Removing phosphate from wastewater: evaluation of the performance of duckweed (*Lemna minor*) operating under cool temperate conditions

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DECLARATION

I confirm that the work submitted is my own except where work that has formed part of jointly authored publications has been included. The contribution of the candidate to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given with the thesis where reference has been made to the work of others.

Chapter 3:

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The candidate was responsible for writing the majority of two sections within the above publication, which are entitled 'Introduction' and 'Chemical and biological technologies for capturing phosphate'.

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ABSTRACT

Reserves of rock phosphate are expiring, increasing the pressure on global agriculture already under stress from growing populations and unsustainable water supplies. The UK imports phosphate for agriculture and primary industries, but subsequently returns much of what it has imported after use as wastes to watercourses through diffuse discharges from agricultural runoff and point discharges from wastewater treatment works. It proves costly to control phosphorus to the low discharge concentrations required in order to avoid eutrophication in water courses.

Duckweed is a free floating macrophyte that has been shown to remove large quantities of nutrients from wastewater under tropical and sub-tropical conditions, but its potential for wastewater remediation and nutrient recovery in cool temperate countries is largely unknown. This thesis explores that potential, by exposing the duckweed *Lemna minor* to simulated and real wastewater treatment conditions of a cool temperate climate, while observing the influence of process variables controlling biomass growth and phosphorus uptake from both growth solution and wastewater.

Under controlled microcosm experiments, it was found that the most influential variables controlling biomass growth and phosphorus uptake were photoperiod and acclimation to phosphorus respectively. When duckweed was acclimated to low phosphorus concentrations, cellular phosphate reduced, causing subsequent periods of rapid phosphate uptake when the inoculum was resupplied with higher phosphorus concentrations. As a result, phosphate in solution was removed from 15 mg P L^{-1} to <0.1 mg L^{-1} in four days while under simulated UK summer and winter conditions. In mesocosm experiments conducted under continual flow conditions with a hydraulic retention time of two days, it was found that two duckweed tanks in series were able to remove phosphate from wastewater at 10 mg P L^{-1} to concentrations similar to that achieved by large wastewater treatment works in the UK (< 0.2 mg P L^{-1}). Preliminary results using an outdoor pilot-scale system helped to better understand the challenges of operating within more realistic conditions, as the entire process is profoundly affected by changes in wastewater characteristics feeding the system; however, time constraints prevented a full study to assess the magnitude of such impacts on phosphorus uptake and biomass growth.

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LISTS OF ABBREVIATIONS, UNITS AND SYMBOLS

ABBREVIATIONS

ANOVA	Analysis of Variance
ATP	Adenosine-Tri-Phosphate
BLAST	Basic Local Alignment Search Tool
BOD	Biochemical Oxygen Demand
BOD_5	5 day Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
DM	Dry Mass
EBPR	Enhanced Biological Phosphate Removal
EC	Electrical Conductivity
FM	Fresh Mass
GS	Growth Solution
H	Hydrogen
H_2PO_4	Phosphate (monobasic)
HRT	Hydraulic Retention Time
K	Potassium
KH_2PO_4	Potassium dihydrogen phosphate
L	Length
L. minor	*Lemna minor*
N	Nitrogen
NH_3	Ammonia
NH_4^+	Ammonium
NO_3^-	Nitrate
^{32}P	Radiolabelled phosphate (heavy isotope with atomic mass of 32)
P	Phosphorus
Pi	Inorganic phosphate (as P)
PO_4^{3-}	Phosphate
PP	Photoperiod
Q	Flow rate
SA	Surface Area

SSC	Steady State Conditions
STP	Sewage Treatment Plant
T	Temperature
t	Time
TP	Total phosphate
UK	United Kingdom
UK TAG	United Kingdom Technical Advisory Group
W	Width
WW	Wastewater
WWTW	Waste Water Treatment Works

UNITS

cm	centimetre
CPM	counts per minute (radioactivity)
d	day
°	degree(s) Celsius
g	gram
H^+	Hydrogen ion (or proton)
h	hour
k	Phosphate removal coefficient
kg	kilogram
K_m	substrate concentration providing $1/K_m$
L	litre
M	molar
m	metre
m^{-2}	square metre
m^{-3}	cubic metre
mg	milligram
mL	millilitre
mm	millimetre
mol	moles

mV	millivolts
ng	nanogram
P	significance value (set to <0.05)
pg	picogram
pH	potential hydrogen ($-\log_{10}[H^+]$)
R^2	Pearson's correlation coefficient
s	second
[S]	substrate concentration
µCi	micro Curie
µg	microgram
µL	microlitre
µM	micromolar
µmol	micromole
µS cm^{-1}	micro Siemens per centimetre
V	velocity
v	volume
V_{max}	reaction rate maximum

SYMBOLS

$	US dollar
Ω	electrical resistance
£	pounds sterling
±	plus/minus
%	percentage
Ø	diameter
<	less than
>	more than

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CHAPTER 1

INTRODUCTION

1.1. Overview

Rock phosphate reserves which are crucial for the production of phosphate fertilisers are finite and are expiring due to constant and necessary extraction. The UK relies on imports for phosphate fertilisers but also loses substantial amounts to the environment via wastewater treatment works (WWTW) (Cooper and Carliell-Marquet, 2013) and agricultural runoff (Withers et al., 2017). A low cost closed loop system is needed to reduce the dependence on imported P and improve the sustainability of this non-renewable resource. Duckweeds (small floating aquatic plants) have been shown to remove phosphorus and other nutrients from waste water (Balla et al., 2014) and accumulate them in tissues for future recovery and reapplication as fertilisers (Yao et al., 2017). There is however a distinct research gap with respect to the performance of duckweed under conditions of a cool temperate climate and clarity is needed on which parameters are likely to be the biggest influence(s) on performance under those conditions.

1.2. The need for sustainable resources

An expanding human population is putting increasing pressure on the Earth and its resources (Rockström et al., 2017). As a result of how the human race has altered the planet we are now in an age of what many refer to as the 'Anthropocene' (Crutzen, 2006). In 2017 the number of people on Earth is approximately seven billion and this number is expected to reach eight to ten billion by 2050 (Lutz and Samir, 2010). The planet cannot expand physically and life expectancies are estimated to increase (Kontis et al., 2017). As populations have risen historically, the production of food and resources have had to rise in tandem (Vance, 2001), (Table 1.1.1), which increases the strain on global resources.

Table 1.1.1. Increasing anthropogenic factors since 1960. Table adapted from (Vance, 2001).

Factor	1960	2000	2030-2040
Food production (Mt)	1.8×10^9	3.5×10^9	5.5×10^9
Population (billions)	3	6	8 to 10
Water stressed countries	20	28	52

Major industries such as manufacturing, agriculture and transport are critical and need raw materials and energy to continue operating to meet the increasing demands of the increasing population (Wiedenhofer et al., 2013). Of paramount importance is the need to provide food for more people each year, with no extra space available with which to do so using agricultural methods to date (Rockström et al., 2017). Publications regularly report on issues such as the lack of access to reliable water sources (Vörösmarty et al., 2010), often termed the 'global water crisis' (Srinivasan et al., 2017a). As a stark example, once the world's 4th largest inland waterbody, the Aral Sea in Central Asia has had its tributary sources abstracted from and diverted for irrigation so heavily that it is now evaporating rapidly and former islands can now be walked to (Micklin, 2016). Rapidly dwindling fossil fuel reserves (Wyman et al., 2011, Kopetz, 2017) have seen political tensions heighten (Klare, 2014), while the unsustainable CO_2 emissions of primary industry (Guan et al., 2009) are continuing to put a strain on the planet, possibly influencing climate change (Solomon et al., 2009). But perhaps less well known and publicised is the fact that the supply of phosphorus, which is mined and produced for agricultural fertilisers is expiring and is in fact non-renewable (Huang et al., 2015, Lu et al., 2017). Global resource sustainability and agricultural practice in particular need to become more efficient and integrated in their use of land, water and crop treatments (fertilisers), possibly by way of sustainable intensification (Rockström et al., 2017), if society is to survive.

Agricultural production may need to double by 2050 (OECD/FAO, 2012) to keep pace with the global demand for meat, dairy and crops (Ray et al., 2013a) and yields are unlikely to reach these targets without radical changes (Srinivasan et al., 2017b). Fertilisers in general consist of varying ratios of nitrogen, phosphorus and potassium. Of these critical plant nutrients, this thesis will focus on phosphorus and to a lesser extent nitrogen by exploring the potential use of duckweed for simultaneous wastewater remediation and nutrient recovery via biological uptake.

Phosphorus is ubiquitous in the world and is found in all organisms. It forms the key component of membrane bilayers as phospholipids; is part of the structure of nucleic acids and nucleotides including ATP (Li et al., 2015) and is involved in metabolic processes such as photosynthesis (Tran et al., 2010). As such this element is critical to life and there is no substitute for it.

1.3. The need for sustainable phosphate use

Phosphorus occurs naturally in sedimentary and igneous deposits in the Earth's crust (Föllmi, 1996). Oceanic sediments also contain phosphorus but not in a form that can be efficiently exploited to date (Puttonen et al., 2014). The rate at which phosphorus is lithified to rock phosphate available for extraction occurs over geological timescales (Föllmi, 1996), which are far slower than rates of anthropogenic extraction. A comparison of the natural and anthropogenic phosphorus cycles is given in Figures 1.3.1 and 1.3.2. The natural biogeochemical phosphorus cycle occurs incrementally over millennia and is a true cycle, while the effects of anthropogenic extractions upset the balance, thus exploiting this resource unsustainably.

Phosphorus for fertiliser is obtained by mining phosphate rock and subjecting the ore to many processes (Elser and Bennett, 2011). Production requires the intensive use of fossil fuels and water (Steiner et al., 2015). To acquire useable phosphate, the ore needs to be excavated and the phosphorus separated from waste rock, processed to fertiliser and then distributed to the customer (Steiner et al., 2015). Excavations require heavy machinery that obviously require diesel or electrical power, then the ore needs to be beneficiated by washing, screening and dissolving in weak acids to increase the proportion of P_2O_5 per unit of mass (Sengul et al., 2006). The result of this process often involves the mass production of phosphoric acid (Steiner et al., 2015) before the product is shipped in this state or as a salt to its destination (by diesel powered transport). It is inherently difficult to acquire accurate data regarding the quantification of global phosphorus reserves or usage, however global extraction in 2010 was estimated at 176 Mt (Schroder et al., 2010), having risen from 46 Mt in 1961 (Kelly et al., 2005). Peak phosphorus extraction was predicted to occur in 2033 (Cordell et al., 2009) before data was re-evaluated and estimates were put back to 2070 (Cordell et al., 2011). Some of the better estimates for longevity of reserves are for four hundred years (Van Kauwenbergh et al., 2013), more conservative estimates are for two hundred years (Schroder et al., 2010) while the worst put it at fifty years (Kelly et al., 2005). Unprecedented price rises in the 1970's and again in 2008 led to the supply of phosphate being re-evaluated (Mew, 2016), but whatever the actual amount is, the critical aspect of the problem is that the supply is non-renewable and methods of recycling should therefore continue to be investigated as a matter of urgency.

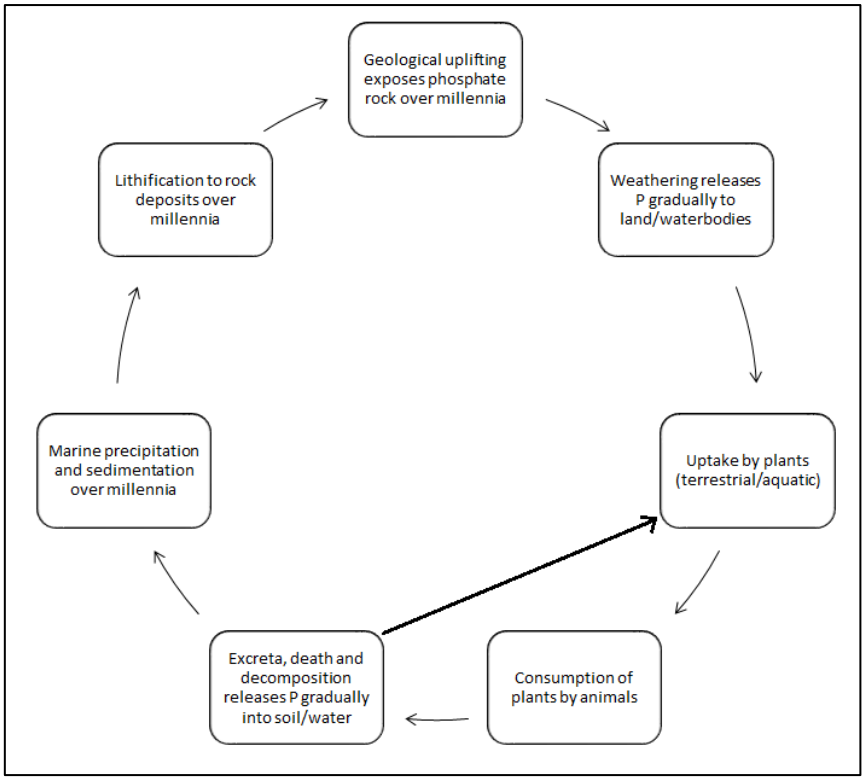

Figure 1.3.1. The natural biogeochemical phosphorus cycle.

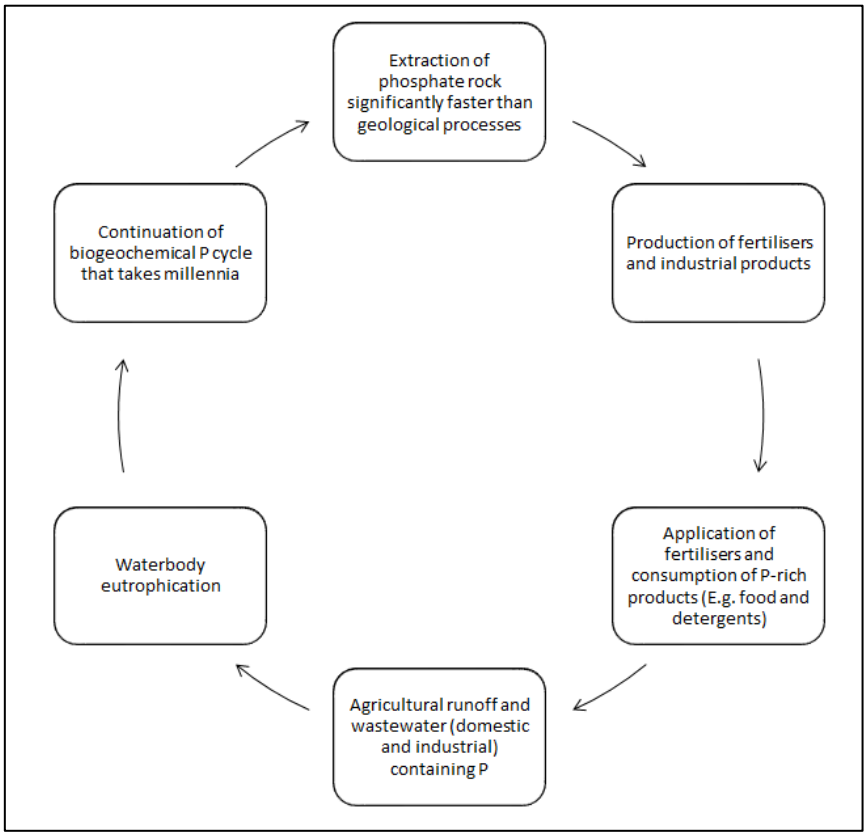

Figure 1.3.2. Anthropogenic aspects of the biogeochemical phosphorus cycle.

Rock phosphate is the source of the vast majority of phosphate fertilisers with biological wastes such as guano making up the rest (Reijnders, 2014). The global demand for rock phosphate used in primary industries has risen for several decades (Mew, 2016). Demand for fertilisers (containing nitrogen, potassium and phosphorus) has also risen since the green revolution of the 1960's and in more recent years this has been due mainly to rapid population growth in developing countries (Figure 1.3.3). The UK has no reserve of rock phosphate (Desmidt et al., 2015) and therefore has to import all the phosphate it requires. In 2009 the UK imported approximately 113.5 kt P for food, feed and fertilisers, with 77.5 kt of this for fertilisers (Cooper and Carliell-Marquet, 2013). At £260 t^{-1} in 2009 (World Bank, 2017), this would have cost almost £30,000,000 and does not include the P imported for industrial processes and detergents, which in itself is significant (Cooper et al., 2011).

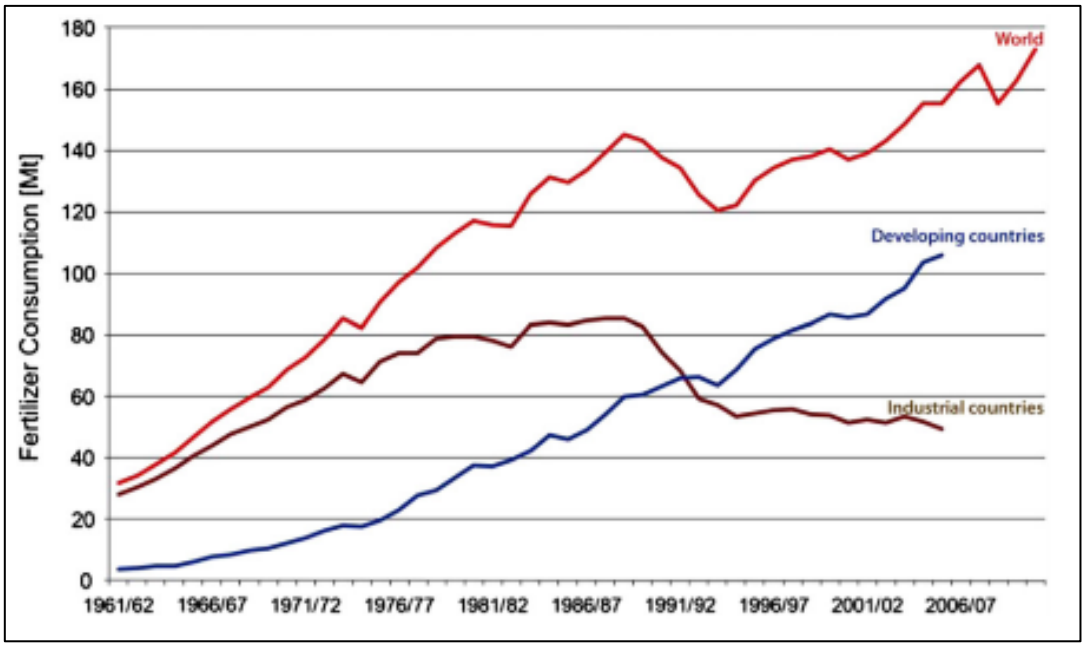

Figure 1.3.3. World fertiliser consumption. (Combined nitrogen, phosphorus and potassium fertilisers). From (Röhling, 2010).

The global phosphorus problem may in the future be exacerbated by the fact that most of the world's reserves reside in only a small number of (sometimes politically turbulent) countries, in particular Morocco and Western Sahara, which are estimated to hold 74 % of global rock phosphate reserves (USGS, 2017) (Figure 1.3.4). It is anticipated that by the end of the 21st century (if reserves are still indeed present), most of the world will rely on Morocco for that supply (Walan et al., 2014). If Morocco is to keep pace with projected demand, it will need to increase production by 700 % by 2100 (Cooper et al., 2011), which in that situation may see it practically controlling the market price. If the sustainable use of phosphate is not addressed soon and wholly, there could be political tensions over the supply of phosphate as seen previously for oil (Klare, 2014).

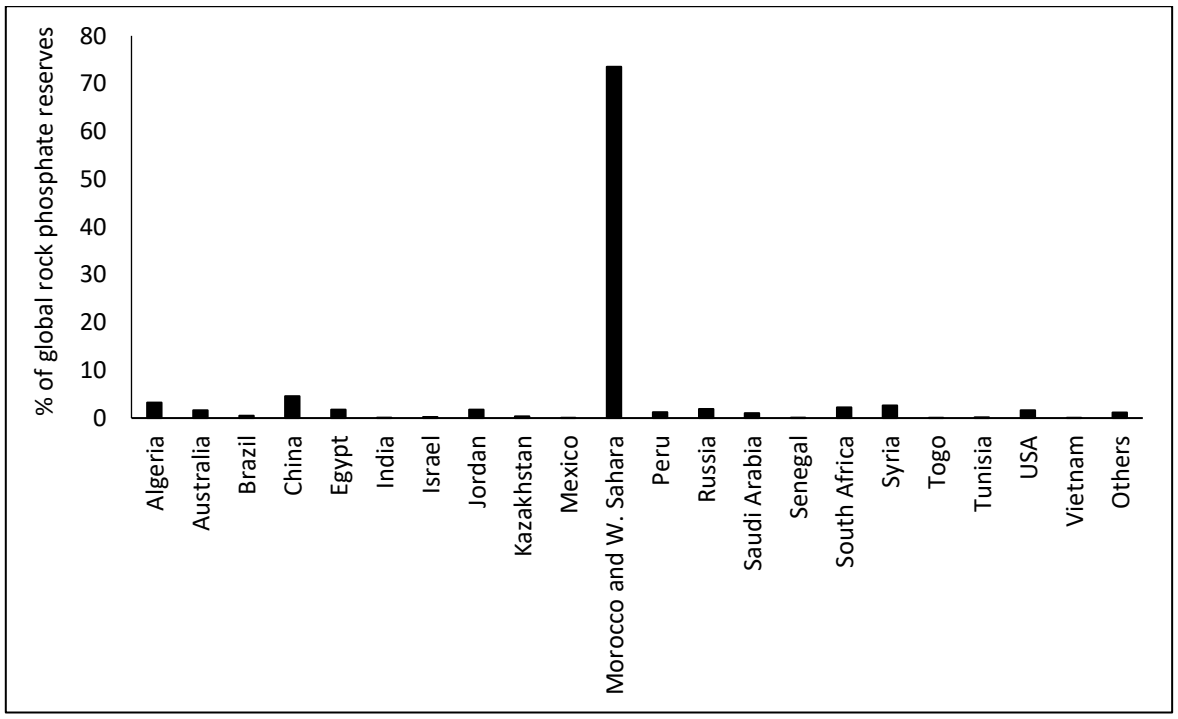

Figure 1.3.4. Global rock phosphate reserves. Figure composed using data from (USGS, 2017).

1.4. Anthropogenic phosphorus inputs to the environment

Phosphate originates from many sources and can make its way into watercourses by several routes such as agricultural surface runoff, wastewater discharges (industrial wastes, human excreta, grey waters and detergents) and P dosing for lead removal in drinking water (Cooper and Carliell-Marquet, 2013). Surface runoff from agricultural fields is a large input, whereby the intensive application of phosphate fertilisers can be washed away by rains into drainage streams or rivers before crops have had time to take up significant amounts (Withers et al., 2017). The efficiency of the uptake of applied phosphorus in fertilisers by agricultural crops has been reported to be as low as just 15 to 30 % (Syers et al., 2008), which does not help this issue. In the UK, the concentration of P in agricultural soils was correlated to concentrations of dissolved P in surface runoff, with reductions in soils causing reductions in runoff concentrations (Withers et al., 2017). At the time of writing, soil P testing is still not compulsory in the UK on farmland but if adopted in the future, this could be an indicator to reduce soil applications. If agriculture is to intensify using current methods then this widespread input may increase in impact.

Industrial point source discharges of P rich wastes have been shown to be a significant source of P inputs to the natural environment, possibly more so than agricultural runoff (Jarvie et al., 2006). In general though in developed countries, industrial and municipal inputs usually flow straight to WWTW via drains and sewer networks. It is a highly complex and difficult task to account for all the fractions of any material throughout a nation over the course of a year so understandably there is a shortage of data current to 2017, but in 2009 a large substance flow analysis of P throughout the UK was attempted in order to estimate mass balances of P and highlight use inefficiencies (Cooper and Carliell-Marquet, 2013). Inputs from industrial detergents were estimated to be 15 kt P; from domestic laundry and dishwasher detergents the value was 11 kt P; and from plumbosolvency additives to drinking water the value was 3.6 kt P. Contributions from human excreta that year was estimated to be 28 kt P and food waste added to sewers was put at 2 kt P. These values are all estimates calculated and presented by Cooper and Carliell-Marquet (2013) based on other assumptions such as the UK population and its estimated consumption or production of various P wastes.

WWTW were reported to have received approximately 60 kt P in 2009, while removing less than 60 %. The remaining P (approximately 25 kt), was released to the environment in the final discharges of WWTW (Cooper and Carliell-Marquet, 2013). Prices of diammonium phosphate peaked at £260 t^{-1} in 2009 (World Bank, 2017) meaning that an estimated £6,500,000 was lost to the environment, which is equivalent to 30 % of the amount imported for fertiliser use. At the time of writing the price of diammonium phosphate was £270 t^{-1} (World Bank, 2017). It is reasonable to expect that consumption

and waste production have increased over the last eight years with little time for technological advances in P recovery, therefore the situation is probably still similar to 2009 if not worse.

1.5. Consequences of excess phosphorus in the environment

P is often the most limiting nutrient in aquatic systems (Conley et al., 2009). When nutrients are allowed to enter oligotrophic or mesotrophic waterbodies they can often cause eutrophication (Comber et al., 2013). Trophic states in aquatic systems can be estimated not just by nutrient concentration, but by levels of biodegradable organic matter, algal biomass content or dissolved oxygen (Xu et al., 2001). Concentrations of P estimated to cause eutrophication in fresh waters however have been estimated to be 0.03 mg P L^{-1} or higher (Table 1.5.1) (Bennett and Schipanski, 2013). Work on purposefully eutrophicating lakes in the 1970's in North America proved the significance of adding P to an oligotrophic aquatic system already in existence (Schindler, 1977). Figure 1.5.1 is an aerial photograph from one of these experiments. The top half of the lake ('Lake 226') in Ontario, Canada, contained supplemented N and C, while the sectioned off bottom half contained supplemented N, C and P which subsequently caused the stark algal bloom (Schindler, 1977).

Table 1.5.1. Trophic state and epilimnetic total P concentration. From (Bennett and Schipanski, 2013).

Trophic state	Total P (μg P L^{-1})
Oligotrophic	< 5
Mesotrophic	5 to 30
Eutrophic	30 to 100
Hypereutrophic	> 100

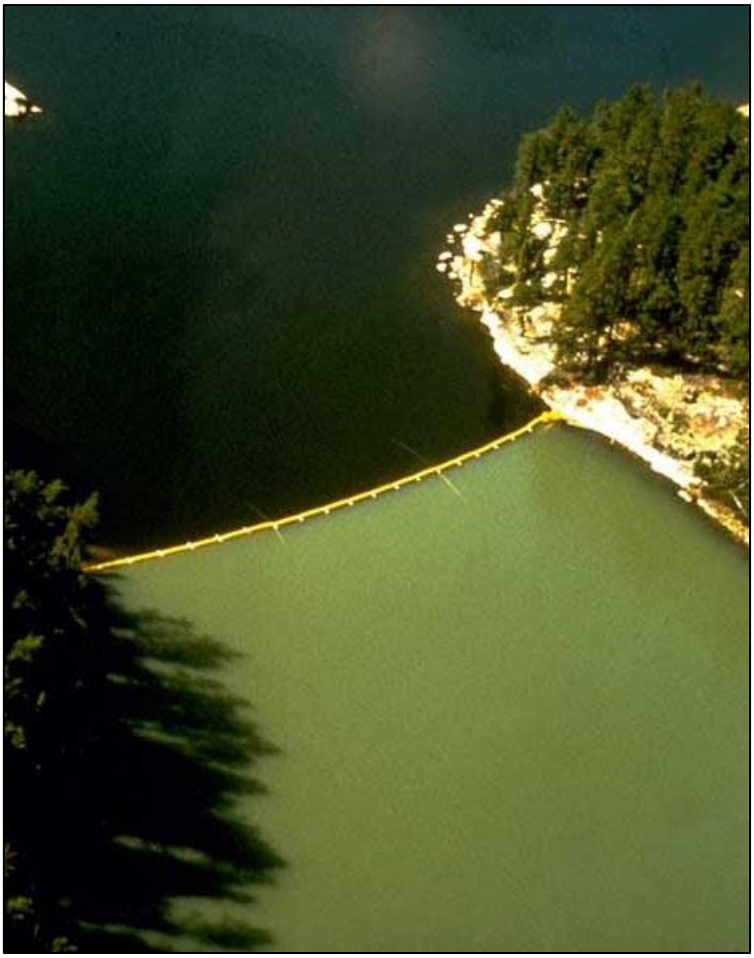

Figure 1.5.1. Facilitated mass eutrophication in Lake 226 Ontario, Canada. From (Schindler, 1977).

Eutrophication can cause rooted and floating macrophytes (the latter including duckweed) to grow extensively in aquatic systems (Quilliam et al., 2015) and can also cause algal and cyanobacterial blooms (Loza et al., 2014), which deplete the concentration of oxygen in the water column (Zhang et al., 2017), reducing biodiversity (Catherine et al., 2013) and causing the death of fish in extreme cases (Robarts et al., 2005). Cyanobacterial blooms in recreational water bodies have been known to be toxic to wildlife and pets (Catherine et al., 2013) and if present in water sources used for drinking water supply they may pose risk to Public health as well due to the potential presence of the microcystin toxin. If both duckweed and unicellular algae happen to be present, then these organisms will compete for the nutrient source in a water body (Szabo et al., 1998), and if the former takes hold sooner, it will spread across the surface and out compete the algae by covering the surface and preventing light from reaching the subsurface (Szabo et al., 1998). Contrastingly it was shown in later

studies that a sub-surface rooted macrophyte (*Elodea nuttallii*) was able to outcompete duckweed under nutrient limiting conditions (Szabo et al., 2010). In wastewater treatment however, the competitiveness of the duckweed could be seen as beneficial, as it would limit the amount of suspended solids (in the form of algae). This would prevent the often onerous task of removing unicellular algae from the entire water column (Michels et al., 2014) and simply require the controlled harvesting of duckweed from the wastewater's surface.

As well as the loss of biodiversity, public users of waterways also suffer from eutrophic duckweed blooms that carpet the surface. Rowers, scuba-divers, anglers and boat users all suffer with cloying duckweed mats interfering with equipment and hiding partly-submerged hazards. An example of this is shown in Figure 1.5.2, which shows the River Ancholme in Lincolnshire, UK during a duckweed bloom. Paradoxically however, the very fact that duckweed will thrive on polluting inputs to waterways is a dual representation not just of an environmental problem, but of a possible solar powered solution.

Figure 1.5.2. Duckweed invasion of the River Ancholme, Lincolnshire, UK. Photograph by J. Paterson, 18th July 2015.

The speed at which duckweed blooms can develop was personally witnessed in summer 2015 on the River Ancholme, Lincolnshire (Figure 1.5.3). This stretch of river was inundated with duckweed and some algal species within just two weeks to an approximate length of 1 km. After numerous water quality tests were conducted measuring parameters including NH_4^+, NO_3^-, dissolved oxygen, conductivity and temperature (as part of a studentship work experience placement), the only difference noted between samples of the river at those time points was an increase in phosphate, with the concentration rising from undetectable amounts of P to 0.08 mg P L^{-1} two weeks later. Specific information regarding the explanation for the increase in P is confidential, but it was concluded to be via anthropogenic inputs.

Figure 1.5.3. Eutrophication in an English waterway. A = River Ancholme, Lincolnshire, UK June 2015. B = River Ancholme, Lincolnshire, UK, July 2015 (two weeks later). Courtesy of Malcolm Bailey.

1.6. Methods of phosphorus removal

1.6.1. Introduction

The fact that elevated concentrations of phosphate released to the environment will cause environmental problems means that most developed countries have limits to how much phosphate is permitted to be released in wastewater discharges. These discharge consents vary between governments but at the outset of this project, the UK Technical Advisory Group (UK TAG) produced a working document that advised the UK government to reduce phosphate discharge consent targets from <1 mg P L^{-1} previously to an average of 0.1 mg P L^{-1} (UK TAG, 2013). As part of the Water Framework Directive in 2006, it was set out to monitor and recommend UK river phosphorus standards to protect the environment (UK TAG, 2008). As knowledge on how species like algae respond to phosphorus concentrations increased and recommendations were received from other EU member states to alter baseline values, the standards were revised (UK TAG, 2012). Further research by environmental agencies showed that factors such as alkalinity or altitude, as well as the presence and variation of nitrogen and sunlight could also affect the utilisation of phosphorus by organisms and thus alter species compositions assessed by trophic indices, thus the standards were revised once more in 2013 (UK TAG, 2013). Annual means of dissolved reactive phosphorus in river samples are to be compared against the standards, generating a local status of high, good, moderate or poor respectively (UK TAG, 2013). Depending on altitude and alkalinity, to achieve 'good' river status for phosphate required annual means to be between 40 to 120 µg P L^{-1} (UK TAG, 2013), with an average of this being 70 µg P L^{-1}. The revised document in 2013 stated that all UK rivers were to achieve 'good' status by 2015 (UK TAG 2013). This was not achieved and the plan was extended for a further five years and is thus ongoing at the time of writing.

Because of the environmental damage that excessive phosphate can cause in waterbodies, many technologies have been and are being developed to capture phosphate from point and diffuse pollutant sources (Dueñas et al., 2003, Ramasahayam et al., 2014). These technologies include both chemical and biological methods, which are used depending upon the available infrastructure of the particular country and its wealth, the available land space and the loads of P applied (Baker et al., 2015). Climate can also have an impact on the choice of technology adopted.

1.6.2. Chemical methods

The addition of metal salts to precipitate out phosphorus has been used for many years (de-Bashan and Bashan, 2004). Ferrous sulphate ($FeSO_4$) will form ferrous phosphate ($FePO_4$) and the use of $FeCl_2$ is also widespread, but the phosphorus can rarely be recovered from the precipitate and as such is disposed of as waste (Wilfert et al., 2015). Fulazzaky et al. (2014) trialled an alternating aerobic/anoxic batch system for municipal wastewater treatment in Toulouse, France. Using iron hydroxyl-phosphate precipitation, the study highlights the complexity of wastewater treatment. Increasing the molar ratio of Fe to P increased precipitation in some cases, but no relationship was discerned. Phosphorus removal efficiency ranged from 20 % to 74 % depending on pH and iron added, with the highest removal corresponding to the lowest phosphorus loading rates, but so many variables were operating that it was inherently difficult to isolate the true effect of variables (Fulazzaky et al., 2014). The molar ratio of Fe to P required for effective phosphorus removal is said to be in the region of two to one (Rittmann et al., 2011). Increasing the ratio can improve phosphorus removal and is sometimes required to maintain an effective removal (Manzouri and Shon, 2011). However, simply adding increasing quantities of iron is not a sustainable or even legal method of phosphorus removal, as the maximum discharge limits on iron are also managed at 3 to 5 mg total iron L^{-1} (UK TAG, 2012a).

Aluminium hydroxide ($Al(OH)_3$ will precipitate readily with phosphorus to form aluminium phosphate ($AlPO_4$), but organic matter can coat the aluminium particles and reduce efficiency (Lazaridis, 2003). Aluminium sulphate ($Al_2(SO_4)_3$ is also used for phosphorus removal from wastewater. In experiments using domestic wastewater collected from Suwon, South Korea, the effect of dosing was found to be pH dependent rather than dose dependent at acidic pH ranges. Increasing doses of aluminium sulphate had no effect on phosphorus removal at pH 4 or pH 5, and only a partial effect at pH 6 (Banu et al., 2008). Increasing doses while maintaining a pH of 8 or 9 increased phosphorus removal, with an optimum described around a dose of 80 mg $Al_2(SO_4)_3$ L^{-1} at neutrality (Banu et al., 2008). Introducing aluminium sulphate to the wastewater reduced the alkalinity and also reduced the pH, causing a reduction in phosphorus removal efficiency. Therefore, the efficiency of dosing with aluminium requires the constant management of multiple solution characteristics, as well as the desorption of phosphorus from the precipitate following treatment.

Adding calcium chloride ($CaCl_2$) causes the direct precipitation of calcium phosphate hydroxyapatite ($Ca_5(PO_4)_3OH$) (Yi and Lo, 2003). This occurs within a Ca to P ratio of approximately 1:7 (Hermassi et al., 2015). Increasing the dose of calcium chloride increased phosphorus removal, and as found for aluminium sulphate, maintaining a steady pH improved this (Hermassi et al., 2015). Maintaining the pH also controlled the amount of calcium in final effluents, but the stable pH adopted in this study

was pH 11.5 (Hermassi et al., 2015) and as such would require further treatment (acidification) to improve quality.

Extraction of phosphorus via struvite ($NH_4MgPO_4 \cdot 6H_2O$) formation is another alternative method that adopts the application of metals (i.e., Mg) and is often used for the recovery of nutrients from anaerobic digestates (Strom, 2006). Struvite precipitation is not new in the wastewater industry as it is known to occur in pipes that purposefully hold waste water with a 1:1:1 molar ratio of NH_4: Mg: PO_4. Under these conditions and at a high pH, phosphate can form crystals which very often clog pipes. The same principle is now conducted under controlled conditions leading to the production of struvite fertiliser.

Substrates such as waste materials (Ramasahayam et al., 2014) or blast furnace slags (Han et al., 2016) are being extensively trialled for their use as phosphorus adsorption and or precipitation materials (Barca et al., 2012, Pratt et al., 2007). A waste product from the steel industry, blast furnace slag has elements of calcium, iron and aluminium (Xue et al., 2009) which were all previously described to precipitate phosphorus. The efficiency of steel slag to remove phosphorus by either precipitative or adsorptive methods depends on many things including wastewater residence time, pH, phosphorus concentration and temperature (Han et al., 2016). A benefit of this material is the content of both Ca and Fe, which were shown to facilitate phosphorus removal at high and low pH values respectively (Han et al., 2016). In addition, the fact that a waste product is being examined for use in the recapture of a key nutrient is extremely positive. Unfortunately, the use of materials such as steel slags all come with the added burden of replacement to maintain efficient removal and intensive processes to attempt to recover the phosphorus as well as the need to monitor parameters of the wastewater applied (Han et al., 2016). Due to the complexity and variations inherent in wastewater over time, predicting when to replace solid substrates such as blast furnace waste is complex.

All the chemical methods reported above carry significant costs that can fluctuate with the cost of iron, magnesium and aluminium (Farchy, 2014) often acquired in the UK from overseas. The processes often require proportionally more dosing metal than phosphorus for its removal, and the need for further chemical treatment (desorption) of produced sludge and or disposal to landfill is unavoidable. In addition, the concentrations of products generated by the chemical reactions during these processes cannot often be predicted by simply using chemical equations, because numerous complex interactions including microbiology and aeration states will alter wastewater composition (Fulazzaky et al., 2014). While many of the chemical processes do provide final effluents containing low phosphorus concentrations, the energy used for dosing units, mixing and pumping stations is a drawback, along with the caveats of extra waste in metal precipitates requiring further attention.

Chemical methods are not a single solution to wastewater treatment, but their appeal to the large scale wastewater treatment plants in the UK and elsewhere is their effectiveness mid-stream for phosphorus removal and capacity for use year-round.

1.6.3. Biological methods

Phosphate can also be removed from wastewater by several biological methods. These include algal systems, microbiological systems and plant assemblages (terrestrial and aquatic), and combinations of these are often used in systems such as wetlands. Some of these biological systems require inputs of energy and others do not. Biological methods of phosphate removal often provide extra benefits in addition to nutrient removal capacities. These include the production of bioenergy crops and animal fodder (Goopy and Murray, 2003, Alaerts et al., 1996).

Microalgae such as *Chlorella* sp. or *Scenedesmus* sp. are used to remove phosphate from wastewater (Larsdotter, 2006). The two most commonly adopted algal systems are waste stabilisation ponds (WSP) and Photobioreactors (PBR) (Chen and Lin, 2006). WSP are used for nutrient capture typically in less developed countries with more available land, less infrastructure and more stable climate (Chopin et al., 2012) and therefore make use of solar energy. Photobioreactors, which are generally more focused on maximal biomass generation (Michels et al., 2014) can also be utilised, but these systems are energy intensive (requiring artificial lights, temperature control and occupy significant space in laboratories).

Algae used in WSP are effective in removing pathogens and improving wastewater quality but the removal of phosphate can sometimes be unreliable (Mburu et al., 2013) or difficult to predict due to interactions and effects of mutual shading, mixing, bacterial competition and environmental conditions (Brown and Shilton, 2014). Algae have historically demonstrated the luxury uptake of phosphate and storage of polyphosphates (Solovchenko et al., 2016). This would be a substantial benefit for phosphate removal from wastewater, but mechanisms that determine luxury uptake are not fully understood and are known to be affected by climatic conditions (Brown and Shilton, 2014). Phosphate storage by unicellular algae was unexpectedly found to be inversely proportional to light intensity under intensities expected to be found naturally, but this was explained by the algae utilising excess phosphate for rapid growth and metabolism (Powell et al., 2009). This could also be seen as a benefit if used for phosphate removal in a non-natural setting, as less energy would be required from artificial lighting for relatively more phosphate stored in the cells (Powell et al., 2009), i.e. the algae wouldn't have to be growing at their highest rates to remove the most phosphate. The application of algal biomass retrieved from WSP to farmland has been shown to provide nutritional benefits to the

land (Ray et al., 2013b), but the efficient retrieval of algal biomass from the water column in the first instance can be extremely difficult (Michels et al., 2014).

Mixed assemblages of algae and bacteria have been shown to capture high concentrations of phosphates in photobioreactors (PBR) (Munoz and Guieysse, 2006). Their symbiotic use under these controlled settings can be highly mutualistic as the algae have been shown to provide the oxygen required for the bacteria to degrade undesired compounds such as organic solvents and phenolic compounds while utilising the CO_2 produced by the bacteria (Muñoz et al., 2004). In addition, microalgae can be sensitive to pollutants such as heavy metals (Chen and Lin, 2006) and therefore require the bacteria to cope with this (Munoz and Guieysse, 2006). Open systems using WSPs have a large footprint and require optimal weather conditions, but in order to overcome such restrictions, the use of photobioreactors (PBRs) has now taken the attention of many researchers. PBR's typically adopt a vertical set up in laboratories or well-maintained pilot systems (Pulz, 2001) and are usually much more expensive to set up and operate than WSP, but are more efficient for wastewater treatment on a biomass to volume basis (Munoz and Guieysse, 2006), therefore the way in which algal reactors are used generally dependent on the wealth and climate of a particular country.

Enhanced Biological Phosphate Removal (EBPR) is commonly adopted at large WWTW in more populated areas and uses bacteria to remove and store high concentrations of phosphate (Brown and Shilton, 2014). Bacterial polyphosphate accumulating organisms (PAO) including *Accumulibacter* sp. and *Tetrasphaera* sp. will accumulate polyphosphates in on site reactors, under alternating aerobic and anaerobic conditions (Barnard, 1975, Mielczarek et al., 2013). During the anaerobic phase, bacteria can take up carbon sources and convert them to polyhydroxyalkanoates, by way of cleaving accumulated polyphosphates and releasing a proportion of phosphate back into the wastewater (Mielczarek et al., 2013). In the subsequent aerobic phase, the microbes utilise the PHA as their energy source to grow and recapture the phosphate that was released in the anaerobic phase (Mielczarek et al., 2013). The method can often require the aeration of waste sludge for the aerobic phase and thus be expensive (Downing and Jeyanayagam, 2016), but phosphorus can also be removed during anoxic conditions by denitrifying bacteria such as *Competibacter* sp., which will use nitrate or nitrite as electron receptors, thus reducing costs (Mielczarek et al., 2013). The EBPR process is preferable to chemical methods due to its utilisation of free biomass, but maintaining conditions for optimum performance and operation can be costly, complex and difficult to predict (Zheng et al., 2014).

Rooted and free-floating varieties of terrestrial and aquatic higher plants (and combinations of these) have been used for the capture of phosphates and other compounds for several years (Vermaat and Hanif, 1998). Natural or engineered and managed wetlands utilising water hyacinth, knotgrass, cattail

and duckweed have been used for the combined purposes of wastewater treatment and biomass production for bioenergy (Fedler and Duan, 2011). In more rural and or developing countries, constructed wetlands play vital role in the treatment of municipal wastewater, where expensive infrastructure is not available (Rai et al., 2013). Constructed wetlands are built in an attempt to mimic natural wetlands, in that they contain a variety of substrates (loams, clays and gravels), microbes (nitrifyers, denitrifyers, algae) and plant forms (reeds, sedges, floating plants and rushes) (Wu et al., 2015, Saeed and Sun, 2013, Vymazal, 2013).

Wetlands can be designed to include sedimentation or filtration zones and wide expanses of aquatic vegetation with extended residence time to remove nutrients, pharmaceuticals and heavy metals (Wu et al., 2014, Wu et al., 2015) and can operate over numerous compartments to enhance performance (Kadlec and Wallace, 2008). Hydraulically the wetlands can operate adopting surface or subsurface flows (Saeed and Sun, 2013), with flow directed horizontally or vertically (Ramprasad and Philip, 2016) and methods of operation can be tailored to suit the types of wastewater received (Wu et al., 2015). Affecting the performance of wetlands are principally the climate, microbial activity and the quality of wastewater that they are exposed to (Meng et al., 2014), with decreases in performance reported in colder months (Vymazal, 2011).

Important characteristics of wetland macrophytes include tolerance to high organic loads, rigid structures to reduce flow, ample substrate for bacterial assemblages and for oxygenation and the ability to be harvested with the minimum of effort (Vymazal, 2011). *Phragmites* sp. (reed), *Typha* sp. (cattail) and *Scirpus* sp. (rush) are all examples of widely adopted genera in constructed wetlands because of these inherent adaptations evolved from natural wetland habitats (Vymazal, 2011). The successful use of constructed wetlands year round has been reported in rural areas of cool temperate countries such as Switzerland (Züst and Schönborn, 2003), Germany (Kern, 2003) and Korea (Ham et al., 2004), where the emergent plants provide protection from snow and help to insulate the water/substrate interface to allow metabolism of wastewater by microbial communities to continue (Vymazal, 2011).

The natural occurrence of duckweed has been reported in wetland systems (Zirschky and Reed, 1988), but its effective use in this capacity has been somewhat disregarded or ignored for temperate or cool temperate countries (Vymazal, 2002). Duckweed shares many traits with reeds and rushes that would make it theoretically suitable for wastewater treatment, in having a fast growth rate under optimum conditions (Lasfar et al., 2007), providing a substrate for bacterial and algal assemblages (Hosselland and Baker, 1979, Reinhold et al., 2010), adding oxygen to the water column (Zirschky and Reed, 1988) and high rates of nutrient uptake (Cheng et al., 2002b). Duckweed is a floating aquatic macrophyte

which has shown promise under warm to tropical conditions for wastewater treatment (including nutrient uptake), both in batch or variable-flow-rate tank systems (Abuaku et al., 2006, Ran et al., 2004), artificial wetlands (Adhikari et al., 2015) and large scale wastewater treatment systems (Alaerts et al., 1996). The large quantities of phytobiomass produced by duckweed systems under optimal conditions (Verma and Suthar, 2014) generally have beneficial by-products that can be used as energy sources such as biogas or biodiesel, when the duckweeds' high starch and low lignin content is exploited (Fujita et al., 1999), or as a high protein feed for fish or cattle (Goopy and Murray, 2003). A clear advantage of being able to use duckweed over chemical methods to remove phosphate from wastewater is that they are solar powered and with the exception of periodic harvesting, can be fairly autonomous and generate biomass as a by-product with value in emerging markets (Alaerts et al., 1996).

1.7. Description of duckweed morphology and taxonomy

Duckweeds are small, floating aquatic macrophytes (Landolt, 1980a). They are monocotyledons of the family Lemnaceae (Landolt, 1986) which contain the world's smallest vascular plants. Being vascular plants of reduced size enables easy prolonged culturing, laboratory manipulation and the applicability of most plant cell models, including those of nutrient uptake and transport. There is often some debate on strict taxonomy of the organism (Les and Crawford, 1999) but it is now widely accepted that there are five genera - *Landoltia, Lemna, Spirodela, Wolffia and Wolfiella* containing 37 species. Depending on localised conditions including catchment pH and nutrient supply, duckweed of the same species can display quite distinct morphological differences such as larger fronds or shorter roots (Wang et al., 2010). Visual identification can be problematic even with microscopic aids, therefore molecular identification via DNA barcoding is extremely useful. To be certain of a species, DNA can be extracted and regions such as the *atpF-atpH* spacer can be amplified and sequenced to provide a positive identification when compared with published duckweed genomic data (Wang et al., 2013). This holds particular importance for this project, where the species of an outdoor duckweed system may vary, leading to variations in performance that would be difficult to predict without accurate knowledge of present species. The interspecific and intra specific differences in duckweed performance were investigated by Bergmann et al. (2000) and large disparities in biomass production were found. Growth experiments at 23°C and 16 h photoperiods, on solution that approximated swine wastewater tested 41 isolates from all the genera of the *Lemnaceae*. These included *Lemna gibba*, *Lemna minor*, *Spirodela polyrrhiza*, *Wolffia Australiana*, *Wolffiella oblonga* and *Spirodela punctata*. During the publication of this particular study, the species *Spirodela punctata* was reclassified into its own genera as *Landoltia punctata*, (after the now late duckweed research pioneer Elias Landolt) (Les and Crawford, 1999). It was shown that there were large disparities between species and between

isolates within species, in terms of frond size to fresh mass ratios, fresh mass increases, dry to fresh mass ratios and protein production (Bergmann et al., 2000). Aspects such as protein production did not always correlate to fresh mass increases, so there was no one 'super isolate' found in these experiments. Subsequent experiments that exposed six of the original forty-one isolates to real swine wastewater were qualitatively described based on visual estimates (Bergmann et al., 2000). Of the six isolates tested, only two managed to survive on the swine wastewater, which contained 172 mg NH_4^+ L^{-1}, 0.6 mg NO_3^- L^{-1}, 14 mg P L^{-1}, 1287 mg COD L^{-1} and a pH of 7.8. The surviving species were *Lemna gibba* and *Spirodela punctata* (now *Landoltia punctata*) but they were reported to grow very little (Bergmann et al., 2000).

Duckweeds are distributed all over the world in all but the most extreme habitats, such as ice sheets or deserts (Landolt, 1986). They are composed of a fused root and frond, strictly termed a collective thallus. There are generally one to two mature fronds with one to two roots that hang down into the upper water column (Hasan and Chakrabarti, 2009). Frond sizes range from 1 to 10 mm across and root length from 5 to 50 mm, depending on species and life stage (Hasan and Chakrabarti, 2009). One or two species occasionally produce tiny white flowers (Priya et al., 2012), but in the main, reproduction is vegetative (Lemon et al., 2001). Reproduction arises by 'daughter' fronds forming at the base of the 'mother' frond where they grow for a period before budding off to start colonies of their own (Lemon et al., 2001). A single plant can survive from a matter of days to several weeks, producing up to 20 daughter fronds during this period. Duckweed compete with algae and other species (Korner and Vermaat, 1998) in the water column by reproducing as fast as possible and colonising the surface to out shade the plankton (Figure 1.7.1). Reproduction can be rapid and duckweed have been reported to double in less than 24 hours under optimum conditions of light, temperature and nutrients (Lasfar et al., 2007).

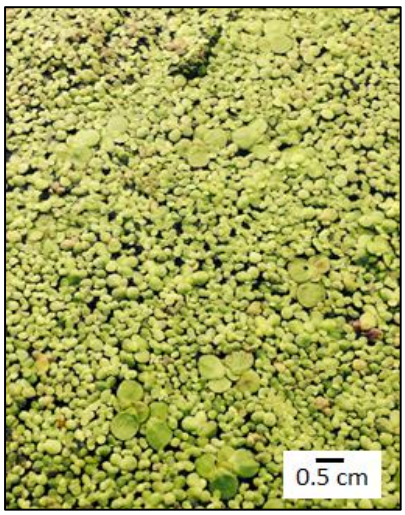

Figure 1.7.1. Mixed assemblage of *Lemna minor* and *Spirodela polyrrhiza*. Photograph by J. Paterson August 2016, at Keadby Canal, Lincolnshire.

1.8. The performance of duckweed on wastewater and growth solutions

1.8.1. Introduction

Experiments referring to the use of duckweed for the treatment of wastewater have been conducted for decades at many scales and under a variety of conditions (Hillman and Culley, 1978, Culley et al., 1981, Culley and Epps, 1973, Filbin and Hough, 1985, Iqbal, 1999, Iqbal et al., 2017, Cheng et al., 2002a, Ran et al., 2004). A reduced size and simple morphology, together with rapid growth rates under optimum conditions of temperature, photoperiod and nutrients (Lasfar et al., 2007) advocates their use as a model plant species (Mkandawire and Dudel, 2005). They have been suggested as trophic indicators of polluted aquatic systems (Linton and Goulder, 1998); used for electrophysiological measurements of membrane transport (Ullrich-Eberius and Yingchol, 1974, Ullrich et al., 1984, Lass and Ullrich-Eberius, 1984); tested for pharmaceutical bioaccumulation (Reinhold et al., 2010); and have had their use as biofuel-producing organism investigated (Aslan, 2016).

Following the removal of nutrients such as N and P, when harvested from wastewater treatment systems, the duckweed biomass is often a beneficial bi-product in itself (Alaerts et al., 1996). Duckweed fronds can be up to 40 % protein as dry mass (Cheng and Stomp, 2009). This is an extremely nutritious source of feed for fish farms and cattle, if heavy metal concentrations are low enough (Bergmann et al., 2000), relieving some of the pressure on land usage for the same requirements. Duckweeds are composed of low amounts of lignin which makes homogenization much easier than for woody plants and bio-ethanol has been produced from the high starch contents (up to 75 % of dry mass) in optimal laboratory conditions (Ge et al., 2012).

Duckweed are reported to survive under a range of chemical or climatic parameters but experimental designs adopted are often different between authors. Every genera of duckweed has been experimented with (Tamot et al., 1987, Cheng et al., 2002a, Abuaku et al., 2006, Shi et al., 2011, Soda et al., 2013); solutions used range from full strength sewage (Cheng et al., 2002b) to autoclaved and buffered growth solutions (Ullrich-Eberius et al., 1978). Experimental scales range from small scale batch cultures where measurements of particular parameters take minutes (Hase et al., 2004), to full scale waste stabilisation pond experiments over extended periods of time (Njambuya et al., 2011). Added to this, several authors who conduct experiments at a particularly small scale have extrapolated results to relate to large scale operations (Al-Nozaily et al., 2000), which makes the inherent magnification of errors an unfortunate but realistic consideration. Other authors simply don't report what certain methods or variables were, for example (Alaerts et al., 1996), who didn't report the species used in that particular publication. These discrepancies make the accurate assumptions or estimations of 'typical' parameter ranges required to maintain healthy duckweed stocks, or estimating

what extremes the duckweed can cope with inherently difficult. The following sub-sections investigate the performance of duckweed under various ranges of environmental parameters and will attempt to synthesise the results from some key experiments to present duckweeds potential for wastewater remediation, while highlighting where potential may lie for further investigations.

1.8.2. Temperature and light exposure

When considering the many differences in experimental setups between publications, an aspect that does unify most publications on this topic is that conditions of temperature and photoperiod are either usually fixed to optimums in the laboratory (Lasfar et al., 2007, Frederic et al., 2006) or when in outdoor settings, are conducted in countries with climates warmer and more stable than cool temperate ones (Ran et al., 2004). This is often the case even when the experimental organisms have been sampled from cooler climates originally for example (Zhao et al., 2014). In fact, authors have either suggested that the use of duckweed in cool temperate countries is not feasible, or where waste stabilisation ponds (WSP) have been in operation in these countries, the presence or use of duckweed is not reported (Vymazal, 2002). Filbin and Hough (1985) stated that duckweed grew up to 45°C before growth was strongly inhibited, while (Cheng et al., 2002b), citing (Classen et al., 2000), hinted at the capacity of duckweed to survive at very low temperatures and be used year-round for wastewater treatment. In that particular publication, it was described anecdotally that the surface of water on which duckweed were growing during experiments froze for several days. Following an increase in temperature and the thawing of the water, the duckweed resumed their growth and removal of nutrients (Classen et al., 2000).

With the exception of (Classen et al., 2000), the small number of publications that have investigated duckweed growth under low temperatures have somewhat written off their capacity for use in the colder and darker months of cool temperate countries, but have provided evidence for their annual survival adaptations. At low temperatures or under other environmental stresses such as high salinities, *Spirodela polyrrhiza* will form starch-rich fronds termed ‘turions’ and will sink to the bottom, lie dormant and refloat once warmer or more clement conditions have resumed (Jacobs, 1947, Kuehdorf and Appenroth, 2012, Appenroth and Nickel, 2010, Appenroth and Adamec, 2015). This could be seen as a dual advantage for this species, in that over winter survival is beneficial for autonomous operation of a remote wastewater system using duckweed, as well as the possibility of forcefully inducing excess starch production for the production of biofuel (Xu et al., 2011). A further adaptation made by plants in response to temperature stress is altering membrane fluidity (Yamori et al., 2014). During cold stress, the ratio of unsaturated fatty acids to saturated fatty acids is increased, making the membranes more fluid, while during heat stress the opposite adaptation has been

observed (Murata and Los, 1997, Murakami et al., 2000, Sung et al., 2003). This has not been specifically investigated in duckweed however.

In attempting to build robust and comprehensive models of duckweed growth Lasfar et al. (2007) maintained *Lemna minor* on Hoagland's growth solution to temperatures of 5°C to 35°C, while fixing photoperiods to 14.5 h and tested photoperiods of 2 h to 20 h while fixing temperature to 27°C. The authors report optimum ranges for growth of 11 h to 14 h photoperiods and temperatures of 23°C to 28°C. For P and N uptake experiments, the authors fixed temperature and photoperiod to 27°C and 14.5 h respectively (Lasfar et al., 2007), so the effect of temperature or photoperiod on nutrient uptake could be analysed in this case. It was noted however that under the conditions applied, P and N (as NO_3^-) uptake was concentration dependent up to approximately 2 mg P L^{-1} and 5 mg N L^{-1} respectively. Conditions adopting 26°C and 12 h to 13 h photoperiods were reported to be the optimum for growth. Temperatures <8°C and >35°C and photoperiods <2 h >20 h were all said to strongly inhibit growth, but unfortunately the effects of simultaneously low temperatures and brief photoperiods were not tested.

Zhao et al. (2014) grew duckweed at temperatures of 20°C, 25°C and 30°C. *Lemna minor* and *Landoltia punctata* sampled from Central China were maintained on growth solution in a laboratory under 16 h photoperiods. The growth rate of *Lemna minor* decreased as temperatures increased from 20°C to 30°C, but confusingly the authors report the 'optimum temperature' to be 25°C (Zhao et al., 2014). *Landoltia punctata* increased growth rates from 20°C to 25°C but then also reduced growth from 25°C to 30°C. Interestingly there was very little difference in the growth rates of *Lemna minor* between phosphate concentrations of 1.5 to 150 mg P L^{-1}, but nutrient uptake was not measured in these experiments (Zhao et al., 2014).

The highest growth rates for *Lemna aequinoctialis*, *Landoltia punctata* and *Spirodela polyrrhiza* were recorded at 25°C by (Li et al., 2016). The duckweed, originally sampled from Eastern China and maintained on Hoagland's solution in a laboratory under 16 h photoperiods were exposed to 20°C, 25°C and 30°C. Growth rates all increased with increasing concentrations of N (as NO_3^-) and P, from 0 to 35 mg N L^{-1} and 0 to 15 mg P L^{-1} respectively, but nutrient removal was again not built into the design of the experiments (Li et al., 2016).

The information gathered from publications on the effects of varying temperatures and photoperiods lacks in two distinct areas. First, there is a dearth of data on the performance of duckweed under low temperatures, or at least temperatures that are relative to annual values in cool temperate countries. Second, the combined and possibly additive effects of how both low temperatures and short photoperiods would impact on the growth and acquisition of nutrients by duckweed has not been

thoroughly investigated. It would therefore be useful to conduct experiments that investigate the effect of low temperatures, while fixing photoperiods on the performance of duckweed, before later combining these parameters at low values that relate to cool temperate climates. This would contribute towards understanding if duckweed use for wastewater remediation in countries like the UK has potential.

1.8.3. Tank dimensions and stocking densities

It was found that increasing the surface area to depth ratio of a tank in a flow through system increased P removal by *Lemna minuta* under 16 h photoperiods and average temperature of 24°C (Alahmady et al., 2013). Concomitantly, increasing depth reduced P removal, possibly owing to the short length of the duckweeds' roots, while not employing mixing within the small tanks. If this were the case, then extending hydraulic retention time (HRT) to allow diffusion would improve P removal and it was indeed shown in that particular publication that decreasing depth while retaining the same hydraulic retention times did improve P removal (Alahmady et al., 2013). In a similar context, Vermaat and Hanif (1998) suggested a depth of less than 10 cm for a duckweed tank to operate at its optimum, which was supported by Alahmady et al. (2013) but in an outdoor setting this would need to be managed to prevent the encroachment of rooted macrophytes. Another significant observation from Alahmady et al. (2013) was that efficiency of P removal was possibly a function of growth. This was suggested to be due to the initial rapid reproduction of the duckweed as it expanded to fill the surface area of each particular tank, followed by a decline in growth due to lack of space at the same time as P removal slowed down. Optimum stocking densities have been suggested by authors (Lasfar et al., 2007, Driever et al., 2005), but this could also depend on factors such as nutrient supply. The reduction in rates of P removal over time seen in Alahmady et al. (2013) could also have been due to possible P limitation, as P removal displayed kinetics similar to the Michaelis-Menten model with asymptotic curves. A partial harvest of biomass during operation to test if reinitiating rapid growth would encourage further P removal was not conducted in that particular study.

The key conclusions drawn from this publication was that with an HRT of 7.5 days, maximum P removal efficiency of 80 % from artificial effluent in a flow through system was achieved by *L. minuta* after 5 days but that this efficiency was reduced to 0 by 20 days, either due to reduced growth rates or P limitation. Increasing surface area to depth ratio improved P removal, but it was not clear whether this was due to increasing the duckweeds' ability to acquire nutrients from deeper in the water column or the fact that it would increase the amount of available biomass per unit volume.

1.8.4. Tolerance to salinity

The salinity of water is related to the amount of dissolved nutrients or metals that it contains and this can be estimated by way of measuring electrical conductivity, although this does not discern between nutrients (El-Kheir et al., 2007). Molar concentrations of NaCl can be multiplied by approximately 100,000x to convert to electrical conductivity (reported in µS cm^{-1}). Salinity stress in higher plants can be managed by the regulation of ionic gradients (expulsion or sequestration of ions) and management of osmotic potentials (E.g. stomatal closure) to cope with changing states of hydration (Ashraf and Harris, 2004), at the expense of energy from ATP (Sikorski et al., 2013) or mediated by abscisic acid. Publications on this topic that refer to duckweed exclusively are scarce. Duckweed is a freshwater organism and any changes in wastewater salinity may have an impact on the plants' growth and nutrition and as such salinity is an important factor to consider.

Recent work on duckweed salinity tolerance revealed that increasing salinity reduced both growth and the uptake of nitrogen and phosphate by *Lemna minor* under laboratory conditions (Liu et al., 2017) and outdoor conditions (Iqbal et al., 2017). At 25°C and 16 h photoperiods, in 100 mL volumes of Hoagland's growth solution containing salinities of 75 mM NaCl, rapid decreases in nutrient removal were noted and at 100 mM NaCl nutrients were released back into growth solutions during the senescence of the duckweed (Liu et al., 2017). The salt concentrations that caused rapid decreases in duckweed performance equated to approximately 7,500 µS cm^{-1}.

Outdoors in Pakistan, experiments with *Lemna minor* in both the summer and winter showed that increasing the electrical conductivity of landfill leachates to above 1,000 µS cm^{-1} caused significant decreases in growth and the removal of total phosphorus, phosphate, ammonium and COD (Iqbal et al., 2017). The authors used 250 mL volume tanks of shallow depth and left them outside for 25 days in each experiment, testing a range of 0 µS cm^{-1} to 3,000 µS cm^{-1}. The authors do not report whether or not they topped up the solutions to counteract evapotranspiration, but this surely would have occurred at the temperatures present, which were an average of 38°C and 30°C for the summer and autumn experiments respectively (Iqbal et al., 2017). Evapotranspiration would have concentrated any salts in the leachate and thus increased conductivity, but this was not reported to be measured during the course of the experiment except at the onset (Iqbal et al., 2017).

