used to obtain the data were Method 10242, Method 10214, and Method 10127 for total nitrogen (TN), total Kjeldahl nitrogen (TKN), nitrates and nitrites (NO_3-N+NO_2-N), ortho-phosphate-phosphorus (o-PO_4-P), and total phosphorus (TP), respectively. These water quality kits were ordered from the manufacture. Suspended solids (SS) was analyzed using Hach Method 8006. Total and volatile solids were analyzed using standard methods (APHA, 2015). A pH, dissolved oxygen (DO) and electric conductivity (EC) analyses were conducted using a Sper Scientific 850049 water meter kit. Batch samples were diluted based on the nutrient

concentration and the range of the test kit. To find the N and P content within the biomass of duckweed, samples were analyzed at the University of Idaho Analytic Laboratory.

To analyze total starch within the biomass of duckweed, wet duckweed strains were dried in an oven at 80°C for 24 hours to obtain a dry weight using a Mettler AE 260 Delta Range balance. The samples were then ball milled to a powder that would pass through a 0.5-µm sieve. The total starch content of the dried duckweed strains was measured using the Megazyme total starch assay kit (Megazyme International, Ireland) using modified AOAC Method 996.11.

A.2.2.1 Method 10242

The Hach test kit is called TNT 880 simplified TKN analysis, formally called Method 10242, where it analyzes TN, TKN, and NO_3-N+NO_2-N. The range of application for this test kit is water and wastewater at a TN range of 0 to 16 mg L^{-1}. The pH and temperature range for the test kit was 3 to 10 and 15 to 25°C, respectively. The principle behind the method uses inorganically and organically bonded nitrogen which is oxidized to nitrate by digestion with peroxodisulphate. The nitrate ions react with 2.6-dimethlyphenol in a solution of sulphuric and phosphoric acid to form a nitrophenol. Oxidized nitrogen is subtracted from TN to result in TKN (Hach, 2005).

To start the test, 1.3 mL of sample, 1.3 mL of 1.54N NaOH solution, and an oxidant tablet was inserted into a test tube of 16 × 120 mm and digested for 1 hour at 100°C in a reactor. After the solution cooled to 20°C, a MicroCap C, from the test kit was inserted into the test tube. The test tube was then inverted until the MicroCap C dissolved within the solution. Slowly, 0.5 mL of solution was taken from the 16 × 120 mm test tube and inserted into a pre-

filled test tube called vial 1 within the test kit. Slowly, 0.2 mL of solution D within the test kit was pipetted into vial 1. Vial 1 was capped immediately and inverted until no streaks were seen within the vial. Slowly, 1.0 mL of undigested sample was pipetted into vial 2 in the test kit. Slowly, 0.2 mL of solution D within the test kit was pipetted into vial 2. Vial 1 and vial 2 were then mixed thoroughly and a period of 15 minutes was given before vial 1 was placed into the spectrophotometer (DR5000, Hach, USA) and the bar code read and absorbance given. Then vial 2 was placed into the spectrophotometer and the bar code and absorbance was read to give a concentration of TN, TKN, and NO3-N+NO2-N in mg L^{-1}. The spectrometer used a built-in program to read the samples (Hach, 2005)

A.2.2.2 Method 10214

The Hach test kit is called TNTplus 846 Molybdovanadate Method, formally called Method 10214, where it analyzes PO_4^{-3} and o-PO_4-P. The range of application for this test kit is water, wastewater, boiler water, surface water, and process water at ranges of 5.0 to 90 mg L^{-1} of PO_4^{-3} and 0 to 30 mg L^{-1} for o-PO_4-P. The pH and temperature range for the test kit was 3 to 10 and 15 to 25°C, respectively (Hach, 2005).

Phosphate ions react with vanadate-molybdate reagent to form yellow dye. Test results are measured at 435 nm. To start the test, 5.0 mL of sample is pipetted into a pre-filled vial. The cap is then placed on the vial and it is inverted many times and left to set for 10 mins. After the time expired, the vials were mixed again. The outside of the vial was cleaned and the results were read on the spectrophotometer in mg L^{-1} (Hach, 2005).

A.2.2.3 Method 10127

The Hach test kit is called the Molybdovanadate Method with Acid Persulfate Digestion, formally called Method 10127, where it analyzes PO_4^{-3}. The range of application for

this test kit is water and wastewater at ranges of 0 to 100 mg L^{-1} of PO_4^{-3}. The pH and temperature range for the test kit was 3 to 10 and 15 to 25°C, respectively. Phosphates present in organic and condensed inorganic forms must be converted to reactive orthophosphate before analysis. Pretreatment of the sample with acid and heat provides the conditions for hydrolysis of the condensed inorganic forms. Organic phosphates are converted to orthophosphates by heating with acid and persulfate. Orthophosphate reacts with molybdate in an acid medium to produce a mixed phosphate/molybdate complex. In the presence of vanadium, yellow molybdovanadophosphoric acid forms. The intensity of the yellow color is proportional to the phosphate concentration. The test results are measured at 420 nm (Hach, 2005).

To begin the test, 5.0 mL of sample is pipetted into a pre-filled vial. A funnel is used to add the pre-measured potassium persulfate to the vial. The vials are inserted into a reactor at 150°C for 30 minutes to digest. After the timer expires, the vials are left to cool until they are 20°C. After the cool down period 2.0 mL of 1.54N NaOH is added to the vials and mixed. After mixing, 0.5 mL of molybdovanadate is added to the vial and mixed. A period of 7 minutes is exhausted before the vials are placed into the spectrophotometer and measured at 420 nm. The results are in mg L^{-1} of PO_4^{-3} (Hach, 2005).

A.2.2.6 Modified AOAC Method 996.11

Thermostable α-amylase hydrolyses starch into soluble branched and unbranched maltodextrins. Amyloglucosidase (AMG) quantitatively hydrolyses maltodextrins to D-glucose. In the procedure, D-Glucose is oxidized to D-gluconate with the release of one mole of hydrogen peroxide (H_2O_2) which is quantitatively measured in a colorimetric reaction employing peroxidase and the production of a quinoneimine dye. The samples are then placed into a spectrophotometer and the absorbance is analyzed at 510 nm (Megazyme, 2017).

The detailed procedure is as follows: 100 mg of the ball milled duckweed sample was weighted and transferred into a glass test tube (16 × 120 mm). The glass test tube was then tapped to ensure that all the plant material was at the bottom. To aid in dispersion, 0.2 mL of aqueous ethanol (80% v/v) was added to the test tube. The mixture was then mixed vigorously on a vortex mixer. Immediately after mixing, 3 mL of thermostable α-amylase was added to the test tube. The test tubes were then transferred to a boiling water bath for a total of 6 min. The tubes were stirred after 2, 4, and 6 min. The tubes were then placed in a bath of 50°C for 5 min. After the period was up, 0.1 mL of amyloglucosidase (330 µl on starch) was added to the test tubes, stirred on a vortex mixer and incubated at 50°C for 30 min (Megazyme, 2017).

