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Optimizing Growth Conditions of *Azolla pinnata* R. Brown for Phytoremediation

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Abstract

Poor agricultural practices involving synthetic fertilizers have caused many environmental issues which leads to the pollution of all biospheres of the earth. In order to meet the ever-increasing demand of fertilizer it has been proposed to turn to eco-friendly biological nitrogen fixation methods. The aquatic fern *Azolla pinnata* has a symbiotic relationship with the nitrogen fixing bacteria *Anabaena azollae*. This relationship allows the plant to live in low nitrogen environments due to the bacteria providing for the plant's nitrogen requirements. Optimally growing the plant would lead to increased use of biological nitrogen fixation, thus decreasing the need for synthetic fertilizers. It is believed that identifying an optimal combination of different growth conditions would be the best way to improve the plants growth and thus it's biological nitrogen fixation capabilities.

An investigation was undertaken to find the optimal growth conditions of the aquatic fern *A. pinnata*. The growth conditions that were investigated were: light intensity, nitrogen presence, pH control and humidity. The light intensity had three settings, i.e. low light (5 000 lx), medium light (10 000 lx) and high light (20 000 lx), nitrogen was either added to the system in the form of potassium nitrate or omitted, pH control to a pH of 6.5 was either done by daily manual dosing or the system was unaltered, and finally there were three humidity settings – low humidity (60 %), medium humidity (75 %) and high humidity at 90 %. A walk-in greenhouse was constructed so that each growth condition could easily be adjusted to the different settings to facilitate a variety of growth condition combinations. Using a 15 % strength Hoagland's growth medium, it was found that a high light intensity of 20 000 lx, pH control and 90 % humidity yielded the highest growth rate of 0.321 d^{-1}. It was found that the pH control must be used in conjunction with the higher humidity values or else algal infection would occur and would negatively affect the growth of the plant. The nitrogen presence did not have a significant effect on the growth rate, this is likely due to the symbiosis between the *A. pinnata* and the *A. azollae*, proving that the diazotroph fixates enough nitrogen to satisfy the plants nitrogen requirements.

A. pinnata has a variety of uses and since the optimal growth conditions study demonstrated that *A. pinnata* can grow in low nitrogen environments, it was then decided to investigate the plant's phytoremediation properties of phosphorus under low nitrogen environments and to observe if there was a pH response when the phosphorus was depleted.

Using the same set-up as in the optimal growth conditions study, the phosphate amounts were varied between 0 ppm to 3.1 ppm in the Hoagland's solution and different pH control schemes were implemented to assess the feasibility of optimising phosphorus uptake in a nitrogen absent environment. It was found that there was no significant difference in growth or phosphorus uptake when the different pH control schemes were used. The pH 5 control scheme caused the *A. pinnata* to uptake phosphorus more readily than the pH 7. This is due to the fact that phosphorus uptake is improved in acidic environments. The *A. pinnata* did gain substantial mass when placed under low and no phosphate levels. There was no significant pH response when the phosphate was depleted. In the natural pH runs it was found that the pH behaved similarly, no matter the amount of phosphorus added to the system. There was only a visual difference in the *A. pinnata* grown in higher concentrations of phosphorus compared to the lower ones. The plants turned a red colour and the fronds were much smaller in size for lower phosphorus levels. Since the *A. pinnata* was grown optimally and the corresponding growth conditions found and that the phytoremediation study proved the plants resilience to varying amounts of phosphorus it is concluded that *A. pinnata* could be used for phytoremediation purposes in nutrient-polluted systems.

Keywords: *Azolla pinnata*; growth; light intensity; humidity; pH; nitrogen; phosphorus

Publication

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"We still do not know one thousandth of one percent of what nature has revealed to us." - Albert Einstein

"Perseverance, the secret of all triumphs." - Victor Hugo

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Nomenclature

μ	Growth rate	d^{-1}
$+ N$	Nitrogen presence	
$+ pHC$	pH control	
$- N$	Nitrogen absence	
$- pHC$	No pH control	
$[PO_4^{-3}]$	Phosphate concentration	ppm
σ	Standard deviation	g
A	Initial phosphate concentration	ppm
Abs	Absorbance	Au
B	Pre-exponential constant	ppm
C	Rate of phosphate uptake	d^{-1}
F	Linear phosphate uptake gradient	$ppm\ d^{-1}$
G	Linear phosphate uptake intercept	ppm
H	Hoagland's growth medium	
HL	High light intensity	lx
I	IRR2 growth medium	
LL	Low light intensity	lx
M_f	Final mass	g
M_o	Initial mass	g
ML	Medium light intensity	lx
P_f	Final phosphate concentration	ppm
R^2	Coefficient of determination	
t	Time	d

1 Introduction

The increase in human population has caused major pressures on the world's agricultural sector. In order to meet rising food demands, synthetic nitrogen fertilizers produced via the Haber-Bosch process have been increasingly applied to crops over the past few decades (Alexandratos, 2005). These highly concentrated mineral fertilizers have caused an array of severe environmental damage. The synthesis of these fertilizers uses vast amounts of fossil-fuel energy, contributing to greenhouse gas emissions and thus climate change (Tellez-Rio *et al*, 2017). When one applies extensive amounts of these nitrogen fertilizers to soil, the soil quality decreases. The reduction of organic matter and humus content in the soil typically acidifies and alters the pH of the soil (Jensen, Carlsson & Hauggaard-Nielsen, 2020). This can stunt growth of plants and even lead to the release of greenhouse gases. In fact, only small percentages of the nutrients that are applied to soils are actually absorbed by the crops; the vast majority of the nutrients are washed into bodies of water via runoff (Moss, 2008). This highly concentrated amount of nutrients entering the water body causes eutrophication and algal blooms (Glibert *et al*, 2014). Both decrease the quality of the water. With the rapid growth of algae in the water body, large amounts of oxygen are absorbed and sunlight is blocked, which kills aquatic fauna and flora (Gibbs, MacKey & Currie, 2009). This loss of biodiversity and accompanying poor water quality negatively affect fragile ecosystems. Ultimately, this is not sustainable, as a loss of biodiversity, pollution and limited natural resources will eventually cause disaster. An environmentally friendly, sustainable and nutrient-recycling solution must be sought.

Azolla pinnata is a species of the genus *Azolla*, a group of aquatic ferns that have a symbiotic relationship with the diazotrophic bacteria *Anabaena azollae* (Wagner, 1997). The *A. azollae* is able to fixate atmospheric nitrogen and create ammonia which is a form of nitrogen the plant can access and use for growth (Stewart, 1976). This special quality allows *Azolla* species to survive in low-nitrogen environments and to produce nitrogen for accompanying crops. This method of inter-cropping to reduce synthetic nitrogen fertilizers has been applied for centuries in parts of Asia (Ventura & Watanabe, 1993). *Azolla* species have incredibly high growth rates and are known as one of the fastest growing plants in the world (Biswas *et al*, 2005). Due to these characteristics *Azolla* has a wide variety of uses: green manure, increase of nitrogen, phytoremediation, animal feed and anaerobic digestion feed, to name but a few. In order to fully utilise *A. pinnata* one must be able to optimally grow the plant.

An experimental investigation was undertaken to find optimal growth conditions for *A. pinnata*. The light intensity, nitrogen presence, pH control and humidity were in-

vestigated to find the conditions that yielded the highest growth rate. An experimental setup was constructed to easily adjust growth medium type and strength, nitrogen presence or absence, pH control, light intensity and humidity control. The plant's mass was recorded throughout the experiment, photographs of the plants were taken for visual reference and the pH of the growth solution was documented. After the optimal growth conditions for the plant were identified, a subsequent investigation was undertaken to study the phytoremediation properties of *A. pinnata* for phosphorus uptake under low nitrogen environments. This was done in an effort to use *A. pinnata* for phytoremediative hydroponic wastewater cleanup. Two different control schemes were used, one using sodium hydroxide and hydrochloric acid and the other using ammonia and nitric acid as the base and acid, respectively. Two different pH set-points were investigated to see if there was an improvement in phosphorus uptake and if there was a pH response to a lack of phosphorus present. Similarly to the growth investigation, photographs and masses were recorded throughout the experiment as well as corresponding phosphate concentrations and pH values.

2 Literature

2.1 Synthetic nitrogen pollution

All plants require particular nutrients for growth, healthy development and reproduction. Before industrialised agriculture, plants obtained these nutrients through biological means; the quality of the soil, soil bacteria and the geographic location played a key role in the determination of plant health and yield (White & Brown, 2010). It was only in 1909 that Fritz Haber formulated the Haber-Bosch process whereby nitrogen and hydrogen are reacted; in the presence of a heterogenous iron catalyst in a high-pressure and high-temperature vessel to form ammonia. The chemical reaction is shown in Equation 1 (Erlt, 2012).

$$N_2 + 3H_2 \rightarrow 2NH_3 \tag{1}$$

Today, this has become standard practice to create ammonia-rich plant fertilizers where approximately 90 % of the product is used as fertilizer (Erlt, 2012). The process creates enough fertilizer to produce 50 % of the world's food production (Boerner, 2019). It no longer mattered what the quality of the soil is, because synthetic fertilizer is used in such abundance that the plant crops will produce faster growth and higher yields. To meet an increasing demand for food, there has been an escalation in the use of synthetic fertilizers and this has caused several devastating environmental issues (Kanter, 2018).

The increase of nitrogen in the nitrogen cycle due to synthetic fertilizers causes major imbalances in the cycle. The reactive nature of ammonia, and the natural conversion of nitrogen in the nitrogen cycle, result in the formation of nitrates, nitrites and nitrous oxide (Mulvaney, Khan & Ellsworth, 2009), which all pollute different biospheres of the world. The atmosphere is greatly polluted in part because the Haber-Bosch process is extremely energy intensive, using fossil fuel energy, and is responsible for over 1 % of the total global atmospheric carbon dioxide, CO_2, emissions (Boerner, 2019). Nitrous oxide, N_2O, enters the atmosphere due to synthetic fertilizers and is a greenhouse gas that is 300 times more potent than CO_2 (Mulvaney *et al*, 2009). By producing and applying synthetic nitrogen fertilizers one increases greenhouse gas emissions and thus expedites climate change (Kanter, 2018). Nitrates are toxic to both humans and animals and enter the hydrosphere as runoff. This excess of nitrogen causes pollution and eutrophication of water bodies (Moss, 2008). This has caused an increase of harmful algal blooms in many parts of the world. These blooms are toxic to aquatic life and greatly decrease the quality of the water by blocking sunlight and decreasing the oxygen content of the water

(Glibert *et al*, 2014). The nitrogen-rich fertilizers that are directly applied to soil, the earth's lithosphere, greatly decrease soil quality (Wang *et al*, 2018). The over-application of fertilizers over periods of time acidifies the soil, altering pH levels and stripping the soil of nutrients and minerals (Virto *et al*, 2015). The organic matter and humus that are normally in soil are no longer applied, ruining the soil ecosystem and rejuvenation (Virto *et al*, 2015). Due to the social, economic and political dependence on sustainable food production and the devastating environmental effects synthetic nitrogen causes, an alternative solution must be sought.

2.2 The aquatic fern *A. pinnata*

The genus *Azolla* is a group of small, floating, freshwater ferns (Kösesakal, 2014). The word Azolla comes from the combination of the two Greek words *Azo* meaning to dry, and *allyo* meaning to kill, implying that the plant would be killed by drought (Roy, Pakhira & Bera, 2016). *Azolla* species are usually green but can turn a distinctive red colour, caused by an anthocyanin pigment located in the vacuole of the plant (Kitoh, Shiomi & Uheda, 1993), which usually indicates the plant is under some environmental stress (Janes, 1998). These stresses can be caused by lower temperatures, pollution, high light regimes, nutrient deficiency, water deficiency or general extreme environmental conditions (Kösesakal, 2014). Each individual plant of the smaller species of *Azolla*, such as *Azolla pinnata*, can range between 1 cm to 2 cm across the circumference of the plant, while the leaves are approximately 1 mm to 1.5 mm in diameter (Hill & Cilliers, 1999). Each plant has a brown, main stem and triangular, lobe-shaped leaves (Wagner, 1997). A photograph of the plant is shown in Figure 1.

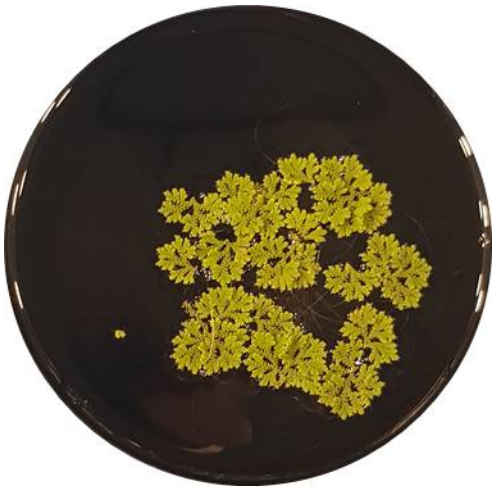

Figure 1: A photograph of *Azolla pinnata* from the University of Pretoria's Manie van der Schijff Botanical Garden.

The leaves of the fern have two lobes; a dorsal lobe and a ventral lobe. The top lobe

contains chlorophyll and is where photosynthesis occurs, while the bottom lobe is partially submerged in water and is colourless (Hill & Cilliers, 1999). The top lobe contains a cavity for the symbiotic bacteria *A. azollae* (Wagner, 1997).

All *Azolla* species have a mutualistic-symbiotic relationship with the diazotrophic cyanobacteria *A. azollae* (Watanabe, 1982). *Azolla* is the only known symbiotic relationship where the cyanobacteria pass straight on to successive offspring via the plant's reproductive sporangia and spores, and the symbiosis has been recorded going back approximately 70 million years (Pereira, 2017). The *Azolla* plant provides the bacteria with mechanical protection from the environment and a fixed carbon source (Wagner, 1997) while the *A. azollae* can fixate atmospheric free nitrogen, thus providing the plant with a nitrogen source in the form of ammonia (Tung & Shen, 1981).

Nitrogen fixation is the process whereby atmospheric free nitrogen (N_2) is converted into ammonia (NH_3) via a diazotrophic bacterium (Stewart, 1976). A diazotroph is a bacterium that contains the enzyme, nitrogenase (Temmink *et al*, 2018). Nitrogen fixation is essentially an enzymatic reduction process where electrons are gained by the nitrogen molecule through the catalysed reaction. Equation 2 shows the chemical reaction representing nitrogen fixation.