An experiment was conducted in West Africa where the duckweed *Spirodela polyrrhiza* was maintained for 10 days on anaerobically pre-treated domestic wastewater ranging in conductivity from 200 to 3,000 µS cm^{-1} (Wendeou et al., 2013). These were 1.5 L volume unrefreshed batch tanks at temperatures of 25°C to 28°C. The authors found an optimum range for duckweed growth of 600 to 1,400 µS cm^{-1}. This range also prompted the best reduction of COD, phosphate, nitrogen and

turbidity. At conductivities of 1,200 µS cm^{-1} and over, relative growth rates decreased and reached their lowest values at 3,000 µS cm^{-1}. At conductivities of 1,600 µS $cm^{-1}$and above, duckweed fronds were reported to reduce in size and turn chlorotic (Wendeou et al., 2013). The authors reported a rapid increase in conductivity after six days in all experimental tanks, due to evapotranspiration of the duckweed concentrating the solutions. Results were reported as averages for the whole 10 day experiment and therefore may be flawed with respect to correlating conductivity with performance as they did not adequately control conductivity for the duration of the experiment, which was the variable they set out to investigate. Topping up the tanks with wastewater or water, recording the conductivity and adjusting with NaCl solution or employing a flow-through setup would have controlled this more appropriately.

The opposite mistake was made by Haller et al. (1974) in their reports of salinity treatments of duckweed. After converting from salinity to conductivity, the authors report decreases in growth rates at conductivities of 9,500 µS cm^{-1}, but solutions were topped up each week to counter evapotranspiration without adding any salts (Haller et al., 1974). As such there would have been differences in the reported and actual values of conductivity. At the end of the four week experiment, growth solutions had been diluted by 40 %, therefore the value of 9,500 µS cm^{-1} reported to cause decreases in growth rates would have possibly been closer to 5,000 µS cm^{-1}. As introduced earlier, the differences in experimental designs often make it difficult to compare authors' results directly, but both Sikorski et al. (2013) and Sree et al. (2015) reported small decreases in the growth rates of duckweed as conductivity rose past 1,000 µS cm^{-1} and both report sharp decreases in growth rates when this value moves above 3,000 µS cm^{-1} (Sikorski et al., 2013, Sree et al., 2015). Thus maintaining wastewater at values well below 3,000 µS cm^{-1} would appear a cautionary but necessary aspect for a duckweed wastewater treatment system, but this may warrant further investigation in a more controlled manner.

1.8.5. Mechanisms of nutrient uptake

Soluble inorganic nutrients are absorbed from solution by both the roots and the fronds of duckweed (Cedergreen and Madsen, 2002, Cedergreen and Madsen, 2003) via transmembrane proteins. Nutrients such as ammonium or potassium can diffuse through the plasma membrane via ion channels, sodium can be transported via antiport proteins and anions including nitrate and phosphate are acquired by hydrogen ion cotransport (Koning, 1994), (Figure 1.8.1). Uptake of phosphorus and nitrate by duckweed is driven along electrochemical gradients (Hase et al., 2004) and requires the membrane potential's regeneration to achieve this (Sakano, 1990). Membrane potentials are created by localised proton concentrations inside and outside of the cell, which in turn are maintained by ATP pumps, therefore the uptake of phosphate depends on these energetic proteins (Krajinski et al., 2014).

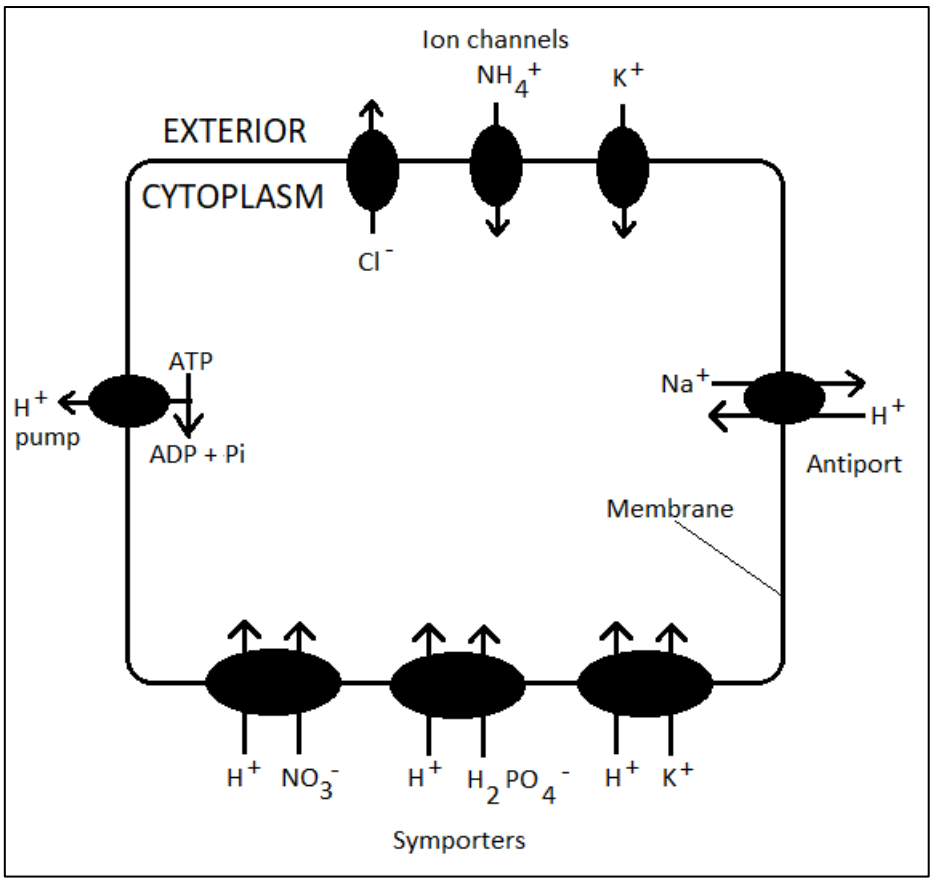

Figure 1.8.1. Model of nutrient uptake by higher plants. Adapted from (Koning, 1994).

Phosphate uptake from the environment in higher plants is known to be facilitated by the phosphate transporter 1 (PHT1) family of proteins (Muchhal et al., 1996). These proteins have been subsequently found in species such as rice (Ai et al., 2009), barley (Rae et al., 2003), foxtail millet (Ceasar et al., 2014), tomato (Liu et al., 1998) and *Arabidopsis thaliana* (Mudge et al., 2002). Homologues were found in the *Spirodela* genome following bioinformatic investigations (unpublished, personal communication from Professor A. Baker, University of Leeds, UK) and as such it is reasonable to suggest that duckweed also employ PHT1 family proteins for this purpose. There is evidence that plants (including duckweed) take up P either by transport proteins with specifically high or low affinities (Rae et al., 2003, Schachtman et al., 1998, Kant et al., 2011, Fan et al., 2013, Ullrich-Eberius et al., 1984), or proteins with the capacity for dual affinity (Ayadi et al., 2015) which can be utilised under conditions of abundance or depletion (Hase et al., 2004). High affinity uptake proteins are typically expressed in external concentrations of 3 to 10 µM phosphate, while low affinity transporters are more abundant when concentrations increase to 50 to 300 µM phosphate (López-Arredondo et al., 2014). Phosphate is taken up as $H_2PO_4^-$ or HPO_4^{2-}, which is determined by the pH of the solution (Kant et al., 2011).

1.8.6. Uptake and tolerance of nitrogen and pH

Nitrogen can vary in wastewater in its concentration and form, the former of which can depend on the fraction or type of wastewater (Körner et al., 2001) and the latter can depend on the pH and temperature (Caicedo et al., 2000). The tolerance of duckweed to nitrogen has been suggested to be as high as 1 g N L^{-1} (Landolt, 1986) (but N specie was not described), with more conservative estimates of <100 mg NH_4^+ L^{-1} dependent on conditions (Cheng et al., 2002b). An optimum approaching 350 mg N L^{-1} was suggested by Landolt (1986) in his extensive study but the form of N applied was not reported. Lasfar et al. (2007) described inhibition of growth rates of at concentrations of nitrate at 120 mg N L^{-1} or higher. Opinion is divided on which form of N (nitrate or ammonium) is the most productive for duckweed growth if present in solution in isolation.

Ammonium (NH_4^+) is absorbed by plants by diffusion into the cell by way of ammonium transporter proteins (AMT) and when present, NH_3 can passively cross the membrane unchecked (Bittsánszky et al., 2015). This does not require the expenditure of energy by the cell as is the case for the uptake of nitrate (Sun et al., 2014) which is taken up by proton symport. This requires the production and regulation of cytosolic pH gradients used to carry nitrate molecules across the plasma membrane as well as energy required to transcend the nitrogen assimilatory pathway. Upon entering the cell, nitrate (NO_3^-) will convert to nitrite (NO_2^-) in the presence of nitrate reductase, then convert to ammonium (NH_4^+) in the presence of nitrite reductase, before glutamine synthetase and glutamate synthetase

convert ammonium to glutamine and glutamine to glutamate respectively (Crawford and Forde, 2002). Amino acids are then subsequently derived from glutamate.

Due the energetic requirements of acquiring nitrate, several authors suggest that duckweed will grow and remove nutrients better when supplied with ammonium either as a sole N source (Caicedo et al., 2000, Porath and Pollock, 1981, Monselise et al., 1987, Mohedano et al., 2012), or when in combination with nitrate (Fang et al., 2007). A drawback to ammonium nutrition is the fact that it can readily dissociate to unionised ammonia (NH_3) , which is toxic to duckweed due to its cell-permeability at concentrations of 8 mg NH_3 L^{-1} or above (Körner et al., 2001) and its dissociation is increased by increases in pH and temperature (Caicedo et al., 2000). The pH at which NH_4^+ and NH_3 are in equal concentrations (the p*K*a), is 9.25 at 25°C (Redruello et al., 2017) and even higher at lower temperatures. Below pH 9.25 NH_4^+ will dominate and above this value NH_3 will be more prevalent. Körner et al. (2003) suggested that high concentrations of ammonium can be tolerated by duckweed as long as solution pH is maintained below 7.8. Britto and Kronzucker (2002) reported a duckweed preference for nitrate however and even ammonium induced toxicity (Britto et al., 2001). In that particular study, when barley cells were exposed to 10 mM NH_4^+, respiration increased by 41 % in order to produce energy to expel the constant influx of cations. Most of the ammonium that diffused into the cell was transported back to the exterior, which resulted in the death of the plants due to expenditure of energy reserves (ATP) (Britto et al., 2001). The phenomenon is not experienced by all plant species however, as rice had apparently evolved a mechanism to lower its membrane potential to greatly reduce the diffusion of ammonium into the cell and thus save energy by not needing to expel as much (Britto et al., 2001).

Ammonium influx was originally shown to supress the uptake of anions by membrane depolarisation by interference with the cellular pH gradient by Löppert (1979) with the duckweed *Lemna paucicostata*. Similar experiments were conducted with the same results in *Lemna gibba* (Ullrich et al., 1984) and together these findings may be an explanation as to why several authors refer to a 'preference' of ammonium over nitrate by duckweed. The same effect was shown for phosphate uptake (Ullrich-Eberius et al., 1984). In small scale laboratory experiments using P starved *Lemna gibba*, adding just 0.2 mM NH_4^+ to growth solution initiated an immediate membrane depolarization and cessation of phosphate uptake that resumed only once all the ammonium had been metabolised (Ullrich-Eberius et al., 1984).

More recent work by Paolacci et al. (2016) showed that duckweed species *L. minuta* and *Lemna minor* both produced their highest growth rates at 30 mg N L^{-1} when provided with either nitrate or ammonium. In small scale laboratory experiments lasting 1 week and adopting 16 h photoperiods and

constant 20°C temperature, *L. minuta* grew significantly more than *Lemna minor* in both nitrate and ammonium solutions. There were no differences however in the growth rates of duckweed between nitrate and ammonium solutions. Interestingly, the authors initially fixed their experimental solutions to pH 4.5, stating is as an 'optimum' (but provided no reference for this) (Paolacci et al., 2016). There was evidence for growth being dependent on nitrate concentration for both species, with growth rates declining from somewhere between 30 mg N L^{-1} and 300 mg N L^{-1}. The duckweed grew better on ammonium than on nitrate from 300 mg N L^{-1} up to 1 g N L^{-1} (Paolacci et al., 2016). The very low pH used in this experiment may have prevented the dissociation of lethal concentrations of free ammonia referred to earlier, and this is also proposed in the paper. The debate in the literature over the preferences of duckweed for N, the difficulty of identifying the true effects of N species independently of pH or temperature and the fact that maximum growth rates of *Lemna minor* were the same between nitrate and ammonium in (Paolacci et al., 2016), mean that more studies in this area are required to identify what duckweed can tolerate if faced with varying conditions of nitrogen in the field.

When nitrogen is present in solution as ammonium, the pH of that solution has an effect on the ratio between ionized and free ammonium which can disturb cellular processes. The dissociation of NO_3 to HNO_3 is not considered a factor in this subject, as the pH at which this occurs is 2. The pH (or hydrogen ion concentration) of a solution will impact on biological systems (Duman et al., 2006), irrespective of nitrogen or other compounds. Photosynthesising organisms maintain a proton gradient to produce the form of energy used by the cell (ATP). Proton gradients are maintained inside the vacuole, cytoplasm, mitochondria, chloroplast and in localised areas external to the plasma membrane and are used for the uptake of nutrients including phosphate and nitrate (Ullrich-Eberius et al., 1984, Ullrich et al., 1984). If the concentration of hydrogen ions present in solution is significantly raised or lowered (decreasing and increasing the pH respectively), then duckweed will in theory need to expend energy in order to combat proton diffusion and maintain cellular homeostasis.

Experiments where duckweed has been exposed to a range of pH values have been conducted since the 1930's. A general consensus of a tolerable pH range is 4 to 8.5 (Hicks, 1932, Hillman, 1961, McLay, 1976) but the survival of the duckweed in these experiments would also be affected by concentrations of N and temperature, if not controlled appropriately. Hicks (1932), collected Lake water from Ohio, US and returned it to the laboratory whereby numerous duckweed species were inoculated at various pH values, as well as making observations in the field. *Spirodela polyrrhiza* died at pH lower than 5 and higher 8, while grew vigorously between pH 6 to 7.5. *Wolffia columbiana* died at pH lower than 5.5 and higher than 8.5, doing the best between pH 6.5 to 8. *Lemna minor* died at a pH lower than 4 or higher than 8 and grew most favourably between pH 6 to 7.5 (Hicks, 1932). Unfortunately the paper

did not report on any conditions applied with regards to the nutritional content of the water used, buffering methods or temperature.

In other work the lower, optimum and higher pH values for the growth of *Lemna minor* were reported to be pH 4, pH 6.2 and pH 10 respectively (McLay, 1976). The experimental temperature was 25°C, there was no ammonium present, conditions were aseptic but photoperiod was not given and no indication of managing evapotranspiration was reported. The author conducted various experiments using Jacob's growth solution which he either buffered, regulated by addition of NaOH or HCl, or left alone. The best results were found from solution that was unbuffered but regulated (McLay, 1976). In these batch conditions, the duckweed rapidly changed the pH of their solution, as a result of growth and nutrient uptake and as a result there is a time limit on survival unless the pH is regulated. When duckweed have been used for wastewater treatment in large flow-through systems, the continuous flow of wastewater and natural buffering capacity means that the effect of pH variations due to duckweed activity can be managed.

More recently Iqbal and Baig (2016) grew *Lemna minor* on leachates with pH values ranging from 4 to 10. 300 mL batch tanks were kept outdoors in Pakistan, with an average temperature and photoperiod of 37°C and 14 h respectively. Solutions were pH-adjusted every day but there is no mention of managing evapotranspiration during the 22 day experiment. Two concentrations of leachate were applied, containing either 20 mg N L^{-1} or 90 mg N L^{-1} as ammonium. The growth of *Lemna minor* as well as nutrient uptake were reported to be the best at pH 7.1 and growth was better in the more diluted leachate. As the pH rose or fell from neutrality, growth decreased and chlorotic fronds were reported at pH 5, with death at pH 4, most likely due to damage of the duckweeds' membrane potential (Iqbal and Baig, 2016). Due to the concentrations of N applied, growth decline of the duckweed at pH values above 8 were most likely due to ammonia toxicity (Iqbal and Baig, 2016).

While the experiments conducted on the pH tolerance of duckweed are varied, they present a general consensus of tolerable limits to growth between pH 5 to pH 8. However, the interactions of pH, ammonium and temperature can be significant and therefore must not be discounted. Where possible these effects need to be examined independently of each other to identify the true effects of either nitrogen or pH.

1.8.7. Removing P and improving wastewater quality

A short study on wastewater remediation was attempted by El-Kheir et al. (2007) using *L. gibba* on primary treated sewage in 25 L batch tanks outdoors in Egypt with an average sewage temperature of 24°C. After an 8 day HRT, removals of total suspended solids (TSS), biochemical oxygen demand (BOD), chemical oxygen demand (COD), NO_3^- and NH_4^+ were at 96 %, 90 %, 90 %, 100 % and 80 % respectively. Electrical conductivity rose by 10 % from 905 µS cm^{-1} to 995 µS cm^{-1} during the experimental period along with total dissolved solids and a selection of metals, but no explanation was offered for why this occurred (El-Kheir et al., 2007). The authors report three fractions of P as 'Phosphorus', 'Phosphate' and 'O. phosphate' (the latter possibly meaning organic phosphate), with P being reduced by the duckweed by 44 % to 64 % depending on the fraction (El-Kheir et al., 2007). Biomass was reported to double in eight days based on fresh weight measurements, but these were achieved by removing just a 1 % surface area harvest every two days and extrapolating the data for the whole tank which could have inherent error. When the biomass increase for the same experiment is analysed based on reported dry weights the increase is adjusted to 60 %. The experiment by El-Kheir et al. (2007) was conducted with only two replicates, not the customary three or more generally accepted as a more robust scientific method. While this particular publication hints at the potential of duckweed for wastewater improvement, unfortunately it also highlights some of the less robust ways in which data can be obtained and analysed. This emphasises the caution that must be employed when inferring from publications in this field.

The list of parameters measured in El-Kheir et al. (2007) highlights just some of the factors present in wastewater that could potentially have an effect on the operation of a biological system used for nutrient removal. It is beyond the scope of this thesis to explore an exhaustive list of biochemical reactions found within wastewater so the focus will be on more fundamental environmental parameters.

1.8.8. Potential pathogens of duckweed

When considering an outdoor duckweed wastewater treatment system, the threat of decimation by pathogen(s) should be considered. Literature on the pathogens of duckweed is extremely rare however, possibly owing to their apparent robustness when expanding across waterbodies under a range of conditions. Fungal species such as *Pythium myriotylum* or *Fusarium monoliforme* are widespread throughout the world and the latter was shown to produce phytotoxic compounds (Abbas et al., 1991). Compounds produced such as fumonisins cause growth inhibition, necrosis and chlorosis, as well as the senescence of plants (Abbas et al., 1992). Large colonies of mixed assemblages of duckweed species were witnessed to be eradicated from wetlands in Louisiana, US by fungal

pathogens (Rejmankova et al., 1986). In that study, samples of duckweed species were returned to the laboratory and exposed to the fungus *Pythium myriotylum*. Two species were resistant – *Spirodela punctata* and *Lemna aequinoctialis*, while others including *Lemna minor*, *Lemna gibba* and *Spirodela polyrrhiza* were all killed (Rejmankova et al., 1986). Temperature also hastened the detrimental effects of the pathogenic fungus. The fact that some species were apparently unaffected would have impact for a duckweed wastewater treatment system, as choices of species could be made provisionally based on resistance, then possibly observed and managed on site if required. Adopting and maintaining a monoculture would be difficult out in a field setting and in any case it is recommended that using mixed duckweed species for wastewater treatment would be more robust than a monoculture (Zirschky and Reed, 1988), with Zhao et al. (2014) reporting more efficient nutrient uptake by mixed duckweed species assemblages.

1.9. Research problem, research gaps and project aims

1.9.1. The research problem

As introduced earlier, the world faces an ensuing phosphorus crisis and many countries (including the UK), adopt phosphorus removal technologies that can be expensive, inefficient and difficult to manage holistically. As agriculture and industries that rely on phosphorus imports continue to develop to meet the demands of increasing populations, and as human generated wastes increase, the need for a low energy closed-loop phosphate recovery system is ultimately of great importance, both economically and environmentally, for developing and developed nations alike.

1.9.2. Gaps in the research to date

Publications documenting the use of duckweed for wastewater remediation are numerous but the body of knowledge as a whole lacks cohesion or even any published or universally adopted experimental protocols. The failings of several publications mentioned earlier make the reinvestigation of some factors that could possibly impact on duckweed performance necessary. These include the possible effects of nitrogen, pH and conductivity. There is also a distinct lack of published work on how duckweed could grow and remediate wastewater under conditions of a cool temperate climate (low temperatures and short photoperiods). In addition to this, at the time of writing, not a single publication or set of publications from any author(s) has been found that has examined the performance of duckweed under both small scale, highly controlled conditions, through to larger scale, real world conditions, with the careful introduction of variables in between to be confident of fundamental impacting influences.

The work of which this thesis represents was an industry partnered research project, aimed at identifying if and how a duckweed system could be operated in the UK to grow and remove phosphate from wastewater to local government standards. With the greatest respect, this means that basing assumptions for a system design on some of the results gathered from the literature and instigating large scale trials immediately contained obvious risks. Therefore this project aimed to design and conduct experiments that transcended the small through to large scale operations.

1.9.3. Project scope and objectives

The overarching aim of this project was to determine the feasibility of a duckweed system for the remediation of wastewater, operating under cool temperate environmental conditions. To realise this ultimate aim, understanding the direct effects that numerous environmental parameters had on the growth and removal of phosphate by *Lemna minor* would need to be identified and as such were the project's main objectives. As a result, the breadth of this project meant that the scope had to be closely managed to maintain a clear direction of research with tangible outcomes. With this in mind the project was divided into three phases.

Phase 1 (Chapter 3), would be the investigation of a selection of parameters (pertinent to UK wastewater treatment) that were assumed from the literature to impact the growth and removal of phosphate by duckweed acquired from a site in the UK. Phase 1 used small scale microcosm experiments and aimed to test one variable at a time while controlling all others. Experiments were conducted with batch systems of duckweed maintained in a growth chamber and allowed a rapid output of experimental data.

Some of the specific research objectives investigated in Chapter 3 include:

- The effects of photoperiod and temperature on the growth and removal of phosphate by *Lemna minor*.
- The effects of salinity, nitrogen species and pH on the growth and removal of phosphate by *Lemna minor*.
- How acclimating *Lemna minor* to varying concentrations of phosphate had an effect on growth and phosphate removal.

The data generated from Phase 1 was used to inform the design of experiments in Phase 2 (Chapter 4). These were conducted with an approximate 100x volumetric scale increase. Phase 2 mesocosm experiments would be conducted outdoors at ambient temperatures, but sheltered from precipitation and provided with artificial lighting to control the photoperiod and light intensity. Specific objectives investigated in Chapter 4 include:

- Identifying the effect of system configuration (tanks in parallel or in series) on phosphate removal by *Lemna minor*.
- Phosphate uptake by *Lemna minor* under continuous flow conditions of both growth solution and real wastewater.
- Assessing the effects of phosphate loading rate and temperature on growth and phosphate removal by *Lemna minor*.

As Phase 2 experiments generated more information they aided the design for work in Phase 3 (Chapter 5). This final phase was the design, build and operation of a pilot scale system that grew duckweed on real effluent outdoors in the UK. It was planned to operate the system through summer, autumn and winter while monitoring duckweed growth and phosphate removal as temperature and photoperiod declined, as well as other wastewater characteristics such as nitrogen, pH, salinity and dissolved oxygen. As this was an iCASE industry-partnered project, it was hoped that results from the pilot system may show potential for further extended trials on a WWTW site and possibly prove commercially viable.

1.9.4. Structure of this thesis

With the exception of this first introductory Chapter (1), this thesis contains five further chapters. Chapter 2 describes all the materials and methodologies adopted for all the experimental work undertaken during this project. Chapters 3, 4 and 5 present the results obtained from microcosm experiments (Phase 1), mesocosm experiments (Phase 2) and pilot system operations (Phase 3) respectively. Chapter 6 is a general discussion that aims to conclude and summarise the key findings from Chapters 3, 4 and 5, while assessing how the aims of the project were met and what recommendations could be made for the continuation of this research.

CHAPTER 2

MATERIALS AND METHODS

2.1. Introduction

The practical work conducted for this research project was divided into three distinct phases (Phases 1, 2 and 3). Phase 1 is reported in Chapter 3. This phase of work consisted of highly controlled, small scale (microcosm) laboratory experiments in which several variables were manipulated in order to assess their effect on the growth and uptake of phosphorus by *Lemna minor,* using growth solution. Phase 2 is reported in Chapter 4. This phase increased the experiments to mesocosm scale and changed the growing environment from 100 mL volume batch pots in Phase 1, to 10 L volume tanks under continuous flow conditions. Phase 2 aimed to assess how a continuous supply of phosphate would affect growth and phosphate uptake under a range of temperatures. The work in this phase also set out to compare the performance of duckweed supplied with both growth solution and real wastewater. The first four experiments used growth solution, before switching to real wastewater collected from Esholt waste water treatment works (WWTW) (Bradford, UK), for the remaining three experiments.

Phase 3 is reported in Chapter 5. This final work phase consisted of the design and build of a large scale duckweed pilot system, consisting of three 330 L tanks connected in series and operating outdoors in the UK, receiving treated effluent from a golf club. Several characteristics of the effluent were measured throughout the system, as well as estimating growth and measuring phosphate removal. Some aspects of the methodologies described below were used throughout all three work phases, such as measuring phosphorus *in planta*. Methods such as measuring phosphorus in solution were different between phases, for example Phase 1 used a previously published laboratory method for cost effectiveness, whereas Phase 3 demanded on-site analyses using portable equipment. In such cases, the different methods were compared using known standards to be confident of later results. Where certain methods are specific to only one work phase, this is made clear in the appropriate sub-section headings.

2.1.1. Water and Chemicals

All water used for solutions and assays was de-ionised (dH_2O) and was purified to <10 MΩ cm^{-1} by filtration (Triple Red).

Chemical salts, acids, bases and reagent chemicals were all procured from Sigma Aldrich or Fisher Scientific at reagent grade.

2.1.2. Glassware and equipment

All glassware and plastic used for maintaining cultures or conducting assays was prepared thus. Washed in detergent and rinsed in dH_2O; soaked in 0.1 M HCl overnight to remove traces of phosphates; rinsed three times in dH_2O and dried upside down on a phosphate free rack.

2.2. Preparation and maintenance of stock cultures

The duckweed isolate used for all experiments in this thesis was sampled by hand from a lagoon in North Lincolnshire (location given in Chapter 3). Sampled duckweed was transferred to sterile Falcon tubes containing lagoon water and kept aerated during transit. On returning to the laboratory, duckweed was rinsed in a sieve before being agitated and settled in buckets of tap water repeatedly to remove detritus and associated biota. Healthy sub samples of the original stock were then transferred to 2 L opaque tubs and grown on autoclaved Hoagland's growth solution, with the duckweed being sieve-rinsed and the growth solution replaced at least every five days.

2.2.1. Axenic culture production

Using aseptic technique, duckweed had roots removed with a razor blade close to the frond base and immersed for two minutes in 10 % (v/v) bleach/dH_2O to kill epiphytic microbial assemblages. Following bleaching, single fronds were transferred to 10 mL autoclaved Hoagland's solution in six well Repli-dishes and covered. Fronds were transferred to fresh sterile solution every two days using aseptic technique until enough fronds had reproduced for experiments.

2.2.2. Growth environments

Duckweed maintenance and Chapter 3 experiments were conducted in a Sanyo MLR-351 growth chamber. Temperature was constantly controlled ±1°C and light was provided by fluorescent tubing at 155 µmol photons m^{-2} s^{-1}. Stock tubs and experimental pots were modified to allow light from only above, to prevent algal proliferation lower in the water column.

Chapter 4 experiments were conducted outdoors to ambient temperatures, but sheltered from wind or rain. Light was provided from overhead fluorescent tubing at 155 µmol photons m^{-2} s^{-1}. The light

intensity was fixed in the experiments reported in Chapters 3 and 4 to control this particular variable, although it could not be controlled outside during experiments reported in Chapter 5.

The pilot system (Chapter 5) was trialled at a confidential location in Lincolnshire, exposed to all weather and receiving effluent from the on-site wastewater treatment facility at a golf club.

2.3. Molecular identification of species

To ascertain species, it was required to extract DNA, amplify a known region via PCR and have the purified products sequenced and compared with known species. Each stage of the process is described below and was derived from the methods of Wang et al. (2010).

2.3.1. Extraction of DNA

Working buffer was made from the following sterilised solutions-

- 5 M NaCl – 28 mL
- 0.5 M EDTA pH 8 – 4 mL
- 1 M Tris HCl – 10 mL
- 2 g Cetyl methylammonium bromide (CTAB)
- 100 mM 2-mercaptoethanol – 100 µL

30 mL dH_2O was added to a 100 mL volumetric flask, using a magnetic stirrer the CTAB was added and dissolved, before adding the NaCl, EDTA and Tris HCl in turn. Once all was dissolved the buffer was made up to 100 mL with dH_2O.

10 mL of the above solution was transferred to a falcon tube and 100 µL 2-mercaptoethanol was added before inverting.

2.3.2. DNA extraction procedure

Duckweed samples were rinsed in dH_2O and patted dry on paper towels. 0.2 g fresh mass (FM) was added to 2 mL Eppendorf tubes containing steel ball bearings. Tubes were flash frozen in liquid N_2 and homogenized in a tissue lyser (Qiagen, UK) for 60 s. 800 µL of extraction buffer was added and samples homogenized once more for one to two minutes. Following this, the tubes were incubated at 60°C for 30 minutes and then vortex mixed. 500 µL of 24:1 chloroform:isoamylalcohol was added and then tubes were centrifuged at 13,000 g for two minutes. The clear upper portion was then transferred to new 1 mL Eppendorf tubes and the remainder was centrifuged again as previously to obtain more supernatant. The supernatant was precipitated in 2/3 volume isopropanol up to 400 µL before being centrifuged at 13,000 g for thirty minutes. The supernatant was removed by pipette and added to 200

µL of 70 % ethanol and gently shaken, before being microfuged for two minutes at 13,000 g. Following removal of the produced supernatant, this step was repeated and the remaining pellet was left briefly to allow remaining ethanol to evaporate. The pellet was then gently re-suspended in 50 µL dH_2O and 0.5 µL RNase. The DNA concentration was then quantified using a Nanodrop spectrophotometer against a combined dH_2O and RNase blank.

2.3.3. PCR and purification procedure

Primers selected for the PCR corresponded to the atpF-atpH non-coding spacer region (579-622 bp) on the *Lemna minor* chloroplast genome, recommended by Wang et al. (2010). Reactions were carried out in a PCR thermal cycler (volumes of solutions below) with an initial denaturing at 94°C for 2 minutes, followed by 35 cycles of 94°C (15 s), 50°C (15 s) and 72°C (40 s) and a final hold at 72°C for 5 minutes. Reactions were carried out in 200 µL PCR tubes and the PCR mix was as follows –

- Autoclaved dH_2O 17.75 µL
- DNA 1 µL
- Forward and reverse primers 1 µL of each at 5 pmol solution
- Dntp MIX AT 10Mm 0.5 µL
- Red Taq PCR buffer 2.5 µL
- Red Taq DNA polymerase 1.25 µL

Primer sequences: **F** – 5’ ACTCGCACACACTCCCTTTCC 3’. **R** – 5’ GCTTTTATGGAAGCTTTAACAAT 3’.

5 µL of PCR product was run on a 1.2 % agarose gel in 1x TAE buffer (100 V for 40 minutes approx.) to check for a corresponding single band of the specified size. Some of the remaining product was then diluted and purified using ExoSap-ITTM (USB Corporation) before being sent for sequencing at Beckman Coulter Genomics Inc., UK.

On the return of sequenced data, consensus sequences were produced in Clustal Omega software, and then run through the BLAST database program to compare for matches with other species.

2.4. Growth solution preparations

All experiments not conducted with sewage treatment works effluent used Hoagland's growth solution. The original recipe used for the regular stock maintenance and bulking up comprised the macronutrients of: $Ca(NO_3)_2 \bullet 4H_2O$ (543 mg L^{-1}), $MgSO_4 \bullet 7H_2O$ (247 mg L^{-1}), KH_2PO_4 (68 mg L^{-1}), KNO_3 (253 mg L^{-1}); and the micronutrients of H_3BO_3 (2.86 mg L^{-1}), $NaMoO_4.2H_2O$ (0.025 mg L^{-1}), $MnSO_4.5H_2O$ (0.025 mg L^{-1}), $ZnSO_4.7H_2O$ (0.025 mg L^{-1}), $CuSO_4.5H_2O$ (0.025 mg L^{-1}), $FeCl_3.6H_2O$ (0.05 mg L^{-1}) and EDTA (0.75 mg L^{-1}) (Cross, 2006). Solutions were autoclaved before use. Prior to all experiments in Chapter 3 excluding P exposure and nitrogen species, all experimental duckweed stocks (taken from the original stock) were maintained for four days on 15 mg P L^{-1} (refreshed daily) before the start of each experiment.

Prior to the P exposure experiments, duckweed stocks were maintained for 10 d at 15 mg P L^{-1} refreshed daily, before (depending on exposure), sub-cultures were removed and maintained for four days on 15, 5, 2, 1 or 0 mg P L^{-1} (refreshed daily). To alter the concentration of P, volumes of KH_2PO_4 were reduced accordingly and the deficit of K was made up with the addition of KCl to maintain nutrient balances.

Experimental solution was topped up each day back to 100 mL with Hoagland's solution including all nutrients listed above except phosphate, to counter evapotranspiration and pH was fixed to 7 at the start of all experiments but not buffered.

2.4.1. Manipulating solution conductivity

Standard solutions made from serially diluted 10 M NaCl (formulated using a heated magnetic stirrer and dH_2O) were measured for conductivity using an HQ40d meter and conductivity probe (Hach Lange, UK). Table 2.1 Shows the corresponding values generated.

Table 2.1. Molarity of NaCl required for corresponding conductivity values.

NaCl (M)	Conductivity (µS cm^{-1})
10	1,000,000
1	100,000
0.1	10,000
0.01	1000
0.001	100
0.0001	10

The modified Hoagland's solution used in all the experiments of this thesis consistently recorded a conductivity of 900 µS cm^{-1} (±50). To obtain conductivities of 4,000 and 10,000 µS cm^{-1} in the culture pots, 0.4 mL and 1 mL of 10 M NaCl was added respectively before making up to 100 mL.

2.4.2 Waste water samples used in Phases 2 and 3.

Waste water samples used in Chapter 4 experiments were collected from the Final Effluent obtained from Yorkshire Water's Esholt WWTW in Bradford, UK. 25 L opaque drums were filled and returned to the University of Leeds weekly before being emptied into a 60 L header tank, analysed and then spiked with P (using KH_2PO_4) for experiments.

The waste water feeding a continuous-flow pilot system (Chapter 5) originated from a golf course complex containing brown and grey waters (waste waters from kitchens, toilets, showers and bar areas); the location of the golf club is confidential. Raw wastewater went through an HPAF sewage treatment system. This was comprised of primary and secondary sedimentation zones, along with forced aeration and nitrate dosing. A pumping system collecting final effluent was put in place to feed the pilot-scale unit tested on site (see Section 2.8 below).

2.5. P analysis in solution (Phase 1)

Pots were agitated with a sterile glass rod to mix the growth solution before a 1 mL sample was removed and syringe filtered (0.45 µm Ø pore size, Fisher Scientific, UK). This was diluted when required for the assay. The assay conducted follows the methods of Pierzynski (2000). In brief, potassium antimonyl tartrate together with ammonium molybdate will react with P_i-containing samples, in an acidic solution, to form an antimony-phospho-molybdate complex. This is reduced by ascorbic acid which turns the solution a shade of blue that is proportional to the concentration of Pi (inorganic phosphorus) present.

2.5.1. Reagent recipe

1. 2.5 M H_2SO_4- add 70 mL concentrated H_2SO_4 to 400 mL dH_2O, cool and make up to 500 mL.
2. Ammonium molybdate solution- dissolve 20 g $(NH_4)6Mo7O_{24}*4H_2O$ in 500 mL dH_2O. Store at 4°C.
3. 0.1 M ascorbic acid- dissolve 1.76 g ascorbic acid in 100 mL dH_2O. Stable for one week in opaque bottle at 4°C.
4. Potassium antimonyl tartrate solution- dissolve 1.3715 g $K(SbO)C_4H_4O_6*0.5\ H_2O$ in 400 mL dH_2O. Make up to 500 mL and store in an opaque bottle.
5. Combined reagent- bring all solutions to room temp and make in the following order to 100 mL while mixing. 50 ml of (**1**), 15 ml of (**2**), 30 ml of (**3**), 5 ml of (**4**) while mixing. Solution is stable for 8 h maximum in an opaque bottle.

2.5.2. Procedure

Add 2 mL of filtered sample to phosphate free test tubes and add 0.32 mL of above reagent. Mix and leave at room temperature for 15 minutes before reading at 880 nm against a reagent/dH_2O blank. Phosphate standard solutions are required to be tested prior to the testing of experimental samples.

2.5.3. Measuring solution characteristics in Phases 2 and 3

PO_4^{3-}, Total P, NO_3^-, NH_4^+, Oxidation Reduction Potential (ORP), Conductivity, pH and Dissolved Oxygen were measured in solutions and wastewaters used in the Phase 2 and 3 experiments using a combination of a portable meter (HQ40d), digestion block (LT200), disposable LCK cuvette tests and spectrophotometer (DR1900) all procured from Hach Lange LTD (Salford, UK).

2.5.4. Chlorophyll analysis

The determination of chlorophylls (*a*, *b* and *total*) was undertaken after the methods of Su *et al*., (2010). 200 mg (fresh mass) samples of duckweed were rinsed in dH_2O and patted dry on paper towels. Samples were immersed into 5 mL 90 % (v/v) reagent grade acetone/dH_2O in opaque Falcon tubes and left to elute in darkness for 24 h at 20°C. Following this 2 mL of the supernatant was read in a Jenway spectrophotometer at 645 nm and 663 nm against reagent blanks. The following equations were used to quantify the chlorophyll(s) present. From Su et al. (2010).

- Chlorophyll *a* ($\mu g\ g^{-1}$ FM) = (12.72 * OD_{663}) – (2.59 * OD_{645})
- Chlorophyll *b* ($\mu g\ g^{-1}$ FM) = (22.9 * OD_{645}) – (4.67 * OD_{663})
- Chlorophyll *total* ($\mu g\ g^{-1}$ FM) = (20.31 * OD_{645}) + (8.05 * OD_{645})

2.5.6. Mass Balances of P (Phase 1)

Overview

Total P was measured in solution, in living duckweed, in detritus and in suspension at time 0 and time t in triplicate destructively sampled pots. Total duckweed biomass (living on the surface) was treated separately from detritus (on the bottom) and suspended particulate matter and all fractions were completely recovered, dried and weighed and assayed.

Process (repeated at days 0 and 4) in this case

1. Total P was measured in solution in 2 mL filtered (0.45 µm Ø pore size) samples.
2. Living (floating) duckweeds were removed from the solution, dried at 70°C for two days, weighed, homogenized and measured for total P.
3. Detritus was carefully recovered from the pot using wide tweezers, dried at 70°C for two days, weighed, homogenized and measured for total P.
4. Remaining solution was poured through a Whatman filter paper (0.45 µm Ø pore size), dried at 70°C for two days, weighed, homogenized and measured for total P (carrying a dried filter paper through the process to tear off the weight).

The following calculations were applied to the data recorded from the above procedures.

- Duckweed P uptake = (P in duckweed * amount of duckweed at time t) – (P in duckweed * amount of duckweed at time 0)
- Change in detritus P = (P in detritus * amount of detritus at time t) – (P in detritus * amount of detritus at time 0)
- Change in suspended P = (P in suspension * amount of suspended matter at time t) – (P in suspension * amount of suspended matter at time 0)

2.5.7. ^{32}P uptake experiments (Phase 1)

P uptake at 30 minutes

Axenic cultures of *Lemna minor* (2.2.1) were maintained on modified Hoagland's solution containing 0.1 mM P for one week prior to experiments. Triplicate 10-20 mg fresh mass (FM) samples were inoculated into 5 mL of experimental perfusion solution containing 0.1 mM P as KH_2PO_4, 0.1 mM $MgSO_4*7H_2O$; 1.8 mM $Ca(NO_3)_2*4H_2O$ and 2.5 mM KNO_3, fixed to pH 5.7 with 1 mM HEPES buffer (Ullrich-Eberius et al., 1984), and acclimated for 30 minutes to the experimental temperature of 22°C with a light intensity of 160 µmol photons m^{-2} s^{-1}. Once acclimated, cultures were spiked with ^{32}P (as $H_3{}^{32}PO_4$, Perkin Elmer, UK, specific activity 285.6Ci mg^{-1}) to a working concentration of 1 µCi mL^{-1} and incubated for 20 minutes in solutions containing concentrations of phosphate from 0-400 µM P (0-12 mg P L^{-1}). Uptake was halted by rinsing the fronds 3x in dH_2O and once in 1 mM phosphate buffer (Hase et al., 2004) and immersing into Opti-Fluor scintillation fluid (Perkin Elmer, UK), before the plants were counted for radioactivity in a scintillation counter. Uptake data was corrected for decay (0.953) and counts per minute were converted to µmol P mg^{-1} (FM) h^{-1}.

P uptake at 5°C, 15°C and 25°C

The same procedure was used as above with the exception of retaining the concentration of P at 0.1 mM while repeating experiments at 5°C, 15°C and 15°C.

2.6. P analysis *in planta*

Total and inorganic phosphate in plant tissues were determined after the methods of Ames and Dubin (1960) and Chen et al. (1956), respectively as described below.

2.6.1. Extraction and homogenization

Plant samples were dried in an oven at 70°C for 48 h in a clean jar. After cooling, samples were fully homogenized with a spatula and added to 2 mL Eppendorfs. These were flash frozen in liquid N_2 and homogenized in a tissue lyser with a ball-bearing. 10 mg of the powdered material was added to new 1.5 mL Eppendorfs containing 1 mL of extraction buffer and homogenized again for three minutes.

2.6.2. Extraction buffer recipe- dissolve chemicals one at a time on a stirring plate on a gentle heat.

Chemical	Molecular Mass (g mol^{-1})	use per 500 mL
• 10 mM Tris-base	121.14	0.61 g
• 10 mM EDTA	372.24	0.19 g
• 100 mM NaCl	58.44	2.92 g
• 1 mM PMSF (dissolved in ethanol)	174.19	0.09 g
• 1 mM 2-mercaptoethanol	78.13	0.5 mL

2.6.3. Preparation

Mix 20 µL homogenized sample with 980 µL 1 % glacial acetic acid in new 1.5 mL Eppendorf tubes. Add 20 µL extraction buffer to 980 µL extraction buffer to run as a blank throughout the procedure. Incubate at 42°C for 30 minutes

2.6.4. Procedure for Total P

Add 100 µL to test tubes. Add 30 µL 10 % $Mg(NO_3)_2$ in 95% ethanol to each. Flame to ash twice over a Bunsen burner. When cooled add 300 µL of 0.5 M HCl and dissolve at 65°C for 30 minutes. Run final assay below.

2.6.5. Procedure for Inorganic P

Spin remaining original homogenized sample from the 'preparation' stage at 13,500 g for five minutes. Add 300 µL of this to test tubes. Run final assay below.

2.6.6. Final assay

Add 300 µL of sample to test tube followed by 700 µL of mixed reagent to each tube and incubate at 37°C for 1 h. Read absorbance at 820 nm against reagent blank. Phosphate standard solutions are required to be tested prior to the testing of experimental samples.

2.6.7. Mixed reagent recipe - 1 part a to 6 parts b (solution can be kept on ice for 8h maximum)

a) 10 % (w/v) ascorbic acid (ok in refrigerator for 1 month)
b) 0.42 % ammonium molybdate in 1 N H_2SO_4 (28.6 mL H_2SO_4, 4.2 g $(NH_4)6Mo7O_{24}•4H_2O$ made up to 1 L in dH_2O- stable at room temperature).

2.7. Ascertaining fresh and dry mass and surface area harvesting

Fresh mass was ascertained by gently blotting the duckweed on paper towels before weighing on an analytical balance. For Phases 2 and 3 the duckweed was rinsed and centrifuged in a salad spinner before being blotted dry and weighed.

Dry mass was obtained by drying the fresh duckweed samples in an oven at 70°C for two days in glass jars then weighing on an analytical balance.

Harvesting duckweed from Phases 2 and 3 was done by partitioning a measured surface area and removing all duckweed with a sieve, before processing for weighing as above. Example photograph of method used in pilot system (Phase 3) shown in Figure 2.8.4.

2.8. Hydraulic set ups for Phases 2 and 3

2.8.1. Phase 2

Figures 2.8.1 and 2.8.2 schematically present the set ups used in Chapter 4 experiments. The location was in the Plant Growth Suite of the Faculty of Biological Sciences, University of Leeds, UK. Three black plastic stationery boxes (Ryman, UK) were used as tanks, each with 20 L capacity (0.4 m x 0.3 m x 0.2 m (L, W, D respectively). A working volume of 10 L was used in each tank for every experiment. A steel frame (1.2 m x 0.8 m (L, W) was fixed 0.2 m above the tanks by a wooden structure and held eight white fluorescent tubes, providing a light intensity of 160 µmol m^{-2} s^{-1} at the water surface.

For experiments that used solutions containing three different concentrations of P, a multi-lined peristaltic pump (Model 205S, Watson Marlow, UK) was used to abstract solutions from separate opaque plastic 10 L drums and delivered to the duckweed tanks 1, 2 or 3 (T1, T2 or T3 respectively, Figure 2.8.1 A) with a hydraulic retention time of three days. For experiments that used only one concentration of P, the configuration was changed from three tanks in parallel to three tanks in series (Figure 2.8.1 B). One 60 L header tank contained the primary solution and a single lined peristaltic pump (Model 504S, Watson Marlow, UK) provided the solution to duckweed tank T1. The duckweed tanks were linked by opaque siphon tubes that caused the flow to move through each tank and on to a drain, with a combined hydraulic retention time of three days (1 d $tank^{-1}$). Siphon inlets and outlets were baffled from the duckweed biomass and used as sampling locations (A, B and C).

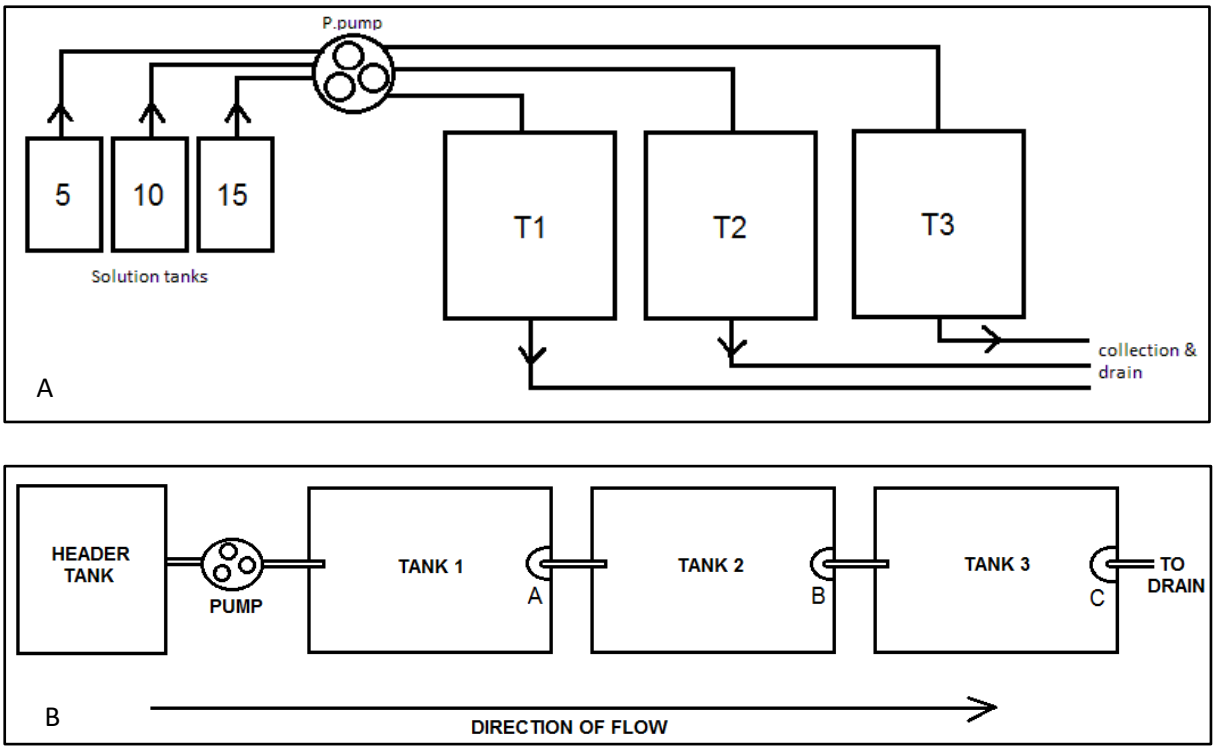

Figure 2.8.1. Hydraulic set ups for Phase 2. Schematics for ranging experiment (A) showing parallel setup and the series setup used in experiments 4.2 to 4.7 (B) in Phase 2. Dimensions given in Section 2.8.1.

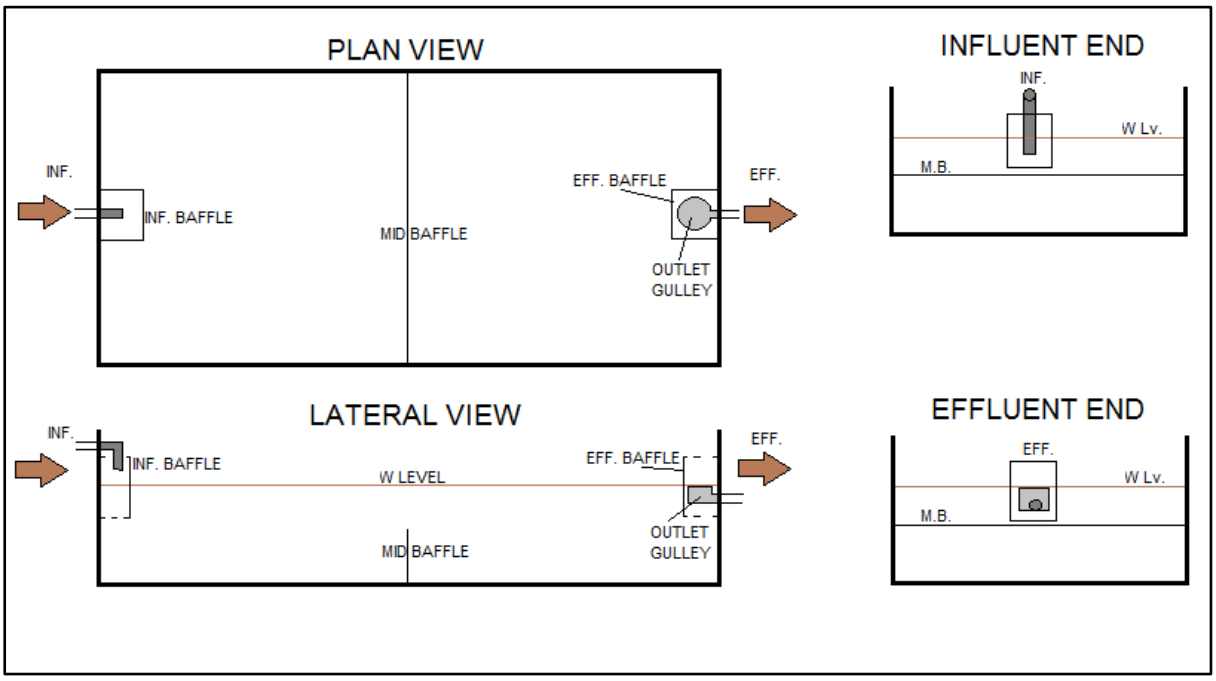

Figure 2.8.2. Section diagrams for tanks used in experiments in Phase 2.

2.8.2 Pilot system set up and build (Phase 3)

To scale up the experimental setup used in Phase 2, a pilot system was designed and built at a confidential location at a golf club in the UK (Phase 3). Hydraulic configurations were designed to be similar to Phase 2 experiments with the same Hydraulic Retention Time (HRT) and flow direction. A weather station (Vantage Pro 2^{TM}, Davis Instruments, Hayward CA, USA) was used to monitor solar radiation, precipitation, evapotranspiration, wind speed and direction, temperature and barometric pressure. Fine plastic pond netting was installed above to prevent interference from wildlife but allow sunlight through.

The system received secondary effluent from a HPAF package treatment plant (STP) that was supplied via a submersible (Clarke CSE400A, Machine Mart, UK) to a 900 L opaque buffer tank (BT). The contents of the buffer tank were pumped back to the HPAF by a submersible pump in order to keep the effluent as fresh and aerated as possible. A Delta ProMinent dosing pump (Germany) sited on top of the buffer tank supplied effluent to duckweed tank 1 whereby it passed through tanks 2 and 3 before returning by gravity to a discharge tank (DT) where a submersible pump (Clarke CSE400A, Machine Mart, UK) expelled effluent to the golf club's original discharge point. All effluent lines were made of opaque hose and either underground or well shaded.

Analysis of the system's performance was carried out by removing grab samples from points A, B, C and D, syringe filtering (0.45 µm Ø pore size, Fisher Scientific) and analysing immediately on site in a small laboratory using equipment described earlier (See Section 2.5.3). Plant samples were harvested, rinsed and dried in an oven also on site.

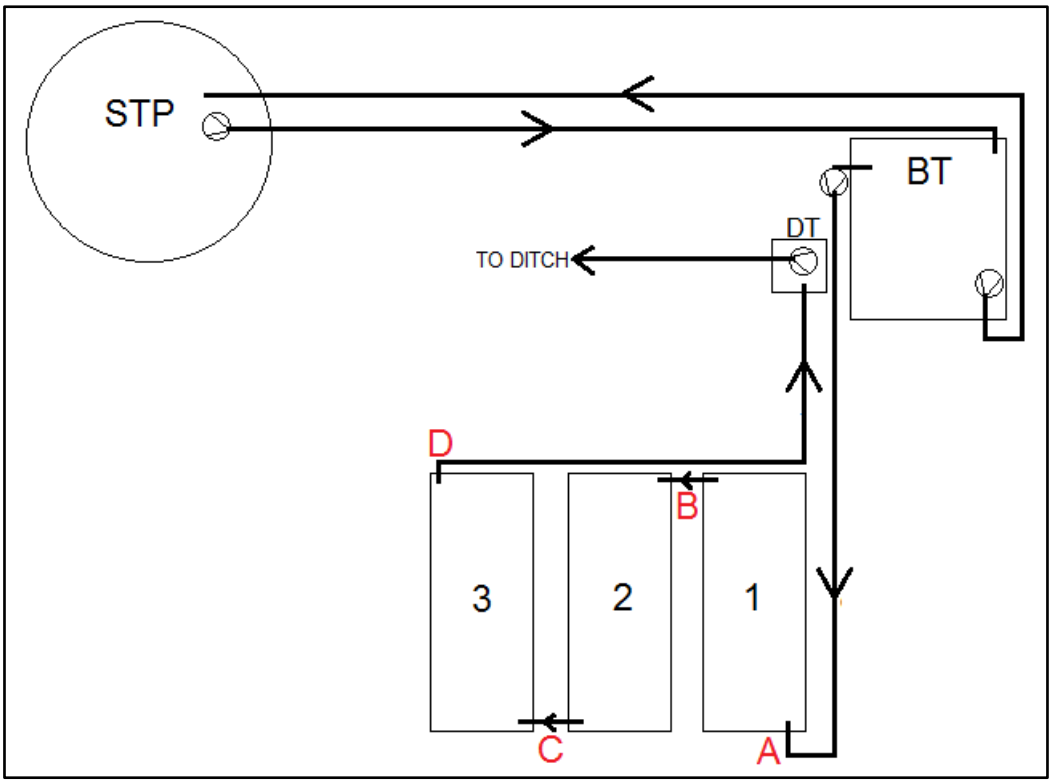

Figure 2.8.3. Hydraulic set up of pilot system. Schematic of hydraulic set up for pilot system trial (Phase 3).

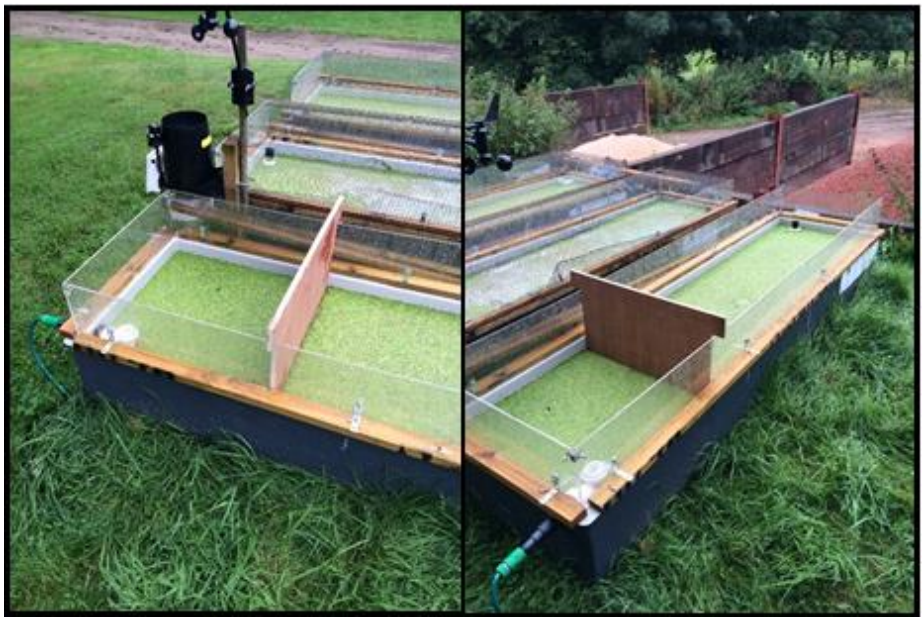

Figure 2.8.4. Pilot system harvesting method. From Phase 3, operational methods in 2.7 above.

2.9. External analyses

Ion Chromatography analysis of the elemental composition of growth solution, Esholt wastewater and pilot system wastewaters were kindly conducted by Dr Adrian Cunliffe in the School of Chemical and Process Engineering (SCaPE), University of Leeds, UK.

Data from Esholt WWTW (Bradford) on wastewater properties (reported in Phases 2 and 3), was kindly provided from the fieldwork of Miss Cigdem Oz and colleagues from the School of Civil Engineering, University of Leeds.

2.9.1. Data analysis

In Phase 1 experiments, phosphate removal coefficients (*K*) were calculated by transforming the mean daily phosphate in solution (n=3) to natural logarithm and plotting against time. Pearson's correlations, *t* tests and One Way ANOVA tests for significance were carried out using SPSS (v. 22, IBM, US). Phosphate uptake kinetics (K_m and V_{max}) were assumed to follow first order Michaelis-Menten terms (Eq. 1) and were calculated by transformation using the Lineweaver-Burk equation (Eq. 2). Values reported from Phase 2 experiments were from triplicate measurements taken during apparent steady state conditions of P removal. Values reported from the pilot experiment (Phase 3) were single grab samples only.

Equation 1. $V = V_{max} * [S] / K_m + [S]$ **Equation 2**. $1 / V = K_m / V_{max} * 1 / [S] + 1 / V_{max}$

Where:

V= Initial phosphate uptake rate

V_{max} = maximal P uptake rate

K_m = P concentration at which 50 % of V_{max} is achieved

[S] = substrate (P) concentration.

Ammonium dissociation calculations (Section 3.3.4, Chapter 3) were derived from equations in Emerson et al. (1975).

Q_{10} calculations (Section 3.2.6, Chapter 3) were derived from equations in Johnson and Thornley (1985).

CHAPTER 3

Identifying and investigating the key variables that influence P uptake and growth of *Lemna minor:* mesocosm experiments with batch cultures.

3.1. Introduction

Duckweeds are vascular plants and as such their production can be dependent on the same factors that influence all higher plants, such as exposure to light or nutrient acquisition. The 1st phase of this project was concerned with investigating how changes in the plants' environment (such as temperature, photoperiod or growth solution chemistry) would impact upon the capacity to grow and/or take up P (herein referred to as 'performance'). Ultimately, data would be required on the duckweeds' performance on real wastewater, but as a controlling factor these 1st phase experiments were conducted on autoclaved nutrient solution only, to omit the organic factor that would be present in wastewater which could significantly contribute to nutrient removal and interactions. The duckweed cultures used may have had minute amounts of microscopic epiphytic algae and bacteria associated with them, but experiments were controlled in a manner that prevented significant algal proliferation by maintaining a dense surface cover of duckweed. As such, no algae was noticed in the water column of the experimental pots or on the duckweed. The initial aim was to understand the contribution of duckweed to P removal under simulated temperate climate conditions

The complexity of the problem could suggest the viable testing of many variables, such as climatic influences, inter-species performance competition or varying several aspects of solution chemistry. However time and objectives forced the focus on those deemed the most likely factors controlling performance from reviewing the literature (Chapter 1). In terms of solution chemistry, the main parameters chosen to test were conductivity (as a proxy for salinity), the concentration of P, the concentration and form of N (ammonium or nitrate) and solution pH. Examining the last three of these variables thus provided a 3^3 matrix from which to randomly choose and test solutions with different combinations of P, N and pH, under fixed conditions of photoperiod and temperature (simultaneously conducting experiments to test nitrate and ammonium in isolation). Results of these ranging experiments are only briefly referred to here, as they were used a guide from which to focus on fewer variables to test and due to their number, were only tested singularly, not in triplicate. Tables of solution composition and results of the experiments are in Appendix I.

Duckweed has been used in tropical and subtropical countries for wastewater treatment for decades, due to the availability of space for large waste stabilisation ponds, warm temperatures, stable photoperiods and the often prohibitive expense of more complex wastewater treatment systems.

Publications on the performance of duckweed under cool-temperate climatic conditions are rare, and at the time of writing there were no peer reviewed articles of duckweed being used exclusively for P removal (or other aspects of wastewater treatment) in the UK. Almost half of the P that the UK imports for fertilisers is lost to watercourses via Wastewater Treatment Works (WWTW), and several duckweed species can be found in eutrophicated water bodies particularly during the calmer months – presumably thriving on these wasted nutrients. Therefore a significant research gap was able to be investigated, in how duckweed survive and take up P under a simulated cool-temperate climate, and if they could do this on wastewater. Incorporated within these research aims was the desire to find out which factors had the most impact – i.e., temperature or photoperiod for example; and if the duckweed could remove P down to discharge consent concentrations (<1 mg P L^{-1}), which would be on a par with the widely used (and often expensive) methods such as metal salt precipitation or Enhanced Biological Phosphorus Removal EBPR.

The ultimate objective of this project was to generate knowledge on how well a duckweed system operating outdoors in the UK could survive and remove P from wastewater year round. Data would be collected by way of research that examined how certain key variables may or may not impact on performance under highly controlled conditions (Phase 1), then increasing the scale and set up (Phase 2), before culminating in a large scale pilot system to be trialled outdoors in a 'real world' situation (Phase 3). The results commence here with the molecular identification of the species used during this project, which was necessary before all of the above experiments could begin.

3.2. Results

3.2.1 Sampling and identification of species

The duckweed isolate used for all experiments reported in this thesis was originally sampled by hand from a small lagoon in Alkborough, North Lincolnshire, UK (Grid reference: 53°41'18"N; 0°40'13"W), (Figure 3.2.1.1). Accurate species identification of duckweed can often be unreliable due to morphological differences owing to external conditions or life stage, therefore reliable genetic identification was required. A system treating waste water with duckweed could change in species composition over time due to environmental interactions beyond control. These changes could affect system performance (Oron et al., 1986, Cheng and Stomp, 2009) and therefore predictability, thus the need to be confident in the species reported on is important.

