Phosphorus Recovery from Wastewater through Enhanced Micro-algal Uptake

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The candidate confirms that the work submitted is his/her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Phosphorus (P) is an important constituent for living organisms as an energy carrier and a component of important biomolecules, which cannot be substituted with another element. Also, it is considered as the main element inducing eutrophication in freshwater bodies. In order to control eutrophication, wastewater treatment works (WWTW) remove P from their final effluent mostly through chemical precipitation and biological bacterial uptake. However, in order to achieve nutrient recovery from wastewaters, it has been suggested alternative biological processes based on biological algal uptake. Microalgae can be used to recover P from wastewaters as they can assimilate high amounts of P for their growth and store any excess as polyphosphate (i.e., luxury P uptake). This study is aimed at examining the potential use of microalgae cultivation for P recovery from wastewater and to identify the implications for its implementation at large WWTW. *Chlamydomonas reinhardtii* 11/32C was chosen as a model organism for this study. With regard to the role of the Nitrogen (N) source (ammonium v. nitrate), results show that there is little impact on P uptake when either ammonium or nitrate is used as the N source; however, by using a mix of ammonium and nitrate, P uptake increases as a result of stress conditions as ammonium is consumed faster than nitrate. Controlling environmental factors for microalgae growth showed that luxurious intracellular P uptake can range between 0.3 and 3.6% dry weight; optimum conditions for algae growth and P uptake where controlled by N concentration (200 mg N L^{-1}, 50:50 NH_4^+:NO_3^-), phosphate concentration (100 mg P L^{-1}), light intensity (250 $\mu Em^{-2}s^{-1}$) and photoperiod (16 hr light: 8 hr dark), resulting in a maximum algal biomass production of 148 mg VSS $L^{-1}d^{-1}$ and intracellular P uptake of 2.8 mg P $L^{-1}d^{-1}$. This study proved that *C.reinhardtii* 11/32C able to store the excess of P as polyphosphate granules located in cell's vacuole. A continuous flow mixotrophic microalgae cultivation system was operated to investigate its performance as a novel biological nutrient control and recovery process. Under tested conditions, the mixotrophic system was able to remove both nitrogen and phosphorus at 97% and 50% respectively, with average P uptake of 2.03 mg P L^{-1} d^{-1} and algal biomass production of 248 mg VSS L^{-1} d^{-1}. This system in operation generated on average 2.6 g L^{-1} of harvested algal biomass per day with a P content of 2.1% dry weight. These results show that a mixotrophic microalgae system has the potential to be implemented as a tertiary wastewater treatment process for biological nutrient removal and hence, a desktop case of study was conducted using data from a full-scale WWTW to assess the feasibility to implement this approach for nutrient recovery.

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List of Acronyms

AD	Anaerobic Digestion
AIC	Agriculture Industries Confederation
AISP	Acid Insoluble Polyphosphate
APHA	American Public Health Association
ASP	Activated Sludge
ASP	Acid Soluble Polyphosphate
ATP	Adenosine Tri Phosphate
BBM	Bold Basal Media
BP	Band Pass
BPR	Biological Phosphorus Removal
CCAP	Culture Collection of Algae and Protozoa
CCD	Charge-coupled Device
CFD	Computational Fluid Dynamic
CFU	Colony Forming Unit
COD	Chemical Oxygen Demand
CPD	Critical Point Drying
D	Dilution rate
DAPI	4’6-Diamidino-2-Phenylindole
DDSA	Dodecenyl Succinic Anhydride
DMP	Dymethilamino-methyl Phenol
DNA	Deoxyribonucleic Acid
DoE	Design of Experiment
DWD	Drinking Water Directive
EA	Environment Agency
EBPR	Enhanced Biological Phosphorus Removal
EC	European Community
EDX	Energy-dispersive X-ray
EU	European Union
FAO	Food and Agriculture Organization
FCM	Flow Cytometry

FE	Final Effluent
FFD	Fractional Factorial Design
FFD	Freshwater Fish Directive
FS	Forward Scatter
FST	Final Settling Tank
FWD	Freshwater Directive
GHG	Greenhouse Gases
HRT	Hydraulic Retention Time
HSM	High Salt Medium
HTR	Heterotrophic Reactor
IC	Ion Chromatography
IFA	International Food Association
iPHEE	Institute of Public Health and Environmental Engineering
ISSR	Inter-Simple Sequence Repeat
L I	Light Intensity
MMT	Million Metric Tonnes
MT	Million Tonnes
N	Nitrogen
NADP	Nicotinamide Adenine Dinucleotide Phosphate
OECD	Organisation for Economic Co-operation and Development
P	Phosphorus
PAO	Polyphosphate Accumulating Organisms
PAR	Photosynthetic Active Radiation
PBR	Photobioreactor
PBS	Phosphate-buffered Saline Solution
PFA	Paraformaldehyde
PPK	Polyphosphate Kinase
PR	Phosphate Rock
PST	Primary Sedimentation Tank
Q	Flowrate
RNA	Ribonucleic Acid
RSM	Response Surface Methodology
SC	Sedimentation Cone

SEM	Scanning Electron Microscope
SRP	Soluble Reactive Phosphorus
SRT	Sludge Residence Time
SS	Side Scatter
ST	Sedimentation Tank
TAG	Triacylglycerol
TEM	Transmission Electron Microscope
Tg	Terra gram
TKN	Total Kjeldahl Nitrogen
TN	Total Nitrogen
TP	Total Phosphorus
TSS	Total Suspended Solids
UK	The United Kingdom
UN	United Nations
US	The United States
UV	Ultraviolet
UWWTD	Urban Wastewater Treatment Directive
V	Volume of reactor
VSS	Volatile Suspended Solids
VSS	Volatile Suspended Solids
WB	World Bank
WWTW	Wastewater Treatment Works

Chapter 1 Introduction

1.1 Background

Phosphorus (P) is a fundamental element for living organisms and very important for high crop yields as it cannot be substituted by another element (Cordell et al., 2009; Cordell et al., 2011). P is a key factor in energy metabolism, mostly found in ATP and DNA, which is vital for the synthesis of lipids, protein and intermediate carbohydrates (Cai et al., 2013). In agriculture, P is an important element and constitutes one of the main components of any fertiliser. Industrial fertilisers support approximately 90% of global agriculture for both food and energy crops (Cordell et al., 2009; Steen, 1998).

Furthermore, the global production of P-based products is predicted to increase up to 100% by 2050, in order to cope with current consumption trends particularly for agriculture. The increment in P demand for fertiliser production is attributed to changes in global population and consumption habits, which are heavily influenced by better economic conditions for habitants in two of the most populated countries in the world (Cordell et al., 2009) – i.e., China and India contribute with 1.39 and 1.29 billion people respectively, which represents nearly 61% of all Asian population (UN, 2015; WB, 2016). Due to the increasing demand for P-based products in all aspects of life, together with the fact that P is a non-renewable resource, it is expected that current global P reserves will be depleted within the next 50 to 100 years (Cordell et al., 2009; Steen, 1998).

In addition, the availability of the P rock reserves worldwide is controlled by a handful of countries; over two-thirds of the world P rock production is under control by Morocco, China and the US (Cordell et al., 2009; Jasinski, 2014). Morocco alone holds for approximately 75% of the remaining global P rock reserves (Cordell and White, 2015). Similar to global oil reserves and production, this “P monopoly” may lead to P scarcity and international political influence on the supply chain as many countries rely

on the limited P reserves controlled by Morocco, China and the US (Cordell et al., 2009; Cordell and Neset, 2014; Cordell and White, 2015).

In 2009, the UK consumed 113.5 kt P that were mainly obtained by importing food, feed and fertilisers; imported fertilisers (78 kt P) accounted for 56% of the total P imports with an estimated of 1.4 kg P per capita per year (Cooper and Carliell-Marquet, 2013).

On the other hand, worldwide human wastewater discharge produces about 3.3 million metric tonnes (MMT) of P per year (Liu et al., 2008). Mihelcic et al. (2011) reported that the recovery of P from urine and faeces could cover 22% of the global P demand. In 2009, the potential recovery and reuse of P from urine could have contributed with approximately 1.68 MMT of P and it is predicted that by 2050, P discharges within urine could increase to 2.16 MMT per year (Mihelcic et al., 2011). Therefore, there are enormous benefits from harvesting even a portion of the nutrients embedded in human waste (N and P), which otherwise will end up stimulating eutrophication in water bodies. Nutrient recovery from waste will help us towards achieving long-term sustainability in agriculture and the improvement of surface water quality.

In the UK, wastewater treatment works (WWTW) receive approximately 55 kt P present in raw wastewater every year, from which 57% end up incorporated into sewage sludge, while the remaining (24 kt P per year) is discharged within the final effluent (Cooper and Carliell-Marquet, 2013). Since the implementation of the EU Urban Wastewater Directive 91/271 and Water Framework Directive (2000/60/EC), aimed at achieving good water quality status in water rivers, the UK water industry has been requested to meet a discharge consent of maximum 1 mg P L^{-1} (Kleemann et al., 2015; UWWTD, 1991; WFD, 2000). That has led to the increase of enormous amounts of energy and chemicals required to achieve the intended P removals from wastewater, but very little has been done to recover P for further reuse and recycle. Only recently, the UK water industry has made an attempt to recover P from wastewater via struvite production (i.e., a slow-release fertiliser) (Bhuiyan et al., 2008; de-Bashan and Bashan, 2004; Kleemann et al., 2015). However, this practice requires the addition of magnesium and the adjustment of pH, which is only economically viable for large wastewater treatment works. Therefore,

it is very important to develop innovative wastewater treatment systems aimed at meeting stringent nutrient discharge consents and to make the wastewater treatment industry more sustainable by including N and P recovery processes.

Microalgae have been identified as one of the most promising source for future renewable energy generation, which have no major impact on food production (Brennan and Owende, 2010). Photosynthetic microalgae utilise energy from the sun and assimilate nutrients like carbon (C), N and P from their environment (i.e., similar to those embedded in wastewaters). Microalgae can grow rapidly to produce biomass that contains valuable products (e.g., proteins, lipids and carbohydrates) and their versatile abilities can be used to produce biofuels, fine chemical products, food supplements and bio-fertilisers (Chisti, 2007; Spolaore et al., 2006). Therefore, microalgae can also be used as part of an integrated approach for wastewater treatment systems aimed not only at meeting the intended discharge consent, but at achieving long-term sustainability in the water sector by encouraging total resource recovery (i.e., nutrient recovery, bioenergy production, greenhouse gas mitigation by CO_2 fixation, etc.).

1.2 Importance of the conducted research

Developments in the microalgae bioengineering field are very important for enhancing current technologies used at wastewater treatment works and hence, it is worth to explore the ability of microalgae systems as tertiary treatment for nutrient control and recovery in existing wastewater treatment processes. Previous studies revealed that a wide range of microalgae species have been employed for nutrient recovery from various wastewater sources, by using either closed photobioreactors or open pond systems. Also, there are extensive studies on microalgae cultivation for nutrient recovery not only in batch cultures, but also in continuous flow systems using different arrangements that include the growth of microalgae under phototrophic, heterotrophic or mixotrophic conditions. This present research work was undertaken under batch and continuous flow culture conditions to study the potential use of mixotrophic microalgae systems for nutrient recovery from sewage via biological algal

accumulation and high algal biomass yields, and therefore suitable to be implemented in the existing wastewater treatment works.

The significance of this study covers the following: (a) this research contributes to improve our understanding of the potential use of microalgae for the recovery of nutrients from nutrient-rich, low-grade waste (i.e., sewage) and to achieve high algal biomass yields, (b) this work studies the potential of microalgae to take up phosphorus from wastewater and to store phosphorus in the form of polyphosphate within algal cells, (c) this study investigates the influence of intracellular phosphorus uptake on overall algal biomass production from wastewater streams, (d) this research also tests the possibility of microalgae to grow under independent heterotrophic and phototrophic conditions to enhance phosphorus recovery from wastewater, and (e) this study intends to provide evidence of alternative and more sustainable concepts for existing nutrient removal processes, with the potential to achieve total nutrient recovery and to eliminate nitrification-denitrification and bacterial phosphorus removal units from existing wastewater treatment works.

1.3 Scope, aim and objectives

This research contributes at reducing current knowledge gaps for the implementation of luxury P uptake by photosynthetic microorganism from wastewater streams. This study is conducted to investigate how environmental factors controlling algal growth are affecting luxury P uptake by microalgae. The study is aimed to examine the potential of engineered microalga systems to recover P in wastewater treatment works under nutrient-rich environmental conditions.

The specific objectives related to this research work are as follows:

1. To identify the environmental factors influencing luxurious P uptake in microalga cultivation using photo-bioreactors.
2. To explore the presence of hyper P accumulators in microalgae cultures, which may have the ability to accumulate high amounts of P in their cells under non nutrient-stressed growing conditions.

3. To develop and test at laboratory scale the potential benefits of an engineered process using photo-bioreactors for microalgae cultivation and P recovery from wastewater.
4. To investigate the potential to implement P recovery from wastewater via biological algal uptake in wastewater treatment works.

1.4 Structure of Thesis

This thesis is organized in nine chapters with Chapter 1 being the introductory chapter highlighting issues about the current P anthropogenic cycle, research hypotheses and objectives. Chapter 2 – Literature Review, which is focused on the environmental factors controlling luxury P uptake and optimisation of P uptake from wastewater using photosynthetic microorganism in continuous flow photo-bioreactors. Chapter 3 – Materials and Methods, describes in detail the research methodology used in this work and the materials and methods used for all experiments, including the use of specific techniques like flow cytometry, cell sorting and microscopy.

Chapters 4 to 7 are dedicated to present a detailed research methodology per each experimental work section and the corresponding results and discussion. They present the experimental work conducted to achieve objectives 1, 2, 3 and 4, described above. The results are reported, critically analysed and compared with previous research works published in the literature. Chapter 4 – Identification of environmental factors influencing optimum luxurious P uptake, investigates several important environmental factors controlling luxury P uptake and provides particular values of each important factor that help to optimise P uptake in *C.reinhardtii* 32C. In this experiment, microalgae were cultivated in a closed system using photobioreactors.

Chapter 5 – Exploring the presence of hyper P accumulators. This chapter includes the search for hyper P accumulators in *C.reinhardtii* 32C, which was cultivated under five different environmental conditions. The presence and location of in-cell polyphosphate granules was observed by Fluorescence Microscopy, Scanning Electron Microscopy (SEM) and Transmission Electron Microscope (TEM).

Chapter 6 – Operation of a continuous flow mixotrophic system with biomass recycle to enhance algal biomass production and luxurious P uptake. This work includes the investigation of algal growth kinetics in a batch culture, which was further used for design of a bench-scale system for studying P uptake by microalgae in a continuous flow photobioreactor; results from that systems are also reported in this Chapter.

Chapter 7 – Assessing the potential for implementing P recovery via biological algal uptake at a large sewage treatment works: this chapter uses a mass flow analysis for P streams in an existing wastewater treatment works. Results help to assess the potential to implement P recovery from wastewater via biological algal uptake in large wastewater treatment works.

Chapter 8 – General Discussion: this chapter summarises the research findings obtained throughout all the experimental work conducted as part of this PhD thesis and presents a deep analysis of how the results may contribute to improve our current knowledge about enhancing biological P uptake using photosynthetic microorganisms. Finally, overall conclusions arising from this study and recommendations for further work are summarised in Chapter 9.

Chapter 2 Literature Review

2.1 Phosphorus cycle

Phosphorus (P) is an essential element for all living organisms. It is fundamental for building genetic material (i.e., DNA and RNA) and an element supporting biochemical energy storage (i.e., ATP) and structural support of microorganisms (i.e., phospholipids) (Ruttenberg, 2003; Westheimer, 1987). However, neither microorganisms nor plants can directly assimilated P originally locked up in bedrocks, soils and sediments and for that reason, it has to be converted into bioavailable chemical species such as dissolved orthophosphate via geochemical and biochemical reactions (Ruttenberg, 2003).

Ruttenberg (2003) noted the presence of four major routes within the global P cycle: (i) weathering of the exposure P bedrock due to tectonic uplift; (ii) chemical erosion and chemical weathering that generate particulate P into rivers; (iii) P transport by rivers into lakes and oceans; and (iv) P sinks in sediments. Initially the origin of the Earth's usable P is derived from phosphate rock through weathering processes, which are the most important routes for P release (Follmi, 1996). Once P species are released from rocks through weathering, it is then transported to soils and sediments by water and air, eventually reaching the oceans. According to Follmi (1996), most phosphate-containing compounds are particulate (95%) in which 40% are organic.

Iron hydroxide ($Fe(OH)_2$) is the most important carrier for solid inorganic phosphate transport, which is very often adsorbed onto clay particles, while some particulate P is also incorporated into organic matter (Filippelli, 2011; Follmi, 1996). Releasing insoluble particulate P into dissolved phosphate phases and vice versa, it is commonly observed in the interphase between water column and river bed; in fact, that is one of the mechanisms controlling P transport in rivers, which is controlled by redox reactions (Follmi, 1996).

In aquatic environments, reactions including P compounds are controlled and catalysed by biota (e.g., bacteria, algae and plants). This process involves

intracellular-extracellular exchange of inorganic P across cellular membranes (Blake et al., 2005). Inorganic P is also obtained from enzymatic release of organic P. Once inorganic P has been taken up by microorganisms, P is transported into higher organisms via the food chain. Eventually the decaying biota will settle to the seafloor, riverbeds and lakebeds as sediments. Then upwelling will generate inorganic P and dissolve it in the water column. Over time, phosphate deposits in the seafloor will be lifted up to the surface becoming phosphate rock due to tectonic movements.

2.2 The importance of Phosphorus and its global availability

Phosphorus (P) cannot be substituted with any another element for food production and therefore, it is vital in agriculture (Jasinski, 2014; Steen, 1998). Current agriculture systems heavily depend on industrial fertilisers, as this sector consumes approximately 90% of all P mined around the world (Chowdhury et al., 2014; Reijnders, 2014). P is also widely used in several industrial products such as food additives, animal feed, detergents and pesticides (Chowdhury et al., 2014; Koppelaar and Weikard, 2013; Van Vuuren et al., 2010).

The global demand for P fertiliser is predicted to increase due to increasing world population, which is it expected to rise to 10.9 billion by 2100 (UN, 2013). This number of people will demand food to fulfil their basic needs and as a consequence, there will be a proportional increment in the demand for P fertilisers (Cordell and Neset, 2014). Moreover, changes in dietary preferences will inevitably add more pressure to fertiliser production; for instance, in emerging economies including China and India, there is an increasing tendency for consuming meat and dairy-based food, which generates a shift from grass- to grain-fed cattle (Cordell et al., 2009). As reported by the International Food Association (IFA, 2011), global P fertiliser consumption has showed an upward trend from 11.1 million tonnes in 1961 to 38.9 million tonnes in 2010 and to 45 million tonnes in 2015 (FAO, 2011). As a consequence, global annual phosphate rock production is forecasted to reach a maximum of 290 MT P by 2033 (Cordell et al., 2009).

In the UK, there is no P-rock (PR) reserves and therefore, this country relies on imported mineral phosphate fertiliser to support UK agriculture (Cooper and Carliell-Marquet, 2013). Recent figures reveal that the UK farming industry consumed 194 Kt of phosphate fertiliser in 2013 (AIC, 2014).

However, the world PR reserves are very limited. In 2014, it was estimated that only 67 million tonnes of high-quality PR are available in few countries including Morocco, China and the US (Cordell et al., 2009; Jasinski, 2014). Morocco controls 75% of the world's PR reserves, followed by China with 6% and the US with about 2% (Kauwenbergh et al., 2013).

Additionally, China has increased the cost of PR export up to 135% to prevent significant trade of their own PR and hence, to secure their access to PR for domestic agriculture demand (Cordell et al., 2009). Historically, the US has been the world's leading consumer, producer and supplier of phosphate fertiliser. However, its global share declined as PR production dropped below 30 million tonnes in 2007, which lead to increments in PR imports in 2008 (99% of imports came from Morocco), in order to fulfil their phosphate fertiliser factories. PR imports from Morocco decreased to 70% of total consumption in the US in 2014 (Jasinski, 2008; Jasinski, 2014).

2.3 Phosphorus in aquatic environments

Phosphorus is considered as one of the main nutrients in aquatic ecosystems and typically, it is found to be the limiting nutrient in freshwater environments (Mounsey, 2004). In fact, there is a threshold level of P concentration in aquatic environments that is used to assess surface water quality. In the UK for instance, the threshold level of P concentration in surface waters is reported as Soluble Reactive Phosphorus (SRP), mainly orthophosphates, and for good ecological status a range between 40 and 50 μg SRP L^{-1} has been set for non-calcareous waters and a set figure of 120 μg SRP L^{-1} for calcareous waters (Duncan et al., 2006). Anthropogenic P sources such as the discharge of wastewater treatment works effluents and agricultural runoff nourish P concentrations in water bodies and therefore, it is important to control those sources (Mounsey, 2004).

P is considered the limiting nutrient controlling eutrophication in water bodies simply because primary productivity in freshwaters is more sensitive to increments of P concentrations than other nutrients like Nitrogen (N) (Cooper and Carliell-Marquet, 2013). This was early proved by Schindler (1977), who found that the increase of P concentration in lake waters developed a proportional increment in net chlorophyll and organic carbon concentrations. Eutrophic conditions are generally related to the concentration of SRP than Total Phosphorus (TP) due to the fact that SRP is more bioavailable and effective at stimulating the growth of photosynthetic microorganisms (White and Hammond, 2009). In the UK, the load of SRP into rivers was estimated to be about 46.7 Kt yr^{-1} with contributions from households (78.3%), agriculture (12.5%), industry (3.5%) and natural sources (5.7%) (White and Hammond, 2009). In the case of sewage, several forms of P enter wastewater treatment works and those vary depending on consumption habits at household level (Table 2.1).

Table 2-1 Chemical forms of P entering Wastewater Treatment Works

Chemical species of P	Chemical formula	Source
Orthophosphate	PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$, H_3PO_4	Household waste (i.e., urine, faeces, greywater), industrial waste
Polyphosphate	$(PO_3)_6^3$, $P_3O_{10}^5$, $P_2O_7^4$	Washing powder, detergents
Organic P (particulate P)	various	Organic matter (i.e., waste solids, human/plant/animal tissue)

Source: Houhou et al., 2009; Jarvie et al., 2006; Metcalf and Eddy, 2004; Millier and Hooda, 2011; Park et al., 2016; Sawyer et al., 2003; Maurer and Boller, 1999; White and Hammond, 2009.