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P \tag{2}$$

Nitrogen fixation is an energy intensive process and occurs under anaerobic conditions. The energy needed is received from the hydrolysis of adenosine triphosphate (ATP), converting it to adenosine diphosphate (ADP). The oxygen levels are heavily regulated through numerous biological and enzymatic processes that react to the environment (S De Vries & J De Vries, 2018). Although the nitrogen fixation process is well documented, there are some unknown steps in the mechanism because biochemists cannot visualise some of the steps in vitro (Stewart, 1976). The enzyme nitrogenase is made up of two soluble proteins: nitrogenase and nitrogenase reductase. The nitrogenase, otherwise known as the MoFe protein, is made up of two Mo atoms, twenty-eight to thirty-four Fe atoms and twenty-six to twenty-eight acid-labile sulfides. The nitrogenase reductase, or Fe protein, is made up of four non-heme Fe atoms and four acid-labile sulfides. The substrate binding and reduction occur at the nitrogenase protein site. The nitrogenase reductase supplies electrons to the nitrogenase protein. The ATP is not hydrolysed to ADP until electron transfer occurs (Stewart, 1976). Nitrogenase bonds 1 nitrogen molecule to 3 hydrogen molecules, forming ammonia while additionally producing hydrogen. This is particularly interesting due to hydrogen being able to be used for alternate energy sources (Stewart, 1976).

2.3 *A. pinnata* chemical make-up

The nutrient composition of *A. pinnata* is of interest. The proximate analysis of *A. pinnata* from two different literature sources is shown in Table 1.

Table 1: Proximate analysis of *A. pinnata* from two literature sources.

Component	(Gupta, Chandra & Shinde, 2018)	(Samad *et al*, 2020)
Moisture (%)	10.00	5.14
Crude protein (%)	22.05	24.82
Ether extract (%)	3.25	2.00
Crude fibre (%)		16.64
Ash (%)	18.94	11.59
Nitrogen free extract (%)		34.95

The cell wall constituents of *A. pinnata* from two different literature sources are shown in Table 2.

Table 2: The cell wall analysis of *A. pinnata* from two literature sources.

Component	(Gupta, Chandra & Shinde, 2018)	(Samad *et al*, 2020)
Neutral Detergent Fibre (%)	48.25	42.52
Acid Detergent Fibre (%)	37.14	31.15
Hemicellulose (%)	11.11	11.37
Lignin (%)	8.07	16.86
Cellulose (%)	28.87	14.29

The mineral content of *A. pinnata* is shown in Table 3.

Table 3: The mineral content of *A. pinnata* from two literature sources.

Component	(Alalade & Iyayi, 2006)	(Gupta, Chandra & Shinde, 2018)
Zn (ppm)	87.59	30.02
Cu (ppm)	16.74	26.29
Mn (ppm)	174.42	348.17
Fe (ppm)	755.73	533.12
Na (ppm)	23.79	
Ca (%)	1.16	0.33
P (%)	1.29	
K (%)	1.25	
Mg (%)	0.25	

2.4 Roles of nutrients in *A. pinnata*

Plants need a variety of nutrients and minerals for healthy growth, development and reproduction (White & Brown, 2010). The primary macronutrients required are nitrogen, phosphorus and potassium which all perform different important functions in the plant (de Bang *et al*, 2021). Nitrogen is often considered the most important macronutrient and that is because it is essential for different roles in plant growth. Nitrogen makes up the primary structures of plant proteins known as amino acids; these monomer structures make up plant tissues, chlorophyll and plant cells (Sun *et al*, 2020). Nitrogen is a part of the nucleic acid structure that forms the nitrogenous bases of the structure. Nucleic acids are the building blocks that form deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Gu *et al*, 2018). Nitrogen is a major part of chlorophyll, which is an organelle that aids photosynthesis and thus food production for the plant (Liang & He, 2018). Plants typically take up nitrogen in the form of nitrates or ammonia (Gu *et al*, 2018). Similarly to nitrogen, phosphorus makes up part of the nucleic acid structure that forms the building blocks of DNA and RNA. This regulates protein synthesis, cell division and the development of new tissue (Kuo & Chiou, 2011). Phosphates are also found in ADP and ATP, the molecules responsible for energy transport in the cell (Rychter & Rao, 2005). Phospholipid molecules are one of the main structural components of plant membranes, and they are important second messengers to regulate plant growth and development as well as cellular responses to environmental change or stress (Paz-Ares *et al*, 2022). The limiting macro-nutrient for *Azolla* growth, according to literature, is phosphorus (Tung & Shen, 1981). There was a marked negative difference in growth rate of *Azolla* grown in the absence of phosphorus (Tung & Shen, 1981). Varying the phosphorus content at different temperatures it was found that 20 mg L^{-1} P at 25 °C gave the highest growth rate for *A. pinnata*, 0.241 d^{-1} (Cary & Weerts, 1992). Potassium is very important for healthy plant growth because it is associated with the flow of water, nutrients and carbohydrates within plant tissues (Xu *et al*, 2020). Potassium aids in enzyme activation inside the plant. This affects ATP, starch and protein production (Prajapati & Modi, 2012).

2.5 Reproduction

Azolla species can undergo sexual and asexual reproduction throughout the year, which explains their fast growth rates (Wagner, 1997). The process of sexual reproduction is much more complex and less common than vegetative reproduction because sexual reproduction can only occur if the environmental conditions are completely optimal for *Azolla* growth (Wagner, 1997). At the site of the ventral leaf lobe, instead of vegetatively

producing another leaf lobe, the fern will produce two types of sporocarps (Watanabe, 1982). These are the megasporocarp and the microsporocarp that differ greatly in size. The megasporocarp will produce one megasporangium that contains a single megaspore. The megaspore contains the *A. azollae* bacteria (Watanabe, 1982). The microsporocarp will produce eight to one-hundred and thirty microsporangia, and each microsporangia contains thirty-two or sixty-four spores (Wagner, 1997). The microsporangia releases the spores, which then cling onto the megaspore that is still situated on the megasporocarp. There is a period of dormancy where the microspore and megaspore germinate to make the male and female gametes respectively (Wagner, 1997). The female gamete is fertilised by the male gamete to create the embryo. The embryo will then germinate and eventually, pushing out of the megasporocarp, will be a plantlet. The spores can be dispersed by the flow of water and via waterbirds or mammals (Wagner, 1997). Advantages to sexual reproduction is that it improves genetic diversity within the population and thus could help the organism adapt to new environments and diseases. However, sexual reproduction is time consuming and energy intensive (Silvertown, 2008).

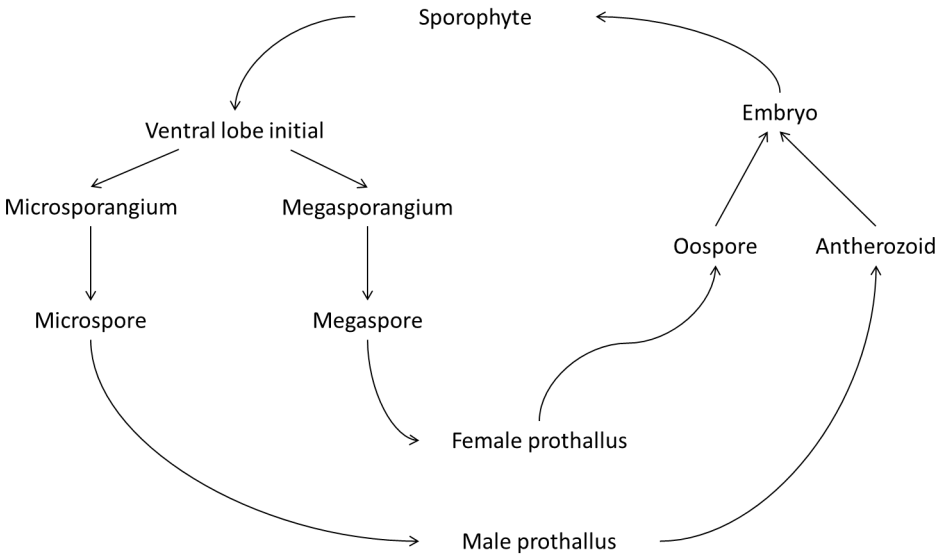

Figure 2: A simplified diagram of the sexual reproduction of *Azolla* (Watanabe, 1982)

Vegetative reproduction is much more common for *Azolla* species, occurring in nature and in agriculture, as the plant does not need optimal conditions to undergo this process. The rhizome leaves form layers that sit on top of one another and these layers naturally break off into fragments, forming entirely new plants that are genetic clones of the parent plant (Janes, 1998). Some advantages of asexual reproduction is that it is a fast process that only requires one parent plant and is generally much less energy intensive (Silvertown, 2008). The problem with asexual reproduction is that it does not lead to genetic variation, therefore a disease can have a devastating effect on the entire population (Silvertown,

2008).

2.6 Growth conditions

One of the main objectives of this study was to find the optimal growth conditions of *A. pinnata*. The following was summarised from *A. pinnata* growth studies from literature focusing on the type of growth medium, the light intensity, pH and humidity.

2.6.1 Growth media

Azolla species, by nature, require little nutrients for growth. This characteristic makes the plant hardy and easy to grow. Many studies' focus was on phytoremediation on specific organic wastewater streams that were in general not properly characterised. The phytoremediation of sewage wastewater streams were done by the following growth studies and yielded the accompanying growth rates; (Chakrabortty & Kushari, 1986) 0.025 d^{-1}, (Sarkar, 1986) 0.056 d^{-1}, (Kumar, 2007) 0.251 d^{-1}. One will note that the growth rates vary quite a bit and this is due to the fact that the sewage streams could be completely different concentrations and have varying nutrients. A growth and phytoremediation study was done on a wastewater stream from a dairy farm by (Goala *et al*, 2021) and the growth rate was found to be 0.100 d^{-1}. The dairy wastewater stream is also organic and will have varying amounts of nutrients. Comparing these growth studies to one another is challenging due to the lack of characteristic information on the growth media and variability of the organic growth media.

Considering synthetic growth media, to try reduce the number of unknowns in the media and thus have a better base-line comparison. One will see that most synthetic media growth studies consulted, used a low strength of Hoagland's medium and the following growth rates were achieved; (Kösesakal & Yildiz, 2019) 0.124 d^{-1}, (Forni *et al*, 2001) 0.0579 d^{-1}, (Rai, Sharma & Rai, 2006) 0.037 d^{-1}. Besides the Hoagland's medium another low-concentration growth medium was used, it was created by (Watanabe, 1982) who achieved a growth rate of 0.065 d^{-1} while other growth studies used this growth medium and achieved higher growth rates due to the nature of the experiment (Kitoh *et al*, 1993) 0.300 d^{-1}. Some other studies did not specify a specific medium but rather just flooded synthetic fertilizer to make the water more nutrient rich (Tung & Shen, 1981) 0.126 d^{-1}, (Tung & Shen, 1985) 0.110 d^{-1}, (Singh & Srivastava, 1985a) 0.085 d^{-1}, (Singh & Srivastava, 1985b) 0.090 d^{-1}. One will note that this does also not properly characterise the growth media but for the flooded fertilizer there is a narrower range of the growth rates.

2.6.2 Light intensity

Light is an important variable in plant growth as it is a determining factor of the rate of photosynthesis (Singh & Srivastava, 1985a). Light and light intensity have major effects on the growth of *A. pinnata*, which seems to be more light sensitive than other species of *Azolla* (Cary & Weerts, 1992). While light intensities are sometimes reported in growth studies, there is a variance between using artificial light and natural light and the manner in which the light intensity is measured is often not discussed.

Most growth studies used natural light but almost all of the studies that did use natural light did not quantify the light intensity (Tung & Shen, 1985), (Chakrabortty & Kushari, 1986), (Cary & Weerts, 1992), (Kumar, 2007), (Gupta, Chandra & Shinde, 2018) and (Kösesakal & Yildiz, 2019). Without proper quantification it is difficult to discern if light intensity has a significant effect on the growth and what intensity one should experimentally plan for. The only two growth studies that varied and quantified the natural light intensity using shade netting was (Singh & Srivastava, 1985a) and (Tung & Shen, 1981), where the light intensity varied between 15 000 lx and 120 000 lx.

When artificial light was used the light intensity was consistently reported but there was no growth study where artificial light was varied to see its effect on the *A. pinnata* growth. Some studies that used artificial light were (Singh & Srivastava, 1985b), (Maejima *et al*, 2002), (Rai *et al*, 2006), (Prasad, Singh & Singh, 2015) and (Goala *et al*, 2021) where the light intensity for the artificial light was generally lower light intensities, and the range was between 2 000 lx and 10 000 lx.

2.6.3 pH of growth media

A. pinnata grows quite well in a pH range of 5 to 8 (Wagner, 1997). This was also noted by (Cary & Weerts, 1992), which reported the highest growth rate of 0.236 d^{-1} when varying the pH to a pH of 7. Higher growth rates have been reported at slightly more acidic pH values. A doubling time of 2.8 days was reported for *A. pinnata* grown in a standard medium with a pH of 5.5 (Tung & Shen, 1981). A doubling time of 2.5 days was reported at a pH value of 5.6 (Watanabe, 1982). (Cary & Weerts, 1992) did an extensive study on the growth rate of *A. pinnata* at different starting pH values. The growth rates for a pH of 4, 7 and 8 were 0.214, 0.236 and 0.233 d^{-1} respectively. Here we can see the *A. pinnata* had higher growth rates at slightly more alkaline pH values. pH control is a variable that has not been properly investigated and compared to non-pH controlled experiments and the vast majority of growth studies did not measure or alter the pH at all, which is a great gap in literature.

2.6.4 Humidity

Azolla species are indigenous to tropical locations that generally have extremely high humidity. *A. pinnata* is native to Southeast Asia and coastal parts of Africa (Watanabe, 1982). This high humidity opens plants' stomata, increasing the uptake of carbon dioxide and thus photosynthesis and growth, but does give rise to insect infestation and mould infections. It is widely accepted that *Azolla* species' optimal humidity is between 85 % and 90 % (Wagner, 1997), and *Azolla* does become dry and fragile at humidity levels less than 60 % (Watanabe, 1982). Most growth studies used a greenhouse to increase humidity levels to above 60 % but did not measure the humidity (Kitoh *et al*, 1993), (Tung & Shen, 1985), (Chakrabortty & Kushari, 1986), (Sarkar, 1986) and (Cary & Weerts, 1992). The growth studies (Kösesakal & Yildiz, 2019) and (Goala *et al*, 2021) measured humidity levels between 55 % and 70 %. A comparative study on the effects of different humidity levels on growth rates has not been investigated.

2.7 Utilisation of *A. pinnata*

Azolla species have several unquestionably advantageous agricultural qualities: a very high biomass productivity if grown in optimal conditions, the ability to fix atmospheric nitrogen and a high crude protein and vitamin content.