Extraction of genomic DNA was successful and primers for the following PCR reactions were designed that amplified a non-coding spacer region (*atpF-atpH*) on the *Lemna minor* chloroplast genome (Wang et al., 2010). The single band shown (Sample A, Figure 3.2.1.2) corresponds to the size range expected (579-622bp) and at this point the PCR products were purified and sent for sequencing. Sequence data

were returned and consensus sequences were generated using Clustal Omega software, before being compared against species through the BLAST programme on the NCBI database. Figure 3.2.1.3 shows the consensus sequence for Sample A (ALKB) compared with the top matching result from the BLAST comparisons (*Lemna minor* chloroplast genome). Following this are the top 10 possible matching species for this sample from the BLAST program (Figure 3.2.1.4). The top 10 BLAST results that were compared with Sample A show a 98 % identity between the submitted ALKB sequence and the top matches with the *atpF-atpH* region of the *Lemna minor* chloroplast genome and an e value of 0.0, therefore we were confident that the species obtained and used for all experiments in this thesis was *Lemna minor*.

Figure 3.2.1.1. Sampling location of duckweed isolate from Alkborough, UK. Latitude 53°41’18”N, longitude 0°40’13”W. (Courtesy of Google Earth).

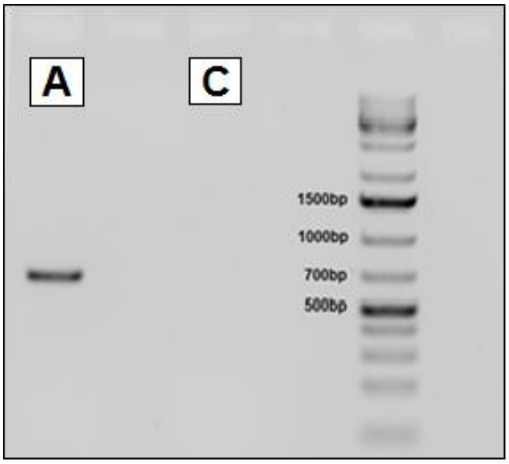

Figure 3.2.1.2. Result of PCR amplification of extracted duckweed DNA on agarose gel. Following successful extraction of total genomic DNA, PCR primers were designed to amplify a specific region on the *Lemna minor* chloroplast genome, the *atpF-atpH* non-coding spacer region. The size of this spacer is from 579-622bp so amplification was successful. Sample A = *Lemna minor* sampled from Alkborough, North Lincolnshire UK. Sample C = negative control containing no DNA.

After the return of sequence data, the consensus sequence was generated using Clustal Omega software, before being compared with other species with BLAST alignment. The comparison shown in Figure 3.2.1.3 is the closest match (98 %), and is from the *Lemna minor* chloroplast genome.

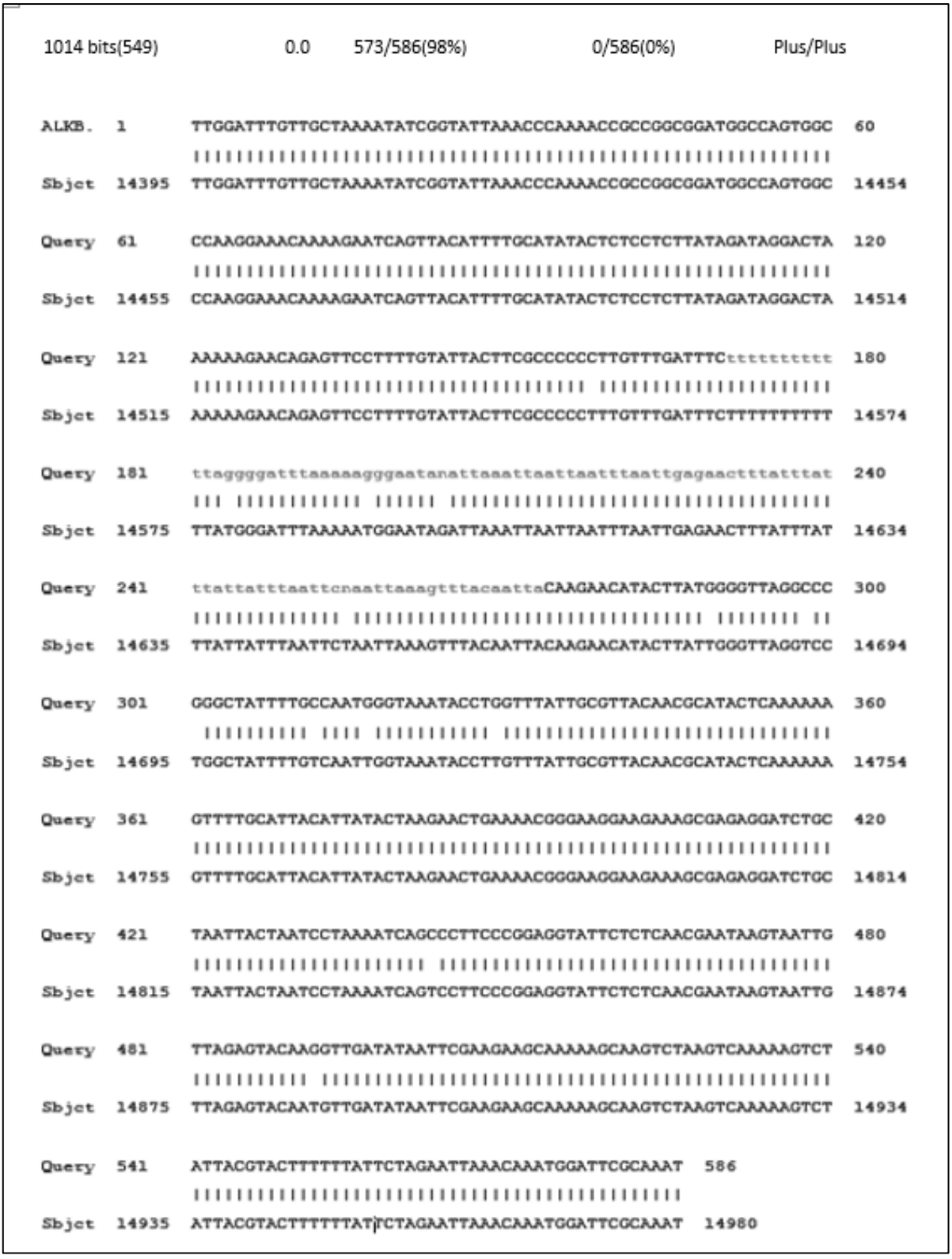

Figure 3.2.1.3. Alignment of Alkborough isolate and *Lemna minor* chloroplast genome.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Lemna minor chloroplast, complete genome	1044	1044	99%	0.0	98%	DQ400350.1
Lemna minor strain 6a ATPase subunit I (atpF) gene, partial cds; atpF-atpH intergenic spacer, complete sequence; and ATPase subunit III (atpH	1038	1038	99%	0.0	98%	KF726150.1
Lemna minor strain 9a ATPase subunit I (atpF) gene, partial cds; atpF-atpH intergenic spacer, complete sequence; and ATPase subunit III (atpH	1038	1038	99%	0.0	98%	KF726154.1
Lemna minor strain 9417 AtpH and ATP synthase subunit I (atpF) genes, partial cds; chloroplast	1031	1031	98%	0.0	98%	GU454231.1
Lemna minor strain 9253 AtpH-like gene, partial sequence; and ATP synthase subunit I (atpF) gene, partial cds; chloroplast	1029	1029	98%	0.0	98%	GU454230.1
Lemna minor strain 7210 AtpH-like gene, partial sequence; and ATP synthase subunit I (atpF) gene, partial cds; chloroplast	1029	1029	98%	0.0	98%	GU454228.1
Lemna minor strain 7136 AtpH-like gene, partial sequence; and ATP synthase subunit I (atpF) gene, partial cds; chloroplast	1029	1029	98%	0.0	98%	GU454227.1
Lemna minor strain 7018 AtpH-like gene, partial sequence; and ATP synthase subunit I (atpF) gene, partial cds; chloroplast	1029	1029	98%	0.0	98%	GU454226.1
Lemna minor voucher AP219 AtpF (atpF) gene, partial cds; and atpH-atpF intergenic spacer, complete sequence; chloroplast	1027	1027	98%	0.0	98%	HQ594751.1
Lemna minor strain 9016 AtpH and ATP synthase subunit I (atpF) genes, partial cds; chloroplast	1026	1026	98%	0.0	98%	GU454229.1

Figure 3.2.1.4 Top ten BLAST alignment matches for the Alkborough isolate consensus sequence. Total genomic DNA was extracted and the non-coding spacer region, *atpF-atpH* of the *Lemna minor* genome was amplified by PCR. Purified product was sequenced and the results produced the consensus sequence in Figure 3.1.1. On comparison, the Alkborough isolate is 98 % likely to be *Lemna minor*.

3.2.2. A mass balance of P throughout *Lemna minor* batch mesocosm systems.

To be confident that P removed from solution was in fact attributable to the duckweed and that significant proportions were not being adsorbed to the pots or used by microbial activity, (both of which could be directly influenced by low or high temperatures respectively), triplicate mass balance experiments were carried out on both axenic and non-axenic cultures at 5°C, 15°C and 25°C. Axenic cultures would be used for ^{32}P uptake experiments (within Sections 3.2.6 and 3.2.7) and non-axenic cultures used for all other experiments. Protocols for axenic culturing, measuring of P and mass balance calculations are in Chapter 2 (Sections 2.2.1, 2.5 & 2.6 and 2.5.6 respectively). In brief, total dissolved P (organic plus inorganic P) was measured in solution at the beginning and end of four day experiments along with total P in the duckweed on the surface, total P suspended in solution and in sub-surface detritus. The latter three fractions were individually calculated as a percentage of what had been removed from solution after four days, which also produced a final (small) percentage unaccounted for.

In axenic experiments the duckweed was responsible for 96 %, 94 % and 95 % of the Total P removed from solution at 5°C, 15°C and 25°C respectively (Figure 3.2.1.1). In triplicate non-axenic experiments, duckweed was responsible for over 90 % of the Total P that had been removed from solution at 5°C, 15°C and 25°C (Figure 3.2.1.2). When considering the margin for error at this experimental scale, the P removed from solution in non-axenic experiments can be said to be similar to that removed by axenic duckweed cultures, therefore P removal from solution in subsequent experiments could satisfactorily be considered as P 'uptake' by duckweed, in either axenic or non-axenic systems.

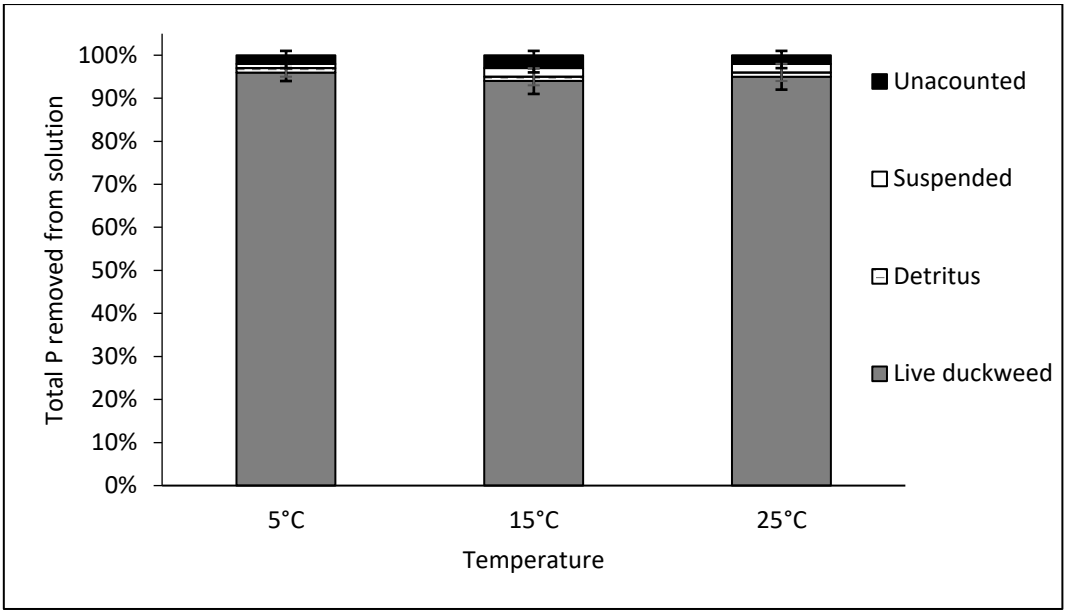

Figure 3.2.2.1. Mass balance of P in 3 axenic batch systems. Triplicate axenic *Lemna minor* cultures were maintained on modified Hoagland's solution for four days at either 5, 15 or 25°C. Initial P was 15 mg P L^{-1} and photoperiod was 12 h. Total P was measured at day 0 and day 4 in suspended matter, detritus and in living duckweed. Concentrations were multiplied by total amounts of each respective fraction. Error bars are standard error of the means.

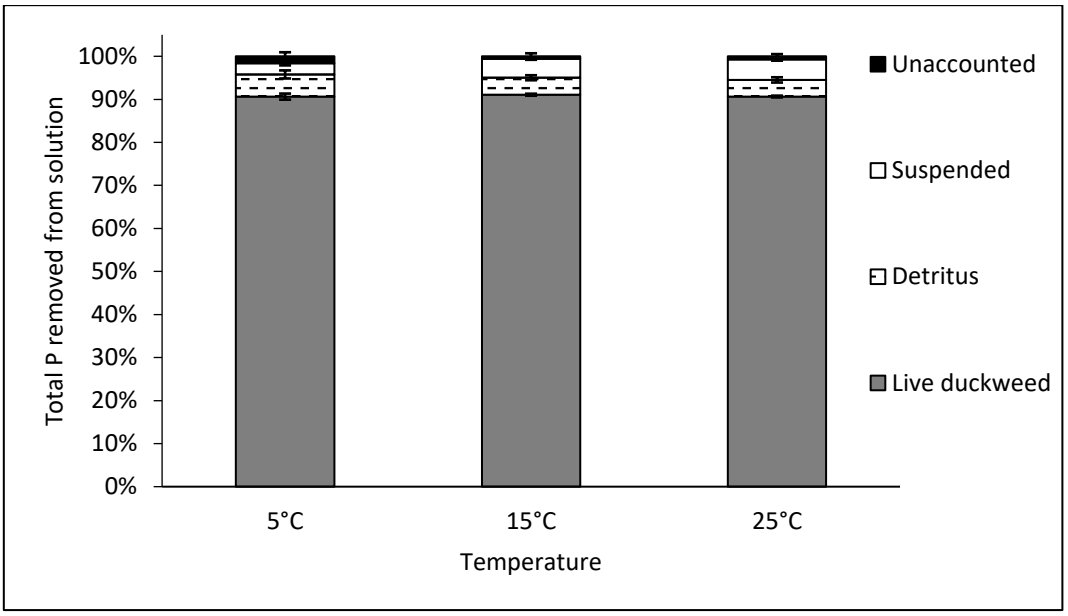

Figure 3.2.2.2 Mass balance of P in 3 non-axenic batch systems. Triplicate non-axenic *Lemna minor* cultures were maintained on modified Hoagland's solution for 4 days at either 5, 15 or 25°C. Initial P was 15 mg P L^{-1} and photoperiod was 12 h. Total P was measured at day 0 and day 4 in suspended matter, detritus and in living duckweed. Concentrations were multiplied by total amounts of each respective fraction. Error bars are standard error of the means.

3.2.3. The effect of conductivity on the performance of *Lemna minor*.

The conductivity of wastewater can vary due to climate and wastewater quality. Conductivity can be used as a proxy indicator of salinity and duckweed is a freshwater macrophyte often used for wastewater treatment in developing countries, yet there is very little in the literature on the salinity tolerance of duckweed. Growth solutions used for experiments in this thesis had a conductivity of 900 µS cm^{-1}, compared with 492 to 2,950 µS cm^{-1} in raw wastewater received at Esholt WWTW from 2014 to 2016 (data collected and provided by colleagues in the School of Civil Engineering, University of Leeds). Understanding how increases in conductivity may affect P uptake and growth by *Lemna minor* is therefore important.

Experiments were designed to test the effect of conductivity on the uptake of P and growth of *Lemna minor* over four days. Triplicate cultures maintained on modified Hoagland's solution were exposed to 900, 4,000 and 10,000 µS cm^{-1}, produced by spiking the latter two treatments with 10 M NaCl solution. Experiments were conducted under 12 h photoperiods and at 15°C, using an initial inoculation of 3 g fresh mass (FM).

At the values used in these experiments, increasing the conductivity reduced P removal (Figure 3.2.3.1). Over four days, plant cultures exposed to 10,000, 4,000 and 900 µS cm^{-1} removed 71 %, 88 % and >99 % of P from solution respectively that all initially contained 15 mg P L^{-1}. This removal correlated highly to conductivity. (R^2 = 0.99, Figure 3.2.3.2). Rates of P removal were the fastest for plants exposed to the lowest conductivity (Figure 3.2.3.3), reaching 580 mg P m^{-2} d^{-1} in 24 h before rates declined for the remainder of the experiment. Plants exposed to the higher conductivities both exhibited reduced P removal rates than those exposed to lower conductivities and removal rates decreased further after two days.

Changes in the amount of biomass produced also correlated highly to conductivity (R^2 = 0.96) and Figure 3.2.3.4 shows decreases of 44 % and 21 % and an increase of 25 % (relative to starting mass), in plants exposed to 10,000, 4,000 and 900 µS cm^{-1} respectively. Growth rates calculated as mg dry mass (DM) pot^{-1} d^{-1} correlated highly to conductivity (R^2 = 0.87, Figure 3.2.3.5). Under these conditions, the regression analysis model in Figure 3.3.5 suggests that in order to maintain positive growth (at least 1 mg DM pot^{-1} d^{-1}), conductivity should be maintained at a threshold of 3,200 µS cm^{-1} or lower, which is almost within the range of conductivity figures reported at Esholt. Relative growth rates [ln base e (mg DM_{t1}/mg DM_{t0})/t], (RGR) were also inversely proportional to conductivity, with plants exposed to 10,000, 4,000 and 900 µS cm^{-1} recording RGR's of -0.15, -0.06 and 0.06 respectively and rates correlating highly to conductivity (R^2 = 0.93).

Increasing conductivity also negatively affected the accumulation of P *in planta*, correlating strongly again (R^2 = 0.99). Plants exposed to 10,000, 4,000 or 900 µS cm^{-1} recorded a -11 %, +46 % and +134 %

change (relative to starting value) of internal Pi (Figure 3.2.3.6). Plant vigour (as assessed by surface coverage and appearance of chlorophylls was much reduced in plants as conductivity was increased (Figure 3.2.3.7). As conductivity was increased, frond number and health were reduced with some chlorosis beginning to occur in the 10,000 and 4,000 µS cm^{-1} treatments after four days.

The phenomenon of active P removal by apparently non-growing cultures exposed to 4,000 or 10,000 µS cm^{-1} is in part explained by a mass balance that measured P at day 0 and day 4 in living (floating) duckweed, detritus (duckweed at the bottom), suspended particulate matter and P dissolved in solution. Figure 3.3.8 describes the fate of P by day 4, showing that almost 100 % of P was accounted for and that plants exposed to the higher conductivities removed P but died and sank at some point between day 1 and day 4. Biomass was not measured in between these points.

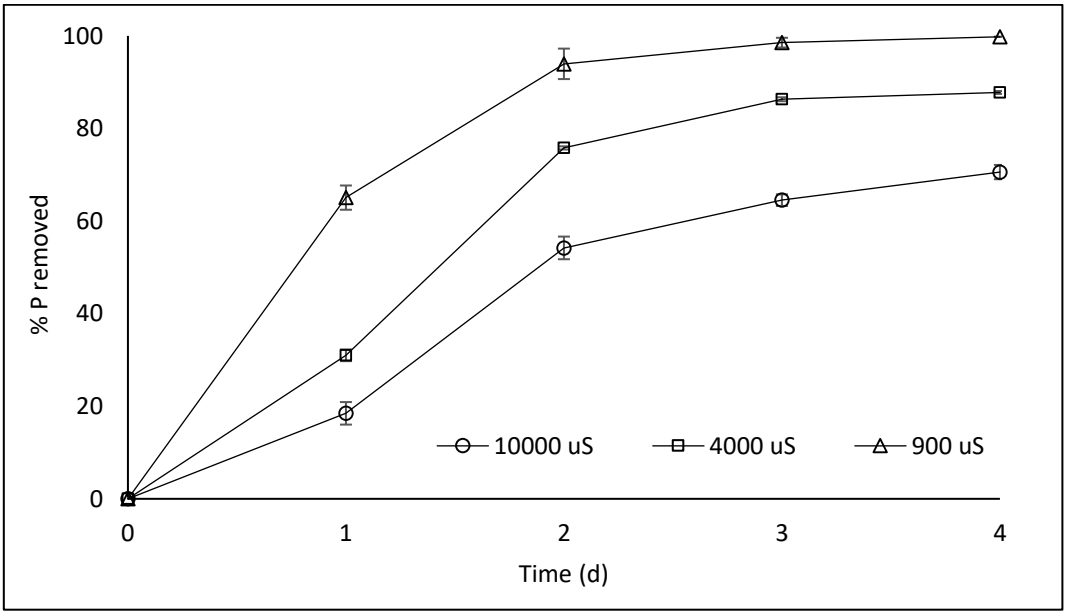

Figure 3.2.3.1. Effect of conductivity on P removal. Triplicate cultures of *Lemna minor* were maintained on modified Hoagland's solution spiked with 10 M NaCl to conductivities of 4,000 and 10,000 µS cm^{-1} for four days under 12 h photoperiods and 15°C. P was initially 15 mg P L^{-1} and un-spiked solution recorded 900 µS cm^{-1}. P in solution was measured daily and converted to % removed. Error bars are standard error of the means.

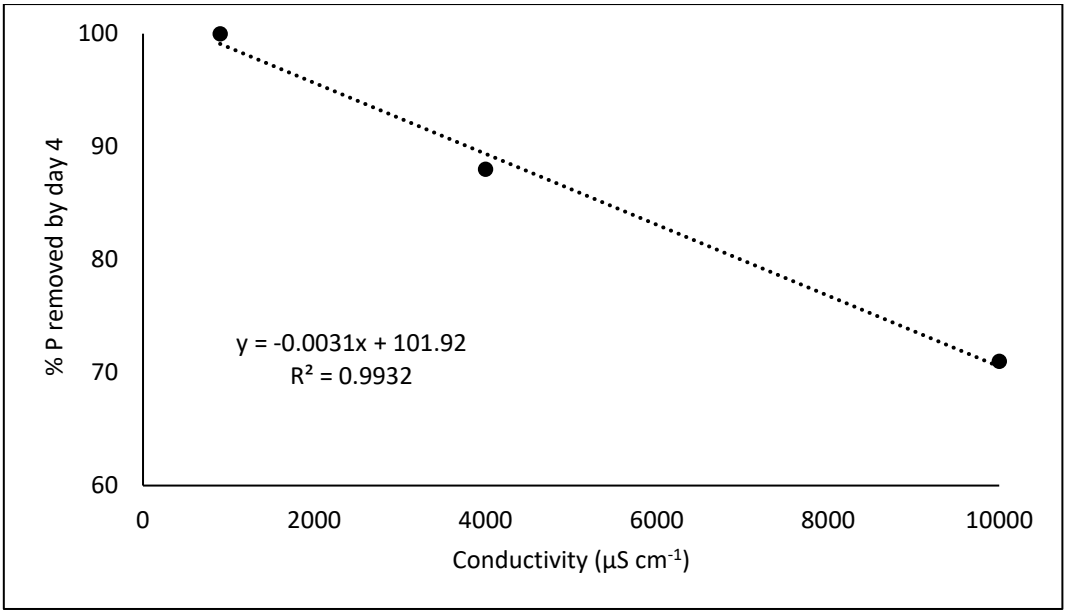

Figure 3.2.3.2. Regression analysis of % of P removed after 4 days versus conductivity. Triplicate cultures of *Lemna minor* were maintained on modified Hoagland's solution spiked with 10 M NaCl to conductivities of 4,000 and 10,000 µS cm^{-1} for four days under 12 h photoperiods and 15°C. P was initially 15 mg P L^{-1} and un-spiked solution recorded 900 µS cm^{-1}. The total % of P removed by day 4 was correlated with conductivity. Error bars are standard error of the means.

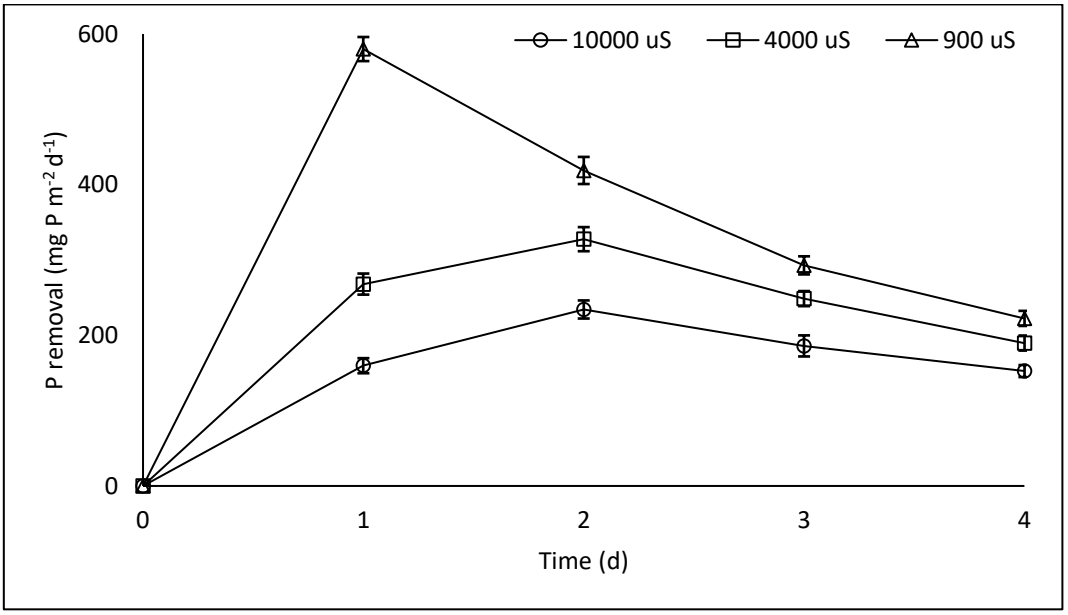

Figure 3.2.3.3. P removal rates of *Lemna minor* exposed to conductivities of 900, 4,000 or 10,000 µS cm^{-1}. Triplicate cultures of *Lemna minor* were maintained on modified Hoagland's solution spiked with 10 M NaCl to conductivities of 4,000 and 10,000 µS cm^{-1} for four days under 12 h photoperiods and 15°C. P was initially 15 mg P L^{-1} and un-spiked solution recorded 900 µS cm^{-1}. P removal rates were calculated as mg P m^{-2} d^{-1}. Error bars are standard error of the means.

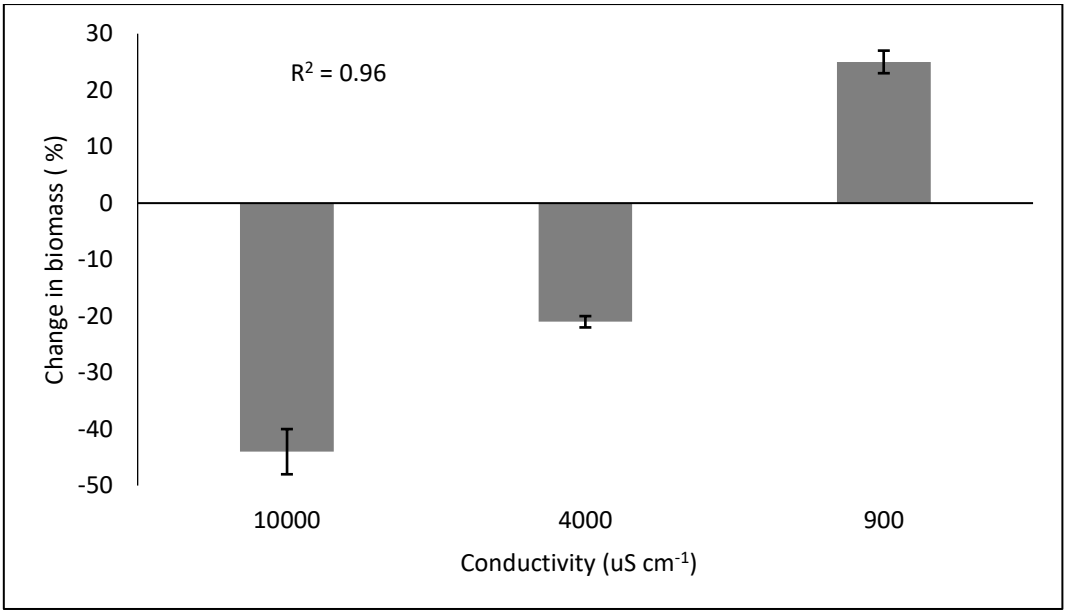

Figure 3.2.3.4. Effect of conductivity on growth of *Lemna minor*. Changes in the amount of remaining live biomass (using dry mass) after 4 days relative to starting dry mass. Triplicate cultures of *Lemna minor* were maintained on modified Hoagland's solution spiked with 10 M NaCl to conductivities of 4,000 and 10,000 µS cm^{-1} for four days under 12 h photoperiods and 15°C. P was initially 15 mg P L^{-1} and un-spiked solution recorded 900 µS cm^{-1}. Dry mass was recorded at day 0 and day 4 in destructively sampled cultures. Error bars are standard error of the means. R^2 value is the correlation of biomass increase (%) to conductivity (µS cm^{-1}).

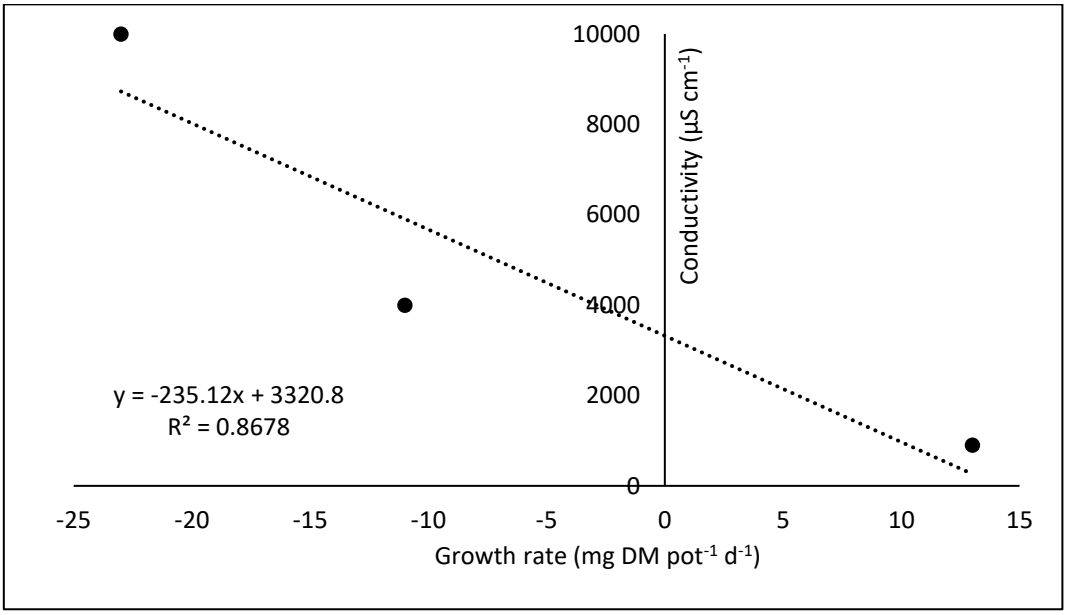

Figure 3.2.3.5. Regression analysis of growth rate versus conductivity. Triplicate cultures of *Lemna minor* were maintained on modified Hoagland's solution spiked with 10 M NaCl to conductivities of 4,000 and 10,000 µS cm^{-1} for four days under 12 h photoperiods and 15°C. P was initially 15 mg P L^{-1} and un-spiked solution recorded 900 µS cm^{-1}. Dry mass increases were recorded after 4 days and growth rates were calculated as mg DM pot^{-1} d^{-1}.

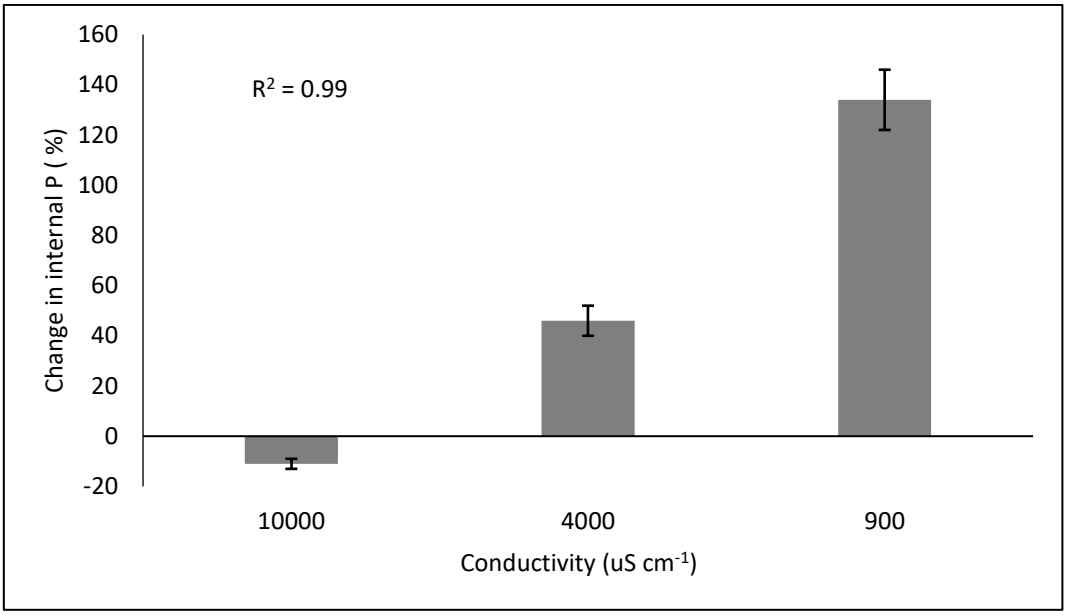

Figure 3.2.3.6. Effect of conductivity on P accumulation in *Lemna minor*. Changes in internal P (% of dry mass) relative to starting values after four days. Triplicate cultures of *Lemna minor* were maintained on modified Hoagland's solution spiked with 10 M NaCl to conductivities of 4,000 and 10,000 µS cm^{-1} for four days under 12 h photoperiods and 15°C. P was initially 15 mg P L^{-1} and un-spiked solution recorded 900 µS cm^{-1}. Inorganic P (Pi) was measured and recorded as a % of dry mass in daily destructively sampled cultures. Error bars are standard error of the means. R^2 value is the correlation of the change in internal Pi to conductivity (µS cm^{-1}).

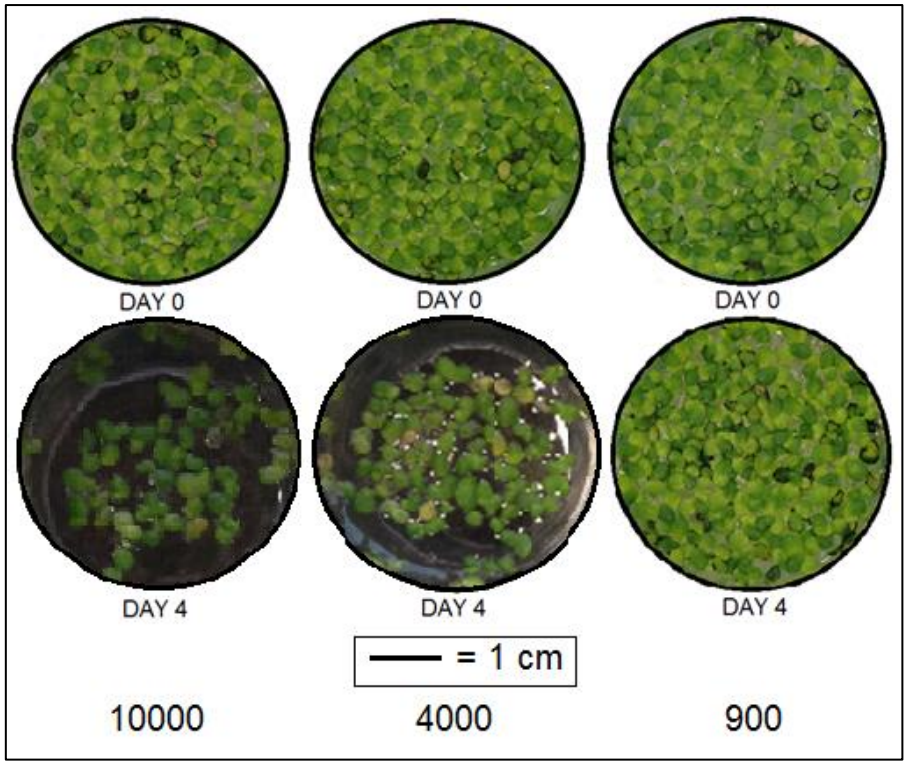

Figure 3.2.3.7. Effect of conductivity on plant vigour. Triplicate cultures of *Lemna minor* were maintained on modified Hoagland's solution spiked with 10 M NaCl to conductivities of 4,000 and 10,000 µS cm^{-1} for four days under 12 h photoperiods and 15°C. P was initially 15 mg P L^{-1} and un-spiked solution recorded 900 µS cm^{-1}. Comparison of plant appearance and density between one randomly chosen pot from each triplicate set on day 0 and day 4.

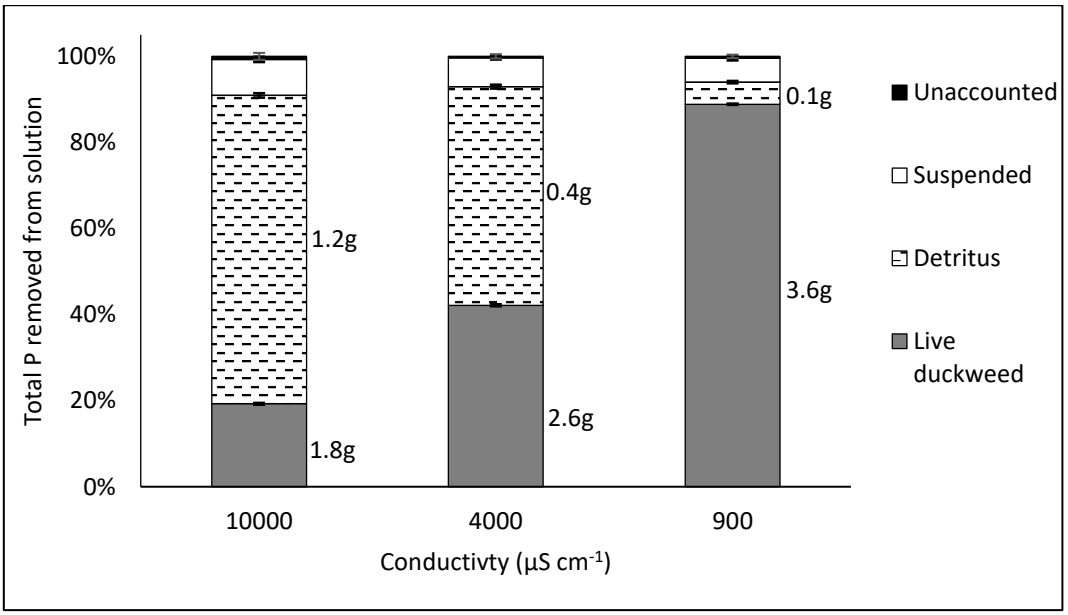

Figure 3.2.3.8. **The fate of P in cultures exposed to conductivities of 900, 4,000 and 10,000 µS cm^{-1}.** Numbers to the right hand centres of live duckweed and detritus fractions are total fresh mass (g, FM) recorded at the end of the experiment. Initial starting masses were 3 g (FM).

3.2.4. The effect of nitrogen species and pH on the performance of *Lemna minor*.

WWTW's in the UK can be nitrifying or non-nitrifying (Gardner et al., 2013) and small works in particular may not have the same buffering capacity as large works if they experience periodic changes in the form of N and its concentration. Systems can experience a failure of nitrification accompanied with spikes in ammonium that would have to be dealt with or passed on to the environment. Published literature is controversial on the N 'preference' of duckweed and also little has been done that separates out the direct effects of N species from pH, which (with ammonium) are known to be related. To test the effect of N species on the growth and uptake of P by *Lemna minor,* cultures were maintained in solutions supplied with only ammonium (set A) or nitrate (set B). Solutions 1A and 1B were initiated with 5 mg P L^{-1}, 10 mg N L^{-1}, at pH 4; and 2A and 2B were initiated with 15 mg P L^{-1}, 50 mg N L^{-1}, at pH 7. The four solution profiles used here were chosen as the best performing ones (in terms of growth and P uptake) from previous ranging experiments referred to in the introduction but not explicitly reported. The results from these experiments are described in Appendix I.

Removal of P was significantly better by duckweed supplied with nitrate as a sole N source over ammonium (P <0.01, Figure 3.2.4.1). After twenty days, solutions 1A and 2A recorded a -2 % (an actual increase of P in solution) and 6 % removal of P, compared with 73 % and 61 % removed by solutions 1B and 2B. For both cultures of set B, P removal increased steadily for 17 days whereas removal by cultures of set A alternated between positive and negative.

This could be explained by data in Figure 3.2.4.2, which shows the changes in biomass produced over the same experiment. On average, solution 1A produced a loss of biomass of 26 % relative to starting Mass. In contrast 1B recorded a significant (P<0.01) 163 % increase. Differences between 2A and 2B were also significantly different, with 2A showing a 35 % increase compared to a 250 % increase by 2B.

P *in planta* described the same trend as for P removal and growth, with decreases in internal Pi of 56 % and 19 % relative to starting values for plants in solutions 1A and 2A respectively, compared with significantly different (P<0.01) increases of 15 % and 117 % recorded in plants grown in solutions 1B and 2B respectively (Figure 3.2.4.3).

Photographs taken at day 0 and day 20 of randomly chosen single pots (from triplicates) show the differences in surface coverage and plant vigour between ammonium and nitrate only supplied cultures (Figure 3.2.4.4). Several chlorotic fronds are seen in both pots of set A by day 20. Surface area coverage was not dissimilar however, leading to the adoption of dry or fresh mass only for the quantification of biomass from this point onwards.

Solution pH changed over the course of the experiment in all 4 solutions (Figure 3.2.4.5). Solution 1A decreased from pH 4.0 to pH 3.2; solution 2A decreased from pH 7.0 to pH 3.3; solution 1B increased

from pH 4.0 to pH 7.6 and solution 2B increased from pH 7.0 to pH 7.7. These changes were presumed to be plant-mediated, as triplicate controls were simultaneously conducted containing no duckweed, which recorded no significant changes in pH at any of the times recorded (Figure 3.2.4.6).

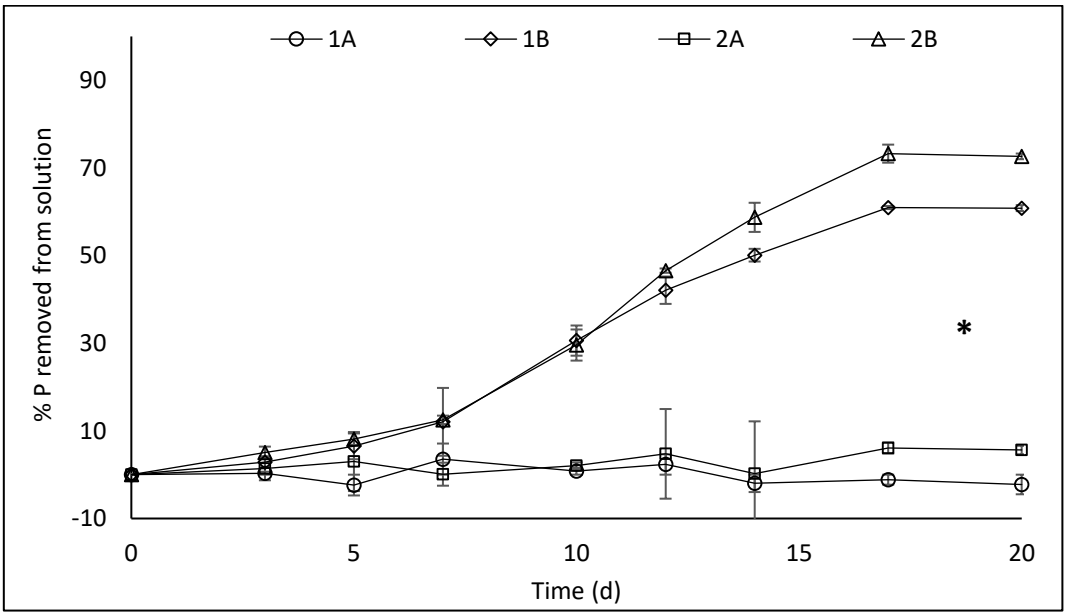

Figure 3.2.4.1. Effect of N species on P removal by *Lemna minor*. Triplicate *Lemna minor* cultures were maintained on modified Hoagland's solution with four different profiles, under 12 h photoperiods and 15°C. 1A and 1B contained 5 mg P L^{-1}, 15 mg N L^{-1}, pH 4 (unbuffered); 2A and 2B contained 15 mg P L^{-1}, 50 mg N L^{-1}, pH 7. Set A was provided with ammonium as the only N source and set B was provided with nitrate. P was measured daily in solution and converted to % removed. Error bars are standard error of the means. '*' denotes a significant difference between both solutions of set A and set B.

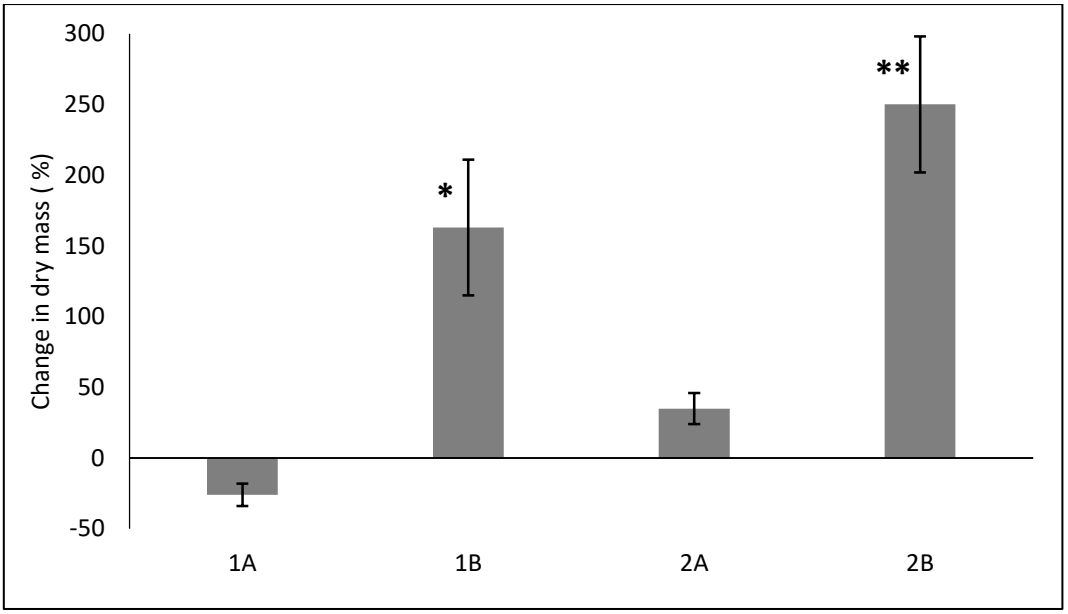

Figure 3.2.4.2. Effect of N species on the production of biomass by *Lemna minor*. Changes in the amount of remaining live biomass after 20 days (% relative to starting dry mass). 1A and 1B contained 5 mg P L^{-1}, 15 mg N L^{-1}, pH 4 (unbuffered); 2A and 2B contained 15 mg P L^{-1}, 50 mg N L^{-1}, pH 7. Set A was provided with ammonium as the only N source and set B was provided with nitrate. '*' indicates a significant difference between 1A and 1B (One way ANOVA, $P < 0.01$); and '**' indicates a significant difference between 2A and 2B (One way ANOVA, $P < 0.01$).

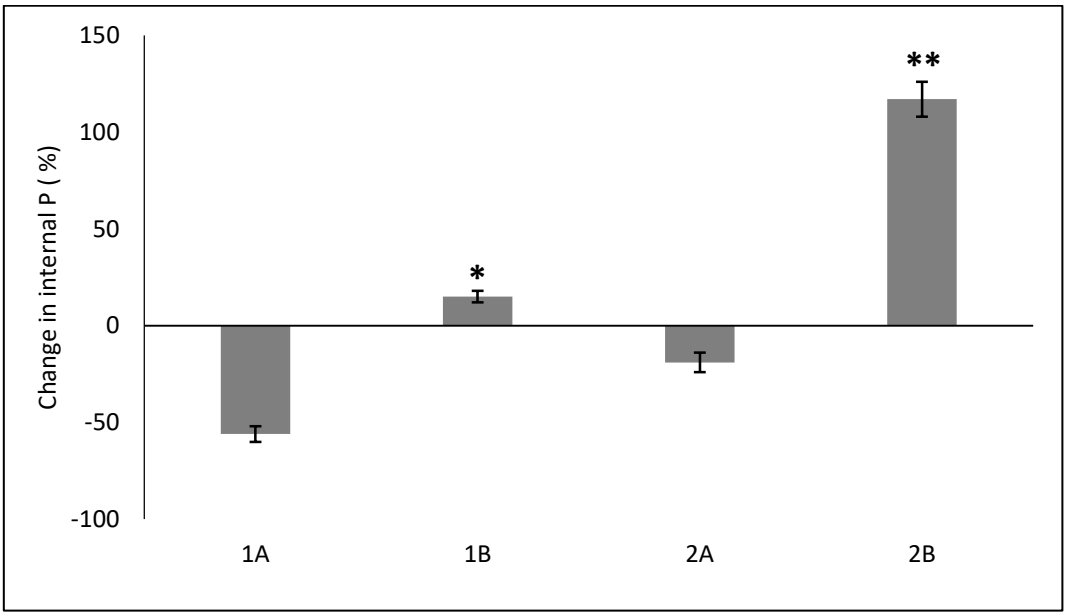

Figure 3.2.4.3. Effect of N species on the accumulation of P in *Lemna minor*. Changes in internal P (% of dry mass) relative to starting values after 20 days between cultures exposed to different growth solutions. Solutions 1A and 1B contained 5 mg P L^{-1}, 15 mg N L^{-1}, pH 4 (unbuffered); 2A and 2B contained 15 mg P L^{-1}, 50 mg N L^{-1}, pH 7. Set A was provided with ammonium as the only N source and set B was provided with nitrate. '*' indicates a significant difference between 1A and 1B (One way ANOVA, $P < 0.01$); and '**' indicates a significant difference between 2A and 2B (One way ANOVA, $P < 0.01$).

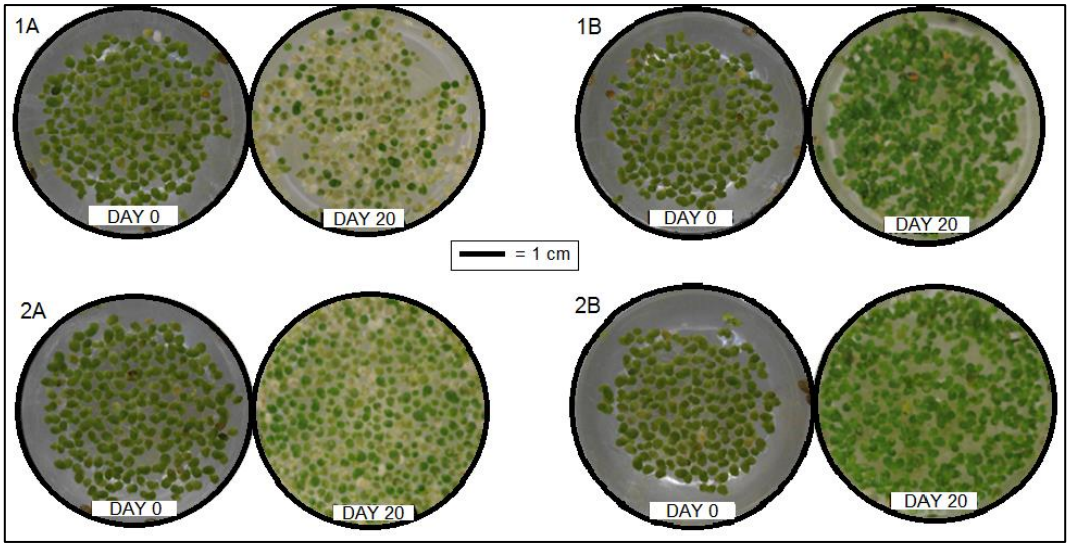

Figure 3.2.4.4. Effect of N species on plant vigour. Changes in appearance and amount of surface cover in triplicate *Lemna minor* cultures exposed to different growth solutions. Solutions 1A and 1B contained 5 mg P L^{-1}, 15 mg N L^{-1}, pH 4 (unbuffered); 2A and 2B contained 15 mg P L^{-1}, 50 mg N L^{-1}, pH 7. Set A was provided with ammonium as the only N source and set B was provided with nitrate.

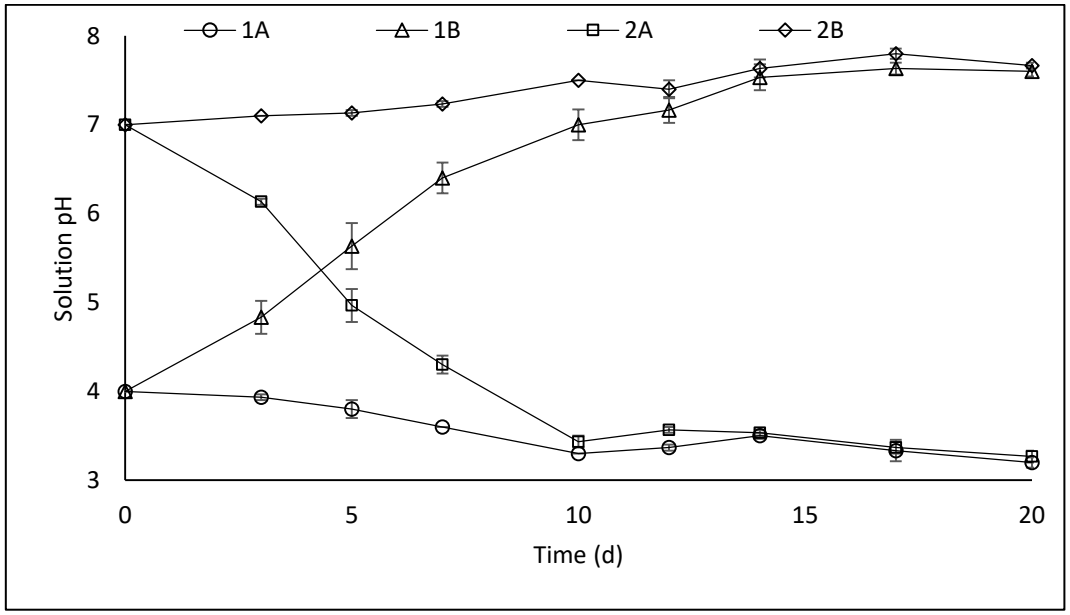

Figure 3.2.4.5. Plant-facilitated changes in solution pH. Changes in growth solution pH brought about by the duckweed cultures. Solutions 1A and 1B contained 5 mg P L^{-1}, 15 mg N L^{-1}, pH 4 (unbuffered); 2A and 2B contained 15 mg P L^{-1}, 50 mg N L^{-1}, pH 7. Set A was provided with ammonium as the only N source and set B was provided with nitrate.

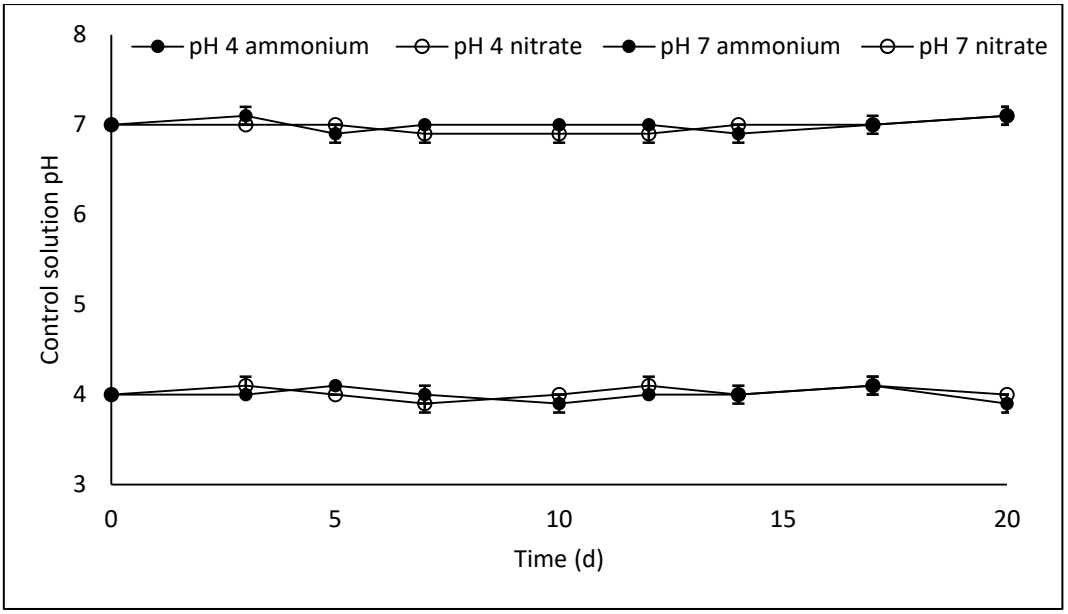

Figure 3.2.4.6. pH values of control solutions. Triplicate control solutions carried through the experiment that were of the same composition as those in Figure 3.2.4.5 but contained no duckweed. Error bars are standard error of the means.

Results generated so far in this section suggest that ammonium was detrimental to growth and P uptake by *Lemna minor* particularly at a low pH, but the sole effects of pH were not able to be considered. Therefore to isolate the effects of pH from N species/concentration and P concentration, triplicate 10 day experiments were conducted with N fixed to 50 mg NO_3^- - N L^{-1} and P initially provided at 15 mg P L^{-1} while testing solutions initially fixed (but not buffered) to pH 4, pH 7 and pH 10.

Altering initial solution pH from 7 to either 4 or 10 had negative effects on P removal and growth. Plants initiated at pH 4 or pH 10 could only manage 38 % and 40 % P removal respectively, in contrast to 60 % by pH 7 plants (Figure 3.2.4.7). Removal rates do appear similar between all three treatments over the first two days before rates of the pH 4 and pH 10 plants level off.

Similar to P removal, growth was negative for pH 4 and pH 10 cultures, recording decreases (relative to starting mass) of -36 % and -42 % respectively. In contrast the pH 7 cultures recorded a 22 % increase (Figure 3.2.4.8).

The negative trends recorded by the high and low pH treatments continued in terms of P accumulation. Cultures of pH 4 and pH 10 showed decreases (relative to starting values) of -17 % and -2 % respectively, while pH 7 cultures increased by 49 % (Figure 3.2.4.9). Duckweed cultures were able to modify their solution pH values from either low or high to more neutral values periodically, before this phenomenon appeared to end and solution pH returned to initial values (Figure 3.2.4.10).

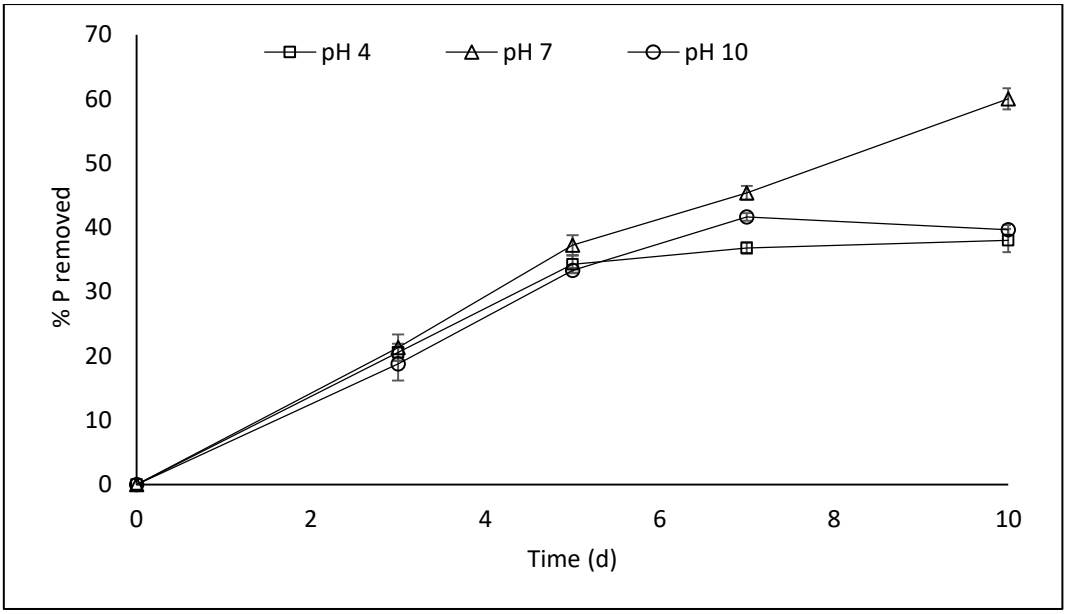

Figure 3.2.4.7. Direct effect of pH on P removal by *Lemna minor*. Triplicate cultures were maintained on modified Hoagland's solution for four days initially containing 15 mg P L^{-1}, 50 mg N L^{-1} as nitrate and initially fixed (but not buffered) to either pH 4, pH 7 or pH 10 with NaOH. P in solution was measured daily and converted to % removed. Error bars are standard error of the means.

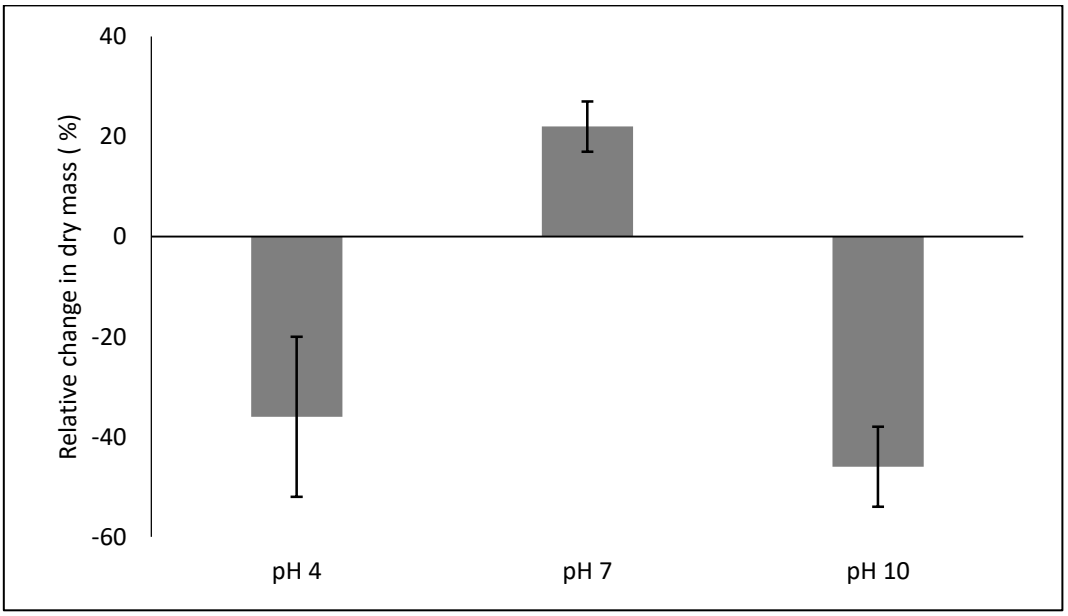

Figure 3.2.4.8. Direct effect of pH on the growth of *Lemna minor*. Triplicate cultures were maintained on modified Hoagland's solution for four days initially containing 15 mg P L^{-1}, 50 mg N L^{-1} as nitrate and initially fixed (but not buffered) to either pH 4, pH 7 or pH 10 with NaOH. Dry mass was measured daily in destructive samples and is reported as % changes relative to starting Mass. Error bars are standard error of the means.