The contents of the test tubes were then transferred to a 100-mL volumetric flask with a glass funnel. The test tubes were rinsed out using a wash bottle of DI water to flush all the solid materials left behind. The volume of the 100-mL volumetric flask was then adjusted to dilute the starch concentration for measurement. After mixing the volumetric flask, a sample of 10 mL was taken from the flask and transferred to a centrifuge vial. The samples were then centrifuged at 3,000 rpm for 10 min. to remove all the solids (Megazyme, 2017).

The clear fluid at the top of the centrifuge vial was used for the assay. Duplicate samples were taken from each centrifuge vial and transferred at 0.1 mL to glass test tubes (16 × 100 mm). Each test tube received 3.0 mL of glucose oxidase/peroxidase (GOPOD) and were incubated at 50°C for 20 min. Each 0.1 mL D-glucose control received 3 mL of GOPOD reagent for testing. Reagent Blank solutions consisted of 0.1 mL of water and 3.0 mL of GOPOD reagent. The absorbance for each sample, and the D-glucose control was read at 510 nm against the reagent blank (Megazyme, 2017).

References

APHA. (2015). *Standard Methods for the Examination of Water and Wastewater.* Washington D.C. : American Public Heath Assosiation.

Hach. (2005). *DR5000 Spectrophotometer: Procedures manual.* November 05 Edition 2.

Megazyme. (2017). *Megazyme Total Starch Assay Procedure.*

Appendix B

Process Pictures and Data

B.1 Process Pictures

Figure B-1 Environmental growth chamber

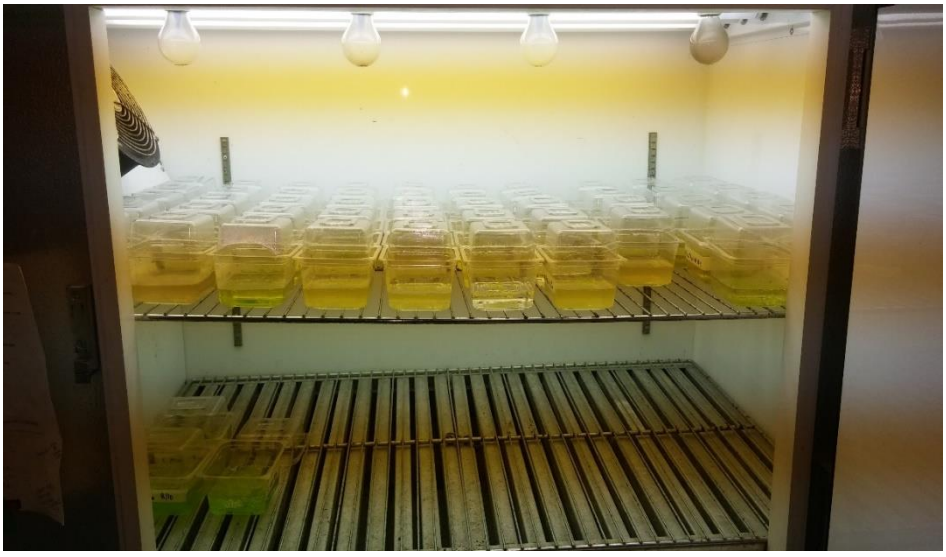

Figure B-2 Duckweed set-up inside environmental growth chamber

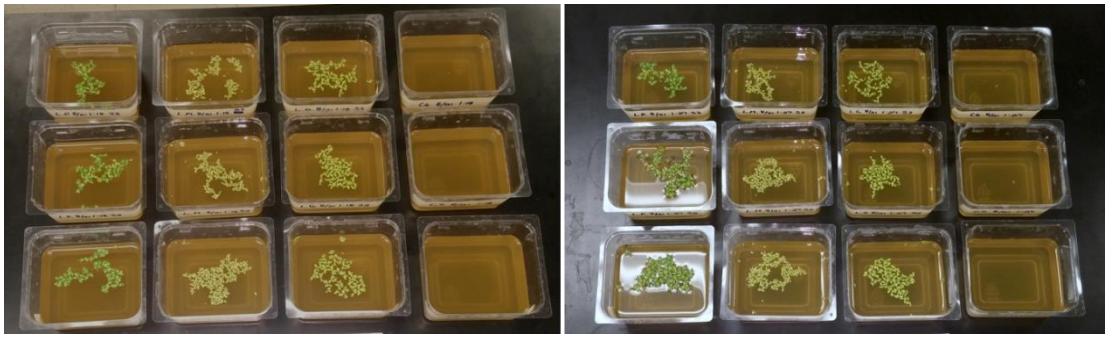

Figure B-3 Duckweed growth at 10,000 lux for L. punctata: Left, L. gibba: Center, and L. minuta: Right at day 0

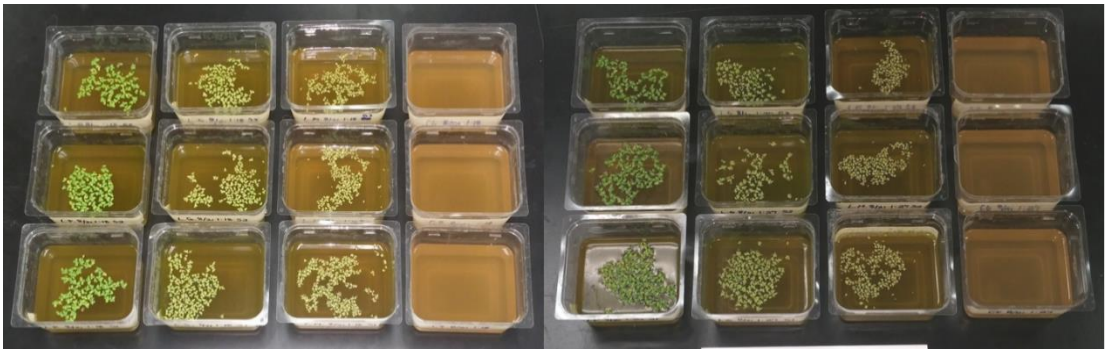

Figure B-4 Duckweed growth at 10,000 lux for L. punctata: Left, L. gibba: Center, and L. minuta: Right at day 4

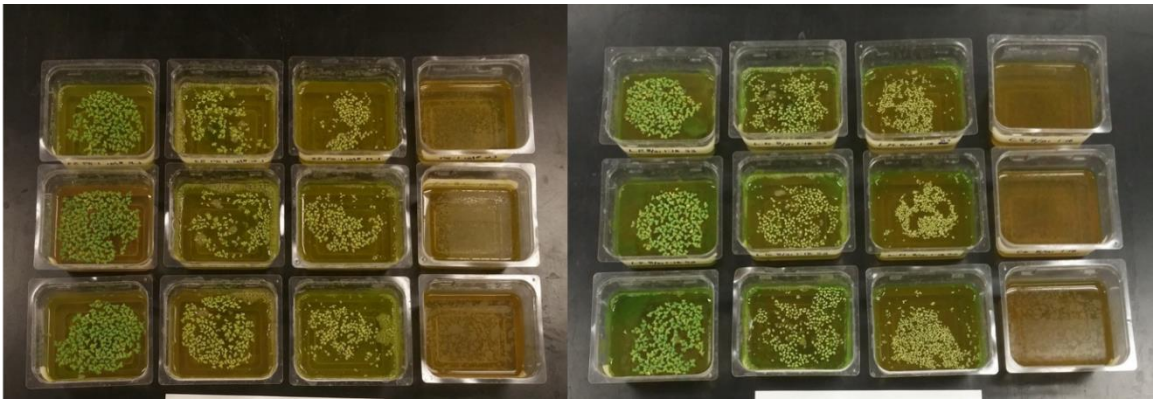

Figure B-5 Duckweed growth at 10,000 lux for L. punctata: Left, L. gibba: Center, and L. minuta: Right at day 8