2.7.1 Biofertilizer

The world wishes to move away from expensive and environmentally-harmful, petrochemical fertilizers (Rosegrant, Roumasset & Balisacan, 1985). *Azolla* species have been used as a biofertilizer in rice paddies in parts of Southeast-Asia for centuries. The *Azolla* is either used as a green manure by mixing it into the soil before the planting of the rice, or it is grown alongside the rice as a secondary crop (Bocchi & Malgioglio, 2010). Intercropping *Azolla* and rice can be realised as a financial alternative to chemical means of fertilisation, if the irrigation system is well developed (Rosegrant *et al*, 1985). The high growth rate and nitrogen fixing ability of *Azolla* makes it an appealing biofertilizer (Tung & Shen, 1985).

Experiments on rice crops showed that yields increased by 44 % to 55 % when grown in the presence of *Azolla* (Tung & Shen, 1985). It was shown through nitrogen mineralisation results that 30 % of the total *Azolla* was converted to ammonia (Tung & Shen, 1985). Experiments were run in the Niger basin to see if *Azolla* could be used as green manure

in rice fields (Kondo, Kobayashi & Takahashi, 1989). The rice yield increased by 27 % with the presence of *Azolla* (Kondo *et al*, 1989). The average nitrogen content in the physical plant *Azolla* was recorded to be 2.55 % (Kondo *et al*, 1989). The mineralisation of nitrogen by *Azolla* was recorded to be 62 to 75 % within a period of six weeks (Kondo *et al*, 1989). Experiments showed that the nitrogen produced by *Azolla* was 70 to 110 kg N ha^{-1} and the rice yield increased by 1.8 to 3.9 t ha^{-1} (Ventura & Watanabe, 1993). Continuous addition of the *Azolla* green manure increased the organic nitrogen content of the soil (Ventura & Watanabe, 1993).

It is important to increase the nitrogen efficiency and decrease the amounts of water pollution through nitrates entering the ecosystem (Bocchi & Malgioglio, 2010). *Azolla* as an organic fertilizer is a viable option in many parts of the world due to the plant's resilience to harsh environments and its extremely fast growth, allowing for large amounts of nitrogen to be fixed (Bocchi & Malgioglio, 2010).

2.7.2 Feed

Azolla has a higher protein content, and especially high lysine concentrations (Shiomi & Kitoh, 2001), than most green fodder crops and aquatic plants and a reasonably high essential amino acid composition favourable for animal nutrition (Hasan & Chakrabarti, 2009). *Azolla* can be a valuable protein supplement for many species such as fish, pigs and poultry (Hasan & Chakrabarti, 2009).

Azolla was used to investigate its potential to be used as a supplement in fish feed. In a feeding experiment *Tilapia nilotica* was given different percentages of *Azolla* in their feed. There was an increase in fish weight, compared to the control, proportional to the higher levels of *Azolla* being fed to them (Shiomi & Kitoh, 2001), proving that *Azolla* could be used as a sustainable, high-protein feed for *Tilapia*. Experiments were done with swine herds and it was discovered that *Azolla* is an excellent source of minerals and amino acids but has low digestibility with swine herds (Leterme *et al*, 2009). Therefore using *Azolla* as a natural mineral supplement with an accompanying high-fibre diet could be beneficial. The final experiment that was of interest was conducted to investigate the inclusion of *Azolla* in chicken feed to determine the effect on the growth of the birds (Samad *et al*, 2020). It was found that an inclusion of 15 % *Azolla* in the feed resulted in the highest overall bodyweight gain. There was no significant change in nutrient digestibility (Samad *et al*, 2020). Overall, including *Azolla* in different farmed animals' diets proves a feasible solution to increase minerals and protein content in a cheap and sustainable manner.

2.7.3 Food

Azolla has been considered as a food source for humans, although no scientific studies have been conducted. *Azolla* does have a high protein content, similar to alfalfa sprouts or spirulina (Sjodin, 2012).

2.7.4 Phytoremediation

The world faces a water-scarcity crisis due to increasing demands on fresh water and an increase in the volume of wastewater. This drives people to start developing more sustainable methods for wastewater management. Wastewater can be improved via biological treatments to decrease the amount of chemical treatment required, which will lower the amounts of pollutants that enter the wastewater stream. *Azolla* removes nitrogen and phosphates that cause eutrophication. A study was conducted in uncontrolled conditions to explore the phytoremediation properties of *Azolla* on sewage water. The conclusion was that *Azolla* can be used to treat secondary-treated sewage during all seasons, which is particularly useful for irrigation purposes (Kumar, 2007). A similar outdoor experiment was conducted where *Azolla* was grown to determine the nitrogen and phosphorus removal ability. The experiment was not in a controlled environment. The plants were grown in sewage water, well water and a Hoagland's growth medium. The nitrate levels in all three mediums decreased. The nitrite concentration decreased in the sewage water but increased in the well water. The phosphate concentration was already low in the sewage and well water but a decrease in concentration was reported in the Hoagland's medium (Forni *et al*, 2001). Another experiment was done where *Azolla* was used as a biofilter for phosphate removal and general wastewater purification. The experiment was conducted outdoors in an uncontrolled environment. The *Azolla* removed approximately 36 % of the phosphates present in the wastewater (Costa, Santos & Carrapiço, 1999). The biomass increase of the fern proves that *Azolla* can grow in partially treated wastewater, but not point-source wastewater (Costa *et al*, 1999).

Azolla can also be used to remove various heavy pollutants from wastewater streams. *Azolla* was grown in a medium that contained 20 mg L^{-1} of Pb^{2+}. The lead content in the entire plant was 1.8 % after six days (Oren Benaroya *et al*, 2004). This proves that *Azolla* can be used in the removal of heavy metals from wastewater streams if the metal content is not too high as to adversely affect the health and growth of the plant. The phytoremediation ability of *Azolla* was investigated with regard to petroleum hydrocarbons. *Azolla* was grown with low percentages of crude oil. The growth of the *Azolla* was only affected negatively when the crude oil percentage was higher than 0.3 %.

Growth was normal between 0.005 to 0.2 % and the biodegradation rate of the crude oil at these concentrations was between 73 to 94 % (Kösesakal, Ünal, *et al*, 2016). The phytoremediation of *Azolla* regarding Azo-dyes (dyes used widely in the textiles, tannery and paper industries and that often pollute water) was investigated. *Azolla* could perform the biodegradation of the dye but it did negatively affect the growth of the *Azolla*, placing the plant under chemical stress (Khataee *et al*, 2013).

3 Experimental

3.1 Materials

3.1.1 *A. pinnata* collection

The *A. pinnata* was housed in the shaded greenhouse at the Manie van der Schijff Botanical Gardens at the University of Pretoria. The *A. pinnata* was grown in a deep pond in a misted greenhouse. The original sample was collected outside Luanda, Angola and was identified as *Azolla pinnata africana*. The subsequent *A. pinnata* are all clones of the original sample due to the asexual reproduction that the plant underwent.

3.1.2 Growth solutions and pH solutions

The Hoagland solution (per litre) contained 0.120 mg of Cu-EDTA-2Na, 0.240 mg of $ZnSO_4 \cdot 7H_2O$, 1.80 mg of $MnCl_2 \cdot 4H_2O$, 0.0490 g of $MgSO_4 \cdot 7H_2O$, 0.0190 g of Fe-EDTA, 0.740 g of $CaCl_2 \cdot H_2O$, 2.88 mg of H_3BO_3, 0.120 mg of $Na_2MoO_4 \cdot H_2O$, and 0.136 g of KH_2PO_4 0.505 g of KNO_3. The exclusion of nitrogen in the Hoagland's medium was achieved by not adding any KNO_3, providing a nitrogen-free growth medium. The phosphorus concentration was altered by adding specific amounts of KH_2PO_4 to obtain the desired phosphorus concentration.

The IRR2 solution (per litre) contained 69.7 mg of K_2SO_4, 98.6 mg of $MgSO_4 \cdot 7H_2O$, 1.90 mg of Fe-EDTA, 58.8 mg of $CaCl_2 \cdot H_2O$, 13.6 mg of KH_2PO_4, 25.0 μg of $CuSO_4 \cdot 5H_2O$, 0.120 mg of H_3BO_3, 36.0 mg of $Na_2MoO_4 \cdot 2H_2O$, 29.0 μg of $ZnSO_4 \cdot 7H_2O$, 24.0 μg of $CoCl_2 \cdot 6H_2O$, 99.0 mg of $MnCl_2 \cdot 4H_2O$, and 14.0 mg of $FeSO_4 \cdot 7H_2O$.

The pH was controlled using a 0.25 M solution of NaOH and a 0.1 M solution of HCl or a 1 M solution of NH_3 and a 0.1 M solution of HNO_3.

3.1.3 Structures

A 143 X 73 X 195 cm walk-in greenhouse was bought from Leroy and Merlin and constructed with a total of six shelves. The greenhouse had an outer plastic cover that could be opened and closed.

Figure 3: Walk-in greenhouse that was utilised as the growth chamber for better humidity control (Leroy & Merlin, 2021).

This allowed for better humidity control inside the greenhouse. A similar cover was constructed, made from blackout material, to not allow any interfering light into the tent.

Three 1 L, black, PVC containers with a diameter of 0.115 m and height of 0.1 m were placed on each shelf. These would act as the three repeats for each experiment. There was a total of 18 containers.

3.1.4 Lighting

Each container had an individual light bulb as the energy source for the *A. pinnata*. Three LED, cool-white, dimmable, 9 W, eurolux light bulbs were soldered together in parallel.

These globes connected to an E27 base connected to a 1 m cabtyre 3-core 1.5 mm wire, which were all soldered to a 2.5 m wire of the same dimensions. The connecting wire was attached to a rotary dimmer and finally connected to a plug. Six of these custom light blub set-ups were constructed. The lights were plugged into a Major Tech-MTD3 programmable 24 h timer. This allowed one to create day/night cycles for the plants.

3.1.5 Humidity control

An Elektra Health 5 L humidifier was used to regulate the humidity of the greenhouse, while the plastic and blackout covers allowed the humidity to remain relatively constant. An Arduino MEGA 2560 was coupled with a Digital Temperature and Humidity- SHT10 sensor. A simple on/off control scheme was employed to achieve the desired humidity set-point. The humidity controls at three different set-points are shown in Figure 4.

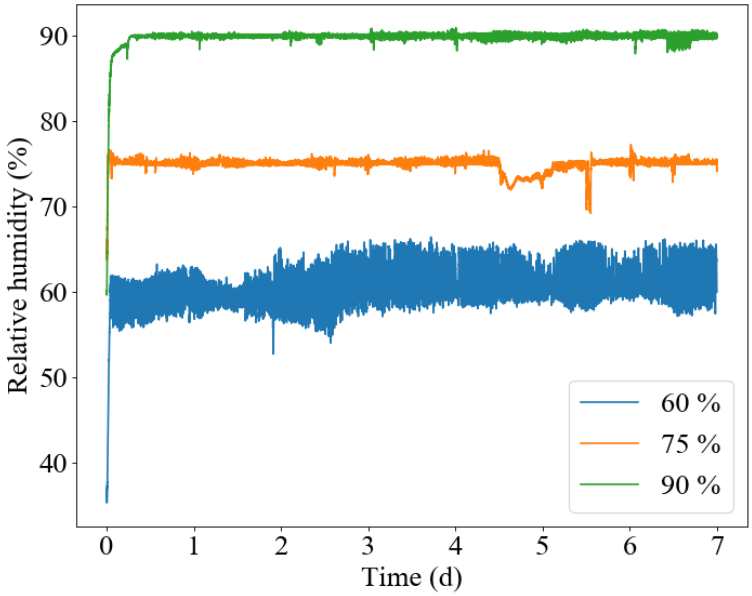

Figure 4: The on/off humidity control data for the three different humidity set-points (da Silva *et al*, 2022).

The 60 % humidity set-point had an average humidity value of 60.81 % and a standard deviation of 2.58 %. The 75 % humidity set-point had an average value of 74.99 % and standard deviation of 0.77 %. Finally, the 90 % set-point value had an average value of 89.83 % and a standard deviation of 1.29 %.

3.2 Methods

3.2.1 Analytical instruments

The wet mass of the *A. pinnata* was measured using an OHAUS Adventurer 2 digital laboratory scale. The light intensity was measured using a handheld digital Victor A1010 lx meter. The daily pH was measured using a digital Bluelab combo pH and EC meter,

Bluelab, Tauranga, New Zealand. The humidity was measured using a Digital Temperature and Humidity sensor (SHT10), Sensirion, Stafa, Switzerland that was coupled with an Arduino MEGA 2560, Smart Projects, Ivera Italy. The phosphate amount was measured using a Spectroquant Phosphate Reagent Test Kit, Merck, Darmstadt, Germany. The absorbance was measured using an UV-Vis spectrophotometer, Agilent Technologies, Santa Clara, USA.

3.3 Experimental procedure

The *A. pinnata* was collected from the greenhouse and washed with tap water. This removed any foreign plant material and dirt ensuring only *A. pinnata* was grown and that the results would be repeatable. The *A. pinnata* would then be placed in the growth medium two days before the run so that the plants could acclimatise to the growth medium before the commencement of the experiment.

The setup of the experiment started with the light intensity adjustments. The hand-held lux meter was used to measure the light intensity that the plants would experience. The light intensity was adjusted by dimming the lights with the rotary switch and moving the bulb closer or further away from the sensor until the desired light intensity was achieved.

The solutions were then made by taking a certain litreage of deionised water and placing a predetermined amount of each stock solution in the deionised water to create the desired solution type and strength. The absence or presence of nitrogen was achieved by adding or withholding the KNO_3 stock solution. The varying amounts of phosphate were achieved by creating a solution with all the stock solutions and then adding the desired amount of phosphate for that growth medium.

Samples were taken by using a 2-10 mL pipette with different pipette tips per container to avoid contamination. A 5 ml sample of the growth solution was taken on days 0, 1, 3, 5 and 7 of the experiment.

The pH was measured daily using the digital pH probe. In cases where the pH was controlled, manual dosing would be done using the 20-200 μL pipette or the 0.2-1 mL pipette. The HCl and HNO_3 would be used to lower the pH and the NaOH and NH_3 would be used to raise the pH to the desired set-point.

The *A. pinnata* that was acclimatised in the growth medium was taken to be weighed. The *A. pinnata* was gently taken out of the growth medium and placed on paper towel to absorb excess water. The initial weight of all the *A. pinnata* was approximately 0.25 g. The mass of the *A. pinnata* was measured on days 0, 1, 3, 5, and 7 of the experiment.