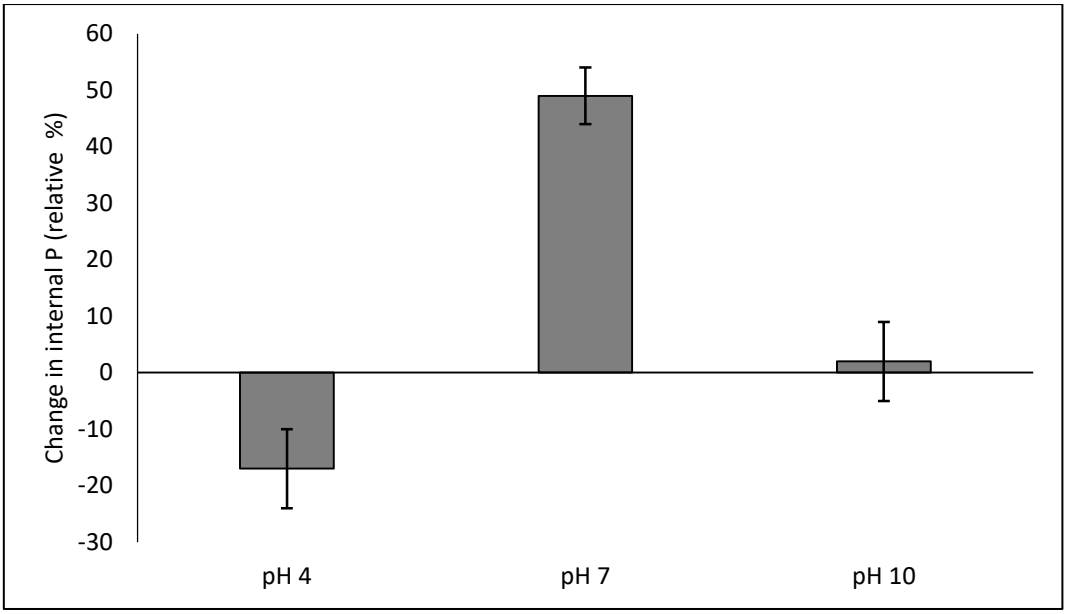

Figure 3.2.4.9. Direct effect of pH on P accumulation in *Lemna minor*. Triplicate cultures were maintained on modified Hoagland's solution for four days initially containing 15 mg P L^{-1}, 50 mg N L^{-1} as nitrate and initially fixed (but not buffered) to either pH 4, pH 7 or pH 10 with NaOH. Inorganic P (Pi) was measured daily in destructive samples and is reported as % change relative to starting values. Error bars are standard error of the means.

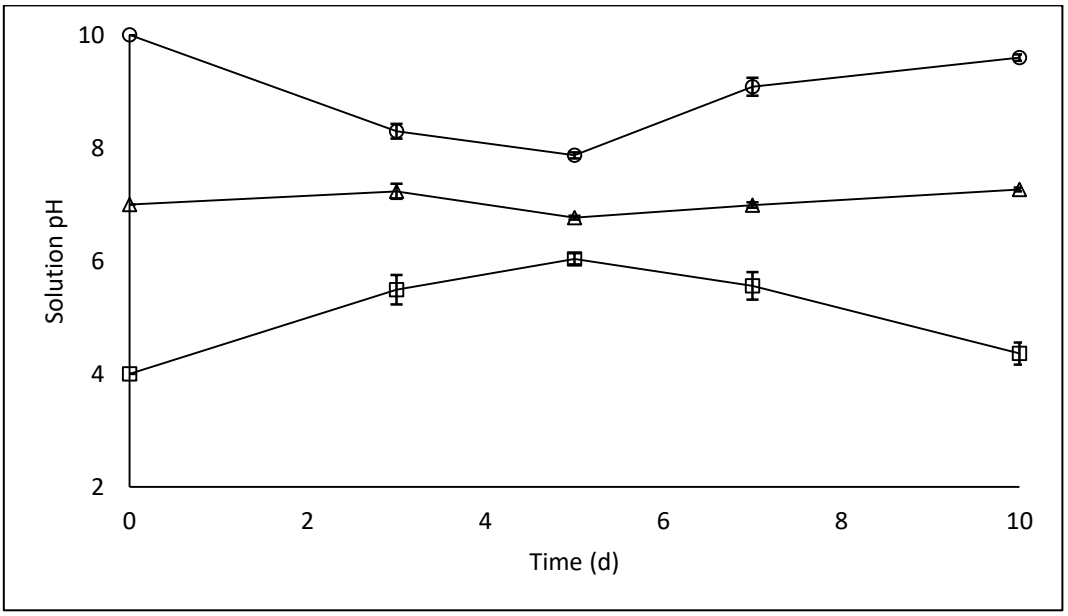

Figure 3.2.4.10. Changes in growth solution pH caused by *Lemna minor*. Triplicate cultures were maintained on modified Hoagland's solution for four days initially containing 15 mg P L^{-1}, 50 mg N L^{-1} as nitrate and initially fixed (but not buffered) to either pH 4, pH 7 or pH 10 with NaOH. Solution pH was recorded daily. Error bars are standard error of the means.

3.2.5. The effect of photoperiod on the performance of *Lemna minor.*

Photoperiod and temperature will affect plant growth and development but the direct effects of each of these variables in isolation have rarely been tested. Plants have evolved various mechanisms to deal with their respective environments, such as C_3, C_4 or CAM pathways of photosynthesis and duckweed is a C_3 plant (Esquível et al., 1998). Duckweed systems have been used for wastewater remediation in the tropics and sub tropics for >30 years where year round photoperiods are close to 12 h and temperatures are warmer and more stable than in northern latitudes. In contrast climate can change significantly from the summer to winter months in temperate countries. British summer and winter 24 h averages of photoperiod and temperature for 2013 were 12 h, 15°C and 6 h, 8°C respectively (MET Office, 2013). There is little published work on the performance of duckweed removing phosphate from wastewater under cool temperate conditions and nothing on their use in the UK. When considering the design of an engineered duckweed system that would operate outdoors in the UK, climatic interactions are important. Added to this the direct effects of photoperiod and temperature should be investigated independently of one another, because in theory, temperature or photoperiod could be modified by way of thermal inputs or LED lighting for example.

To examine the direct effect of photoperiod on phosphate uptake and growth, photoperiod was tested at 0 h (darkness), 6 h, 12 h and 24 h under a fixed (constant) temperature of 15°C. Light intensity was 160 µmol m^{-2} s^{-1}. Experiments were initiated with 15 mg P L^{-1} and conducted for four days, with P removal, internal Pi and biomass production being measured daily in triplicate 100mL batch pots.

From an initial concentration of 15 mg P L^{-1}, plants kept in darkness removed 32 % of P in two days before removal ceased (Figure 3.2.5.1). Plants under constant illumination removed 67 % of P over 2 days then also ceased uptake. The plants exposed to 6 h and 12 h photoperiods continued to remove P for four days, removing up to 80 % and 79 % respectively and after 2 days removal appears to slow down. There was no difference in P removal at day 4 between the plants grown with 6 h and 12 h photoperiods but at this same time point there were differences in the amounts of biomass produced (Figure 3.2.5.2).

There was a clear influence of photoperiod on growth, with a reduction in remaining live biomass in the plants kept in darkness (0h), no change in the plants maintained in 24 h light and increases of 15 % and 25 % (relative to starting mass) by the plants grown in 6 h and 12 h photoperiods respectively (Figure 3.2.5.2). All final values of biomass were significantly different from one another (One way ANOVA, P = <0.01 in each case).

Internal inorganic phosphate (Pi) increased overall in all plants over four days (Figure 3.2.5.3). Both the plants kept in darkness and constant illumination increased their Pi to 0.8 % and 1.1 % of dry mass

respectively from day 0 to day 2, before these values plateaued and decreased to 0.7 % and 0.9 %. Plants exposed to 6 h and 12 h photoperiods increased their Pi to 1.1 % and 1.2 % respectively by day 3 where these values remained.

P removal occurred independently of light for two days and P removal was the same for plants producing different amounts of biomass, therefore these results suggest that growth and P removal may not be exclusively linked. In addition, under these conditions P removal for the plants exposed to 6 h and 12 h photoperiods was limited to 80 % over four days (reaching 3 mg P L^{-1}). To examine if temperature was a controlling factor of this removal limitation, in Section 3.2.6 temperature was varied and tested against a fixed photoperiod of 6 h.

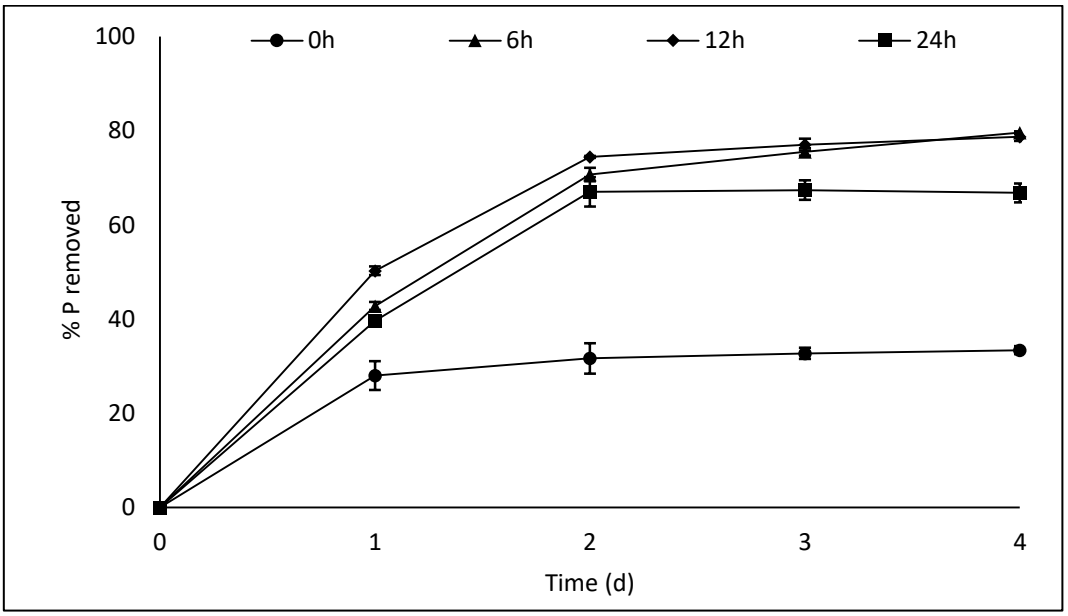

Figure 3.2.5.1. Effect of photoperiod on P removal by *Lemna minor*. Triplicate cultures grown on modified Hoagland's solution initially containing 15 mg P L^{-1} were exposed to 0 h, 6 h, 12 h or 24 h photoperiods at a constant temperature of 15°C for four days. P in solution was measured daily and converted to % removed. Error bars are standard error of the means.

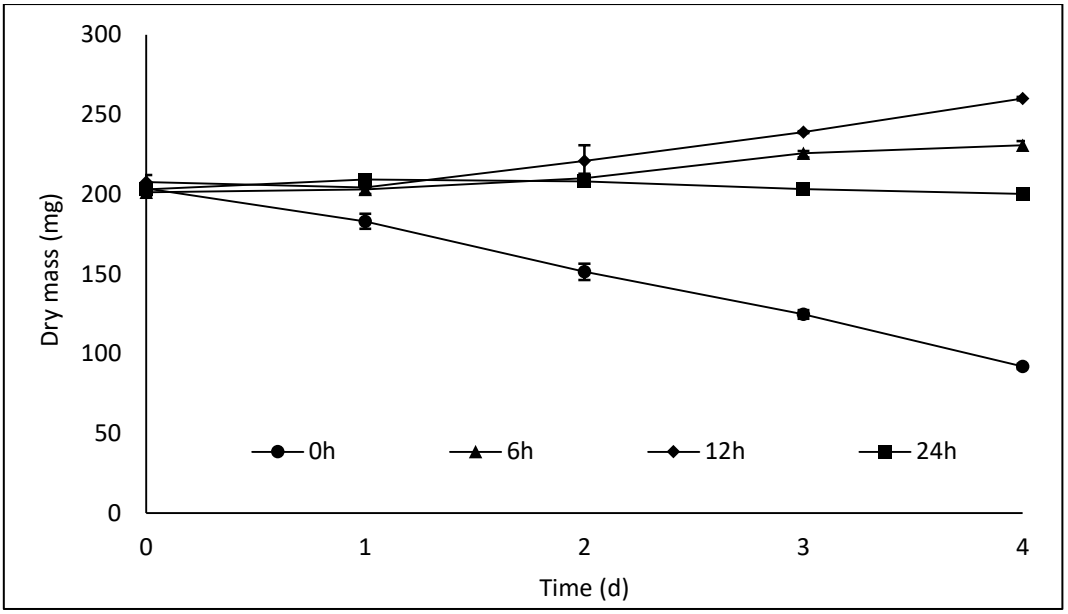

Figure 3.2.5.2. Effect of photoperiod on the growth of *Lemna minor*. Triplicate cultures grown on modified Hoagland's solution initially containing 15 mg P L^{-1} were exposed to 0 h, 6 h, 12 h or 24 h photoperiods at a constant temperature of 15°C for four days. Total dry mass was measured daily in destructive samples. Error bars are standard error of the means.

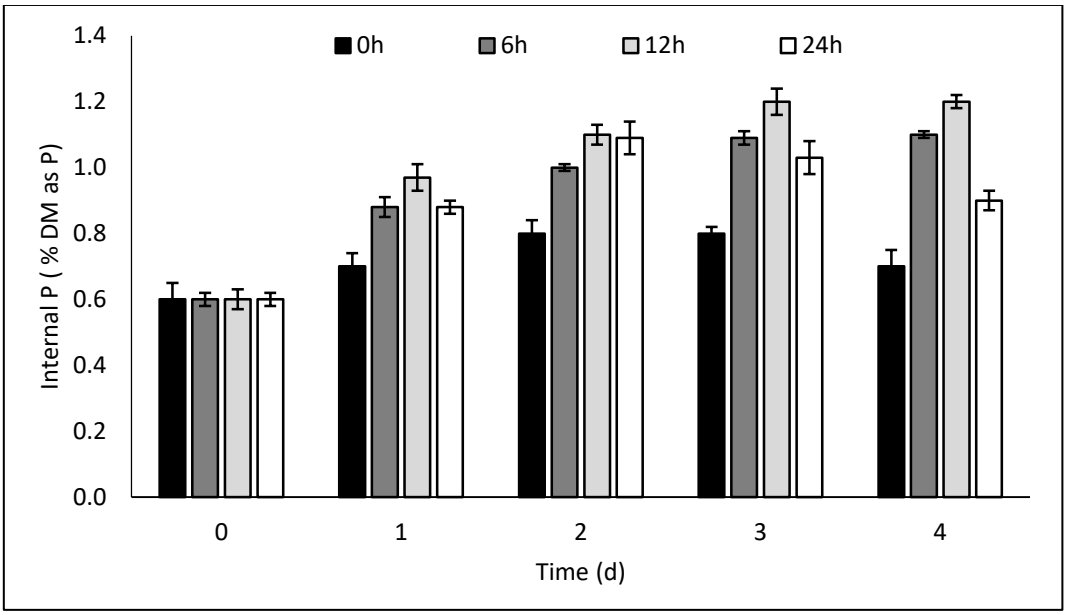

Figure 3.2.5.3. Effect of photoperiod on P accumulation in *Lemna minor*. Triplicate cultures grown on modified Hoagland's solution initially containing 15 mg P L^{-1} were exposed to 0 h, 6 h, 12 h or 24 h photoperiods at a constant temperature of 15°C for four days. Inorganic P (Pi) was measured daily in destructive samples and reported as a % of total dry mass. Error bars are standard error of the means.

3.2.6. The effect of temperature on the performance of *Lemna minor*.

To continue examining the effect of climate on P uptake and growth, the next step was to investigate temperature, independently of photoperiod. The basic experimental set up was the same as previously, but now temperature was tested at 8°C, 15°C and 25°C while keeping photoperiod fixed to 6 h. In addition to these experiments direct uptake of radiolabelled phosphate was also measured at 5°C, 15°C and 25°C. These latter experiments were conducted in real time with axenic duckweed to completely rule out the relatively small contribution of microbial uptake or sedimentation/precipitation and to quantify specific transport, as opposed to uptake after several days that could involve many steps through the cell. Data would also provide kinetic information on linear phase uptake.

P removal by *Lemna minor* was clearly temperature dependent (Figure 3.2.6.1). After four days, plants exposed to 8°C, 15°C and 25°C removed 61 %, 81 % and 97 % of the P in solution respectively from an initial concentration of 15 mg P L^{-1}. Figure 3.2.6.1 shows that P removal was more rapid over the first two days than for the second two days by the 15°C and 25°C plants. P removal coefficients correlated highly to temperature (R^2 = 0.97) (Table 3.2.6.1).

Rates of P removal increased with temperature but decreased over time proportionally between temperatures (Figure 3.2.6.2). Q_{10} values for overall P removal rates were similar between plants grown at 15°C and 25°C (Table 3.2.6.2), suggesting a limitation to P removal in the 15°C and 25°C experiments.

There was no significant difference in biomass produced between the plants grown at 15°C and 25°C by day 4 (*P* = >0.05), (Figure 3.2.6.3) and dry mass correlated positively to temperature (R^2 = 0.72), but there was a significant difference between these plants in terms of P removed from solution at the same time point (*P* = <0.01). The plants grown at 8°C increased their original dry mass by only 3 % while removing 61 % of P from solution.

Pi increased in the plants maintained at 8°C from 0.6 % to 1 % by day 2 where this value remained (Figure 3.6.4). The plants maintained at 15°C and 25°C increased internal Pi from 0.6 % to 1.1 % and 1.2 % respectively by day 3. Q_{10} values were similar for P accumulation between plants grown under 15°C and 25°Cat day 4 (Table 3.2.6.3).

Transport of ^{32}P by axenic duckweed was clearly temperature dependent (Figure 3.2.6.5) and correlated highly (R^2 = 0.91, Figure 3.2.6.6). Q_{10} values for ^{32}P uptake were more than double those for 'cold' P uptake (5°C) but were similar again between plants maintained at 15°C and 25°C (Table

3.2.6.4). Transport rates were double that of *in planta* accumulation at 15°C and more than 3x higher at 25°C (Table 3.2.6.5).

This data again suggest that growth and P removal may not be directly linked temporally and that something other than temperature was limiting P removal to 80 % for the plants tested at 15°C.

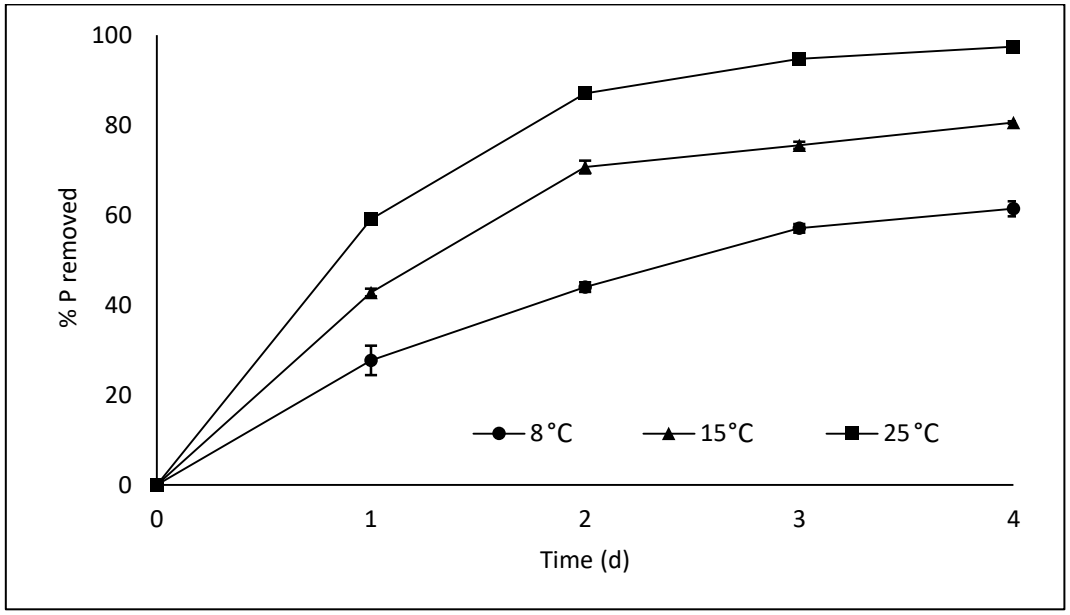

Figure 3.2.6.1. Effect of temperature on P removal by *Lemna minor*. Triplicate cultures grown on modified Hoagland's solution initially containing 15 mg P L^{-1} were exposed to 8°C, 15°C or 25°C under a 6 h photoperiod for four days. P in solution was measured daily and converted to % removed. Error bars are standard error of the means.

Table 3.2.6.1. Relationship between temperature and P removal by *Lemna minor.* Triplicate cultures grown on modified Hoagland's solution initially containing 15 mg P L^{-1} were exposed to 8°C, 15°C or 25°C, under a 6 h photoperiod. P was measured in solution daily and removal coefficients (*K*) were calculated by transforming the mean daily P in solution to natural logarithm (base e) and plotting against time.

Temperature (°C)	*K*	R^2 value
8	-0.24	0.98
15	-0.41	0.94
25	-0.94	0.99

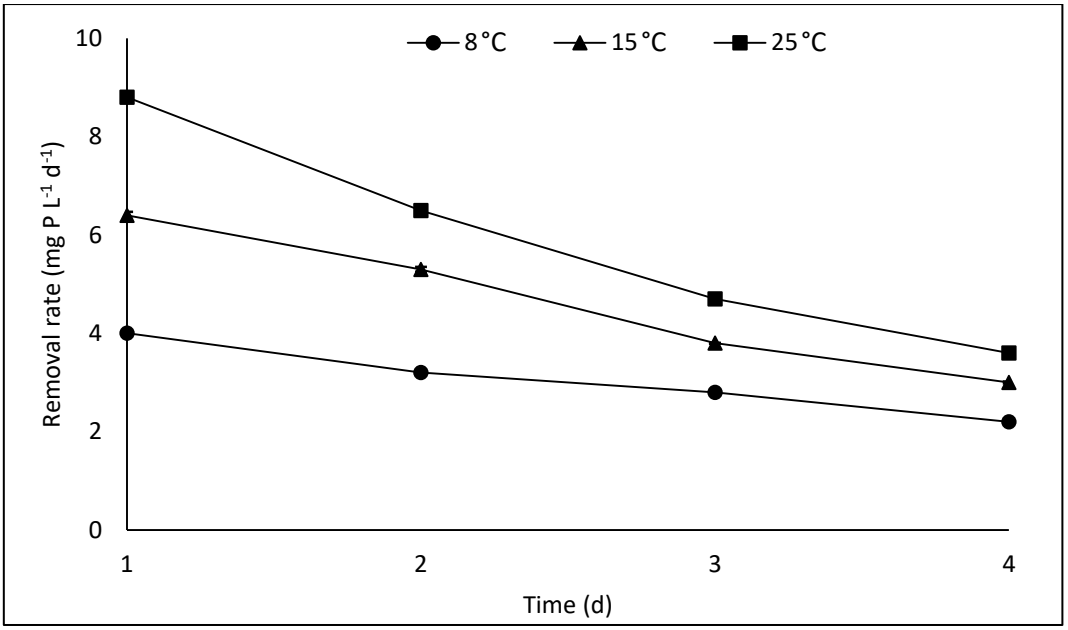

Figure 3.2.6.2. P removal rates of *Lemna minor* cultures. Triplicate cultures were maintained on modified Hoagland's solution for four days at 5°C, 15°C and 25°C at 6 h photoperiods. Plants were initially provided with 15 mg P L^{-1} in 100 mL batch pots. Error bars are standard error of the means.

Table 3.2.6.2. Q_{10} values for day 1 P removal rates.

Temperature (°C)	P removal rate (mg P L^{-1} d^{-1})	Q_{10}
8	4	
15	6	1.8
25	9	1.5

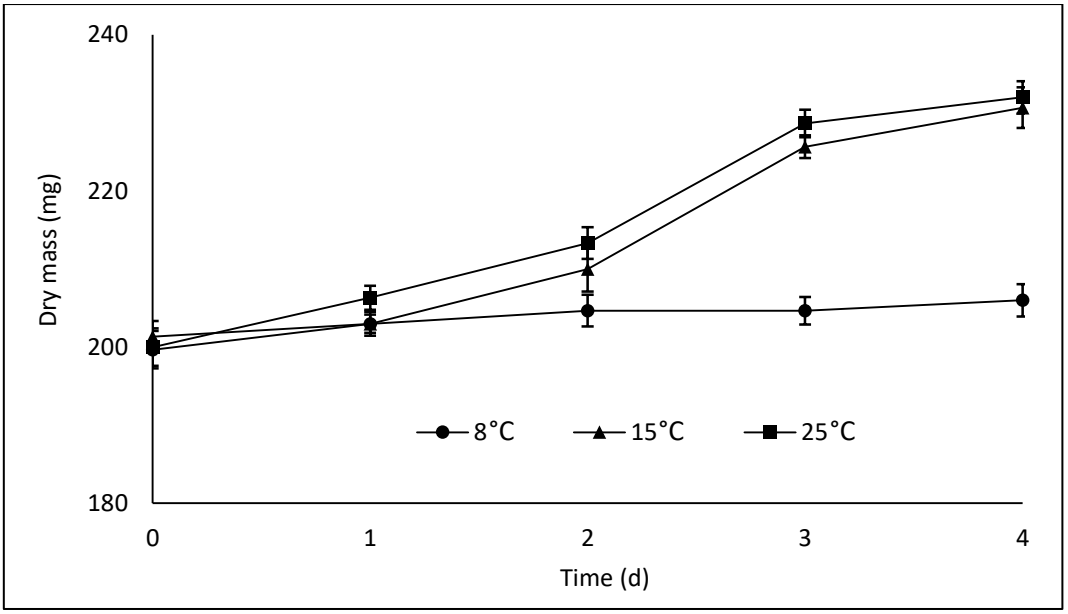

Figure 3.2.6.3. Effect of temperature on the growth of *Lemna minor*. Triplicate cultures grown on modified Hoagland's solution initially containing 15 mg P L^{-1} were exposed to 8°C, 15°C or 25°C under a 6 h photoperiod for four days. Total dry mass was measured daily in destructive samples. Error bars are standard error of the means.

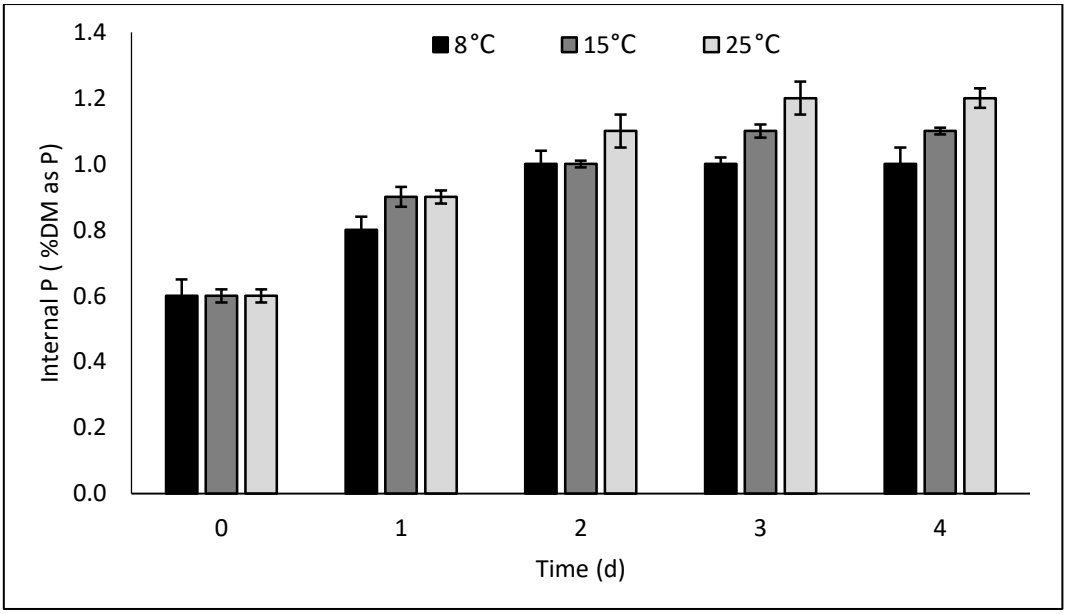

Figure 3.2.6.4. Effect of temperature on P accumulation in *Lemna minor*. Triplicate cultures grown on modified Hoagland's solution initially containing 15 mg P L^{-1} were exposed to 8°C, 15°C or 25°C under a 6 h photoperiod for four days. Inorganic P (Pi) was measured daily in destructive samples. Error bars are standard error of the means.

Table 3.2.6.3. Q_{10} values for inorganic P accumulation *in planta*.

Temperature (°C)	Accumulation rate (mg P mg DM^{-1} d^{-1})	Q_{10}
8	0.0012	0
15	0.0013	1.1
25	0.0015	1.2

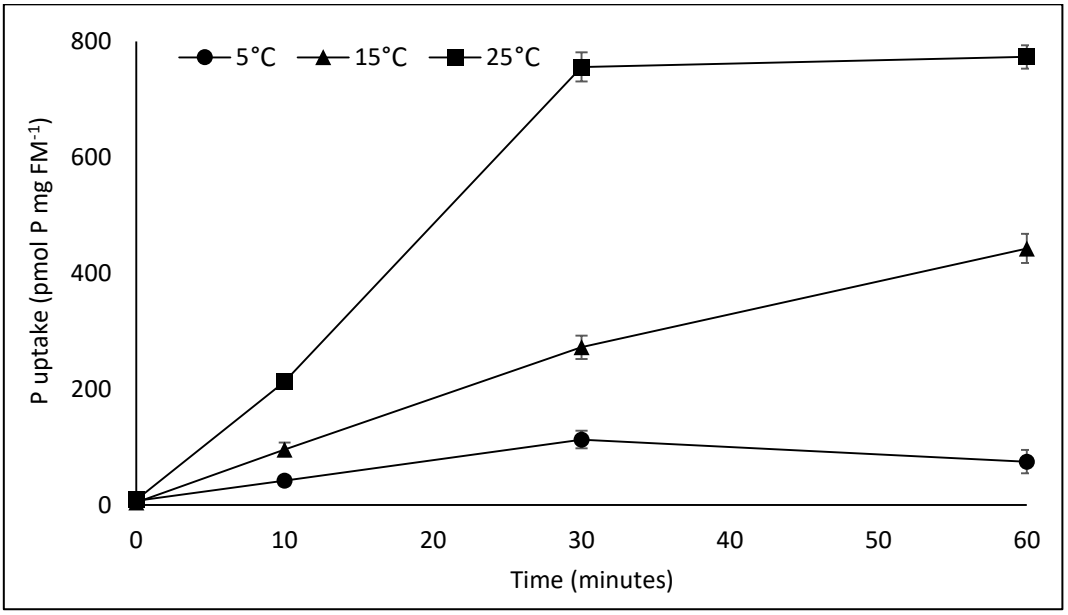

Figure 3.2.6.5. Temperature dependent P uptake in *Lemna minor*. Triplicate 10-20 mg (fresh mass) axenic cultures of *Lemna minor* were incubated for 1 hour in perfusion solution containing 0.1mM P (3 mg P L^{-1}) at either 5, 15 or 25°C, pH 5.7 and spiked with $[^{32}P]H_2PO_4^-$ to a final working concentration of 1µCi mL^{-1}. At 0, 10, 30 and 60 minutes, reactions were halted by rinsing and immersion into scintillation fluid, before being read in a scintillation counter for radioactivity. Allowing for decay constants and correction factors (Chapter 2, Section 2.5.7), counts per minute (CPM) were converted to uptake. Error bars are standard error of the means.

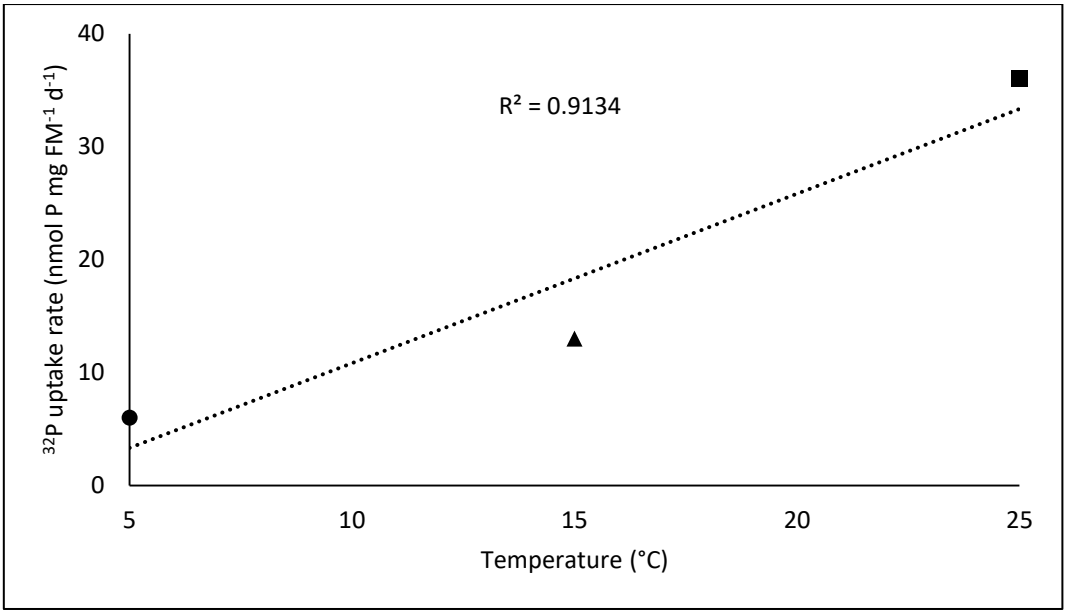

Figure 3.2.6.6. Relationship between temperature and ^{32}P uptake by *Lemna minor*. Pearson's correlation between temperature and P uptake data from Figure 3.2.6.5.

Table 3.2.6.4. Q_{10} values for ^{32}P uptake rates at 30 minutes.

Temperature (°C)	^{32}P uptake rate (pmol P mg FM^{-1} d^{-1})	Q_{10}
5	113	
15	272	2.4
25	756	2.8

Table 3.2.6.5. ^{32}P transport rates versus accumulation rates in both temperature experiments. Rates of P accumulation and ^{32}P transport in temperature experiments. Rates for accumulation were calculated from P removed at day 1 (Figure 3.2.6.1) divided by biomass. Rates for transport were calculated from ^{32}P uptake (converted to mg), divided by biomass at 30 minutes (linear phase, Figure 3.2.6.5).

Temperature (°C)	Accumulation (mg P mg FM d^{-1})	^{32}P transport (mg P mg FM d^{-1})
15	0.2	0.4
25	0.3	1.1

3.2.7. The effect of the provision of P on the performance of *Lemna minor*.

In the photoperiod and temperature experiments, P removal was limited to 80 % when maintained under simulated summer conditions and this limitation was proposed to be either internal storage being at full capacity or low concentrations of P in solution towards the end of the experiments reducing uptake.

In addition to this finding, as a consequence of weather or wastewater quality variations, small WWTW without extensive infrastructure can sometimes experience variations in P loadings. Samples of effluent from Alkborough WWTW (adjacent to where the duckweed used was sampled from) during 2014 were tested personally and were a mean of 7 mg P L^{-1}. Phosphate in raw wastewater received at Esholt WWTW (a large works) ranged from 0.9 to 7.1 mg PO_4^{3-} - P from 2014 to 2016. It is important therefore to know how the external P concentration may affect P uptake and what the uptake kinetics are, as this may aid to inform models predicting a hypothetical duckweed system.

To go towards investigating this, 0.2g (FM) triplicate cultures were maintained on modified Hoagland's solution for ten days initially containing either 5, 15 or 30 mg P L^{-1}; 50 mg N L^{-1} as nitrate and set to pH 7. Photoperiod was 12 h and the temperature was 15°C. P in solution, P *in planta* and biomass production (as dry mass) were all measured periodically throughout.

Increasing initial P in solution from 5 mg P L^{-1} to 15 mg P L^{-1} increased uptake as an overall amount but this appears somewhat in proportion with the amount supplied, as rates are similar from day 3 onwards (Figure 3.2.7.1). There was little difference between the two higher loadings in the amount of P removed by day 10, because the 30 mg P L^{-1} treatments' uptake rates reduce and are the same at day 10 (Figure 3.2.7.3). As an overall %, this equates to 80 %, 44 % and 22 % of all P removed from solution by the 5, 15 and 30 mg P L^{-1} cultures respectively. The data suggests a P removal saturation point under these conditions is somewhere between 15 and 30 mg P L^{-1}.

Removal coefficients derived from the Ln (base e) of P remaining in solution correlated highly to P load (R^2 = 0.87, Figure 3.2.7.2). Increasing the initial load of P also increased internal P and these two variables correlated highly (R^2 = 0.99). Relative to starting values of 1.4 % (dry mass represented by P), when supplied with 5, 15 or 30 mg P L^{-1}, internal P increased by 14 %, 49 % and 80 % respectively (Figure 3.2.7.4). Relative increases in biomass from an average initial 10 mg dry mass were similar between all P load treatments (Figure 3.2.7.5), which suggests that vigorous growth of the duckweed is not necessary for P uptake and not stimulated by increased provision of P in this case.

In order to generate kinetic data on P uptake by *Lemna minor*, 20 mg (FM) cultures were incubated at 22°C in solutions ranging from 0 to 12.4 mg P L^{-1} (0 to 400 µM P) for twenty minutes spiked with ^{32}P. Radioactivity was counted in a scintillation counter and converted to uptake per unit biomass.

Evidence for P concentration limiting P uptake is apparent (Figure 3.2.7.6). There are two concentration ranges in which different K_m and V_{max} values were calculated, suggesting different P uptake affinities. Low capacity high affinity uptake occurred between 0.05 to 0.80 mg P L^{-1} and high capacity low affinity uptake occurred between 0.8 to 12.4 mg P L^{-1} (Table 3.2.7.1).

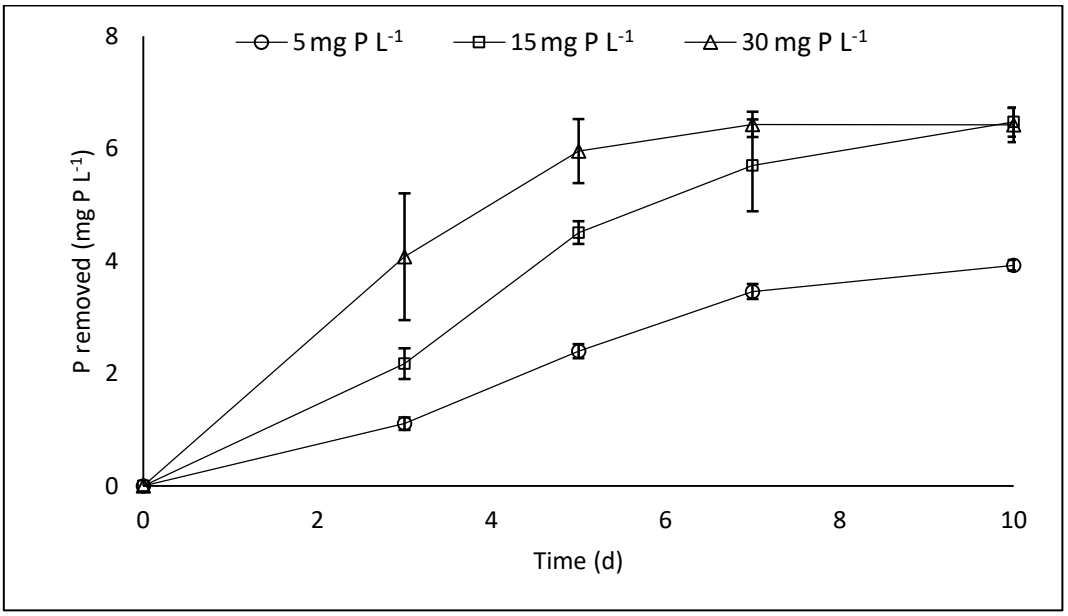

Figure 3.2.7.1. Effect of P load on P removed by *Lemna minor*. Triplicate cultures were maintained on modified Hoagland's solution for ten days at 15°C and 12 h photoperiods. Plants were initially provided with 5, 15 or 30 mg P L^{-1} in 100 mL batch pots. P removed from solution was measured every two days. Error bars are standard error of the means.

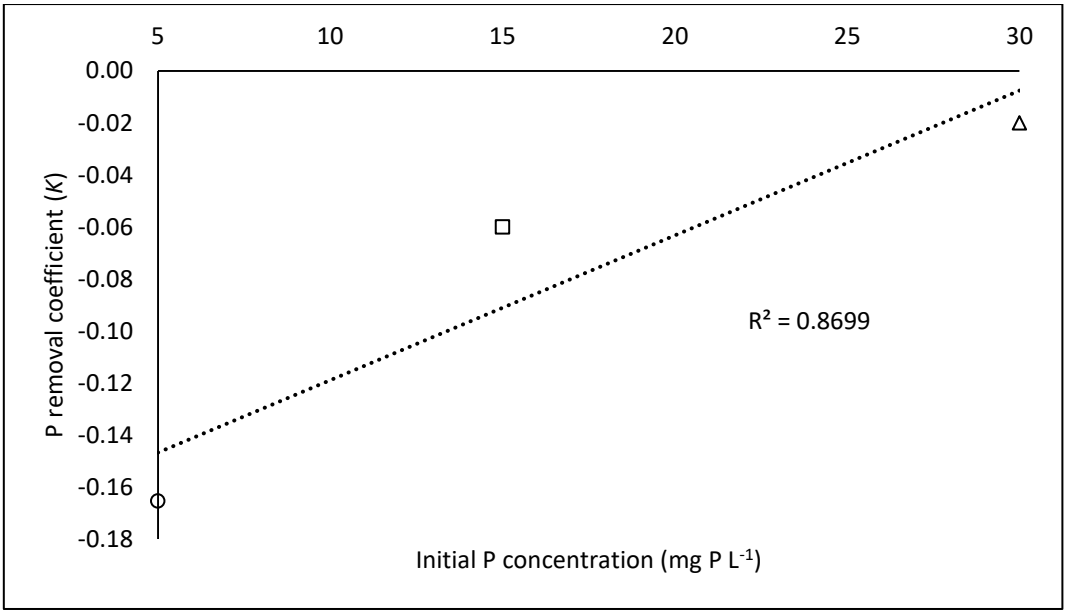

Figure 3.2.7.2. Relationship between initial P load and P removal coefficients. A Pearson's correlation was run between initial P concentrations (mg P L^{-1}) and P removal coefficients, derived from Ln to base e of remaining P concentration.

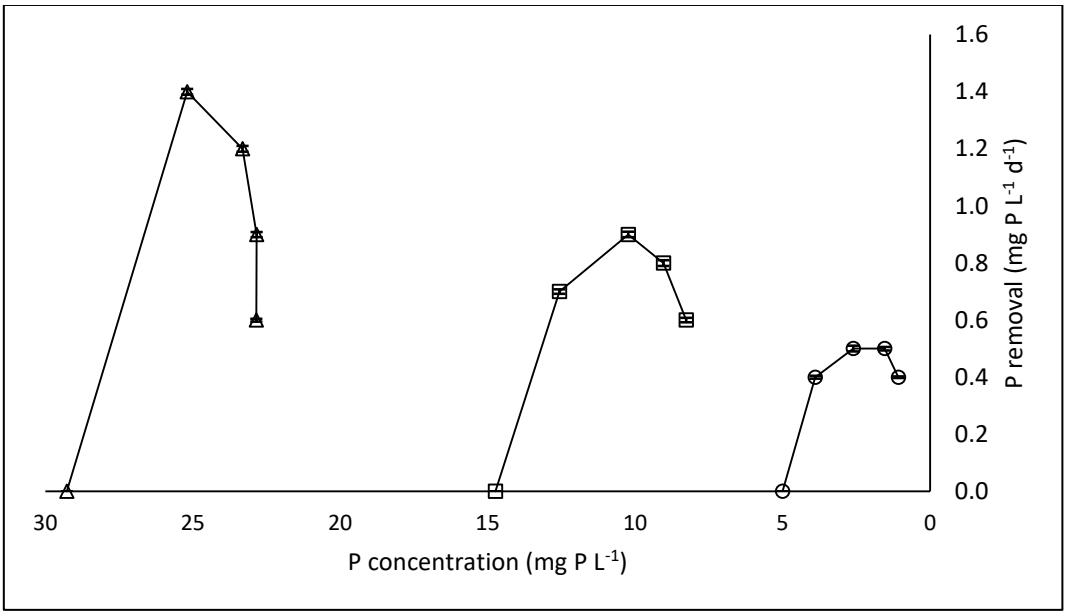

Figure 3.2.7.3. P removal rates by *Lemna minor* cultures. Triplicate cultures were maintained on modified Hoagland's solution for ten days at 15°C and 12 h photoperiods. Plants were initially provided with 5, 15 or 30 mg P L^{-1} in 100 mL batch pots. Error bars are standard error of the means.

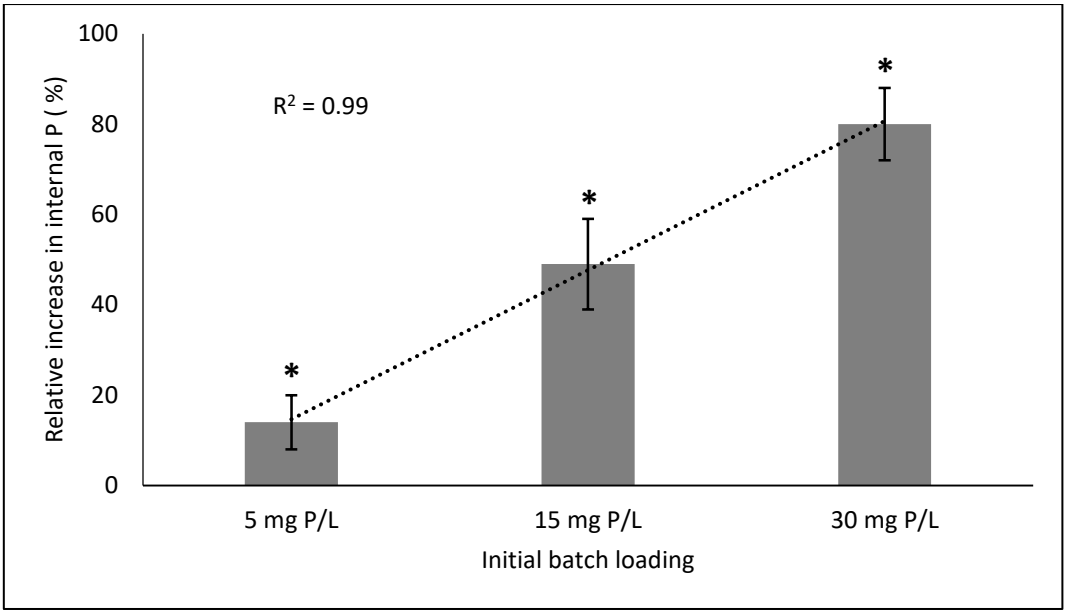

Figure 3.2.7.4. Effect of initial P loading on P accumulation in *Lemna minor*. Triplicate cultures were maintained on modified Hoagland's solution for ten days at 15°C and 12 h photoperiods. Plants were initially provided with 5, 15 or 30 mg P L^{-1} in 100 mL batch pots. Internal inorganic P (Pi) was measured at day 0 and day 10 and the % change (relative to average starting values of 1.4 % of dry mass) were recorded. All cultures were significantly different from one another in terms of % change (all $P < 0.01$). R^2 value is the correlation between increase in Pi (relative %) and initial P load (mg P L^{-1}).

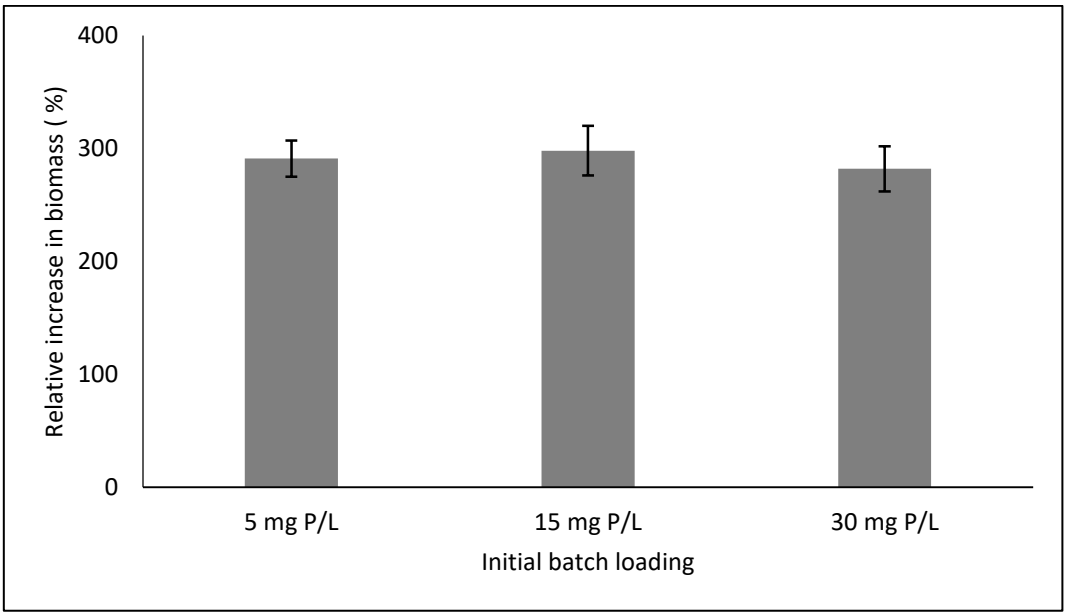

Figure 3.2.7.5. Effect of initial P load on the growth of *Lemna minor*. Triplicate cultures were maintained on modified Hoagland's solution for ten days at 15°C and 12 h photoperiods. Plants were initially provided with 5, 15 or 30 mg P L^{-1} in 100 mL batch pots. Dry masses were recorded at day 0 and day 10 and the differences recorded as % changes relative to starting values. Error bars are standard error of the means.

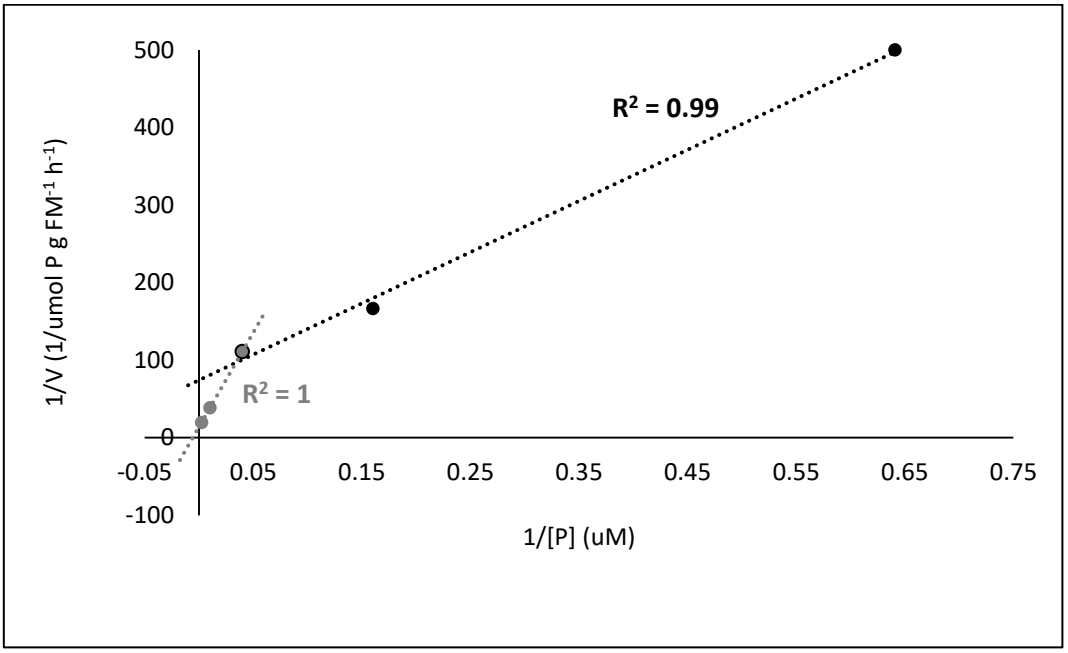

Figure 3.2.7.6. Bi-phasic P uptake kinetics of *Lemna minor.* Lineweaver Burk plot of ^{32}P uptake (exposed to 1.56-400 µM P). Triplicate 10-20 mg (fresh mass) cultures of *Lemna minor* were incubated for twenty minutes in perfusion solutions containing 0-400 µM P at 22°C, pH 5.7 and spiked with $[^{32}P]H_2PO_4^-$ to a final working concentration of 1 µCi mL^{-1}. 1.56-25 µM P is in grey; 25-400 µM P is in black.

Table 3.2.7.1 High and low affinity P uptake in *Lemna minor*. K_m and V_{max} values derived from Lineweaver-Burk transformations of uptake data (from Figure 3.2.7.6).

Concentration range	K_m	V_{max}
1.56-25 µM P (4.7-75 µM PO_4) (0.05-0.8 mg P L^{-1})	7 µM P (21 µM PO_4) (0.2 mg P L^{-1})	0.01 µmol P g^{-1} (FM) h^{-1} (0.03 µmol PO_4 g^{-1} (FM) h^{-1}) (0.3 µg P g^{-1} (FM) h^{-1})
25-400 µM P (75-1200 µM PO_4) (0.8-12.4 mg P L^{-1})	170 µM P (510 µM PO_4) (5.3 mg P L^{-1})	0.07 µmol P g^{-1} (FM) h^{-1} (0.21 µmol PO_4 g^{-1} (FM) h^{-1}) (2 µg P g^{-1} (FM) h^{-1})

3.2.8. The effect of P acclimation on the performance of *Lemna minor*.

In these P acclimation experiments, there was a clear impact of P acclimation on P accumulation *in planta* and subsequently on P removal from solution.

Plant Pi changed rapidly when cultures were immersed into growth solutions containing varying concentrations of P. Following incubation in 15 mg P L^{-1} for 10 days, internal Pi was an average of 0.93 % (of DM). Transferring sub-cultures into solutions containing 0, 1, 2, 5 and 15 mg P L^{-1} for 4 days caused Pi to drop to 0.1 %, 0.2 %, 0.3 %, 0.7 % and 0.8 % (of DM) respectively under simulated UK average (24 h) summer conditions of 12 h photoperiods and 15°C (Figure 3.2.8.1, Points A-E, days 0-4) and to 0.2 % under simulated UK average (24 h) winter conditions of 6 h photoperiods and 8°C (Figure 3.2.8.1, point F). Internal Pi at day 4 correlated well to external P concentration (R^2 = 0.69).

Immersing the cultures back into solutions all containing 15 mg P L^{-1} for a further 4 days caused Pi to increase to 0.4 %, 0.5 %, 0.6 %, 1 % and 1 % respectively for plants under simulated UK average (24 h) summer conditions of 12 h photoperiods and 15°C (Figure 3.2.8.1, points A-E, days 4-8) and to 0.6 % for plants under simulated UK average (24 h) winter conditions of 6 h photoperiods and 8°C (Figure 3.2.8.1, point F).

In addition to plant Pi, during days 4 to 8 of the experiment P removal from solution was also recorded. Plants pre-acclimated to 0, 1 and 2 mg P L^{-1} (including the simulated winter treatment) were all able to remove >99 % of P from solution in 4 days or less (Figure 3.2.8.2, points A-C and point F respectively). In previous experiments under the same photoperiods and/or temperatures, plants could only remove up to 80 % (under simulated summer conditions, Figure 3.2.8.1) or 61 % (under simulated winter conditions, Figure 3.2.8.1). Plants pre-acclimated to 5 and 15 mg P L^{-1} removed just 88 % and 81 % of P from solution respectively (Figure 3.8.2, points D and E). The reciprocal of P removal coefficients (1/K) correlated strongly to initial internal Pi (R^2 = 0.95) (Figure 3.2.8.3). P removal was dependent on internal Pi, which in turn was dependent on the external P within which the plants were immersed.

Production of biomass was directly compared between plants maintained under simulated summer and winter conditions, both previously acclimated to 1 mg P L^{-1} (Figure 3.2.8.4). Plants under simulated summer conditions increased biomass by 21 % while plants under simulated winter conditions did not increase at all and day 4 values were significantly different (P = <0.01). Both the summer and winter grown plants removed >99 % of P from solution, therefore this is strong evidence that P uptake can occur in the absence of active growth. Results also show that under these controlled conditions, the duckweed removed P to lower than UK discharge consents of 1 mg P L^{-1}, but additional considerations

have to be taken when assessing the low uptake rate and the residence time required to achieve that target.

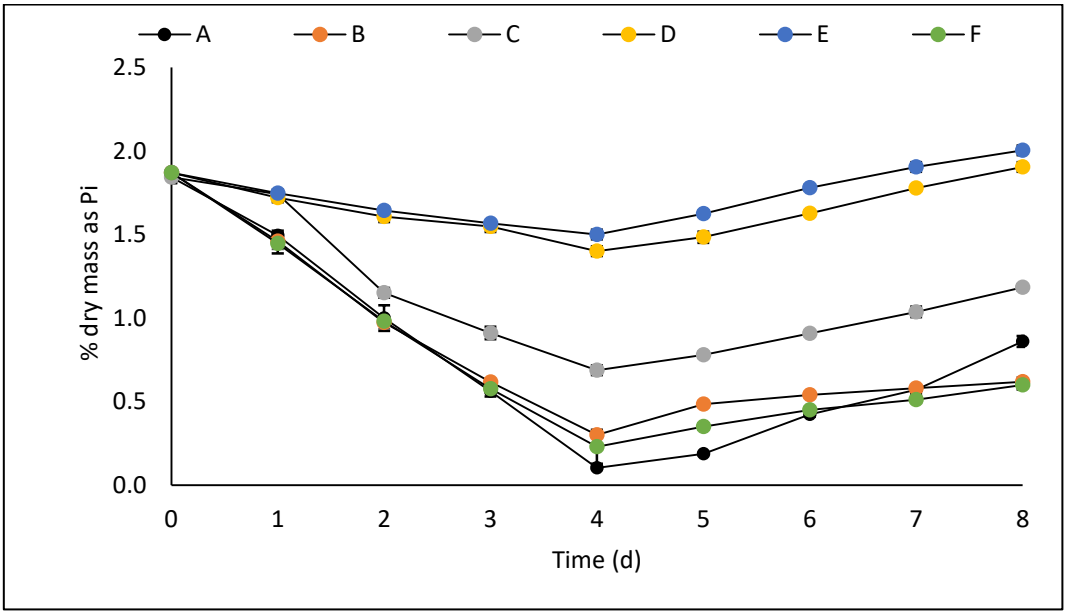

Figure 3.2.8.1. P acclimation alters internal P in *Lemna minor*. Triplicate sub cultures were immersed into modified Hoagland's solution initially containing 0, 1, 2, 5 or 15 mg P L^{-1} (A-E respectively) and 1 mg P L^{-1} (F) for four days (d0-d4), then transferred to fresh solutions all containing 15 mg P L^{-1} (d4-d8). All day 0 sub-cultures were acquired from one original stock maintained on 15 mg P L^{-1} for 10 d (refreshed every two days). A-E were kept at 15°C under 12 h photoperiods and F plants were kept at 8°C and 6 h photoperiods. Inorganic P (Pi) was measured daily in destructive cultures and reported as % of dry mass. Error bars are standard error of the mean.

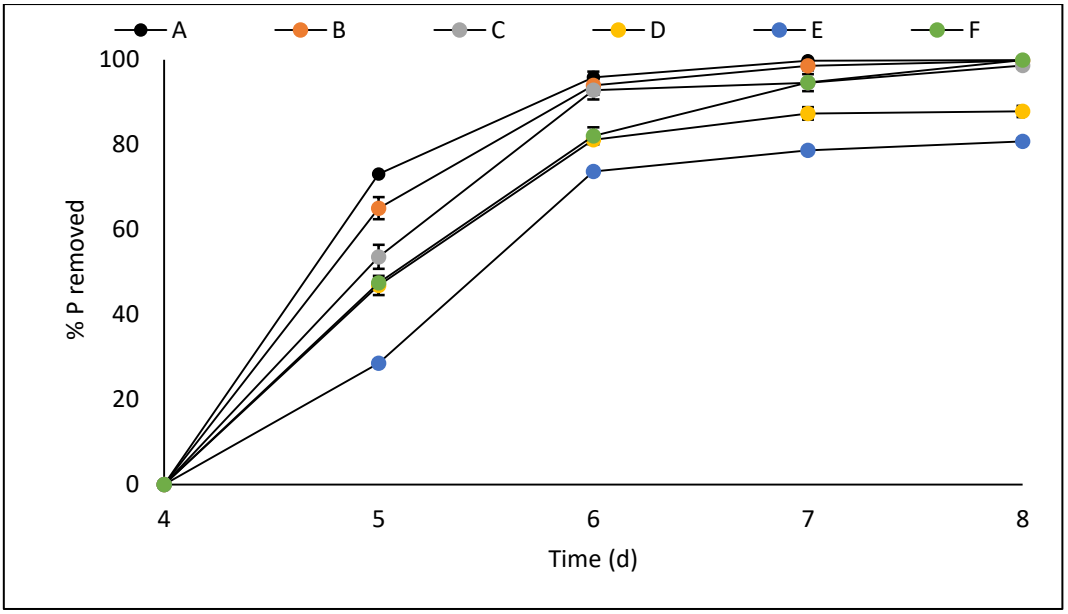

Figure 3.2.8.2. Internal P affects P removal by *Lemna minor*. Triplicate sub cultures previously immersed into modified Hoagland's solution initially containing 0, 1, 2, 5 or 15 mg P L^{-1} (A-E respectively) and 1 mg P L^{-1} (F) for four days, were then transferred to fresh solutions all containing 15 mg P L^{-1} for a further four days. Days 4-8 on the x axis correspond to days 4-8 in Figure 3.9.1. A-E were kept at 15°C under 12 h photoperiods and F plants were kept at 8°C and 6 h photoperiods. P in solution was measured daily and converted to % removed. Error bars are standard error of the mean.

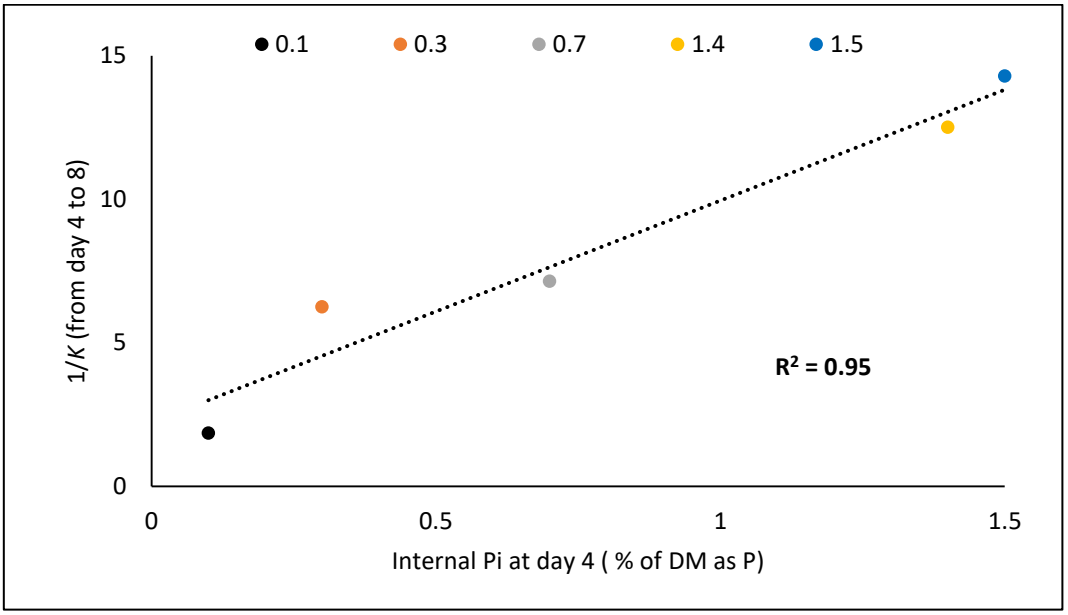

Figure 3.2.8.3. Relationship between internal P and P removal by *Lemna minor*. Pearson's correlation of the reciprocals of removal coefficients (*K*) over four days (days 4-8 in Figure 3.2.8.1) and the respective starting internal P content in cultures A-E measured on day 4.