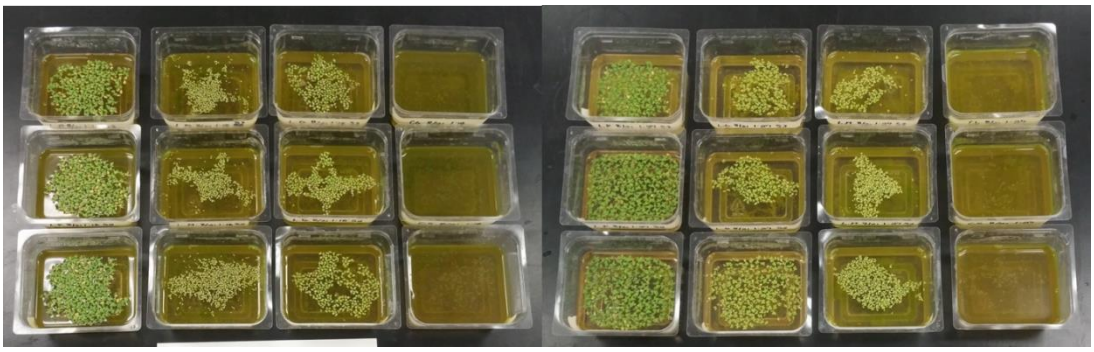

Figure B-6 Duckweed growth at 10,000 lux for L. punctata: Left, L. gibba: Center, and L. minuta: Right at day 12

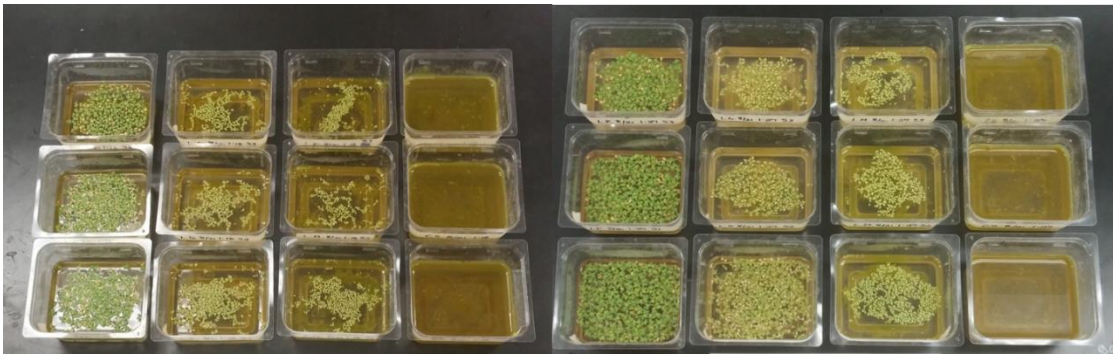

Figure B-7 Duckweed growth at 10,000 lux for L. punctata: Left, L. gibba: Center, and L. minuta: Right at day 16

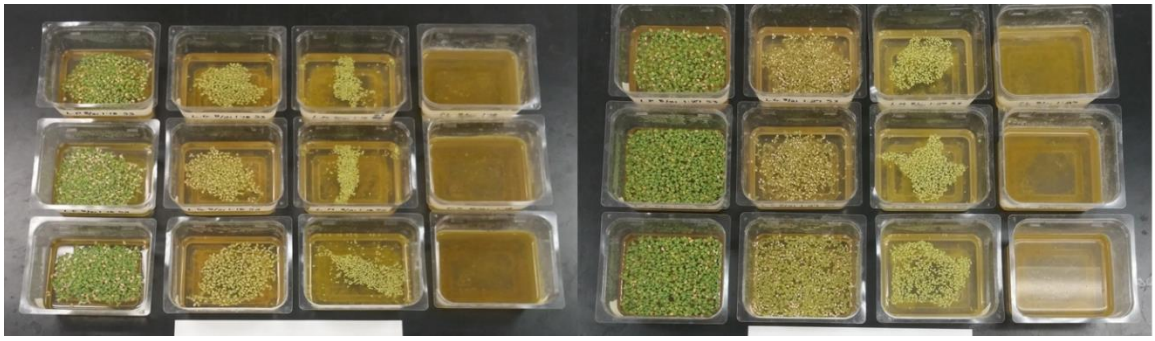

Figure B-8 Duckweed growth at 10,000 lux for L. punctata: Left, L. gibba: Center, and L. minuta: Right at day 20

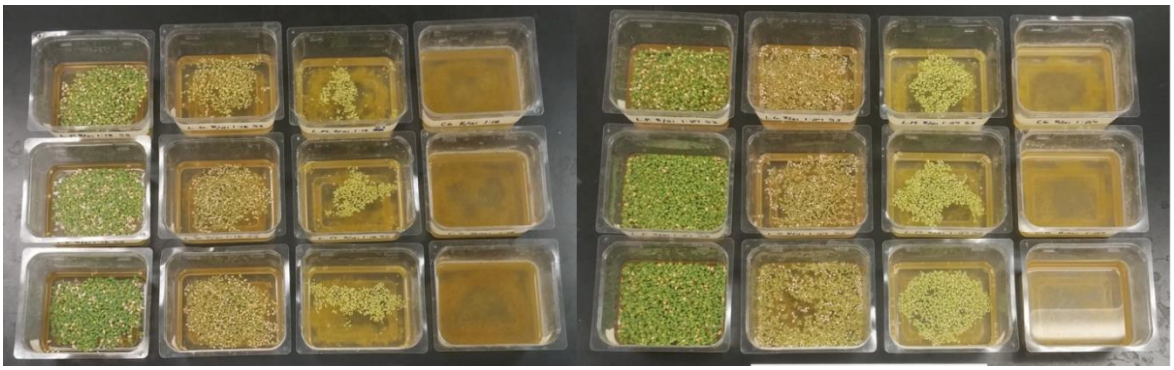

Figure B-9 Duckweed growth at 10,000 lux for L. punctata: Left, L. gibba: Center, and L. minuta: Right at day 24