The *A. pinnata* was transferred to the growth container with the particular growth medium and was then placed under a light bulb.

The humidifier was filled with tap water and switched on. The walk-in greenhouse was then closed and the black out cover placed over it. The humidity could now be set using the Arduino code. The Arduino code was run and the humidifier would be on until set-point was reached and would then switch off and on to maintain the desired set-point. The tent would be opened daily to perform pH measurements and dosing, sampling, mass measurements and to take photographs of the plants.

3.4 Fitting curves

In order to calculate the growth rate, μ d^{-1}, of the plants, the mass of the plants was recorded on days 0, 1, 3, 5 and 7. These data points were placed as an input into the *scipy.optimize curve_fit* function in Python® along with Equation 3. The M_f (g) is the final mass or the mass calculated at time t (d), M_o (g) is the initial mass and t (d) is the time. The curve_fit function minimises the error of the fit against Equation 3 and calculates the growth rate that gives the best fit.

$$M_f = M_o e^{\mu t} \tag{3}$$

Samples were taken of the growth medium on days 0, 1, 3, 5, and 7. The samples were reacted with a Spectroquant test kit and then placed in the UV-Vis spectrophotometer where the absorbance was measured. A calibration curve was created to convert the absorbance measurements into phosphate concentrations. A total of nine repeat samples were created with the following range of phosphate concentrations: 3.1, 1.8, 1.2, 0.9, 0.6, 0.3 and 0 ppm phosphate. The absorbance was measured for these known concentrations and the average was taken. The curve shown in Figure 5 was calculated in Python® by assuming a linear correlation and minimising the error of the fit.

The straight line equation for the calibration curve is shown in Equation 4.

$$Abs = 0.357 * [PO_4^{-3}] + 0.097 \tag{4}$$

The curve_fit function in Python® was employed to calculate the phosphate uptake rate. Similarly to the growth curve the data points were placed into the curve_fit function and an initial guess was used to get a minimisation of error fit. Equation 5 was used to fit

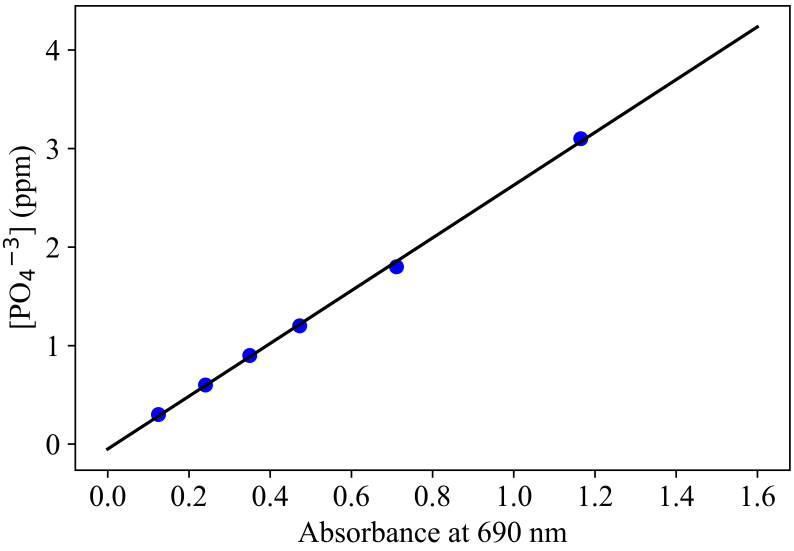

Figure 5: The phosphate calibration curve, relating absorbance 690 nm to phosphate concentration (ppm) with a coefficient of determination of 0.9991.

the data. In some instances the phosphate uptake went to zero before the seven days, in which case the curve was fit to the previous data points and not the last data point since the phosphate was already at a zero point. A was the initial phosphate concentration (ppm), B pre-exponential constant (ppm), C the rate of phosphorus uptake (d^{-1}) and t time (d).

$$P_f = A - Be^{ct} \tag{5}$$

In some cases the curve_fit function was not able to fit the phosphate data to Equation 5. Instead a straight line curve was used to fit the data shown in Equation 6. F is the gradient (ppm d^{-1}) and G is the intercept (ppm).

$$P_f = Ft + G \tag{6}$$

3.5 Experimental schedule

3.5.1 Growth conditions of *A. pinnata*

When the optimal growth conditions of *A. pinnata* were being investigated, the following growth conditions were to be evaluated: nitrogen presence in the growth medium, pH control, light intensity and humidity.

Firstly the medium type and strength were decided upon. The medium type was between the Hoagland's medium and the IRR2 medium at various different strengths which is shown in Table 4.

Table 4: Experimental schedule for the investigation of two different growth mediums and different strengths.

Growth medium	Strengths (%)
Hoagland's growth medium	1, 5, 10, 15
IRR2 growth medium	100, 500

Once the medium was decided upon one could investigate the different growth conditions.

The following was set as the different levels for each growth condition. Nitrogen was either present or absent in the medium. The pH was either controlled at a pH of 6.5 or was not controlled. The light intensity had three settings: high 20 000 lx, medium 10 000 lx or low 5 000 lx. Finally the relative humidity had three settings: 60 %, 75 % and 90 %. Every combination of growth condition was explored to be properly analysed and compared, as shown in Table 5.

Table 5: The experimental schedule for the investigation of the optimal growth conditions for *A. pinnata* evaluating the light intensity, humidity, nitrogen presence (+ N), nitrogen absence (- N), pH control (+ pHC) and no pH control (- pHC).

Light intensity (lx)	Relative humidity (60 %)	Relative humidity (75 %)	Relative humidity (90 %)
5 000	+ N, - N, + pHC, - pHC	+ N, - N, + pHC, - pHC	+ N, - N, + pHC, - pHC
10 000	+ N, - N, + pHC, - pHC	+ N, - N, + pHC, - pHC	+ N, - N, + pHC, - pHC
20 000	+ N, - N, + pHC, - pHC	+ N, - N, + pHC, - pHC	+ N, - N, + pHC, - pHC

3.5.2 Phosphate uptake of *A. pinnata*

After optimal growth conditions were investigated, these conditions were used to investigate different pH regimes effect on phosphate concentration uptake. Different phosphate concentrations were tested: 0, 0.3, 0.6, 0.9, 1.2, 1.8 and 3.1 ppm. Three different pH

levels were tested: pH control at 5, 7 and no pH control. Different pH control schemes were tested using sodium hydroxide NaOH and hydrochloric acid HCl as the base and acid or ammonia NH_3 and nitric acid HNO_3 as the base or acid. Every combination of pH control scheme and phosphate uptake concentration was explored to be properly analysed and compared and is shown in Table 6.

Table 6: The experimental schedule of the phosphate uptake experiments showing the pH control scheme, the initial phosphate concentrations and the different pH set-points.

pH Control	Phosphate concentration (ppm)						
	0	0.3	0.6	0.9	1.2	1.8	3.1
No control	pH	pH	pH	pH	pH	pH	pH
NaOH and HCl	pH 5, pH 7	0pH 5, pH 7	pH 5, pH 7	pH 5, pH 7	pH 5, pH 7	pH 5, pH 7	pH 5, pH 7
NH_3 and HNO_3	pH 5, pH 7	0pH 5, pH 7	pH 5, pH 7	pH 5, pH 7	pH 5, pH 7	pH 5, pH 7	pH 5, pH 7

4 Results and discussion

4.1 Growth conditions of *A. pinnata*

Each experiment was conducted in triplicate to obtain more accurate results. Figure 6 shows the coefficient of determination (R^2) for each experimental repeat. The R^2 value shows a value of 0 to 1 and it is a measure of how statistically well the model fits the data. The standard deviation (σ) was calculated for each experimental set, using the three repeats.

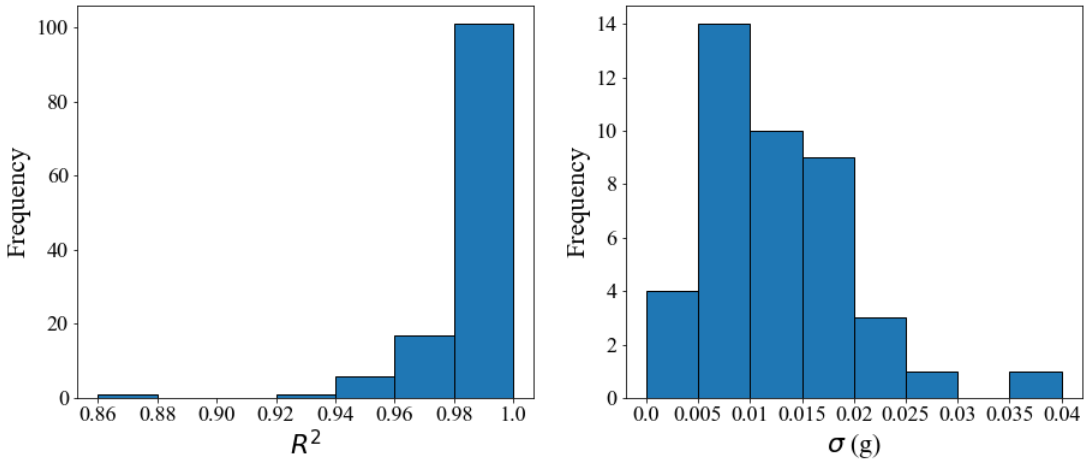

Figure 6: The coefficient of determination, R^2, a value between 0 and 1 shows how accurately a model, shown by Equation 3, fits the data. The R^2 was calculated for each repeat experiment. The standard deviation σ (g) was calculated for each experimental set and shows how closely the set varies from the average of the repeats (da Silva *et al*, 2022).

One will note that the R^2 are almost all above 0.9. There was one experimental set that did not fit the model that well with an R^2 value of 0.87. Since 99.2 % of the rest of the R^2 values were above 0.9 and 96.03 % of the values were above 0.95, one can conclude that the model accurately fitted each of the repeats. The maximum standard deviation value was 0.04 g while the majority of the standard deviations lay between 0.005 and 0.01 g. This proves that the growth rate of the repeated experiment tended to be close to the average of the three repeats. This high repeatability allowed one to use averages for the growth rates.

The first experiment was conducted to find the optimum medium and strength for the growth of *A. pinnata*. This medium type and strength would then be standardised for the rest of the experiments. The media that were investigated were Hoagland's medium and the IRR2 medium. Both media proved to yield high growth rates for *A. pinnata*

in literature. The strengths that were investigated were 1 %, 5 %, 10 % and 15 % for the Hoagland's medium and 100 % and 500 % for the IRR2 medium. The Hoagland's medium has much higher concentrations of nutrients than the IRR2 medium, which is why it can be diluted while the IRR2 medium concentration can be increased.

The other growth conditions were kept constant. There was nitrogen present in all of the media and pH control was not implemented. The light intensity was set to 10 000 lx, the humidity was set to 75 %. These conditions were chosen to be intermediary for the testing conditions and not putting any additional stress to the plant by taking away nutrients or altering pH. Figure 7 shows the average mass data (g) and the growth model predicted using Equation 3 with the accompanying growth rate value, μ d^{-1}.

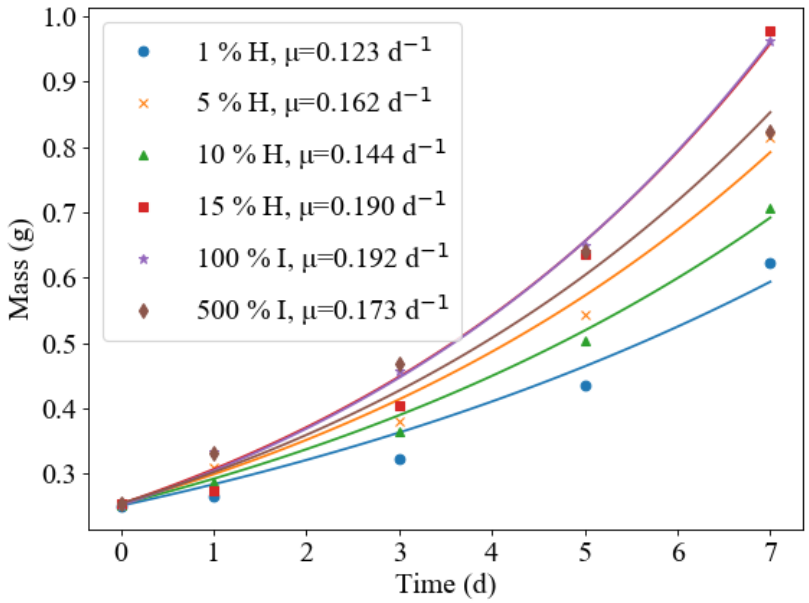

Figure 7: The growth solution experiment results showing the average mass values taken on days 0, 1, 3, 5, and 7 and the growth model with the corresponding growth rate μ d^{-1}. The Hoagland's medium (H) and the IRR2 (I) growth mediums are shown with the strength (da Silva *et al*, 2022).

One can see from Figure 7 that the 100 % IRR2 solution achieved a growth rate of 0.192 d^{-1}, the highest growth rate. The 15 % Hoagland's medium achieved a growth rate of 0.190 d^{-1}, which is just a 1.05 % difference in growth. Figure 8 shows the initial and final photographs taken of the *A. pinnata* in the two different mediums.

In Figure 8 one will note a distinct colour difference between the ferns on day 7. The *Azolla* species have an anthocyanin pigment that becomes visible in the plants by displaying a red colour when the plant is under stress. One can come to the conclusion that this stress response has become visible because of the growth solution, since all the

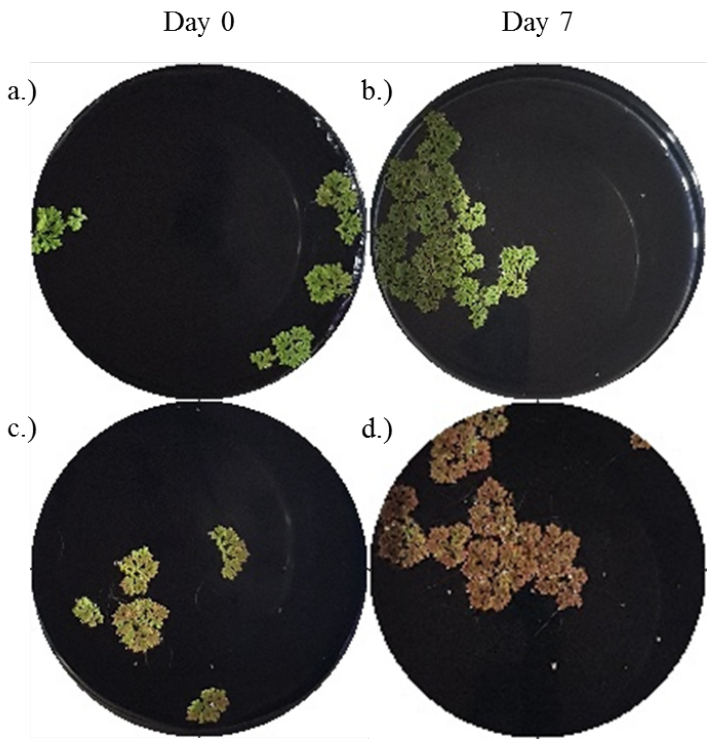

Figure 8: Photographs were taken of the *A. pinnata* in the Hoagland's medium (a. and b.) and the IRR2 medium (c. and d.). The ferns were under the same environmental conditions except for the growth medium (da Silva *et al*, 2022).

other environmental conditions were kept constant. Since the IRR2 solution did not significantly increase the growth rate and the Hoagland's medium did not produce a stress response in the plant, it was decided to select the 15 % Hoagland's growth medium for the growth solution for the future experiments.