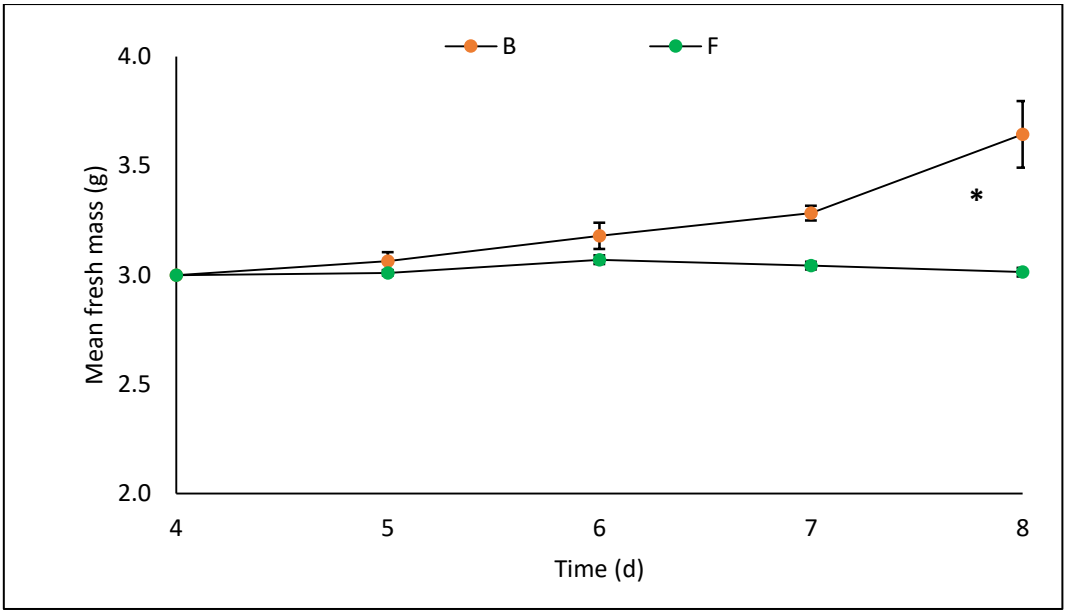

Figure 3.2.8.4. Change in fresh mass of whole cultures B and F. '*' indicates a significant difference (P = <0.01). B plants were kept at 15°C under 12 h photoperiods and F plants were kept at 8°C and 6 h photoperiods. Total fresh mass was measured daily in triplicate destructive samples. Days 4-8 correspond to days 4-8 in Figure 3.2.8.1. Error bars are standard error of the means.

3.3. Discussion

3.3.1. Sampling and identification

The variables investigated in this Chapter were seen as possible factors that would likely play a role in the field during the operation of a large scale duckweed system. Understanding how fundamental variables (such as temperature or P in solution) affected P uptake and growth at this point in a controlled setting was important, before the scale of experiments grew and considerably more variables (such as microbiological interactions) would be introduced by default.

It was important to obtain and identify a model species that was pertinent to this project's aims. An isolate was sourced adjacent to a WWTW at Alkborough, North Lincolnshire, which satisfied requirements of it being naturally existing in the UK and able to grow in a lagoon that received final effluent.

Being able to identify the isolate was important as morphological differences between and even within species can be apparent and identification is generally made by observing frond size or root number (Landolt, 1980a). In terms of performance (P removal and growth), differences have also been reported (Bergmann et al., 2000) therefore the need to know accurately what species was being used throughout this project would be valuable, as the ultimate aim was a pilot system outdoors, where species could change due to interactions of wildlife.

Positive identification as *Lemna minor* was seen as beneficial due to it being widely researched with several authors publishing articles using this species (Harvey and Fox, 1973, Obek and Hasar, 2002, Cheng et al., 2002b) and as such may provide comparisons on performance (dependent on experimental design).

3.3.2. The fate of phosphorus in microcosm experiments.

Obtaining a mass balance of P throughout the microcosm experiments in this Chapter was important, as P needed to be accounted for as the duckweed were not maintained in sterile cultures. Therefore there would have been microbial assemblages and unicellular algae present that may have had some influence on nutrient depletion in the experimental pots. Experiments were designed in a manner to prevent algal proliferation by only providing illumination from directly above and inoculating enough duckweed to thoroughly cover all of the growth solution surface.

The vast majority of P could be accounted for at three different temperatures in both axenic and non-axenic cultures (Figure 3.2.2.1 and 3.2.2.2). The results gave the confidence to attribute P removal data to the duckweed and negated considerations of pot adsorption/precipitation at lower temperatures; or microbial uptake at higher temperatures. The results also allowed for increased data

output as maintaining an axenic culture and using sterile technique for all experiments was not necessary.

In conductivity experiments (Section 3.2.3) where portions of some cultures died and sank to the bottom, a mass balance was conducted again and P was accounted for once more in all fractions of the experimental mesocosm (Figure 3.2.3.8). This further upheld the robustness of the experimental design and gave confidence in the results generated for the experiments that were to follow.

3.3.3. The effect of conductivity

Plants are known to deal with periods of elevated salinity by way of regulating ionic gradients and dealing with osmotic stresses (Ashraf and Harris, 2004), but there are very few peer reviewed publications with regard to the conductivity or salinity tolerance of duckweed. Conductivity can fluctuate in wastewater and duckweed is a freshwater macrophyte, therefore it is important to know how a proposed duckweed system treating wastewater would perform under varying conductivities and if there was a threshold value that could not be tolerated, in terms of survival and P removal.

Under these conditions, the data presented suggest a threshold for growth at 3,200 µS cm^{-1}. *Spirodela polyrrhiza* maintained on domestic wastewater under batch cultures outdoors in a West African climate increased RGR in solutions with conductivities from 600 to 1,200 µS cm^{-1}, before declining and reaching their lowest RGR at 3,000 µS cm^{-1} (Wendeou et al., 2013). *S. polyrrhiza* fronds were reported to be green and healthy at conductivities of 800 to 1,600 µS cm^{-1}, while from 1,800 µS cm^{-1} and higher, fronds turned yellow, which is similar to the this study (Figure 3.2.3.7). Maximum phosphate removal was observed at 1,200 µS cm^{-1} which if correct, is close to the lower conductivity solution used presently (900 µS cm^{-1}) , as well as the average for Esholt WWTW of 918 µS cm^{-1} between 2014 and 2016.

Overall P removal rates in this study were inversely proportional to conductivity (Figure 3.2.3.2). During the experiment, removal rates in the 4,000 and 10,000 µS cm^{-1} cultures declined after 2 days, initially assumed to be attributed to unhealthy plants not growing (Figure 3.2.3.3). However, P removal in the 900 µS cm^{-1} culture also decreases, more sharply and sooner (after day 1, Figure 3.3.3), in healthy growing cultures, therefore this phenomenon may not be explained by conductivity alone. The mass balance conducted showed that almost all of the P removed was attributable to the duckweed, either healthy living plants or plants that had removed P and subsequently died (Figure 3.2.3.8). Reduced rates of P removal after day 1 by the plants grown at 900 µS cm^{-1} may be due to P limitation, as the cultures had had little time to grow in 24 h, yet removed 65 % from solution and by day 2 had removed 94 % (Figure 3.2.3.1). The reduction in removal rates from day 2 onwards is

proportional between all treatments (Figure 3.2.3.3). This may be coincidence and be explained by two separate phenomena. The explanation for the 900 µS cm^{-1} treatment's decline in removal may be P limitation, while the explanation for the proportionality between the two higher conductivity treatments may be a proportional decrease in living biomass. If correct, this latter proposal would suggest that conductivity was not directly responsible for the differences in P removal, but that increasing conductivity negatively affected growth (Figure 3.2.3.4), which had a knock on effect on P removal. Biomass was not measured between day 0 and day 4, but overall amounts of detritus were measured during the mass balance (Figure 3.2.3.8) and this revealed that both higher conductivity treatment cultures did not increase in biomass at any point and when detritus + remaining live biomass were measured, total values were the same as the initial day 0 inoculations of 3 g (fresh mass).

Haller et al. (1974) report a decrease in growth rate of *Lemna minor* when exposed to salinities of 6.6 ‰ or higher, which corresponds to 9,500 µS cm^{-1}. However the authors state that initial salinities were set (and left), while growth solutions were topped up weekly with dH_2O to counter evapotranspiration. By the end of the experiment, the solutions had been diluted by 40 %, although this is not taken into account, meaning that any salinity reported in that particular study would be grossly over estimated. Calculations for conductivity based on this dilution factor suggest the value where RGR begins to decline is actually closer to 5,000 µS cm^{-1}.

Changes in internal P were also highly correlated to conductivity. There was a decrease (relative to starting values) in the plants exposed to 10,000 µS cm^{-1} which means that Pi must have been mobilised and either excreted directly from the cells or used for other cellular processes while succumbing to higher salinities, such as using ATP to provide the energy for producing osmoprotective compounds and/or regulating ionic balances (Sikorski et al., 2013). Using energy in this way would also show a decrease in growth, which is exactly what occurred. P in solution did not increase in those cultures therefore the latter is the most likely. Pi increased in the plants exposed to 4,000 µS cm^{-1} which is evidence for conductivity at this value not preventing P uptake. The increase described by the plants exposed to 900 µS cm^{-1} was almost 3x higher than those of the 4,000 µS cm^{-1} treatments however so there was a negative effect between 900 and 4,000 µS cm^{-1}, which was proposed earlier to be 3,200 µS cm^{-1}.

Direct comparisons of authors' reports of duckweed salinity tolerance are difficult due to differences in experimental design, however Sree et al. (2015) show a reduction in RGR by *Lemna gibba* exposed to conductivities over 1,100 µS cm^{-1}. The decline is followed by a sharp decrease at 3,000 µS cm^{-1} and a RGR of zero at 45,000 µS cm^{-1} (Sree et al., 2015), although the authors did not measure RGR between

30,000 and 45,000 µS cm^{-1} so this cessation of growth could have been at a lesser value. It is important to mention that as well as the difficulty of comparing experimental results, species performance cannot necessarily be compared due to variance in inter-clonal cultures. Duckweed can survive under a range of conditions and clones of the same species can often perform differently, owing to their acclimation to respective environments (Sree et al., 2015, Bergmann et al., 2000).

Sikorski et al. (2013) showed incremental decreases in *Lemna minor* culture growth rates when exposed from 391 to 3,125 µS cm^{-1}, before a sharp decrease is seen when increasing from 3,125 to 6,250 µS cm^{-1}. Growth is not negative however until conductivity reaches 25,000 µS cm^{-1}, which shows a much higher tolerance of duckweed than the isolate used in this Chapter. Explanations for why duckweed in the present study succumbs to increasing conductivity are difficult to suggest as no other variables than those reported were measured. However it is known that salinity stress in plants is related to several defence mechanisms including increased abscisic acid (ABA) and increases in reactive oxygen species (ROS). If ABA is transported to the guard cells of stomata, it can cause these to close and prevent the gaseous exchanges of photosynthesis (Chaves et al., 2009). Chlorophyll synthesis is also negatively affected by salinity stress, drastically disturbing photosynthetic mechanisms (Keppeler, 2011). ROS species include hydrogen peroxide (H_2O_2), which when over produced will bleach plant cells and lead to mortality (Chang et al., 2012), which may be the explanation for the paler fronds of the higher conductivity treatments in Figure 3.2.3.7.

The main conclusions to draw from this experiment are that P uptake appeared to be affected by conductivity to a lesser extent than growth. In this situation, occasional spikes in conductivity may be absorbed by a duckweed P removal system, but prolonged exposure to higher values (over 3,000 µS cm^{-1}) would cause plants to die and P removal to cease. The conductivity value to which the duckweed seems to cope with is much higher than would be expected throughout any stage of a wastewater treatment process which would be positive for possible future large scale trials. Intraspecific variation in tolerance and performance may allow for duckweed isolates to be acclimated to various conditions in the field, but it is hard to predict performance at the large scale using this data from mesocosm studies. To more specifically explain exactly how the plants exposed to higher conductivities were affected would require the measurement of chlorophylls and/or ROS, among other things.

3.3.4. The effect of nitrogen species and pH

As well as conductivity, the concentration and form of nitrogen found at different stages of a wastewater treatment plant can vary (Körner et al., 2001). If a nitrifying plant fails periodically, then ammonium could overtake nitrate as the dominant form of N in the system; ideally, if a new biological system is able to take ammonium as the main nitrogen source and take up phosphorus at the same time, this would represent substantial savings regarding operation costs. A review of the literature found that opinion is divided on what N species duckweed will take up or exist on preferentially, but several of these experiments are hard to evaluate, as authors do not report or control variables such as temperature or pH, both of which would affect the equilibrium of NH_3 and NH_4^+ in solution. It is important to attempt to identify the effect of N species on duckweed performance, while controlling pH and temperature.

Results from the present experiments showed that the duckweed grew more, removed more P from solution and accumulated more P when supplied with nitrate only, when compared to ammonium only supplied plants. Fronds grown on both ammonium solutions had mostly turned yellow and unhealthy by the end of the experiment whereas those grown on nitrate were not.

Several authors state that duckweed (and terrestrial plants) will grow better and take up nutrients with ammonium as a sole N source over nitrate (Monselise & Kost, 1993; Caicedo et al., 2000; Porath & Pollock, 1981). Authors also suggest a preferential uptake of NH_4^+ over NO_3^- when combined in solution (Fang et al., 2007). This is often hypothesised due to the much lower energy required to assimilate NH_4^+ into amino acids when compared to the additional reduction of NO_3^-. An overview of the N assimilatory pathway is given in Table 3.3.4.1, rewritten from Crawford and Ford (2002).

Table 3.3.4.1. The nitrogen assimilatory pathway.

	Via		Via		Via		Via			
NO_3^-	Nitrate reductase	NO_2^-	Nitrite reductase	NH_4^+	Glutamine synthetase	Glutamine	Glutamate synthetase	Glutamate	Amino acids	Proteins & nucleotides

Some authors report nitrate being preferred however (Britto & Kronzucker, 2002) and even toxicity to plants when NH_4^+ is supplied as the sole source of N (Britto et al., 2001) especially in a high pH solution as this will drive the dissociation of more NH_3. It has been reported that for duckweed growing in an acidic solution (such as 1A and 1B), nitrates are preferred (Britto & Kronzucker, 2002); whereas in neutral or alkaline solutions ammonium is taken up preferentially (Mohedano et al., 2012). The

proportion of NH_3 of total ammonium is low, relative to NH_4^+, but this equilibrium shifts with increases in pH (Caicedo et al., 2000) and values of up to 8 mg NH_3 – N L^{-1} were reported to suppress duckweed growth (Körner et al., 2001). NH_3 is said to be toxic due to its permeability to cell membranes and ability to diffuse into the cell unchecked, disrupting intracellular pH gradients (Caicedo et al., 2000). As the pH of both 1A and 2A dropped throughout the present experiment (and at least some NH_4^+ is assumed to have been taken up and assimilated), toxicity from NH_3 is unlikely to explain what happened to these cultures. Calculated starting values of 0.04 and 175 µg NH_3 L^{-1} would have only decreased over time – i.e. using the equation from Emerson et al. (1975).

It has been suggested that ammonium will supress the uptake of anions by duckweed, by way of NH_4^+ cationic influx in to the cell and subsequent depolarisation of the membrane (Ullrich et al. 1984), caused by a loss of the cellular pH gradient. This was shown to be the case in *L. paucicostata* by Löppert (1979). It is not unreasonable to suggest that an ammonium mediated mechanism that prevents the influx of anionic nitrate would not act in the same way for phosphate. Ullrich et al. (1984) has shown this to be the case for both NO_3^- and $KH_2PO_4^-$, with the addition of 0.2 mM NH_4^+ to the growth media of *Spirodela polyrrhiza*, promoting an immediate depolarisation and loss of P uptake in P starved plants. Crucially, P uptake resumed once the ammonium supply was consumed. Concentrations of NH_4^+ - N applied in this experiment were 0.55 mM for culture 1A and 2.78 mM N as NH_4^+ for 2A, way above what Ullrich et al. (1984) found to be inhibiting, therefore membrane disruption from ammonium may be a plausible explanation. Although the presently used plants were not starved of nutrients beforehand, the stock cultures used had not been exposed to ammonium for several months following their original sampling. There would also be an expenditure of energy (ATP) as the plants attempted to regulate their cytosolic pH. The lack of nutrients and energy would then lead to senescence. It is suggested here that duckweed (and other species) may not necessarily take up ammonium preferentially over nitrate as several authors (and those that cite them) suggest, but moreover that when combined, ammonium disrupts the uptake of nitrate and the plants' membrane channels have no way to combat the influx of ammonium, thus appearing to have a 'preference' for ammonium.

Britto et al. (2001) reported a 'futile cycling' of NH_4^+ across the cell membrane of *Hordeum vulgare* (Barley) when exposed to high concentrations (10 mM) of NH_4^+. Positron tracer experiments showed that *c.* 80 % of the NH_4^+ diffusing into the cell was forced out again against steep external concentration gradients in an energy draining cycle. This constant flux of cationic ammonium and the necessity to expel it came at a high energy cost to the plant, increasing respiration by 41 % and resulting in the death of the plants (Britto et al., 2001). In the same experiments, rice did not show these adverse effects however. The rice plants decreased their membrane potential to reduce NH_4^+

accumulation and conserve energy (Britto et al., 2001). Internal phosphate reserves of the ammonium supplied cultures of the present experiment also declined, which is evidence for this process occurring and would contribute to a reduced energy supply and plant vigour, resulting in their yellowing appearance. The combination of low N and low pH in solution 1A may preclude the effects of NH_4^+ toxicity and the poor performance of these cultures may be due exclusively to low pH.

The pH in 1A remains low (<4) and beneath the proposed survival threshold of pH 4 (Hicks 1932; Hillman 1961; McLay 1976). Hicks (1932) reported senescence of 7 species of duckweed at a pH lower than 4.5 or higher than 7.5. Hillman (1961) states pH ranges including 'outer limits' for duckweed growth to be 3.5-8.5. McLay (1976) grew *Lemna minor* and *Spirodela polyrrhiza* on unbuffered Jacob's media which contained nitrate and no ammonium, and recorded very similar final pH values to this experiment (Figure 3.2.4.5), with initial values of pH of 4 and 7 being increased by the duckweed to *c*. pH 7.7 and 7.8 respectively (McLay 1976). Controls were carried out alongside the treatments in the present experiment which recorded no changes in solution P or pH therefore changes are due to the duckweed and any associated microbes (Figure 3.2.4.6). A further point to note is that at the pH values tested in this Chapter (pH 4, 7 and 10), the NO_3^- in solution would not have dissociated to HNO_3 as the p*K*a for this is 2. In contrast, the dissociation of NH_4^+ to NH_3 as pH rises (p*K*a for this is 9.25) makes NH_4^+ solutions less stable.

The pH of 1B began to increase immediately from 4 at day 0 to 7.6 by day 20 (Figure 3.2.4.5). This would have prevented H^+ toxicity to the cultures and allowed growth, metabolism and the uptake of nutrients. The removal of anions such as phosphate is said to be by way of H^+ cotransport (Ullrich-Eberius et al., 1981). In a batch reactor with a finite supply of protons, this removal activity seen by the plants of 1B would add to the increase in alkalinity (Figure 3.2.4.5). The only difference between 1A and 1B is the form of N and the pH of solution 1B rose instantly and constantly in this experiment, therefore the combined presence of ammonium and a low pH may have contributed to plants of solution 1A dying. To isolate the effects of pH, ammonium and ammonia is not practical as all 3 aspects are joined in equilibrium and would require even more extremes of pH to be tested. As well as affecting ionic forms, changes in solution pH would change the amount of protons available for nutrient cotransport and transmembrane proton gradients (affecting ATP synthesis and usage). This highlights the complexity of trying to isolate what the direct effects that solution pH has as well as the downstream effects.

The pH of solution will affect duckweed survival by impacting on cellular pH gradients and nutrient acquisition. If a plant is unable to regulate its balance of protons it will die. P uptake is by way of proton symporters and plants grown in the present experiment on nitrate solutions were able to take up

phosphate in tandem with protons, as evidenced by the increases in pH of those cultures (Figure 3.2.4.5). The ammonium supplied cultures were provided with the same concentrations of phosphate at the same pH, yet took up significantly less phosphate while solution pH fell dangerously low. This could be due to the plasma membrane ATPase pump expelling protons to regulate cytoplasmic at a high energy cost. Therefore ammonium toxicity has most likely played a role in disrupting the regulation of the duckweeds' cytosolic pH, expending their energy supplies and restricting nutrient acquisition.

When the direct effect of pH was tested independently of other variables (including N), results show that *Lemna minor* will grow and remove P better when kept close to pH 7 (Figures 3.2.4.7, 3.2.4.8 and 3.2.4.9). A more concise pH value range for healthy growth and P removal cannot be obtained from this data due to the small number of pH values tested. To be able to approximately model and predict this value would involve testing two or more values in between both pH 7 and pH 10 and between pH 4 and pH 7, providing linear points with which to run regressions.

P removal was similar between all 3 treatments for the first five days. After this the high and low pH treatments stopped removing P (Figure 3.2.4.7). As these particular cultures were supplied with nitrate only as the source of N, this may suggest the ability to cope with H^+ extremes for several days before succumbing to the conditions. Growth and internal P was not measured in between end points for this experiment, however the pH in solution was (Figure 3.2.4.10). This showed that (as in solutions 1B and 2B earlier), the plants were able to manipulate the H^+ of the solution periodically bringing it closer to neutrality, before this capacity was reduced and the plants senesced. The ability of H^+ anion symport is therefore crucial not only for nutrition but can be beneficial to maintain a healthy localised pH also.

3.3.5. The effect of photoperiod

Exploring the effect of photoperiod on the performance of duckweed was seen as an important variable to consider due to the range of photoperiod recorded in the UK year round. From an engineering design perspective, it could also be useful to investigate how the duckweed would perform under constant (24 h) or no (0 h) illumination. Data on photoperiod also needed to be gathered independently from the effects of temperature, which were assumed to play a role in performance and would be considered in the next section. Growth, P removal and P accumulation were measured daily under a fixed temperature of 15°C while testing photoperiods of 0 h, 6 h, 12 h and 24 h. This photoperiod range includes UK winter and summer 24 h averages (6 h and 12 h photoperiods respectively, (MET Office, 2013)). 3 g (FM) triplicate cultures were initially provided with 15 mg P L^{-1} in 100 mL pots and observed for four days.

Photosynthesis is required for plant growth and the light-dependent reactions provide ATP for plants to use as an energy source in fixing carbon and other energy-requiring processes such as nutrient uptake. ATP is indirectly required for the energetic uptake of P across the plasma membrane by way of generating the transmembrane proton gradient to which H^+ cotransport of P is linked (Ullrich-Eberius et al., 1984). This allows P accumulation against a usually steep concentration gradient. Therefore a difference in P uptake would be expected between plants exposed to 6 h and 12 h light but this was not the case with both treatments removing 80 % of P (Figure 3.2.5.1), even though plants grown under 12 h photoperiods produced significantly more biomass than those under 6 h (Figure 3.2.5.2). Plants maintained in darkness did not grow but also removed P for up to two days. These findings suggest that P uptake and photosynthesis are not exclusively linked temporally and that maintaining exponential or even constant linear growth is not necessary for P removal. The duckweed must be kept alive and healthy, but the accepted paradigm that duckweed could only be used in tropical or sub-tropical conditions (promoting the best growth) for P recovery may warrant further scrutiny (temperature effects notwithstanding).

Lasfar et al. (2007) reported no significant effects on growth rates by photoperiods between 2 to 20 h, although they exposed their plants to an average light intensity of 371 µmol m^{-2} s^{-1}, compared to 155 µmol m^{-2} s^{-1} in the present study. 371 µmol m^{-2} s^{-1} is higher than the stated light saturation point of *Lemna* at 342 µmol m^{-2} s^{-1} (Lasfar et al., 2007) and this was more than double the energy supplied in this study, therefore this may have been an unwitting controlling factor in that publication. These results could have impact for the possibility of using duckweed for phytoremediation in the UK, where average (24 h) summer and winter photoperiods are 12 h and 6 h respectively (MET Office, 2013). It is difficult to estimate light intensity throughout the seasons, as this depends on cloud cover which of course can vary at any point. Therefore to estimate that light intensity will be better in winter than in summer is not recommended. Cold clear days are often allow more solar radiation to penetrate down to the Earth's surface than warm humid days. Full sunlight has been reported from 1400 to 2200 µmol m^{-2} s^{-1} (Ritchie, 2010, Wedge and Burris, 1982) with photodegradation of *Lemna* sp. occurring from 300 to 600 µmol m^{-2} s^{-1} depending on temperature (Wedge and Burris, 1982). Therefore some degree of shade protection may be required on bright days for a large scale system operating outdoors, although the fast reproduction of duckweed when all other conditions are optimal may would see any photo damaged fronds rapidly replaced.

Respiration occurs in the cell continuously, but under times of stress becomes more important in providing ATP independently of photosynthesis (Rebeille et al., 1984) and this could explain how during darkness the plants were able to continue removing P from solution for 48 h. This energy source would have expired in the absence of light, explaining why P removal did not continue. Conversely,

plants exposed to constant illumination would over time be expected to sustain damage to photosystems from over excitation (if light intensities were high enough), typically bleaching the plants, reducing ATP production and concomitantly the proton gradient required for P uptake . Yin et al. (2015) found a decrease in growth rates of duckweed grown for 39 days under 24 h photoperiods when exposed to light intensities above 110 µmol m^{-2} s^{-1}. The apparent lack of growth (Figure 3.2.5.2) and cessation of P uptake (Figure 3.2.5.1) after two days may be due to a combined effect of prolonged photoperiod and the particular exposure of 160 µmol m^{-2} s^{-1}. Phosphorylation of ADP to produce ATP required to maintain proton gradients under times of stress (constant darkness or illumination) would use up cytosolic P. This appears to be the case in Figure 3.2.5.3, where for plants exposed to 0 h or 24 h photoperiods, P accumulation *in planta* increases initially but internal concentrations decrease from day 2 onwards. Therefore while some P removal can occur independently of normal photosynthetic reactions, the plants cannot be forced to tolerate constant or zero illumination and a balance must be observed to maintain the health of the plants and prolong growth and P removal.

The relatively low light energy required for growth and P removal activity however (daylight is in the 1,000's of µmol m^{-2} s^{-1}), could be facilitated by LED lighting for example incorporated into layers or stacks of duckweed tanks, reducing the footprint of a system drastically. As temperature is known to fluctuate annually in cool temperate countries at the same time as photoperiod, this variable would need to be tested next independently of photoperiod to negate synergistic or antagonistic effects.

3.3.6. The effect of temperature

In the previous section it was shown that photoperiods between 6 h and 12 h had no effect on P removal when the temperature was fixed at 15°C, and that providing constant or zero illumination limited P removal to two days. Temperature will fluctuate in cool temperate countries as will photoperiod, therefore it was important to test this variable under a photoperiod pertinent to the UK and one that was not limiting to performance. Experiments were conducted over four days, with a fixed photoperiod of 6 h, 160 µmol m^{-2} s^{-1} light intensity and triplicate 3 g (FM) cultures inoculated into 100 mL of solution initially containing 15 mg P L^{-1}. P removal, growth and P accumulation *in planta* were measured at 8°C, 15°C and 25°C. Temperatures were chosen to include simulated UK averages (24 h) of winter and summer (8°C and 15°C respectively, (MET Office, 2013)).

P removal was clearly temperature dependent (Figure 3.6.1) but growth of plants kept at 15°C and 25°C was similar (Figure 3.2.6.3). Lasfar et al. (2007) report a significant decrease in duckweed growth at temperatures <10°C which is what was also observed in the present experiment. Conversely however they state an optimum range for growth of between 23°C and 28°C, with rates decreasing significantly as temperature dropped to 15°C. However the authors used densities of 0.5 to 1 kg

duckweed (FM) m^{-2}, which would leave more room for growth than the densities used in this study. Thus a possibly unwitting controlling factor for growth could be the stocking density used presently. Lasfar et al. (2007) and Driever et al. (2005) both claim a maximum limit density of duckweed to be 180 g dry mass m^{-2}. As duckweed dry mass can be 3-14 % of fresh mass (Landolt and Kandeler, 1987), this would mean a fresh mass limit range of 6-1.3 kg m^{-2} fresh mass respectively. Initial inoculations used in the present experiments were 3 g (FM) and experimental pots had a surface area of 1735 mm^{-2}, so stocking density extrapolates to 1.73 kg fresh mass m^{-2} and falls within this proposed limitation range. Therefore growth in the 15°C and 25°C experiments may have been halted by lack of space, unwittingly controlling this variable and preventing a viable conclusion as to how temperature affects growth under these conditions. Even if growth was unwittingly controlled in this manner however, the suggestion that growth and P removal are not constantly linked is still viable due to differences in P removal and could even be a beneficial aspect for a large scale system. The less biomass required for P removal the better as this would mean a smaller land footprint of a system and less biomass processing to maintain the system.

This taken with the results from the previous section suggests that the mechanism for P removal does not rely on the plants' rapid growth but the activity of uptake transporters in outer cell membranes alone. These chemical/biochemical reactions would be expected to increase in rate with an increase in temperature which is exactly what happens. If photoperiod and therefore growth (within extreme limits) does not limit P removal then the limiting factor may be a nutrient (N or P). Experimental pots were topped up daily with fresh growth solution containing all nutrients (including N) and therefore N was not expected to be the limiting factor, whereas P was removed from the solution by all cultures in this experiment, which would reduce the external supply so the limiting factor could be P supply or internal stores being at full capacity.

Transport of P is highly temperature dependent (Figure 3.2.6.6). Q_{10} values calculated for transport were much higher than those for accumulation, the latter measurements being much cruder and less specific. This would suggest that initial transport into the cell is more temperature dependent than accumulation, probably owing to the many steps in transporting P from the external environment to the vacuole (known for storage of P). Membrane fluidity is affected by temperature (Murata and Los, 1997) which in turn affects the rate of conformational changes that transport proteins must undergo during uptake. This is supported by the increased rates of transportation over the rates of accumulation (Table 3.2.6.5).

Transport rates are comparable with (Ullrich-Eberius et al., 1984) who reported (following conversions for comparison) uptake of 52,000 nmol P mg $(FM)^{-1}$ h^{-1}, in comparison to 67,000 nmol P mg $(FM)^{-1}$ h^{-1}

in the present experiment. Both experiments were conducted at the same temperature (25°C) but used distinct species (*L. gibba*) and different concentrations of P in experimental perfusion solutions (Ullrich et al used 3x less). The results for uptake rate reported by Hase et al. (2004) (0.005 nmol P mg $(FM)^{-1}$ h^{-1}) are not comparable to the present study, possibly owing to their using duckweed of a different genera (*Spirodela oligorrhiza*), maintaining plants in more than 10x more P (possibly saturating transport capacity), illuminating their plants at just 80 µmol m^{-2} s^{-1} and fixing perfusion solutions to pH 8.5.

Taking the photoperiod and temperature experiments together, simulated conditions of UK winter and summer were applied (6 h PP, 8°C and 12 h PP, 15°C respectively, (MET Office, 2013)). The results so far showed that under these conditions the duckweed could only remove 60 % to 80 % of P under winter and summer conditions respectively. This would not be low enough for a hypothetical system that would need to meet low discharge consents (0.1 mg P L^{-1}) and the energy required to maintain a system at 25°C would be prohibitively expensive. As previously referred to, P supplied in external solution may have been the limiting factor to P removal. To investigate the possibility of P supply dictating P removal, the capacity of duckweed to store P and to see if the duckweed were able to reduce concentrations of P further, performance needed to be measured within a range of P concentrations while temperature and photoperiod were fixed.

3.3.7. The effect of P provision

To investigate the effect of P supply on the performance *Lemna minor*, triplicate 3 g (FM) cultures were inoculated into 100 mL pots initially containing 5, 15 or 30 mg P L^{-1} and observed for 4 days. It was not practical to replenish P in the batch pots back to these initial values, so it is accepted that concentrations of P would reduce once more as the experiments continued. The effect of a constant P supply would be investigated in the next Chapter. Temperature during this experiment was 15°C and photoperiod was 12 h which simulated UK summer averages. The stocking density of 3 g (FM) in the previous experiments was changed to 0.2 g in an attempt to find out if increasing P supply increased growth and if the same density as previous experiments would be reached by the end of this one. If final stocking densities were similar, this would suggest that a maximal density was controlling growth (as discussed in the previous section). P removal, growth and P accumulation *in planta* were measured. Following this experiment, ^{32}P uptake experiments were conducted that measured direct uptake of radiolabelled P for 30 minutes (linear uptake phase) at 22°C when supplied with 0 to 12.4 mg P L^{-1} (0 to 400 µM P).

Increases in biomass were all similar (Figure 3.2.7.5), increasing from 10 mg (DM) to between 33 and 42 mg (DM), which provided a density that covered the surface of experimental pots fully. These

similar increases suggest that increasing P provision under these conditions either did not increase growth or that once 100 % coverage is reached, growth is constrained by lack of space. The first suggestion cannot be addressed appropriately as growth was not measured in between the start and end of the experiment, therefore it is not known if growth rates were faster between P treatments towards the beginning of the experiment. The latter point is not thought to be significant in this particular experiment as previous experiments (photoperiod, temperature and conductivity) have shown that initial 3 g (FM) cultures can increase their density further (by up to 20 %), so it would appear that a space limiting factor has not been reached in the present experiment. The density reached presently extrapolated to between 420 and 520 g (FM) m^{-2}, which is well below the maximal densities proposed by (Lasfar et al., 2007) as discussed earlier.

Under the conditions applied, increasing external P increased accumulation *in planta*, but final removal rates were similar between the 15 and 30 mg P L^{-1} provided plants (Figure 3.2.7.3). As a consequence, a higher overall proportion of P was removed from solution by the plants provided with less P (5 mg P L^{-1}). This suggests that if a (hypothesised) duckweed system was required to meet low P discharge consents (0.1 mg P L^{-1}), then under these conditions concentrations would need to be less than 5 mg P L^{-1}. It is accepted that small scale controlled experiments are far from an outdoor pilot system, but this is stated simply for tentative informative purposes. If P recovery (for later recycling) was the aim of a system, then increasing the P in solution would be more favourable. Therefore design considerations at this stage appear less straight forward than a single duckweed tank removing P to very low concentrations. The transport capacity of the duckweed appears to be saturated but the actual storage capacity has not yet been reached.

Concentrations of P *in planta* increased with external P (Figure 3.2.7.4) and values were high in comparison to other species. All plants commenced at 1.4 % P (% of dry mass represented by P) and plants provided with 5, 15 or 30 mg P L^{-1} increased their content to 1.6 %, 2.1 % and 2.6 % respectively. Kant et al. (2011) state the % dry mass of plants as P is typically 0.2 %. An average range is said to be 0.1 to0.5 % as Dry matter by Mahler (2004), with critical and toxic values of 0.14 and 0.36 % dry mass as P in Maize suggested by Zia et al. (1988). In Kant et al. (2011), *Arabidopsis* plants accumulated P up to *c*. 2 % of Dry matter, whereby the plants developed chlorosis and necrosis from leaf margins before dying. The negative side effects from the over accumulation of P in terrestrial plants can be a result of iron deficiency (Nichols, 1988). Depending on pH, iron is often low in availability in soils due to being locked up with metal cations and organic compounds (as is often the case for P) (Nichols, 1988). If plants that are adapted to low Fe and P suddenly take up excess P, this can form $FePO_4$ in the plant tissues, rendering the Fe unavailable (Hendreck, 1991). Symptoms include interveinal chlorosis and reddening of the leaf margins (Nichols, 1988). All growth solutions used in this experiment utilised the

iron chelator EDTA, which keeps iron available to the plant in a pH range of approximately pH 4 to pH 8 (Reed, 1996). Interestingly, Kant et al. (2011) also used EDTA in mineral growth solutions and still reported toxic symptoms, yet no negative side effects of P accumulation were found in the present study. This has positive impact for a proposed P recovery system that could in theory be accumulate more P as a % than current plants used for wastewater treatment (such as reed beds or water hyacinth).

In general plants respond to phosphate fluctuations by varied strategies to increase uptake of this essential macronutrient. This includes induction of phosphate transporters that have high affinity (i.e. can scavenge P at low concentration) and secretion of phosphatases to release P from organic molecules. While this has not been studied extensively in duckweeds, induction of high affinity P transport capacity (Ullrich-Eberius et al., 1984) and a Pi transporter (Hase et al., 2004) by low Pi has been reported. Measurement of Pi uptake presently also demonstrated low and high affinity uptake modes (Figure 3.2.7.6).

Ullrich-Eberius et al. (1984) reported dual uptake affinities in *Lemna gibba*, with Km's of 2 to 27 µM P and 21 to 24 µM P for apparent high and low affinity systems respectively. They also reported no difference in Km between light and dark maintained plants, which would support the results in Figure 3.2.5.1. In the present study, Km's of 7 µM P and 170 µM P (Table 3.2.7.1) were calculated from a Lineweaver-Burke plot also describing high and low affinity uptake capacity (Figure 3.2.7.6). These numbers are reasonably comparable taking into account the necessary numerous conversions, differences in experimental methodologies and species used, but most importantly, the authors' plants had been starved of P for several days before those experiments. Interestingly, a large difference between P starved and P replete plants in uptake of P was reported, with replete plants taking up 52 nmol P g $(FM)^{-1}$ h^{-1} and starved plants taking up 406 nmol P g $(FM)^{-1}$ h^{-1} (Ullrich-Eberius et al., 1984). This suggests that reducing external provision of P promotes P uptake activity. Experiments conducted in the present study typically exposed plants to 15 mg P L^{-1} (with the exception of P provision experiments), and stock plants used in all experiments were maintained on solutions containing 15 mg P L^{-1} and which was regularly refreshed. This was seen as a necessary control for experiments but was now proposed as factor that prevented the duckweed from removing P to the very low concentrations that would be required in a hypothetical P removal system.

3.3.8. The effect of P acclimation

As results so far had suggested a possible limitation of P removal by the amount of P provided, it was seen as necessary to investigate the effect of P acclimation on internal P concentrations (*in planta*) and in turn how this may affect P uptake. Triplicate 3 g (FM) cultures were inoculated into solutions containing 0, 1, 2, 5 or 15 mg P L^{-1} and internal P was measured for 4 days. Following this, all plants were re-inoculated into solutions all containing 15 mg P L^{-1} and internal P was measured once more, with the addition of P in solution being measured also. Conditions were 12 h photoperiods and 15°C to simulate UK summer conditions and one additional treatment acclimated to 1 mg P L^{-1} was maintained at 8°C and 6 h photoperiods to simulate UK winter conditions.

The pre-incubation of *Lemna minor* at lower P concentrations resulted in depletion of internal reserves (Figure 3.2.8.1), most likely due to the recycling of vacuolar or cytosolic P for energy supply and biosynthesis. The vacuole is known to be a large storage compartment in the plant cell that sequesters P for later remobilisation back into the cytosol (Shen et al., 2011), and was shown in ^{31}P NMR experiments (Lauer et al., 1989). In Arabidopsis, expression of a vacuolar phosphate transporter protein (VPT1) localised in the tonoplast was induced under conditions of high Pi, but when mutants lacking this transporter were grown, the ability to adapt to low and high P conditions by way of vacuolar influx or efflux of P was reduced, causing growth defects (Liu et al., 2015). It was suggested that the majority (>90 %) of Pi found in plant cells is in the vacuole (Bieleski, 1973), and although analysis of Pi *in planta* in the present study was not selective for cell organelles, but rather a whole-plant extraction from fully homogenized samples, it seems likely that the decline in internal Pi is principally from vacuolar stores. LEE and Ratcliffe (1993) grew Maize roots at 3µM P (0.09 mg P L^{-1}) and estimated more P in the cytosol than the vacuole, while P sufficient Barley leaves showed continued vacuolar accumulation dependent on concentration up to 6.4mM (Mimura et al., 1990). Using a Pi analog (MeP), Pratt et al. (2009) showed accumulation of Pi into the cytosol initially blocked Pi efflux from the vacuole, but a continued supply of Pi into the cytosol initiated transport across the tonoplast for storage. Pratt et al. (2009) suggested that the management of Pi homeostasis initially comes from signals in the cytosol. Interestingly internal Pi concentration was very strongly correlated with P uptake (R^2 = 0.95, Figure 3.2.8.3) suggesting this is an important sensor that stimulates P uptake when the plants are returned to conditions of plentiful P, allowing more rapid and complete P removal (Figure 3.2.8.2).

Internal Pi depletion facilitated the removal of P to well below UK discharge consent concentrations of <1 mg P L^{-1} (UK TAG, 2013), even when provided with 15 mg P L^{-1}. This was achieved under simulated summer and winter conditions of combined photoperiod and temperature and is a significant step in highlighting duckweed's potential for P recovery in the UK or any cool temperate country.

Concentrations of P in secondary effluent are often 1-2 mg P L^{-1} and hypothetically, this would provide the appropriate media to maintain a duckweed stock with a low internal Pi, for transferral and use in P removal from upstream wastewater fractions (higher in concentration) down to concentrations of µg P L^{-1}.

3.3.9. Main conclusions

Under the conditions applied the duckweed has shown promise to warrant further examination of performance at a larger scale with more operational variables and more realistic environmental conditions in which axenic cultures are hard to keep, particularly when using wastewater. The results are considered in the context of the duckweed's viability for use as a P removal (or recovery) system operating outdoors, therefore the conclusions drawn here are tentative and simply lead in the right direction for further development of the research project. An appropriate species was selected and positively identified and methods to track P in experimental systems were tested and accomplished. *Lemna minor* appears to be able to cope with conductivities higher than those found typically in most fractions of wastewater, with an apparent growth threshold of approximately 3,000 µS cm^{-1} but positive P removal for several days under more saline conditions. Nitrate appears to be more favourable to the duckweed when present as the only source of N, but experiments containing a mixture of nitrate and ammonium may inform more, as would experiments that used a neutral pH with lower concentrations of ammonium than tested presently. As most WWTW's in the UK are nitrifying, the results are positive as there would not be the need for denitrifying process and a duckweed system would also help to control ammonium discharges if nitrification units were to underperform. It is difficult to separate out the effects of ammonium and pH, but it was shown that the duckweed will not tolerate a low pH and small amount of ammonium simultaneously, as the ammonium may interrupt the regulation of the cellular H^+ gradient.

Photoperiods and temperatures pertinent to a cool temperate climate were favourable to the duckweeds' existence and removal of P, with increases in temperature promoting increased P removal. As P removal occurred both during the dark (where plants did not grow) and under constant illumination (where plants would eventually become light-stressed) periodically, it was suggested that active reproduction and P removal were not exclusively linked, or that P removal could at least occur independently of growth. This hints at the potential for using duckweed year round in cool temperate climates. The relatively low light energy required for the duckweed to operate would also favour cloudy dull days in the field or open the possibility of 'stacked' duckweed tanks illuminated by low energy LED lamps to reduce the physical footprint. Evidence for *Lemna minor* employing high and low affinity P uptake systems was found, which has been previously shown in *L. gibba.* This proves that manipulating the external P causes a physiological change to the plants' uptake activity and could be

better quantified by conducting experiments that characterised which transport proteins were being expressed under variations of P provision.

A major breakthrough in terms of the project's aims was the discovery that reducing the supply of external P (to concentrations regularly found in wastewater) reduced internal reserves, which acted as a signal to upregulate P uptake. This upregulated activity resulted in UK P discharge consent concentrations being beaten under highly controlled simulated conditions of both summer and winter. The effect of reducing P on P removal was stronger than the effect of raising the temperature, which is positive from a cost point of view when hypothesising a duckweed treatment system.

In terms of the project's aims the results were positive and provided the confidence with which to move to Phase 2 and develop the project, by increasing to mesocosm scale and introducing a constant flow of growth solution before replacing this with real wastewater collected from Esholt WWTW (Bradford, UK) and reassessing performance.

CHAPTER 4

Phosphate removal by *Lemna minor* in a continuous flow treatment system at mesocosm scale: investigating the effect of P loading rates and temperature while comparing growth solution and wastewater.

4.1. Introduction

In the previous chapter it was shown that under the conditions applied, the duckweed would remove more P from solution when the amounts of P stored in the seeding duckweed inoculum were reduced. This internal reduction was achieved by acclimating duckweed to a reduced concentration of P in the external solution. When pre-incubated to 2 mg P L^{-1} or less, the duckweed was able to remove P in small batch systems from mg L^{-1} down to µg L^{-1} concentrations in four days under photoperiods and temperatures pertinent to cool temperate climates. Also apparent was that phosphate transport capacity could be saturated by high concentrations of P but internal cellular concentrations continued to rise and a toxicity threshold was not reached. This data was important to realise in the context of a hypothetical duckweed system appended to a WWTW receiving a continued supply of P. Questions remained such as how much of a constant P supply could the duckweed cope with and remove? How much of an effect did temperature have on performance under these conditions? Photoperiod and temperature did have an effect on P uptake and growth in batch experiments but only at the extremes tested (extremes relative to a cool temperate climate). The duckweed could cope with conductivity similar (and higher) than that regularly found in wastewater and providing the pH was close to 7, could metabolise various forms and concentrations of nitrogen. This information along with that of numerous studies published previously under tropical and sub-tropical conditions, suggested that the duckweed would grow on wastewater and remove nutrients. But for the present project it was important to know how they would do this under conditions more similar to a cool temperate climate. The results from the previous chapter were positive in view of the progression of this industry-partnered project and helped to inform the design of the next phase reported in this chapter.

The next logical step was to identify how the duckweed would cope when exposed to a continuous supply of phosphate containing solution (as opposed to the previous batch conditions); and how they would perform on real wastewater. While exploring this, conditions of temperature and P loading rates would need to be fixed and tested. As in the previous chapter, there were several other variables that are alluded to in the literature that were worthy of investigation, such as varying and testing a duckweed harvesting regime, stocking densities or organic loading rates. But changing each of these variables would require an acclamatory period before measurements could start to be made.

Therefore due to limited time, the variables that were considered the most important to test were temperature, P loading rates and comparing performance on growth solution with wastewater under conditions pertinent to the UK and cool temperate regions in general.

It is difficult to compare the many publications relating to the use of duckweed for nutrient removal or production of biomass, due to the variability in conditions during experiments, the range of parameters tested and methods used in generating data (Alaerts et al., 1996, Mohedano et al., 2012, Soda et al., 2013) (Table 4.1.1). However, the capacity of duckweed to take up nutrients from wastewater and produce biomass exponentially, under tropical and sub-tropical conditions is well documented (Verma and Suthar, 2014, Fujita et al., 1999, Hammouda et al., 1995). Experiments on waste stabilisation ponds have been conducted in some temperate countries with average winter air temperatures of 8°C (Faleschini et al., 2012), but the authors did not report the presence of duckweed. Duckweed has been reported (anecdotal) to die off in Northern Italy during winter months (Bonomo et al., 1997). What is presently lacking in the literature are robust empirical experiments on how duckweed would perform under cool temperate conditions including those of the UK.

Experiments conducted for the present chapter required a moderate upscaling of the experimental dimensions and volumes used earlier, but as many variables as possible were retained from the previous chapter in order to provide results that were as related as possible. The same duckweed isolate was used, photoperiod was fixed to 12 h, the same modified Hoagland's growth solution was prepared in bulk, light intensity was 160 µmol m^{-2} s^{-1} and temperatures tested were 5°C, 8°C and 15°C. Following (and with exception to) an initial ranging experiment (see Section 4.2.1) which set out to test various experimental methodologies and generate initial performance data, experiments were conducted using growth solution at 15°C, 8°C and 5°C (see Sections 4.2.2, 4.2.3 and 4.2.4 respectively); and then they were switched to real wastewater at 5°C, 8°C and 15°C (see Sections 4.2.5, 4.2.6 and 4.2.7 respectively). These six experiments all used the same flow rates, hydraulic retention times and P loading rates. Section 4.2.8 then collates the main data in Tabular form for ease of final comparisons. Figure axes for all experiments use the same scales for visual comparison.

Table 4.1.1. Comparison of P removal performance in various experiments using wastewater. Data provided or derived from information given in each publication listed.

Removal rate (mg P m^{-2} d^{-2})	Loading rate (mg P m^{-2} d^{-2})	Temperature (°C)	Photoperiod (h)	Stock density (kg FW m^{-2} d^{-2})	Species	Author(s)	Critique
40	Max. 123	19-28	11	1.6	*	(Alaerts et al., 1996)	Poor FW determination method. Large temperature range. Duckweed species not reported.
360	390	*	*	0.2	*L. punctata*	(Mohedano et al., 2012)	No specific climate data reported.
0.08	78	20	16	0.2	*W. arrhiza*	(Soda et al., 2013)	Influent flow rates variable.
0.04	40	20	16	0.2	*W. arrhiza*	(Soda et al., 2013)	FW determination not clarified.
0.02	20	20	16	0.2	*W. arrhiza*	(Soda et al., 2013)	

*No data provided

4.2. Results

4.2.1 Ranging experiment

The ranging experiment was conducted to allow the duckweed time to acclimate to the new conditions and to estimate growth and P removal performance. This was required before subsequent experiments (see Sections 4.2.2 to 4.2.7) so that they could be designed to be more focused and allow the measurement of more parameters while within similar time frames. The ranging experiment set up used three modified Hoagland's solutions (See Chapter 2 for composition), each with the same composition except the concentration of P, which was 15, 10 and 5 mg P L^{-1} respectively provided to tanks T1, T2 and T3 (Figure 4.2.1.1). Nitrogen was supplied as nitrate at 50 mg N L^{-1} and the pH was initially fixed to 7 but not buffered. A multi-lined peristaltic pump supplied the solutions to three separate 10 L tanks, at a flow rate of 3.3 L d^{-1}, providing a theoretical hydraulic retention time (HRT) of three days. Tanks had a small mid-baffle fixed to the bottom and were assumed to be complete mixing reactors. Effluent exited by gravity overflows that prevented the loss of duckweed by a small baffle (see Chapter 2 for detailed designs). 100 g (fresh mass) of *Lemna minor* was originally seeded into each tank from one large original stock. Over the 20-day experiment, effluent phosphate was measured every two days and the amount of biomass was recorded at days 10 and 20. Temperature was recorded every 4h in each tank remotely using waterproofed data loggers (Thermochron iButton, Ref: DS1922L-F5#, Embedded Data Systems, Lawrenceburg, KY, USA).

The bulk formulation of each influent was successful and the specified concentrations of P supplied to each tank were within acceptable ranges (Figure 4.2.1.2). As the duckweed grew and acclimated to conditions, P was removed steadily until steady state conditions were reached at day 12 for all three tanks and removal rates were similar between all tanks also (Figure 4.2.1.3). Tanks T1, T2 and T3 removed 4 4%, 71 % and 87 % of their respective P loads (Figure 4.2.1.4) with tank T3 producing an effluent with a 97.5th percentile value of <1 mg P L^{-1} (Table 4.2.1.1). On a surface (m^{-2}) basis, tank T2 removed more P than tank T1, while being supplied with 50 % less (Table 4.2.1.1).

Increasing the provision of P increased the production of biomass, with tanks T1, T2 and T3 increasing by 160 %, 152 % and 120 % after the first 10 days respectively. This pattern was similar over the next 10 days, with tanks T1, T2 and T3 increasing by 172 %, 160 % and 140 % respectively (Figure 4.2.1.5). The original stocking density of 100 g was returned to this value at day 10 to maintain healthy growth while retaining a full surface cover to minimize algal proliferation. Production correlated highly to P supply at day 10 (R^2 = 0.89) and more so at day 20 (R^2 = 0.98).

The experimental setup meant that temperature was stable throughout the experiment with an average range of just 3°C between day and night within the tanks over the whole 20 days (Figure

4.2.1.6). The results from this initial ranging experiment informed the design that was retained for all experiments that follow in this chapter. It was decided to change the configuration to connect the three tanks in series, with tank T1 receiving an effluent containing 10 mg P L^{-1}. This was chosen as it promoted the most P removal on an area basis, was the highest concentration to be expected in a UK WWTW following secondary treatment (including nitrification) and also sustained growth while leaving space for more (Tank T1 produced more over the same area), should this be facilitated by changing variables in subsequent experiments. An apparent steady state was expected to be reached in approximately ten days, therefore the experimental duration was kept at twenty days.

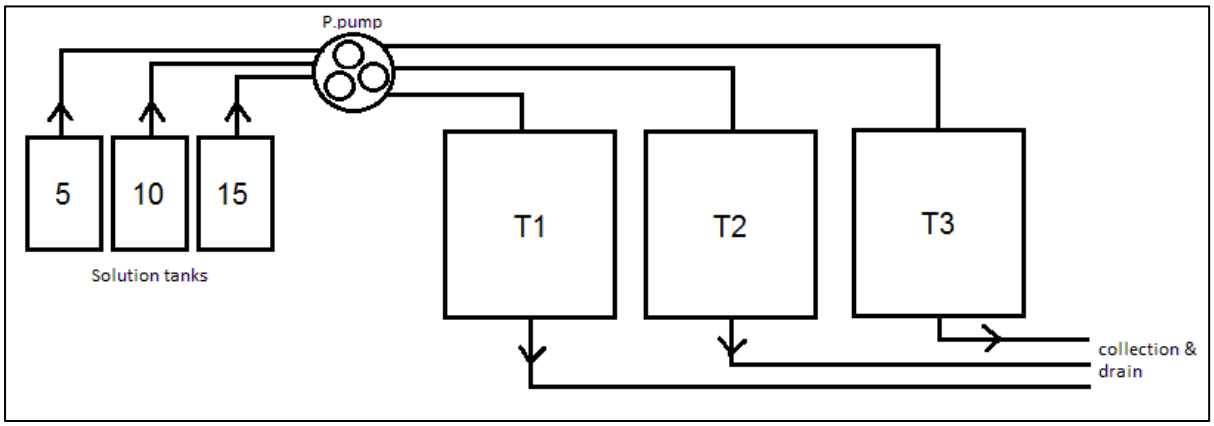

Figure 4.2.1.1. Hydraulic set up for ranging experiment. Hoagland's solution was prepared in separate opaque plastic drums at 5, 10 or 15 mg P L^{-1} ('solution tanks' '5', '10' and '15' respectively) and supplied at the same flow rate of 3.3 L d^{-1} by a multi lined peristaltic pump to opaque plastic duckweed tanks 1, 2 and 3 respectively. Tank volumes were 10 L with a surface area of 0.1 m^{-2}.

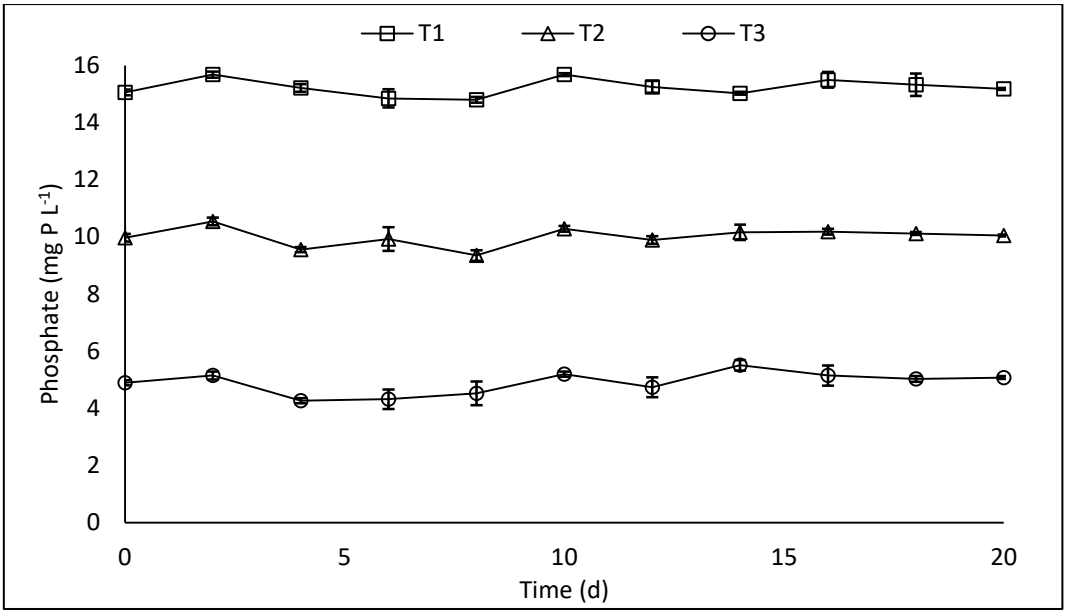

Figure 4.2.1.2. Influent phosphate (mg P L^{-1}) supplied to duckweed tanks T1, T2 and T3 for ranging experiments. Influents were made in bulk every few days but monitored to discount any sedimentation or adsorption in the drums. Error bars are standard error of the means.

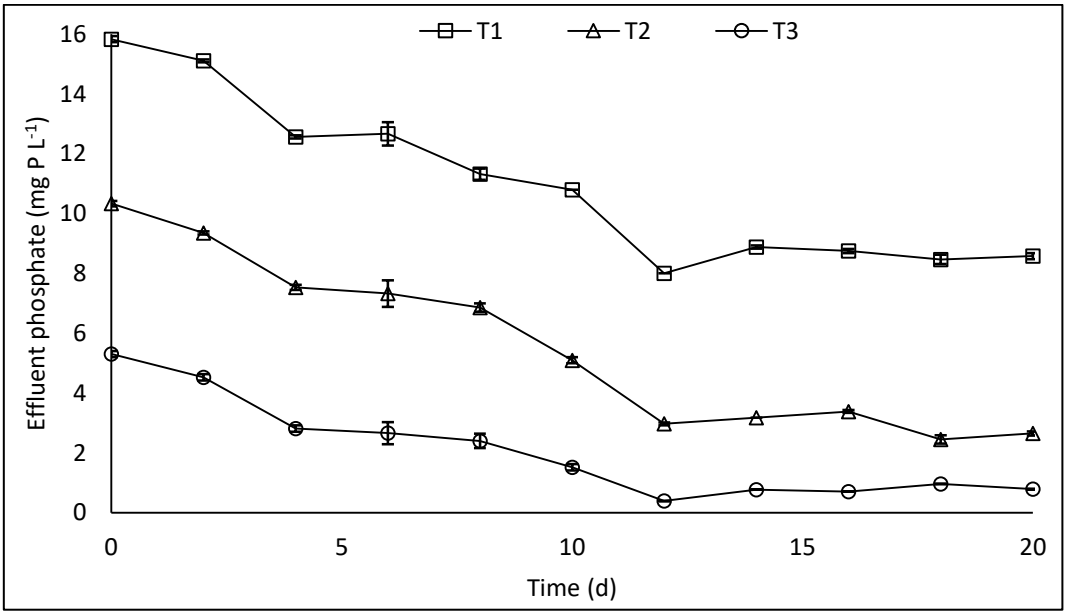

Figure 4.2.1.3. P concentration in the effluent of tanks T1, T2 and T3 from ranging experiments using *Lemna minor* at 15°C. Effluent samples were taken prior to drain discharge (Figure 4.2.1.1), syringe-filtered (0.45 µm Ø pore size) and tested using phosphate cuvette tests (LCK phosphate test kits and DR1900 spectrophotometer, Hach Laing, UK). Error bars are standard error of the means.

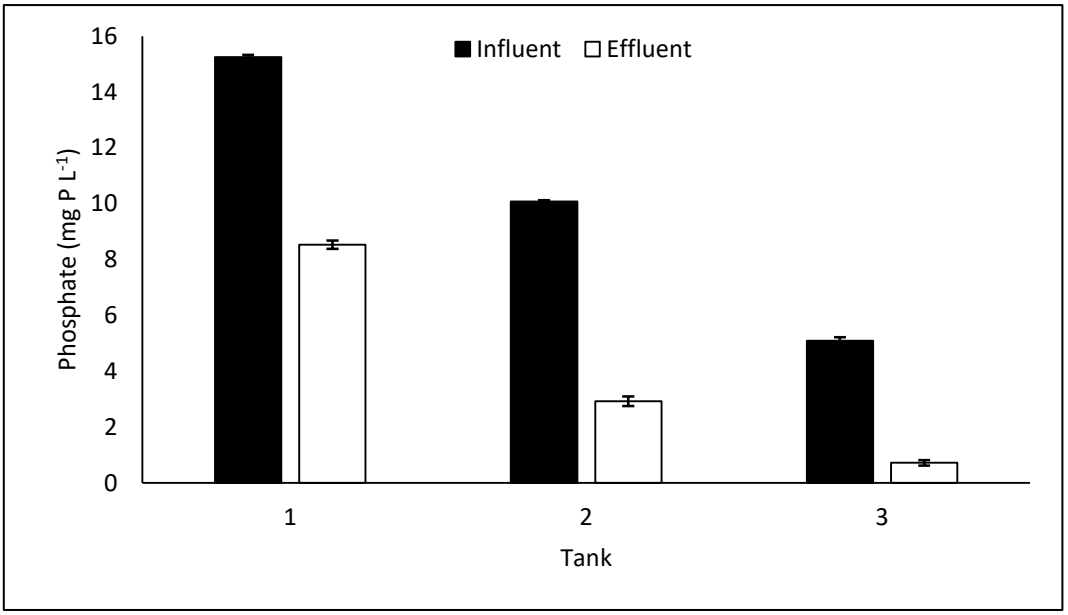

Figure 4.2.1.4. P removal by *Lemna minor* under steady state conditions with variable P supply. Phosphate (mg P L^{-1}) was measured every 2 days during steady state conditions (days 12 to 20) in both influent and effluent fractions and data presented are the means from this period. Error bars are standard error of the means.

Table 4.2.1.1. Mean P loading rates, removal rates and 97.5th percentile effluent values during steady state conditions (days 12 to 20).

	Tank T1	**Tank T2**	**Tank T3**
P effluent concentration, 97.5th percentile (mg P L^{-1})	8.9	3.4	0.9
P surface loading rate (mg P m^{-2} d^{-1})	503	333	168
P surface load removal (mg P m^{-2} d^{-1})	222	236	144
P load removal efficiency (%)	44	71	86

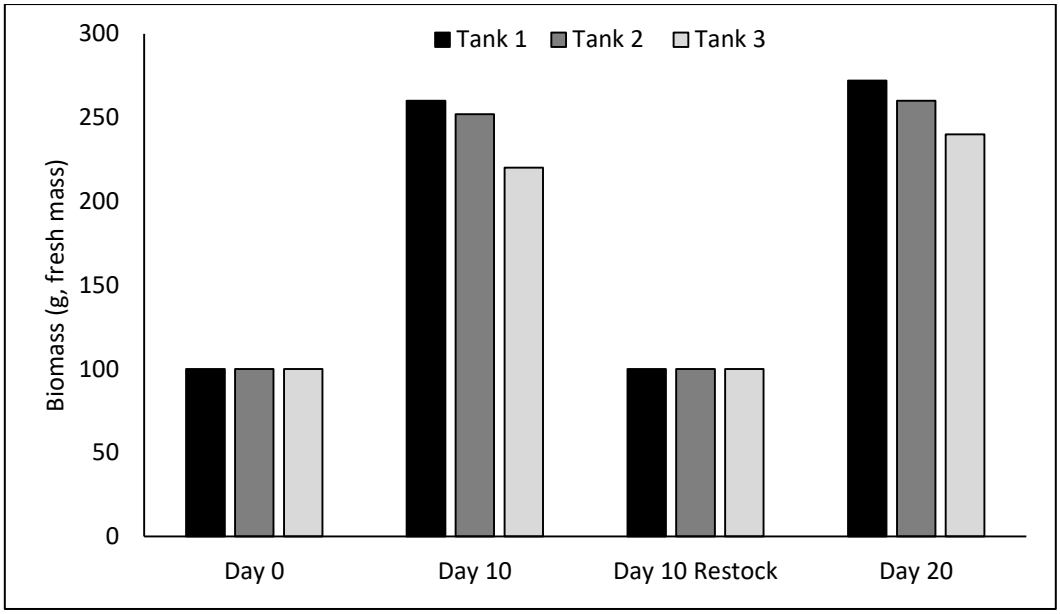

Figure 4.2.1.5. Production of *Lemna minor* biomass at 15°C with varying P supply. 100 g (fresh mass) was originally seeded into each tank at day 0. At day 10, total biomass was estimated and 100 g (FM) was retained in each tank. At day 20 total biomass was estimated once more. Estimations were made by removing 25 % of surface cover, removing excess moisture by spinning and blotting and weighing on an analytical balance and multiplying by 4. Tanks T1, T2 and T3 received 15, 10 and 5 mg P L^{-1} respectively at 3.3 L d^{-1} each. Tank volume was 10 L and surface area was 0.1 m^{-2}.