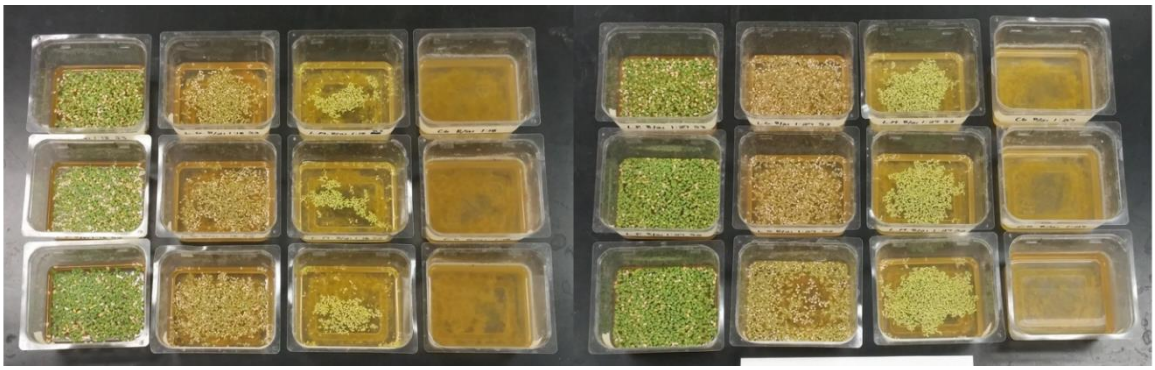

Figure B-10 Duckweed growth at 10,000 lux for L. punctata: Left, L. gibba: Center, and L. minuta: Right at day 28

Figure B-11 Conducting simplified TKN analysis on diluted samples

Figure B-12 Conducting TP and o-PO_4-P analysis on diluted samples

Figure B-13 Conducting a pH test using a water quality test kit: Before and After pH adjustment

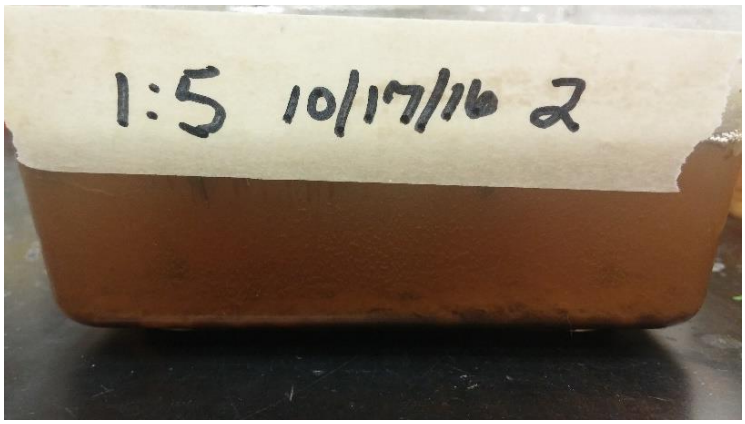

Figure B-14 Sedimentation of anaerobically digested dairy manure at dilution 1:5 at 3000 lux

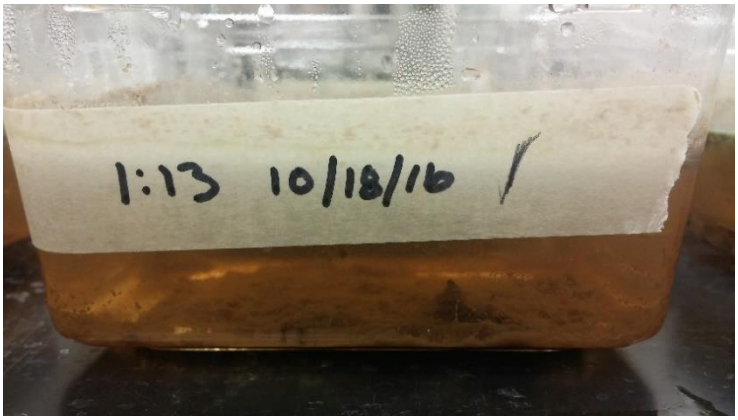

Figure B-15 Sedimentation of anaerobically digested dairy manure at dilution 1:13 at 3000 lux

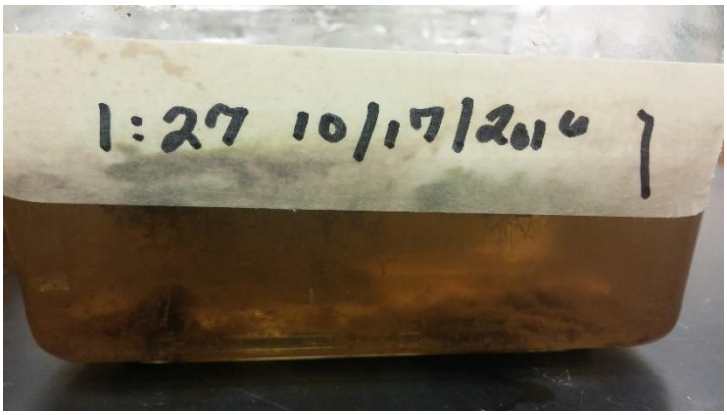

Figure B-16 Sedimentation of anaerobically digested dairy manure at dilution 1:27 at 3000 lux

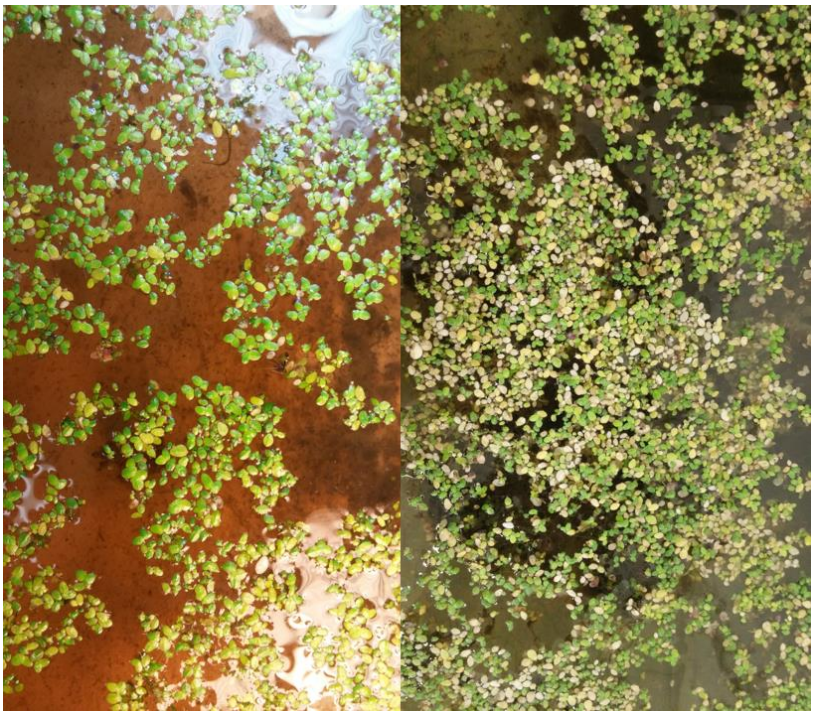

Figure B-17 Starch content of Landoltia punctata: healthily duckweed on the left and high starch content on the right

Figure B-18 Dried and ball milled duckweed: higher starch content shows a white pigmentation

Table B-1 Raw growth data for duckweed cultivated on anaerobically digested dairy manure at a light intensity of 10,000 lux