In terms of the actions taken at improving surface water quality in the UK, the Environment Agency (EA) has mainly focused on tackling point pollution sources by implementing regulations such as the EU Urban Waste Water Treatment Directive (91/271/EEC) and the Water Framework Directive (2000/60/EC). The UK water industry has invested £13.9 billion between 2000 and 2010, in order to meet EU regulations regarding sewage discharges (point sources) (Defra, 2012), but in the same period river water quality has not substantially benefited from such interventions, as current levels of nitrate and phosphates are still a major matter of concern (NAO,

2010). In fact, as pollution from point sources has been reduced, the impact of diffuse pollution is becoming more evident and effective actions are urgently needed. Despite efforts from the EA to persuade the farming sector to recognise their responsibilities for diffuse pollution, the sector's awareness of the problem remains low. Water pollution imposes not only environmental costs through its effects on aquatic life and human health (i.e., eutrophication, harmful toxins from algal blooms, etc.), but also financial costs from the treatment of water for drinking. The cumulative cost of water pollution in England and Wales has been estimated at up to £1.3 billion per annum (NAO, 2010).

2.4 Phosphorus control in wastewater treatment works

The most common practices for P control at wastewater treatment works, mainly focus on removal with very little recovery, are chemical precipitation, biological uptake or a combination of both methods (Manyumba et al., 2009). Chemical precipitation of phosphate includes the addition of metal salts by using alum, iron or lime (de-Bashan and Bashan, 2004). Those processes are certainly effective at removing phosphate from wastewaters to very low levels of SRP (<0.01 mg P L^{-1}) and TP (<0.1 mg P L^{-1}) (Smith et al., 2008). However, they lead to significant costs particularly for the addition of ferric salts and eventually, they generate P-bonded chemical sludge (Likosova et al., 2013). As a consequence of this practice, further chemical processes are needed in order to recover P from the resulting sludge. P control via chemical precipitation is unsustainable and makes P recovery and reuse as a fertiliser in agriculture almost impossible to achieve.

Biological P removal processes are generally considered to be a more sustainable alternative, which is achieved at conventional wastewater treatment works by using activated sludge units through a process known as enhanced biological phosphorus removal (EBPR). In this biological process, microorganisms known as polyphosphate accumulating organisms (PAO) extract phosphorus from the wastewater and accumulate it as intracellular polyphosphate granules (Acevedo et al., 2014). A nearly complete P removal through a series of anaerobic and aerobic cycles in modified activated sludge units is achieved by wasting P-rich sludge from the final sedimentation

tank (Manyumba et al., 2009). This practise is well developed and has been extensively applied at large treatment works, but alternatives are now needed as that process on its own would not be able to meet future P discharge consents (<0.1 mg SRP L^{-1}). Moreover, the used of anaerobic digestion for renewable energy generation from secondary sewage sludge has led to the production of nutrient rich waste streams (i.e., digestate liquor), that require further treatment.

Another alternative to promote P recovery at wastewater treatment works (WWTW) is through processing P-rich waste side-streams like digested from anaerobic digesters. This nutrient-rich waste stream is originated from the anaerobic digestion of primary and secondary sludge and contains very high concentrations of nitrogen (mainly ammonia and organic nitrogen) and phosphorus (SRP and TP) (Munch and Barr, 2001). The liquid phase of this waste stream (digestate liquor) can be used to precipitate crystals of a mineral called struvite ($MgNH_4PO_4.6H_2O$) by adding magnesium and controlling the pH. Uncontrolled struvite precipitation within pipes in WWTWs was reported as an issue causing blockages and leading to increase energy costs for sludge pumping and maintenance costs for pipe replacement (Jaffer et al., 2002). Struvite can be used in agriculture as a fertiliser with several added benefits such as slow P release, less frequent application, and contain two or three fold lower heavy metals than commercial fertilisers. However, struvite production requires the addition of magnesium and adjusting of pH that increases operation costs (Bhuiyan et al., 2008). Struvite production is well-developed and applied at large wastewater treatment works.

As an alternative to conventional processes for P control, alga-based processes have been suggested as a viable alternative. Microalgae has been extensively used for the treatment of domestic wastewater worldwide; in particular, the use of the symbiotic relationship between microalgae and bacteria in Waste Stabilization Ponds (WSPs) has been the main important feature of this low-cost, natural wastewater treatment system. In simple terms, heterotrophic bacteria metabolise organic carbon and other nutrients under the presence of oxygen for growth and energy, and produce oxidised forms of carbon (CO_2), nitrogen (N under the form of NH_4^+, NO_2^- and NO_3^-) and P (PO_4^{3-}); in return, photosynthetic microalgae utilise CO_2 and nutrients (NH_4^+,

NO_3^- and PO_4^{3-}) for growth and the production of oxygen to support bacterial activity (Craggs, 2005). As a result of the biological and chemical processes taking place both in the water column and sludge layers, it has been identified that the main mechanisms for phosphate removal in WSPs include: (a) biological uptake by microalgae and bacteria, followed by in-cell accumulation of Poly-P; and (b) chemical precipitation of inorganic phosphate complexes (i.e., struvite and hydroxyapatite), which is induced by high pH conditions (pH>8.2) and the presence of key cations (Ca^{2+}, Mg^{2+}, Al^{3+} and Fe^{3+}) (Arceivala, 1981; Van Haandel and Lettinga, 1994).

WSPs have been used as a natural wastewater treatment process for over 3,000 years. In the USA, the first 'modern' WSP system was constructed in San Antonio, Texas (1901) and nowadays, one third of all secondary wastewater treatment facilities in the USA (>7,300 systems) include a pond system of one type or another (USEPA, 1983; USEPA, 2002). In Europe, the first WSP was built in 1920 in Munich (Germany) for polishing a secondary effluent from the local sewage treatment works (Vuillot and Boutin, 1987). Nowadays, WSPs are very widely used for small and rural communities (<2,000 p.e.) with about 3,000 systems in France and 1,100 in Germany only (Bucksteeg, 1987; Gratziou and Chalatsi, 2013; Racault and Boutin, 2005). In some cases WSPs have also been used to serve larger communities, particularly in Mediterranean France (50,000 p.e., Rochefort sur Mer), Spain (95,000 p.e., Murcia), Cyprus (300,000 p.e., Anthoupolis and Mia Milia WSP systems in Nicosia), and Greece (4,000 p.e., Moudros, Island of Limnos) (Gratziou and Chalatsi, 2013).

However, it is fair to acknowledge that the performance of WSPs is still seasonal; heavily dependent on weather conditions; demands extensive use of land; and the opportunities for P recovery and reuse are limited to the nearby demand of treated wastewater for irrigation. Such constraints represent the main challenges for developing alga-based technologies to retrofit existing (or to compete with new) large wastewater treatment plants (WWTPs) currently using bacterial-based technology (e.g., activated sludge process).

Nevertheless, microalgae have been identified as a viable option for meeting energy and nutrient recovery goals in large, highly mechanised WWTPs, but the development of efficient technologies for high-cell-density algal biomass cultivation in wastewater is yet to be proven. Modern WWTPs with energy recovery via AD of sewage sludge have great potential for allowing the implementation of algae cultivation, as they provide: (a) continuous source of nutrients for algae growth – i.e., CO_2 contained in biogas from AD reactors and flue gas from combined heat and power (CHP) units; and N and P compounds in both wastewater and digestate; (b) downstream processes in place for harvesting algal biomass – i.e., polymer dosing for improving sedimentation of algal biomass, sludge thickening and dewatering; and (c) advanced digestion systems that will render the algal biomass suitable for mesophilic AD with subsequent generation of heat and power through CHP units (Horan and Camargo-Valero, 2013).

Microalgae have proven their ability to effectively remove both ammonium and phosphate from wastewater even under temperate climate conditions (up to 10 % N and 3 % P in dry algal biomass) (Powell et al., 2008; Camargo-Valero et al., 2010), therefore they may provide a new route for biological nutrient recovery and potentially removing all together the need for nitrification/de-nitrification processes and phosphate removal by P-accumulating bacteria in large WWTPs. In the long term, there is a real opportunity to develop a novel, relatively simple and sustainable technology to harvest nutrients via organic N and Poly-P accumulation by microalgae in wastewater treatment works.

2.5 Microalgae and wastewater

Microalgae are identified as one of the oldest microorganisms containing chlorophyll *a* as their primary photosynthetic pigment (Lee, 1980); photosynthetic organisms require relatively simple substances for growth. Through the process of photosynthesis, algae convert water, carbon dioxide and light into oxygen and biomass that contain added-value products (Pilon et al., 2011). Microalgae are more energy efficient than land-based plants due to their single cell structure (Dismukes et

al., 2008) and the added-value products to biomass ratio is higher than aquatic or terrestrial plants, because algae do not require energy to build up plant organs like roots or callus (Rupprecht, 2009). In order to describe the function of microalgae for bioremediation and biomass production, their different types, current uses, and the requirements for cultivation will be explained in the following sub sections.

2.5.1 Taxonomical classification of microalgae

Microalgae are part of a diverse group of photosynthetic organisms that have different types of cell organisation ranging from unicellular, colonial and filamentous (Tomaselli, 2007; Vassilev and Vassileva, 2016). Unicellular microalga is categorised as motile or non-motile algae and their motility depends on the presence of flagella. Species of *Chlamydomonas* have been defined as unicellular chlorophyte algae with two anterior flagellas (Harris, 2001; Tomaselli, 2007). In addition, *Botryococcus braunii* has been identified to have a colonial cell organisation. The colony develops as a new daughter cell, which is generated on the surface of the colony and layers of a new extracellular biopolymer will be formed after cell division (Ashokkumar et al., 2014; Furuhashi et al., 2016). Filamentous algae occur in green masses floating on surface waters. Some cyanobacteria such as *Anabaena, Anabaenopsis, Nadularia, Oscilatoria, Spirulina,* and *Nostoc* are categorised as filamentous microorganism (Markou and Georgakakis, 2011).

In addition, according to the characteristics of their cell membrane, microalgae can be divided as prokaryotic and eukaryotic cells (Tomaselli, 2007). Prokaryotic cells lack of a membrane-bound organelles (i.e. plastids, mitochondria, nuclei, Golgi and flagella) and they are more similar to bacteria than algae (Brennan and Owende, 2010). The DNA and photosynthetic membranes are not organised in chromosomes and they are freely lied in the cytoplasm (Tomaselli, 2007). Eukaryotic microalgae have a true membrane-bounded nucleus such as plastids, mitochondria, nuclei, Golgi bodies and flagella which can control the functions of the cell for survival and reproduction (Brennan and Owende, 2010).

Biologists have categorised eukaryotes into a variety of classes according to cell pigmentation. Based on that, the most abundant microalgae are: green algae (*Cholophyceae*), red algae (*Rhodophyceae*), diatoms (*Bacillariophyceae*) and golden algae (*Chrysophyceae*) (Brennan and Owende, 2010; Vassilev and Vassileva, 2016). The chlorophyta cells have chlorophyll a and b and a single chloroplast surrounded by two envelop membranes (Heimann and Huerlimann, 2015). Cell walls of green algae generally contain cellulose, although some alga species are naked and the chloroplast may have an eyespot and pyrenoids (Tomaselli, 2007).

Furthermore, algae can either be cultivated in autotrophic, heterotrophic or mixotrophic conditions. Autotrophic algae require inorganic compounds such as CO_2, salts and a light energy source for growth. They can convert solar energy into algal biomass (Davis et al., 2011; Mata et al., 2010). Autotrophic algae can be cultivated either in open systems (i.e., raceway ponds) or in closed photobioreactors (Borowitzka, 1999; Davis et al., 2011). Under heterotrophic cultivation, microalgae do not require the use of light for photosynthesis and as a consequence, they need an external source of organic compounds as an energy source, as well as other nutrients like N and P (Mohan et al., 2015; Perez-Garcia et al., 2011). Moreover, some algae are mixotrophic, which means they have the ability to carry out photosynthesis and require exogenous organic nutrients. However, light is not a limiting factor for growth as mixotrophic microalgae can enhance their growth over autotrophic and heterotrophic cultures (Brennan and Owende, 2010; Mohan et al., 2015).

2.5.2 Integration of microalgae for biomass production and phosphorus recovery in wastewater treatment systems

Microalgae provide dual desirable functions of bioremediation to various types of wastewater and biomass production for commercial products based on their chemical composition (Chiu et al., 2011). A number of microalgae species have been used in numerous studies and have shown to be effective at removing N and P from different wastewater streams (Pittman et al., 2011). There are various research works reported in the scientific literature aim at optimising N and P uptake by microalgae, either for nutrient control or to maximize nutrient content in algal cells for biomass feedstock

production. According to the US Department of Energy, the concept of the use of wastewater as a medium and nutrient source for algae production was due to the need for alternative fuels during the energy crisis in the 1970's. That approach provided multiple opportunities for both environmental protection and renewable energy production (Sheehan et al., 1998). In addition, bioremediation of wastewater by using microalgae is considered to be more sustainable and provides several advantages such as: (a) low energy demand as symbiosis between bacteria and microalgae can reduce the aeration requirement (Oswald, 2003), (b) microalgae can assimilate N and P from wastewater for their nutrient growth (Pittman et al., 2011), (c) microalgae cultures can help reducing GHG emissions (Gouveia et al., 2016; Judd et al., 2015), and (d) algal biomass contains high amount of nutrients and carbon that are useful for bioproducts and bioenergy (Gouveia et al., 2016; Heimann and Huerlimann, 2015).

Previous studies showed positive results regarding the potential of utilising wastewater as a source of nutrients for microalgae growth and the integration of nutrient recovery and biomass production. Various species of microalgae provide very high nutrient removal efficiencies from municipal wastewaters, including *Chlorella kessleri*, *Chlorella vulgaris* and *Scenedesmus obliquus* (Caporgno et al., 2015; Mennaa et al., 2015; Ruiz-Marin et al., 2010).

Furthermore, green microalgae have been investigated for their nutrient removal ability in a wide range of waste streams ranging from low to high strength wastewaters. Wang et al. (2010) observed that *Chlorella* sp. could remove nutrient from settled domestic sewage more efficient than activated sludge and proposed that it will be more economically to introduce an algal-based process as a secondary treatment rather than a tertiary treatment. In addition, other studies have evaluated the use of secondary and tertiary effluents for algal cultivation (Ruiz et al., 2013; Samori et al., 2013). Also the effluent from primary sedimentation, commonly high in nutrient concentration, especially for ammonia and phosphorus, proved to be very good at supporting algal growth and even better than using the final effluent (Tam and Wong, 1990; Wang et al., 2010). Wang et al. (2010) reported that the specific growth rate for microalgae in wastewater can reach up to 0.412 d^{-1} with ammonia and phosphorus concentrations higher than 35 mg N L^{-1} and 7 mg P L^{-1}, respectively.

Moreover, it has been found that algal growth was significantly better in digestate liquor, which is a nutrient-rich effluent stream from the anaerobic digestion of sewage sludge, due to a much higher level of N and P (Dalrymple et al., 2013a); nutrient content in digestate have been reported to be in the order of 67-250 mg NH_3 L^{-1}, 120-250 mg TKN L^{-1}, and 120-300 mg TP L^{-1} (Dalrymple et al., 2013a; Kong et al., 2010b; Wang et al., 2010). Dalrymple et al. (2013a) reported that the highest NH_4^+ removal from digested liquor was 94%, whilst Li et al. (2011) observed 89% of total nitrogen removal. In the case of *C.reinhardtii*, Kong et al. (2010b) found that their growth varies depending on the sort of wastewater and cultivation conditions. For instance, the highest biomass yield (2.0 g $L^{-1}d^{-1}$) occurred when *C.reinhardtii* grown in 100% digested liquor, while a much lower figure was achieved when using the final effluent due to limited N and P availability.

Microalgae also have proved their ability to effectively treat wastewater from animal farming; for example, *Chlamydomonas reinhardtii* showed high specific growth rate (1.286 d^{-1}) and nutrient removal (89% N and 49% P removals) when cultivate in swine wastewater (Hasan et al., 2014). Another species like *Tetraselmis suecica*, cultivated in a tubular photobioreactor on fish farm wastewater, reported total nutrient removal (Michels et al., 2014). These studies show that there is a possibility for integrating residues from animal farming industry and municipal wastewater for bioremediation and algal biomass production, which can be highly beneficial in rural communities.

High percentage of P removal (> 80%) has been also reported from microalgae cultivation using digestate wastewater (Li et al., 2011; Wang et al., 2010; Zhou et al., 2012a). This could indicate that luxury P uptake might be present in such systems. However, Li et al. (2011) reported that although the portion of P removal from the digestate was high, that did not mean that algae were able to take up most of the P present in wastewater, because the high pH found in the system had also played an important role supporting phosphate precipitation. Due to the fact that about 76-96% of the phosphorus was removed by sedimentation and only 3-23% was assimilated into algae biomass (Li et al., 2011). The use of digestate as a nutrient-rich wastewater require preliminary pre-treatment, due to the presence of fine particles and the very

dark colour impeding light penetration (Rusten and Sahu, 2011). Additionally, it also contains toxic substances which may inhibit microalgae growth (Munoz and Guieysse, 2006a).

The reported literature review reveals that microalgae have an important potential for nutrient control and recovery from a wide range of wastewater, with the added benefit of producing high algal biomass yields. However, the recovery of phosphate has not been fully studied and much more work is needed to understand the mechanisms controlling that process (Mallick, 2002). The efficiency of phosphate removal and algal growth depend on many environmental conditions including nutrient composition (micro and macro nutrient), light, temperature, pH, carbon sources and oxygen (Pittman et al., 2011). Therefore, it is significantly important to take into account how those key environmental factors may enhance luxury P uptake by microalgae in a real wastewater treatment plant.

2.5.3 Microalgae bioproducts and bioenergy

Microalgae are versatile organisms which have an importance feature to develop a wide range of products application. They have high photosynthetic capability to convert solar energy into valuable biomass with different biochemical composition (Noue and Pauw, 1988). Mostly photosynthetic microorganisms utilize H_2O as their electron source, sunlight as source of energy and CO_2 as carbon source. The end products of their photosynthetic process are oxygen, carbohydrates, protein and lipids contained within the cells (Pilon et al., 2011; Spolaore et al., 2006).

The early attempt of microalgae utilization was conceived by Germans in the second world war as a source of food supplement (Ugwu et al., 2008). Then, the interest to use microalgae biomass as a bio-fuel and bio-fertiliser started in the 1970's during the first oil crisis. It was followed by utilization of microalgae as a source of fine chemicals in 1980's (Mata et al., 2010; Noue and Pauw, 1988). In Table 2.2, there is a list of the chemical composition of different microalga based on the work reported by Spolaore et al. (2006) and Demirbas (2011).

Table 2-2 Chemical composition of algae (%, dry mass)*

Species of microalgae	Proteins	Carbohydrates	Lipids	Nucleic acid
Chlamydomonas reinhadrtii	48	17	21	-
Chlorella vulgaris	51-58	12-17	14-22	4-5
Dunaliella salina	57	32	6	-
Scenedesmus obliquus	50-56	10-17	12-14	3-6
Spirulina maxima	60-71	13-16	6-7	3-4.5
Synecoccus sp.	63	15	11	5

*Source: Spolaore et al. (2006) and Demirbas (2011).

Apart from the chemical composition listed above, microalgae are also able to utilise surplus of P, which is stored and accumulated within their cells in the form of polyphosphate granules – i.e., a mechanism known as luxury P uptake (Larsdotter, 2006b; Powell et al., 2008). Polyphosphate is a linear polymer of hundreds of orthophosphate residues linked by high-energy phosphoanhydride bounds (Figure 2.1). Furthermore, Table 2.3 presents the chemical forms of P in microalgae cells as polyphosphate and phospholipids (Janero and Barrnett, 1981; Kornberg et al., 1999; Solovchenko et al., 2015; Ruiz et al., 2001a).

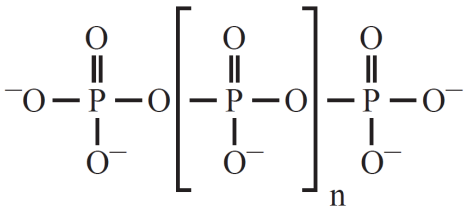


Figure 2-1 Inorganic polyphosphate (Kornberg et al., 1999)

Table 2-3 Chemical forms of P in microalgae cells

Chemical forms of P	Chemical formula
Phospholipids	Phosphatidylglycerol (PG), Phosphatidylethanolamine (PE), Phosphatidylchloline (PC)
Polyphosphate	$(PO_3)_6^{3}$, $P_3O_{10}^{5}$, $P_2O_7^{4}$
Pyrophosphate (PPi)	

The importance of polyphosphate in a variety of industrial applications has been reported by Rao et al. (2009), including bioremediation, biomining of copper, food additive, therapeutic drugs, insulating fibre for construction, non-flammable clothing and substitute of costly ATP.

2.6 *Chlamydomonas reinhardtii* as a model organism to study phosphorus recovery

C.reinhardtii is a unicellular biflagellate green algae which has been explored as a genome project organisms for decades (Proschold et al., 2005; Stauber and Hippler, 2004), including fundamental biological research covering numerous morphological, physiological, and genetic studies including photosynthesis processes, the function of chloroplast and the assimilation and utilisation of nutrients, that have been elucidated by using *C.reinhardtii* as a model organism (Grossman et al., 2007; Stauber and Hippler, 2004).

Another important feature of *C.reinhardtii* is that they have been established as an excellent model organism to study the improvement of biofuel and bio-product production from green microalgae (Scranton et al., 2015). In addition, a variety of environmental conditions lead to understand N and P utilisation in *C.reinhardtii* has been reported by Grossman (2000). This work also described the synthesis of a number of P species in *C.reinhardtii* that help to understand in-cell P uptake and storage, which in conjunction with the work reported by Borchardt and Azad (1968), has demonstrated the potential of the polyphosphate storage mechanism in algal cells particularly for implementing P recovery from low-grade waste streams.

Microalgae can retain their polyphosphate granules in their cells longer than bacteria (Currie and Kalff, 1984). This ability gives a superior advantage to microalgae for potential applications in wastewater treatment works where P recovery via biological algal uptake may be implemented. Previous studies conducted to observed luxury P uptake by *Scenedesmus* spp., in a continuous flow waste stabilisation pond system using synthetic wastewater, found that *Scenedesmus* spp. were able to accumulate

within a range between 0.41 and 3.16% P (dry weight) in their cells (Powell et al., 2008). Luxury P uptake mechanisms lead to the storage of P reserves in the form of polyphosphate granules (Powell et al., 2009). Intracellular polyphosphate accumulation has been also reported in *Chlamydomonas reinhardtii* (Werner et al., 2007). The polyphosphate bodies in *C.reinhardtii* are located in the vacuole that provides a secretory function and acts as an intracellular digestive compartment (Komine et al., 2000; Ruiz et al., 2001a). However, the size of polyphosphate granules in microalgae is not uniform and their particle size highly depends on the surrounding environmental conditions. That was also observed in *Trypanosoma cruzi* (Ruiz et al., 2001b). In fact, after 48 hours of starvation, it was identified that in a culture of *Chamydomonas* the size of polyphosphate granules decreased within the vacuoles, confirming the in-cell storage of P as reserves (Aksoy et al., 2014).