Figure 9 shows a comparison between the following growth conditions: light intensity, humidity, pH control and the absence or presence of nitrogen. The light intensity was changed to three different levels: low light (5 000 lx), medium light (10 000 lx) and high light (20 000 lx). The relative humidity was changed to three different set-points: 60 %, 75 % and 90 %. The pH control was either present, controlling the pH to a value of 6.5 with daily dosing, or not present. Finally, nitrogen was either added to the growth medium or it was not.

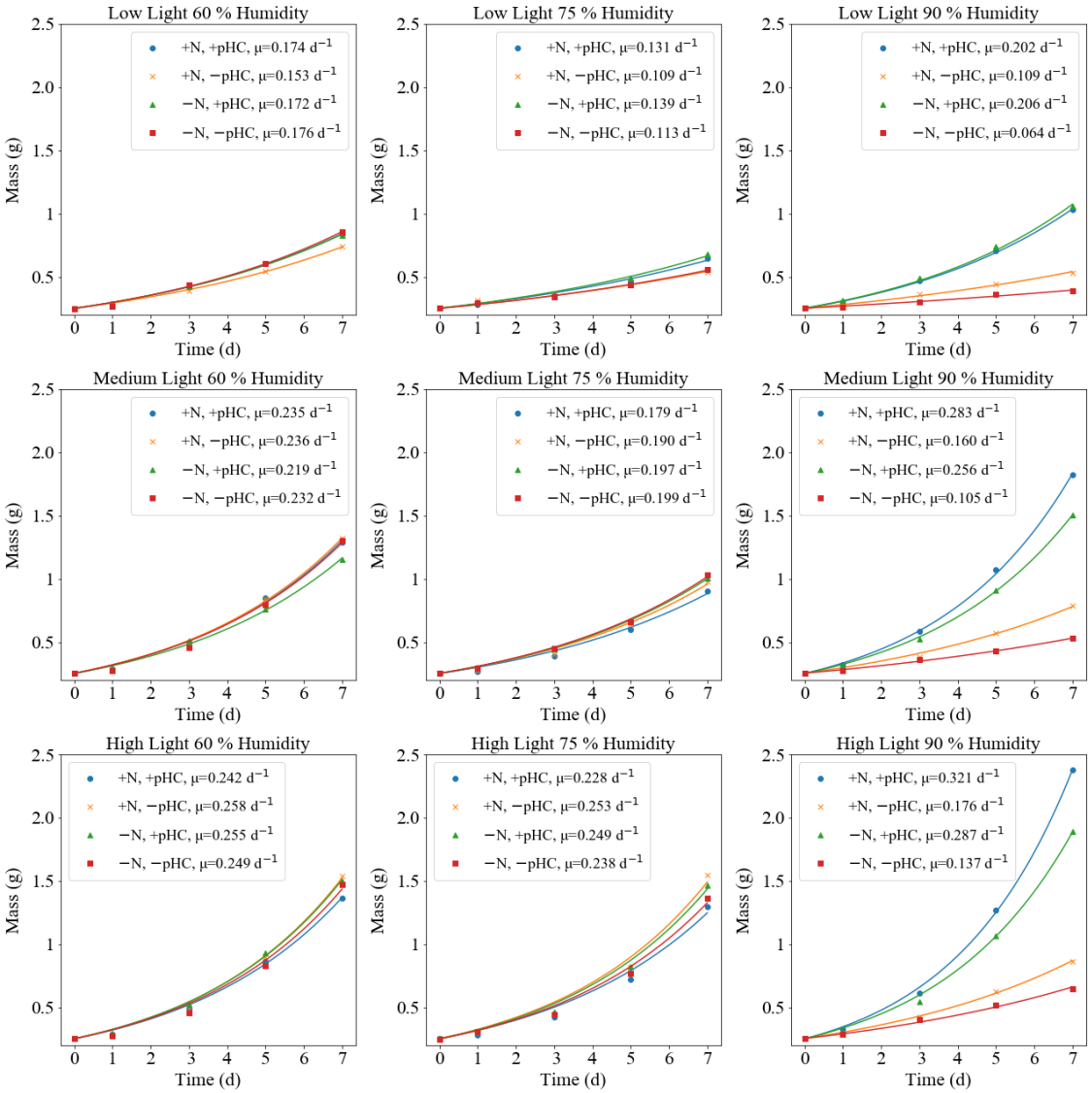

Figure 9: The average mass (g) results with the predicted growth model and corresponding growth rate for different growth conditions. The humidity values increase with 15 % increments per column while the light intensity doubles down the rows. The presence (+ N) and absence (- N) of nitrogen as well as the presence (+ pHC) or absence (- pHC) of pH control are shown on each subplot (da Silva *et al*, 2022).

One can observe from Figure 9 that the light intensity had a major effect on the growth of *A. pinnata*. There was a general trend that increasing the light intensity increased the mass of the *A. pinnata* and thereby increased the growth rate.

The high light intensity of 20 000 lx in combination with nitrogen absence at all humidity values turned the ferns a red colour. This is a stress response to the higher light intensities and lack of nutrients, which will cause *A. pinnata* to turn a characteristic red colour under these conditions; this is discussed in literature. This did not seem to affect the growth rate because, besides the visual stress response, that plants grew extremely well in comparison to the lower light intensities. Comparing the low light to the medium light intensity, there

was on average a 46 % increase in mass. The medium light intensity compared to the high light intensity mass increased on average by 17 %.

Based solely on light intensity, while other conditions were constant, the largest growth difference was 76 %. This was between the low and medium light intensity with no pH control, no nitrogen present and 75 % humidity.

The presence or absence of nitrogen did not significantly affect the growth rate of the *A. pinnata*. One will see this in each subplot of Figure 9. The nitrogen presence compared to the nitrogen absence growth was not significantly different. Comparing the experiments with the same conditions except for nitrogen presence, the largest difference in growth rate was 0.055 d^{-1}, which related to a 6.3 % average difference. This value was considered too small to be a significant difference. The nitrogen presence in the medium is not a rate-limiting step for the growth of *A. pinnata*. This is because of the symbiotic relationship between *Azolla* species and the nitrogen-fixing cyanobacteria *A. azollae*. The bacteria supply the plant with all its nitrogen requirements so that it can live in completely nitrogen-free environments.

pH control was a binary factor that was investigated. Each combination of growth condition was pH controlled to 6.5 via daily dosing, or left to naturally run while still measuring the pH daily. One will note the pH control versus non-pH control on each subplot of Figure 9. One will note that the pH control does not make a significant difference in the growth results for humidity values of 60 % and 75 %. It was only for the 90 % humidity that one can clearly see that the pH control did make a significant difference for the growth rate for the *A. pinnata*. The low light intensity pH control did 85.32 % (nitrogen present) and 221.88 % (nitrogen absent) better than the non-pH control. The medium light pH control did 76.88 % (nitrogen present) and 143.81 % (nitrogen absent) better and the high light did 82.39 % (nitrogen present) and 109.49 % (nitrogen absent) better. The reason for this vast improvement in growth is that the high humidity environment promotes algal and fungal growth. An algal infection did occur for the non-pH controlled experiments while it did not for the controlled experiments. One can believe that the pH control prevented the algal infection and the high humidity promoted high growth.

The humidity results can be compared in Figure 9 by comparing the columns to one another. High humidity promotes plant growth because the stomata remain open at higher humidity. This is important for photosynthesis. If the stomata remain open, more CO_2 can be absorbed and better temperature regulation can occur. One will note that the 60 % humidity had slightly higher growth rates than the 75 % humidity. We are unsure why this occurs. The 90 % humidity with the combination of the pH control proved to be the best growth conditions for the *A. pinnata*. One can see on each of

the 90 % humidity subplots that the pH controlled runs vastly out perform the non-pH controlled runs. This is due to the lack of algal infection that was discussed previously. The highest growth rate achieved was 0.321 d^{-1}, which is a doubling time of 2.16 days. The conditions that yielded this growth rate was: high light intensity, pH control, 90 % humidity and nitrogen presence.

Figure 10 shows the pH data of all the experiments. The pH was measured before dosing occurred to show how the pH drifted from the set point.

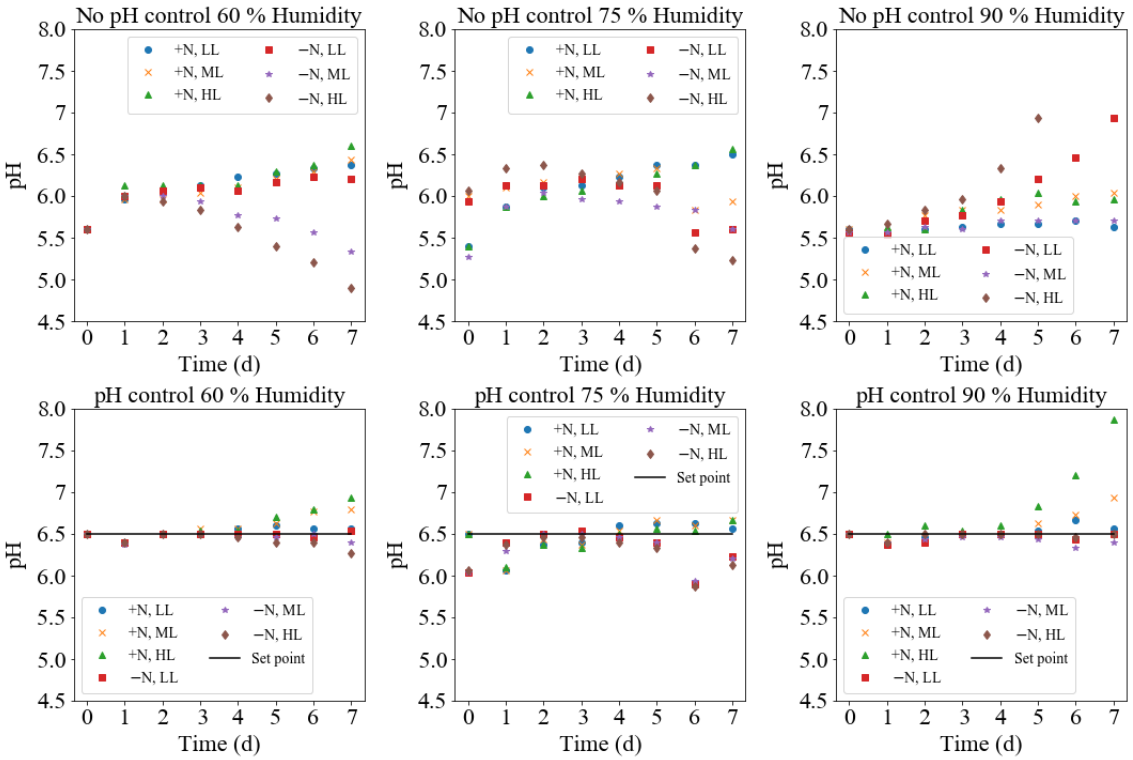

Figure 10: The average pH data for each experimental set. The pH was recorded before dosing to get to set-point. The different light intensities: 5 000 lx (LL), 10 000 lx (ML) and 20 000 lx (HL) and nitrogen presence (+ N) or absence (- N) are also shown. The set-point for the controlled pH was 6.5 (da Silva *et al*, 2022).

The starting pH of the experiments ranges between 5.5 and 6. One will note that there was a trend where the pH of the nitrogen-present experiments increased while the pH of the nitrogen-absent experiments decreased. The potassium nitrate that was used as the nitrogen source dissociates within the medium, creating a potassium ion (K^+) and a nitrate ion (NO_3^-). The nitrate ion creates the increase in the pH seen in the mediums that contain nitrogen.

One will note in the non-pH control and 90 % humidity subplot that the pH increased rapidly. This is due to the algal infection that caused the poor growth of the *A. pinnata*.

The pH results of the highest growth can be observed in subplot pH control and 90 %

humidity (+ N HL). The pH did increase to 8 and was then dosed down to the set-point again but no algal infection was present.

Considering all the growth conditions that were assessed, a one-way ANOVA analysis was performed between the triplicate biomass measurements taken on day one and seven for all the experimental conditions tested. The results from the first day showed that no significant difference could be observed between any of the runs. The results for the day 7 biomass measurements are shown in the heat map Figure 11. The colours are scaled according to the significance level with white representing an insignificant difference and dark green showing a very significant difference in the measurements.