Units	Biomass Yield (g wet. m^{-2})								
Dilution	1.6 g L^{-1} Hoagland E-Medium			1:18			1:27		
Treatment	**L. punctata**	**L. gibba**	**L. minuta**	**L. punctata**	**L. gibba**	**L. minuta**	**L. punctata**	**L. gibba**	**L. minuta**
0d	36.9	39.4	60.4	40.2	49.4	53.3	40.2	49.0	53.6
4d	77.3	97.0	91.2	94.0	100.4	67.2	108.7	97.8	59.7
8d	134.7	119.9	90.1	132.6	100.8	62.8	171.0	106.4	79.9
12d	179.9	151.8	90.0	129.6	161.6	83.2	247.1	180.0	97.7
16d	231.0	183.5	96.2	189.3	167.1	107.1	302.2	310.7	107.7
20d	270.5	199.8	101.0	219.6	207.5	113.8	344.8	376.4	132.4
24d	292.7	203.2	115.6	234.6	252.6	115.5	350.2	451.6	142.5
28d	382.3	248.2	157.1	358.6	357.9	171.4	425.6	576.8	204.4

Table B-2 Raw data for the reduction of total nitrogen from anaerobically digested dairy manure from duckweed treatments at 10,000 lux

Units	Total Nitrogen (mg L^{-1})							
Dilution	1:18	1:27	1:18			1:27		
Treatment	**Control**	**Control**	**L. punctata**	**L. gibba**	**L. minuta**	**L. punctata**	**L. gibba**	**L. minuta**
0d	83.1	55.8	83.1	83.1	83.1	55.8	55.8	55.8
4d	44.1	53.4	43.8	40.1	42.6	36.4	34.6	45.7
8d	44.4	29.5	24.7	26.3	25.5	14.5	19.4	16.0
12d	21.3	15.8	14.3	18.0	15.8	11.6	16.0	12.5
16d	15.9	13.6	13.2	10.8	13.8	8.6	10.6	12.2
20d	12.6	12.6	7.8	9.9	10.0	7.8	9.9	10.0
24d	13.6	13.6	7.1	8.9	11.8	7.1	8.9	11.8
28d	15.9	12.9	6.3	9.3	12.5	8.5	11.8	12.0

Table B-3 Raw data for the reduction of total Kjeldahl from anaerobically digested dairy manure from duckweed treatments at 10,000 lux

Units	Total Kjeldahl Nitrogen (mg L^{-1})							
Dilution	1:18	1:27	1:18			1:27		
Treatment	**Control**	**Control**	**L. punctata**	**L. gibba**	**L. minuta**	**L. punctata**	**L. gibba**	**L. minuta**
0d	78.3	53.6	77.6	77.3	77.3	55.9	55.9	55.9
4d	39.4	54.6	39.0	35.2	38.2	31.9	30.3	40.8
8d	40.0	26.3	20.2	21.7	20.6	11.6	16.5	12.9
12d	18.5	13.3	20.4	15.4	13.2	9.1	13.2	9.7
16d	14.5	7.3	11.7	9.2	12.1	7.0	9.0	10.8
20d	15.8	10.8	11.5	10.3	7.5	6.7	8.3	8.0
24d	13.8	11.4	12.7	13.7	11.8	5.8	7.1	9.8
28d	13.4	10.6	12.4	11.3	11.9	7.0	9.8	9.9

Table B-4 Raw data for the reduction of total phosphorus from anaerobically digest dairy manure from duckweed treatments at 10,000 lux

Units	Total Phosphorus (mg L^{-1})							
Dilution	1:18	1:27	1:18			1:27		
Treatment	**Control**	**Control**	**L. punctata**	**L. gibba**	**L. minuta**	**L. punctata**	**L. gibba**	**L. minuta**
0d	9.9	6.7	9.9	9.9	9.9	6.7	6.7	6.7
4d	6.7	6.3	5.0	3.5	3.9	2.9	3.7	4.8
8d	5.4	4.8	4.3	3.2	3.8	1.6	3.1	2.9
12d	5.4	4.8	4.3	3.2	3.8	1.6	3.1	2.9
16d	2.7	2.9	1.3	1.0	1.9	0.5	0.4	1.0
20d	3.8	0.8	2.6	2.0	3.2	1.5	2.0	3.4
24d	6.0	6.6	4.7	3.9	5.0	3.1	5.7	4.4
28d	7.7	5.0	2.5	2.7	3.3	0.6	1.6	1.6

Table B-5 Raw data for the reduction of ortho-phosphate-phosphorus from anaerobically digest dairy manure from duckweed treatments at 10,000 lux

Units	ortho-Phosphate-Phosphorus (mg L^{-1})							
Dilution	1:18	1:27	1:18			1:27		
Treatment	**Control**	**Control**	**L. punctata**	**L. gibba**	**L. minuta**	**L. punctata**	**L. gibba**	**L. minuta**
0d	8.9	6.0	8.9	8.9	8.9	6.0	6.0	6.0
4d	6.0	5.9	4.3	3.8	3.9	2.6	1.2	2.8
14d	0.8	0.5	0.20	0.20	0.5	0.5	0.5	0.5
28d	0.3	0.6	0.1	0.20	0.3	0.3	0.4	0.3

Table B-6 Duckweed controls of 1.6 g L^{-1} of Hoagland E-Medium for the parameters of TN, TKN, TP, and o-PO_4-P at a light intensity of 10,000 lux

Days	L. punctata	L gibba	L. minuta
Dilution	1.6 g L^{-1} Hoagland E- Medium (TN)		
0	257.6	257.6	257.6
4	137.1	146.6	147.0
8	186.3	169.8	154.0
12	161.7	155.5	157.7
16	148.5	149.2	155.5
20	121.8	141.2	144.1
24	89.9	90.8	113.7
28	46.5	36.5	68.1
Dilution	1.6 g L^{-1} Hoagland E- Medium (TKN)		
0	69.0	69.0	69.0
4	39.3	22.7	38.0
8	40.0	26.8	39.6
12	41.4	24.3	24.3
16	34.7	29.3	32.2
20	48.0	47.8	59.9
24	44.1	31.8	38.5
28	32.4	27.5	42.4
Dilution	1.6 g L^{-1} Hoagland E- Medium (TP)		
0	100.00	100.00	100.00
4	24.37	18.53	25.33
8	5.60	2.80	3.37
12	6.80	15.80	8.53
16	3.07	7.93	9.47
20	0.47	2.23	1.77
24	1.80	5.20	1.67
28	0.15	0.38	0.17
Dilution	1.6 g L^{-1} Hoagland E- Medium (o-PO_4-P)		
0	94.97	94.97	94.97
4	43.89	45.80	55.00
14	6.35	11.66	11.35
28	0.15	0.38	0.17

Table B-7 Raw data for Landoltia punctata for the parameters of growth, TN, TKN, NO_3-N+NO_2-N, and biomass uptake of N at a light intensity of 3,000 lux