Other strains of *Chlamydomonas* sp. (YG04 and YG05) have been reported to be very effective at removing phosphate from secondary wastewater effluents; Rasoul-Amini et al. (2014) reported that these strains were able to remove approximately all the orthophosphate present in a batch culture with the initial concentration of 19.11 mg PO_4^{3-} L^{-1}. In that work, environmental conditions including nutrient concentration, light cycle (dark/light) and alga species were study to assess their influence on P uptake. Deng et al. (2010) studied the effect of NH_4^+-N, TP concentration and N/P ratio on N and P removal from wastewater using *Chlamydomonas reinhardtii* and their results showed that both NH_4^+-N and TP were entirely removed from a wastewater containing an initial concentration of 55 mg NH_4^+-N L^{-1} and 7 mg TP L^{-1}. Generally speaking, research works using batch cultures at lab scale have demonstrated a promising performance of microalgae for both N and P removal and recovery, but similar performances are hard to replicate in larger reactors or under continuous flow conditions.

C.reinhardtii showed their ability to grow in the effluent from sludge dewatering units in a sewage treatment works (also known as centrate) by using a pilot-scale recirculating helical photobioreactor (BIOCOIL) (Kong et al., 2010b); however, the reported removal efficiency was only 83% for N and 15% for P, with a net algal biomass production of 2 g L^{-1} d^{-1}, which confirms the difficulties of high-density

microalgae cultures where algal growth and other processes may be affected by poor light penetration. In synthetic wastewater *C.reinhardtii* mixed with *Chlorella* and bacteria could eliminate N and P for more than 90% when cultivated in semi-batch culture with recycle (Ashok et al., 2014). These outcomes demonstrate that *C.reinhardtii* is able to assimilate nutrients at a high rate, but there is still a need for further developments improving reactor design and operational conditions.

C.reinhardtii also have the ability to grow in photoautotrophic, heterotrophic and mixotrophic conditions, which is explained by examining genes and proteins critically involved in photosynthesis and respiration and their responses to different light and nutrient environments (Grossman, 2000; Grossman et al., 2003; Rupprecht, 2009). A carbon flux balance analysis (carbon assimilation) in *C.reinhardtii* was published by Boyle and Morgan (2009) in which algal growth under autotrophic, heterotrophic and mixotrophic culture were compared. It was found that biomass yields were higher under autotrophic conditions followed by mixotrophic and heterotrophic cultures. However, *C.reinhardtii* relied on acetate as organic carbon source when they grow under non photosynthetic conditions (Boyle and Morgan, 2009; Harris, 2001; Therien et al., 2014).

Along with the potential for N and P removal and recovery from liquid waste streams using microalgae, it has been demonstrated that there is an added benefit as microalgae is also able to simultaneously accumulate lipids, which is potentially beneficial when considering the opportunities for bioenergy generation from harvested algal biomass. Deng et al. (2012) successfully isolated wild-type strains of *C.reinhardtii* CC124 to obtain mutants (*C.reinhardtii* CC124-M3, CC124-M3 and CC124-M25) able to optimize lipid accumulation. Their results showed that the mutant strain was able to produce higher lipid content when grown under nutrient-stress conditions, which also works in favour for in-cell P accumulation. Other studies also demonstrated that a wild-type of *C.reinhardtii* and its mutants (*C.reinhardtii cw15 and cw15 sta16*) accumulated high in-cell lipid content, starch and oil when grown in a nutrient starved medium (Siaut et al., 2011; Wang et al., 2009).

This review, and the fact that *Chlamydomonas* is one of the most commonly occurring microalgae in sewage treatment ponds in the UK (along with *Euglena* and *Chlorella* – (Abis and Mara, 2005)), supports the selection of *C.reinhardtii* as a suitable model organism to study the potential use of microalga in wastewater treatment not only for nutrient recovery (mainly P), but also for biomass feedstock production. Therefore, in order to better understand P uptake and enhanced algal biomass production from wastewater, it is still required to study the influence of important environmental factors controlling those processes and the appropriate reactor design and operational conditions.

2.7 Influence of environmental factors on algal P uptake and biomass growth

A number of environmental factors have been reported to affect nutrient uptake rates and algal growth for various species of microalgae including: initial nutrient concentration, photoperiod, light intensity, pH, temperature, mass transfer coefficients and density of inoculum. Among these parameters, nutrient concentration and light intensity have direct effect on nutrient uptake rates, which control enhanced algal biomass growth, while the others may have an indirect influence on the removal of nutrients from wastewater. The influence of the main environmental factors controlling algal P uptake and biomass growth are explained in the following sections.

2.7.1 Light Intensity

Microalgae cultivation strongly depends on the availability of light and its intensity for supporting photosynthetic processes (Goncalves et al., 2014). In a photosynthetic process the fixation of 1 molecule of CO_2 require 8 einsteins (1.92 MJ) of photosynthetically active radiation (PAR) which is approximately 48% of the solar light incident (Amaro et al., 2011). As a photosynthetic microorganism, microalgae require light to meet their energy requirements and that in particular forces P uptake in microalgae (Borchardt and Azad, 1968). Light intensity is not only an essential component to support algae growth not only under phototrophic conditions, but under mixotrophic conditions as well (Goncalves et al., 2014; Smith et al., 2015), simply

because in a mixotrophic culture the algae is also capable to utilise light simultaneously while synthesising organic carbon as an energy source.

In a phototrophic culture, a given light intensity of 60 $\mu Em^{-2}s^{-1}$ influenced P uptake in *Scenedesmus* to perform luxurious uptake and the algae grew faster using acid-soluble polyphosphate (Powell et al., 2008). Under higher light intensity conditions (180 $\mu Em^{-2}s^{-1}$) and continuous illumination, *C.vulgaris* showed to assimilate 68% of P and 99% of N (Goncalves et al., 2014). That work also conveyed that an increase of light irradiance and the time of exposure have contributed to enhance specific algal growth on several alga strains. However, the excess of the light intensity beyond a limit threshold will cause photoinhibition and damage to chlorophyll pigments (chlorophyll a). That effect was investigated in *Chlamydomonas reinhardtii* CC125 using different light intensities from 1000 to 2000 $\mu Em^{-2}s^{-1}$; it was found that oversaturation of light caused a significant decrease in photochemical yield (Nama et al., 2015). A wild-type of *C.reinhardtii* also bleach and vanish at very high light intensity (1500 and 2000 $\mu Em^{-2}s^{-1}$) as reported by Forster et al. (2005) and therefore to reduce the effect of photoinhibition in *C.reinhardtii,* the recommended light intensities for cultivation were sugested be between 200 and 400 $\mu Em^{-2}s^{-1}$ (Harris, 2008).

2.7.2 Photoperiod

Photoperiod is an important parameter for microalgae growth because the culture will be exposed to a fluctuated light regime for better photosynthetic efficiency. The effect of the light/dark cycle on the growth of *Chlamydomonas reinhardtii* was investigated by Takache et al. (2015), who found that pigment adaptation is a key effect influencing microalgae growth. Goncalves et al. (2014) also compared three different light cycles of 14:10, 10:14 and 24:0 on nutrient removal and algal growth rates in a batch culture. They reported that a 24:0 light/dark cycle generated the highest specific growth rate coefficient (1.2 d^{-1}) in *C.vulgaris*, with a net P removal of 68% and 100% N removal from OECD medium. The effects of the photoperiod were also evaluated by Zhang et al. (2015) under different illumination cycles from 8:16 to 24:0, with a light intensity of 100 $\mu Em^{-2}s^{-1}$. They suggested that illumination for at least 16 and 20 hours were the

most efficient regime for N and P removal respectively, while maximum algal biomass yields was reported at a 24:0 light/dark cycle.

Sforza et al. (2012) studied the effects of the light/dark cycle on photosynthetic efficiency of *Nannochloropsis salina* and found that the alternation of light and dark cycles allow the microalgae for re-oxidation of electron transporters in the photosynthetic apparatus, which otherwise will cause radiation damage and decrease the photosynthetic productivity. Continuous illumination will damage the photosystem II (PSII) and therefore, the algae will require to constantly repair such damage (Murata et al., 2007). Krzeminska et al. (2014) found that continuous illumination (24:0) highly influenced specific growth rate and biomass production of *Botryococcus braunii* and *Scenedesmus obliquus*, while a shorter light exposure (12:12) made a significant influence on *Neochloris* strains (*Neochloris conjuncta, Neochloris terrestris* and *Neochloris texensis*). Thus, the effect of the photoperiod in a given culture varied among microalgae species and therefore, it is important to identify the right length of photoperiod and its effect on microalgae strains commonly found in wastewater treatment systems regarding nutrient removal and net algal growth.

2.7.3 Macronutrients

The biochemical composition and specific growth of microalgae are dependent on the variation of nutrient availability (Juneja et al., 2013). N and P are essential macronutrients for microalga growth and cell metabolism. They are found in a variety of biological substances such as peptides, proteins, lipids, pigments (chlorophyll), energy transfer molecules (ADP, ATP) and in genetic substances (RNA) (Cai et al., 2013). The variation of N and P concentrations creates an effect on nutrient uptake and microalgae growth. Such relationship was investigated in *Scenedesmus* sp. and it was found that the specific growth rate was $0.98\pm0.12 \times 10^6$ and $1.34\pm0.12 \times 10^6$ cells/ml/d when a N/P ratio of 5/1 and 20/1 were used respectively, while the total P uptake was 99% when the initial TP was 1.3 mg L^{-1} (Li et al., 2010a).

P concentration can also have an effect on biomass composition and algal growth. Markou et al. (2012) examined various P concentrations (10, 50, 250 and 500 mg P L^{-}

^{1}) at different light intensity values (24, 42, and 60 $\mu Em^{-2}s^{-1}$), in order to identify the changes on maximum algal biomass production and P removal. The maximum algal biomass yield was 3,592±392 mg/l, which was achieved at 250 mg P L^{-1} and light intensity of 60 $\mu Em^{-2}s^{-1}$, while the highest P removal occurred at the lowest P concentration and higher light intensity. This indicates that high biomass yields not necessarily coincide with high P removal (or uptake) (Wang et al., 2013a). However, if we consider the effect of N concentration on P uptake and algal biomass growth, it seems that both are directly dependent (Beuckels et al., 2015), which implies that both N and P create a combined effect not only on algal biomass production, but also on nutrient uptake and hence, it is important to consider these two nutrients as independent variables.

2.7.4 Carbon

Carbon is a non-mineral nutrient very important for microalgae photosynthesis and growth (Juneja et al., 2013). In photoautotrophic conditions, algae require an inorganic carbon source and rely on solar energy to perform photosynthesis and growth (Juneja et al., 2013; Smith et al., 2015). Whereas under heterotrophic conditions, algae depend on an organic carbon to supply both carbon and energy requirements (Bumbak et al., 2011; Mohan et al., 2015).

Metabolic activity is an important factor that influences carbon uptake rates in microalgae. Microalgae are capable to utilise different sources of carbon including CO_2 from atmosphere, exhaust gases from industry (i.e., flue gas) and soluble carbonates from water (Sydney et al., 2010) and therefore, the potential to act as a sink to CO_2 by fixing carbon in their biomass (Sayre, 2010). As a result of carbon biofixation, considerable amounts of organic substances are produced and locked in the resulting biomass (Kurano et al., 1995).

Different concentrations of CO_2 for maximising algal biomass production have been reported by Fu et al. (2012) in *C.vulgaris.* Their results shown that 2.5% (v/v) CO_2 was adequate to support growth for *C.vulgaris*, while a range between 2.5% and 9.5% (v/v) CO_2 inhibited their growth. This optimum carbon dose (2.5%, v/v) achieved a

maximum algal biomass production of 1.61 g DW L^{-1} d^{-1}. The influence of organic and inorganic carbon on the growth of *Chlamydomonas reinhardtii* (wild type and starch-less *sta6* mutant) and Triacylglycerols (TAG) production under low N concentration have been investigated by Davey et al. (2014); they reported that TAG production was higher when the microalgae used an organic carbon source (acetate) under mixotrophic conditions, but net biomass growth was affected, particularly for the mutant. However, the wild type showed higher growth under phototrophic conditions. The need for a carbon sources is evident and considering that the amount of carbon incorporated in algal cells can reach up to 56% of dry weight (Gatenby et al., 2003), it is important to assess the right balance between C, N and P for optimum growth and nutrient uptake. Therefore, it is important to adjust the ratio of nutrient and carbon in order to enhance biomass production that leads to the increase in nutrient uptake from the growth media (or wastewater in this case).

2.8 Research problem and current research gaps

The current processes for nutrient control in large wastewater treatment works contributes to uphold a linear approach to resource consumption with limited opportunities for nutrient recovery and reuse as fertilisers. For instance, ammonium control includes the use of slow-growing nitrifying bacteria for transforming ammonium into nitrate and if total N removal is required, nitrate is removed via de-nitrification. Nitrification requires additional energy inputs for aeration and mixing, longer retention times and reduces net sludge production for methane production via Anaerobic Digestion. P removal is often achieved by biological uptake and/or chemical precipitation, although at larger treatment works the biological route is usually preferred over the chemical path. However, the growth of P-accumulating bacteria is limited by organic carbon and a supplemental carbon source in the form of acetate is often added as part of the treatment (i.e., 30 – 48 mg acetate per litre of sewage) (Manyumba et al., 2009).

The UK water sector has been required to continue reducing the impact of sewage discharges, mainly from NO_3^-, PO_4^{3-} and priority substances. The challenge to achieve good water status in surface waters is still on going and sits on top of

ambitious goals aim at reducing net energy consumption and greenhouse emissions. Energy consumption in the UK water industry will continue increasing in order to achieve stringent discharge consents and to deal with increasing sewage and sludge production (Ofwat, 2005; Palmer, 2010). For instance in England and Wales, more than 10 billion litres of sewage are generated daily, which require approximately 2,800 GWh of energy for its treatment; the carbon footprint of the energy consumed in the sector contributes annually with nearly 1.7 million tonnes of GHG emissions and therefore, improving energy efficiency and in-house energy generation are desperately needed in order to reduce net power consumption from the grid (Ofwat, 2006; Water-UK, 2007a). Therefore, the development of innovative wastewater treatment based on alternative processes like microalgae systems may play an important role to meet stringent discharge consents, to reduce energy consumption and to achieve a more sustainable way to recover P from waste streams.

According to the current literature review, it is found that there is insufficient research in the development of high-cell density, continuous flow microalgae systems for enhancing intracellular P recovery coupled with the increase of biomass production of microalgae from wastewater, in particular the use of separate bioreactors for photoautotrophic and heterotrophic cultivation. Also, the effect of various environmental conditions that contribute to the optimisation of both potential outcomes (optimum P uptake and algal biomass production) are still poorly understood. Moreover, in regard to the study of hyper P accumulation in microalgae, no previous works were found at isolating hyper P accumulators from *C.reinhardtii* 11/32C. Therefore, this research will focus on the identification of the optimum environmental conditions that trigger intracellular P accumulation and biomass production in *Chlamydomonas reinhardtii,* and investigate the potential of its implementation in wastewater bioremediation.

Chapter 3 Materials and Methods

This chapter describes the materials and methods used in the study of Phosphorus (P) recovery from wastewater through enhanced micro-algal uptake. The methods described in the following sections are arranged into four main parts, which are in line with the four research objectives proposed in this work: (i) identification of environmental factors influencing optimum luxury P uptake using photobioreactors; (ii) exploring the presence of hyper P accumulators; (iii) operation of a continuous flow mixotrophic system with biomass recycle to enhance algal biomass production and luxurious P uptake; and (iv) assessing the potential for implementing P recovery via biological algal uptake at a large sewage treatment works.

3.1 Identification of environmental factors influencing optimum P uptake

This optimisation study was conducted to investigate the relationship between four important factors namely N and P concentrations, light intensity and photoperiod to enhance P uptake by microalgae, using *C.reinhardtii* 11/32C as a model organism. In this set of experiments, carbon (C) concentration is not included as a variable and its presence in culturing media was always in abundance, thus it was not considered to be a limiting factor for microalgae growth. The strain of *C.reinhardtii* was chosen for this study because of several important reasons: (a) there is an extensive knowledge gather from its genome project (Proschold et al., 2005; Stauber and Hippler, 2004); (b) it can be cultivated under photoautotrophic, heterotrophic or mixotrophic growing conditions (Boyle and Morgan, 2009; Grossman, 2000; Grossman et al., 2003; Therien et al., 2014); and (c) it is able to store the excess of P as polyphosphate granules (Komine et al., 2000; Ruiz et al., 2001a).

Several tests were conducted to achieve the objective of this work. Firstly, the microalgae were cultivated in ideal environmental conditions using a standard Bold's Basal Media (BBM) with the addition of $NaHCO_3$ as an inorganic carbon source. In this

study, the ability of the algae to uptake nutrients was monitored and the content of N and P in the algal cells was validated by using an energy-dispersive X-ray spectroscopy (EDX) technique. Secondly, the algae were cultivated in a modified BBM culture with different nitrogen sources (NO_3^-, NH_4^+ and a combination of both nitrogen) to observe which nitrogen source was more suitable for enhancing P uptake. The results of this investigation were key in order to determine the best conditions in the synthetic media for optimising algae cultivation and P uptake. Finally, an optimisation study was carried out following a fractional factorial experimental design (FFD). That set of experiments would allow us to identify the influence of important environmental factors that stimulate P storage in algal cells and also trigger algal biomass productivity. Results from the optimisation test were analysed using a response surface methodology (RSM) and a validation test were conducted based on the RSM analysis.

3.1.1 Microalgae cultivation

Stock cultures of *C. reinhardtii* 11/32C were purchased from the Culture Collection of Algae and Protozoa (CCAP), Scottish Association for Marine Science. Approximately 5 ml of a pure *C. reinhardtii* 11/32C was inoculated into 100 ml of sterile BBM enriched with 7.5 ml of 45g L^{-1} $NaHCO_3$ as a carbon source and cultivated in 250 ml conical flasks. The cultures were stored in a shaking incubator (Infors Multitron, Figure 3.1) and sub-samples were maintained under similar conditions and used as a seed inoculum for subsequent experiments using Photobioreactors (PBRs).

In the incubator, the culture was maintained under the following environmental conditions: temperature 20±2^oC; photoperiod 12 hr light: 12 hr dark; and light intensity 40 µE/m^2s. These environmental conditions were recommended by the CCAP. Two replicates were run per test and all the experiments were conducted at the Public Health Engineering Laboratory, in the Institute for Public Health and Environmental Engineering (iPHEE), School of Civil Engineering, University of Leeds. Algae cultures were monitored by microscopic examination on a weekly basis and fresh replicates were prepared every 4 weeks following the same inoculation technique. Also, Petri

dishes with stock samples in an agar of BBM were stored in the fridge (≈4°C) as back-up cultures.

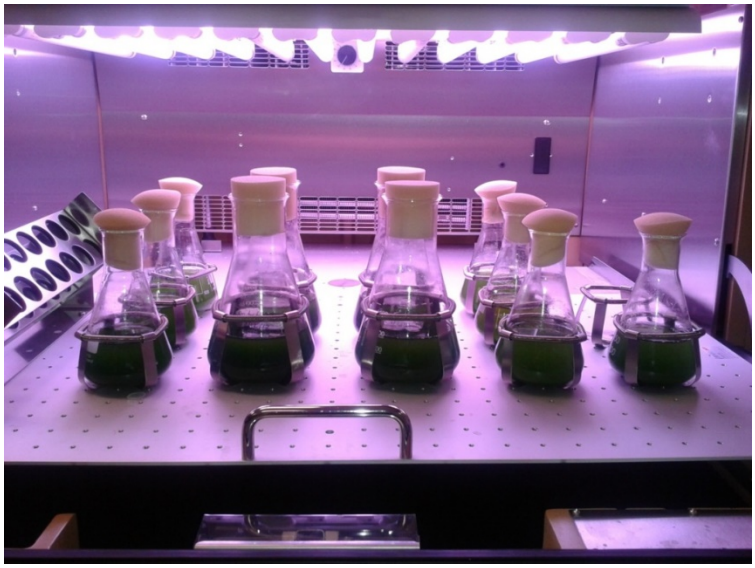


Figure 2-2 *C.reinhardtii* 11/32C culture in an Infors Multitron incubator

3.1.2 Culture medium for microalgae cultivation

a. Standard Bold's Basal Medium (BBM)

BBM was used as a culture medium for microalgae cultivation, following the protocol recommended by CCAP. This medium contains macro- and micro-nutrients that are required to support algae growth. The preparation of the BBM includes two steps, the preparation of a stock solution containing all macro-nutrients and the preparation of other stock solutions containing micro-nutrients. The stock solution containing macro-nutrients was prepared by dissolving the following chemicals reagents in 1 litre of distilled water: 25g $NaNO_3$, 2.5g $CaCl_2.2H_2O$, 7.5g $MgSO_4.7H_2O$, 7.5g K_2HPO_4, 17.5g KH_2PO_4, and 2.5g NaCl. The stock solutions containing micronutrients include: (i) Alkaline EDTA (50g L^{-1} EDTA anhydrous, 31g L^{-1} KOH); (ii) Acidified Iron (4.98g L^{-1} $FeSO_4.7H_2O$, 1mL H_2SO_4); (iii) 11.42g L^{-1} H_3BO_3; and (iv) Trace metals (8.82g L^{-1} $ZnSO_4.7H_2O$, 1.44g L^{-1} $MnCl_2.4H_2O$, 0.71g L^{-1} MoO_3, 1.57g L^{-1} $CuSO_4.5H_2O$, 0.49g L^{-1} $Co(NO_3)_2.6H_2O$). This standard nutrient medium contains 42mg N L^{-1} and 53mg P L^{-1}.

A closed cultivation system using a 2.5-Litre photobioreactor was chosen because PBRs are flexible systems that allow optimisation of biological and physiological characteristics of cultivated algae. This can reduce the risk of contamination and enhance algal biomass productivity (Mata et al., 2010).

b. Modified BBM

The concentrations of N and P in this modified BBM medium were adjusted to represent similar concentrations found from nutrient rich streams in a sewage treatment works. Such concentrations were determined from monthly water quality surveys conducted at the Yorkshire Water's Esholt Wastewater Treatment Works (WWTW). Three levels of nutrient concentrations were used for this modified medium: low, medium and high, and those levels are referred to the concentrations found in the final effluent, raw wastewater and the digestate liquor from the anaerobic digestion of sewage sludge, respectively. They were prepared as shown in Table 3.1.

In addition, 75 ml of a 45g L^{-1} $NaHCO_3$ stock solution were added per litre of modified BBM as a sole source of inorganic carbon to meet a final concentration of 500 mg C L^{-1}, which guarantees that carbon was always available for algal. The concentrations of the remaining chemicals were the same as in the standard BBM described in section 3.1.2.