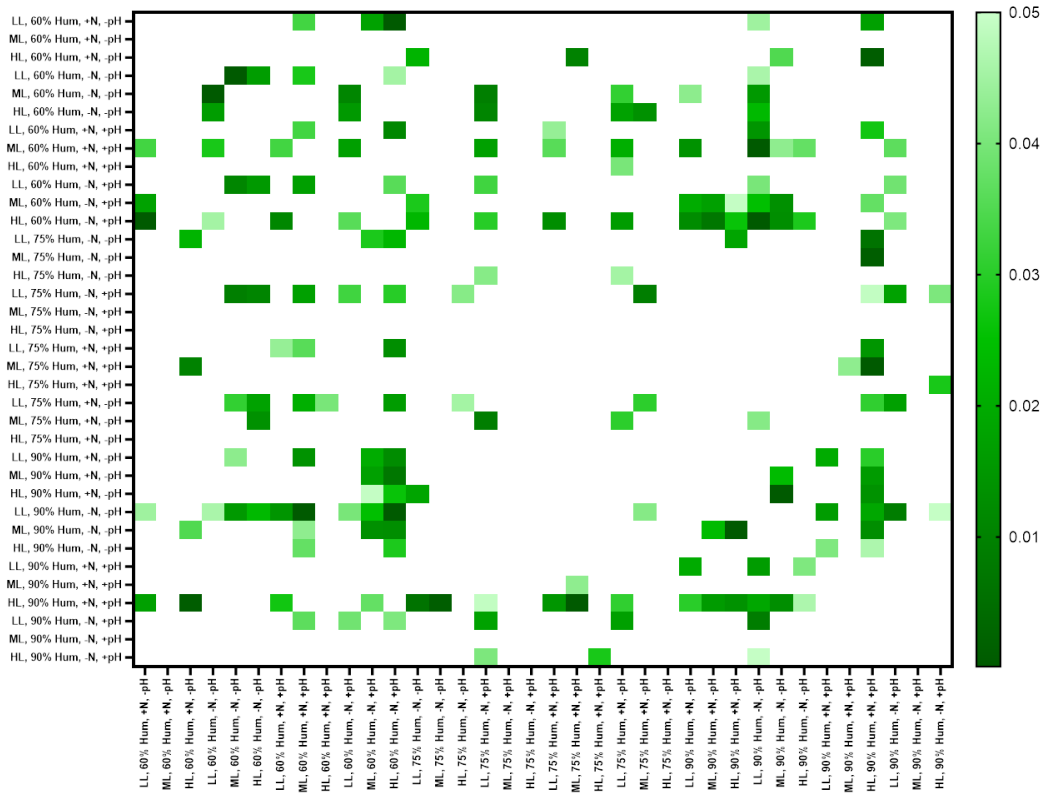

Figure 11: A heat map showing the adjusted P values when comparing the repeated results from the individual experimental conditions. The colour scale shows comparisons with no significant differences, shown as white, to most significant differences, shown in dark green (da Silva *et al*, 2022).

4.2 Phosphate uptake of *A. pinnata*

The mass of the *A. pinnata* was measured by weighing the plants on days 0, 1, 3, 5 and 7. A growth curve was fitted to the average mass data points of the triplicate experiment.

Figure 12 shows the average discrete mass data points and corresponding growth rate of the *A. pinnata* at different initial phosphate amounts and at a constant pH control scheme in each subplot.

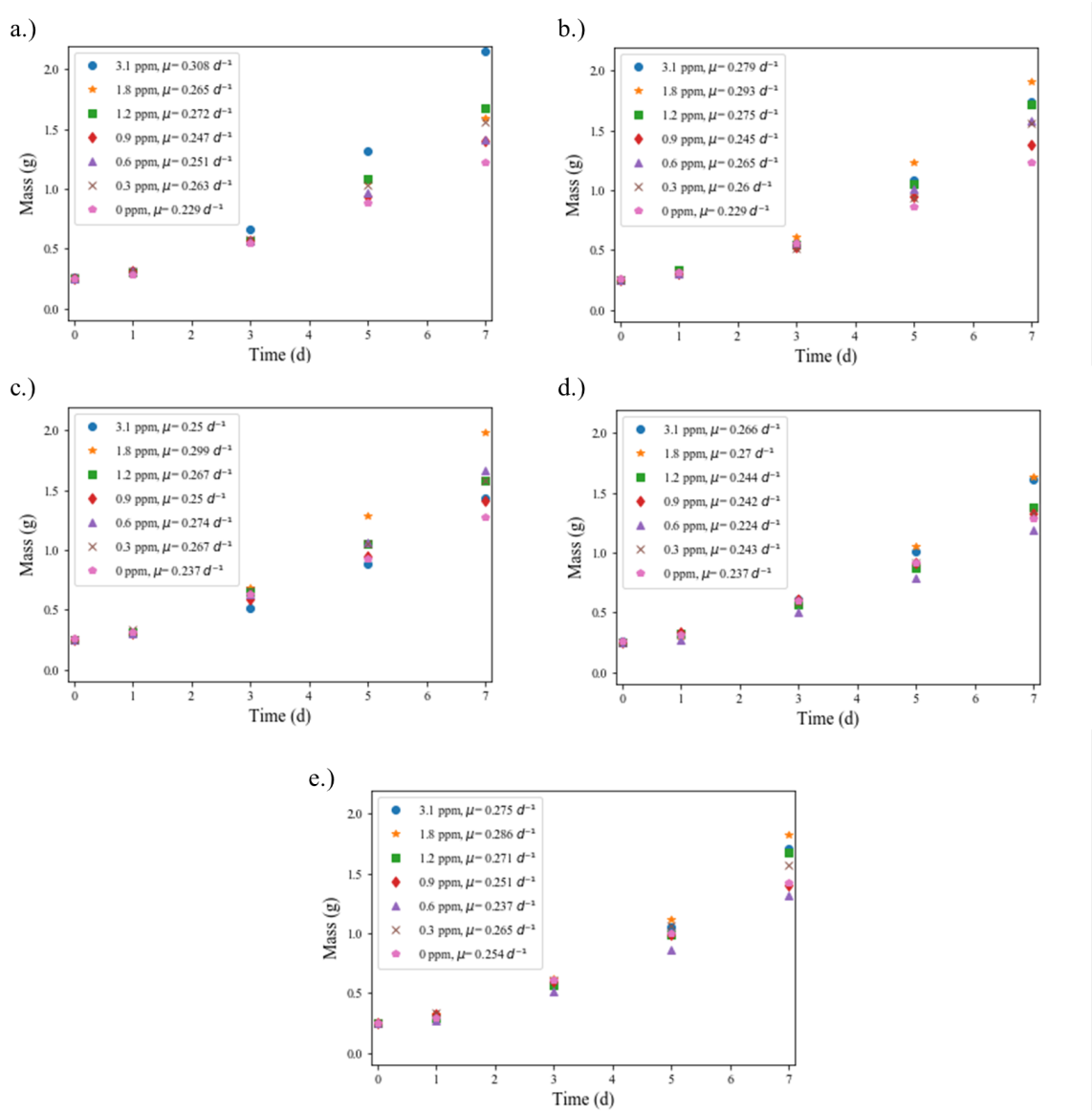

Figure 12: The average discrete mass (g) results for the varying phosphate amounts (ppm) with the corresponding average growth rate μ (d^{-1}) calculated using the *scipy.optimize curve_fit* module in Python®. The different conditions are shown in each subplot a.) pH 5 NaOH and HCl b.) pH 7 NaOH and HCl c.) pH 5 NH_3 and HNO_3 d.) pH 7 NH_3 and HNO_3 and e.) No pH control.

Figure 12 shows one that overall the different initial phosphate values did not have such a significant effect on the growth of *A. pinnata* as originally thought. This is especially true for the 0 ppm phosphate amount. There was a slight trend of a decrease in growth as the initial phosphate level decreased. The average growth rate values of each subplot and the range between the maximum and minimum growth rate of that subplot are shown in Table 7. One will note that the average values are very similar, except for subplot d.)

pH 7 NH_3 and HNO_3, proving the different pH control schemes did not have much of an effect on growth either.

Table 7: The average growth rates and range of growth rates for each subplot shown in Figure 12.

Growth condition	Average μ (d^{-1})	Range (d^{-1})
a.) pH 5 NaOH and HCl	0.262	0.079
b.) pH 7 NaOH and HCl	0.264	0.064
c.) pH 5 NH_3 and HNO_3	0.263	0.062
d.) pH 7 NH_3 and HNO_3	0.247	0.033
e.) No pH control	0.263	0.049

Table 7 shows that subplot a.) did have the largest range of growth rates between the highest and lowest phosphate amount. One will note on subplot a.) that the 3.1 ppm initial phosphate amount coupled with the pH 5 NaOH and HCl scheme produced the highest growth rate overall of 0.308 d^{-1}. This correlates with the idea that more available nutrients would result in a higher overall mass and growth rate. If one compares the growth of the NaOH and HCl scheme to the NH_3 and HNO_3 scheme at pH 5, the growth rate was 0.25 d^{-1}. The counterparts at 3.1 ppm were not the highest growth rates for the other pH control schemes. The 1.8 ppm initial phosphate amount produced the highest growth rates for all the other control schemes.

The addition of nitrogen into the nitrogen-free system had a varying effect on the growth rate at the pH 5. There was not a trend present in the pH 5 results as it alternated between which is better or worse. Comparing the pH 7 NaOH and HCl scheme to the NH_3 and HNO_3, one will note that the non-nitrogen scheme almost always did better. Due to the *A. azollae* symbiosis, the addition of nitrogen did not have an advantageous effect on growth.

The slowest growth observed was 0.229 d^{-1} under the 0 ppm phosphate amount and at the NaOH and HCl pH control scheme at both the 5 and 7 pH. The slowest growth rates were between the 0 ppm and the 0.6 ppm. This relates to the idea that lower nutrients available would hinder growth, but a growth rate of 0.229 d^{-1} is a doubling time of 3.03 days which is not by any means slow. It is interesting to note that under no pH control the growth rate of the 0 ppm phosphate is 0.254 d^{-1}, which is higher than some phosphate containing experiments with pH control. Some plants, including *A. pinnata*, have phosphorus starvation strategies to protect themselves from nutrient starvation. This mainly involves re-purposing internal phosphorus to where it is most needed for survival.

Figure 13 compares different pH conditions in each subplot while keeping the initial phosphate amount the same.

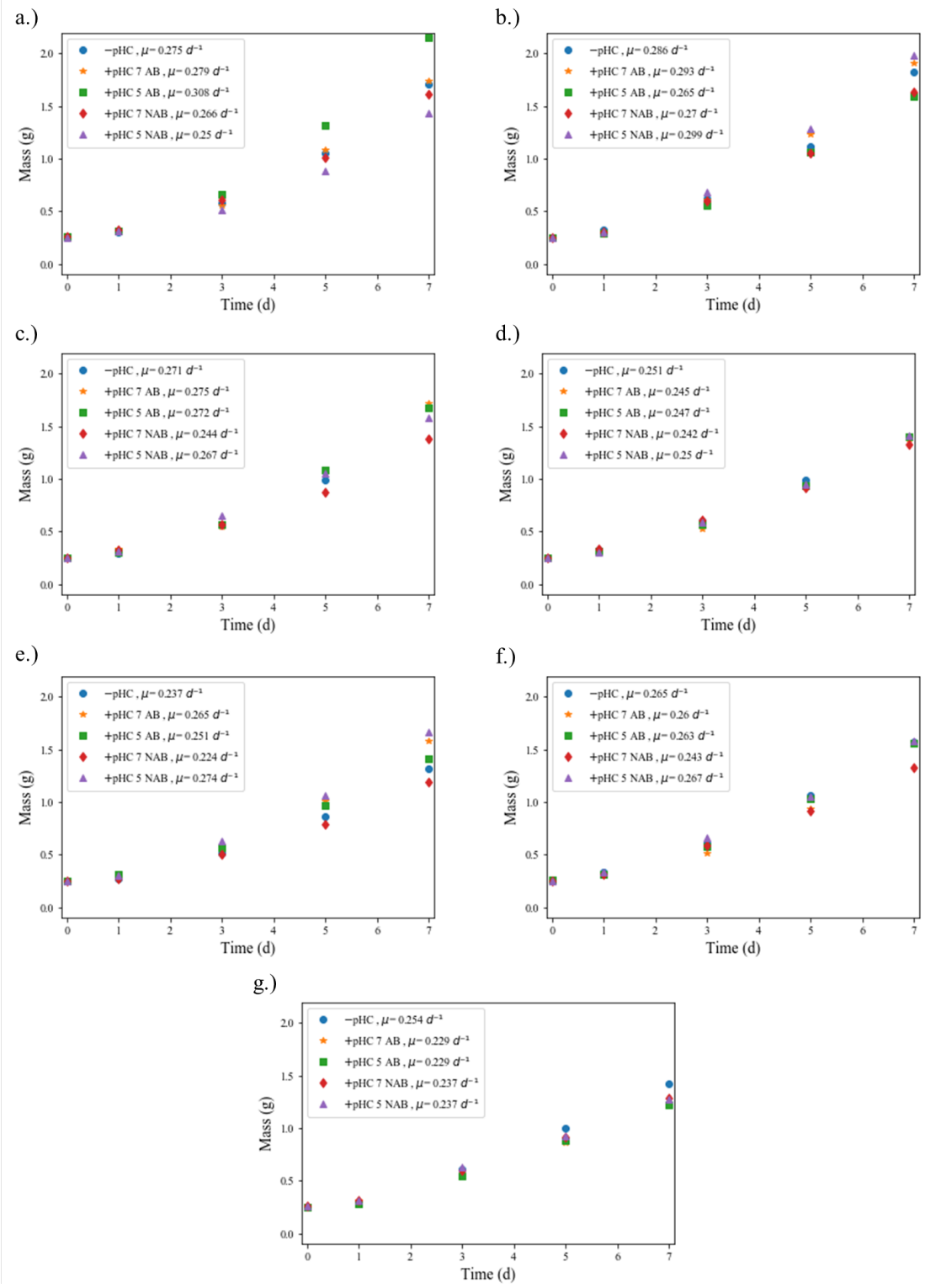

Figure 13: The average discrete mass (g) results for the same phosphate amounts (ppm) with the corresponding average growth rate μ (d^{-1}) calculated using the *scipy.optimize curve_fit* module in Python®. The different conditions are shown −pHC for no pH control, +pHC for pH control, AB for NaOH and HCl and NAB for NH_3 and HNO_3. The subplots have the following phosphate starting amounts a.) 3.1 ppm b.) 1.8 ppm c.) 1.2 ppm d.) 0.9 ppm e.) 0.6 ppm f.) 0.3 ppm and g.) 0 ppm.

One can see in Table 8, which shows the average growth rates and range of the growth rates for each subplot in Figure 13, that there is a decreasing trend in the growth rates as the phosphate amount decreases. This is true except for the 1.8 ppm and 0.3 ppm phosphate amounts. These average growth rates were unexpectedly higher than the previous larger phosphate amount. This could be due to a more desirable nutrient ratio that was met at these particular values than at higher nutrient levels.

Table 8: The average growth rates and range of growth rates for each subplot displayed in Figure 13.

Initial phosphate amount (ppm)	Average μ (d^{-1})	Range (d^{-1})
a.) 3.1	0.276	0.058
b.) 1.8	0.283	0.034
c.) 1.2	0.266	0.031
d.) 0.9	0.247	0.009
e.) 0.6	0.25	0.05
f.) 0.3	0.26	0.024
g.) 0	0.237	0.025

Although on average the 0 ppm phosphate amount was 0.046 d^{-1} lower than the 1.8 ppm phosphate amount, it was interesting that the *A. pinnata* still grew quite quickly regardless of the pH control scheme. One will note in Figure 13 that the pH 5 schemes generally produced higher final masses than the counterpart schemes.