Unit	**Biomass yield (g wet. m^{-2})**		
Dilution	**1:5**	**1:13**	**1:27**
0d	36.8	37.0	39.3
4d	67.8	71.8	107.3
8d	71.1	133.2	166.2
16d	120.2	216.7	155.7
20d	162.8	259.7	185.8
24d	187.6	263.9	185.8
Unit	**Total Nitrogen (mg L^{-1})**		
0d	266.6	114.3	57.1
4d	103.2	48.6	17.1
8d	61.0	19.2	18.6
16d	49.0	20.7	7.0
20d	26.3	12.2	7.0
24d	26.4	12.2	7.8
Unit	**Total Kjeldahl Nitrogen (mg L^{-1})**		
0d	250.0	107.1	53.6
4d	97.2	46.2	10.1
8d	46.4	9.4	17.0
16d	23.4	10.6	5.8
20d	23.3	10.9	6.0
24d	23.2	10.5	6.7
Unit	**Nitrates and Nitrites (mg L^{-1})**		
0d	16.7	7.1	3.6
4d	5.9	2.5	2.6
8d	14.7	7.7	1.5
16d	30.9	10.1	1.3
20d	3.0	1.3	1.0
24d	2.9	1.7	1.0
Unit	**Biomass uptake of N (mg m^{-2})**		
0d	116.9	123.9	78.7
4d	215.3	240.6	215.0
8d	225.9	446.0	332.9
16d	381.9	725.6	311.9
20d	517.3	869.5	372.3
24d	595.9	883.6	372.3

Table B-8 Mass balance on nitrogen for the parameters of total nitrogen removal, biomass removal, nitrification/denitrification, and nitrogen sedimentation

Units	Mass Balance on N (mg)				
Day	**Dilution**	**TNR**	**BMR**	**N_2**	**NS**
24	1:5	41.0	5.5	5.6	29.9
24	1:13	19.0	9.5	1.7	7.8
24	1:27	7.7	2.9	0.0	4.8

Table B-9 Starch accumulation on the duckweed strains L. punctata, L. gibba, and L. minuta for a duration of 28 days

Treatment	**Dilution 1:18**	**Dilution 1:27**	**Control 1.6 g L^{-1} HE medium**
L. punctata	26.1	30.2	20.5
L. gibba	19.1	17.7	10.1
L. minuta	26.0	33.0	15.0

Table B-10 Starch content of Landoltia punctata over a cultivation period of 30 days on dilution ratios 1:27, 1:149, and a control of 355 mg L^{-1} of Hoagland E-Medium

Units	**% Starch (m/m)**		
Dilution	**1:27**	**1:149**	**355 mg L^{-1} Hoagland E-medium**
0d	16.8	16.8	16.8
5d	22.6	16.6	26.1
10d	17.9	13.1	14.7
15d	14.0	11.7	12.3
20d	11.8	15.4	15.7
25d	16.0	18.4	17.1
30d	30.1	20.3	20.3

B.3 Commercial Lab Data

NORTHWEST LABS, LLC.
150 BRIDON WAY
JEROME, IDAHO 83338
208-324-7511

Sample Analysis Report

Customer No.	U032	Date:	8/17/2015
Name:	U of I Twin Falls R & E Center	Sample #	89031
Address:	315 Falls Avenue	Date Received:	7/24/2015
	Twin Falls, ID 83301		
ATTN:	Lide Chen		
Sample Desc:	Digested Dairy Manure		

	As Received	As Received lbs/ton		As Received	As Received lbs/ton
Dry Matter %	1.24	24.8	NO_3-N ppm	0.33	0.1
Moisture %	98.76	1975.2	P_2O_5 %	0.02	0.4
Total Nitrogen (N) %	0.13	2.5	K_2O_5 %	0.09	1.8
Total Carbon (C) %	0.89	17.8	Ca %	0.04	0.8
TOC %	0.33	6.7	Mg %	0.03	0.6
C:N	7.11		S %	0.01	0.2
Crude Protein %	0.78	15.6	Zn ppm	2.00	0.0
Crude Fiber %	0.02	0.3	Fe ppm	13.0	0.0
Crude Fat %	0.02	0.4	Mn ppm	1.00	0.0
ADF %	0.07	1.4	Cu ppm	0.50	0.1
NDF %	0.09	1.7	B ppm	0.30	0.1
Ash %	0.44	8.8	Na %	0.07	1.4
Sugar %	0.25	5.0	Cl %	0.04	0.8
Starch %	0.68	13.6			
Lignin %	0.02	0.5			

Signed: M. Atkinson

THANKS FOR YOUR BUSINESS

Northwest Labs LLC accepts no responsibility nor makes any claims or inferences as to the origin of the sample or the sampling technique, unless certified by our lab.

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Certificate of Analysis

Prepared For: Kevin Kruger
Biological & Agricultural Engineering
University of Idaho
PO Box 442060
Moscow, ID 83844-2060

Case ID: **PNOV16-002**
Report Date: 20-Dec-16
Date Received: 29-Nov-16
Client Ref.: BFF469
Project ID:

1st Level QC: Lydia Osborne Date: 12-20-16
2nd Level QC: [signature] Date: 12-20-16

Case Comments:

ND = Not Detected NA = Not Applicable RL = Reporting Limit QNS = Quantity Not Sufficient

20-Dec-16

Analytical Sciences Laboratory
Certificate of Analysis

Case ID: PNOV16-002

Client SampleID: 1:5 S1 Site/Location:
ASL Sample ID: P1600488 Matrix: Solid - Dry Weight

Percent Carbon & Nitrogen	Method: Combustion, ASA 29-2.2	Prep: N/A		Analysis Date: ########
	Results	RL	Pres.: None	Filter? N/A
Total Nitrogen	4.0 %	0.010		

Client SampleID: 1:5 S2 Site/Location:
ASL Sample ID: P1600489 Matrix: Solid - Dry Weight

Percent Carbon & Nitrogen	Method: Combustion, ASA 29-2.2	Prep: N/A		Analysis Date: ########
	Results	RL	Pres.: None	Filter? N/A
Total Nitrogen	3.5 %	0.010		

Client SampleID: 1:5 S3 Site/Location:
ASL Sample ID: P1600490 Matrix: Solid - Dry Weight

Percent Carbon & Nitrogen	Method: Combustion, ASA 29-2.2	Prep: N/A		Analysis Date: ########
	Results	RL	Pres.: None	Filter? N/A
Total Nitrogen	3.6 %	0.010		

Client SampleID: 1:13 S1 Site/Location:
ASL Sample ID: P1600491 Matrix: Solid - Dry Weight

Percent Carbon & Nitrogen	Method: Combustion, ASA 29-2.2	Prep: N/A		Analysis Date: ########
	Results	RL	Pres.: None	Filter? N/A
Total Nitrogen	4.2 %	0.010		

Client SampleID: 1:13 S2 Site/Location:
ASL Sample ID: P1600492 Matrix: Solid - Dry Weight

Percent Carbon & Nitrogen	Method: Combustion, ASA 29-2.2	Prep: N/A		Analysis Date: ########
	Results	RL	Pres.: None	Filter? N/A
Total Nitrogen	3.5 %	0.010		

Client SampleID: 1:13 S3 Site/Location:
ASL Sample ID: P1600493 Matrix: Solid - Dry Weight

Percent Carbon & Nitrogen	Method: Combustion, ASA 29-2.2	Prep: N/A		Analysis Date: ########
	Results	RL	Pres.: None	Filter? N/A
Total Nitrogen	4.0 %	0.010		

ND = Not Detected NA = Not Applicable RL = Reporting Limit QNS = Quantity Not Sufficient