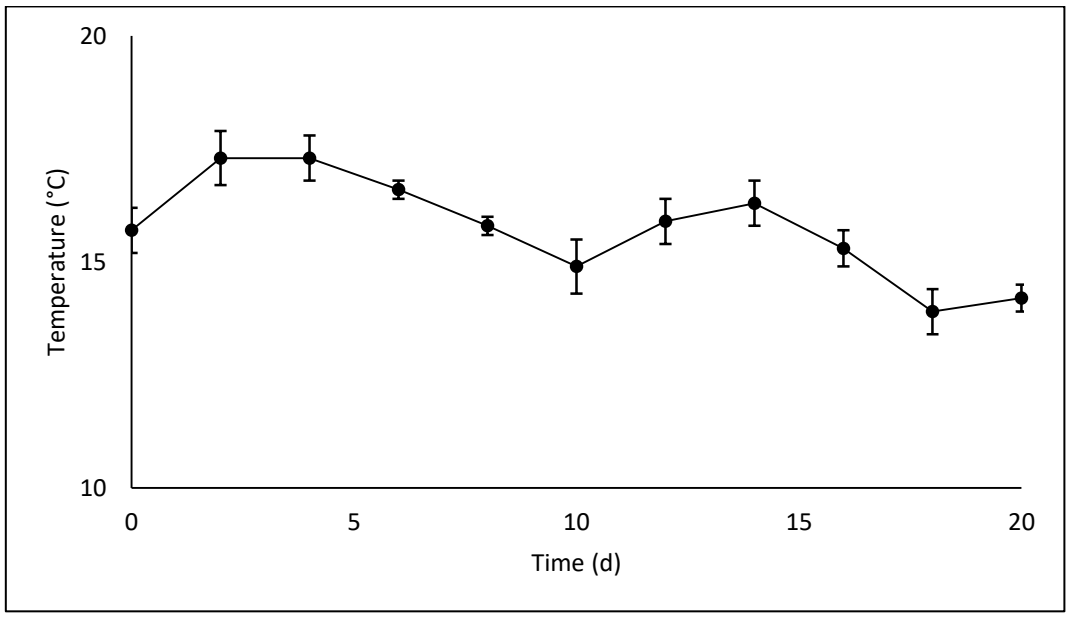

Figure 4.2.1.6. Growth solution temperature. Data are daily means from loggers kept in tanks that recorded temperature every 4 h. Error bars are standard error of the means.

4.2.2. Growth solution (GS) experiments at 15°C

Results from the ranging experiments were encouraging from an applied perspective, in that on average 86 % of P was removed from the solution provided to T3 (5 mg P L^{-1}). This is a concentration that was similar to an average of 4.5 mg P L^{-1} recorded in raw wastewater at Esholt WWTW between 2014 and 2016. A duckweed system hypothetically appended to a WWTW would expect to be regularly exposed to this kind of concentration but variances (spikes from high loads or dilution from heavy rainfall) are also to be considered.

Building on the knowledge gained in Phase 1, where reducing P exposure was shown to improve P removal, experiments were designed to alter the system configuration to series (Figure 4.2.2.1). This allowed three tanks to be connected as an assumed plug flow reactor-like system and would identify if increasing the number of tanks would reduce P in effluents downstream. By default, this set up allows the testing of three P loading rates (at the start of tanks T1, T2 and T3), and when left to operate over several months, allowed the effect of temperature to be monitored, providing temperature remained relatively stable. In reference to the possibility of P load variances in real wastewater, the chosen initial concentration of P to be provided for all subsequent experiments was 10 mg P L^{-1} which was seen as the highest concentration that a UK WWTW would encounter downstream of primary settlement.

Experiment 4.2 was conducted over twenty days with a 24 h average tank temperature during this time of 15°C (with a range of 2°C). For this and all other following experiments of this chapter, flow rate was increased to 10 L d^{-1}, providing an HRT of 1d $tank^{-1}$ or 3d for the whole system. Nitrogen (as nitrate) and pH were the same as previously (50 mg N L^{-1}, pH = 7) and photoperiod was 12 h. P was measured every 2 days in the header tank and points A, B and C of the system (Figure 4.2.2.1). Biomass was estimated at days 10 and 20 and restocked to 100 g (FM) at day 10 as previously. P *in planta* was also recorded at days 0, 10 and 20 in triplicate sub-samples. This experimental setup was replicated for all experiments that follow (4.2.2 to 4.2.7) with the exception of 4.2.5 to 4.2.7 using wastewater instead of growth solution.

Formulation of influent with a stable initial concentration of P was again successful and steady state conditions were again apparent from day 12 onwards (Figure 4.2.2.2). Rates of P removal throughout the whole experiment were similar between tanks T2 and T3 but these were both different from tank T1 (Figure 4.2.2.2 and Table 4.2.2.1). Moving through the system tank by tank, removal of applied P loads was 69 %, 96 % and 35 % respectively by tanks T1, T2 and T3. Performance by tank T1 was similar to tank T2 of the ranging experiment that received the same amount of P (10 mg P L^{-1}) and removed 71 % (Table 4.2.1.1). In the present experiment tank T2 removed 96 % of its load of 3 mg P L^{-1}. This

may suggest that high concentrations of P relative to WW fractions post pre-treatment were not required for luxury uptake and later recovery. Tank T3 had little impact on further P reduction as shown in Figure 4.2.2.3 and by the 97.5th percentile values given in Table 4.2.2.1. If shown consistently this would reduce the hypothesised footprint by 1/3.

Production of biomass was similar between all three tanks which was somewhat unexpected. Tanks T1, T2 and T3 receiving progressively less P increased biomass by 152 %, 140 % and 152 % by day 10, and by 140 %, 152 % and 152 % by day 20 respectively, following the stocking density at day 10 being returned to 100 g (Figure 4.2.2.4). Biomass production was similar between all tanks but duckweed in tank T1 continued to increase P accumulation whereas duckweed in tanks 2 and 3 reduced their accumulation from day 10 to day 20 (Figure 4.2.4.5). The decrease in internal concentrations may be explained by the restocking at day 10 promoting the growth of new biomass with relatively less available P as you move through the system.

From an applied perspective, results were encouraging as the proposed UKTAG target of a <0.1 mg P L^{-1} consent was met continuously under steady state conditions with two duckweed tanks in series, under photoperiods and temperatures of a simulated cool temperate summer. The next objective was keep all conditions the same with the exception of allowing the temperature to fall and repeat the experiments at an average tank temperature of 8°C.

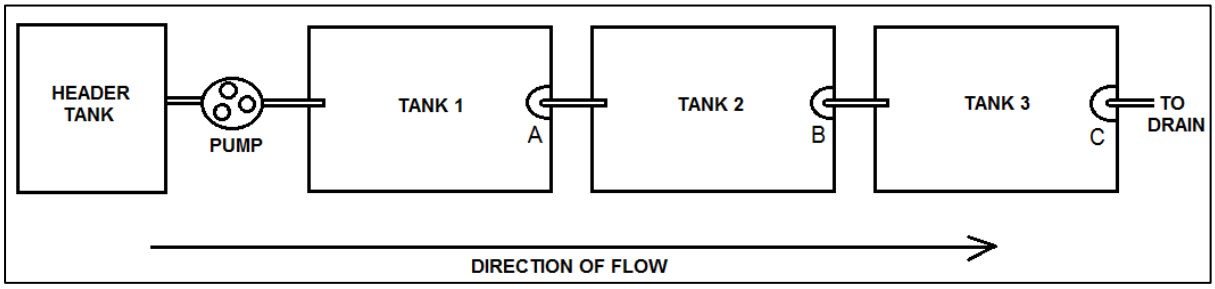

Figure 4.2.2.1. Hydraulic set up for experiments 4.2.2 to 4.2.7. Solution containing 10 mg P L^{-1} was supplied from an opaque header tank via a peristaltic pump through the three tank system connected in series. Each tank had a volume of 10 L and surface area of 0.1 m^{-2}. A, B and C were the baffled points for sampling and analysis.

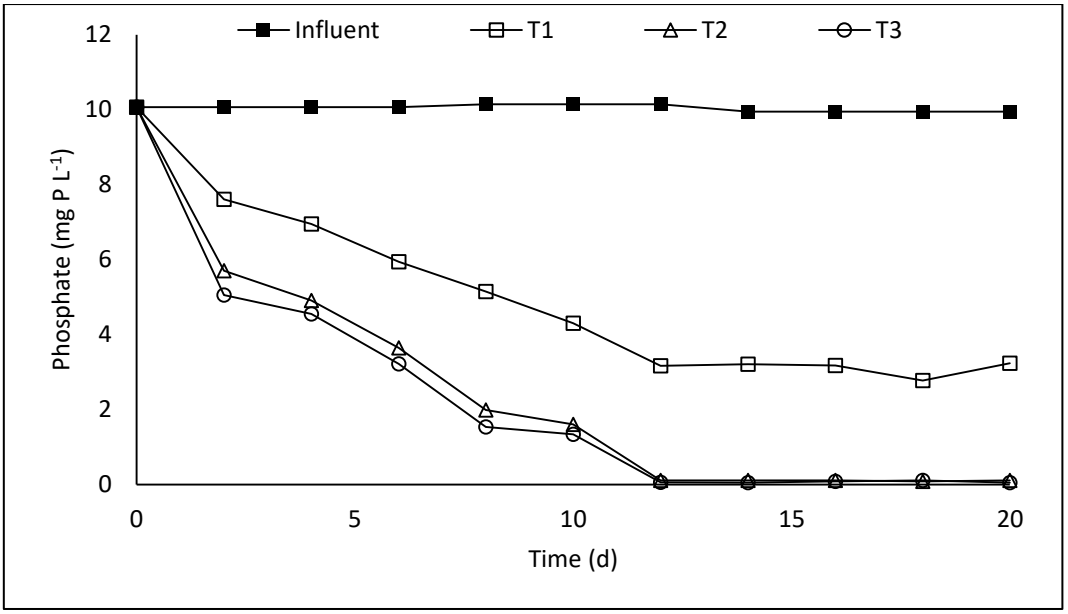

Figure 4.2.2.2. P removal from growth solution by *Lemna minor* at 15°C. Influent and subsequent effluents P concentrations (mg P L^{-1}) produced by duckweed tanks T1-T3 connected in series. Effluent samples were removed from points A, B and C (Figure 4.2.2.1), syringe-filtered (0.45 µm Ø pore size) and tested using phosphate cuvette tests (LCK phosphate test kits and DR1900 spectrophotometer, Hach Laing, UK). Influent was added to a header tank and spiked with KH_2PO_4 to 10 mg P L^{-1}.

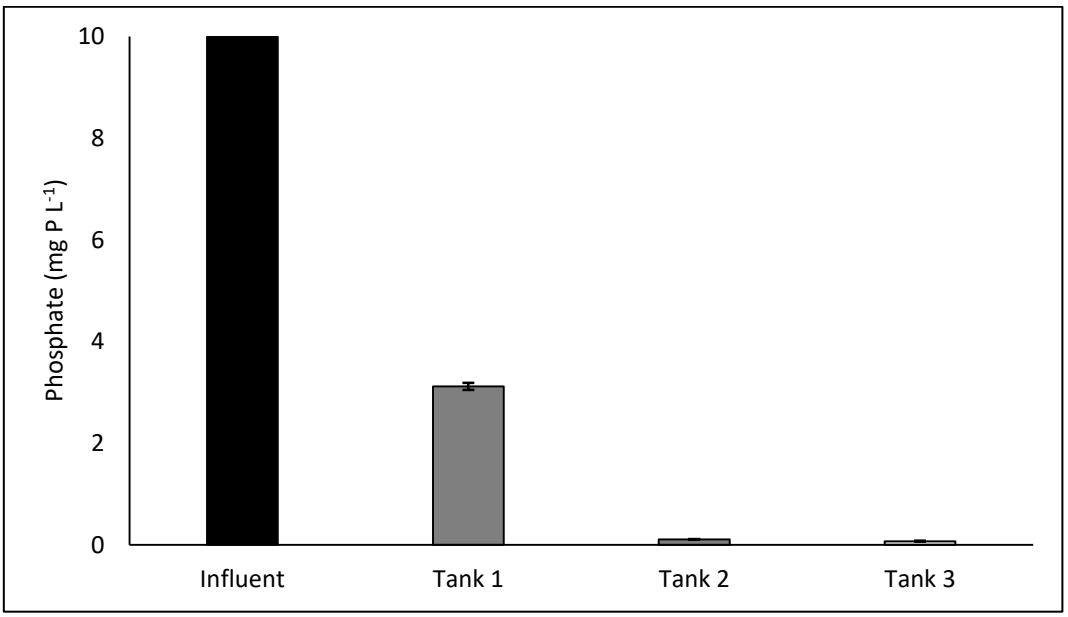

Figure 4.2.2.3. P removal from growth solution by *Lemna minor* at 15°C under steady state conditions. Mean phosphate measured in the primary influent and subsequent effluents produced by each duckweed tank in series. Samples were removed from points A, B and C (Figure 4.2.2.1), syringe-filtered (0.45 µm Ø pore size) and tested using phosphate cuvette tests (LCK phosphate test kits and DR1900 spectrophotometer, Hach Laing, UK). Error bars are standard error of the means.

Table 4.2.2.1. Mean P loading rates, removal rates and 97.5th percentile effluent values during steady state conditions (days 12 to 20).

	Tank T1	Tank T2	Tank T3
P concentration, 97.5 percentile (mg P L^{-1})	3.2	0.1	0.1
P surface loading rate (mg P m^{-2} d^{-1})	999	312	11
P surface load removal rate (mg P m^{-2} d^{-1})	687	301	4
P load removal efficiency (%)	69	96	35

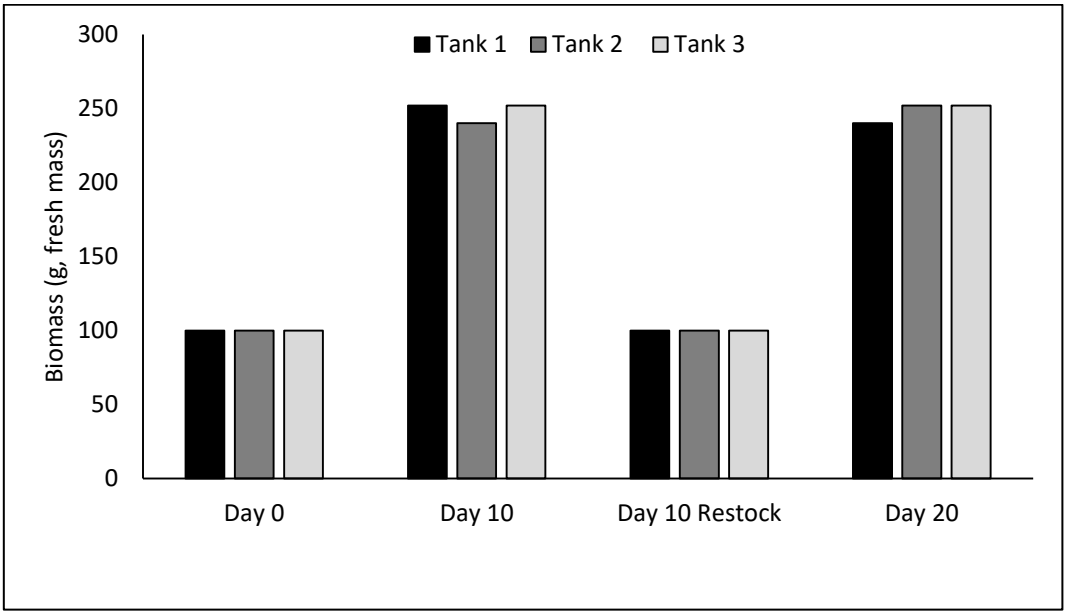

Figure 4.2.2.4. Production of *Lemna minor* biomass grown at 15°C on growth solution. 100 g (fresh mass) was originally seeded into each tank at day 0. At day 10, total biomass was estimated and 100 g (FM) was retained in each tank. At day 20 total biomass was estimated once more. Estimations were made by removing 25 % of surface cover, removing excess moisture by spinning and blotting and weighing on an analytical balance and multiplying by 4. Tank volume was 10 L and surface area was 0.1 m^{-2}.

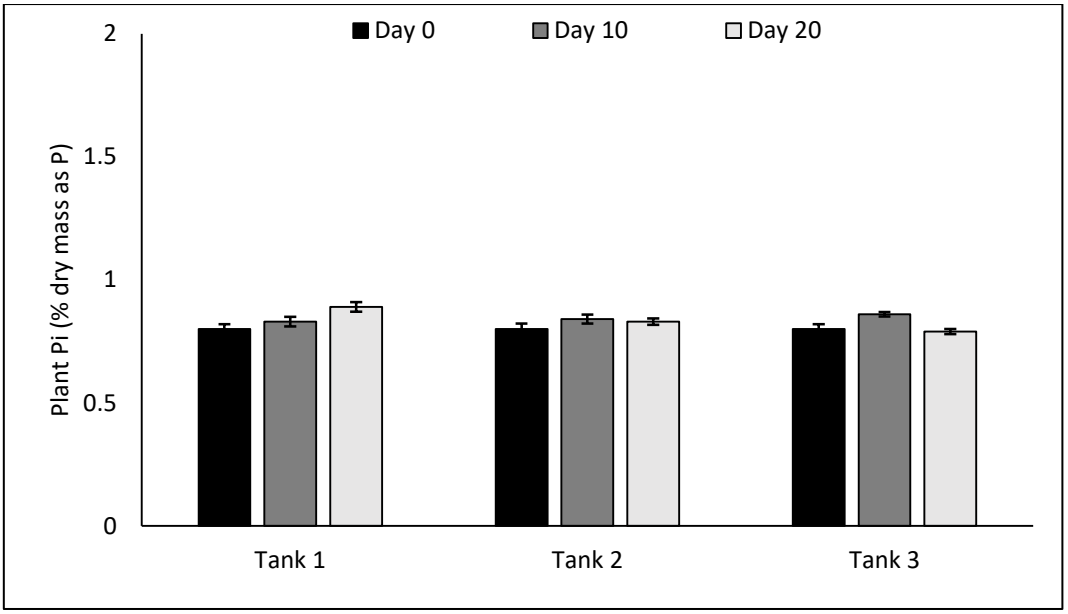

Figure 4.2.2.5. P accumulation of *Lemna minor* grown at 15°C on growth solution. Inorganic P was measured in triplicate duckweed samples taken at days 0, 10 and 20 from each tank. Data is presented as the % of dry mass represented by P. Analysis of P *in planta* is described in Section 2.6 of Chapter 2.

4.2.3. Growth solution (GS) experiments at 8°C

Tank temperatures were stable at 8°C with a total range of 2.4°C. Steady state conditions arose once more from day 12 onwards and influent P was consistent (Figure 4.2.3.1). Rates of P removal were again similar between tanks T2 and T3 while being different to tank T1 (Figure 4.2.3.1). Tanks T1, T2 and T3 removed 67 %, 97 % and 26 % of P loads respectively which was similar to that at 15°C. Values for 97.5th percentiles of P were encouraging once more with tanks 2 and 3 describing 0.2 and 0.1 mg P L^{-1} respectively (Table 4.2.3.1).

Production of biomass at 8°C was similar between tanks once more but less than previously at 15°C (Figure 4.2.3.3). Tanks T1, T2 and T3 increased by 140 %, 132 % and 132 % by day 10 and by 152 %, 140 % and 132 % by day 20 respectively, following the restock of 100 g (FM) at day 10.

Accumulation of P *in planta* followed the same pattern as previously with tank T1 duckweed increasing steadily but tanks T2 and T3 levelling off or decreasing by day 10 respectively (Figure 4.2.3.4). Similarities in the amount of biomass produced while being exposed to different amounts of P would explain this. Overall concentrations of P in the biomass were slightly higher than in the previous experiment, possibly explained by the duckweed being in situ constantly throughout the period that covered all of these experiments.

Concentrations of P produced were once more encouragingly low for the hypothesised pilot system that was to be built but comparisons were needed with lower temperatures. Therefore, the next experiment (and final one conducted using growth solution) retained all conditions with the exception of a lower temperature, stable at 5°C.

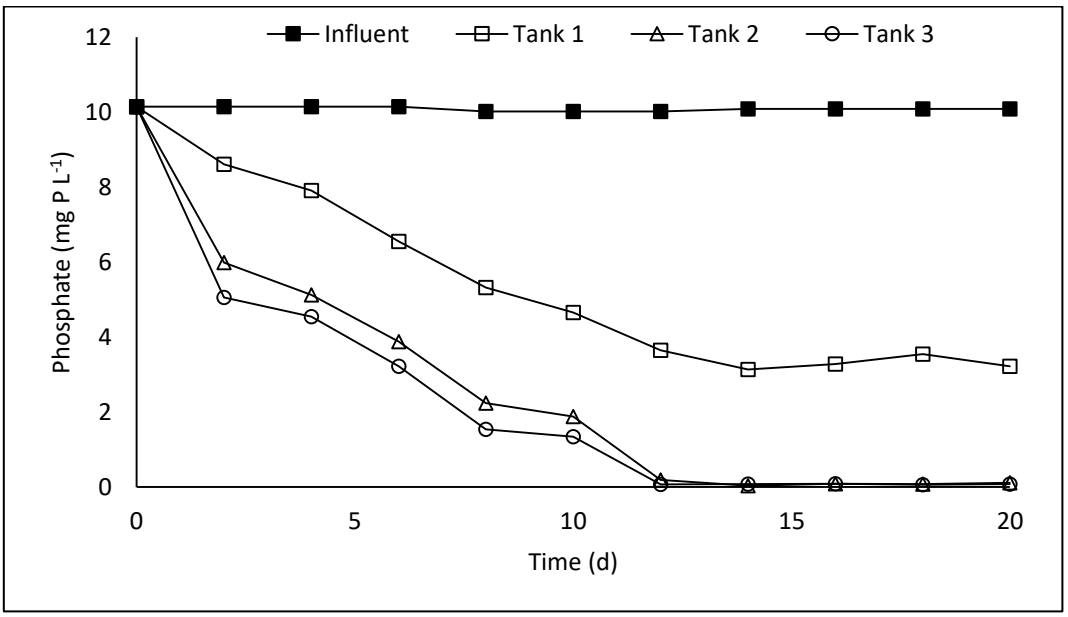

Figure 4.2.3.1. P removal from growth solution by *Lemna minor* at 8°C. Influent and subsequent effluents P concentrations (mg P L^{-1}) produced by duckweed tanks T1-T3 connected in series. Effluent samples were removed from points A, B and C (Figure 4.2.2.1), syringe-filtered (0.45 µm Ø pore size) and tested using phosphate cuvette tests (LCK phosphate test kits and DR1900 spectrophotometer, Hach Laing, UK). Influent was added to a header tank and spiked with KH_2PO_4 to 10 mg P L^{-1}.

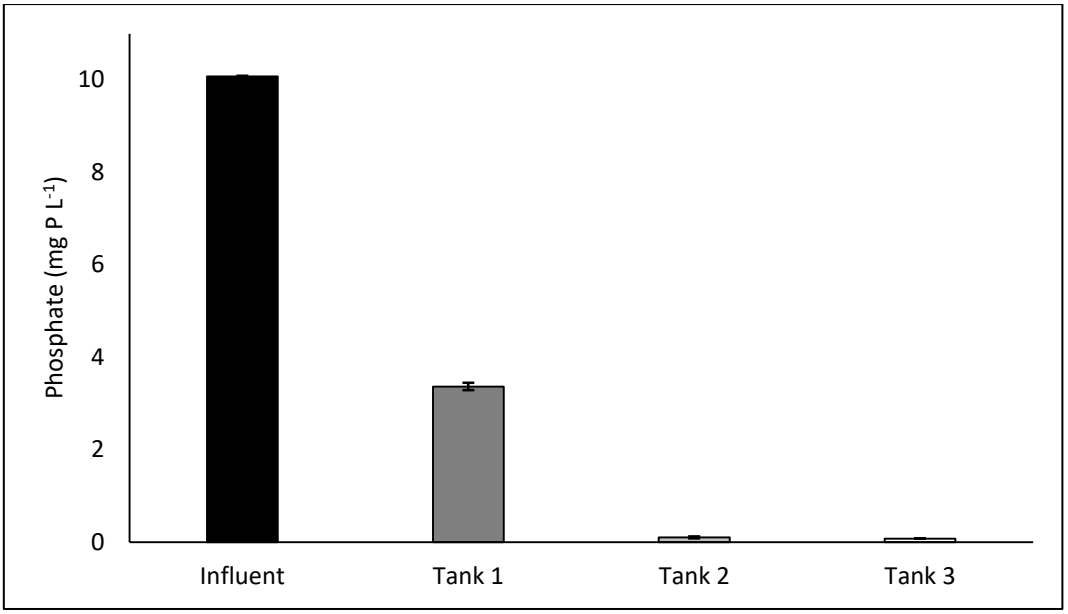

Figure 4.2.3.2. P removal from growth solution by *Lemna minor* at 8°C under steady state conditions. Mean phosphate measured in the primary influent and subsequent effluents produced by each duckweed tank in series. Samples were removed from points A, B and C (Figure 4.2.2.1), syringe-filtered (0.45 µm Ø pore size) and tested using phosphate cuvette tests (LCK phosphate test kits and DR1900 spectrophotometer, Hach Laing, UK). Error bars are standard error of the means.

Table 4.2.3.1. Mean P loading rates, removal rates and 97.5th percentile effluent values during steady state conditions (days 12 to 20).

	Tank T1	**Tank T2**	**Tank T3**
P concentration, 97.5 percentile (mg P L^{-1})	3.6	0.2	0.1
P surface loading rate (mg P m^{-2} d^{-1})	1008	337	10
P surface load removal rate (mg P m^{-2} d^{-1})	671	326	3
P load removal efficiency (%)	67	97	26

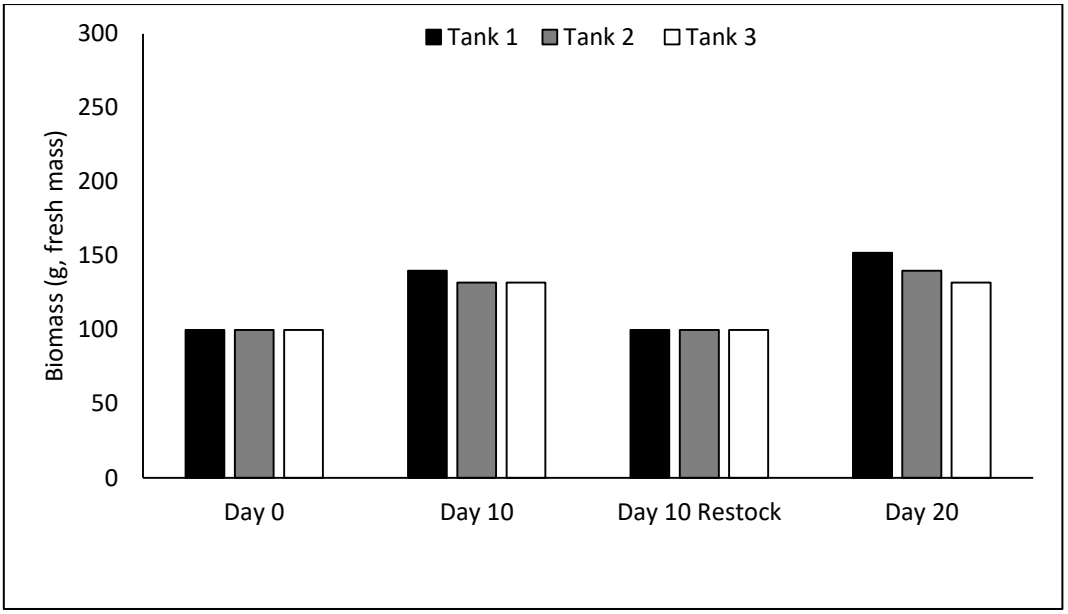

Figure 4.2.3.3. Production of *Lemna minor* biomass grown at 8°C on growth solution. 100 g (fresh mass) was originally seeded into each tank at day 0. At day 10, total biomass was estimated and 100 g (FM) was retained in each tank. At day 20 total biomass was estimated once more. Estimations were made by removing 25 % of surface cover, removing excess moisture by spinning and blotting and weighing on an analytical balance and multiplying by 4. Tank volume was 10 L and surface area was 0.1 m^{-2}.

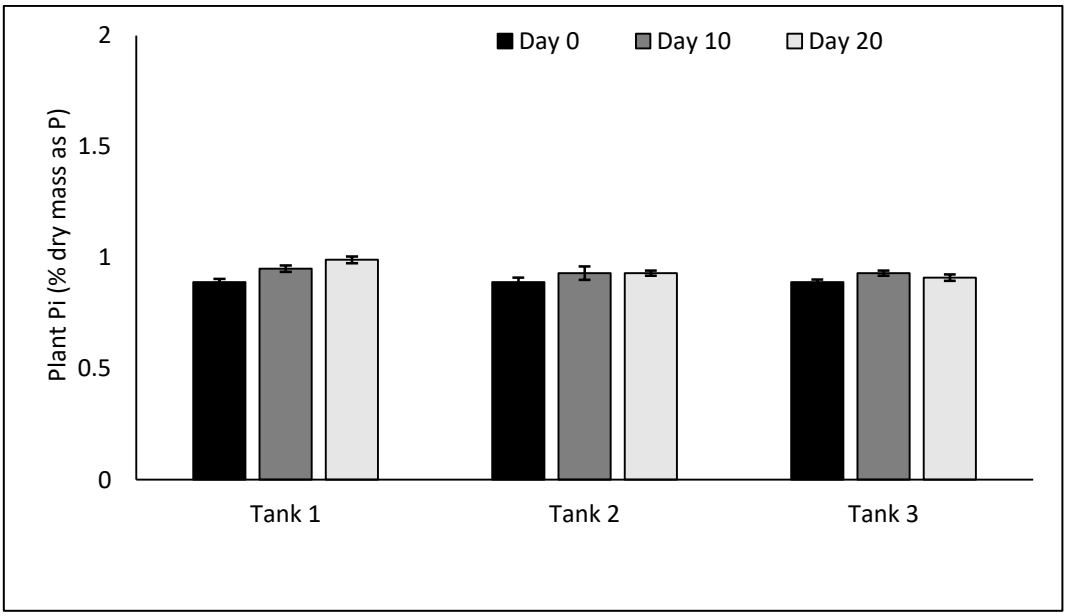

Figure 4.2.3.4. P accumulation of *Lemna minor* grown at 8°C on growth solution. Inorganic P was measured in triplicate duckweed samples taken at days 0, 10 and 20 from each tank. Data is presented as the % of dry mass represented by P. Analysis of P *in planta* is described in Section 2.6 of Chapter 2.

4.2.4. Growth solution (GS) experiments at 5°C

Tank temperatures were again stable at 5°C with a total range of 1.1°C. Influent composition was stable and steady state conditions arose at day 12 (Figure 4.2.4.1). There was again little difference between tanks 2 and 3in terms of P removal rates (Figure 4.2.4.1) and effluent values of P (Figure 4.2.4.2). Even at 5°C the 97.5th percentile values of P for tanks 2 and 3 were both 0.2 mg P L^{-1} (Table 4.2.4.1). P load removal percentages followed the same pattern and held similar values as for the previous 8°C and 15°C experiments. Removal for tanks 1, 2 and 3 was 66 %, 96 % and 24 % respectively (Table 4.2.4.1).

Production of biomass was significantly lower than it was at 8°C, with tanks 1, 2 and 3 increasing by just 8%, 4% and 0% by day 10 and by 4 %, 0 % and 0 % by day 20 respectively (Figure 4.2.4.3). As P removal was similar to the previous experiments, this was continued evidence for the duckweed's capacity for P uptake not being exclusively driven by vigorous growth, as was shown in Chapter 3. P accumulation *in planta* increased steadily in all tanks (Figure 4.2.4.4), probably due to the continued uptake of P during a period that saw very little growth.

At this point it was decided to repeat experiments 4.2.2 to 4.2.4 but use real wastewater in the place of growth solution, in order to investigate if an organic (or other) constituent would affect the performance of the duckweed. As time was progressing it was seen as hopefully beneficial to do it this way in order to attempt to have experiments at comparable temperatures. All other aspects of the experimental design (stocking density, photoperiod, flow rate etc.) were retained.

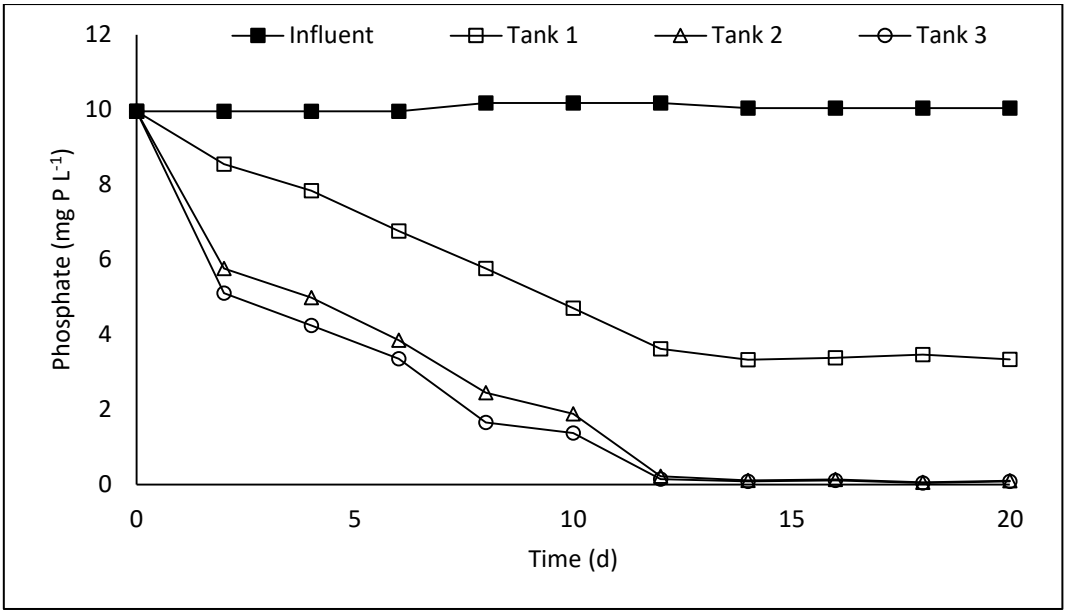

Figure 4.2.4.1. P removal from growth solution by *Lemna minor* at 5°C. Primary influent and subsequent effluent P (mg P L^{-1}) produced by duckweed tanks 1-3 connected in series. Effluent samples were removed from points A, B and C (Figure 4.2.2.1), syringe-filtered (0.45 µm Ø pore size) and tested using phosphate cuvette tests (LCK phosphate test kits and DR1900 spectrophotometer, Hach Laing, UK). Influent was added to a header tank and spiked with KH_2PO_4 to 10 mg P L^{-1}.

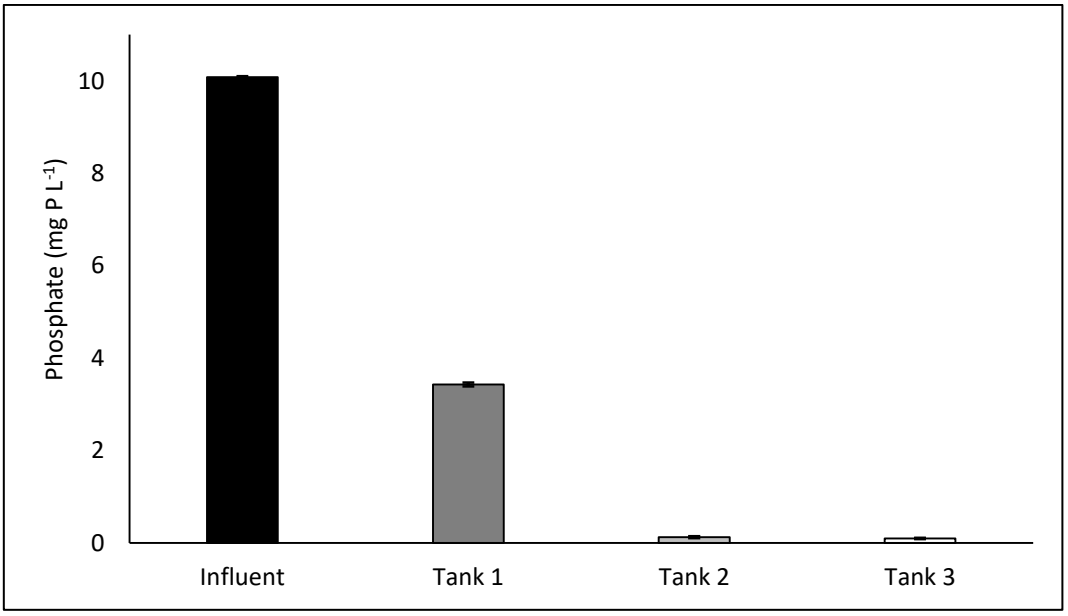

Figure 4.2.4.2. P removal from growth solution by *Lemna minor* at 5°C under steady state conditions. Mean phosphate measured in the primary influent and subsequent effluents produced by each duckweed tank in series. Samples were removed from points A, B and C (Figure 4.2.2.1), syringe-filtered (0.45 µm Ø pore size) and tested using phosphate cuvette tests (LCK phosphate test kits and DR1900 spectrophotometer, Hach Laing, UK). Error bars are standard error of the means.

Table 4.2.4.1. Mean P loading rates, removal rates and 97.5th percentile effluent values during steady state conditions (days 12 to 20).

	Tank T1	Tank T2	Tank T3
P concentration, 97.5 percentile (mg P L^{-1})	3.6	0.2	0.2
P surface loading rate (mg P m^{-2} d^{-1})	1008	343	13
P surface load removal rate (mg P m^{-2} d^{-1})	665	330	3
P load removal efficiency (%)	66	96	24

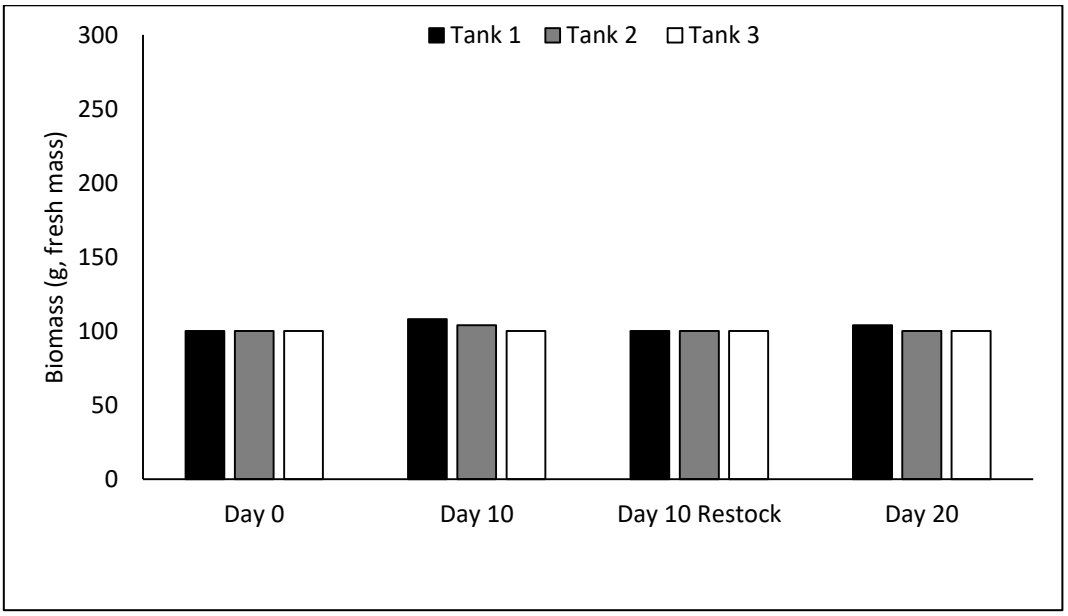

Figure 4.2.4.3. Production of *Lemna minor* biomass grown at 5°C on growth solution. 100 g (fresh mass) was originally seeded into each tank at day 0. At day 10, total biomass was estimated and 100 g (FM) was retained in each tank. At day 20 total biomass was estimated once more. Estimations were made by removing 25 % of surface cover, removing excess moisture by spinning and blotting and weighing on an analytical balance and multiplying by 4. Tank volume was 10 L and surface area was 0.1 m^{-2}.

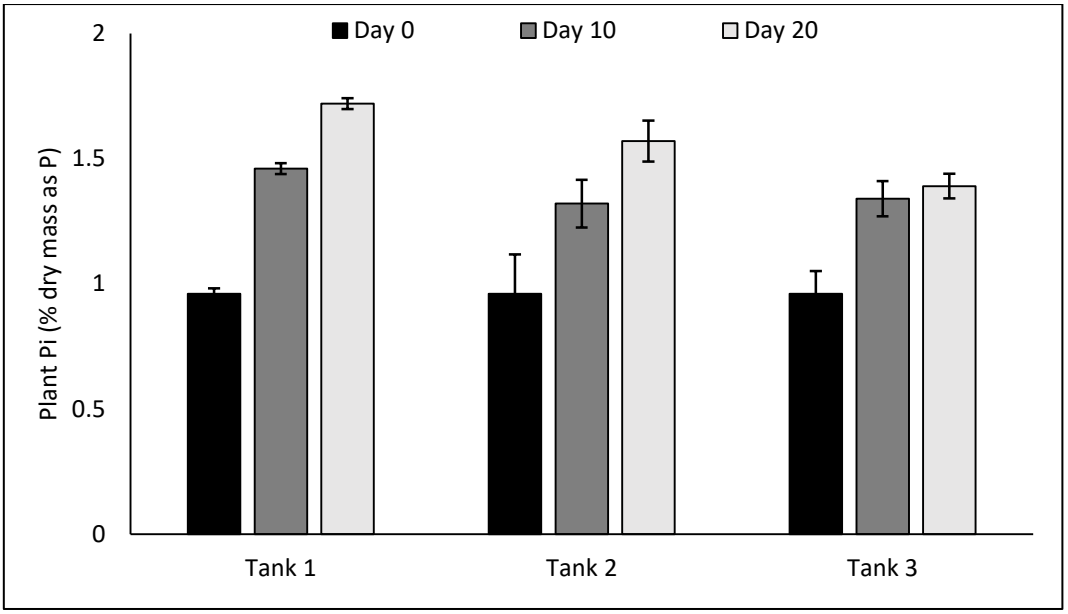

Figure 4.2.4.4. P accumulation of *Lemna minor* grown at 5°C on growth solution. Inorganic P was measured in triplicate duckweed samples taken at days 0, 10 and 20 from each tank. Data is presented as the % of dry mass represented by P. Analysis of P *in planta* is described in Section 2.6 of Chapter 2.

4.2.5. Wastewater (WW) experiments at 5°C

At this point the media used for experiments was changed from growth solution to real wastewater collected from Esholt WWTW (Bradford, UK) to introduce an organic fraction and allow for comparison with growth solution. All other experimental conditions applied in experiments 4.2.2 to 4.2.4 were maintained (E.g. stocking density, flow rate, photoperiod, nitrate and pH etc.). The wastewater was final effluent, collected periodically from the final discharge point of the WWTW, returned and added to a large header tank and analysed, before being spiked with the appropriate amount of KH_2PO_4 to a concentration of 10 mg P L^{-1}. Experiments were conducted when temperatures were stable at the same values as previous experiments.

Temperatures recorded in the tanks showed an average of 5°C and values for organic matter (i.e., measured as Chemical Oxygen Demand – COD) and nitrate reduced as solution moved through the system (Table 4.2.5.1). In this experiment steady state conditions were apparent from day 14 onwards, taking 2 days longer than previously (Figure 4.2.5.1). P removal during steady state conditions was 58 %, 70 % and 33 % by tanks T1, T2 and T3 respectively with 97.5th values for effluent P being 4.3, 1.3 and 0.8 mg P L^{-1} respectively (Table 4.2.5.2). This was not as low as for growth solution under the same temperature (Table 4.2.4.1).

Tanks T2 and T3 recorded similar mean effluent P (Figure 4.2.5.2) but concentrations were 13x and 8x higher than those recorded in growth solution (Figure 4.2.4.2). Production of biomass was very little compared to duckweed maintained on growth solution. Tanks T1, T2 and T3 increased by 4 %, 0 % and 0 % by day 10 and by 8 %, 0 % and 4 % by day 20, following the restock (Figure 4.2.5.3). P accumulation *in planta* rose steadily in the duckweed in all 3 tanks from 1.2 % of dry mass to 1.6 %, 1.2 % and 1.4 % respectively in tanks T1, T2 and T3 (Figure 4.2.5.4).

P removal of the duckweed at 5°C on wastewater was not as positive as when kept on growth solution but internal concentrations were still increasing to nearly 2 % of dry mass without showing any negative side effects. The next experiment would be conducted at 8°C in order to compare with experiment 4.2.3.

Table 4.2.5.1. Characteristics of wastewater collected from Esholt WWTW used in experiment 4.2.5. Mean values of WW parameters recorded in the influent prior to application and in the effluents of each tank during steady state conditions (SSC).

	Mean	SSC Means		
	Influent	**Tank T1**	**Tank T2**	**Tank T3**
pH	7.5	7.5	7.2	7.6
Conductivity ($\mu S\ cm^{-1}$)	1,400	1,300	1,200	1,250
COD ($mg\ L^{-1}$)	40	35	30	30
Dissolved Oxygen ($mg\ L^{-1}$)	8	9	9	9
ORP (mV)	200	210	200	220
Temperature (°C)	5.0	5.3	5.2	5.2
Nitrate ($mg\ N\ L^{-1}$)	60	55	45	35
Ammonium ($mg\ N\ L^{-1}$)	0.05	0.03	0.02	0.02

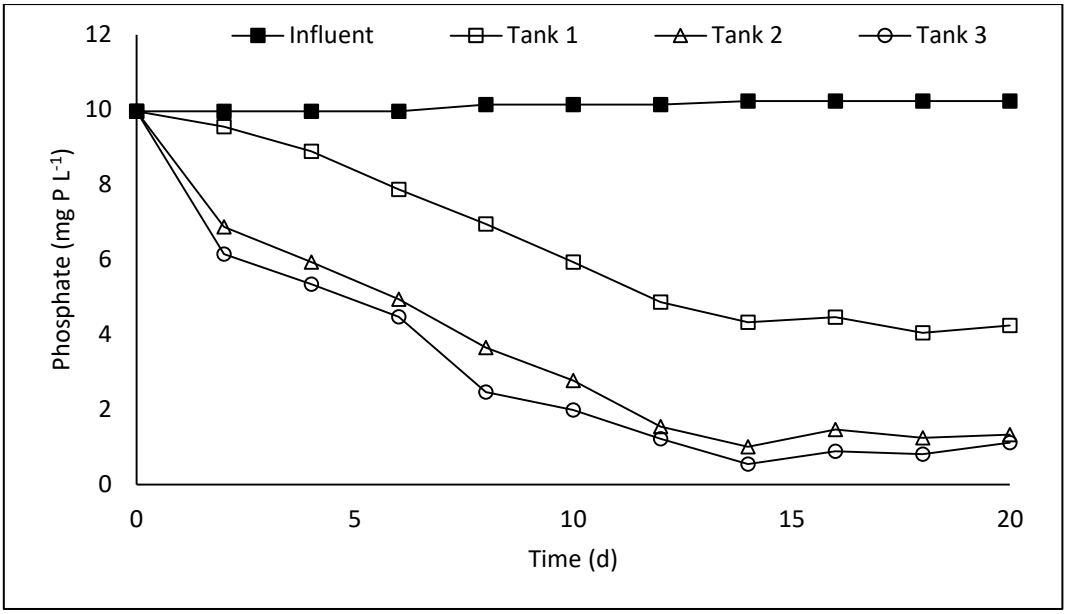

Figure 4.2.5.1. P removal from wastewater by *Lemna minor* at 5°C. Influent and subsequent effluent P (mg P L^{-1}) produced by duckweed tanks T1-T3 connected in series. Effluent samples were removed from points A, B and C (Figure 4.2.2.1), syringe-filtered (0.45 µm Ø pore size) and tested using phosphate cuvette tests (LCK phosphate test kits and DR1900 spectrophotometer, Hach Laing, UK). Influent was added to a header tank and spiked with KH_2PO_4 to 10 mg P L^{-1}.

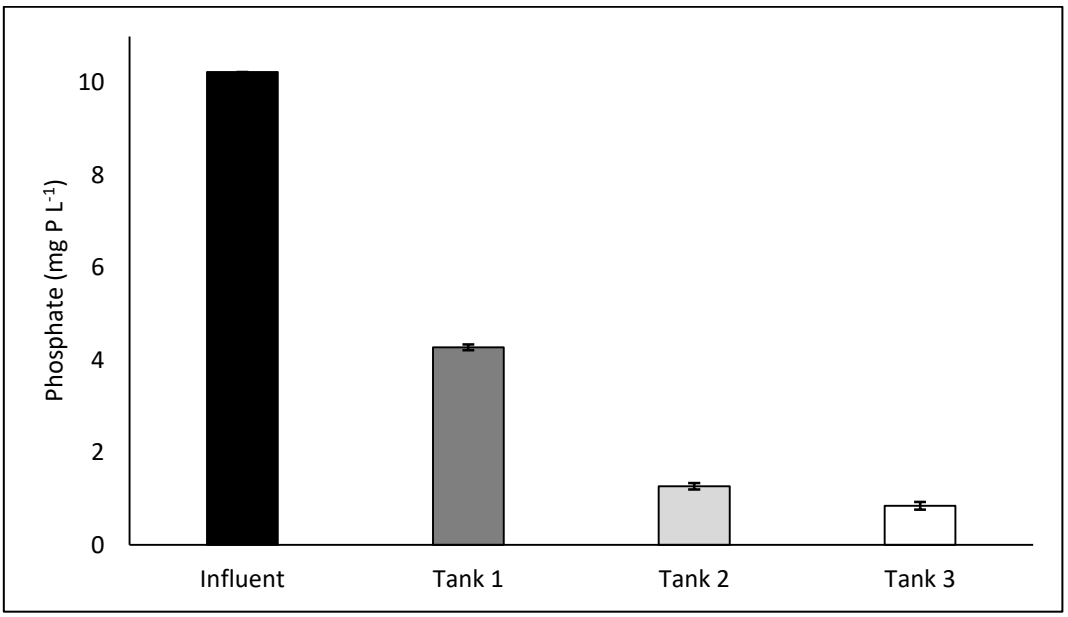

Figure 4.2.5.2. P removal from wastewater by *Lemna minor* at 5°C under steady state conditions. Mean phosphate measured in the primary influent and subsequent effluents produced by each duckweed tank in series. Samples were removed from points A, B and C (Figure 4.2.2.1), syringe-filtered (0.45 µm Ø pore size) and tested using phosphate cuvette tests (LCK phosphate test kits and DR1900 spectrophotometer, Hach Laing, UK). Error bars are standard error of the means.

Table 4.2.5.2. Mean P loading rates, removal rates and 97.5th percentile effluent values during steady state conditions (days 14 to 20).

	Tank T1	Tank T2	Tank T3
P concentration, 97.5 percentile (mg P L^{-1})	4.46	1.46	1.10
P surface loading rate (mg P m^{-2} d^{-1})	1023	428	127
P surface load removal rate (mg P m^{-2} d^{-1})	596	301	42
P load removal efficiency (%)	58	70	33

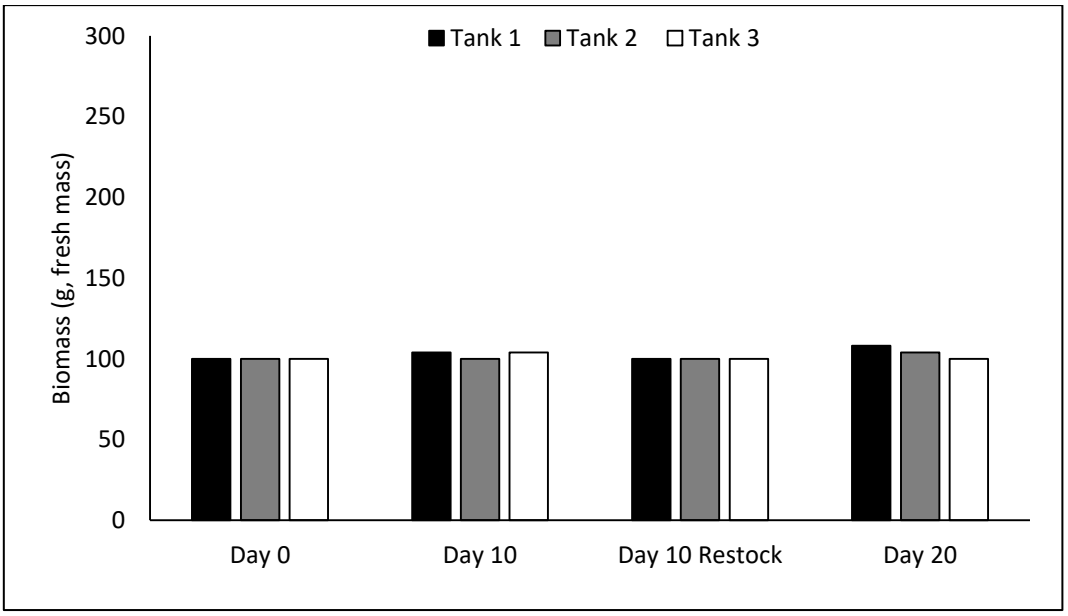

Figure 4.2.5.3. Production of *Lemna minor* biomass grown at 5°C on wastewater. 100 g (fresh mass) was originally seeded into each tank at day 0. At day 10, total biomass was estimated and 100 g (FM) was retained in each tank. At day 20 total biomass was estimated once more. Estimations were made by removing 25 % of surface cover, removing excess moisture by spinning and blotting and weighing on an analytical balance and multiplying by 4. Tank volume was 10 L and surface area was 0.1 m^{-2}.

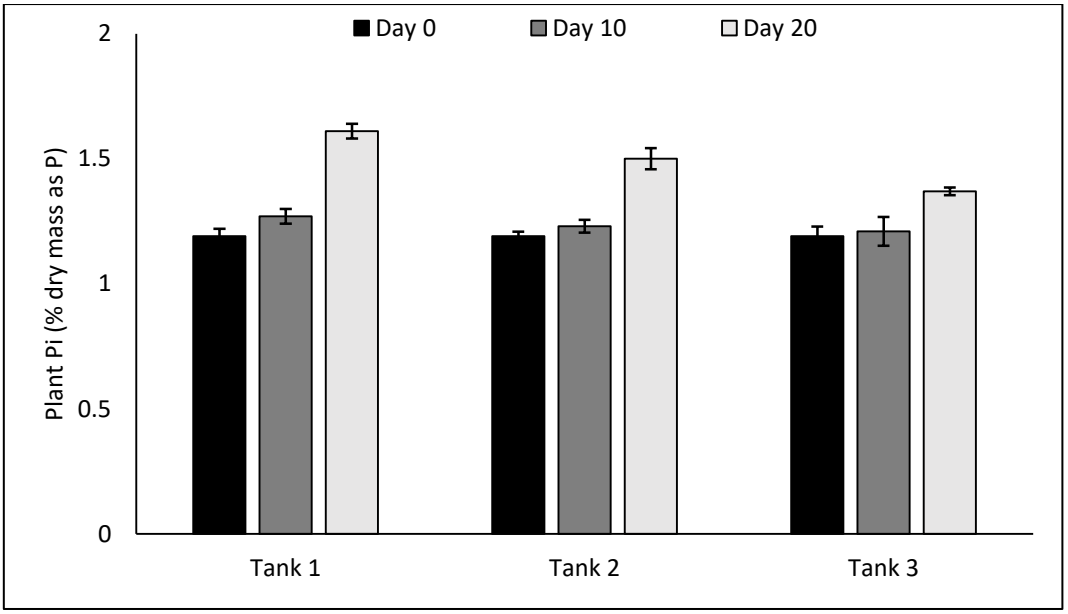

Figure 4.2.5.4. P accumulation of *Lemna minor* grown at 5°C on wastewater. Inorganic P was measured in triplicate duckweed samples taken at days 0, 10 and 20 from each tank. Data is presented as the % of dry mass represented by P. Analysis of P *in planta* is described in Section 2.6 of Chapter 2.

4.2.6. Wastewater (WW) experiments at 8°C

This experiment was conducted over a 20-day period where the temperature was stable at 8°C. COD, nitrate and ammonium were all reduced by the system (Table 4.2.6.1). Steady state conditions arose at day 14 once again and the wastewater was accurately spiked to a stable 10 mg P L^{-1} (Figure 4.2.6.1). Mean effluent P recorded in tanks T1, T2 and T3 was 4.0, 1.4 and 0.7 mg P L^{-1} respectively (Figure 4.2.6.2), which was similar to values recorded in the wastewater experiment at 5°C (Figure 4.2.5.2) and significantly higher than values at 8°C using growth solution (Figure 4.2.3.2). Removal capacity was 60 %, 64 % and 48 % for tanks T1, T2 and T3 respectively (Table 4.2.6.2). Performance at the same temperature was better by duckweed kept on growth solution for tanks T1 and T2, but not by tank T3 (Table 4.2.3.1). In the present experiment P removal by tank T3 was 22 % better.

Biomass in the present experiment increased in tanks T1, T2 and T3 by 40 %, 20 % and 20 % by day 10 and by 80 %, 60 % and 60 % by day 20 respectively (Figure 4.6.3). Overall this is better than the same results conducted with growth solution (Figure 4.3.3). P accumulation increased in the duckweed of tanks T1, T2 and T3 from 1.3 % of dry mass to 1.6 %, 1.5 % and 1.4 % respectively, decreasing as available P decreased. To complete the set of experiments for this chapter the temperature was left to increase until it was stable at 15°C and the experiment could be repeated.

Table 4.2.6.1. Characteristics of wastewater collected from Esholt WWTW used in experiment 4.2.6. Mean values of WW parameters recorded in the primary influent prior to application and in the effluents of each tank during steady state conditions (SSC).

	Mean	SSC Means		
	Influent	Tank T1	Tank T2	Tank T3
pH	7.2	7.4	7.6	7.5
Conductivity ($\mu S\ cm^{-1}$)	1,600	1,400	1,300	1,200
COD ($mg\ L^{-1}$)	60	45	40	30
Dissolved Oxygen ($mg\ L^{-1}$)	9	10	10	9
ORP (mV)	210	220	210	200
Temperature (°C)	8.0	8.2	8.3	8.1
Nitrate ($mg\ N\ L^{-1}$)	55	45	30	25
Ammonium ($mg\ N\ L^{-1}$)	0.01	0.01	ND	ND

BDL = Below Detection Limit

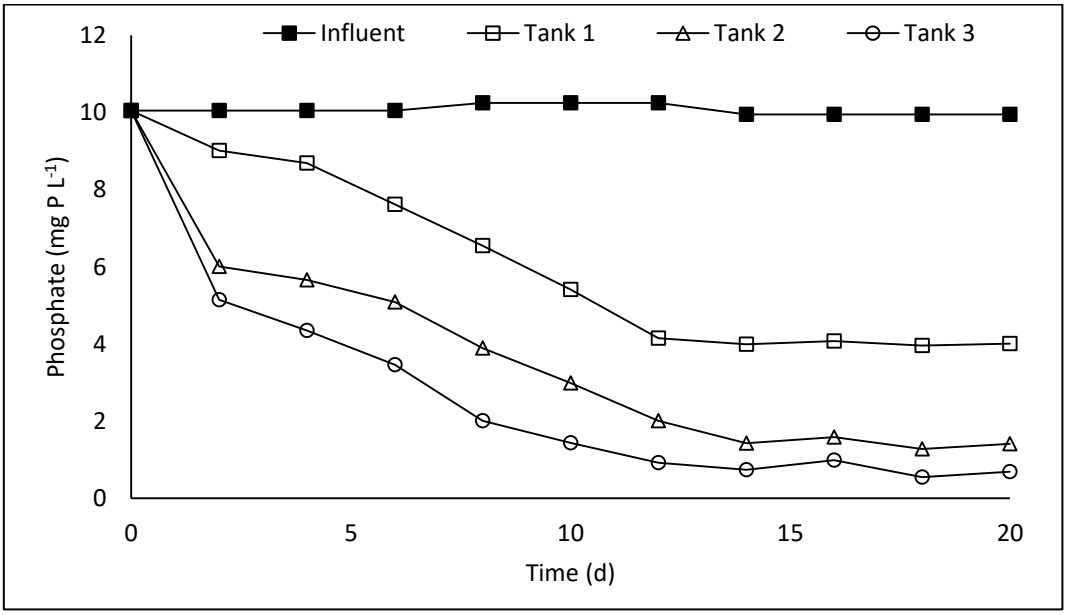

Figure 4.2.6.1. P removal from wastewater by *Lemna minor* at 8°C. Influent and subsequent effluent P ($mg\ P\ L^{-1}$) produced by duckweed tanks 1-3 connected in series. Effluent samples were removed from points A, B and C (Figure 4.2.2.1), syringe-filtered (0.45 µm Ø pore size) and tested using phosphate cuvette tests (LCK phosphate test kits and DR1900 spectrophotometer, Hach Laing, UK). Influent was added to a header tank and spiked with KH_2PO_4 to 10 $mg\ P\ L^{-1}$.

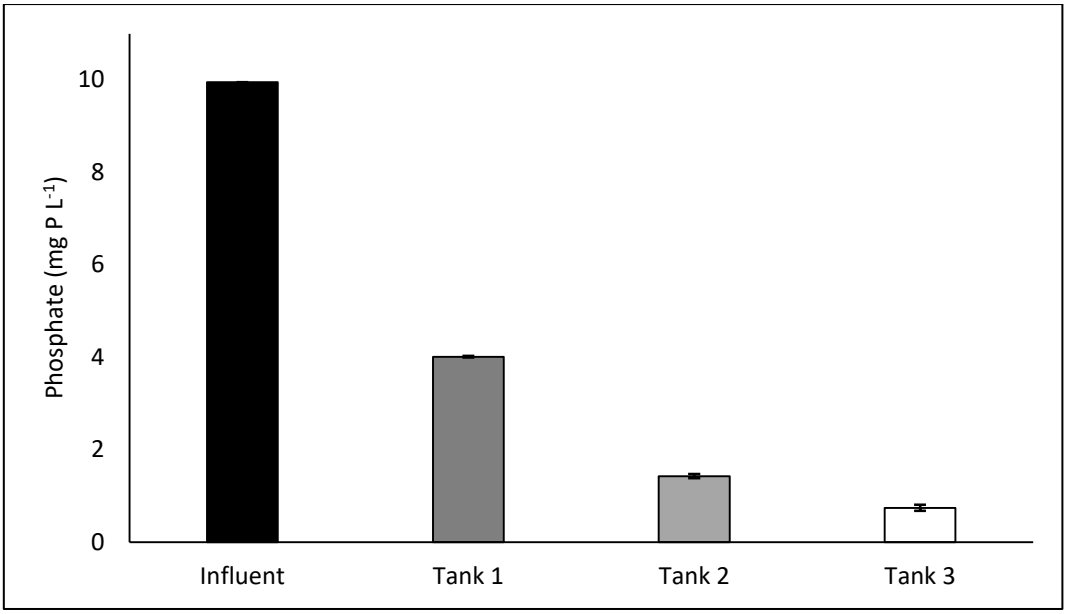

Figure 4.2.6.2. P removal from wastewater by *Lemna minor* at 8°C under steady state conditions. Mean phosphate measured in the primary influent and subsequent effluents produced by each duckweed tank in series. Samples were removed from points A, B and C (Figure 4.2.2.1), syringe-filtered (0.45 µm Ø pore size) and tested using phosphate cuvette tests (LCK phosphate test kits and DR1900 spectrophotometer, Hach Laing, UK). Error bars are standard error of the means.

Table 4.2.6.2. Mean P loading rates, removal rates and 97.5th percentile effluent values during steady state conditions (days 14 to 20).