Table 2-4 Modified algal culturing media (Modified BBM) for the optimisation P uptake

Nutrient	Level	25 gL^{-1} of $NaNO_3$ (ml)	10 gL^{-1} of NH_4Cl (ml)	K_2HPO_4 (mg)	KH_2PO_4 (mg)	Final concentration mg N, P L^{-1}
Nitrogen	Low	2.43	3.82	-	-	10
	Medium	24.29	38.21	-	-	100
	High	121.45	191.05	-	-	500
Phosphorus	Low	-	-	1.41	3.29	1
	Medium	-	-	14.08	32.86	10
	High	-	-	140.83	328.63	100

3.1.3 Algal growth and nutrient uptake in photo-bioreactors

Preliminary experiments conducted to observe algal growth and nutrient uptake of microalgae were carried out in BBM using photobioreactors (PBRs). The alga culture was cultivated in a 2.5L tubular PBRs (diameter: 7 cm; height: 61 cm), see Figure 3.2, under the following conditions: room temperature (aprox. 20^{o}C); photoperiod 16 hr light: 8 hr dark; and light intensity 250 µE/m^{2}s. The light cycle period and intensity were used according to recommend environmental conditions for the growth of *C.reinhardtii* (Elizabeth, 2008). All the experiments were conducted in triplicates.

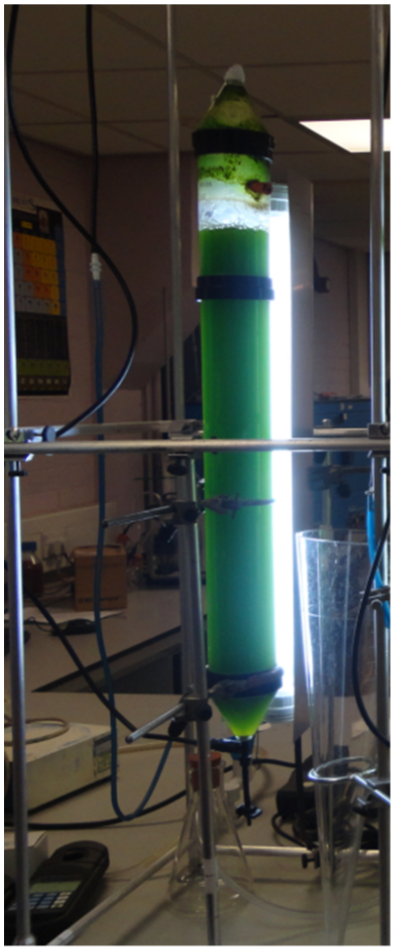


Figure 2-3 *C.reinhardtii* 11/32C cultivation in a tubular photobioreactor

Standard methods for water quality characterization (APHA, 2012) were used to monitor algal growth, biomass productivity, nutrient utilisation, and P uptake as reported in Table 3.2.

Table 3-2 **Analytical methods used for monitoring algal growth, nutrient utilisation and P uptake**

Parameter	Un-filtered sample	Method*	Filtered sample	Method*
Phosphorus	Total Phosphorus	4500-P.B	Total Phosphorus	4500-P.B
	Intracellular P	Yao et al. (2011)	Soluble Reactive Phosphorus	4500-P.E
Nitrogen	TKN	4500-N_{org}	TKN	4500-N_{org}
	-	-	NH_4^+	4500-NH_3 B and C
	-	-	NO_3^-	850 Professional IC Metroohm
Algal growth and biomass productivity	TSS/VSS	2540 D,E	-	-

***** Standard Methods for Examination of Water and Wastewater (APHA, 2012), unless otherwise stated

3.1.3.1 **Algal Growth and Biomass Production**

Determination of algae growth over its lifecycle was required to obtain specific growth rates under favourable environmental conditions. The algae were cultivated in BBM and placed in PBRs. Under controlled laboratory conditions, *C. reinhardtii* 11/32C have shown that the batch cultures reached stationary phase after 14 days and therefore, the batch experiments in this set of experiments were conducted for that period of time. During the 14 days, samples were taken from the algal PBRs every two days for analysis. The algal growth was measured in terms of biomass increments (dry weight basis), in which a known quantity of sample was filtered through a pre-weighed 47mm GF/C filter paper for VSS determination. In terms of monitoring biomass increments, total suspended solids (TSS) represent a general physico-chemical property of the sample and therefore, volatile suspended solids (VSS) was used as a representative parameter to describe the actual dry algal biomass content, which was used to calculate algal growth and biomass production (Becker, 1994). TSS and VSS were measured as described in Table 3.2.

In addition, the specific algal growth rates and biomass production were measured using data collected from the exponential growth phase in the batch cultures because in this phase the algae grow under conditions that truly represent their behaviour under the set environmental conditions.

The algal biomass (X) was calculated by using equation 3.1 (APHA, 2012) and the specific growth rate (μ) was analysed using linear regression to determine the gradient of the line representing algal biomass concentration (natural logarithm) against time (Schnurr et al., 2014).

$$\mathrm{X}\ (\mathrm{mgl}^{-1}) = \frac{(\mathrm{B}_1 - \mathrm{B}_2)\times 1000}{\text{sample volume, ml}} \qquad \text{Eq. 2-1}$$

Where B_1 is weight of residue + 47mm GF/C filter paper before ignition (mg), B_2 is weight of residue + 47 mm GF/C filter paper after ignition (mg), X is algal biomass, and t is cultivation time.

3.1.3.2 **Phosphorus uptake**

This set of experiments were aimed to determine the portion of added P in the medium which is assimilated by *C.reinhardtii* 11/32C. P is typically measured as total phosphorus (TP) which is a sum of the particulate and dissolved fraction of P (Patel et al., 2012). It is analysed into two steps: (a) conversion of the particulate P into dissolved orthophosphate, and (b) quantification of the total dissolved orthophosphate.

In this study the conversion of the particulate P into dissolved orthophosphate was conducted by using the ammonium persulphate digestion method (4500-P.B) and the total orthophosphate content was measured by using the ascorbic acid method (4500-P.E) (APHA, 2012). The fraction of suspended organic P in algal cells was measured by calculating the difference between TP from unfiltered and filtered samples. Additionally, in order to determine the remaining P concentration in the algal medium, filtered samples were processed for soluble reactive phosphorus (SRP) using the ascorbic acid method (4500-P.E; APHA, 2012).

P uptake rates were calculated from linear regression, in which the fraction of suspended organic P (mg P L^{-1}) was plotted against cultivation time (days). The rate of P taken up within algal cells was determined from data falling under the exponential growth phase.

The method developed by Yao et al. (2011) was used to determine intracellular P in microalgae. This method introduces a different protocol for sample filtration aimed to split P absorbed on the algal cell walls (and often counted as the P that has been taken up by the microalgae, if reported as suspended organic P), from the actual intracellular P accumulated within algal cells (Yao et al., 2011).

3.1.3.3 **Nitrogen uptake**

Total organic nitrogen was measured by using the Total Kjeldahl Nitrogen (TKN) method (4500-N_{org}; APHA, 2012). There are three major steps in Kjeldahl nitrogen determinations: (a) organic nitrogen bounded in the algal sample is converted into NH_4^+ through digestion process, (b) acidic sample is neutralised by concentrated NaOH, thus NH_4^+ is converted into NH_3 and eventually collected in the receiving vessel which contain boric acid solution during sample distillation and (c) the concentration of the captured NH_4^+ is then titrated using 10mM concentrated H_2SO_4 till the colour of the solution is changed to pale lavender. The fraction of the suspended organic nitrogen was produced from the difference between unfiltered and filtered algal samples. Then, nitrogen uptake rates in the forms of mg N L^{-1} d^{-1} were obtained from a linear regression analysis of data including suspended organic nitrogen (mg N L^{-1}) against cultivation time (days). In order to calculate the remaining N in the culture medium, NH_4^+ and NO_3^- were measured over the cultivation period.

The measurement of NH_4^+ concentration in the culture medium was carried out using the standard method 4500-NH_3 B and C (APHA, 2012). This analysis was carried out using a Buchi distiller, which was equipped with a steam generator, a distiller and a concentrate NaOH dispenser, followed by titration with boric acid.

NO_3^- concentrations in the medium were measured by using Ion Chromatography (IC) in a 850 Professional IC Metroohm. Samples were filtered through a 47 mm GF/C filter paper, then filtered through 0.45 μm Minisart ® Syringe Filter, before injection in the IC equipment.

3.1.3.4 Energy Dispersive X-ray spectroscopy (EDX) analysis

An EDX analysis was conducted to determine the elemental content of *C.reinhardtii* 11/32C as a background information for further experiments. The algal samples were prepared following the protocol set for SEM sample preparation (Section 3.2.1).

An EDX Carl Zeiss EVO MA15 (Oxford Instruments) coupled to the microscope was used for elemental analysis of algal samples. This analysis was conducted in the Leeds Electron Microscopy and Spectroscopy Centre (LEMAS), School of Chemical and Process Engineering, University of Leeds.

3.1.4 Influence of the nitrogen source on algal phosphorus uptake

This study was conducted to investigate the influence of the nitrogen source on algal P uptake rates. Three PBRs were set containing (i) NO_3^-, (ii) NH_4^+ and (iii) a mix of NO_3^- and NH_4^+ as nitrogen sources in BBM (Figure 3).

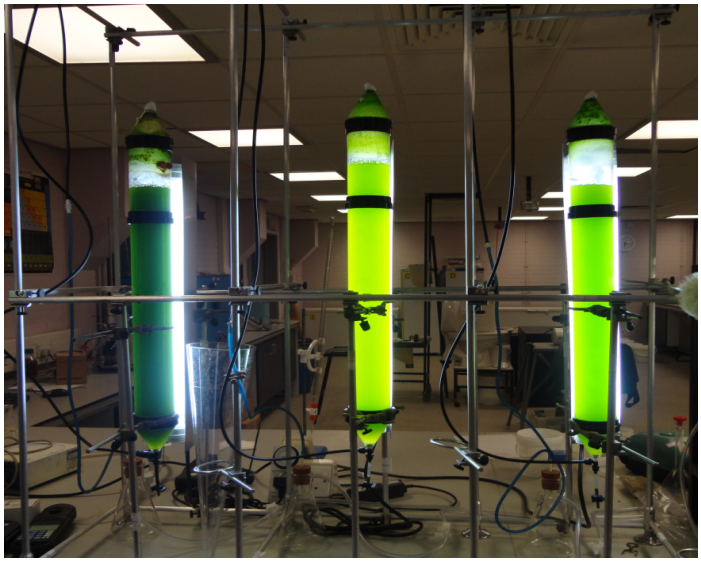


Figure 2-4 *C.reinhardtii* 11/32C cultivation with different nitrogen sources

The following environmental conditions were set to these experiments: 100 mg N L^{-1}, 1 mg P L^{-1}; photoperiod 12 hr light: 12 hr dark; and light intensity 250 µE/m^2s. These selected environmental conditions were set based on the FFD test number 14, which is explained in the following section. The same procedures as described in section 3.1.3 were used to determine the algal specific growth rate, biomass productivity and P uptake.

3.1.5 Identification of environmental factors to optimise algal P uptake

This experiment was conducted to explore the relationship between key environmental factors controlling alga growth (i.e., N, P, light intensity and photoperiod), for the optimisation of P uptake. The algae were cultivated in a wide range of different nutrients as described in Table 3.1. In addition, the software STATISTICA version 7 was used to construct the design of experiments. A Fractional Factorial Design (FFD) was chosen for the P uptake optimisation test in this study. FFD is a simplified full factorial design that can reduce the number of experiments without affecting the robustness/quality of the results (Barton, 1998; Montgomery, 2013). A modified environmental conditions applied in these experiments are described in Table 3.3.

Table 2-5 Environmental variables used for the optimisation of P uptake

		VARIABLE OF THE ENVIRONMENTAL FACTORS			
		Nitrogen mg N L^{-1}	Phosphorus mg P L^{-1}	Light Intensity µEm-^{2}s^{-1}	Photoperiod light : dark
LEVEL	Low	10	1	100	8:16
	Medium	100	10	250	12:12
	High	500	100	400	16:8

The experiments conducted for this optimisation test are listed in Table 3.4. The total number of experiments was 27 (3^{k-p}) in which represent 1/3 of the experiments required for a Full Factorial Design (81 experiments); each batch culture was run for

14 days. The specific growth rate, algal biomass, and the amount of P in the algal cells were measured and the resulting data was analysed using a response surface methodology (RSM). Moreover, validation of the optimum specific growth rate, biomass productivity, and P uptake found from the RSM analysis were tested in the lab. Three different P concentrations were tested in triplicate (80, 100, 120mg P L^{-1}). The same N concentration, light intensity and photoperiod of 200mg N L^{-1}, 250 $\mu Em^{-2}s^{-1}$ and 16 hr light: 8 hr dark respectively, were used in these experiments.

Table 2-6 Experimental design - Fractional factorial design (FFD) (3^{k-p})

Test number	Nitrogen, mg N L^{-1}	Phosphorus, mg P L^{-1}	Light Intensity, $\mu E/m^2s$	Photoperiod, light:dark
1	10	1	8:16	100
2	100	100	8:16	100
3	500	10	8:16	100
4	10	100	12:12	100
5	100	10	12:12	100
6	500	1	12:12	100
7	10	10	16:8	100
8	100	1	16:8	100
9	500	100	16:8	100
10	10	100	8:16	250
11	100	10	8:16	250
12	500	1	8:16	250
13	10	10	12:12	250
14	100	1	12:12	250
15	500	100	12:12	250
16	10	1	16:8	250
17	100	100	16:8	250
18	500	10	16:8	250
19	10	10	8:16	400
20	100	1	8:16	400
21	500	100	8:16	400
22	10	1	12:12	400
23	100	100	12:12	400
24	500	10	12:12	400

Test number	Nitrogen, mg N L^{-1}	Phosphorus, mg P L^{-1}	Light Intensity, µE/m^2s	Photoperiod, light:dark
25	10	100	16:8	400
26	100	10	16:8	400
27	500	1	16:8	400

3.1.6 Statistical Analysis

Data analysis was conducted using SPSS version 20 and Microsoft Excel 2013. Both software packages were used to analysed all growth kinetics data, nutrient uptake and removal rate. The growth kinetics data and nutrient uptake rates were analysed using linear regression to determine the slope of the line of algal biomass in forms of VSS and nutrient uptake rate versus cultivation time.

In addition, data from the optimisation test was analysed using the software STATISTICA version 7. The accuracy of the model was determined by the regression coefficient R^2 and adjusted R^2 (adj R^2). Furthermore, the statistical significance of the correlation between environmental factors variation was identified by the analysis of variance (ANOVA). The significance of regression coefficients for each environmental factors and correlation between variables were determined with a confidence level of 95%.

3.2 Exploring the presence of hyper P accumulators

This study aims to isolate individual cells of *C.reinhardtii* 11/32C that has high content of in-cell P in the form of polyphosphate. It is assumed that hyper P accumulators naturally appear and therefore, this section describes the methods for identifying the presence of such organisms in alga cultures.

In order to observe cell surface morphology of *C.reinhardtii* 11/32C, a scanning electron microscope (SEM) was used at the Faculty of Biological Sciences, University of Leeds. Then, fluorescence microscopy was used to verify the reliability of DAPI

staining in the algal cells. DAPI helps to visualise the presence of polyphosphate granules. Transmission electron microscopy (TEM) was also used to examine in detail cell organelles and localise the presence of polyphosphate granules. The presence of polyphosphate in algal cells confirmed and that was followed by another test using flow cytometry and cell sorting in order to isolate hyper P accumulators from a culture of *C.reinhardtii* 11/32C.

3.2.1 Scanning Electron Microscope

The surface morphology and cell shape of *C.reinhardtii* 11/32C were observed using a scanning electron microscope (SEM). In order to do this, a sample containing the microalgae was fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer for 2.5 hours, and then rinsed twice with 0.1M phosphate buffer for 30 minute, each. Algal cells were then post fixed overnight with 1.0% osmium tetroxide (OsO_4) (Jensen, 1968). Then, the specimens were rinsed twice for 30 minutes each with 0.1M phosphate buffer. Afterwards, they were dehydrated using a series of rinses with ascending concentrations of acetone (20%, 40%, 60%, 80%, and 2x100% for 20 to 60 minutes for each change). The dried samples were subjected to the process of critical point drying (CPD) to replace the acetone with liquid CO_2 under controlled temperature conditions. Eventually, the dried samples were mounted on the stubs and coated with platinum before proceeding to SEM observation. The examination used FEI Quanta 200F FEGESEM at voltage of 3 to 5 kV.

3.2.2 Observation of Polyphosphate Granules in *C.reinhardtii* 11/32C

3.2.2.1 Fluorescence Microscopy

Fluorescence microscope was used to verify the reliability of DAPI staining in algal cells. This staining is aimed to visualise the presence of polyphosphate granules in *C.reinhardtii* 11/32C (Ruiz et al., 2001a). The algal cells concentration of 5×10^7 per ml were spun down in a Centrifuge Technico Maxi at 7000 rpm for 3 minutes. The cells were rinsed three times with phosphate-buffered saline solution (PBS) to remove unacceptable materials in the solution. The specimens then were fixed with 3% paraformaldehyde (PFA) and re-suspended before storing at 4^oC for 30 minutes.

Afterwards, the algal pellets were re-spun down at the same velocity and rinsed three times with PBS.

The clean fixed algae then was stained with 50µg mL^{-1} of 4',6'-diamidino-2-phenylindole (DAPI) in a dark environment and incubated for 30 minutes at room temperature (Aschar-Sobbi et al., 2008; Gomes et al., 2013). Furthermore, the stained cells were mounted on a microscope slide and the polyphosphate granules observed using a confocal laser scanning microscope (LSM 510 META, Carl Zeiss). A magnification of 100x was used for an oil objective lens. DAPI-polyphosphate spots in *C.reinhardtii* 11/32C can be excited at 405 nm (DAPI Hoechst, ECFP) and the emitted light filtered using a 535-590 nm band pass (BP) in an emission filter.

3.2.2.2 **Transmission Electron Microscope**

Transmission electron microscopy (TEM) was conducted to examine the detail of organelles and to localise the presence of polyphosphate granules in *C.reinhardtii* 11/32C cells. There were several sample preparations to localise the polyphosphate granules in algal cells using this technique. The fixation and dehydration procedures used the same techniques as described in Section 3.2.1. However, instead of acetone dehydration used for SEM, samples for TEM analysis were dehydrated by using ascending series of alcohol, with the same concentrations and times.

Furthermore, the dehydrated samples were further processed by using propylene oxide for 20 minutes, then continued with a different ratio of propylene and Araldite (i.e., 50%-50%, 75%-25% of the propylene oxide-Araldite mix) for several hours overnight. The mixture of the anhydride-epoxy at ratio of 0.7 was done by employing the following chemicals: 27ml of Araldite CY212, 23ml of Dodecenyl succinic anhydride (DDSA) and 1ml tris (dymethilamino-methyl) phenol (DMP-30 reported as the best composition of resin for biological specimen (Luft, 1961; Spurr, 1969). Then, the specimens were embedded with neat fresh Araldite for 3 to 8 hours before polymerising overnight at 60^{o}C.

The next step was ultra-thin sectioned which is done by using microtomes. The c.100nm thick sections were picked up on 200 mesh copper, thin bar, Formvar coated grids. This thickness would allow electrons pass through the specimens. Afterwards, the samples were stained with concentrated 8% Uranyl Acetate for 1 hour, followed by Reynold Lead Citrate for 15 minutes (Reynolds, 1963). Eventually, the arrangements of the organelles particularly polyphosphate inside stained cells were studied using TEM (JEOL JEM 1400 TEM) with a tungsten filament running at 120kV (Marine et al., 2004). The TEM used was also equipped with a AMT 1K charge-coupled device (CCD) for imaging.

3.2.3 Flow cytometry and cell sorting

The flow cytometry (FCM) analysis is an important approach to determine a particular cell features (Hyka et al., 2013). In this study, FCM was used to identify the hyper polyphosphate accumulator in *C.reinhardtii* 11/32C. The FCM was equipped with a sorting facility that was able to isolate individual polyphosphate accumulators from a set population of algae cells.

Alive and fixed algae were used for FCM analysis. Algae samples with a concentration of a 5 x 10^7 cells/ml were spun down in an Eppendorf centrifuge at 3000 rpm for 5 minutes. The same sample preparation was carried out as mentioned in Section 3.5.1 was applied for FCM analysis. However, the live cells were processed without fixation because the target was to keep the sorted algae alive for future cultivation. A concentration of 50 μg/mL DAPI was used as well for FCM analysis, because DAPI would also stain polyphosphate granules and can help with sorting hyper P accumulators (Kawaharasaki et al., 2002).

The FCM and cell sorting was conducted using a Becton Dickinson FACSAria II cell sorter that was fitted with 405, 488 and 633 nm lasers. A 405 laser with BP filter 450/40 was used for detecting DAPI stained cells. It detected all the wavelengths between 410 and 490 nm. Furthermore, a 488 laser with BP 530/30 filter, that detected all the wavelengths between 500 and 560 nm, was used to obtain a green shift of the DAPI bind polyphosphate in the algal samples.

A flow cytometer consists of three main systems: fluidics, optics, and electronics (Biosciences, 2000). The fluidics system transport of single particles into a stream that can be interrogated by the laser beam. The algal samples were filtered through 50 μm Cell Trics® before loaded them into the FCM equipment. They were introduced into a sample stream and directed into a hydrodynamic focusing stream that is produced by sheath stream (Figure 3.4). At this point, the algae passed through the nozzle in single cells. In a certain point, which is called interrogation point, the algae and the laser interact and generate a scattering light to all directions.

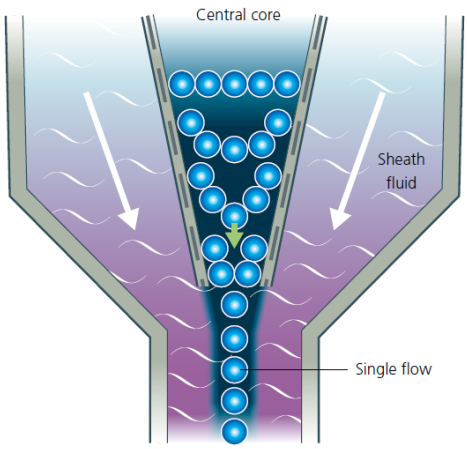


Figure 2-5 A single cell passed through the hydrodynamics focusing (Rahman)

Forward scatter (FS) illuminated the alga in the forward direction. The amplitude of the FS light is proportional to the size of algal cells. FS light was collected by the detector in which they amplified and converted into voltage pulses (Gunther, 2011).

In addition, side scatter (SS) which is located 90^{o} from the laser path illuminated the individual alga and scattered the light to all the directions. This light represented the granularity of the algal cell content (i.e., polyphosphate). The scatter light was delivered back to the SS path and captured by several filters. The schematic overview of the flow cytometry is shown in Figure 3.5. Eventually, the appropriate wavelength that represents the content of polyphosphate was delivered into a particular detector and transferred into voltage pulses. The voltage pulses information is then converted into digital information using a specific software (BD FACSDiva).