20-Dec-16

Analytical Sciences Laboratory
Certificate of Analysis

Case ID: PNOV16-002

Client SampleID: 1:27 S1 Site/Location:
ASL Sample ID: P1600494 Matrix: Solid - Dry Weight

Percent Carbon & Nitrogen	Method: Combustion, ASA 29-2.2	Prep: N/A		Analysis Date: ########
	Results	RL	Pres.: None	Filter? N/A
Total Nitrogen	3.5 %	0.010		

Client SampleID: 1:27 S2 Site/Location:
ASL Sample ID: P1600495 Matrix: Solid - Dry Weight

Percent Carbon & Nitrogen	Method: Combustion, ASA 29-2.2	Prep: N/A		Analysis Date: ########
	Results	RL	Pres.: None	Filter? N/A
Total Nitrogen	3.5 %	0.010		

Client SampleID: 1:27 S3 Site/Location:
ASL Sample ID: P1600496 Matrix: Solid - Dry Weight

Percent Carbon & Nitrogen	Method: Combustion, ASA 29-2.2	Prep: N/A		Analysis Date: ########
	Results	RL	Pres.: None	Filter? N/A
Total Nitrogen	3.5 %	0.010		

Samples will be discarded one month after date of final report unless otherwise requested

ND = Not Detected NA = Not Applicable RL = Reporting Limit QNS = Quantity Not Sufficient

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Certificate of Analysis

Prepared For: Kevin Kruger
Biological & Agricultural Engineering
University of Idaho
PO Box 442060
Moscow, ID 83844-2060

Case ID: PJAN17-001
Report Date: 24-Jan-17
Date Received: 17-Jan-17
Client Ref.: BFF469
Project ID:

1st Level QC: Tyelia Osborne Date: 1-24-17
2nd Level QC: Date: 1-24-17

Case Comments:

ND = Not Detected NA = Not Applicable RL = Reporting Limit QNS = Quantity Not Sufficient

24-Jan-17

Analytical Sciences Laboratory
Certificate of Analysis

Case ID: PJAN17-001

Client SampleID: 1:5 S1 Site/Location:
ASL Sample ID: P1700001 Matrix: Solid - Dry Weight

Macro Element Screen	Method: ICP	Prep: Nitric Digest	Analysis Date: 24-Jan-17
	Results	RL	Pres.: As Received / Filter? N/A
Phosphorus	26000 µg/g	80	

Client SampleID: 1:5 S2 Site/Location:
ASL Sample ID: P1700002 Matrix: Solid - Dry Weight

Macro Element Screen	Method: ICP	Prep: Nitric Digest	Analysis Date: 24-Jan-17
	Results	RL	Pres.: As Received / Filter? N/A
Phosphorus	26000 µg/g	20	

Client SampleID: 1:5 S3 Site/Location:
ASL Sample ID: P1700003 Matrix: Solid - Dry Weight

Macro Element Screen	Method: ICP	Prep: Nitric Digest	Analysis Date: 24-Jan-17
	Results	RL	Pres.: As Received / Filter? N/A
Phosphorus	30000 µg/g	40	

Client SampleID: 1:13 S1 Site/Location:
ASL Sample ID: P1700004 Matrix: Solid - Dry Weight

Macro Element Screen	Method: ICP	Prep: Nitric Digest	Analysis Date: 24-Jan-17
	Results	RL	Pres.: As Received / Filter? N/A
Phosphorus	26000 µg/g	40	

Client SampleID: 1:13 S2 Site/Location:
ASL Sample ID: P1700005 Matrix: Solid - Dry Weight

Macro Element Screen	Method: ICP	Prep: Nitric Digest	Analysis Date: 24-Jan-17
	Results	RL	Pres.: As Received / Filter? N/A
Phosphorus	20000 µg/g	20	

Client SampleID: 1:13 S3 Site/Location:
ASL Sample ID: P1700006 Matrix: Solid - Dry Weight

Macro Element Screen	Method: ICP	Prep: Nitric Digest	Analysis Date: 24-Jan-17
	Results	RL	Pres.: As Received / Filter? N/A
Phosphorus	31000 µg/g	40	

ND = Not Detected NA = Not Applicable RL = Reporting Limit QNS = Quantity Not Sufficient

24-Jan-17

Analytical Sciences Laboratory
Certificate of Analysis

Case ID: PJAN17-001

Client SampleID: 1:27 S1 Site/Location:
ASL Sample ID: P1700007 Matrix: Solid - Dry Weight

Macro Element Screen	Method: ICP	Prep: Nitric Digest	Analysis Date: 24-Jan-17
	Results	RL	Pres.: As Received / Filter? N/A
Phosphorus	22000 µg/g	40	

Client SampleID: 1:27 S2 Site/Location:
ASL Sample ID: P1700008 Matrix: Solid - Dry Weight

Macro Element Screen	Method: ICP	Prep: Nitric Digest	Analysis Date: 24-Jan-17
	Results	RL	Pres.: As Received / Filter? N/A
Phosphorus	24000 µg/g	500	

Client SampleID: 1:27 S3 Site/Location:
ASL Sample ID: P1700009 Matrix: Solid - Dry Weight

Macro Element Screen	Method: ICP	Prep: Nitric Digest	Analysis Date: 24-Jan-17
	Results	RL	Pres.: As Received / Filter? N/A
Phosphorus	22000 µg/g	140	

Samples will be discarded one month after date of final report unless otherwise requested

ND = Not Detected NA = Not Applicable RL = Reporting Limit QNS = Quantity Not Sufficient

Appendix C

Calculations

C.1 Sample Calculations for Cultivation of *Landoltia punctata*

C.1.1 Biomass productivity

The sample calculation for biomass productivity is as follows:

$$\boldsymbol{BP = \frac{DW_f - DW_i}{t}}$$

Where,

BP	=	Biomass Productivity, g wet wt. m^{-2} d^{-1}
DW_f	=	Duckweed Weight Final, g wet wt. m^{-2}
DW_i	=	Duckweed Weight Initial, g wet wt. m^{-2}
t	=	time, d

$$\boldsymbol{BP = \frac{250.8\ g\ wet\ wt.m^{-2} - 37.5\ g\ wet\ wt.m^{-2}}{30\ d} = 7.1\ g\ wet\ wt.m^{-2}d^{-1}}$$

C.1.2 Relative growth rate

The sample calculation for first order version of relative growth rate is as follows:

$$\boldsymbol{RGR = \frac{ln\left(\frac{m_f}{m_i}\right)}{t}}$$

Where,

RGR	=	Relative Growth Rate, d^{-1}
m_f	=	Duckweed Weight Final, g wet wt. m^{-2}
m_i	=	Duckweed Weight Initial, g wet wt. m^{-2}
t	=	time, d

$$\boldsymbol{RGR = \frac{ln\left(\frac{50.8\ g\ wet\ wt.m^{-2}}{37.5\ g\ wet\ wt.m^{-2}}\right)}{30\ d} = 0.063\ d^{-1}}$$