	Tank T1	**Tank T2**	**Tank T3**
P concentration, 97.5 percentile (mg P L^{-1})	4.1	1.6	1.0
P surface loading rate (mg P m^{-2} d^{-1})	995	401	143
P surface load removal rate (mg P m^{-2} d^{-1})	594	258	69
P load removal efficiency (%)	60	64	48

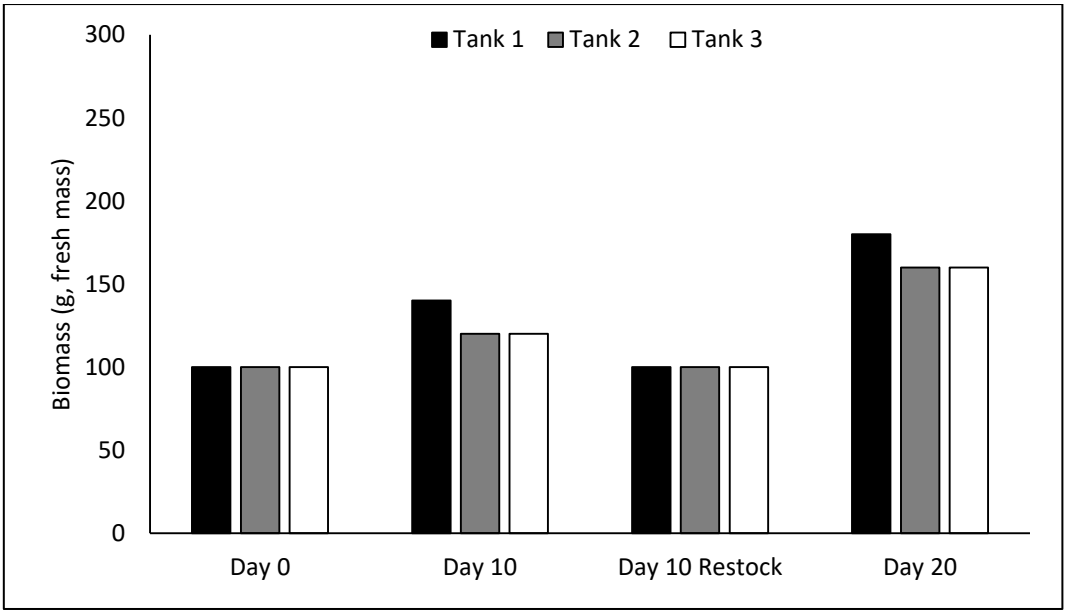

Figure 4.2.6.3. Production of *Lemna minor* biomass grown at 8°C on wastewater. 100 g (fresh mass) was originally seeded into each tank at day 0. At day 10, total biomass was estimated and 100 g (FM) was retained in each tank. At day 20 total biomass was estimated once more. Estimations were made by removing 25 % of surface cover, removing excess moisture by spinning and blotting and weighing on an analytical balance and multiplying by 4. Tank volume was 10 L and surface area was 0.1 m^{-2}.

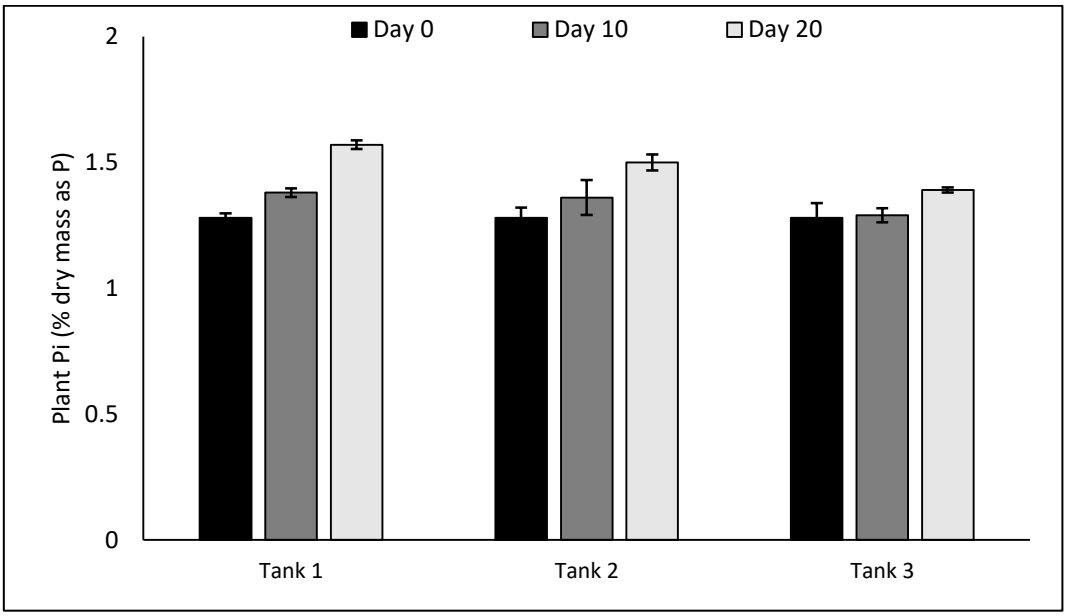

Figure 4.2.6.4. P accumulation of *Lemna minor* grown at 8°C on wastewater. Inorganic P was measured in triplicate duckweed samples taken at days 0, 10 and 20 from each tank. Data is presented as the % of dry mass represented by P. Analysis of P *in planta* is described in Section 2.6 of Chapter 2.

4.2.7. Wastewater (WW) experiments at 15°C

The mean temperature recorded in the tanks over the experimental period was 15°C. Concentrations of nitrate, ammonium and COD were all improved as influent moved through the system (Table 4.2.7.1). Steady state conditions were apparent from day 14 onwards as previously and the spiked influent wastewater was stable at 10 mg P L^{-1} (Figure 4.2.7.1). Mean effluent P produced by tanks 1, 2 and 3 was 4.1, 1.2 and 0.6 mg P L^{-1} respectively (Figure 4.2.7.2). The 97.5th percentile values for effluent P were 4.2, 1.3 and 0.8 mg P L^{-1} and removal capacity was 59 %, 72 % and 47 % for tanks T1, T2 and T3 respectively (Table 4.2.7.2). This removal capacity is poorer than that recorded by the duckweed at the same temperature with growth solution (Table 4.2.2.1), with the exception of tank 3 which in this case was 12 % better.

Production of biomass in the present experiment was 30 % better than when duckweed was kept on growth solution at the same temperature (Figure 4.2.2.3). On wastewater, the duckweed in tanks T1, T2 and T3 increased their initial amounts by 180 %, 172 % and 180 % by day 10 and by 172 %, 180 % and 172 % by day 20 respectively (Figure 4.2.7.3). P accumulated by the duckweed in tank T1 increased in concentration from 1.4 % of dry mass to 1.7 %. In tanks 2 and 3 however concentrations remained at 1.4 % for the duration of the experiment.

To provide a more rapid and clear analysis of the data generated from experiments 4.2.2 to 4.2.7, comparison Tables are presented in Section 4.2.8.

Table 4.2.7.1. Characteristics of wastewater collected from Esholt WWTW used in experiment 4.2.7. Mean values of WW parameters recorded in the primary influent prior to application and in the effluents of each tank during steady state conditions (SSC).

	Mean	SSC Means		
	Influent	Tank T1	Tank T2	Tank T3
pH	7.1	7.3	7.2	7.4
Conductivity ($\mu S\ cm^{-1}$)	1,700	1,400	1,300	1,300
COD ($mg\ L^{-1}$)	65	50	40	30
Dissolved Oxygen ($mg\ L^{-1}$)	8	8	8	9
ORP (mV)	190	200	190	210
Temperature (°C)	15	15.3	15.2	15.4
Nitrate ($mg\ N\ L^{-1}$)	65	45	35	20
Ammonium ($mg\ N\ L^{-1}$)	0.1	0.05	0.02	0.01

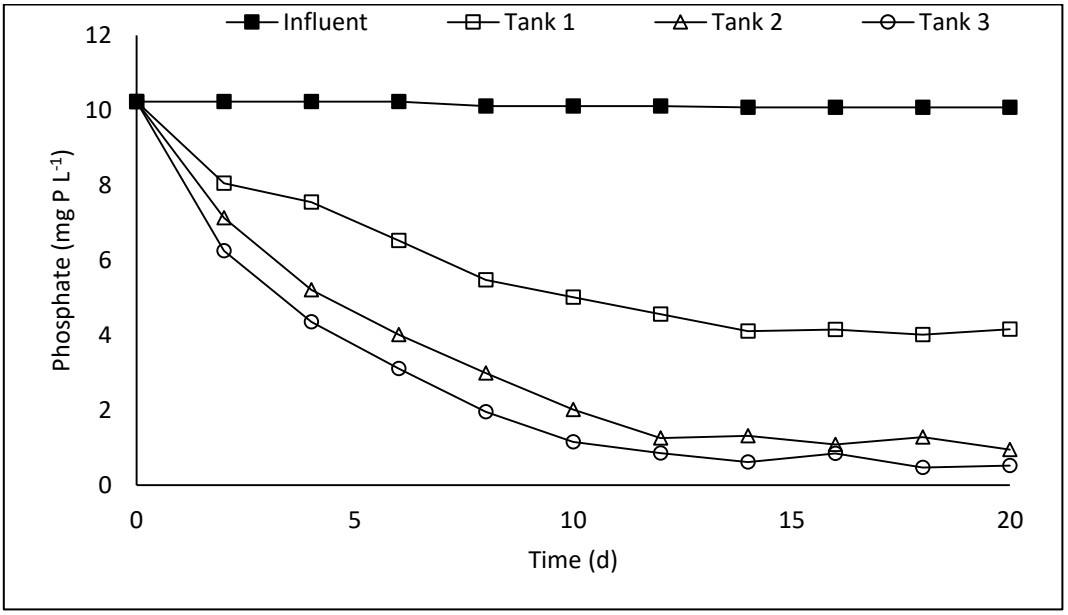

Figure 4.2.7.1. P removal from wastewater by *Lemna minor* at 15°C. Primary influent and subsequent effluent P ($mg\ P\ L^{-1}$) produced by duckweed tanks 1-3 connected in series. Effluent samples were removed from points A, B and C (Figure 4.2.2.1), syringe-filtered (0.45 µm Ø pore size) and tested using phosphate cuvette tests (LCK phosphate test kits and DR1900 spectrophotometer, Hach Laing, UK). Influent was added to a header tank and spiked with KH_2PO_4 to 10 $mg\ P\ L^{-1}$.

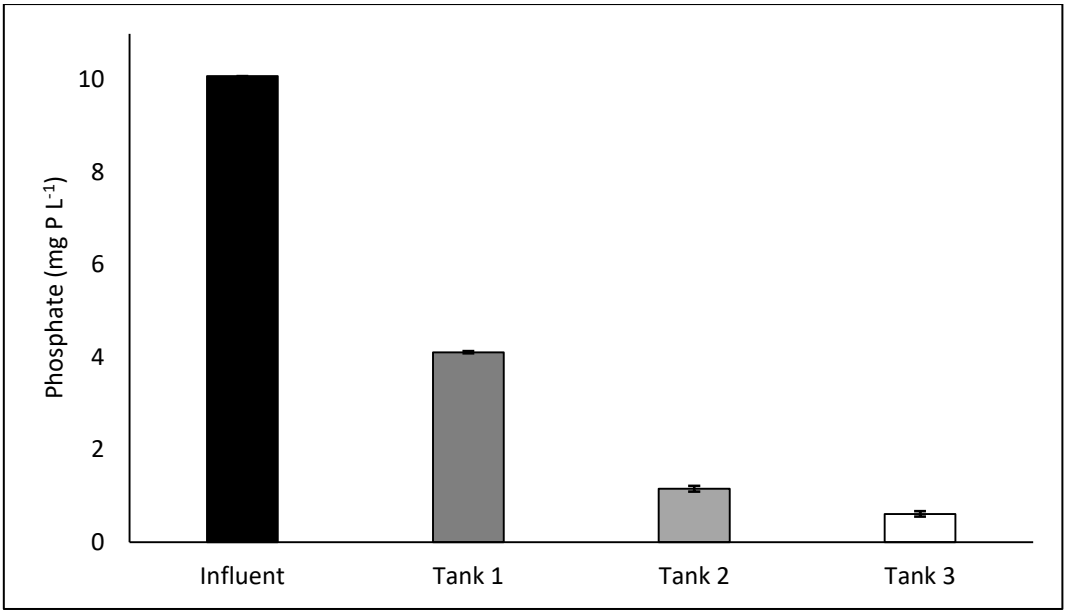

Figure 4.2.7.2. P removal from wastewater by *Lemna minor* at 15°C under steady state conditions. Mean phosphate measured in the primary influent and subsequent effluents produced by each duckweed tank in series. Samples were removed from points A, B and C (Figure 4.2.2.1), syringe-filtered (0.45 µm Ø pore size) and tested using phosphate cuvette tests (LCK phosphate test kits and DR1900 spectrophotometer, Hach Laing, UK). Error bars are standard error of the means.

Table 4.2.7.2. Mean P loading rates, removal rates and 97.5th percentile effluent values during steady state conditions (days 14 to 20).

	Tank T1	**Tank T2**	**Tank T3**
P concentration, 97.5 percentile (mg P L^{-1})	4.2	1.3	0.8
P surface loading rate (mg P m^{-2} d^{-1})	1008	411	116
P surface load removal rate (mg P m^{-2} d^{-1})	597	295	55
P load removal efficiency (%)	59	72	47

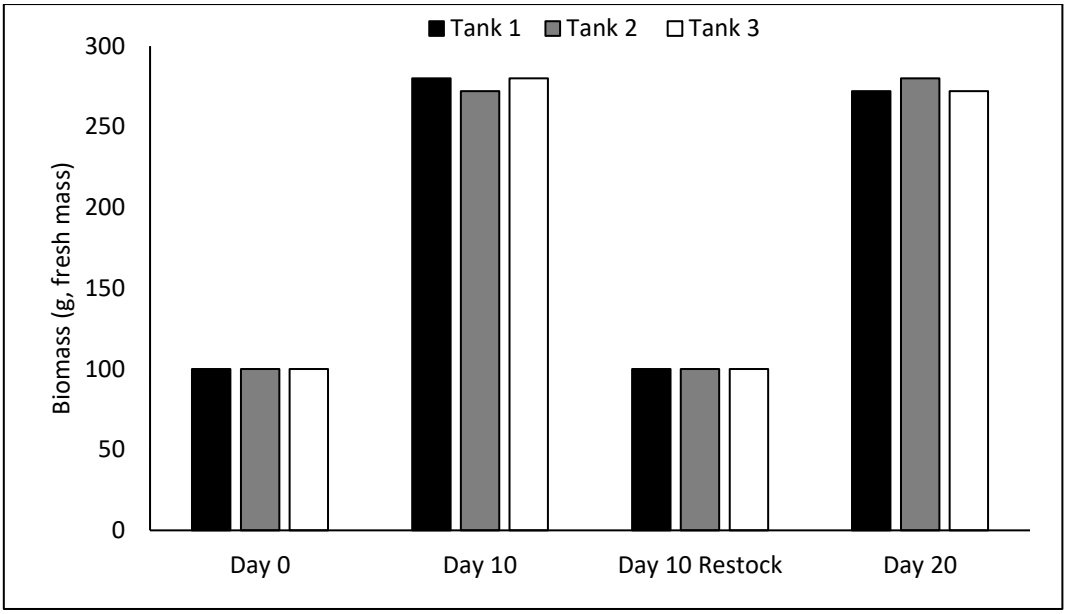

Figure 4.2.7.3. Production of *Lemna minor* biomass grown at 15°C on wastewater. 100 g (fresh mass) was originally seeded into each tank at day 0. At day 10, total biomass was estimated and 100 g (FM) was retained in each tank. At day 20 total biomass was estimated once more. Estimations were made by removing 25 % of surface cover, removing excess moisture by spinning and blotting and weighing on an analytical balance and multiplying by 4. Tank volume was 10 L and surface area was 0.1 m^{-2}.

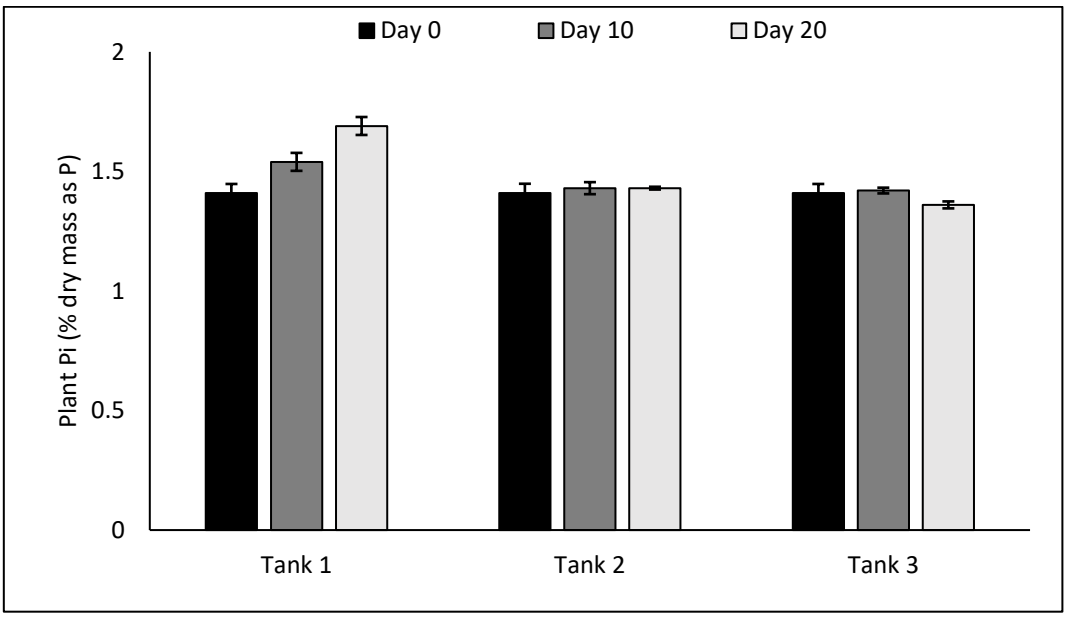

Figure 4.2.7.4. P accumulation of *Lemna minor* grown at 15°C on wastewater. Inorganic P was measured in triplicate duckweed samples taken at days 0, 10 and 20 from each tank. Data is presented as the % of dry mass represented by P. Analysis of P *in planta* is described in Section 2.6 of Chapter 2.

4.2.8. Collation of results

The following Tables present the key data from experiments 4.2.2 to 4.2.7 for comparison. Table 4.2.8.1 shows that tanks T1 and T2 provided with growth solution produced a lower 97.5th percentile effluent P than those using wastewater, irrespective of temperature. Tanks T2 and T3 were always significantly different ($P < 0.01$ every time) between growth solution (GS) and wastewater (WW) for 97.5th percentile values. Values for tank 3 97.5th percentiles correlated well to temperature, with R^2 values being 0.91 and 0.84 for GS and WW respectively.

P load (mg P m^{-2} d^{-1}) correlated well to P removal for each 3-tank system at every temperature (Table 4.2.8.2). This suggests that increasing the load of P would increase the amount removed overall, which is good for a P recovery system. P loads removed by tanks T1 and T2 were always higher for GS than WW, but Tank T3 of each WW experiment removed more loads than tank T3 of GS experiments, probably due to P limitation in the low P GS tank T3's (Table 4.2.8.3).

In contrast to P removal results, the mean increase in biomass production was almost exclusively more when duckweed was grown on wastewater than on growth solution (Table 4.2.8.4). Numerous stocks kept on wastewater increased significantly more than their growth solution counterparts ('*' = $P <$ 0.01).

The maximal density reached by wastewater-grown duckweed at 15°C was 2.8 kg (fresh mass) m^{-2}, which is in the middle of the reported range for maximum duckweed density (1.3 to 6 kg (FM) m^{-2}) (Lasfar et al., 2007, Driever et al., 2005). Increases in biomass correlated well to temperature, with R^2 values for GS and WW being 0.78 and 0.83 respectively.

The values for P *in planta* cannot be compared between growth solution and wastewater, as the duckweed was in situ for several months constantly growing and removing P. Therefore, the duckweed in experiments towards the end of this chapter had had significantly more time to accumulate P than those at the onset and as such do contain more. Values can however be compared between tanks of the same system to analyse P load and P uptake. The largest increase of internal P during an experiment was for growth solution at 5°C, probably due to the lack of growth at that temperature with continued P removal (Table 4.2.8.5 A, B and C).

Table 4.8.6 provides a comparison of GS with WW in terms of elements and other characteristics measured during both 15°C experiments. It highlights that there were some notable differences in micronutrients present, particularly Fe and Na which were 500x and 128,000x more concentrated in the WW than in GS.

Under the conditions applied it was apparent that P removal was better by duckweed kept on growth solution but that production of biomass slightly favoured wastewater. P was removed by 2 tanks in series down to concentrations that would be acceptable in a real WWTW discharge under 12h photoperiods and temperatures of 15°C, 8°C and 5°C.

Table 4.2.8.1. Comparison of media performance at fixed temperatures. 97.5th percentile values for phosphate (mg P L^{-1}) measured in the effluents of all 3 tanks in each experiment from Sections 4.2.2 to 4.2.7. GS = Growth solution; WW = wastewater. '*' denotes a significant difference between the same tank number of different solutions ($P < 0.01$).

Effluent P (mg P L^{-1}) (97.5th percentile)	**15°C**		**8°C**		**5°C**	
	GS	**WW**	**GS**	**WW**	**GS**	**WW**
Tank T1	3.2	4.2	3.6	4.1	3.6	4.5
Tank T2	0.1*	1.3	0.2*	1.6	0.2*	1.5
Tank T3	0.1*	0.8	0.1*	1.0	0.2*	1.1

Table 4.2.8.2. Correlation of P surface loading rate to P removal. P loading (mg P m^{-2} d^{-1}) was correlated to each respective tanks' P removal (mg P m^{-2} d^{-1}) to generate an R^2 value for each experiment.

R^2 values for P load vs P removal

Temperature (°C)	**GS**	**WW**
15	0.98	0.98
8	0.97	1.00
5	0.97	0.98

Table 4.2.8.3. Comparison of mean P loading and removal rates (mg P m^{-2} d^{-1}) from each experiment. A = growth solution (GS) experiments and B = wastewater (WW) experiments.

A. GS

Temp. (°C)	15	15	8	8	5	5
	P load	**P removal**	**P load**	**P removal**	**P load**	**P removal**
Tank T1	999	687	1,008	671	1,008	665
Tank T2	312	301	337	326	343	330
Tank T3	11	4	10	3	13	3

B. WW

Temp. (°C)	15	15	8	8	5	5
	P load	**P removal**	**P load**	**P removal**	**P load**	**P removal**
Tank T1	1,023	596	995	594	1,008	597
Tank T2	428	301	401	258	411	295
Tank T3	127	42	143	69	116	55

Table 4.2.8.4. Comparison of biomass production at fixed temperatures. Mean biomass increase (mean of day 10 and day 20 increase, g, fresh mass) by each tank. GS = Growth solution; WW = wastewater. '*' denotes a significant difference between the same tank number of different solutions.

Mean biomass increase (%)

	15°C		**8°C**		**5°C**	
	GS	**WW**	**GS**	**WW**	**GS**	**WW**
Tank T1	146	172*	46	60*	6	6
Tank T2	146	172*	36	40	2	2
Tank T3	152	172*	32	40*	0	2*

Table 4.2.8.5. Mean P in planta accumulated by duckweed. A = day 0; B = day 10; and C = day 20. Values are the % of dry mass represented by P.

Mean P *in planta* at day 0 (% dry mass as P)

A	15°C		8°C		5°C	
	GS	**WW**	**GS**	**WW**	**GS**	**WW**
Tank T1	0.8	1.4	0.9	1.3	1	1.2
Tank T2	0.8	1.4	0.9	1.3	1	1.2
Tank T3	0.8	1.4	0.9	1.3	1	1.2

Mean P *in planta* at day 10 (% dry mass as P)

B	15°C		8°C		5°C	
	GS	**WW**	**GS**	**WW**	**GS**	**WW**
Tank 1	0.8	1.5	1	1.4	1.5	1.3
Tank 2	0.8	1.4	0.9	1.3	1.3	1.2
Tank 3	0.9	1.4	0.9	1.3	1.3	1.5

Mean P *in planta* at day 20 (% dry mass as P)

C	15°C		8°C		5°C	
	GS	**WW**	**GS**	**WW**	**GS**	**WW**
Tank 1	0.9	1.7	1	1.6	1.7	1.6
Tank 2	0.8	1.4	0.9	1.5	1.6	1.2
Tank 3	0.8	1.4	0.9	1.4	1.4	1.4

Table 4.2.8.6. Comparison of mean values for constituent elements and other characteristics for GS and WW primary influents. Elements were measured by ion chromatography and characteristics were measured with water quality testing apparatus (Hach Lange, UK). Possible important differences are in bold type.

Element (mean mg L^{-1})	GS	WW
P	10	10
Mg	24	4
S	32	71
Ca	72	40
K	118	21
Na	0.003	383
Cl	290	874
Mo	0.006	0.003
Mn	0.05	0.01
Cu	0.004	0.01
Zn	0.008	0.07
B	0.2	0.02
Fe	0.0004	0.2
Al	ND	0.03
Characteristic (unit)		
NO_3^- - N	50	65
NH_4^+ - N	ND	0.1
COD (mg O_2 L^{-1})	ND	65
ORP (mV)	200	190
DO (mg O_2 L^{-1})	10	8
Conductivity (µS cm^{-1})	900	1700
pH	7	7.1
Temperature (°C)	15	15
Colour	Clear	Clear

BDL = Below Detection Limit

4.3. Discussion

4.3.1. P removal, P accumulation and growth between GS and WW

Duckweed supplied with growth solution removed more P than duckweed grown on wastewater at every temperature and at every point in a specific 3-tank system as indicated by 97.5th percentile values for effluent P (Table 4.2.8.1). 97.5th percentile values are referred to presently as they were the maximum concentration of P recorded in the effluents for 97.5 % of the time, which is a better indicator of performance than the mean. Characteristics such as macronutrients, pH and temperature were similar between GS and WW experiments, but samples from the 15°C experiments were additionally analysed for elemental composition and this highlighted several differences in the concentrations of micronutrients present (Table 4.2.8.6). Sulphur (S), Chlorine (Cl), Iron (Fe) and sodium (Na) were particularly notable in that they were 2x, 3x, 500x and 128,000 higher in WW than in GS. These concentrations are not assumed to have had a detrimental effect to the duckweed as reported in the literature (Landolt and Kandeler, 1987, Frick, 1985) and in any case they appeared healthy and grew more on WW than on GS (see subsequent sections). However, having elevated concentrations of (unchelated) Fe could have promoted the precipitation of a proportion of the P supplied in the WW to a form unavailable to the duckweed, leading to the poorer P removal seen. The assays used presently to measure P did not discriminate between various phosphate salts. As metals such as $FeCl_2$, $FeCl_3$, and Al_2O_4 are purposefully used in this manner to remove phosphorus from wastewater in WWTW, this could be an explanation for the reduced uptake from WW in this case. This would not necessarily be a negative for a hypothetical system however, as in theory this could be managed by more metal additions for P removal if there was a problem with the biological system, or less metal addition to promote more P uptake, depending on whether a system was to be for P removal or P recovery (in the biomass). The preferred option from an environmental and economic perspective would however be less metal addition and more biological uptake.

The other obvious difference between GS and WW is the organic content of the latter. As there was very little algae or bacterial assemblages present in the GS in comparison to WW, one would expect more P removal due to the aid of these microorganisms in WW. Any released organic P from dying duckweed could also be made bioavailable to living duckweed by P solubilizing bacteria, promoting uptake as suggested by Alaerts et al. (1996), but this does not appear to be the case. As stated above the health of the duckweed grown on WW was not in question, due them outgrowing duckweed kept on GS, so the answer for poorer P removal in WW may be a chemical one as discussed above. Duckweed have also been suggested to take up small organic molecules such as sucrose (Frick, 1994), and can contribute to the reduction of organic material found in wastewater (Korner et al., 1998). COD

was not measured through the whole system, only in the system influent as an indicator of quality. If heterotrophic growth of the duckweed was indeed occurring, this would in theory use some of the cell's energy supply (ATP) for organic molecule uptake by way of proton antiport. This energetic demand would not be required in the GS as it contained no organic supply. With the exception of tanks T1 and T2 at 5°C, WW promoted more % biomass increases than GS in every experiment (Table 4.2.8.4) and this could have been aided by a ready supply of organic molecules. The finding that the most P removal was not described by the best growing duckweed cultures also echoes some of the results from the previous chapter, which suggested that active growth and P removal were not exclusively linked at least temporally.

A further possible explanation for the poorer P removal of WW compared to GS was the unfortunately necessary experimental set up. Experiments 4.2.2 to 4.2.7 were conducted in sequence throughout the seasons in order to maintain similar conditions and to reduce the time implication of restarting experiments. This caused a gradual increase in internal P concentration of the plants as experiments progressed from GS to WW. At the beginning, internal concentrations were 0.8 % P as dry mass for the GS 15°C experiment. By the final experiment (WW 15°C), concentrations were up to 1.4 % P as dry mass – nearly double. Experiments in the previous chapter showed how manipulating the duckweeds' internal P content resulted in increased or reduced P uptake activity, therefore this aspect of the experimental design may have had an unwitting influence on the performance of the WW grown duckweed cultures. This also prevents the direct comparison of P accumulation between GS and WW and between temperatures, as these were variables tested at different times throughout the course of these experiments.

4.3.2. P removal, P accumulation and growth between P loading rates of GS and WW

Reducing the applied load of P by way of using two tanks in series promoted the removal of P down to low concentrations on a par with large, energy intensive WWTW in the UK. Maintaining a higher loading resulted in more P removed as an absolute amount. This means that the footprint of a hypothetical multi-tanked system could be justified, with an initial tank used for luxury P recovery (for later recycling) and downstream tanks for effluent polishing to low concentrations. In theory the value of duckweed biomass (high starch and high protein content) could provide a cost benefit for the system, and low P containing harvests from downstream tanks could be added to an on-site anaerobic digester for energy production. This could have impact for the several hundred small sewage treatment works in the UK that reside in rural areas, with less infrastructure or resources, but more available land for (hypothetically), large-tank biological wastewater remediation systems.

As temperature did not significantly affect P removal rates, mean values removed by the same tanks at different temperatures were calculated (From Table 4.2.8.3). The highest mean P removal values for GS and WW were 674 and 596 mg P m^{-2} d^{-1} respectively. Lowest P removal values were 3 and 55 mg P m^{-2} d^{-1} respectively for GS and WW. It is difficult to assimilate and compare data from the literature on this topic, due to the many different experimental setups and conditions applied, methodologies used and simply how data are reported. Attempting a comparison, data in Table 4.1.1 show that the present data for WW experiments compare most closely with Alaerts et al. (1996) and with Mohedano et al. (2012). The former author reported a loading rate of 123 mg P m^{-2} d^{-1} (maximum) with a removal rate of 40 mg P m^{-2} d^{-1}. This corresponds to the average values for WW tank 3 loads and removals of 130 and 55 mg P m^{-2} d^{-1} respectively. The latter author reports loading and removal rates of 390 and 360 mg P m^{-2} d^{-1} respectively, while average tank T2 load and removal values for WW experiments were 410 and 290 mg P m^{-2} d^{-1} respectively. This suggests that performance by the duckweed on WW in this study is similar to that by systems extensively used on larger scales in temperate and sub-tropical climates. If the duckweed could be manipulated to remove P to lower concentrations than those reported here, there would be justification for extensive trials and continuation of research.

P removal correlated highly to P loading rates for all experiments (Table 4.2.8.2). However, P loading rate only had a notable effect on P *in planta* at 5°C and this was more pronounced for GS than WW (Table 4.2.8.5 C). This was most likely due to the lack of growth with continued P uptake concentrating removed P into the remaining biomass. Evidence in the previous chapter was shown for P removal occurring independently of expansive growth and this seems to be case in tank T3 at 5°C. This does have positive implications from an engineering point of view as duckweed tanks could quite easily be sheltered from wind exposure and potentially covered during the cooler nights, to increase the local air temperature. In addition, depending on travel time via pipe networks and the HRT in each tank, WW temperature may never be as low as 5°C in any case.

P loading rate had no apparent effect on biomass increases with the exception of the 5°C experiment, where tank T1 produced 3x more biomass than tanks T2 and T3 for WW and 3x and 6x more than tanks T2 and T3 for GS respectively (Table 4.2.8.4). This suggests (in the absence of testing other variables) that in this case P (or other nutrients) could have been a limiting factor for growth in tanks T2 and T3 while metabolism was reduced.

4.3.3. The effect of temperature on duckweed growth

Unlike P removal, under the conditions applied, temperature had a significant effect on biomass growth for both GS and WW (Table 4.2.8.4). This was expected to be the case due to physiological response of duckweed to temperature and the correlation of growth to temperature in the previous chapter. Growth decreased as temperatures decreased from 15°C to 5°C and this is supported by Lasfar et al. (2007) who report a significant decrease in growth rates of duckweed at temperatures below 10°C. At 15°C, the mean increases by tanks T1 to T3 using GS and WW were 146%, 146% and 152 %; and 172 %, 172 % and 172 % respectively (Table 4.2.8.4). There was more variation in the amounts of biomass produced at 8°C and 5°C, therefore the reason for the inter-tank similarity at 15°C needed to be explained. Initial and day 10 stocking densities were set to 100 g (FM) which provided a fully covered yet uncrowded surface of each tank of $0.1 m^{-2}$ area containing enough duckweed to out-shade unicellular algae that may appear but leave room for growth. The maximum amount produced by the 15°C WW cultures was 172 % increase, or a total of 272 g (FM) per tank. This extrapolates to 2.7 kg (FM) m^{-2} of duckweed, a value within the maximum density range proposed by both Lasfar et al. (2007) and Driever et al. (2005). They proposed a maximum density limit of 180 g dry mass (DW) and duckweed has been reported to have a dry mass ratio of 3 % to 14 % of fresh mass. Therefore 2.7 kg (FM) could be 80 g to 380 g (DW). As all three tanks have exactly the same value for the 15°C WW experiment, this looks likely. Harvesting the duckweed at more regular intervals than 10 d would have provided more data with which to generate growth rates, but as harvest regimes have been shown to influence duckweed system performance (Xu and Shen, 2011, Vermaat and Hanif, 1998), it was seen necessary to maintain the same method throughout experiments 4.2.2 to 4.2.7 to allow for direct comparisons.

4.3.4. Conclusions

P removal was far better by duckweed grown on GS than on WW and this may be due to excesses of metals in WW rendering some of the P unavailable to the plant or competition (and the energy expended) at duckweed uptake sites between phosphate and organic molecules. However, it may also be explained by the fact that after months of being in situ, duckweed in the WW experiments had accumulated almost double the amount of P that GS experimental duckweed had. This internal concentration had been shown in the previous chapter to indeed have an effect on P removal and therefore it is difficult to draw a direct conclusion when comparing GS and WW in this way. Removal rates demonstrated by the WW grown duckweed were comparable to those of systems used abroad, and if duckweed had their internal P content manipulated to the same value as that of the GS experiments it could in theory improve the P removal capacity further. A two-tank GS system removed

P to concentrations on a par with expensive WWTW operations under simulated UK summer conditions and at temperatures of 8°C and 5°C under 12 h photoperiods, but the WW systems were not far behind in some cases. In theory, adopting a duckweed system for wastewater remediation would have a larger footprint and longer HRT than large scale chemical systems, but the footprint could well be offset by the benefits of less running costs and the production of beneficial biomass.

Increasing P loads increased P uptake as an overall amount and no toxicity symptoms were displayed under these conditions. The addition of a second tank vastly improved P removal and the reduction of supplied P downstream did not adversely affect the production of biomass with exception to experiments conducted at 5°C. This information was positive and important to identify for a system that could contain numerous tanks/aspects that could therefore be designated for either P recovery or P removal, depending on conditions and economic interests.

Duckweed grew better on WW than on GS and this may have been due to increased nutrition from small organic molecules and an increased internal concentration of P (relative to GS cultures) being available for this activity. Maximum density was probably reached by the 15°C WW cultures which was a mean of 2.7 kg (FM) m^{-2}. This density was important to realise and could be referred to when assessing the density of a large scale system to maintain health and performance.

Although there were some variables that time constraints prevented the testing of and some aspects of experimental design that prevented further important analyses, the results were taken as a positive on the whole. The experiments provided more data and confidence with which to develop the project to the final phase which would see the co-design and self-build of a large scale duckweed pilot system treating wastewater outdoors in the UK, as yet unpublished.

CHAPTER 5

A Pilot scale trial system to assess the growth of *Lemna minor* and removal of P from wastewater in a cool temperate climate.

5.1. Introduction

Results from the previous chapter showed that *Lemna minor* could be maintained on and remove P from wastewater (WW) under temperatures pertinent to UK summer and winter averages (and lower). Growth on WW was better than on growth solution (GS) and continued until space availability probably prevented more production, while P removal from GS was on a par with the more expensive and energy intensive methods adopted by large WWTW with 97.5th percentile values for P at 0.1 mg P L^{-1} in the effluent of a systems with two tanks in series. P removal was better by duckweed kept on GS than it was for those kept on WW, but this may have been down to elevated internal concentrations of P in the duckweed trialled on WW in comparison to those used with GS. Duckweed kept on WW still removed P down to low concentrations, therefore the need from a research and from the industry partners' perspective to identify if and how a duckweed system could operate outdoors in the UK and remove P from WW still needed to be addressed.

As introduced in the previous two chapters, duckweed have been shown to remove P and grow on WW in countries with warmer climates and more land availability at lower costs than here in the UK. The research in the present project initially provided data on the effects that particular variables such as temperature, photoperiod and P acclimation had on duckweed growth and P removal under highly controlled microcosm batch conditions (Chapter 3). Following on from that experiments were designed to introduce flow-through conditions at mesocosm scales and test the duckweed on real WW to examine if the duckweed had potential to operate under more robust conditions. As the results were all informative and in the main positive as to the direction of this project, the concluding chapter of the research project examines performance in a duckweed pilot system treating wastewater under a natural cool temperate climate.

Designs to upscale the system for a final time were made and a site was found by the industry partner that had a ready supply of treated WW and space to use. The location was at a golf club in rural Lincolnshire, UK. The design aimed once again to retain as much similarity with previous experiments where possible for continuity. The same *Lemna minor* isolate was used; Hoagland's growth solution was originally prepared in bulk to acclimate the duckweed before switching to live effluent and the

delivery pump flow rate was set to provide the same hydraulic retention time (HRT) as in the previous chapter (1 d $tank^{-1}$). Due to the scope and scale of this project, various aspects could not be controlled such as WW characteristics (pH, nutrients, organics and temperature etc.) and climate. These were to be monitored to identify what affect they may have on duckweed performance.

The objective was to design, build and monitor a pilot system treating WW throughout autumn and winter of 2016/2017. Once the system was operating, it was planned to monitor several characteristics of the WW (P, NO_3^-, NH_4^+, temperature etc.) and the biomass (P *in planta*, chlorophylls, growth etc.) and when steady state conditions (SSC) of P removal were apparent, maintain the system with a regular harvest.

This final results chapter initially describes the design and build of the pilot system, before reporting the results from start up through to SSC. The planned duration of operation was prematurely ended after 80 days due to the viability of duckweed health from around day 40 onwards. This is discussed later in the chapter and possible reasons for the poor performance of the pilot are discussed.

5.2. Results

5.2.1. System design and build

Before reporting on the operating performance, it is necessary to report the results of the design and build of the pilot system itself as this took a significant portion of the projects' time to organise and deliver. Although two tanks proved sufficient in the previous chapter, there were still several unknowns and the financial/time costs of possibly altering things on site at a later date meant that a three tank system was opted for. This would also maintain continuity with the previous chapter in terms of hydraulic setup. Principal design considerations included the surface area to depth aspect ratio; organic loading, HRT and temperature (Körner et al., 2003). On the ground the system had to be robust towards weather/wildlife; easily accessible for sampling/harvesting; the tanks needed to retain a large width to length aspect ratio and be impervious to light; and the hydraulics needed to be controlled. The site itself needed to have a ready supply of WW, relatively easy access, adequate security and an electrical power source.

The projects' industry partners secured an available site for the practical work for Phase 3. The site was a golf club in rural Lincolnshire, UK which along with weekly attending golfing members, held regular public functions at weekends and engagements that would involve catering, hospitality and post-function cleaning. The site treated its sewage by way of an HPAF package sewage treatment plant (STP) that was approximately 15 years old (Figure 5.2.1.1). The STP operated by way of primary and secondary settlement zones, an aeration and nitrate dosing zone to combat septicity and final gravity

discharge via sub surface ducting to drainage ditches on the site's borders. The nature of this site meant that there would be brief periods where no effluent flowed (at night and during quiet periods) and so abstracting directly from the sewage outflow would not be possible. Therefore, a collection or buffer tank would be required to hold several days' WW to supply for the duckweed tanks during these quiet periods. Figure 5.2.1.2 shows where the STP, discharge ditch and pilot system locations were, highlighting the distances between them and thus the work involved to link the system up hydraulically. Final effluent produced by the pilot system itself could not travel by gravity back to the drainage ditch, therefore a discharge trap containing a submersible pump was located adjacent to the buffer tank which periodically expelled final effluent back to the drainage ditch.

Figure 5.2.1.3 shows the hydraulic setup of the whole system (not to scale) and Table 5.2.1.1 gives dimensions. A submersible pump abstracted treated effluent from the final compartment of the STP and pumped it to the buffer tank via 20mm Ø hose lines. The buffer tank itself contained a submersible pump that returned the effluent to the STP at the same rate via the same hose type, to prevent freezing in the winter months, keep the WW fresh and to maintain the WW level in the buffer tank. The buffer tank (Figure 4.2.1.4) held a working volume of approximately 1,000L which would provide three days' worth of WW to the duckweed tanks during quiet times if needed. A Prominent Delta pump abstracted WW from the buffer tank and supplied it to duckweed tank 1 at 336 L d^{-1} which gave the system an HRT of 1 d $tank^{-1}$ or 3d overall. Tanks were connected by sub-surface 50 mm Ø pipes that included ventilation to the surface to prevent siphoning (Figure 5.2.1.5). WW travelled through the duckweed system and exited via gravity through 50 mm Ø MDPE piping to the discharge trap (100 L). Once the trap filled to a point that activated an internal submersible pump it was then transported back to the original discharge ditch via 20mm Ø hose lines. At numerous points, the approximate total of 600 linear metres of hose lines had to be laid inside ducting in sub surface excavations, or pulled through existing sub surface ducting. A Davis Vantage Pro weather station was installed between tanks 1 and 2 to record numerous parameters including air temperature and rainfall (Figure 5.2.1.6).

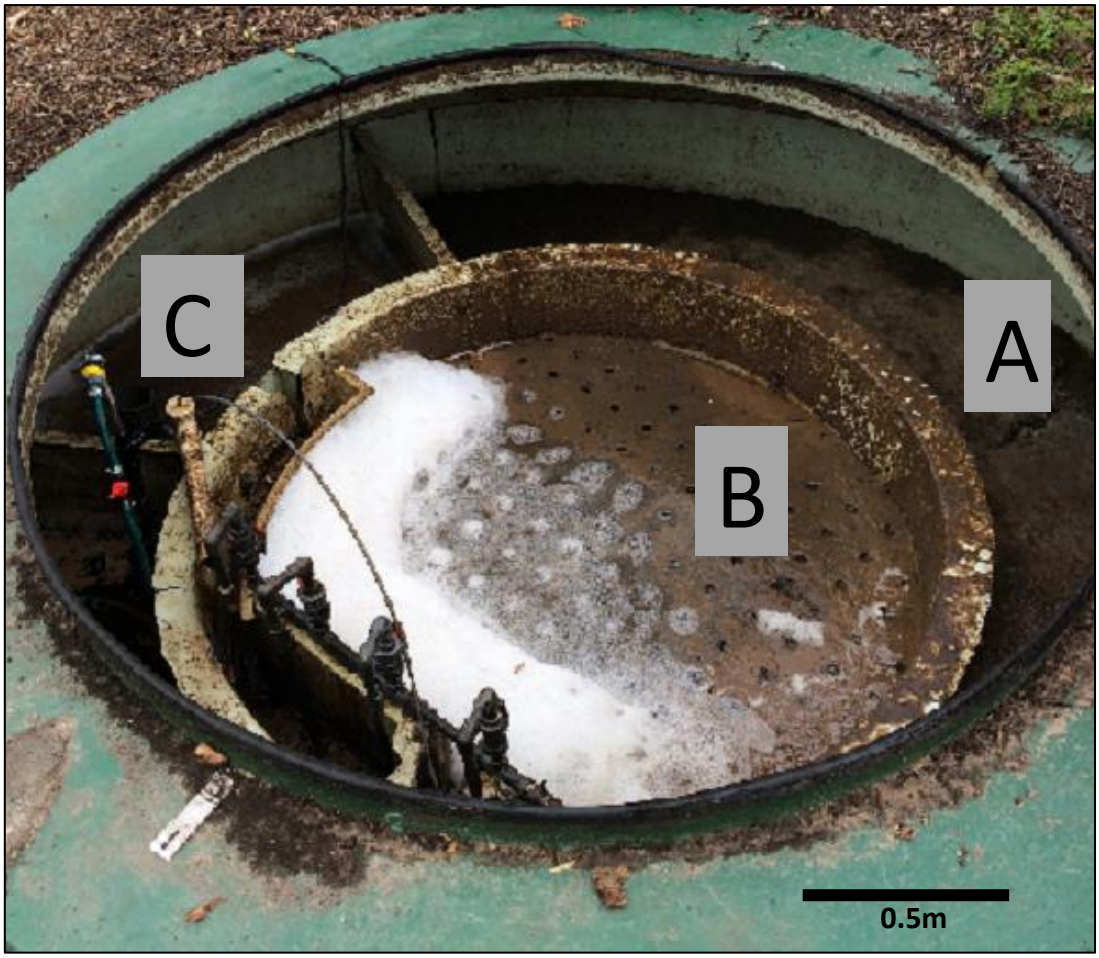

Figure 5.2.1.1. HPAF Sewage treatment plant used by golf club. A = primary and secondary settlement zones; B = aeration zone and nitrate dosing point (thin grey pipe); and C = final discharge and location of abstraction pump (red valve visible). Lid removed for photograph.

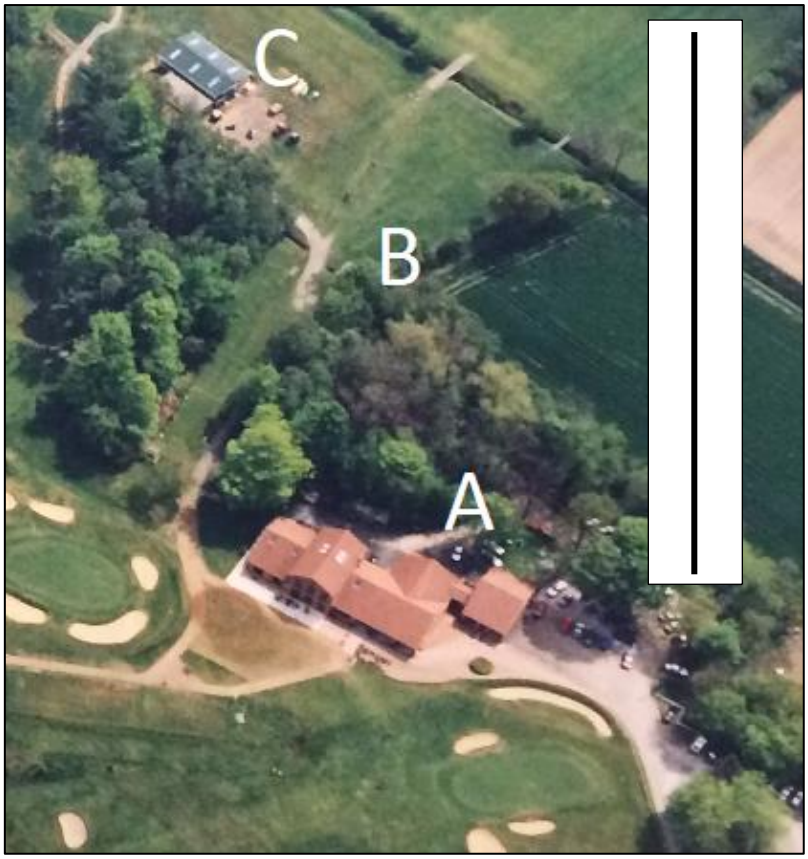

Figure 5.2.1.2. Aerial view of pilot site location in Lincolnshire, UK. A = Clubhouse and STP location; B = discharge drainage ditch; and C = buffer tank and duckweed system location. Scale bar on the right of picture represents approximately 150 m.

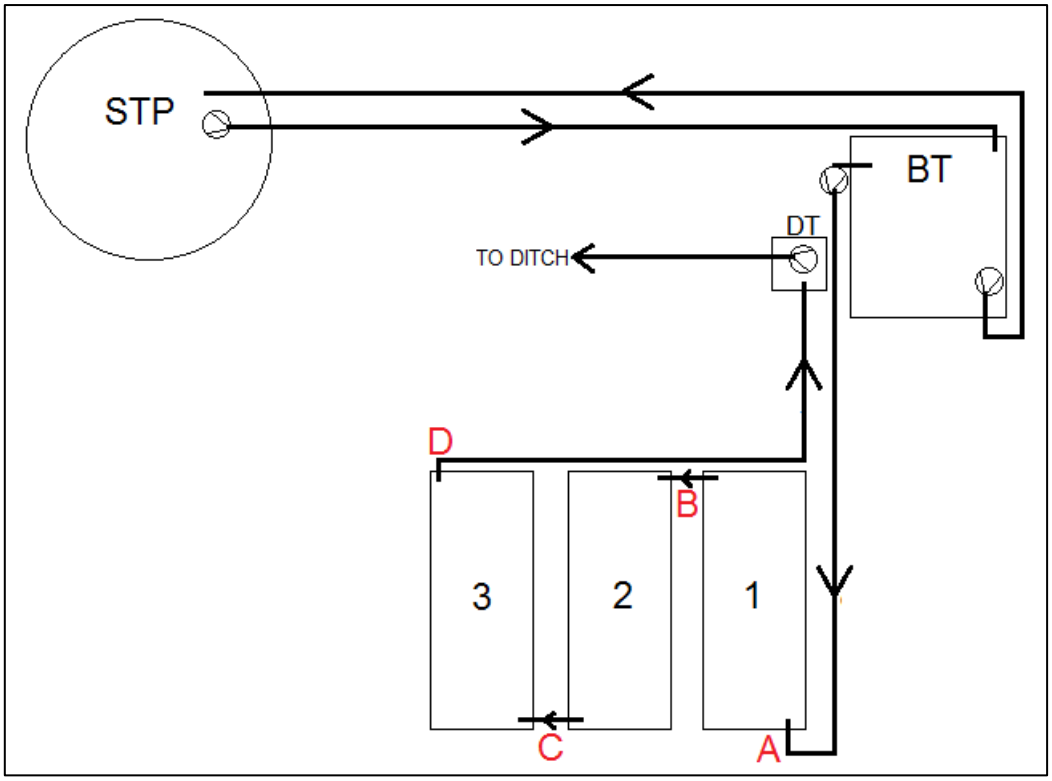

Figure 5.2.1.3. Pilot system hydraulic setup. STP = sewage treatment plant; BT = buffer tank; DT = discharge trap; 1, 2 and 3 are duckweed tanks 1, 2 and 3; and A, B, C and D were the location of sampling points for WW analysis. Drawing not to scale.

Table 5.2.1.1. Volumetric and hydraulic parameters set in the pilot system.

Aspect	Value
Surface area (m^{-2} $tank^{-1}$)	1.4
Volume (L $tank^{-1}$)	336
Flow rate (L d^{-1})	336
HRT (d $tank^{-1}$)	1

Figure 5.2.1.4. Pilot system buffer tank, delivery and discharge hydraulics. A = buffer tank; B = Prominent Delta delivery pump (to duckweed tank 1); and C = discharge trap containing submersible pump.

Figure 5.2.1.5. Duckweed tank layout. WW entered tank 1 inlet (bottom right corner) and continued through tanks 2 and 3 via ventilated sub-surface connections where gravity returned WW back to discharge tank. Clear Perspex screens were installed for wind protection and netting to prevent wildlife (birds/small mammals) from invading.

Figure 5.2.1.6. Weather station location. Sited between tanks 1 and 2.

5.2.2. System run in and acclimation

Materials were procured and the system was built, adapted and tested hydraulically throughout April and May of 2016. During this time the relatively small amounts of duckweed that had been transported from Leeds to site were allowed to bulk up and acclimate to outdoor conditions while being provided with Hoagland's growth solution formulated in bulk in the buffer tank. After several weeks of this the system was switched to allow the real WW to flush out the GS gradually. On 25th July the system was receiving full strength treated WW and conditions were monitored to observe when steady state conditions of P removal would ensue. A 25 % surface harvest was taken every 10d to maintain vigorous growth but retain enough cover to outcompete algae. The aim was to maintain steady state conditions and monitor performance as the year progressed through summer to autumn to winter. Results on WW characteristics and duckweed performance for the whole experimental period as well as during SSC are reported in Section 5.2.3.

5.2.3. System performance

Figure 5.2.3.1 describes the quality of several aspects of the WW that was delivered to duckweed tank 1 throughout the entire experiment. The pH remained relatively stable throughout the experiment but was higher than expected with a mean of 8.4. Over the 80 days from 25th July onwards, temperature inside the tanks ranged from 17°C to 11°C (24 h averages) and became slightly less stable from day 55 onwards. The influent was relatively more aerated during the 1st half of the experiment than the 2nd but fairly stable and not anoxic. Phosphate rose from 10 mg P L^{-1} at day 10 to 17 mg P L^{-1} at day 20 and fell to 5 mg P L^{-1} by day 40 and then became relatively stable with a mean of 8 mg P L^{-1}. At the same time as the spike in phosphate, ammonium in the primary influent also described a spike at day 20 reaching 45 mg N L^{-1} from 14 mg N L^{-1} in just 5 days. Ammonium decreased back to 7 mg N L^{-1} over the next 10 days before spiking once more to 24 mg N L^{-1}. After this second spike, concentrations fell right down to 1 mg N L^{-1} or less where they remained for the duration of the experiment. The ammonium spikes were proposed to be due to a failure of nitrification in the STP. From day 0 to day 35 there was also a decline in ORP from +150 mV to +10 mV and at this point the nitrate dosing unit at the STP was increased to prevent anoxic conditions ensuing and possible septicity. This was done by an industry operative working for the benefit of the golf club, but it was done without prior warning or discussion regarding an appropriate dosing concentration or introducing a more gradual increase. As a result the concentration of nitrate went from an average of 65 mg N L^{-1} from the previous 35 days to 605 mg N L^{-1} in just 5 days. Nitrate then fell by day 45 and recorded an average of 370 mg N L^{-1} for the rest of the experiment. Many of these spikes and declines made for an unpredictable experimental solution but from day 45 onwards the effluent appeared to stabilise for most characteristics.

As P removal and biomass production were the factors that were to be monitored more closely in the system, P was measured at all four points and biomass estimated in all three tanks from day 0 to be able to identify when steady state conditions (SSC) of P removal occurred. Conditions of P removal stabilised at day 40 in both the primary influent and in all three duckweed tank effluents (Figure 5.2.3.2). Throughout the experiment the effluent produced by the duckweed tanks did not describe any predictable pattern, such as lower P effluents in downstream tanks. Spikes of P were not typically absorbed by tank 1 and reduced for tank 2 and so on as expected and in several cases, downstream tanks actually recorded higher P effluents than upstream ones (Figure 5.2.3.2). This was in contrast to the clear effect that additional tanks had on P removal in the previous chapter and other publications.

Tables 5.2.3.1 and 5.2.3.2 present the 2.5th and 97.5th percentile values for all WW characteristics measured during SSC for all four points of the system. Both values are reported because certain

characteristics (such as pH or P) were notably and unexpectedly high, so it is important to know what the value was above for 97.5 % of the time as well as what values were below, to provide a complete range rather than just the means. During SSC, temperature, ORP and pH were relatively stable throughout the system, but the latter never fell below pH 8.1 (Table 5.2.3.1). Conductivity was never below 3,500 µS cm^{-1}, which was higher than the value at which previous experiments in Chapter 3 showed to be detrimental to growth and P uptake. Some nitrate and ammonium was taken up through the system and the low concentrations of ammonium during SSC were fortunate given the high pH values recorded, as very little ammonia would have been dissociated. Steady state conditions for ammonium are considered from day 45 onwards to omit a significantly high outlying value at day 40 as concentrations were still falling.

Most importantly for the project and unfortunately, P was never taken lower than 5 mg P L^{-1} even though it never went above 9 mg P L^{-1} in the primary influent during SSC. The mean value for final effluent (produced by Tank 3) during steady state conditions was 7 mg P L^{-1} (Figure 5.2.3.3.A). As the primary influent mean value was 7.7 mg P L^{-1} this is a mean removal of just 9 %. As the surface area of the whole system was 4.2 m^{-2} and the flow rate was 336 L d^{-1}, this calculates to mean P load and removal rates during SSC of 616 mg P m^{-2} d^{-1} and 56 mg P m^{-2} d^{-1} respectively. This is much less than what was calculated for WW experiments at 15°C in the previous chapter which were mean P load and removal rates of 1,000 mg P m^{-2} L^{-1} and 950 mg P m^{-2} L^{-1} respectively.

Figures 5.2.3.3, 5.2.3.4 and 5.2.3.5 describe all the mean values for each WW characteristic measured at each point of the system during SSC (means of nine samples from each system point, sampled every five days from day 40 to day 80 with the exception of NH_4^+ which was calculated from day 45 to day 80). Phosphate has already been described, pH and ammonium showed very little change while nitrate showed a gradual decrease as it moved through the system (Figure 5.2.3.3 D, C and B respectively). Dissolved Oxygen remained low but aerobic throughout the system, conductivity remained close to 4,000 µS cm^{-1} in each tank, while COD and TSS actually increased downstream through the system (Figure 5.2.3.4 A, B, C and D respectively). ORP remained between +160 mV and +200 mV (Figure 5.2.3.5 A).

In analysing the biomass, tank 1 produced more fresh mass, had a higher chlorophyll concentration and more internal phosphate than tanks 2 and 3 (Figure 5.2.3.5 B, C and D respectively). Unexpectedly, on average the duckweed in tank 3 produced more biomass and chlorophyll than tank 2. Tank 3 duckweed recorded slightly less internal P than tank 2 but the difference was not significant. As a result of the behaviour of most WW or biomass characteristics moving downstream through the system, no robust correlations or regressions could be made.

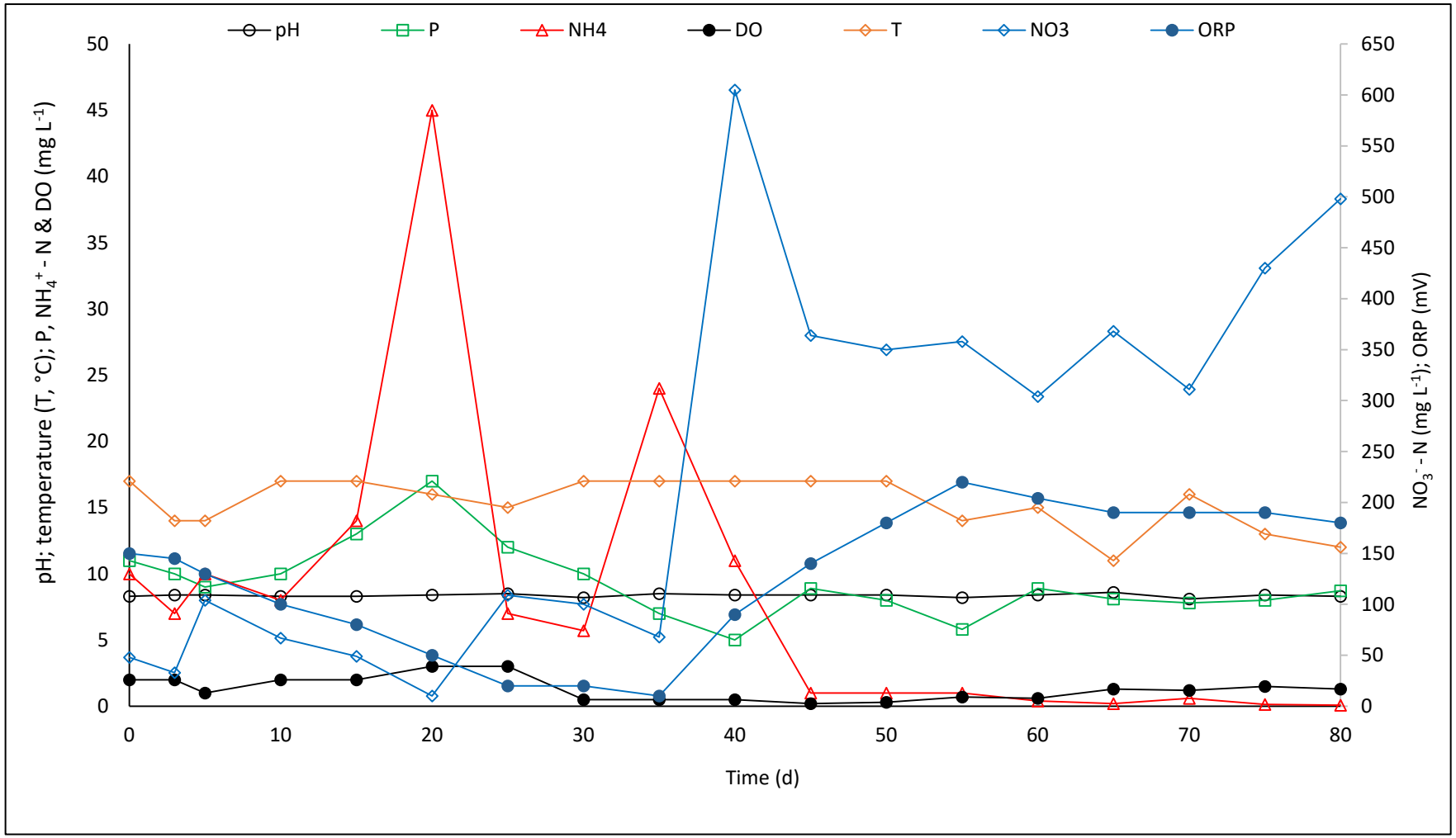

Figure 5.2.3.1. Influent wastewater characteristics. Grab sample values for pH, P (mg P L^{-1}), NH_4^+ (mg N L^{-1}), Dissolved Oxygen (mg O_2 L^{-1}) (Y axis) and (Z axis) NO_3^- (mg N L^{-1}) and Oxidation-Reduction Potential (ORP, mV). Temperature (Y axis, °C) was recorded remotely by temperature loggers in situ. Samples were taken (n=1), filtered (0.45µm Ø pore size) and processed on site immediately. Data presented for entire experimental duration of 80 days.