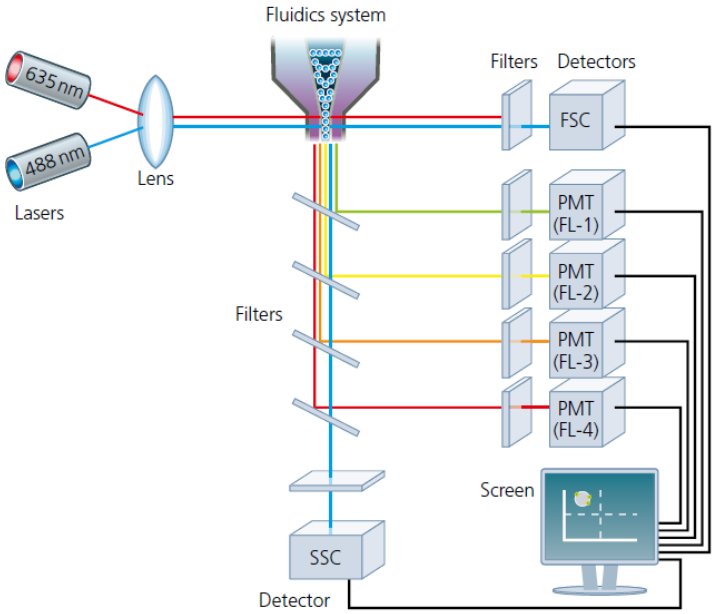


Figure 2-6 A schematic overview of the flow cytometry process (Rahman, 2015)

3.3 Operation of a continuous flow mixotrophic system with biomass recycle to enhance algal biomass yields and P uptake

This study was conducted to assess the possibility to develop an algal-based process that could overcome the limitations for simultaneous nutrient recovery and control found in current wastewater treatment plants using activated sludge process (ASP). A continuous flow microalgae cultivation systems was used for this experiment, in which the algae culture was fed continuously with fresh modified media at different hydraulic loading rates to identify the best conditions to generate high algal biomass production and P recovery. The system consisted of five main compartments: (i) inlet (feeding tank), (ii) heterotrophic reactor (HTR), (iii) autotrophic photobioreactor (PBR), (iv) sedimentation tank (ST) and (v) final effluent tank (FE) (Figure 3.6). The characteristics of the fresh medium were analysed regularly once the feeding media was changed. Moreover, several parameters as listed in Table 3.2 were also analysed from samples collected every two days from the effluent the following units: HRT, PBR, ST and FE. Those analyses were needed to monitor algal biomass production and P recovery over the time at different HRTs.

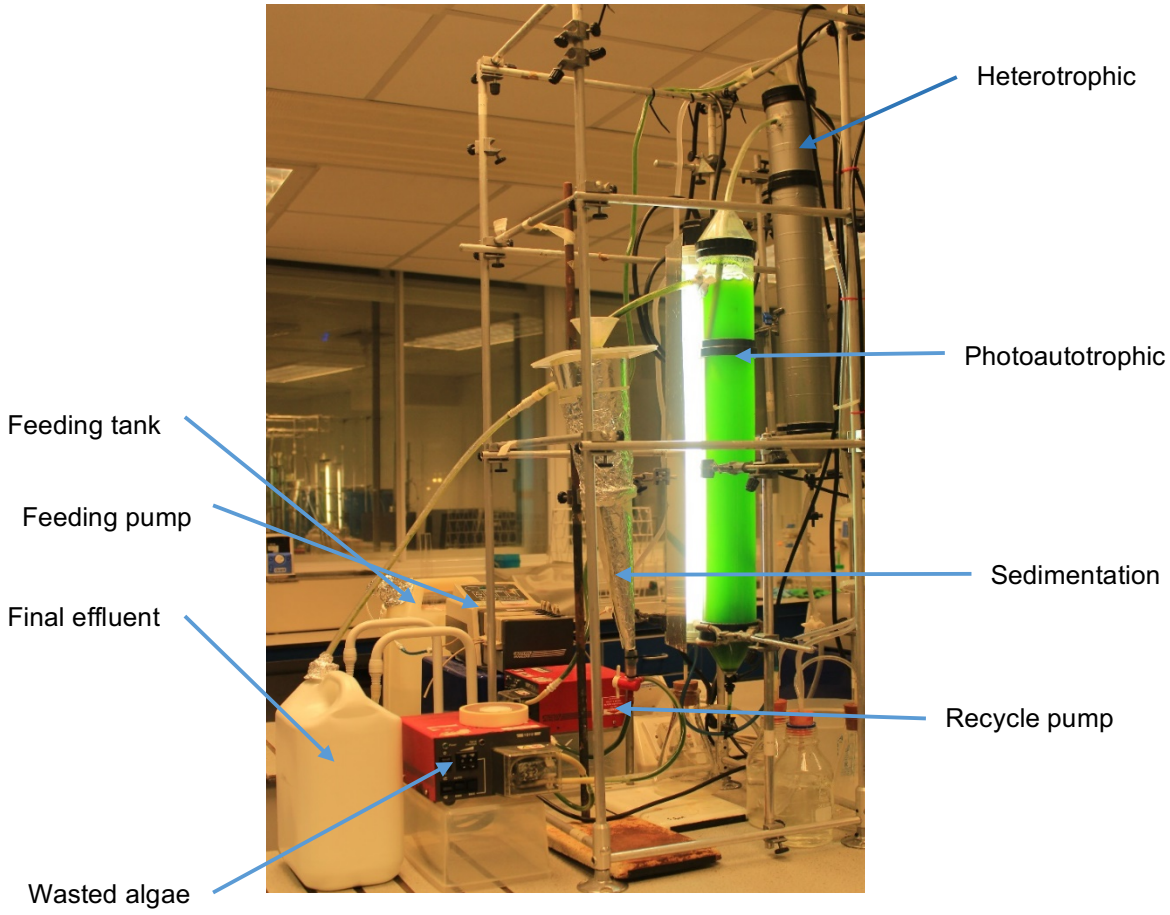


Figure 2-7 Continuous flow microalgae cultivation system

3.3.1 Design parameters for a continuous flow system

3.3.1.1 Growth kinetics in batch cultures

Algae cultivation in batch cultures was conducted under different environmental conditions. These experiments aimed to obtain the characteristics for growth kinetics controlling the growth of *C.reinhardtii* 11/32C. This study is useful not only to compare specific growth rates of *C.reinhardtii* 11/32C in batch cultures, with previous published works, but can also be used to predict growth rate (and potentially P uptake rates) in continuous flow systems (Ruiz et al., 2013).

Monod (1949) equation is the most common model used to examine the effect of a single growth-limiting substrate concentration on the growth of microorganisms. That equation could be expressed as follows:

$$\mu = \mu_{max} \frac{S}{(K_s + S)} \quad \text{Eq.2-2}$$

Where μ is a specific growth rate (day^{-1}), μ_{max} is the maximum growth rate (day^{-1}), S is the substrate concentration (mg L^{-1}), and K_s is the half saturation coefficient (mg L^{-1}).

The calculation of the specific growth was based on the increment of algal biomass concentration (mg VSS L^{-1}). The growth fitting model gave the value of μ_{max} and K_s which can be used to predict the specific growth rate in a continuous flow system.

3.3.1.2 Nutrient growth concentration

The optimisation test was conducted to investigate the relationship between environmental factors (N, P, Light Intensity, and Photoperiod) their impact on algal biomass concentration and intracellular P uptake. Previous results from optimisation tests shown that the most favourable nutrient concentration to cultivate *C.reinhardtii* 11/32C was 100 mg P L^{-1} and 200 mg N L^{-1}. However, it is very unlike to find similar nutrient concentrations in real wastewater treatment works as high N and P concentrations only exist in the digestate generated from anaerobic digesters. The digestate is pitch black and require pre-treatment to improve light penetration for microalgae growth and the removal of potential toxicants like H_2S and NH_3 (Sahu et al., 2013). Alternatively, it can be used to support heterotrophic algae growth, but that approach was not part of the current study.

In order to determine the most feasible conditions is a more realistic wastewater treatment scenario, a wastewater quality survey and characterisation was conducted from May 2014 to February 2015 at the Yorkshire Water's Esholt Wastewater Treatment Works in Bradford, UK. The average values of wastewater characteristics are reported in Table 3.5.

Table 2-7 Wastewater characterisation at Esholt

Nutrient source	TKN, mg N L^{-1}	TP, mg P L^{-1}	COD, mg O_2 L^{-1}
Digested liquor	1,727	24	8,900
Raw wastewater	61	8	664
After primary sedimentation	24	2.4	258
After secondary clarifier	3	1.1	55
Final effluent	2.5	0.4	42

Considering the results from Table 3.5, the concentration of nutrients chosen for testing a continuous flow system were 50 mg N L^{-1}, 15 mg P L^{-1} and 500 mg C L^{-1}. However, real wastewater may also contains unpredicted substances such as organic or inorganic compounds, toxins, pathogenic microorganism, heavy metal, etc. (Chiu et al., 2015). These substances were difficult to control at lab scale and although they may affect algal growth in a continuous flow system, it was decided to use synthetic wastewater with focus on its nutrient content. As a result, a modified synthetic wastewater was used for the cultivation of *C.reinhardtii* 11/32C for the study of biomass production and P recovery. The modified media is described in Table 3.6. In order to help algal biomass sedimentation, 20 mg L^{-1} of polymer concentration (i.e., Zetag 50) was added into the fresh modified media.

Table 2-8 Modified algal culture for continuous flow cultivation

Nutrient	**Volume of stock solutions used for media preparation**					**Final Conc. mg N,P,C L^{-1}**
	25 g L^{-1} $NaNO_3$ (mL)	**25 g L^{-1} $C_2H_3O_2NH_4$ (mL)**	**K_2HPO_4 (mg)**	**25 g L^{-1} $C_2H_3NaO_2.3H_2O$ (mL)**	**45 g L^{-1} $NaHCO_3$ (mL)**	
Nitrogen	6.1	5.5	-	-	-	50
Phosphorus	-	-	84	-	-	15
Carbon (org.)	-	5.5	-	13.6	-	100
Carbon (inorg.)	-	-	-	-	62	400

3.3.1.3 Hydraulic retention time (HRT)

In a continuous flow system, the flowrate of the feeding medium (Q) is the most important factor that controls algal growth (Becker, 1994). Since there were two reactors in series used in the system, the total working volume (V) was 4,510 mL. The amount of the feeding volume (Q) passed through the system was initially set at 4,510 ml day^{-1} (1 day HRT). The hydraulic retention time of the medium in the system could be calculated using the following equation:

$$\frac{1}{HRT} = \frac{Q}{V} \qquad \text{Eq. 2-3}$$

$$\frac{Q}{V} = D \qquad \text{Eq.2-4}$$

Where D is the dilution rate and HRT is the hydraulic retention time

In this system, microalgae growth under steady state conditions, where algal growth remained constant and the net change of the amount of algal biomass is determined by the specific algal growth rate and the wash out of algal biomass (Becker, 1994).

$$\frac{dX}{dt} = \mu X - (\frac{Q}{V}) \qquad \text{Eq. 2-5}$$

$$\frac{dX}{dt} = \mu X - DX \qquad \text{Eq. 2-6}$$

at the steady state condition

$$\frac{dX}{dt} = 0, \ \ and \ \ \mu = D \qquad \text{Eq. 2-7}$$

3.3.1.4 Sludge Residence time (SRT)

Based on (Metcalf and Eddy, 2004), the sludge residence time (SRT) is also related to μ as given by Equation 3.8. In the continuous flow microalgae system tested in this study, the algal cell residence time can also be referred as SRT under steady state conditions.

$$\mu = \frac{1}{SRT}$$ Eq. 2-8

Since

$$SRT = \frac{V\,X}{(Q - Qw)\,Xe + Qw\,Xr}$$ Eq. 2-9

Then, the amount of the wasted algae (Q_w) is given in the equation 3.10.

$$Qw = \frac{V}{(SRT.Xr)} - \frac{(Qe.Xe)}{Xr}$$ Eq. 2-10

Where:

V = reactor volume, mL

Q = feeding flowrate, mL d^{-1}

X = expected algal biomass concentration, mg VSS L^{-1}

Qw = flowrate of wasted algae, mL d^{-1}

Qe = flowrate of the effluent, mL d^{-1}

Xe = concentration of algal biomass in effluent, mg VSS L^{-1}

Xr = concentration of recycled algal biomass, mg VSS L^{-1}

3.3.2 Operation of a continuous flow microalgae cultivation system

The continuous flow system was initiated by running the reactors under batch conditions for heterotrophic and autotrophic growth until the net algal biomass concentration reached the stationary phase. Then, the continuous flow operation of the system was initiated by supplying fresh media to the reactors in series.

The flowrate was set according to the hydraulic retention time (HRT) set for the experiment. Three different HRTs were used: 12, 18 and 24 hours applied to each reactor. Two types of reactors in series were used in this system; the total working volume for each reactor was 2,255 mL (4,510 mL in total). The fresh media was fed into the heterotrophic reactor (HTR), which also received the recycle of settled algal biomass from the sedimentation tank (ST); the overflow from the HTR was taken into the autotrophic photobioreactor (PBR), which was connected to a sedimentation tank

(ST). The supernatant or final effluent, was collected into the final effluent tank (FE). Settled algae were recycled into the HTR every 2 hours for 15 minutes. The average volume of settled algal biomass recycled into the HTR was 60 ml per each recycling cycle (i.e., 15 minutes, every 2 hours). Moreover, in order to maintain algal density at the particular set value, settled algae collected in an Imnhoff cone was purged twice per day (≈300 ml per day) (Figure 3.7).

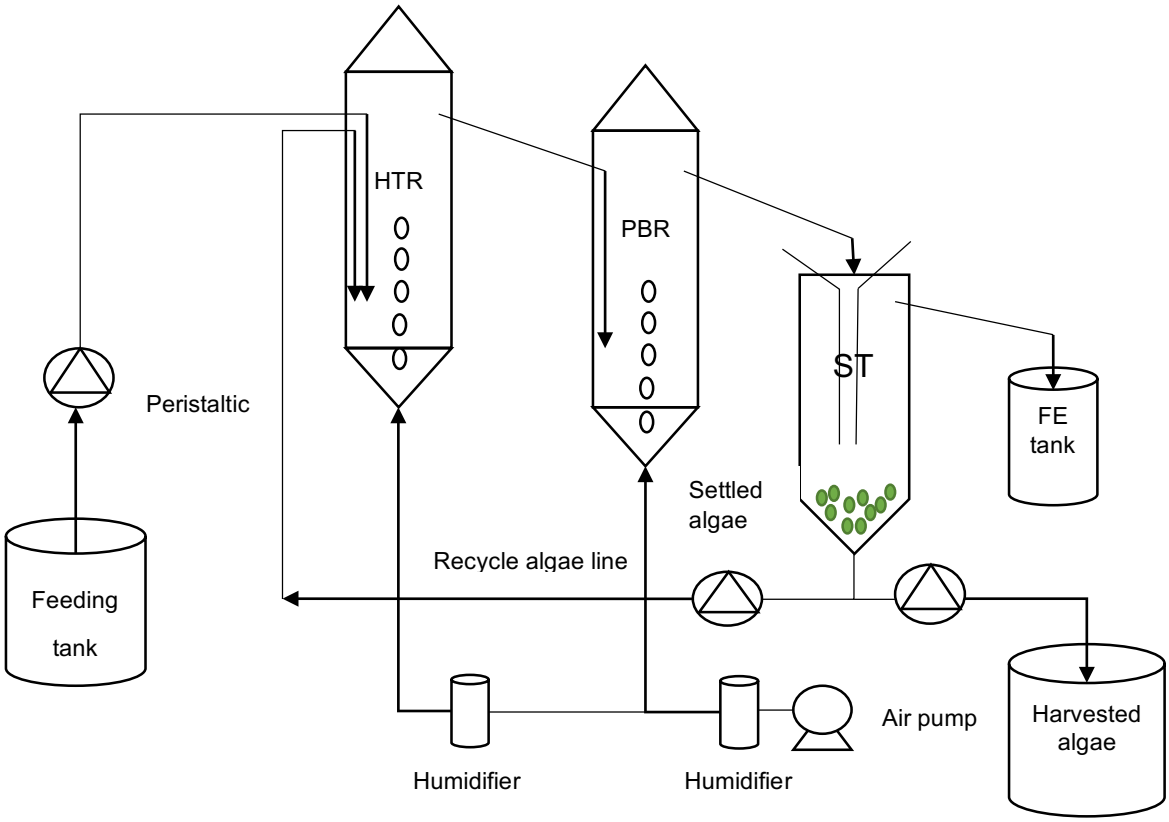


Figure 2-8 Diagram of the continuous flow mixotrophic cultivation system

In order to monitor algal biomass growth, P recovery and the performance of the system, water samples were collected every two days from HTR, PBR, ST and FE. Collected samples were processed for VSS, TP, SRP, Intracellular P, NH_4^+, NO_3^-, TKN, Chlorophyll-a and bacterial counts. The analytical methods used were explained in Section 3.1. Chlorophyll-a and bacterial counting will be described in the next section. In addition, to identify the presence of polyphosphate granules in the algal cells during the operation of the continuous flow system under different HRTs, TEM analysis and bio-imaging of DAPI-stained algae were also conducted.

3.3.3 Algal chlorophyll-a and bacterial population monitoring

Chlorophyll-a content in algal cells was measured to monitor algae growth over the operation period of the continuous flow photobioreactor system. This parameter is important to consider as the content of chlorophyll-a can be used to demonstrate that the algae grew in the favourable conditions and could compete with bacteria present in the system. The chlorophyll-a content was analysed as described in (Pearson et al., 1987) and calculated by using the following equation:

$$Chlorophyll - a\ \left(\frac{\mu g}{l}\right) = \frac{(Abs\ 663 - Abs\ 750)}{77} \times \frac{10}{vol} \times 10^6 \qquad \text{Eq. 2-12}$$

Where:
vol is the volume of sample filtered in mL
Abs 663 and Abs 750 are the absorbance reading at 663 and 750nm, respectively

The number of heterotrophic bacteria was closely monitored to control any culture contamination. A series of dilutions of collected samples were cultured in agar plates and colony counts after incubation were reported as Colony Forming Units (CFU) per volume of sample. 1 mL of water sample taken from the heterotrophic reactor was poured into 9 ml of autoclaved ringer solution. Then, 1mL of diluted sample was transferred into another 9 mL of ringer solution and repeated subsequently until a 10^5 serial dilution was obtained. 100 µl of the diluted sample was pipetted onto an agar plate under sterile environmental conditions. Afterwards, agar plates were stored at 60^o in an incubator for 24 hours before bacterium colonies were counted.

3.4 Assessing the potential for implementing P recovery via biological algal uptake at a large sewage treatment works

This research activity introduces the potential application of microalga-based system in existing sewage treatment works. An analysis of energy and mass balances based on the results from lab-scale experiments using a continuous flow microalgae cultivation system were used to verify the hypothesis that microalgae could be considered as a sustainable alternative for P recovery and control in wastewater

treatment systems. In addition, to validate this hypothesis, wastewater samples were collected from an existing large-scale sewage treatment facility (Yorkshire Water's Esholt Wastewater Treatment Works). Results were used for calculating energy and mass balances in a wastewater treatment works, and then compared with a hypothetical scenario where nutrient control was exclusively attributed to the implementation of microalgae systems.

Chapter 4 Identification of Environmental Factors Influencing Optimum P Uptake

4.1 Introduction

Wastewaters generated as a by-product of human activities including domestic water consumption, agriculture, farming and industrial processes, contain various pollutants including nitrogen (N) and phosphorus (P) compounds (Cai et al., 2013). In the 20th century, the global discharge of N and P into surface waters was 7.7 and 1 Tg year^{-1}, respectively. The contribution of human waste in terms of N and P to this large quantity of nutrient-rich waste was 87 and 88%, respectively (Moree et al., 2013). Therefore, it is worth to explore the possibility to harvest any quantity of N and P from waste streams and utilise them as fertilisers to support agriculture and to achieve food security, otherwise such nutrients will be wasted into water courses stimulating eutrophication process. Nutrient recovery from waste will help us to achieve long-term sustainability and to improve surface water quality.

In this Chapter, synthetic media was used as a source of nutrients for microalgae cultivation to mimic wastewater. Considering that microalgae can effectively assimilate the nutrients from wastewater for their growth (Larsdotter, 2006a), the assimilation can convert inorganic N into amino acids in intracellular fluids and utilise inorganic P to generate ATP as an energy input (Cai et al., 2013).

The work reported herein focuses then on enhancing P uptake in microalgae cells. Typically, microalgae used for wastewater treatment systems can take up to 1% P (dry-weight); however, under the right favourable environmental conditions, microalgae may incorporate P into their cells above 1% (i.e., luxury P uptake) and accumulate P reserves inside their cells as polyphosphate (Poly P) granules (Borchard et al.,1968). However, total content of P inside algal cells is not directly correlated to algal biomass and hence, when there is high algal biomass production that does not necessarily mean higher in-cell P accumulation; although higher algal biomass production helps to harvest the accumulated P in algal cells. However, biological intracellular P uptake is relatively constant and generally increases with increasing P

in the growth medium in which the algae can accumulate intracellular P up to 89% of their total P content (Saxton et al., 2012; Werner et al., 2007).

Such environmental conditions promoting both luxury intracellular P uptake and enhanced algal biomass production have not been observed yet; in fact, luxury P uptake is actually poorly understood. Therefore, it is important to explore further in order to better understand how environmental factors set for microalgae cultivation can simultaneously stimulate and enhance intracellular P uptake and algal biomass growth. This study studies key environmental factors controlling luxurious intracellular P uptake and algal biomass production.

4.2 Methodology

The aim of this research section was to identify the most important environmental factors that stimulate luxurious intracellular P uptake and support high algal biomass production. The selection of the microalgae strain as a model microorganism and environmental factors influencing algal growth and nutrients uptake were searched from existing published literature. *Chlamydomonas reinhardtii* (strain 11/32C) was selected as a model microorganism in this study due its ability to grow under photoautotrophic and heterotrophic conditions and has a high capacity to store polyphosphate (Werner et al., 2007). The strain was propagated in sterile Bold Basalt Media (BBM) and kept in an incubator as inoculum for future experiments. The cultivation method was described in Section 3.1.1.

In order to calculate the algal growth and nutrient uptake rates under ideal conditions, *C.reinhardtii* 11/32C were cultivated in a sterile standard BBM using 2L tubular Photobioreactors (PBRs) for 14 days. Samples were collected every two days and analysed for algal biomass and nutrient uptake as described in Table 3.2 (except for intracellular P). All the tests were carried out in triplicate using simultaneously three 2L PBRs (Figure 3.3). Furthermore, the algae culture was also cultivated using different nitrogen sources (NO_3^-, NH_4^+ and a NO_3^- + NH_4^+ mix) to examine the effect of N on P algal uptake.