Figure 14 shows the phosphate uptake rates of the *A. pinnata* over the course of the experiment and corresponds to the growth rates in Figure 12. Each subplot shows the different starting concentrations at one type of pH control scheme.

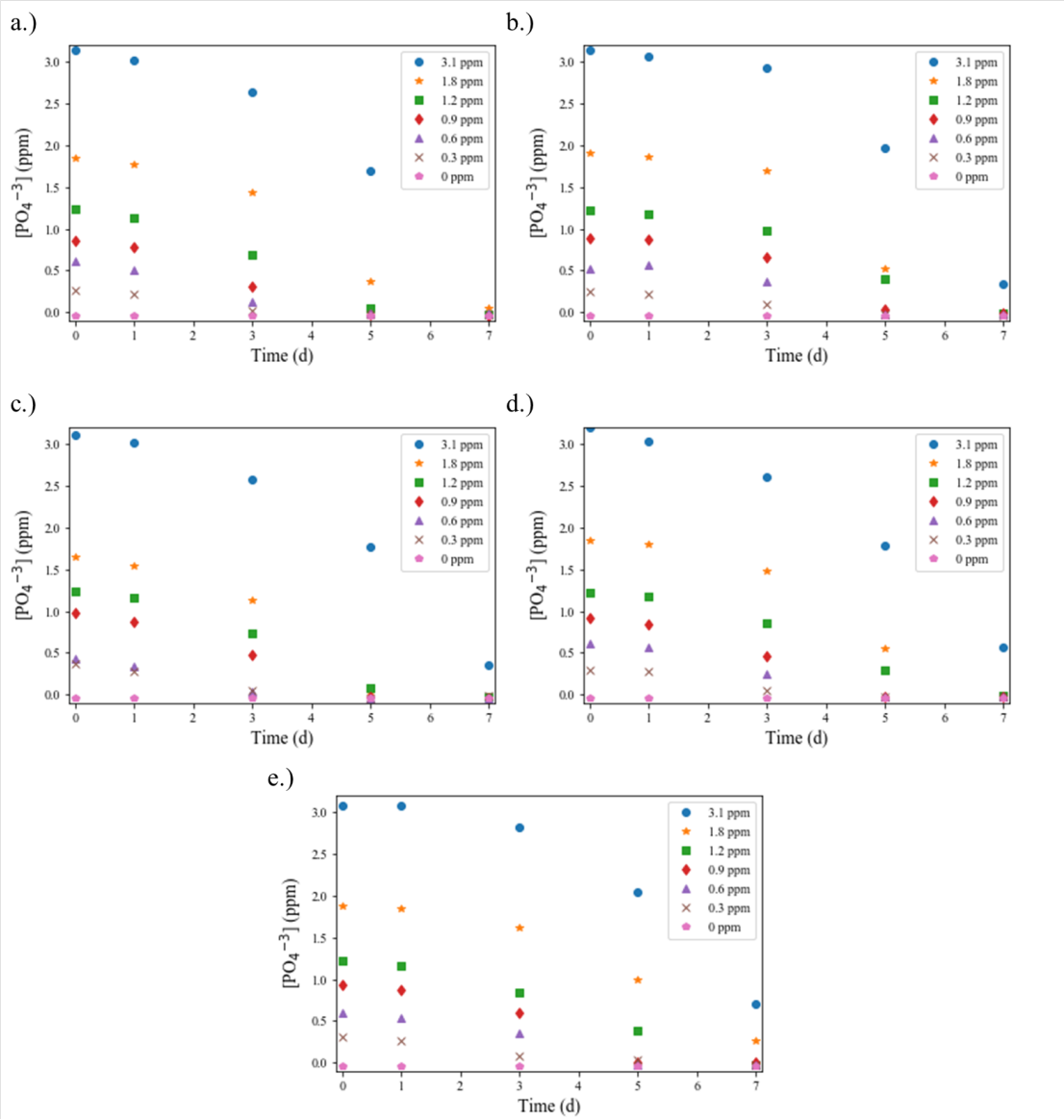

Figure 14: The average discrete phosphate (ppm) uptake results for the varying phosphate amounts. The different conditions are shown in each subplot a.) pH 5 NaOH and HCl b.) pH 7 NaOH and HCl c.) pH 5 NH_3 and HNO_3 d.) pH 7 NH_3 and HNO_3 and e.) No pH control.

One can clearly see an inverted exponential trend of the *A. pinnata* growth rates shown in Figures 12 in the phosphate uptake rates. Generally, for the 0.3 ppm to the 1.8 ppm phosphate concentrations, all the phosphate was depleted by the end of the seven day experiment. The only 3.1 ppm phosphate concentration that was completely consumed in the seven days was for the pH 5 NaOH and HCl, which had the highest growth rate. This must mean that the higher growth rate was due to the higher uptake of the nutrients. Several factors affect the uptake of phosphorus for plants. One of the factors is the pH of the soil or medium that the plants are grown in. Typically, more acidic soils promote the take up of phosphorus in plants. If one compares the pH 5 subplots (a. and c.) to their pH 7 counterparts (b. and d.) one will note that the pH 5 graphs uptake the phosphate

faster than the pH 7.

Figure 15 shows the phosphate uptake rates of the *A. pinnata* by comparing the different pH controls on each subplot and each subplot having a different initial phosphate amount.

One will note in Figure 15 that the pH control strategies take up phosphate at similar rates for the lower concentration of phosphates. Subplots a.) 3.1 ppm and b.) 1.8 ppm, are the two conditions where one can see a split of the results because of the pH strategies. It seems that the pH 5 strategies take up phosphate quicker than the pH 7 strategies and the no pH control.

Figure 16 shows the pH responses of the *A. pinnata* at different phosphate amounts at the same pH control scheme. The pH was measured before corrective dosing took place and subplot e.) had no pH control.

When one compares the pH 5 to their pH 7 counterpart at the different pH control schemes, one can see that the pH 5 results are more volatile than the pH 7. Although the phosphate uptake seems to be better at a lower pH, the *A. pinnata* seems to make less pH adjustments with the pH 7. The neutral pH seems to be preferable for the plant. The different phosphate amounts for the pH 7 experiments had similar trends in pH while the 0 ppm phosphate seemed to be the more volatile experiment to control. This could be a buffer capacity problem. Subplot e.), the non-pH controlled system, was particularly interesting because even with no pH control present and with varying phosphate amounts the pH trend was similar. The pH for all the phosphate amounts, increased until day 3 and then decreased to day 4 and then increased until day 6.

Figure 17 shows different pH control strategies at the same initial phosphate level.

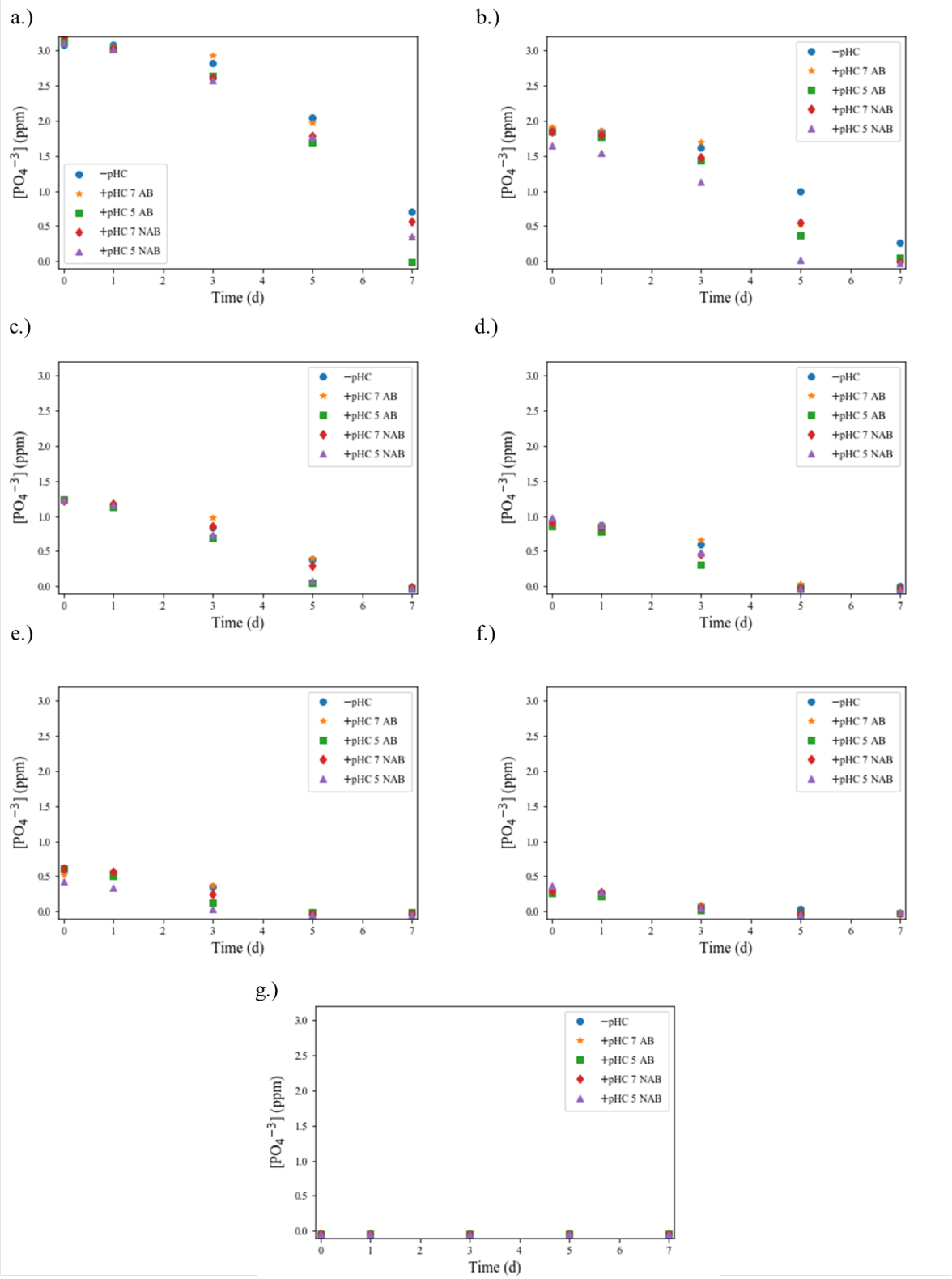

Figure 15: The average discrete phosphate (ppm) uptake results for the same phosphate amounts. The different conditions are shown −pHC for no pH control, +pHC for pH control, AB for NaOH and HCl and NAB for NH_3 and HNO_3. The sub-plots have the following phosphate starting amounts a.) 3.1 ppm b.) 1.8 ppm c.) 1.2 ppm d.) 0.9 ppm e.) 0.6 ppm f.) 0.3 ppm and g.) 0 ppm.

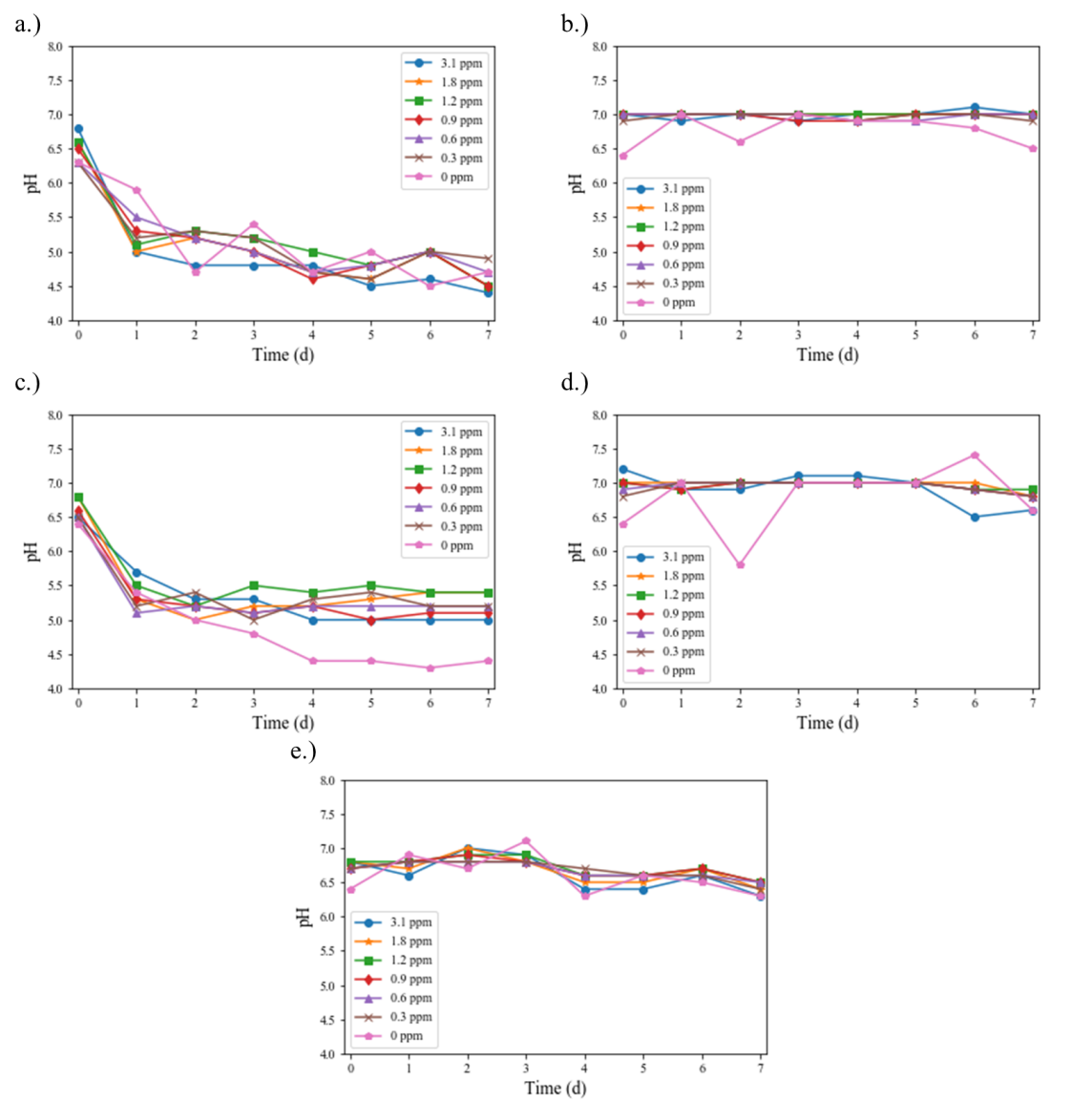

Figure 16: The average discrete pH results for the varying phosphate amounts. The discrete pH values are connected to better visually illustrate trends. The different conditions are shown in each subplot a.) pH 5 NaOH and HCl b.) pH 7 NaOH and HCl c.) pH 5 NH_3 and HNO_3 d.) pH 7 NH_3 and HNO_3 and e.) No pH control.