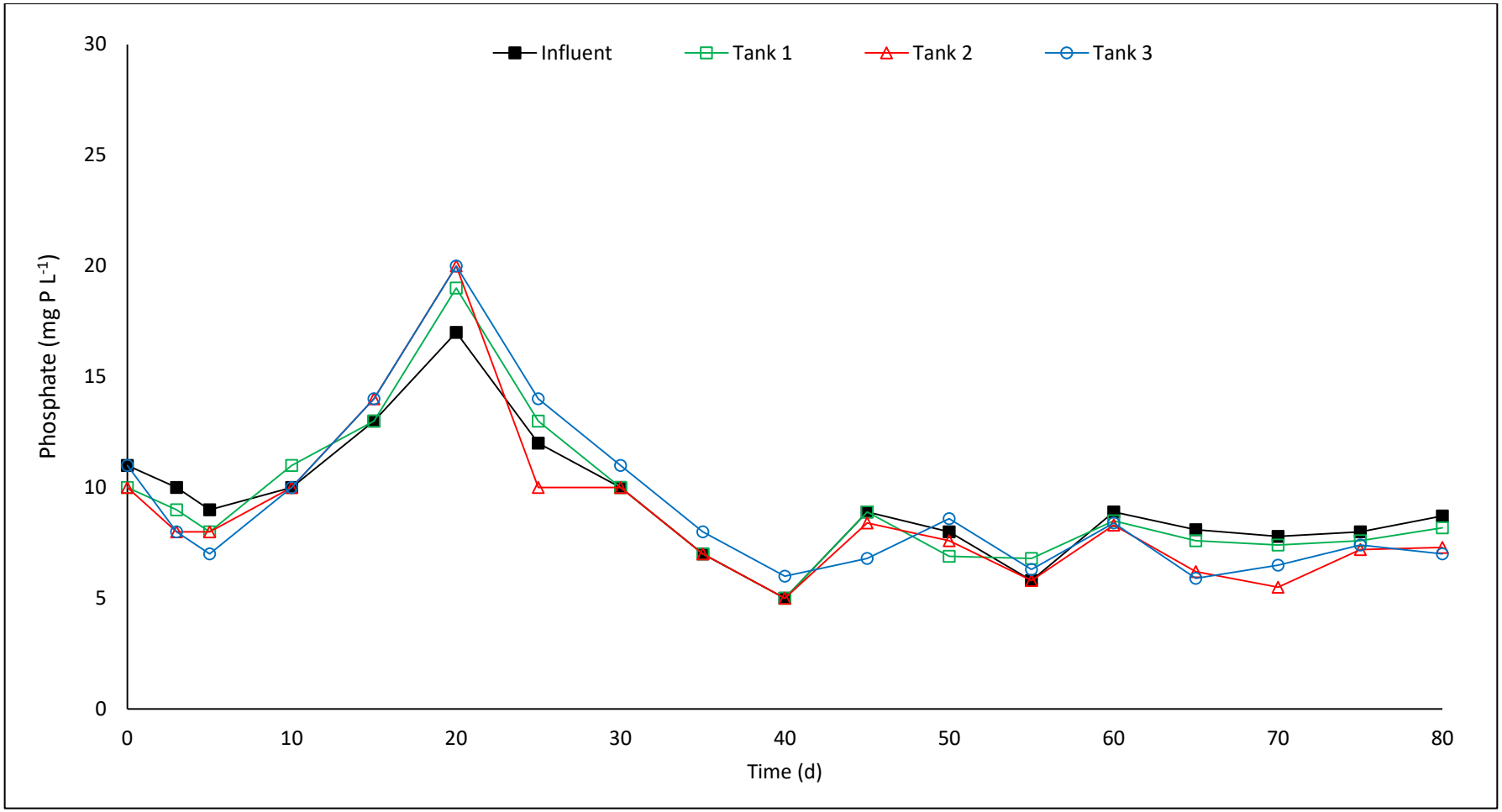

Figure 5.2.3.2. Pilot system P removal. Concentrations of phosphate (mg P L^{-1}) measured in the primary influent and in the effluents of duckweed tanks 1, 2 and 3. Samples were taken (n=1), filtered (0.45µm Ø pore size) and processed on site immediately. Data presented for entire experimental duration of 80 days.

Table 5.2.3.1. 2.5th percentile values for all parameters measured during steady state conditions (days 40 to 80). Samples were taken, filtered and processed immediately on site. *Values for ammonium were calculated from day 45 to omit an outlying value.

2.5th percentile values

PARAMETER	Buffer Tank	Tank 1	Tank 2	Tank 3
P (mg P L^{-1})	5.2	5.4	5.1	5.9
NO_3^- (mg N L^{-1})	300	300	280	200
NH_4^+ (mg N L^{-1})*	0.1	ND	ND	ND
DO (mg O_2 L^{-1})	0.2	0.3	0.2	0.2
COD (mg O_2 L^{-1})	60	60	60	40
TSS (mg L^{-1})	20	20	25	40
pH	8.1	8.2	8.4	8.3
Conductivity (µS cm^{-1})	3,900	3,500	3,700	3,800
ORP (mV)	160	160	150	150
Temperature (°C)	11	10	11	11

BDL = Below Detection Limit

Table 5.2.3.2. 97.5th percentile values for all parameters measured during steady state conditions (days 40 to 80). Samples were taken, filtered and processed immediately on site. *Values for ammonium were calculated from day 45 to omit an outlying value.

97.5th percentile values

PARAMETER	Buffer Tank	Tank 1	Tank 2	Tank 3
P (mg P L^{-1})	8.9	8.8	8.4	8.6
NO_3^- (mg N L^{-1})	580	470	450	450
NH_4^+ (mg N L^{-1})*	1	1	1	1
DO (mg O_2 L^{-1})	1.5	4.0	2.1	3.4
COD (mg O_2 L^{-1})	200	170	160	130
TSS (mg L^{-1})	30	35	45	65
pH	8.6	8.5	8.8	8.6
Conductivity (µS cm^{-1})	4,800	4,200	4,300	4,400
ORP (mV)	220	210	220	210
Temperature (°C)	18	17	17	17

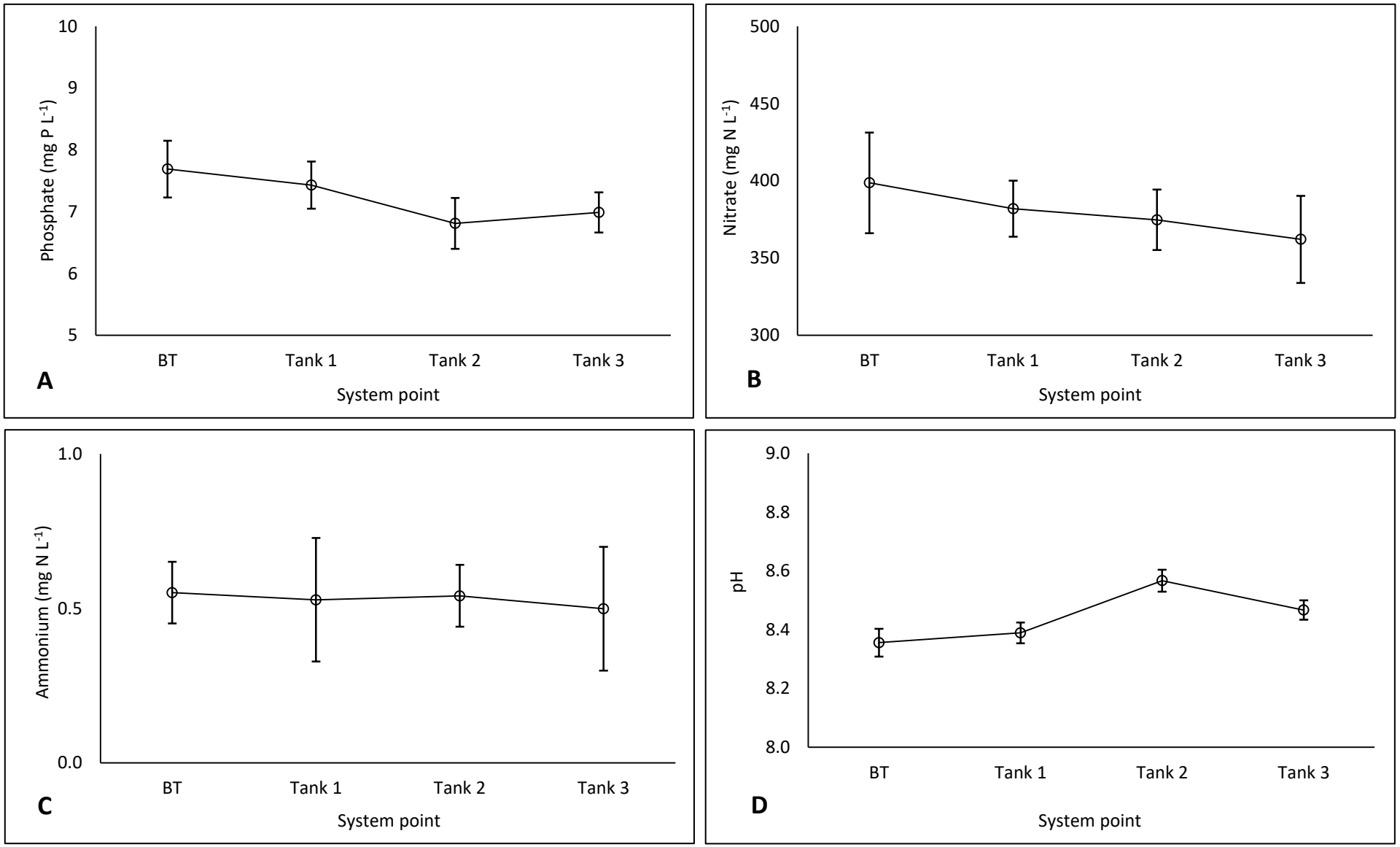

Figure 5.2.3.3. Influent and effluent quality of duckweed pilot system. BT = buffer tank; Tanks 1 to 3 are effluents produced by duckweed tanks 1 to 3. A = mean phosphate (mg P L^{-1}); B = mean nitrate (mg N L^{-1}); C = mean ammonium (mg N L^{-1}); and D = the mean pH. Samples were taken, filtered and analysed from a 3 tank duckweed system operating for 80 days during July to October 2016, receiving treated wastewater from a golf club in rural England. Samples were taken during apparent steady state conditions (days 40 to 80, n = 9). Error bars are standard error of the means.

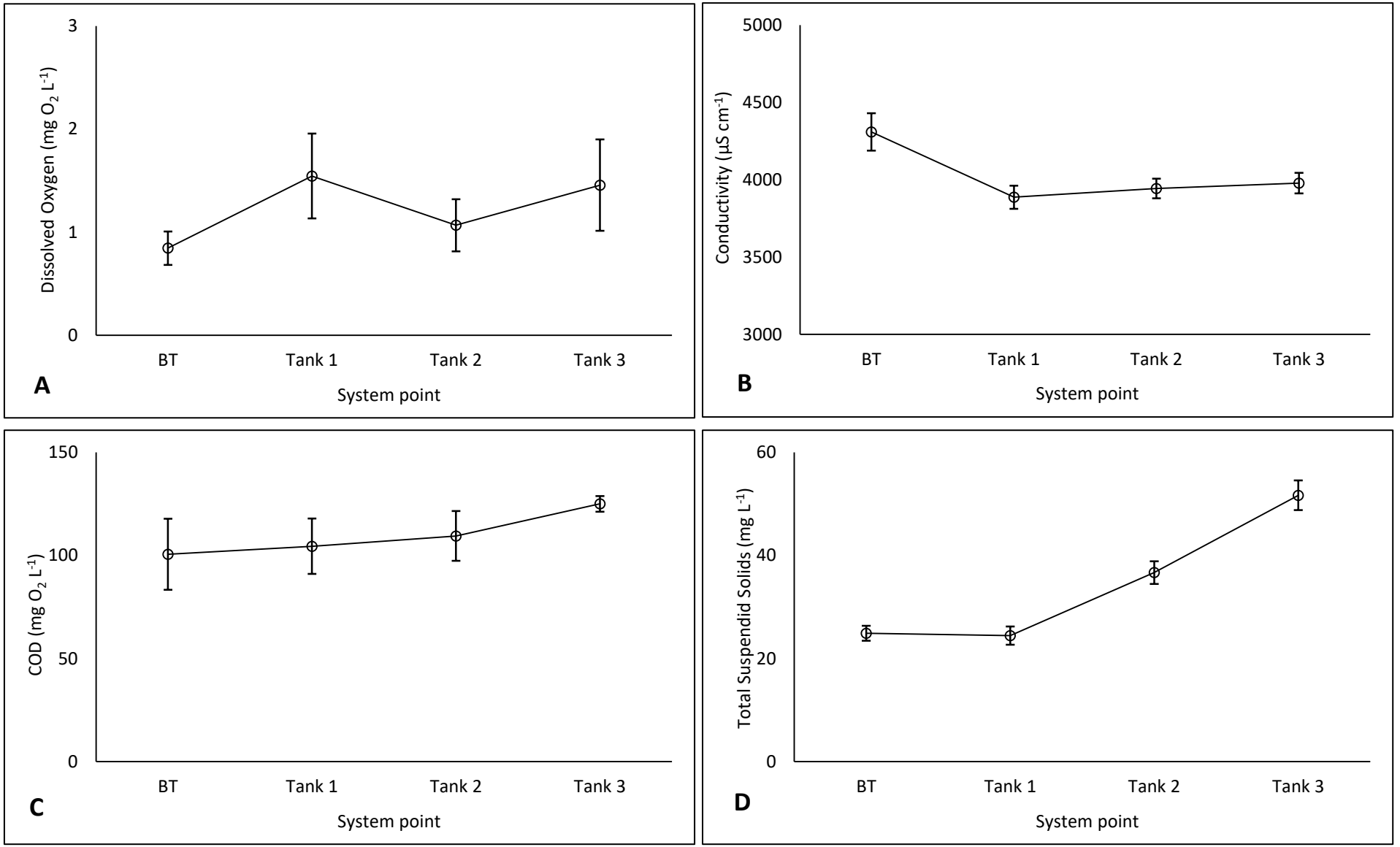

Figure 5.2.3.4. Influent and effluent quality of duckweed pilot system. BT = buffer tank; Tanks 1 to 3 are effluents produced by duckweed tanks 1 to 3. A = mean dissolved Oxygen (mg O_2 L^{-1}); B = mean conductivity (µS cm^{-1}); C = mean Chemical Oxygen Demand (mg O_2 L^{-1}); and D = mean Total Suspended Solids (mg L^{-1}). Samples were taken, filtered and analysed from a 3 tank duckweed system operating for 80 days during July to October 2016, receiving treated wastewater from a golf club in rural England. Samples were taken during apparent steady state conditions of P removal (days 40 to 80, n = 9). Error bars are standard error of the means.

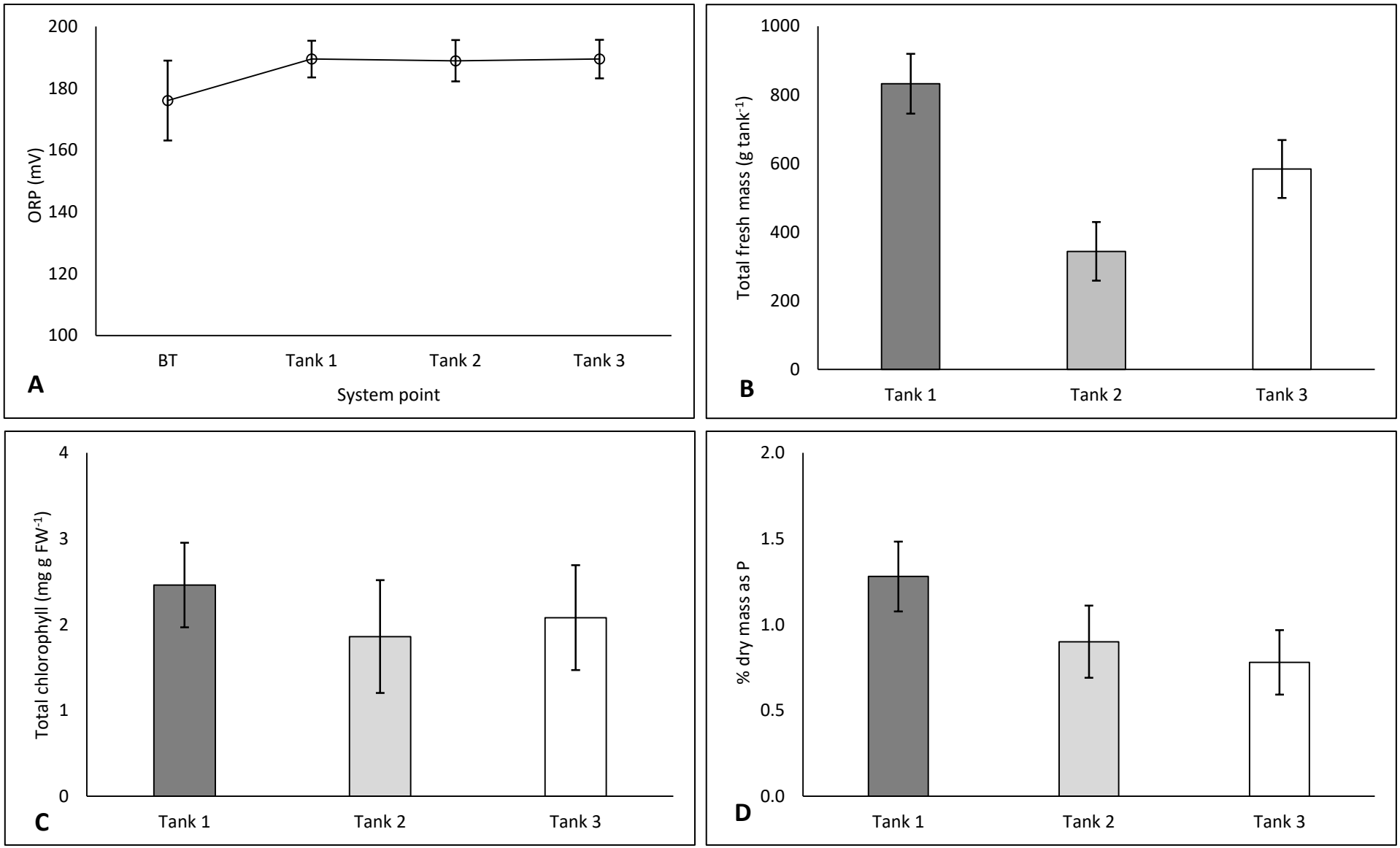

Figure 5.2.3.5. Effluent quality (cont.) and biomass analysis of duckweed pilot system. BT = buffer tank; Tanks 1 to 3 are effluents produced by duckweed tanks 1 to 3. A = Mean oxidation-reduction potential (mV); B = mean total fresh mass (g $tank^{-1}$); C = mean total chlorophyll (mg g FW^{-1}); and D = mean % of dry mass represented by inorganic P. Samples were taken and analysed from a 3 tank duckweed system operating for 80 days during July to October 2016, receiving treated wastewater from a golf club in rural England. Samples were taken during apparent steady state conditions of P removal (days 40 to 80, n = 9). Error bars are standard error of the means.

5.2.4. Decline of the duckweed system

At day 40 during the experiment, it was noticed that the duckweed in tank 2 was becoming visibly more chlorotic and smaller in frond size than duckweed in the adjacent tanks (Figure 5.2.4.1). After carefully removing as much of the unhealthy duckweed as possible over the next 10 days, the same phenomenon began to occur in tank 3 (Figure 5.2.4.2). By day 60 most of the duckweed in tank 2 was unhealthy looking (Figure 5.2.4.3), algae were beginning to encroach and the duckweed in tank 3 was continuing to decline also. At day 70 the duckweed in tank 1 had also started to rapidly deteriorate while those in tanks 2 and 3 had got even worse (Figure 5.2.4.4) and most of what live biomass still remained was unhealthy. At day 80 it was decided to conclude the pilot at that stage and start to dismantle, sterilise and decommission all the equipment and hose-lines before winter made this task even harder. As well as the visual signs of the duckweed's declining health, measurements of biomass production (Figure 5.2.4.5), chlorophyll concentration (Figure 5.2.4.6) and internal P concentrations (Figure 5.2.4.7) all described a steady decline of the vigour and health of the duckweed.

Final WW samples from each system point at day 80 were taken and analysed for element profiles to compare with the GS and WW used in the previous chapter (Table 5.2.4.1). There are some notable differences between the pilot's WW (Primary influent) and that of the previous chapter's WW in concentrations of elements. There was more than double the amount of Calcium (Ca) and potassium (K) in the pilot WW. There was much less sodium (Na) but still enough for the duckweed's requirements. In terms of micronutrients there was 3x less molybdenum (Mo) and 50x less manganese (Mn) in the Pilot's primary influent. There was also 3x more copper (Cu) in the pilot WW but it was not at concentrations suggested to be toxic to duckweed of 1.6 mg Cu L^{-1} or higher (Prasad et al., 2001). There was notably very little difference in the amount of elements measured between tanks (Table 5.2.4.2), which does not explain why the duckweed in tanks 2 and 3 began to decline and get worse far sooner than the duckweed in tank 1. In almost every case, the duckweed in tank 1 reduced the concentration of a particular element slightly, but concentrations in the effluents produced downstream were always higher, possibly due to decaying duckweed biomass.

In other characteristics, nitrate was 6x higher in the pilot's WW than in the previous chapter but not at a concentration thought to be toxic by itself (Table 5.2.4.1). Ammonium was 10x higher than the previous chapter's WW but again not at toxic concentrations. COD was much higher and the colour was brown in comparison to the clearer WW used in the previous chapter. As previously stated the conductivity was elevated and the dissolved oxygen was low, but as duckweed are argued to produce O_2 into the water column this was not seen as a significant issue.

The discussion for this chapter attempts to assimilate the data generated and provide reasons for the decline of the duckweed system, but given the unstable nature of the primary influent, the many biochemical and chemical reactions that could have been taking place at any point of the system and the sheer number of variables to consider, a definitive answer was not found and only recommendations could be made.

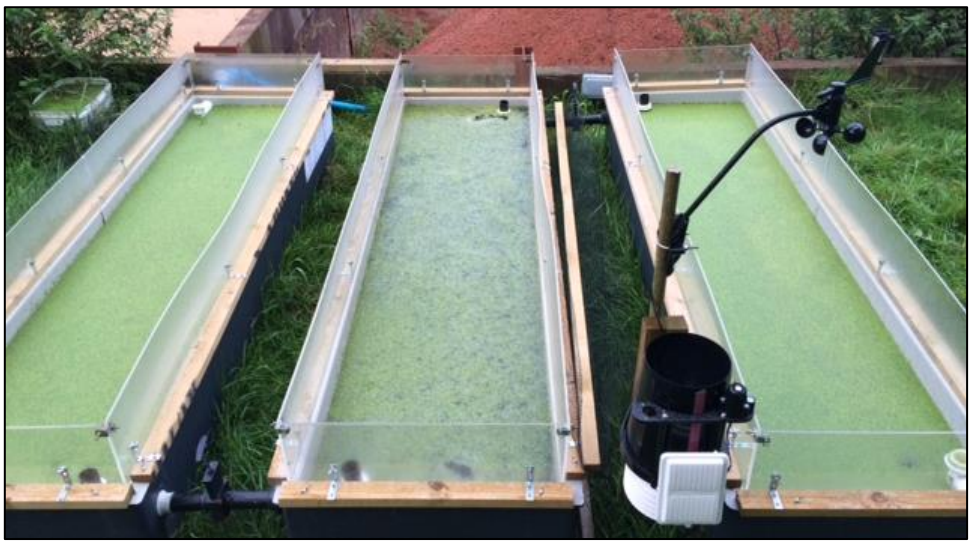

Figure 5.2.4.1. Unhealthy duckweed in tank 2 at day 40. Duckweed tanks 1 to 3 are right to left in this picture.

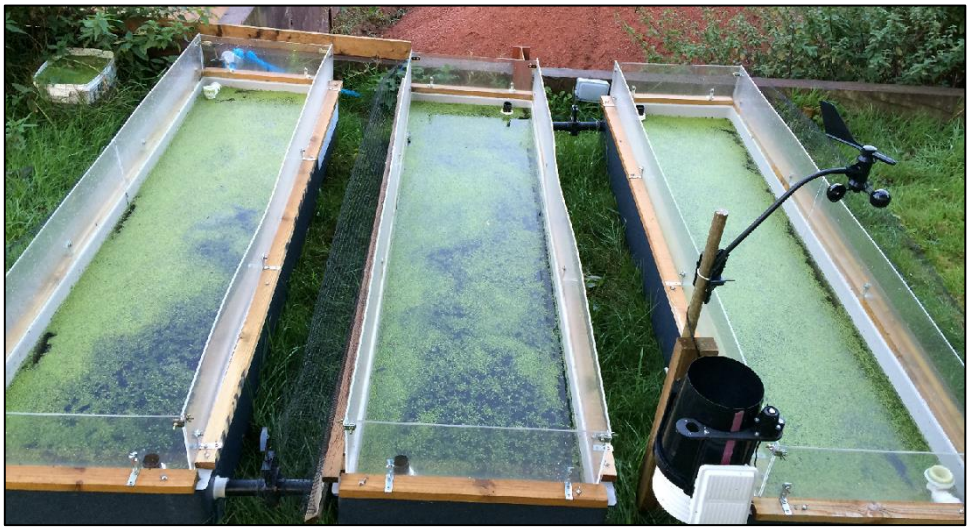

Figure 5.2.4.2. Declining duckweed at day 50. Tanks 1 to 3 are right to left in this picture.

Figure 5.2.4.3. Unhealthy duckweed at day 60. Tanks 1 to 3 are left to right in this picture.

Figure 5.2.4.4. Close up of duckweed in Tank 2 at day 70. Bleached, chlorotic and black fronds were witnessed as well as overall size reduction.

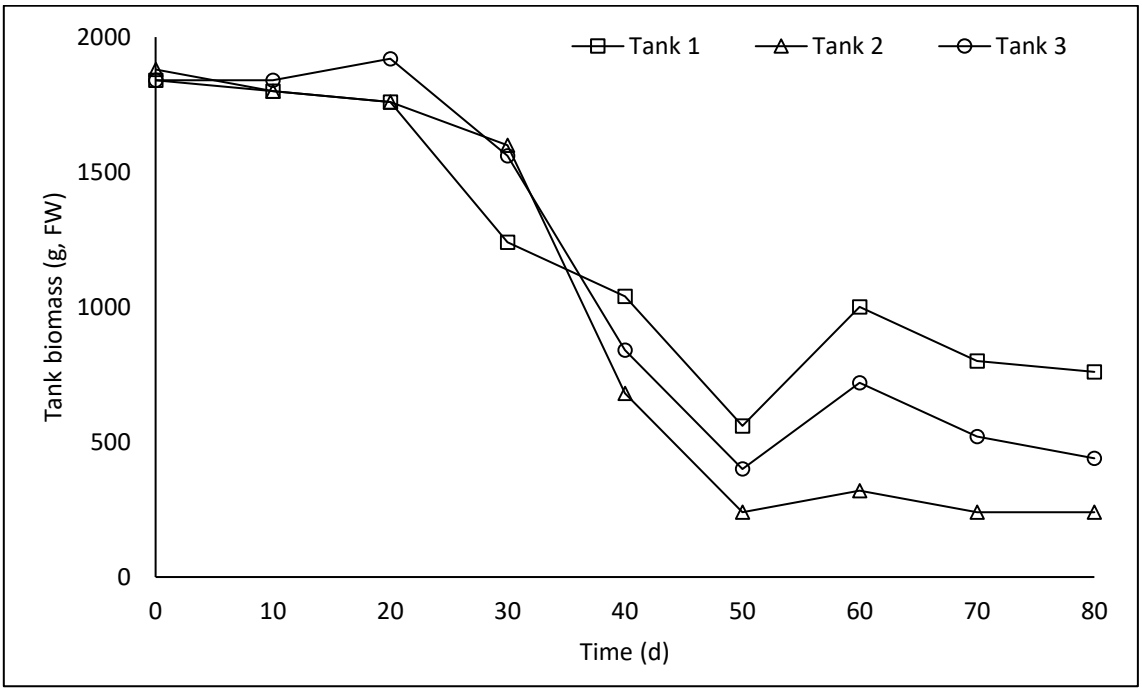

Figure 5.2.4.5. Decline in duckweed biomass production. Estimated total fresh mass (g) in each tank from day 40 to day 80. A 25 % surface area harvest was removed, span to remove excess WW and weighed on an analytical balance. The result was multiplied 4x.

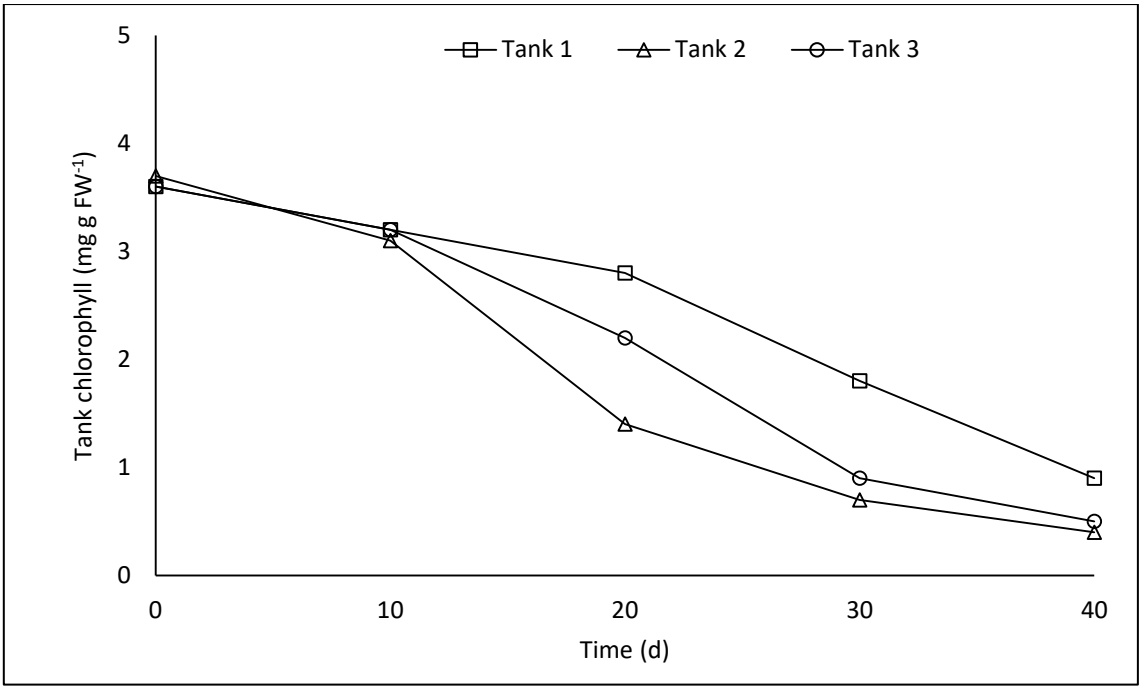

Figure 5.2.4.6. Decline in duckweed chlorophyll. Total chlorophyll (mg g FW^{-1}) was recorded every 10 d from day 40 to day 80 in random samples from each duckweed tank taken during harvests.

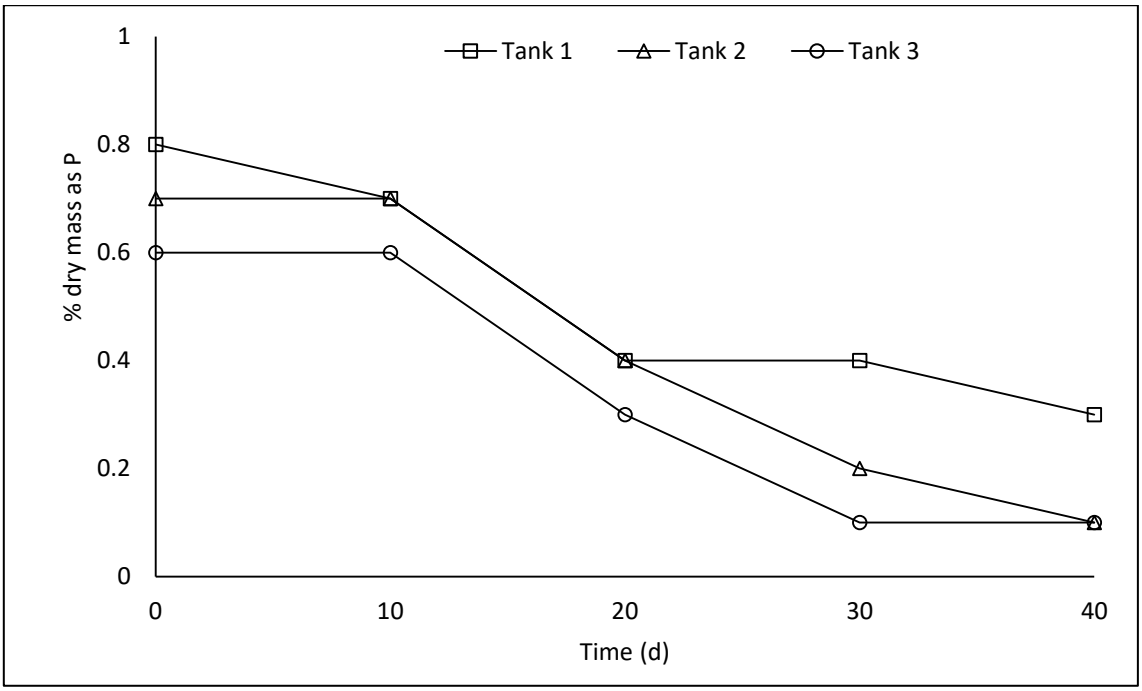

Figure 5.2.4.7. Decline in duckweed P content. Internal P content (% dry mass as P) was measured in the same random samples removed during harvests.

Table 5.2.4.1. Comparison of primary influent received by pilot system and the growth solution (GS) and wastewater (WW) used in Chapter 4.

Element (mg L^{-1})	GS (Chapter 4)	WW (Chapter 4)	WW (Pilot site)
P	10	10	8
Mg	24	4	7
S	32	71	26
Ca	72	40	97
K	118	21	47
Na	0.003	383	7
Cl	290	874	906
Mo	0.006	0.003	0.001
Mn	0.05	0.01	0.0002
Cu	0.004	0.01	0.03
Zn	0.008	0.07	0.03
B	0.2	0.02	0.3
Fe	0.0004	0.2	0.4
Mean characteristic (unit)			
NO_3^- - N	50	65	400
NH_4^+ - N	ND	0.1	1
COD (mg O_2 L^{-1})	ND	65	100
ORP (mV)	200	190	190
DO (mg O_2 L^{-1})	10.0	8.0	0.8
Conductivity (µS cm^{-1})	900	1700	4300
pH	7.0	7.1	8.4
Temperature (°C)	15	15	14
Colour	Clear	Clear	Brown

BDL = Below Detection Limit

Table 5.2.4.2. Elemental comparison between pilot primary influent and effluents produced by duckweed tanks 1, 2 and 3.

Element (mg L^{-1})	Primary Influent	Tank 1	Tank 2	Tank 3
P	8	7	7	7
Mg	7	6	7	7
S	26	21	23	25
Ca	97	73	86	91
K	47	29	31	33
Na	7	7	8	9
Cl	906	760	870	820
Mo	0.0010	0.0003	0.0004	0.0005
Mn	0.0002	0.0002	0.0003	0.0003
Cu	0.03	0.03	0.03	0.03
Zn	0.03	0.02	0.02	0.02
B	0.3	0.2	0.2	0.2
Fe	0.4	0.3	0.3	0.4

5.3. Discussion

5.3.1. Possible factors affecting duckweed health and system performance

The premature end to this pilot system trial came about due to the decline of duckweed health. While a definitive reason for this occurrence was not reached, there may be several plausible explanations and the possibility of an additive combination of these factors may also have played a role. Attempts were made to identify if the unpredictability of the primary influent and the spikes of ammonium and phosphate could be correlated to the number of people attending the golf club (more at functions and weekends, less during the week etc.) but robust and reliable data could not be found as no procedures were in place to record people numbers at the site. Several aspects of the quality of the primary influent were considered as reasons for the system's decline. Based on evidence generated in Chapters 3 and 4 of this thesis, the most likely candidates were assumed to be the relatively high conductivity and pH, the spikes of ammonium shocking the system at days 20 and 35 and the spike in nitrate that increased 10 fold between days 35 to 40.

Duckweed were shown previously in this thesis to deteriorate and remove only small amounts of P when exposed to conductivities of more than 3,000 µS cm^{-1} or when supplied with ammonium at 50 mg N L^{-1} at a pH of 7. The former could have disrupted osmoprotective compounds (Sikorski et al., 2013) and the latter could have periodically dissociated enough NH_4^+ to NH_3 and H^+ to disrupt cell proton gradients (Ludewig et al., 2007) or depleted cell energy levels by futile ammonium cycling (Britto et al., 2001). However, if the death of the duckweed was due to poor effluent quality or shocks, one would expect the duckweed in tank 1 to succumb to these effects first as it should have received the full strength of the influent. This was not the case as it was the duckweed in tank 2 that deteriorated the worst, followed by those in tank 3 and finally those in tank 1. It was also proposed that some of the elevated amounts (relative to Chapter 4 WW) of calcium, potassium and iron (Table 5.2.4.1) may have precipitated portions of the phosphate rendering it unavailable to the duckweed. This proposal would mean that the duckweed in tank 1 would take up any remaining nutrients and downstream tanks would be depleted. However, the elemental analyses between tanks (Table 5.2.4.2) showed that although tank 1 did reduce the concentration of several elements, nutrients were still available throughout the system and were at similar concentrations between tanks 2 and 3, therefore this reason does not appear to be a likely explanation.

Taken together, a short circuit causing a bypass of tank 1 and poor influent quality going straight to tank 2 may explain why duckweed in that tank deteriorated before that of tank 1. However, the HRT and the shallow and oblong shaped tanks make this unlikely. Baffles are used in larger scale WW treatment systems and are shown to increase effectiveness (Balla et al., 2014) by reducing dead zones

and short circuiting, but they were not installed in the present system. Tests were conducted during the run in phase however that added dye to the buffer tank and its course was followed as it migrated through the system (before duckweed was added) with no apparent short circuiting. This is a personal visual account only however and due to time pressures, hydraulic modelling was seen as beyond the scope of the project at the time. If further research were conducted in the future, hydraulic modelling and nutrient transect profiles in biomass-free systems would be recommended prior to biomass inoculations.

5.3.2. Phosphorus removal performance

In Chapter 3 it was shown that reducing the internal P concentration of duckweed promoted P uptake from solution and it was suggested that the internal P concentration of the duckweed acted as a signal for P uptake. When experiments were conducted between GS and WW in Chapter 4, it was noted that the internal P content of the duckweed stocks used for separate experiments was allowed to increase to nearly double the amount between the 1st and last experiments from 0.8 % to 1.5 %, in stocks grown on GS and WW respectively. This increase was tentatively suggested to have had an effect (a reduction) of P uptake capacity in the WW experiments. However in this chapter, duckweed in tanks 1, 2 and 3 began the experiment with internal P concentrations of 0.8 %, 0.7 % and 0.6 % (DM as P) respectively (Figure 5.2.4.7). This value only reduced over time and did not exhibit the same increases in P uptake as witnessed in Chapter 3 when those cultures were pre-incubated on low P concentrations (Section 3.2.8, Chapter 3). The discrepancy between the pilot's P removal performance and that of previous experiments conducted on WW in Chapter 4 was further apparent in terms of P surface load removal rates. In the previous chapter, at a similar temperature to what the pilot was exposed to (15°C), 95 % of the applied surface load of P was removed (Section 4.2.7, Chapter 4). This is in stark contrast with just 9 % removed by the pilot system's three tanks. If internal P content is indeed a signal for P uptake in *Lemna minor*, then in the pilot plant other factors may have been responsible for the disruption of this mechanism, either in the organism directly (E.g. the plants' chlorosis and senescence); or in the external environment of the wastewater (E.g. the presence of metals possibly rendering P unavailable).

As phosphate did not reduce downstream through the system, the contribution of decaying organic matter must not be ignored in terms of nutrient inputs to each duckweed tank. Szabó et al. (2000) described net fluxes of TN (82 %) and TP (73 %) from duckweed matter to water during decomposition experiments with *L. gibba*. Nitrate, phosphate, conductivity and redox potential were all showed to increase as the plant material was processed, using microbes found in waste water (Szabó et al., 2000). The authors reported that nitrate rose from trace concentrations to *c*. 70mg L^{-1} and phosphate rose

from *c*. 1 to *c*. 22 mg L^{-1} over 200 days. The fact that there would have been decaying matter, organic loading and duckweed dying in all the tanks unexpectedly could all have contributed to the poor results of P removal reported here. However, when biomass was analysed for internal P content, this was shown to reduce over time. It has been shown in the previous chapters of this thesis that constant P removal by the duckweed increases internal P concentrations. This is not the case in this chapter (Figure 5.2.4.7), therefore the lack of P removal throughout the system is likely due to poor P uptake in this case and not significant additions from decaying biomass. As the concentration of P did not rise significantly downstream, the uptake of this additive input may have been facilitated by bacteria and algae. There is evidence for this with the increase of total suspended solids (TSS) in the system downstream (Figure 5.2.3.4, D).

5.3.3. Wastewater quality

Reasons for the low dissolved oxygen content of the WW (measured in situ underneath the duckweed) could have been a high BOD content. BOD was not measured in this experiment but the effluent was brown in colour (suggesting a significant organic content). A duckweed cover that was too dense could prevent gaseous exchange between the WW and the atmosphere or bacteria associated with the duckweed mat could have consumed all available Oxygen during growth and aerobic respiration but the density used in these experiments was not considered to be extreme and was calculated to be within maximum density limits recommended by Lasfar et al. (2007) and Driever et al. (2005).Brix and Schierup (1989),Culley and Epps (1973) and Zirschky and Reed (1988) all suggest anaerobic conditions (< 1 mg O_2 L^{-1}) directly underneath duckweed mats. However Stowell et al. (1981) suggest that duckweed actually produce Oxygen at the root-water interface. It may not definitively correlate with BOD in this case but TSS measured at points A to D rose along with COD levels, both of which are also related to organic content of WW.

Fats, oils and grease (FOG) and detergents have been suggested to hinder duckweed growth and uptake of nutrients (Iqbal, 1999). The FOG could possibly facilitate a hydrophobic barrier that adheres to the plants' root and frond surfaces, preventing uptake or transfers (Iqbal, 1999). Detergents may damage the outer cells of the plants' interface with their environment, again disrupting uptake and reproduction (Skillicorn et al., 1993), however Gijzen and Khonker (1997) report duckweed tolerance to detergents. No quantitative data on the amounts of FOG and or detergents present in the primary influent was able to be gathered, however regular foaming occurs in the STP during aeration (as seen in Figure 5.2.1.1) and information from the cleaning staff on site suggested that although they did not use bleach, the kitchen staff did (daily) and the waste lines from the kitchen run into the STP. In addition, the fat trap outside the golf club kitchen rarely performs adequately as it is located so close

to the kitchen outlets and also receives very warm dishwasher waste, thus probably emulsifying the fats before the trap can do its job. These fats could possibly cool and re-harden later in the system. Occasionally a slight sheen on the surface of the contents of the buffer tank was noted, but as explained above, in the absence of a short circuit, one would expect duckweed tank 1 to bear the brunt of any potentially harmful properties of the WW before tanks 2 or 3 and this was not the case.

5.3.4. Possible fungal infection and heterogeneity

A further potential explanation for the decline in health of the duckweed, and one that goes towards answering why the decline began downstream in the system, could be a fungal infection. There is extremely little published literature on the fungal pathogens of duckweed, possibly a testament to their robustness. However, a publication by Rejmankova et al. (1986) describes dense stands of duckweed decimated by the fungus *Pythium myriotylum* outdoors in Louisiana, US. Anaerobic conditions ensued under the duckweed mat and gas bubbles formed (as was witnessed occasionally on site in this study). The authors (Rejmankova et al., 1986) later inoculated several species of duckweed in the laboratory and correlated temperature with rates of infection. They concluded that at 22°C or over, dense colonies would spread the infection exponentially and die rapidly. The duckweed would still die at less than 22°C but at a slower rate. Of the species investigated, *Lemna minor* (used in this thesis), *L. gibba and S. Polyrrhiza* were killed outright. *L. valdiviana* showed symptoms and died but was more resistant. *L. aequinoctialis* and *S. punctata* did not show any symptoms and survived.

If the duckweed in the pilot system did indeed succumb to a fungal infection this may have been exacerbated by the monoclonal content of the tanks. Zirschky and Reed (1988) suggested that mixed species systems may combat infection by way of heterogeneity and competition. Selective harvesting of the duckweed present in the pilot system was practically impossible as the unhealthy plants were so well dispersed and this would have meant a remaining inoculum of just a few healthy fronds, leaving the system open to rapid algal proliferation from the lack of shading. The infection may have been allowed to spread upstream from tank 1 to tank 2 during two brief periods of a pumping failure at days 20 and 30. This was an overnight failure of the delivery pump that would have created batch conditions in the tanks and possibly allow the fungus to spread back to tank 1. Once normal delivery resumed the fungal pathogens would have been dispersed throughout the system. The dearth of information in the literature on this topic and the lack of available mycologists for opinion at the time mean that this is still speculation however, but would be the best explanation for it not being a primary influent quality issue.

Not all the duckweed present in the system deteriorated and died, at closing the system down on day 80, approximately 1 % of all remaining biomass still appeared green and healthy, relative to the rest. This may suggest a certain heterogeneity and resistance in the duckweed, which in retrospect was also apparent in nitrogen experiments in Chapter 3. In that case most of the duckweed exposed to ammonium died off if the pH was at 4 or 7 but there were several fronds remaining that still appeared healthier than the rest (Figure 3.2.4.4, Chapter 3). This may indicate that a degree of selective cultivation may be possible to produce intraspecific isolates with varied resistance to certain parameters. The performance of duckweed in terms of growth and nutrient removal has been shown to be different between and within species by Bergmann et al. (2000) who found differences between *Lemna* species in terms of growth and protein content. This finding could have beneficial impact for the design of a similar duckweed system to the one reported here, but that system would then need to be highly controlled to retain conditions for the particular duckweed isolate to thrive on. This is likely to be extremely difficult by being prohibitively costly and/or complicated to put into practice in a real world setting.

5.3.5. Conclusions

Moving from the relatively controlled experimental conditions of Chapters 3 and 4 to conduct a large scale long term experiment outdoors revealed insights in to the numerous parameters which would need to be considered for a duckweed P removal system. The number and complexity of biochemical and chemical reactions that could be taking place at any one time is daunting, without the additive effects of temperature and climatic fluctuations. For these reasons and those of projects' scope and resources, it was not possible to pinpoint an exact explanation for the death of the duckweed in the pilot system and its lack of P removal capacity.

If the lack of performance was due to an issue with primary influent quality, then one would expect tank 1 to succumb before downstream tanks but this was not the case. If a short circuit of tank 1 led to an overloading of some description to tanks 2 and 3, one would expect to see evidence for this in inter-tank comparisons but again this was not the case. Airborne fungal spores could have landed randomly in tank 2 and initiated the decline. This would have required a spread against the regular flow of WW through the system which would not be possible, however two brief hydraulic failures could in theory have allowed this. More airborne spores could of course also have landed on tank 1 at a later date than those of tank 2. Resistance of a small percentage of duckweed to whatever afflicted the system was evidence for the possibility of duckweed screening and acclimation to produce more resistant clones, but the variability and number of parameters that a hypothetical 'super isolate' would have to be acclimated to and tested against prevents the practicality of this.

In the final chapter, the general discussion will pick out key findings from each results chapter and discuss what implications they may have for this field of research. Retrospective ways to investigate some of the outcomes presented in this thesis are suggested along with possible future directions for this area of research.

CHAPTER 6

General discussion and conclusions

6.1. Introduction and recap of objectives

The ultimate aim of this project was to explore the possibility of using duckweed for wastewater treatment, primarily phosphate removal, under conditions of a cool temperate climate. The lack of publications on duckweed use under conditions of a cool temperate climate and a disparate body of knowledge necessitated this research. Closer to home, the UK imports large quantities of phosphate for use in agriculture and industry, but often loses significant proportions to agricultural runoff and inefficient wastewater treatment processes. These losses not only have a direct economic impact, but also disrupt the ecological status of aquatic systems.

The breadth of this project meant that the selection of potential variables to test was enormous, the scale of experimental setups could in theory range from Petri dish to pond-sized and the number of wastewater characteristics that could influence performance was somewhat daunting. What this research intended to do therefore was select and test a set of variables proposed in the literature to be the most impacting on the performance of a duckweed based treatment system, while controlling as many experimental aspects as possible, but increasing the research in scale incrementally to approximate more realistic conditions as the project developed.

Retrospectively, there were numerous other aspects to this research that would have been interesting and informative to explore, such as observing performance of species other than *Lemna minor*, or identifying the expression of particular proteins during varied exposure to nutrients. However, due to this project being directly linked with an industry partner, experiments were often time-limited and required to answer fixed hypotheses before moving on to another aspect, always with the final goal of constructing and testing a large scale pilot system in the latter stages.

6.2. Significant and novel results from the project

The culmination of this research saw a pilot system successfully built and trialled outdoors in the UK receiving real wastewater and this has not been reported or published to date at the time of writing. The operation and observations of the system were prematurely ended due to an inexorable decline and subsequent death of the duckweed stocks *in situ*, possibly due to abrupt changes in the quality of the influent attributed to poor performance of the wastewater treatment system feeding the pilot plant. While unfortunate, in itself this ultimate result was significant, in that it showed that more

consideration was needed in respect to the constituents and quality of wastewater that duckweed would encounter in a real system. Some of the results generated in the research leading up to the pilot trial did however reveal some clues as to what factors can impact on duckweed performance under the conditions applied.

6.2.1. Importance of duckweed selection

Acquiring and using a duckweed isolate naturally occurring in the UK was preferable to obtaining a laboratory-cultured clone from elsewhere, as this avoided the ethical impacts of possibly introducing a non-native species to the environment in the later pilot trials. It was also important to retain this particular isolate for use in all experiments for continuity, as previous publications have highlighted inter- and intraspecific variation in terms of growth rates and protein production (Bergmann et al., 2000). During work placement activities, a duckweed isolate was located occurring naturally in close proximity to the proposed pilot site. This was successfully identified as *Lemna minor*, initially by taxonomical keys (Landolt, 1980b) and later by molecular barcoding methods (Wang et al., 2010). It may not be realistic to expect one particular isolate to remain in situ in a large scale outdoor system, as the introduction of more species could occur by way of wildlife interactions. However, for many of the highly controlled experiments conducted in Chapter 3, adopting a single species isolate, devoid of epiphytic assemblages was appropriate and necessary. Duckweed stocks adopted for any proposed outdoor system would likely change due to introduction by birds and subsequent species competition if not managed carefully, for example by installing fine mesh netting above the tanks. Time pressures of this project prevented the exploration of this aspect but it has been suggested that a mixed species assemblage may well prove to be more vigorous than a duckweed monoculture (Zirschky and Reed, 1988, Zhao et al., 2014). This could be seen as important as reducing management and increasing the autonomy of a system would increase its benefits to would be users (i.e. water companies).

6.2.2. Nitrogen preferences of duckweed

The literature is somewhat divided with respect to which species of nitrogen duckweed will take up ‘preferentially’ when both forms of ammonium and nitrate are present (Fang et al., 2007), or which nitrogen species promotes better overall performance when supplied in isolation (Caicedo et al., 2000, Mohedano et al., 2012). The results from experiments in Chapter 3 suggested that when provided in isolation, nitrate facilitated better growth and phosphate uptake, while ammonium-supplied solutions actually caused chlorosis and impeded phosphate uptake. Reasons suggested for this were ammonium induced toxicity, with influx disturbing membrane proton gradients required for nutrient uptake and exhausting energy supplies while the duckweed tried to maintain cellular homeostasis, as found in Britto et al. (2001). The experiments conducted in Chapter 3 examined two nitrogen concentrations

(10 and 50 mg N L^{-1}) and two pH values (pH 4 and 7), but did not control and test nitrogen and pH independently. The dissociation of ammonium to ammonia is affected by the pH (Caicedo et al., 2000) and in these experiments the pH of the ammonium solutions rose significantly, which would have altered the chemistry of the experimental solutions. In addition, a pH of 4 is reported to be at the lowest limit for duckweed survival (McLay, 1976, Iqbal and Baig, 2016). With hindsight it would have been preferable to extend the range of nitrogen concentrations applied (E.g. 5, 10, 30, 50 mg N L^{-1}) while buffering the pH, to identify the true effect of nitrogen. The experiments that followed this did test pH (at 4, 7 and 10), while fixing nitrate to 50 mg N L^{-1} and this showed the true effect of pH, with results being as expected and duckweed performing better at or close to neutral pH values. The results of the nitrogen experiments describing preference for nitrate agree with the minority of the literature, but did not provide a definitive explanation or suggest an ammonium threshold value for duckweed tolerance. As wastewater can vary in the form and concentration of nitrogen due to local treatment processes and primary waste sources, this is still an important area of research. In the future, inoculating duckweed into solutions containing a combination of ammonium and nitrate at various concentrations, while appropriately controlling all other variables and measuring N uptake may go towards discerning true duckweed 'preferences' for nitrogen. Tracking the fate of the heavy isotope ^{15}N nitrogen through the cell would also provide clues to its residence time and metabolism. In the meantime, it seems that a wastewater treatment system with nitrification would benefit from having a duckweed system for total nitrogen and phosphorus control via biological uptake, removing the need for energy intensive bacterial processes.

6.2.3. Suitable tolerance to salinity

Electrical conductivity can be used as a proxy measure of salinity and is measured by wastewater management technicians for this reason. Duckweed is a freshwater macrophyte and as such is not expected to survive in waters with brackish or saline conditions. In Chapter 3 it was shown that the duckweed could cope with conductivities of approximately 3,000 µS cm^{-1} and lower. Similar values for salinity tolerance in duckweed were also found by Wendeou et al. (2013) but values much higher than this were said to be tolerated by duckweed in Liu et al. (2017). A conductivity of 3,000 µS cm^{-1} does not represent brackish or saline waters, but is much higher than good quality effluent should be when leaving a WWTW. Measurements made personally of wastewater at large WWTW in the UK including Esholt, Bradford, UK and at other WWTW during studentship work experience activities revealed conductivity levels were always way below this in secondary treated or final effluents. Due to disparities in the literature over an accepted tolerance limit for duckweed, empirical research for this was necessary and results not only agreed with several published studies, but highlighted a robust tolerance of *Lemna minor* for salinity, not expected due to their freshwater origins. While conductivity

does not discern between various ion species, results are a good indicator that *Lemna minor* would not be troubled by the salinities found in most UK municipal wastewater effluents.

6.2.4. Significant drivers of phosphate removal

As expected, temperature increased the phosphate uptake capacity of the duckweed, but the effect of photoperiod was negligible under the range tested. Increasing photoperiods from 6 h to 12 h increased the production of biomass, but did not increase phosphate uptake, therefore the rate at which phosphate removal occurs under these conditions was not limited by growth, a result which has not been previously reported in the literature.

When acclimated to low phosphate conditions, duckweed stocks maintained under short photoperiods and low temperatures (6 h and 8°C), removed the same proportion of phosphate from solution as those maintained under conditions of longer photoperiod and higher temperature (12 h and 15°C, see Chapter 3). These conditions were chosen to represent 24 h averages of British winter and summer respectively (MET Office, 2013) and research on duckweed performance under these combined simulated winter conditions has not been previously reported.

Starving duckweed cultures of phosphate in order to investigate the effects on membrane transport was carried out by Ullrich-Eberius et al. (1984), but purposefully acclimating duckweed to a varying range of phosphate, in order to manipulate internal concentrations and promote subsequent uptake has not been previously published in the context of wastewater treatment. It was shown in Chapter 3 that reducing phosphate in solution reduced internal stores, this in turn increased uptake significantly and the effect was similar to increasing temperatures from either 15°C to 25°C or increasing temperature from 8°C to 15°C. Under batch conditions, when acclimated for four days at 2 mg P L^{-1} or less, the duckweed could reduce phosphate in solution by 99%, taking it from 15 mg P L^{-1} to less than 0.1 mg P L^{-1} and achieving the UK TAG recommended concentrations under both summer and winter simulated conditions of photoperiod and temperatures. This has not been published previously and is a significant indicator of the potential of this species for phosphate removal in cool temperate countries like the UK.

In Chapter 4, placing duckweed tanks in series had the effect of reducing downstream exposure to phosphate, which in turn resulted in high removal percentages even under low temperatures, therefore the effect of phosphate deprivation to increase phosphate uptake can be designed into a flow through system. This showed that while low concentrations could be achieved, the system would not just be a simple one tank design. A large scale system appended to an existing WWTW would

require numerous tanks and design aspects to consider such as harvesting methods. These aspects would all have an impact on the systems overall area footprint and capital and operating costs.

If the results described above could be demonstrated at the large scale, it would mean that duckweed systems could be adopted in cool temperate countries as well as warmer ones. Investment and experience of operation in more developed countries such as the UK could then be fed back to less wealthy nations to improve their sustainable wastewater treatment practices as an international partnership.

Experiments that measured radiolabelled phosphate uptake in Chapter 3 suggested that duckweed displayed dual affinity for phosphate uptake, similar to previous publications (Ullrich-Eberius et al., 1984, Hase et al., 2004). Changes in the rates of phosphate uptake depending on concentration suggested that a low affinity high capacity system was adopted in times of phosphate abundance, while a high affinity low capacity system was engaged when phosphate was scarce. Taken together, the results from phosphate acclimation and kinetic experiments could have practical implications for a duckweed system design. Hypothetically, the phosphate removal capacity of a duckweed system operating within a real WWTW could have its performance improved by way of adopting a multi tank system. In theory, duckweed stocks in tanks downstream in a system would be exposed to less phosphate. Therefore these stocks could periodically be used by selective harvesting to inoculate the front end of the system to promote rapid phosphate removal and operate under semi-batch conditions. It was not possible to trial this with the pilot system reported in Chapter 5 due to the premature conclusion of the fieldwork, but this had been in discussion to possibly be trialled at some point. In addition, realising that uptake capacities and affinities change with changing concentrations could lead to a phosphate polishing tank downstream with longer residence time than bulk removal phases upstream, to assure current discharge standards were being met. Identifying the specific proteins involved during phosphate uptake in duckweed would ascertain if they are indeed members of the PHT1 family, which are present in numerous plant species. This would increase understanding of uptake mechanisms, allow for direct comparisons with other model plant species and possibly aid in predicting performance by way of uptake models and subsequently streamlining a system's design and footprint.

6.2.5. Full scale system complexities

There are a number of wastewater characteristics that if left unchecked could negatively impact on a biological system. Nutrient loads, pH values, organic loads and more hazardous chemicals could all play havoc with the density and number of species present, from microbes to macrophytes. What makes this even harder to manage is the number of treatments required at WWTW to remove aspects such as heavy metals, nutrients, biological oxygen demand and pathogens. Some of these methods have negative consequences for the biology in a system and as such need to be controlled carefully. Periodic shocks of ammonium, nitrate and redox potential to the pilot system and effluent of generally poor quality reported in Chapter 5, may all have played a synergistic or antagonistic part in the poor performance output. Due to the number of possible factors at play, it was not possible to provide a specific explanation for the death of the duckweed in the system, but likely candidates may also have included a fungal pathogen, constant high pH values and conductivities or short circuiting. Diluting the effluent in the buffer tank prior to entering the duckweed system may have helped, as adopted by Cheng et al. (2002b) following duckweed senescence in full strength wastewaters previously (Cheng et al., 2002a). To provide further suggestions as to what occurred at the pilot trial and for duckweed performance in general, it may be useful to conduct further mid-scale experiments with real wastewater and vary the quality during operation. This could involve using a relatively strong, secondary effluent, sampled immediately following sludge removal for example and then adopting serial dilutions of this while observing duckweed performance under controlled conditions.

6.3. Conclusions and future perspectives

The objectives of this research project were to conduct highly controlled experiments that identified the impacts of certain environmental variables, increase the scale and operational variables before culminating in the design, construction and monitoring of a large scale outdoor pilot system. These objectives were achieved and knowledge has therefore been added to the community of duckweed research. Results highlighted the complexity of biological wastewater treatment systems and the daunting number of factors that can affect systems that receive real wastewater. There are numerous ways in which the research presented in this thesis could be expanded and continued. Suggestions for these avenues include conducting more microcosm investigations on the interactions of nitrogen and pH, examining the length of time that duckweed could go without phosphate (or other nutrients) and what concentrations or length of exposure would prove toxic. Conducting more experiments at the mesocosm scale and manipulating the strengths or characteristics of wastewater to identify operational ranges of parameters like ammonium, pH or photoperiod would be highly beneficial. In addition, trialling a pilot system on more stable wastewater of a higher quality than that used in Chapter 5 and for longer periods than those tested, would be informative to investigate the effect of outdoor seasonal variations of climate. Assessing the performance of different duckweed monocultures or mixed species assemblages may also prove informative while helping to combat potential pathogens of duckweed, such as fungal species discussed previously.

This research project has highlighted the complexity of trying to manage a biological system to improve the sustainability of a crucial and finite resource. Hypothetically, if a portion of the duckweed in a system could be kept healthy while having their phosphate supply significantly reduced, then this duckweed could be transferred for rapid phosphate removal as well as low phosphate polishing. Pinning down an efficient method in how to specifically achieve this has not yet been realised, but results from this research have shown potential in *Lemna minor* and further research would be viable and important. The benefits from the biomass produced in a duckweed system such as high starch or protein content, or on site energy feedstock production would further support their utilisation in more developed countries, where a cost benefit analysis would be required when comparing with proven methods of wastewater treatment.

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APPENDIX I

A series of ranging experiments were initially designed and conducted at the beginning of this project to identify optimal growth solution profiles for the production of biomass and removal of P from media. A range of pH, P and N values (three values for each variable) were chosen to be investigated. These were: pH 4, 7 and 9; 5, 15 and 30 mg L^{-1} P and 10, 30 and 50 mg L^{-1} N and were chosen to represent waste water profiles reported in the literature. All twenty seven possible combinations (3^3 factorial design) of pH, P and N were tabulated (Table I, A), and nine were subsequently chosen (3^2 simplified factorial design) by a random number generator (Table I, B). As an aim of this project was to grow duckweed on waste water, it was agreed to consider the influence of both ammonium N and nitrate N on biomass production and P uptake separately, as waste water treatment outflows can vary in this respect. To achieve this, the nine chosen media combinations were replicated, with set A designated as the NH_4^+ and set B the NO_3^- treatments respectively.

Experiments were carried out on the nine growth solution combinations (Table I, B), to identify which solution promoted the production of the most biomass and/or the greatest removal of P from solution. These results are presented in Table II.

Table I. Solution combinations of pH, P and N used in preliminary ranging experiments. **A** = all possible combinations of pH (4, 7 & 9); P (5, 15 & 30 mg P L^{-1}); and N (10, 30 & 50 mg N L^{-1}). **B** = nine possible combinations of the original twenty seven, chosen randomly. Of the nine media combinations displayed here, two sets were made. Set A was formulated using NH_4^+ as the sole source of N and set B used NO_3^- only.

A		**mg P L^{-1}**	**mg N L^{-1}**	**mg N L^{-1}**
Media	**pH**	**(as P)**	**(as NH_4^+)**	**(as NO_3^-)**
1	4	5	10	10
2	4	5	30	30
3	4	5	50	50
4	4	15	10	10
5	4	15	30	30
6	4	15	50	50
7	4	30	10	10
8	4	30	30	30
9	4	30	50	50
10	7	5	10	10
11	7	5	30	30
12	7	5	50	50
13	7	15	10	10
14	7	15	30	30
15	7	15	50	50
16	7	30	10	10
17	7	30	30	30
18	7	30	50	50
19	9	5	10	10
20	9	5	30	30
21	9	5	50	50
22	9	15	10	10
23	9	15	30	30
24	9	15	50	50
25	9	30	10	10
26	9	30	30	30
27	9	30	50	50

B		**mg P L^{-1}**	**mg N L^{-1}**	**mg N L^{-1}**
Media	**pH**	**(as P)**	**(as NH_4^+)**	**(as NO_3^-)**
1	4	5	10	10
5	4	15	30	30
8	4	30	30	30
15	7	15	50	50
16	7	30	10	10
17	7	30	30	30
18	7	30	50	50
21	9	5	50	50
25	9	30	10	10

Table II. Top performing culture solutions from preliminary ranging experiments. Experiments conducted on the nine chosen solutions (Table II, B) produced the two best solution combinations below (1B & 15B) in terms of P removal and growth. These two solutions were duplicated to include a set containing ammonium and used in the more focused nitrogen experiments (Chapter 3, Section 3.4).

	Solution	% media surface area covered	% media P removed	% FW biomass increase
SET A	1	33	19	40
Ammonium	5	31	10	27
	8	28	3	40
	15	27	13	53
	16	55	24	220
	17	50	27	173
	18	55	23	160
	21	50	51	200
	25	57	42	280
SET B	**1**	**71**	**70**	**320**
Nitrate	5	47	35	180
	8	46	25	240
	15	**64**	**75**	**280**
	16	59	65	253
	17	46	39	187
	18	56	46	227
	21	52	64	207
	25	52	38	220

Cultures grown in solutions from set B (nitrate treatments) produced more biomass and removed more P from the media than set A (ammonium treatments). Data in Table II was recorded on the final day of 7 day experiments. Data in bold type are the top two performing growth solutions (1B and 15B) in terms of the percentage of solution surface area covered by plants; percentage of P removed from respective solutions and percentage increases in fresh mass. For the nitrogen experiments (Chapter 3, Section 3.4) these solutions were renamed 1B and 2B respectively (for labelling order clarity) and duplicates of these utilising ammonium instead of nitrate were labelled 1A and 2A respectively.