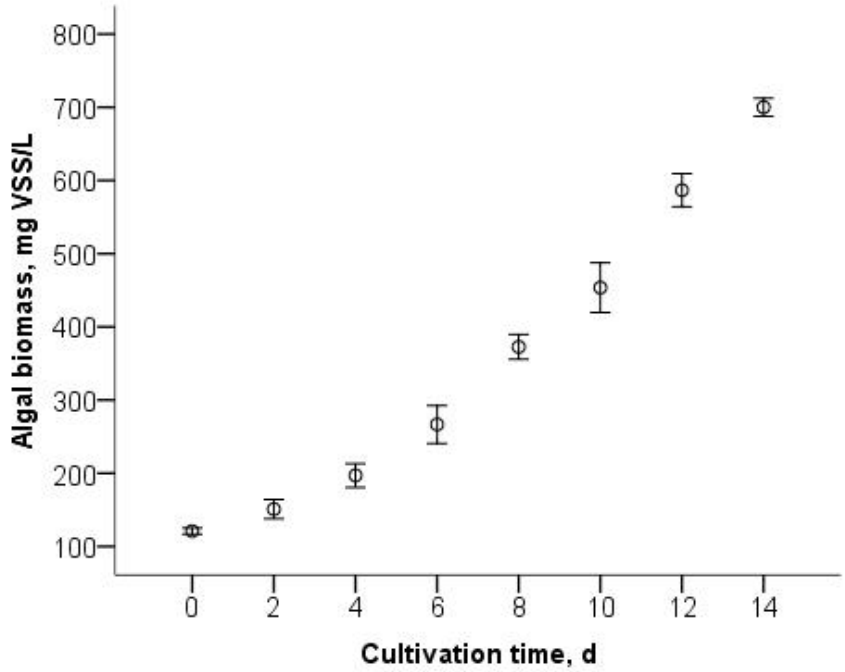
Several environmental conditions were used for this cultivation as described in Section 3.1.4. Then, the selected nitrogen source, which stimulated a higher P uptake and biomass production was used for future studies.

Moreover, to identify which level of each selected environmental factors influence luxurious P uptake and biomass production in *C.reinhardtii* 11/32C, a fractional factorial experimental design (FFD) was used to run a series of tests using four different environmental factors at three different levels each. The design of this experiment is described in Section 3.1.5.

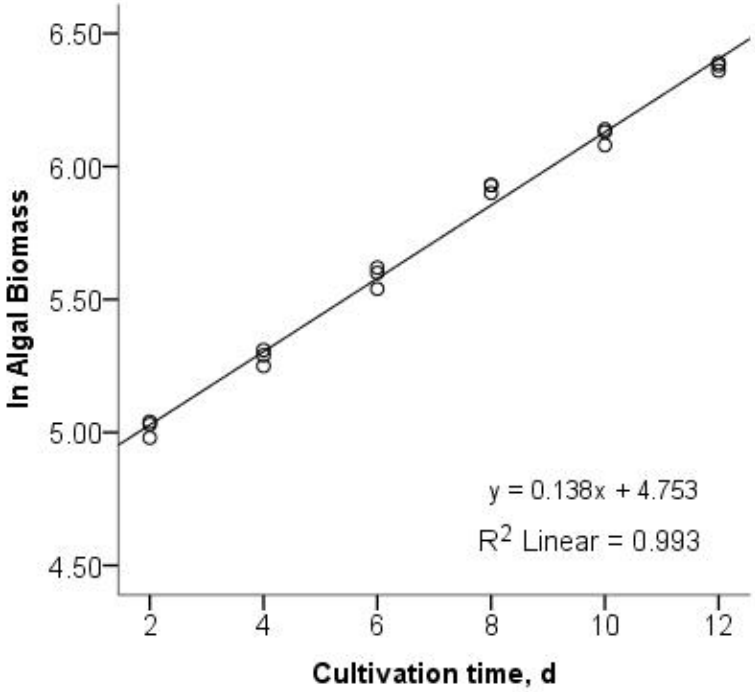
4.3 Results

4.3.1 Algal growth and nutrient assimilation under laboratory controlled conditions

A well-defined growth pattern of *C.reinhardtii* 11/32C cultivated in a standard BBM is shown in Figure 4.1a. Once the cultivation started, there was a lag phase of about two days that was required for the algae to acclimatise to the new environmental conditions. Then the algal biomass concentration increased exponentially from day 2 until day 12 that followed a linear regression when Ln VSS was plotted against cultivation time; see Figure 4.1b (regression coefficient, $R^2 = 0.99$). Using data from the exponential growth phase, the specific algal growth rate (μ) was 0.14 d^{-1} and biomass productivity was 44 mg VSS L^{-1} d^{-1}. Moreover, the increase in algal biomass was followed by an increase in N and P uptake in algal cells, as the nutrient concentrations in the media declined over time due to the assimilation by microalga (Figure 4.2).

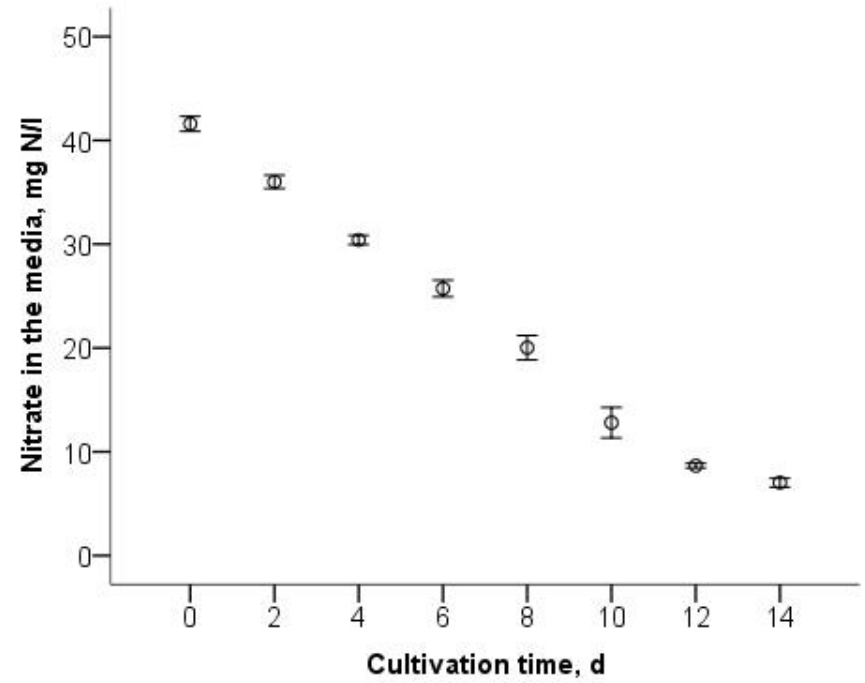


a. Algal biomass

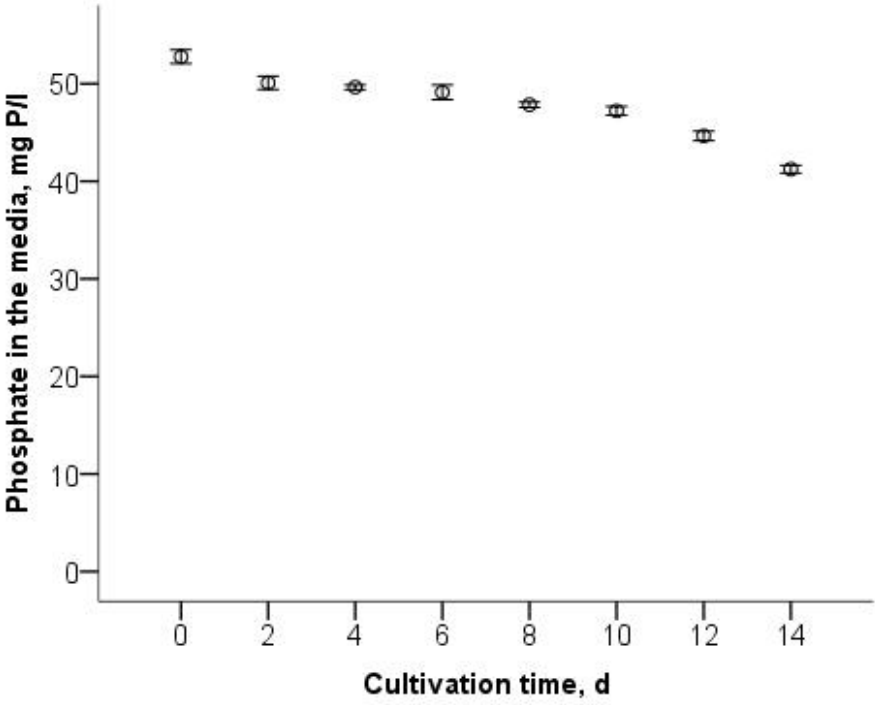


b. ln Algal biomass (ln VSS)

Figure 4-1 Algal biomass growth and specific growth rate for *C.reinhardtii* 11/32C using standard BBM



a. Utilisation of NO_3^- by *C.reinhardtii* 11/32C (NO_3^--N L^{-1})



b. Utilisation of PO_4^{3-} by *C.reinhardtii* 11/32C (PO_4^{3-}- P L^{-1})

Figure 4-2 Nutrient assimilation by *C.reinhardtii* 11/32C using standard BBM

Figure 4.2a above shows that 83% of the NO_3^- contained in the media (mean initial concentration of 42 mg N L^{-1}) was taken up and incorporated in the algal biomass after 14 days. Simultaneously, PO_4^{3-} concentration slightly decreased from 53 to 41 mg P L^{-1} from which only 22% of PO_4^{3-} was taken up by *C.reinhardtii* 11/32C (Figure 4.2b).

Under tested conditions, *C.reinhardtii* 11/32C was able to uptake NO_3^- at 2.13 mg N L^{-1} d^{-1} and PO_4^{3-} at 0.5 mg P L^{-1} d^{-1}, which indicates that the microalgae was able to accumulate 5.8% N and 1.6% P (dry weight). The content of N and P in the algal cells was validated by Energy-dispersive X-ray spectroscopy (EDX) analysis. Figure 4.3 below shows that N and P contents in algal cells cultivated after 14 days were 5.9% and 1.3% respectively, confirming experimental results.

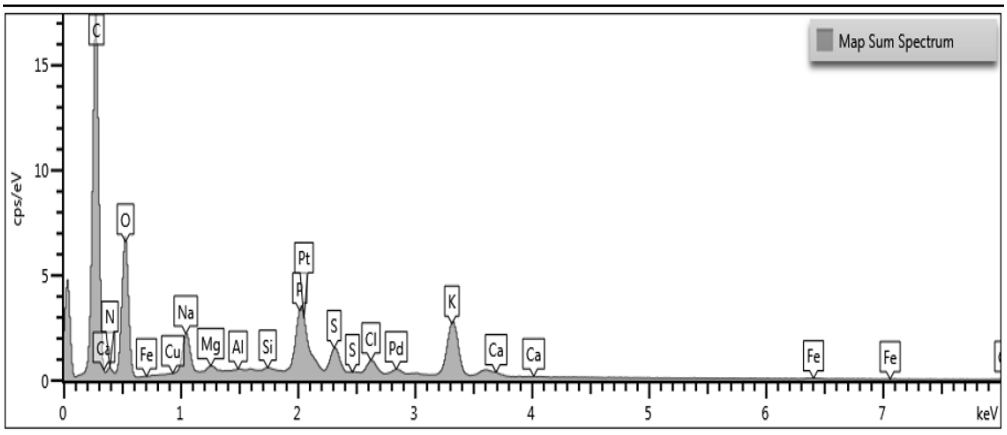


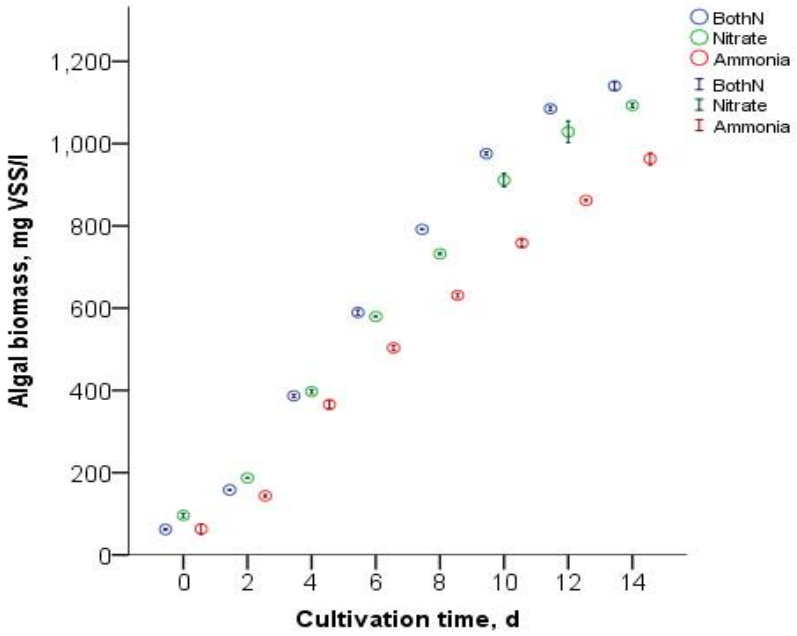
Figure 4-3 EDX analysis for N and P content in *C.reinhardtii* 11/32C

4.3.2 Effect of inorganic nitrogen source on algae growth and biomass productivity

Different N sources (e.g., (1) NO_3^- and NH_4^+; (2) NO_3^-; and (3) NH_4^+) were used to investigate the effect of N source on algal growth rate, P uptake and biomass production. As shown in Figure 4.4, algal growth rate and biomass productivity increased during the exponential growth phase (between days 4 and 12). The exponential growth phase was confirmed by linear correlation of transformed data (Ln VSS v time) with a resulting R^2 value of 0.99, which gave confidence when calculating specific algal growth rates.

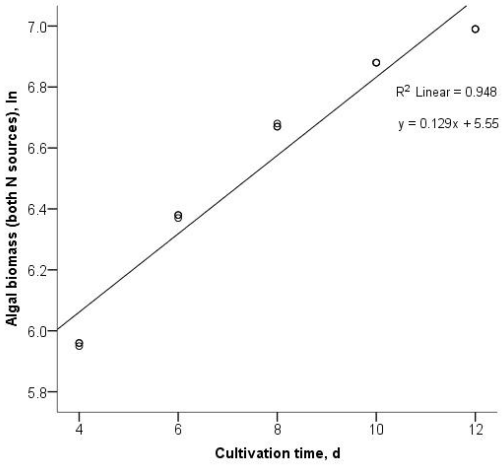
Results showed that there was no significant difference on microalgae growth and biomass production under different N sources (p>0.05). However, specific algal growth rates and biomass productivity cultivated in a media containing both NO_3^- and NH_4^+ (experiment 1) was always higher than the figures reported for NO_3^- and NH_4^+ on their own (experiments 2 and 3, respectively). The specific algal growth rates were 0.128 d^{-1}, 0.117 d^{-1}, and 0.106 d^{-1} for experiments 1, 2, and 3 respectively.

Algal biomass productivity for experiment 1 (NO_3^- and NH_4^+ mix) was 89 mg VSS l^{-1} d^{-1}, while the figures for algae grown in media containing only NO_3^- or NH_4^+ were 79 and 62mg VSS l^{-1} d^{-1}, respectively. In practical terms, it seems that *C.reinhardtii* 11/32C grew better when using a combination of NO_3^- and NH_4^+.

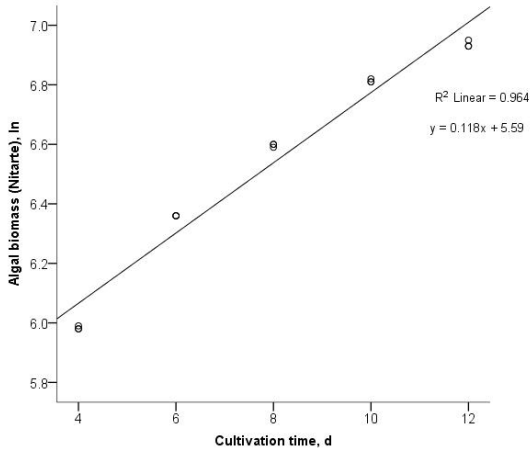


a. Algal biomass concentration of *C.reinhardtii* 11/32C in different N sources

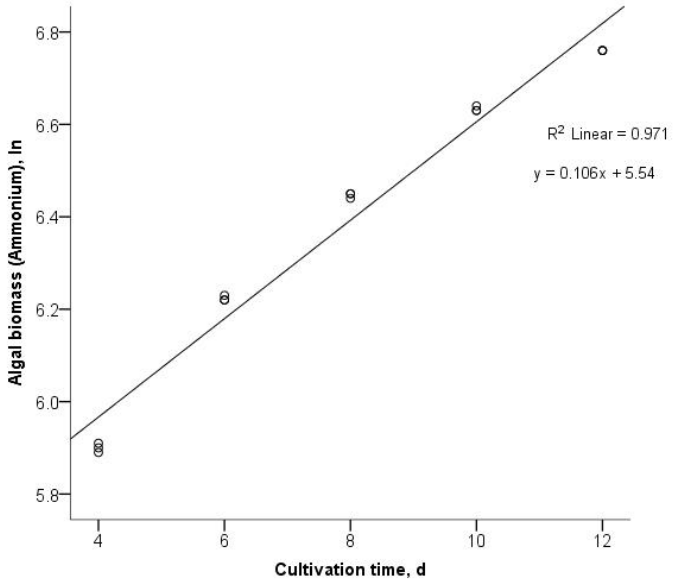
Figure 4-4 Algal biomass and specific growth rate of *C.reinhardtii* 11/32C using different nitrogen sources



b. Specific growth rate of *C.reinhardtii* 11/32C in both N sources



c. Specific growth rate of *C.reinhardtii* 11/32C in Nitrate



d. Specific growth rate of *C.reinhardtii* 11/32C in Ammonium

Figure 4-4 Algal biomass and specific growth rate of *C.reinhardtii* 11/32C using different nitrogen sources

Figure 4.5 shows that *C.reinhardtii* 11/32C preferred to take up NH_4^+ over NO_3^- with a total ammonium removal from the media of 97%. However, the specific growth rate and biomass productivity of *C.reinhardtii* 11/32C grown found in experiment 3 is lower than those found in experiments 1 and 2. In addition, algae have shown to consume less NO_3^- from the media with an average N reduction of 45%. This study thus has shown that *C.reinhardtii* 11/32C produced the higher specific growth rate and biomass

productivity when cultivated with a mix of NO_3^- and NH_4^+ in the culture media (Experiment 1). That could imply the convenience of using the effluent from a conventional activated sludge process (i.e., no extended aeration for full nitrification) as the perfect culture media with regard to algal biomass production.

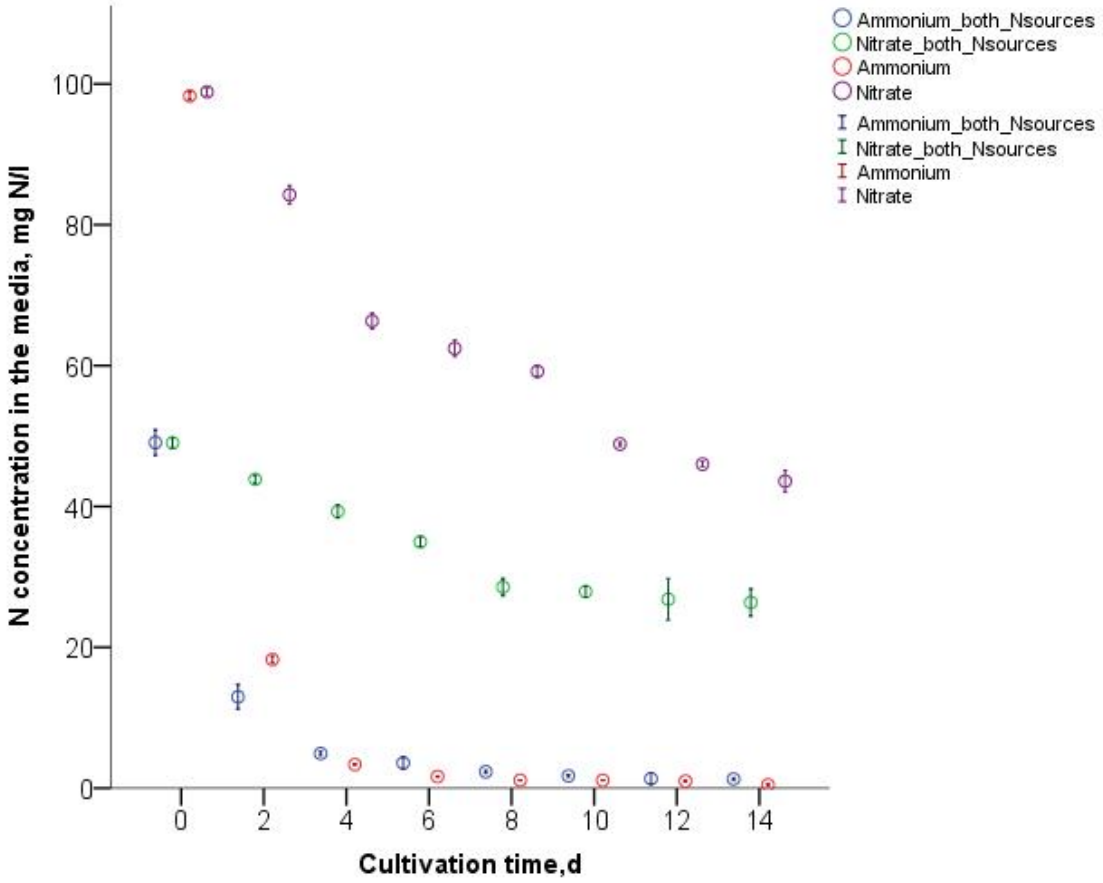


Figure 4-5 Nitrogen species concentration in the culture media over time

In terms of P assimilation, the concentration of PO_4^{3-} declined rapidly within the first 2 days of the cultivation period in all the media tested independently of the N source used (Figure 4.6); the microalgae were able to remove more than 99% of PO_4^{3-} in all three experiments. PO_4^{3-} concentration was reduced from approximately 1 mg P L^{-1} to 0.012 - 0.063 mg P L^{-1} during the first 4 days and then completely removed below the analytical detection limit by day 14.