One can see in Figure 17 that the mediums that were dosed with nitrogen, thus introducing nitrogen into the system, usually resulted in a lower pH than the nitrogen-free counterpart. One can see in subplots a.) through to f.) that the pH 5 counterparts are an inverse of one another. As discussed in Figure 16 one will note that the pH 7 controlled pH has less dramatic fluctuations than the pH 5. Subplot g.), the 0 ppm phosphate, was extremely volatile compared to the other phosphate-containing subplots. This must be a buffering capacity issue.

Figure 18 displays the growth rate, phosphate uptake and pH of the 3.1 ppm phosphate amount with no pH control.

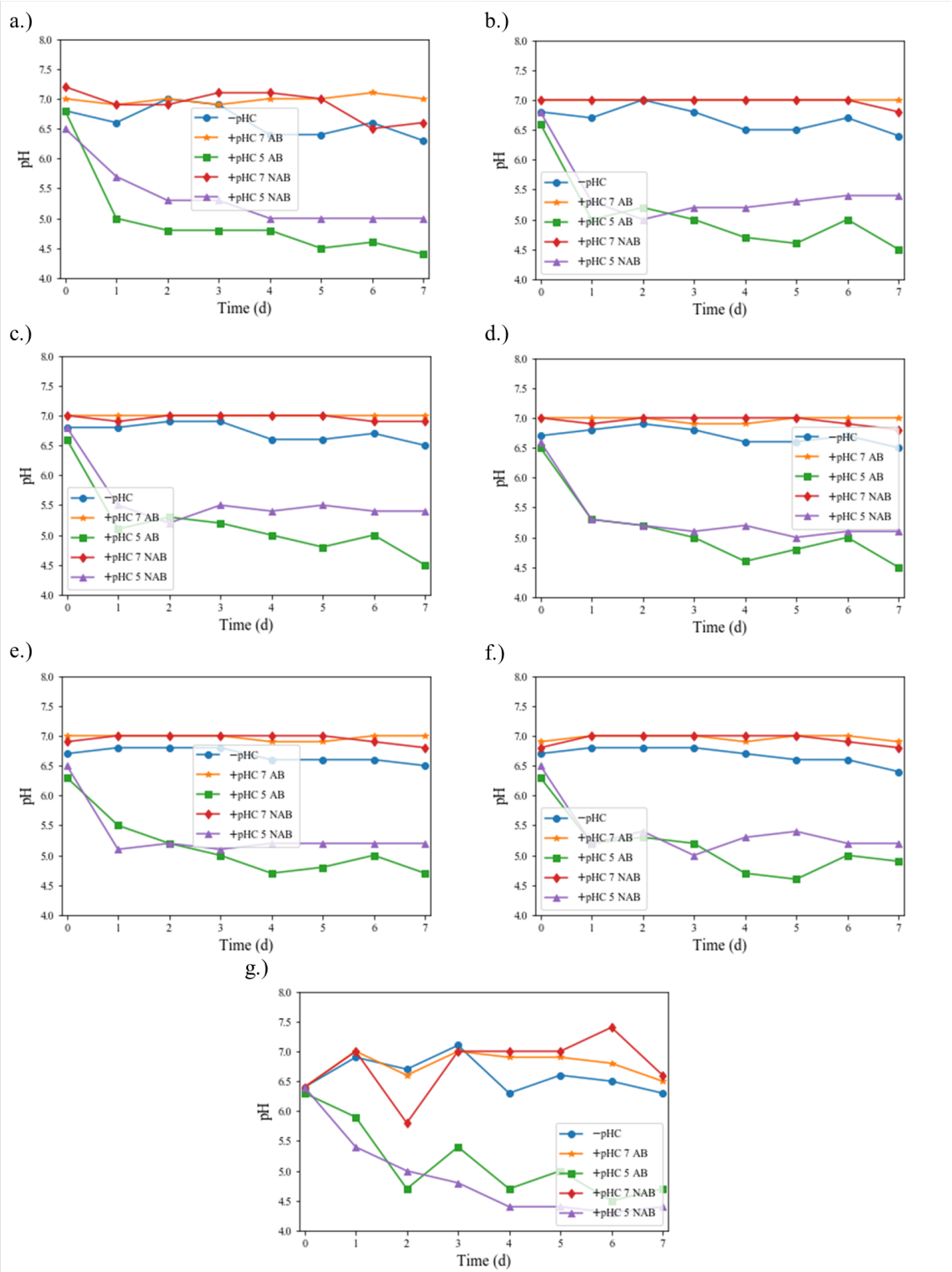

Figure 17: The average discrete pH results for the same phosphate amounts. The discrete pH values are connected to better visually illustrate trends. The different conditions are shown −pHC for no pH control, +pHC for pH control, AB for NaOH and HCl and NAB for NH_3 and HNO_3. The subplots have the following phosphate starting amounts a.) 3.1 ppm b.) 1.8 ppm c.) 1.2 ppm d.) 0.9 ppm e.) 0.6 ppm f.) 0.3 ppm and g.) 0 ppm.

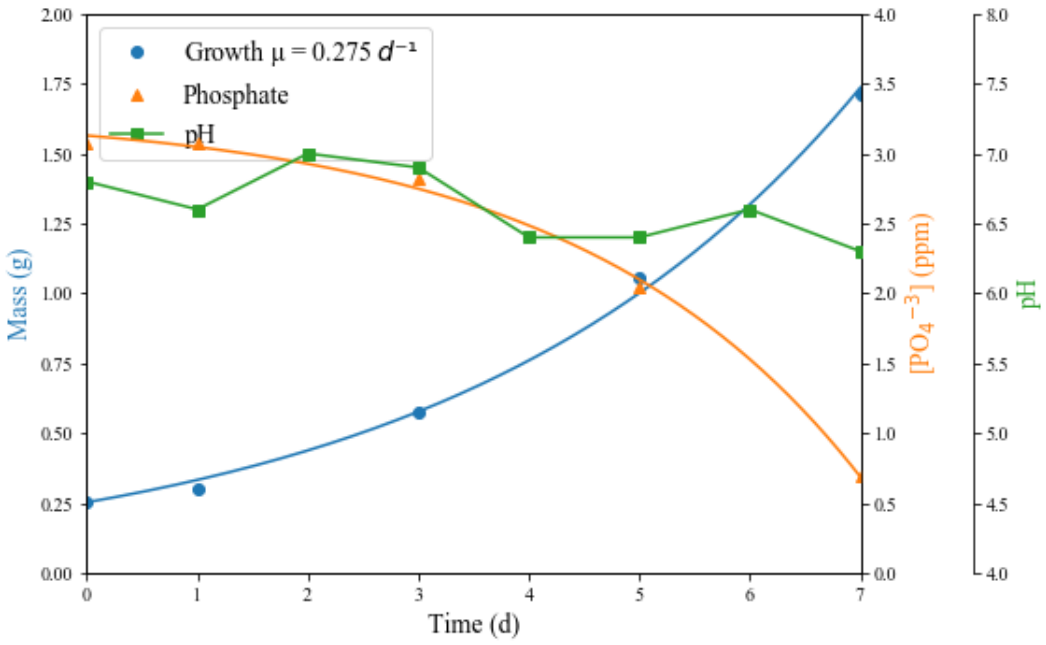

Figure 18: Grouped depiction of phosphate uptake, pH and growth for no pH control and a starting phosphate concentration of 3.1 ppm.

Figure 19 shows the growth rate, phosphate uptake and pH of the 0 ppm phosphate amount with no pH control.

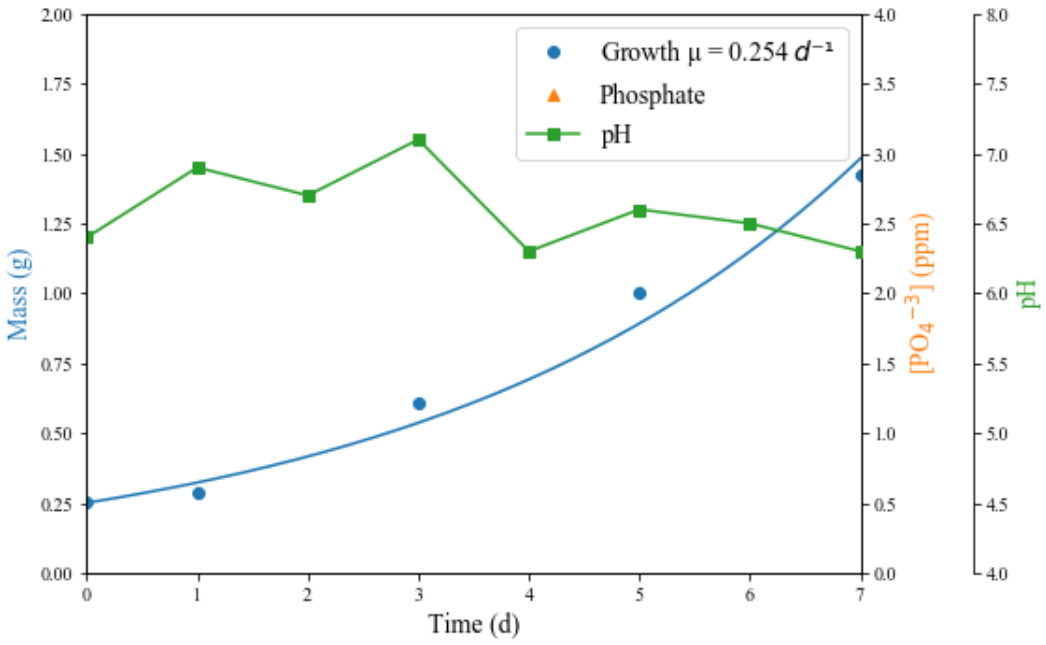

Figure 19: Grouped depiction of phosphate uptake, pH and growth for no pH control and a starting phosphate concentration of 0 ppm.

Comparing Figures 18 and 19 one will note that growth rates are 0.275 d^{-1} and 0.254 d^{-1} respectively. This is a difference of only 0.021 d^{-1}. Comparing the highest phosphate amount to no phosphate it is interesting that the growth rates are not that different to one another. One can see the inversion of the phosphate uptake rate compared to the growth rate on Figure 18. The pH on both figures increases until day 3 and decreases on day 4, not really giving much of an indication when phosphate levels are becoming low. This is clear because the pH behaves similarly whether there is phosphate or not.

Figure 20 shows a photograph of one of the triplicate runs of the non-pH controlled *A. pinnata* on day 7 with image a.) containing 3.1 ppm phosphate and image b.) containing 0 ppm phosphate.

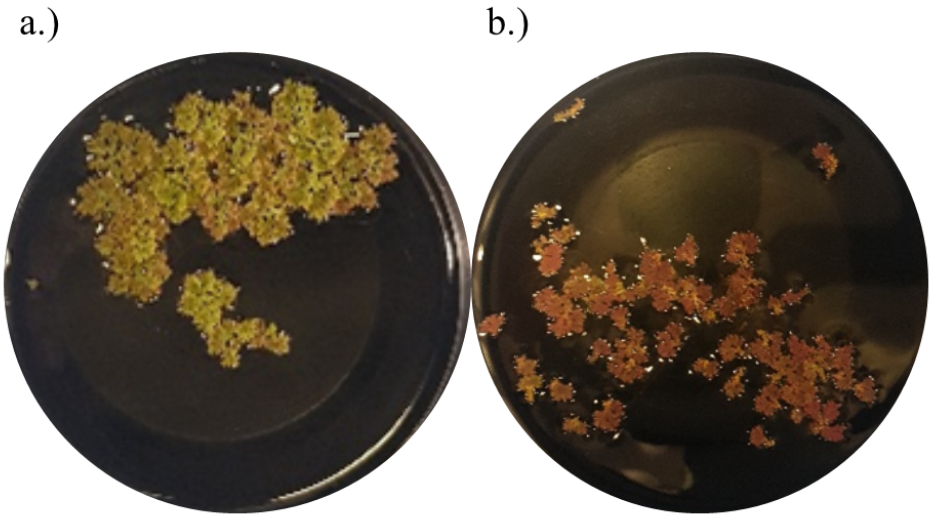

Figure 20: Photographs of the *A. pinnata* on day 7 of the non-pH controlled experiments. Image a.) contained 3.1 ppm of phosphate and image b.) contained 0 ppm of phosphate.

One can see in Figure 20 that even though these two different phosphate conditions yielded similar growth rates and pH trends, there is a clear visual difference in colour and frond size. This puts into question the health of the plant and sustainability of the low phosphorus environment.

5 Conclusions and recommendations

The optimal growth conditions for *A. pinnata* in a laboratory environment are 20 000 lx (HL), 90 % humidity coupled with pH control. It was found that adding nitrogen to the medium did not significantly affect the growth rates of the plant because the symbiosis between *A. azollae* provided all the nitrogen requirements. The highest growth rate achieved was 0.321 d^{-1}, which results in a doubling time of 2.16 days.

A. pinnata still gained mass when there was no phosphorus present; the growth rates were not particularly slow either but there was a slight decrease in growth rate with a decrease of available nutrients. The presence of nitrogen as the base and acid did not have significant effects on the growth rate or phosphorus uptake rate. This is because of the symbiotic relationship with *A. azollae*. The pH 5 did seem to cause a faster uptake rate of the phosphorus than the pH 7 because acidic pH does improve phosphorus uptake in soils. The pH 7 results showed that the *A. pinnata* seemed to be more adjusted to a neutral pH because the pH results of the pH 5 data were much more volatile than the pH 7 data. There was a clear correlation between the growth rate data and the phosphate uptake data. There was no distinct pH response when *A. pinnata* depleted its phosphorus source. There was a distinct visual change in the plants when comparing higher phosphorus amounts to lower phosphorus amounts. The *A. pinnata* turned a red colour and the frond size decreased drastically at lower phosphorus levels.

A recommendation for future experiments is to correlate the colour change of *A. pinnata* to the phosphate depletion using an RGB analysis in Python®. This way one can create a continuous wastewater cleaning system by use of colour and dose feed when a certain degree of colour difference is present to introduce more phosphorus into the system. A further recommendation is to digest the produced *A. pinnata* biomass in digestion systems to make the captured nitrogen more bioavailable and thereby determine the potential impact of the biomass as a biofertilizer.

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