C.1.3 Doubling time

The sample calculation for doubling time is as follows:

$$\boldsymbol{DT = \frac{ln\ (2)}{RGR}}$$

Where,

DT = Doubling Time, d
RGR = Relative Growth Rate, d^{-1}

$$\boldsymbol{DT = \frac{ln\ (2)}{0.063\ d^{-1}} = 11\ d}$$

C.2 Batch Sample Relative Growth Rate

The sample calculation for zero order version of relative growth rate is as follows:

$$\boldsymbol{C_t = k \times t + C_o}$$

Where,

C_t = Biomass Yield at time t, g wet. m^{-2}
k = Relative Growth Rate, g wet. m^{-2} d^{-1}
t = time, d
C_o = Initial Biomass Yield, g wet. m^{-2}

A generated equation from Microsoft Excel was produced from the growth data:

$$\boldsymbol{C_t = 13.340 \times t + 61.967}$$

C.2.1 Nutrient rate constant

The sample calculation for first order version of nutrient rate constant is as follows:

$$\boldsymbol{A = A_o \times e^{-zt}}$$

Where,

A	=	Biomass Yield at time t, g wet. m^{-2}
A_o	=	Relative Growth Rate, g wet. m^{-2} d^{-1}
z	=	time, d
t	=	Initial Biomass Yield, g wet. m^{-2}

A generated equation from Microsoft Excel was produced from TN reduction data:

$$\boldsymbol{A = 52.191 \times e^{-0.122t}}$$

C.3 Batch Efficiency

The sample calculation for batch efficiency is as follows:

$$\boldsymbol{TNR = BMR + NH_3 + NS + N_2}$$

Where,

TNR	=	Total Nitrogen Removal, mg
BMR	=	Biomass uptake of N, mg
NH_3	=	Ammonia volatilization, mg
NS	=	N sedimentation, mg
N_2	=	Nitrification/denitrification, mg

C.3.1 Total nitrogen removed

$$\boldsymbol{TNR = (TN_i - TN_f)\times V}$$

Where,

TNR	=	Total Nitrogen Removal, mg
TN_i	=	Initial TN concentration, mg L^{-1}
TN_f	=	Final TN concentration, mg L^{-1}
V	=	Volume, L

$$\boldsymbol{TNR = (114.3\ mg\ L^{-1} - 16.01\ mg\ L^{-1})\times 0.2\ L = 19.0\ mg}$$

C.3.2 Biomass uptake of N

$$\boldsymbol{BMR = NUR\times V\times t}$$

Where,

BMR	=	Biomass Uptake of N, mg
NUR	=	Nitrogen Uptake Rate, mg m^{-2} d^{-1}
SA	=	Surface area, m^2
T	=	time, d

$$\boldsymbol{BMR = 34.01\ mg\ m^{-2}\times 0.0116\ m^2\times 24\ d = 9.48\ mg}$$

C.3.3 Ammonia volatilization

$$\boldsymbol{pKa = 0.09108 + \frac{2729.2}{273.2 + T}}$$

Where,

pKa	=	Ammonia Dissociation Constant
T	=	Temperature, °C

$$\boldsymbol{pKa = 0.09108 + \frac{2729.2}{273.2 + 20°C} = 9.4}$$

Then inserting pKa into the next equation:

$$\boldsymbol{\%NH_3 = \frac{100}{1 + 10^{(pKa - pH}}}$$

Where,

$\%NH_3$	=	Percent Ammonia in System, %
pKa	=	Ammonia Dissociation Constant, unitless
pH	=	pH, unitless

$$\boldsymbol{\%NH_3 = \frac{100}{1+10^{(9.4-7.0}} = 0.4\%}$$

Ammonia volatilization is seen as negligible in this system.

C.3.4 Nitrification/denitrification

$$\boldsymbol{N_2 = (NO3_i - NO3_f)\times V}$$

Where,

N_2	=	Nitrification/denitrification, mg
$NO3_i$	=	Initial NO3 concentration, mg L^{-1}
$NO3_f$	=	Final NO3 concentration, mg L^{-1}
V	=	Volume, L

$$\boldsymbol{N_2 = (7.0\ mg\ L^{-1} - 1.0\ mg\ L^{-1})\times 0.2\ L = 1.2\ mg}$$

C.3.5 N sedimentation

Solving for NS gives:

$$\boldsymbol{NS = TNR - BMR - N_2}$$

$$\boldsymbol{NS = 19.0\ mg - 9.48\ mg - 1.2\ mg = 8.32\ mg}$$

C.3.5 Percentages of total nitrogen removed

$$\%\boldsymbol{BMR} = \frac{\boldsymbol{BMR}}{\boldsymbol{TNR}}\times\boldsymbol{100}$$

$$\%\boldsymbol{N_2} = \frac{\boldsymbol{N_2}}{\boldsymbol{TNR}}\times\boldsymbol{100}$$

$$\%\boldsymbol{NS} = \frac{\boldsymbol{NS}}{\boldsymbol{TNR}}\times\boldsymbol{100}$$

$$\boldsymbol{BMR = \frac{9.48\ mg}{19.0\ mg} \times 100 = 50\%}$$

$$\boldsymbol{\%N_2 = \frac{1.2\ mg}{19.0\ mg} \times 100 = 6.3\%}$$

$$\boldsymbol{\%NS = \frac{8.32\ mg}{19.0\ mg} \times 100 = 43.8\%}$$

Note: There is error associated with this analysis as the final percentages are not quite the same as in the analysis within chapter four. That error can be seen in Table 4-10. The nitrification/denitrification part of the mass balance is the most sensitive out of the pathways of nitrogen

C.4 Starch Content Analysis

Starch content was found through the manufactures equations and Microsoft Excel spreadsheet to determine percent starch content. The main equation used in the analysis is as follows:

$$\boldsymbol{\%Starch = \Delta A \times F \times \frac{V}{0.1} \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180} \times D}$$

Where,

%Starch	=	Starch, %
ΔA	=	Absorbance read against blank
F	=	Conversion from absorbance to µg
V	=	Sample volume
100	=	Conversion to mL sample volume
0.1	=	Volume of sample analyzed
1/1000	=	Conversion from µg to mg
100/W	=	Factor to express starch as percentage of flour weight
W	=	weight of sample in mg
162/180	=	Adjustment from free D-Glucose to anhydro D- Glucose
2	=	Dilution of the sample solution on incubation with AMG
D	=	Dilution Factor (if needed)

$$\%\boldsymbol{Starch} = \mathbf{0.378} \times \mathbf{86} \times \frac{\mathbf{100\ mL}}{\mathbf{0.1}} \times \frac{\mathbf{1\mu g}}{\mathbf{1000\ mg}} \times \frac{\mathbf{100}}{\mathbf{100\ mg}} \times \frac{\mathbf{162}}{\mathbf{180}} = \mathbf{29.25}\%$$

Adjusting for moisture content (MC). The MC of the duckweed sample was 8% m/m. The equation to determine starch content on a dry basis is as follows:

$$\%\boldsymbol{Starch}\ (\boldsymbol{Dry\ basis}) = \%\boldsymbol{Starch} \times \frac{\mathbf{100}}{\mathbf{100} - \%\boldsymbol{MC}}$$

$$\%\boldsymbol{Starch}\ (\boldsymbol{Dry\ basis}) = \mathbf{29.25}\% \times \frac{\mathbf{100}}{\mathbf{100} - \mathbf{8}\%} = \mathbf{29.27}\%$$