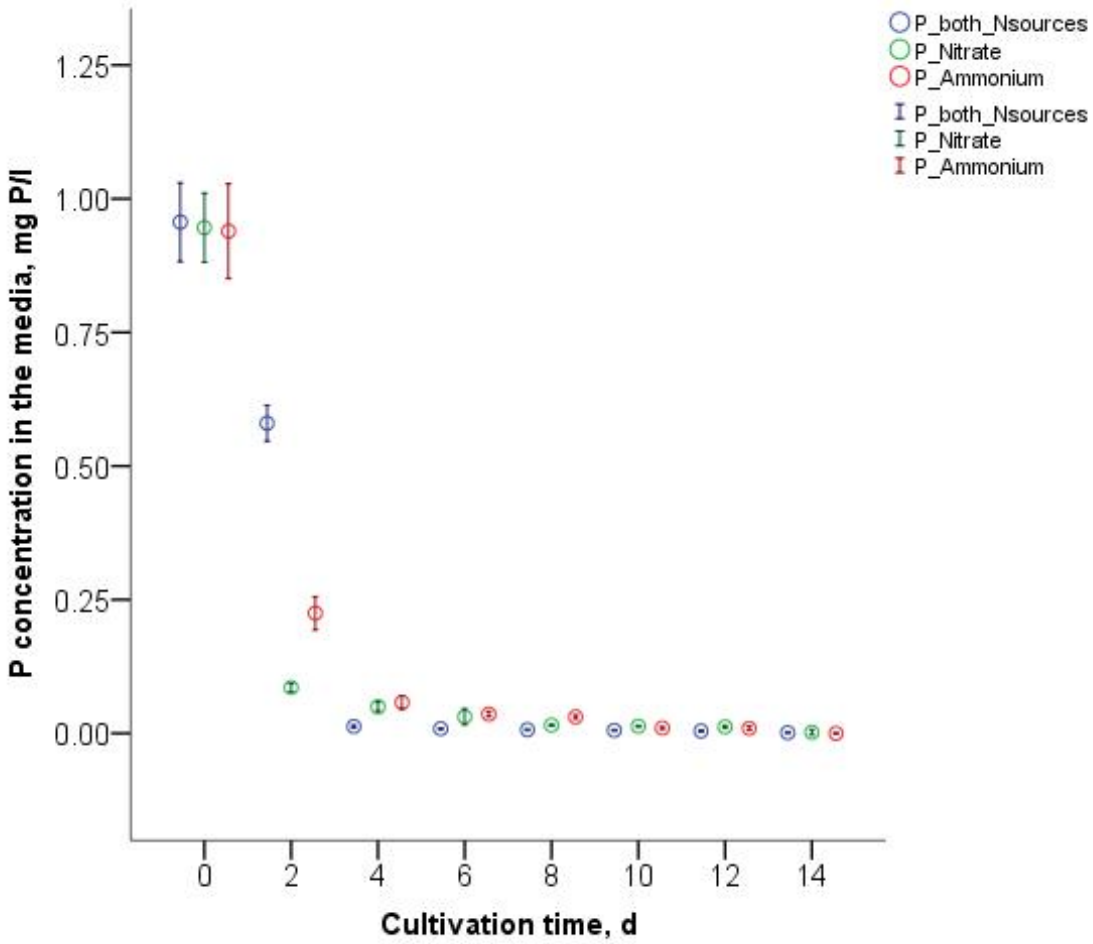
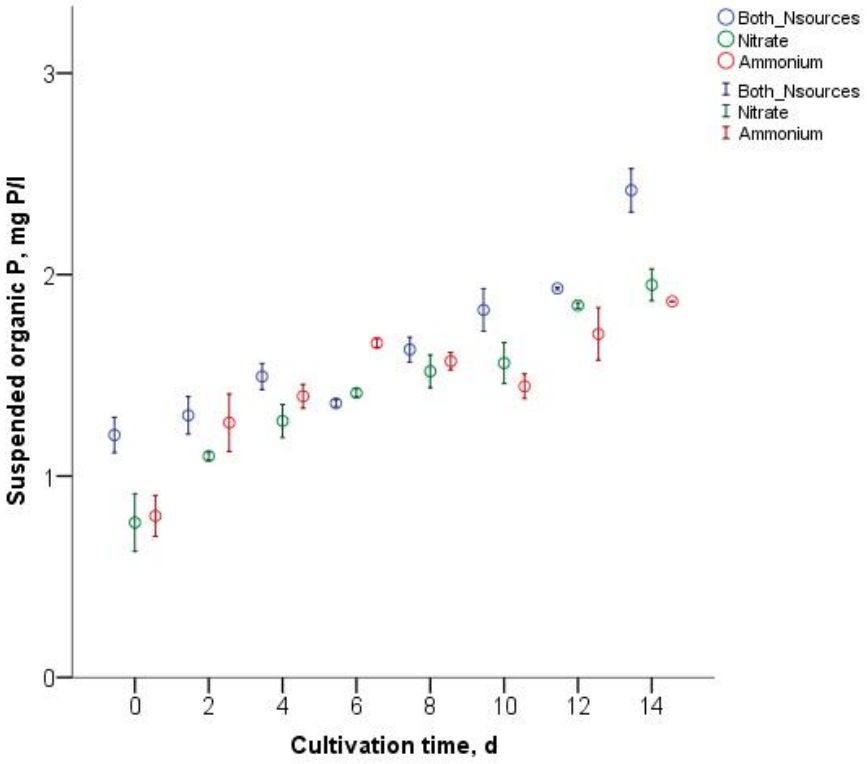


Figure 4-6 Remaining P concentration under different nitrogen sources

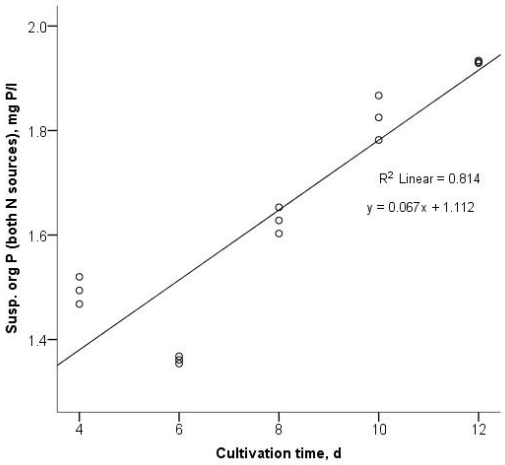
4.3.3 Effect of different inorganic nitrogen sources on P uptake rate of *C.reinhardtii* 11/32C

P uptake by *C.reinhardtii* 11/32C during the exponential growth phase occurred from day 4 to 12 (Figure 4.7). The net P uptake rate was calculated from data reporting suspended organic P in the media over time. Exponential regression coefficients (R^2) for the three experiments testing different nitrogen sources were 0.8, 0.9, and 0.5, for experiments 1, 2 and 3 respectively. The combination of NO_3^- and NH_4^+ as N source influenced higher P uptake and produced higher concentrations of suspended organic P than using NO_3^- and NH_4^+ on their own, although a short lag phase was found from day 4 to 6.

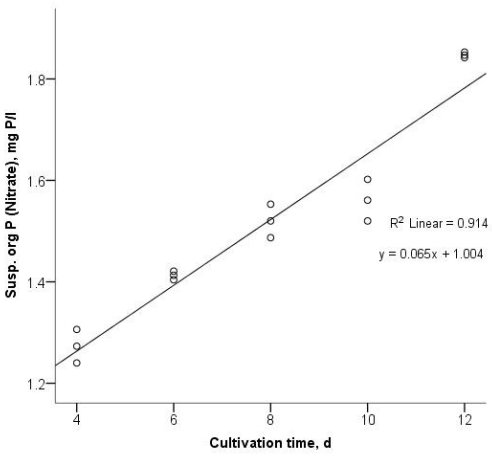
The effects caused by switching NH_4^+ utilisation in the first four days for NO_3^- afterwards enhanced net P uptake rates (Figure 4.7b). This stressful conditions triggered faster P uptake by microalgae, which continue day 14. After day 4, P uptake rate in Experiment 1 was 0.067 mg P $L^{-1}d^{-1}$, which is higher than P uptake rates found for experiments using NO_3^- or NH_4^+; P uptake rates for experiments 2 and 3 were 0.065 and 0.020 mg P $L^{-1}d^{-1}$ respectively.



a.

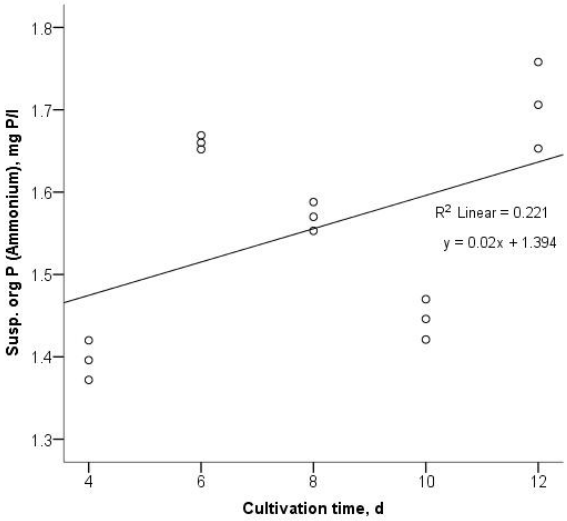


b.



c.

Figure 4-7 Suspended organic P over 14 days (a) and in exponential phase (b-d)



d.

Figure 4-7 Suspended organic P over 14 days (a) and in exponential phase (b-d)

Interestingly, when the content of P was plotted against the corresponding N content in dry biomass, P content varied from 0.2 to 2.3%. The higher P content was about 1.8 to 2.3% when algae were cultivated in the media containing both NO_3^- and NH_4^+ and it corresponded to N content ranging from 3.0 to 4.2% (Figure 4.8). From that, it is possible to infer that by using a media combining NO_3^- and NH_4^+ as nitrogen source, ammonium is rapidly consumed by microalgae and then the shift into nitrate as N source accelerates P uptake and in-cell storage.

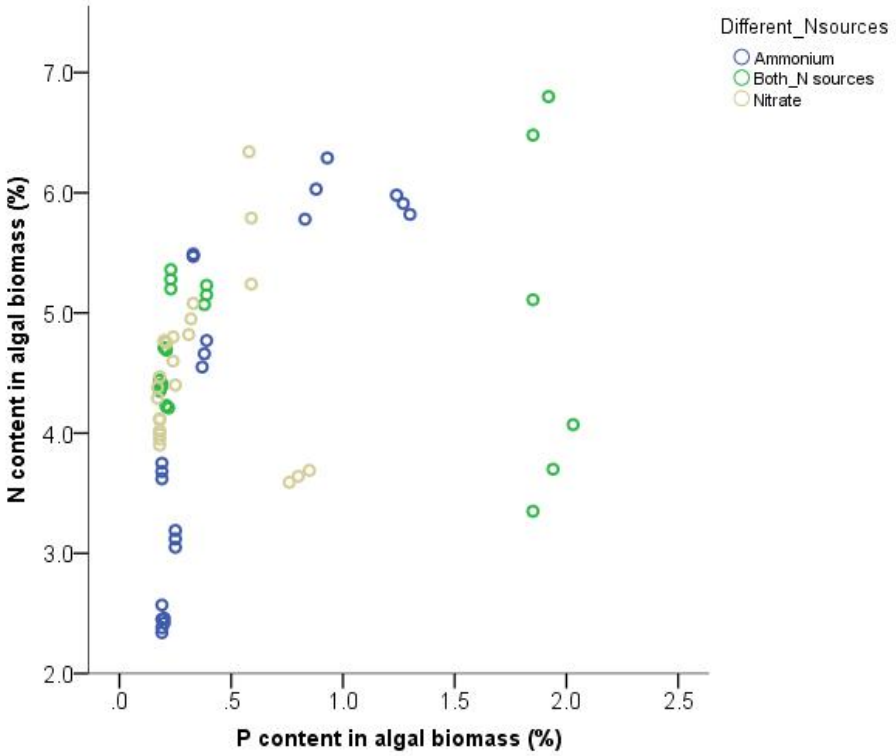


Figure 4-8 Phosphorus v. Nitrogen content in algal biomass (% dry weight)

4.3.4 Identification of optimum environmental factors enhancing intracellular P uptake and algal biomass productivity

The algal biomass production of *C.reinhardtii* 11/32C was studied using the following key environmental factors as independent variables: N and P concentration, photoperiod and light intensity. An equal ratio of NO_3^- and NH_4^+ was used as a nitrogen source (50:50). The experimental design for the impact of environmental factors affecting intracellular P uptake and algal biomass productivity are reported in Table 4.1, which also include both observed (experimental) and predicted (response surface model) values.

Previous optimisation studies reported in the literature have used uniform values for each level of the variables tested as part of their experimental design. Uniform values indicate the same range value for each level of the tested variables, while not uniform values indicated the opposite. In this study, the selected values of two variables (i.e., N and P concentrations) are not uniform and therefore, more realistic values were

chosen to analyse experimental and predicted results. The reason of having non-uniform concentrations of N and P is that these values represent wastewater characteristics found during the water quality survey conducted at Esholt Wastewater Treatment Works (WWTW). Thus, tested values are based on the concentration of N and P found in the final effluent (low level), raw wastewater (medium level), and digested liquor (high level) at Esholt WWTW.

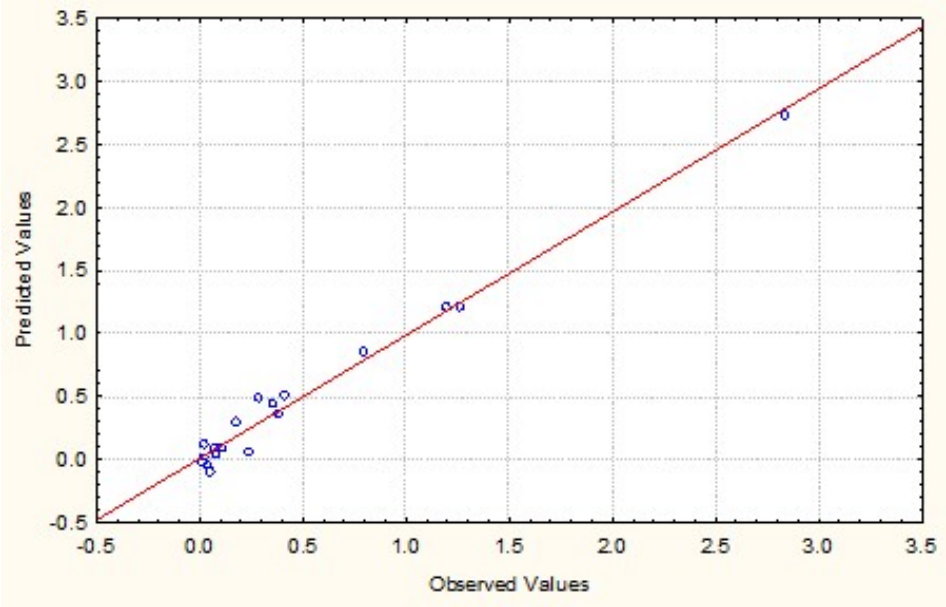
The experimental results shown that the intracellular P uptake rates varied between 0.009 and 2.84 mg P $L^{-1}d^{-1}$. The lowest intracellular P uptake rate was obtained using the following environmental factors: Nitrogen concentration, 100 mg N L^{-1} (50:50 of NO_3^- : NH_4^+); Phosphorus concentration, 1 mg P L^{-1}; Photoperiod, 16 hr light and 8 hr dark; and Light Intensity, 100 $\mu Em^{-2}s^{-1}$. Whereas the largest intracellular P uptake rate was obtained at 100 mg L^{-1} of N concentration, 100 mg L^{-1} of P concentration, 16 hr light:8 hr dark of photoperiod, and 250 $\mu Em^{-2}s^{-1}$ of light intensity.

Algal biomass productivity yield varied between 3 and 149 mg VSS L^{-1} d^{-1}. The lowest algal biomass productivity found was obtained using the following environmental factors parameters: 10 mg L^{-1} of N concentration, 10 mg L^{-1} of P concentration, 16 hr light:8 hr dark of photoperiod, and 100 $\mu Em^{-2}s^{-1}$ of light intensity. Whereas the largest algal biomass productivity rate was obtained at 100 mg L^{-1} of N concentration, 100 mg L^{-1} of P concentration, 16 hr light:8 hr dark of photoperiod, and 250 $\mu Em^{-2}s^{-1}$ of light intensity.

Table 4-1 Results from the 3^{k-p} fractional factorial experimental design for optimum intracellular P uptake and algal biomass productivity

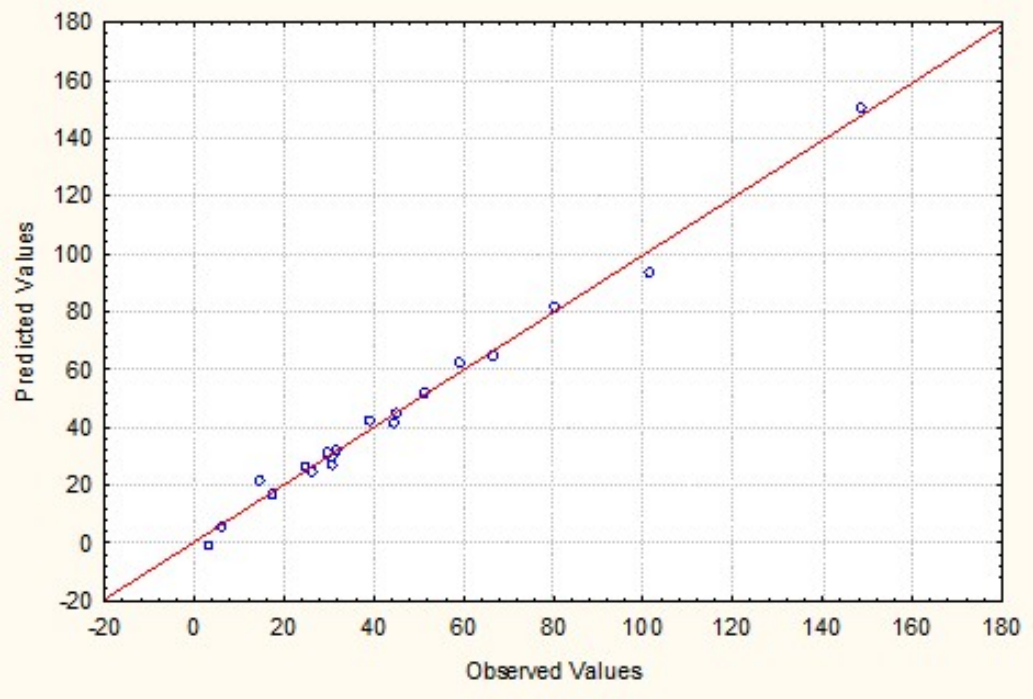
	Environmental variables				Biomass productivity mg VSS L^{-1} d^{-1}		Intracellular P uptake mg P $L^{-1}d^{-1}$	
Test no.	N, mg N L^{-1}	P, mg P L^{-1}	Photoperiod light:dark	Light Intensity, $\mu Em^{-2}s^{-1}$	Observed	Predicted	Observed	Predicted
1	10	1	8:16	100	17.22	17.22	0.02	0.02
2	100	100	8:16	100	38.96	42.11	0.23	0.07
3	500	10	8:16	100	-45.86	-	-0.02	-
4	10	100	12:12	100	30.75	29.60	0.28	0.50
5	100	10	12:12	100	44.89	44.73	0.26	0.15
6	500	1	12:12	100	-9.83	-	-0.04	-
7	10	10	16:8	100	2.96	1.05	0.10	0.10
8	100	1	16:8	100	59.00	62.28	0.01	0.03
9	500	100	16:8	100	26.24	25.13	0.38	0.37
10	10	100	8:16	250	30.61	27.22	1.19	1.21
11	100	10	8:16	250	66.32	64.80	0.41	0.51
12	500	1	8:16	250	-2.99	-	-0.03	-
13	10	10	12:12	250	14.30	21.38	0.08	0.05
14	100	1	12:12	250	101.24	94.00	0.08	0.09
15	500	100	12:12	250	-15.46	-	-0.57	-
16	10	1	16:8	250	24.63	26.73	0.04	0.05
17	100	100	16:8	250	148.53	150.30	2.84	2.73
18	500	10	16:8	250	51.04	52.25	0.35	0.45
19	10	10	8:16	400	16.46	15.75	0.05	0.08
20	100	1	8:16	400	29.57	31.43	0.02	0.13
21	500	100	8:16	400	31.83	31.73	0.79	0.86
22	10	1	12:12	400	10.09	15.33	0.02	0.020
23	100	100	12:12	400	80.37	81.84	1.26	1.22
24	500	10	12:12	400	-62.18	-	0.05	0.04
25	10	100	16:8	400	6.26	5.63	0.07	0.10
26	100	10	16:8	400	44.21	41.61	0.18	0.31
27	500	1	16:8	400	-7.60	-	-0.04	-

In order to obtain sensible results from the optimisation tests, several negative values for P uptake and biomass yields (see Table 4.1), which indicate that the microalgae decreased their biomass or net P uptake, were excluded from this analysis. These were caused by toxic environment in high level of N (250 mg NO_3^--N L^{-1} and 250 mg NH_4^+-N L^{-1}). After removing those values, the residuals between predicted and observed results were very low. Figure 4.9 presents predicted and observed results of intracellular P uptake and algal biomass productivity. Regression linear coefficient (R^2) for the two response (i.e., Intracellular P uptake and Biomass productivity) were 0.92 and 0.96 respectively.



a. Intracellular P uptake

Figure 4-9 Observed versus predicted results for intracellular P uptake (a) and algal biomass productivity (b)



b. Algal biomass productivity

Figure 4-9 Observed versus predicted results for intracellular P uptake (a) and algal biomass productivity (b)

4.3.4.1 Effect of nitrogen and phosphorus concentration, light intensity and photoperiod on intracellular P uptake

Intracellular P uptake in *C.reinhardtii* 11/32C under different N and P concentrations, light intensity and photoperiod revealed that the microalgae was able to grow in a culture media containing up to 100 mg L^{-1} of P concentration and those conditions stimulated higher P uptake when compared with other tests using 1 and 10 mg P L^{-1}. The intracellular P uptakes of the algae grown in 100 mg P L^{-1} ranged from 0.07 to 2.84 mg P $L^{1}d^{-1}$, whilst the algae grown in 1 and 10 mg P L^{-1} could only take up P at rates ranging from 0 to 0.08 mg P $L^{-1}d^{-1}$ and from 0.05 to 0.41 mg P $L^{-1}d^{-1,}$ respectively. Figure 4.10 presents five different patterns of intracellular P increments of tested microalgae under selected environmental conditions with P media concentration of 1, 10 and 100 mg P L^{-1} (tests 8,11, 17, 19 and 26; see Table 4.1).

The algae cultivated under the conditions set for Test 17 reported a short lag phase and increasing intracellular P in the culture over time, which reached the highest point of 37 mg P L^{-1} in 14 days. This test shows that the surplus of P concentration in the media helped to enhance the intracellular P uptake rate (2.84 mg P $L^{-1}d^{-1}$). On the other hand, the algae cultivated in the media with lower P concentrations (e.g., 1 mg P L^{-1}, Test 8; and 10 mg P L^{-1}, tests 11, 19 and 26) stored less intracellular P in their cells. Figure 4.10 illustrates that in test 11, the algae slowly accumulated P only within the first 10 days with an intracellular P uptake rate of 0.41 mg P $L^{-1}d^{-1}$ before reaching the stationary phase. The other three tests (8, 19 and 26) seem to take up smaller amounts of P and to store less P in their cells. The five different environmental conditions presented in detail in this section set conditions for P storage in algal cells with a P content of 0.4%, 0.7%, 0.8%, 1.0% and 2.1% DW for tests 8, 19, 26, 11 and 17, respectively. These five different tests were randomly selected based on the lowest to the highest intracellular P uptake rates from the conducted FFD experimental design.

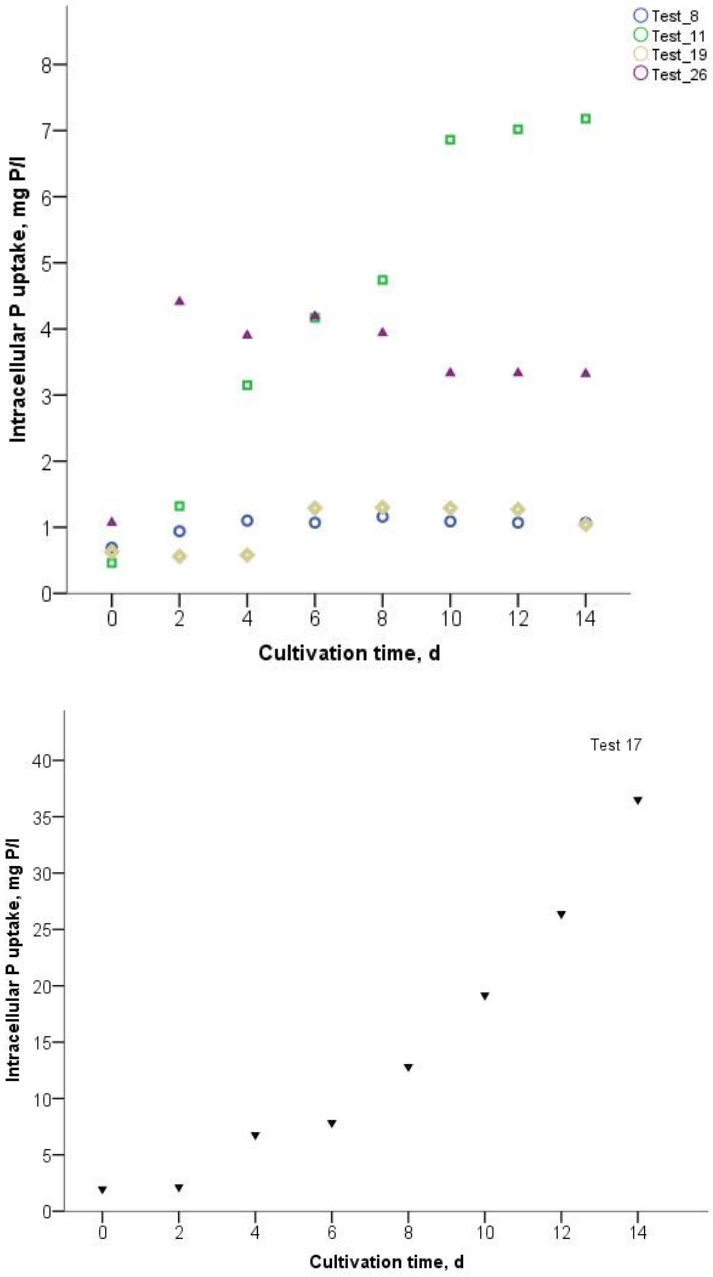


Figure 4-10 Intracellular P in *C.reinhardtii* 11/32C grown under selected environmental conditions with different P media concentrations

4.3.4.2 Effect of N and P concentration, light intensity and photoperiod on algal biomass production

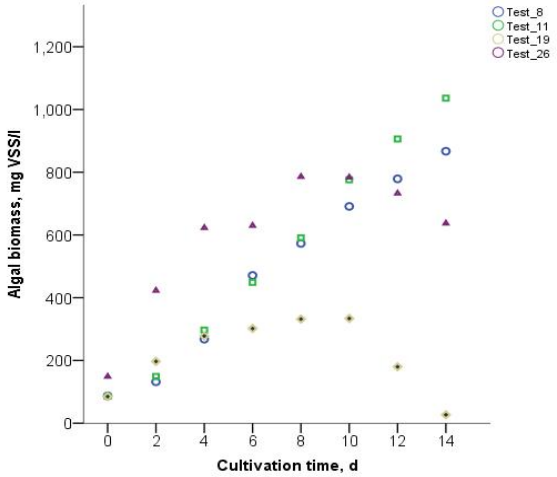
To explain the effect on algal biomass production and N and P assimilation due to tested environmental conditions, five different cultures (test 8, 11, 17, 19 and 26) were

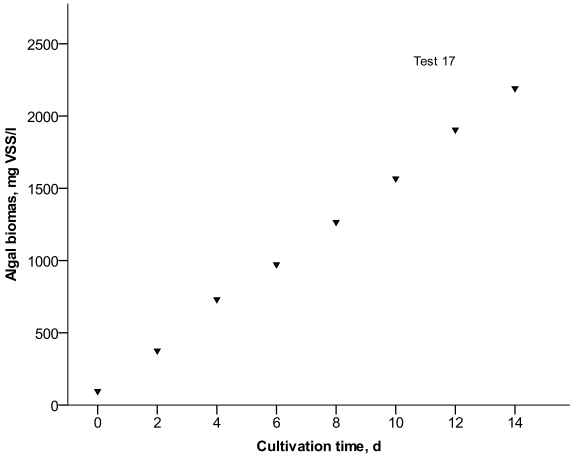
selected to demonstrate experimental findings, see Figure 4.11. The dry algal biomass production in Test 8 and 11 shows a steady increase up to day 10 and then continue to grow at a slower rate until day 14 (Figure 4.11a). The alga culture in test 11 produced a higher amount of biomass than Test 8, as more N was assimilated for algae growth.

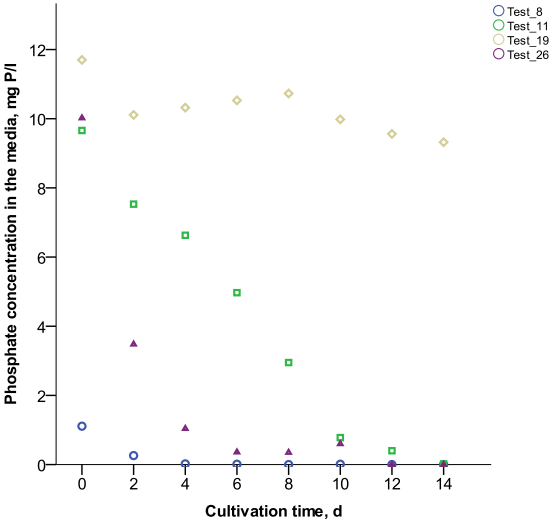
Biomass production in Test 11 was 66 mg VSS $L^{-1}d^{-1}$, while in Test 8 was 59.0 mg VSS $L^{-1}d^{-1}$. Additionally, microalgae cultivated in test 19 and 26 have a similar pattern with biomass production of 16.5 and 44.2 mg VSS $L^{-1}d^{-1}$ respectively (Figure 4.11a). It is because the concentration of nitrogen in Test 19 was limiting algae growth. As a consequence, algae growth declined after day 8 in Test 19. Test 17 revealed to be the most favourable for enhancing algal biomass growth a maximum production of 149 mg VSS $L^{-1}d^{-1}$ (Figure 4.11a).

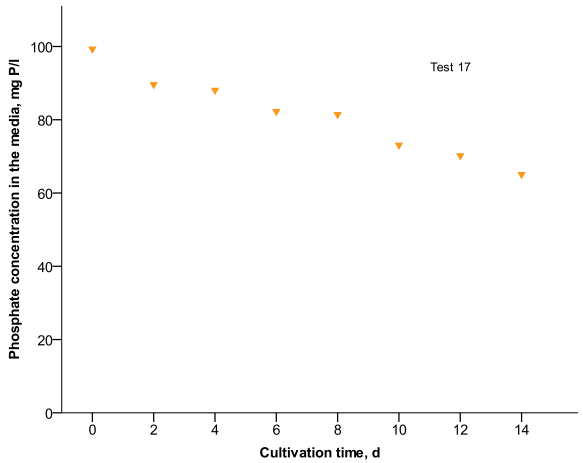
Figure 4.11b presents the profile of remaining P in the culture media for the same set of tests. In Test 8, with the lowest P concentration (1 mg P L^{-1}), P concentration was completely depleted by day 4. Whilst in tests 11 and 26, with 10 mg P L^{-1}, P was completely removed after day 8. Microalgae in these three tests nearly consumed 100% of initial P. Therefore, P was the limiting nutrient controlling biomass productivity. Test 17 had an initial concentration of 100 mg P L^{-1}, resulting in a P consumption of 34% in 14 days; P did not limit algae growth in this experiment.

Figure 4.11 c and d present the profile of N concentration in the culture media for tests 8, 11, 17, 19 and 26. In Test 8, the algae removed only 9% of NO_3^- but 98% of NH_4^+. This result was interesting as microalgae prove to have a higher preference for NH_4^+ over NO_3^-. On the other hand in Test 19, nearly 100% of all supplied N was rapidly consumed and was the nutrient limiting algal growth. Therefore, the algal growth declined after day 8 (Figure 4.11 a). The same pattern was also observed in Test 26. In test 17, the NH_4^+ concentration was rapidly consumed in only 2 days, followed by NO_3^- consumption at similar pace, but P ran out at day 6. That contributed to rapid biomass production during the first 6 days, followed by a decline in biomass concentration after day 8 due to P limiting conditions.


Test_8
Test_11
Test_19
Test_26
Algal biomass, mg VSS/l
1,200
1,000
800
600
400
200
0
0
2
4
6
8
10
12
14
Cultivation time, d


Test 17
Algal biomas, mg VSS/l
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
Cultivation time, d


Test_8
Test_11
Test_19
Test_26
Phosphate concentration in the media, mg P/l
12
10
8
6
4
2
0
0
2
4
6
8
10
12
14
Cultivation time, d


Test 17
Phosphate concentration in the media, mg P/l
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Cultivation time, d

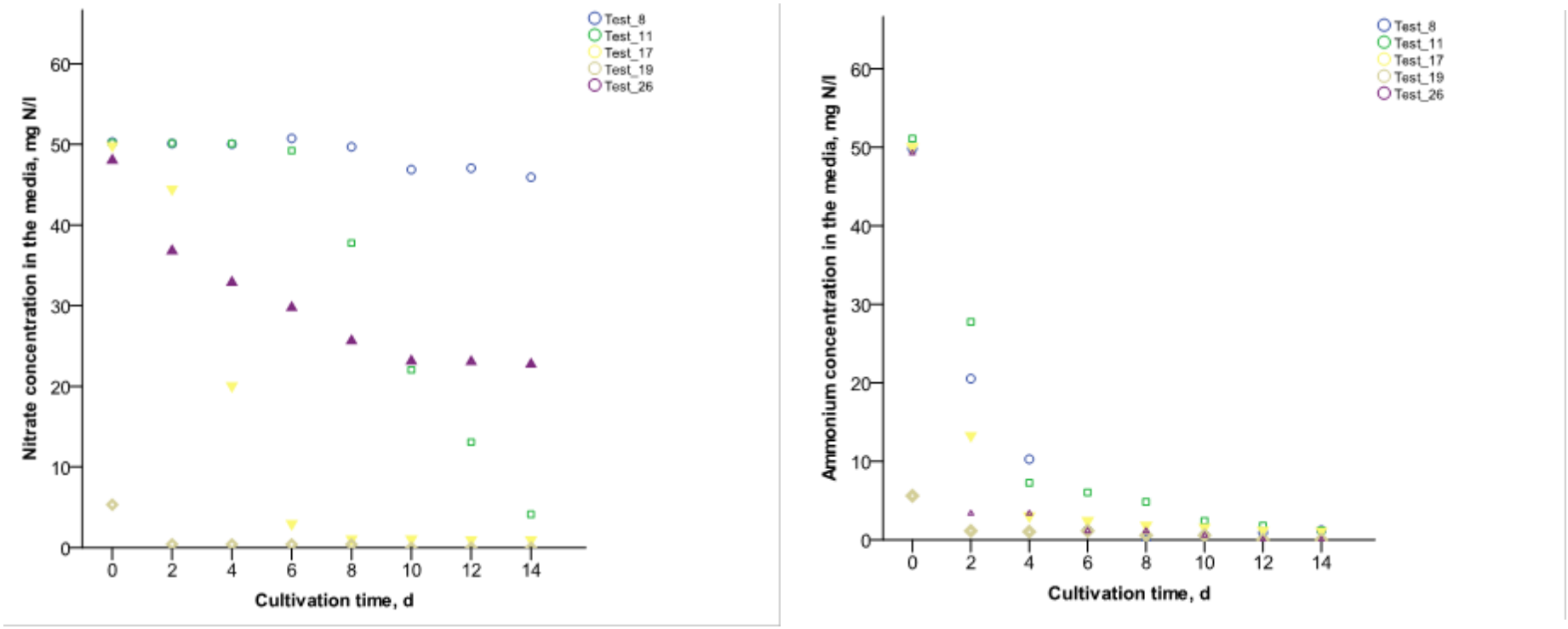


Figure 4-11 The increment of dry algal biomass and the utilisation of N and P in the media

4.3.4.3 **Optimisation of intracellular P uptake**

Optimum intracellular P uptake in *C.reinhardtii* 11/32C was investigated by using the Response Surface Methodology (RSM). Once the optimisation model was fitted from the natural values then the predicted and the residual values were calculated. The following equation (eq. 4.1) represents a second-order response polynomial equation that calculate the predicted intracellular P uptake (Z) as a function of all the variables tested in previous sub-sections (see variables in Table 4-2).

$$Z = 2.3265 - 0.0258X_1 + 0.0076X_2 - 0.6454X_3 + 0.0186X_4 + 0.00006X_1X_2 + 0.0015X_1X_3 + 0.00005X_1X_4 + 0.00115X_2X_3 + 0.000003X_2X_4 - 0.00044X_3X_4 - 0.000022{X_1}^2 - 0.00012{X_2}^2 + 0.0273{X_3}^2 - 0.00003{X_4}^2$$

Eq. 4-1

The variance analysis presented in Table 4.2 helped to determine regression coefficients of each variable and its correlation affecting the objective function. Low *p*-values indicate that the parameter significantly influence the response (Z).

Table 4-2 Regression coefficients for tested environmental variables and *p*-values determined by the fit of the response surface model

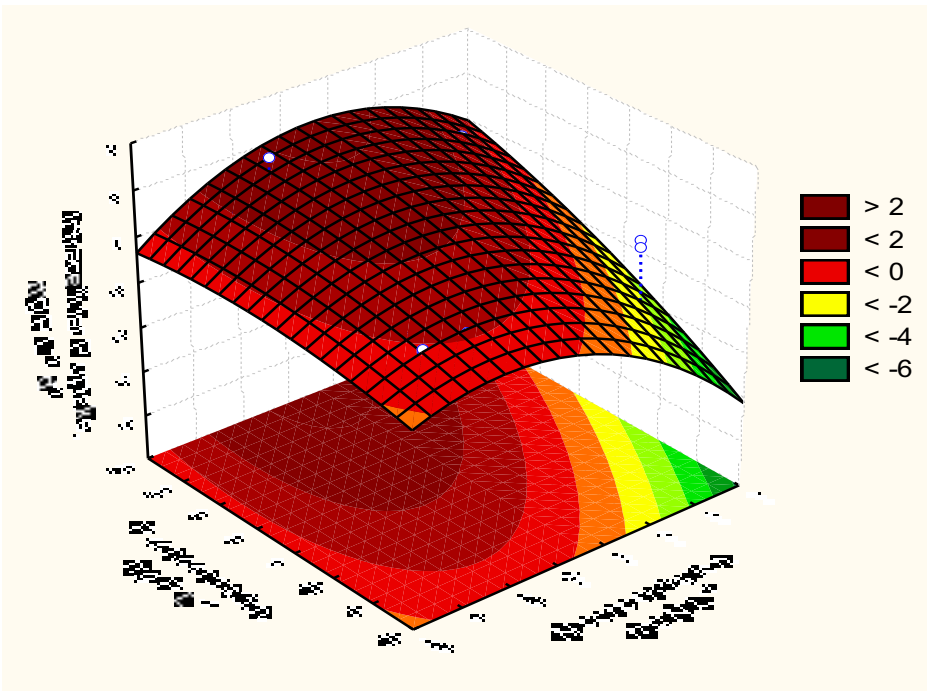
Variable	Regression coefficient	t statistic	*p*-value
β_0	2.32648	2.13127	0.08627
X_1; N	-0.02577	-5.49957	0.00272
X_2; P	0.00758	0.46770	0.65967
X_3; Photo	-0.64538	-3.67106	0.01443
X_4; LI	0.01855	6.68301	0.00113
X_1X_2; *N P*	0.00006	5.14879	0.00362
X_1X_3; *N Photo*	0.00152	6.68329	0.00113
X_1X_4; *N LI*	0.00005	7.98399	0.00049
X_2X_3; *P Photo*	0.00115	3.49246	0.01742
X_2X_4; *P LI*	0.000003	0.30396	0.77340
X_3X_4; *Photo LI*	-0.00044	-3.51030	0.01710
X_1^2; *N N*	-0.00002	-7.36128	0.00073
X_2^2; *P P*	-0.00012	-0.77279	0.47457
X_3^2; *Photo Photo*	0.02726	3.81795	0.01239
X_4^2; *LI LI*	-0.00003	-7.40925	0.00071
R^2	0.98		
R^2 adj	0.92		

where: N: Nitrogen; P: Phosphorus; Photo: Photoperiod; LI: Light Intensity

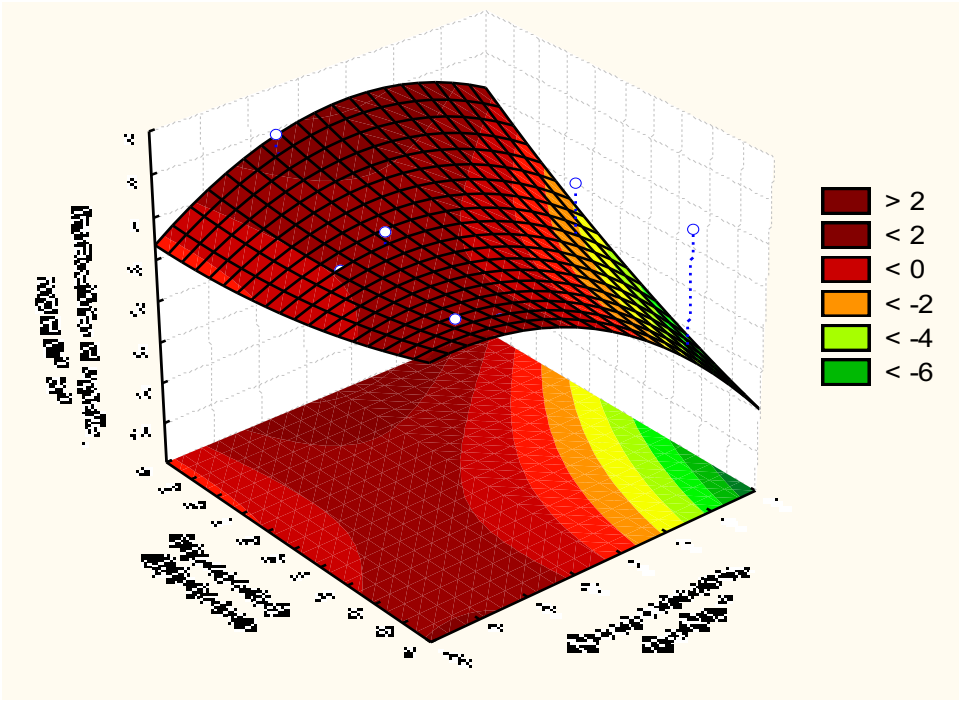
It can be seen that three of the linear coefficients (i.e., nitrogen concentration, photoperiod and light intensity) were statistically significant at $p < 0.05$. This indicates that by increasing the value of these variables, microalgae can stimulate P uptake and store P reserves in their cells. Interestingly, P concentration on its own did not have an effect on increasing the concentration of intracellular P. However, the interaction between P concentration with two other variables (i.e., nitrogen concentration and photoperiod) was statistically significant ($p < 0.05$) and affected intracellular P uptake in *C.reinhardtii* 11/32C, in which the interaction between P and N concentrations was stronger than the interaction between P and photoperiod. Additionally, another interaction between the variables P concentration and photoperiod also revealed a significant effect on intracellular P uptake. The interaction of *N-N, Photo-Photo, LI-LI* were not a real interaction, nevertheless the value of that interaction influenced the quadratic effect on the maximum/optimum response (Martinez et al., 2011).

The response surface plots for intracellular P uptake (Z) in microalga are presented in Figure 4.12. The figures represent a second order polynomial model (Equation 4.1). The surface plots show the significant influence of the interaction among the tested variables. Interestingly, the interaction between N and photoperiod was 15 times higher than P concentration and photoperiod (Table 4.2). This means that N concentration had more influence on increasing intracellular P uptake than P concentration.

The results from the RSM predict that the maximum intracellular P uptake would be achieved under the following environmental conditions: 200 mg N L^{-1}, 120 mg P L^{-1} and 16 hours of light period (Figure 4.12a and b). Furthermore, light intensity showed a wide range of potential values (i.e., 100 - 400 $\mu Em^{-2}s^{-1}$) when interacting with N concentration (Figure 4.12c). A combine effect of light intensity and photoperiod shows a saddle point and a wide range of potential values for photoperiod (i.e., less than 8 hours of light or longer than 16 hours of light). This effect was more evident when light intensity was 250 $\mu Em^{-2}s^{-1}$.



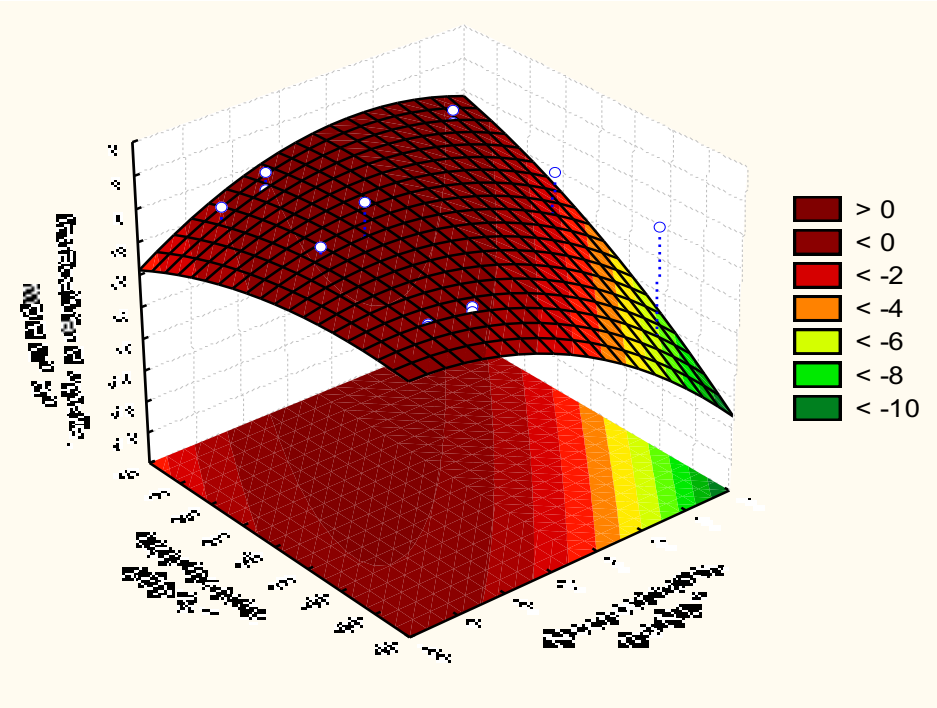
a



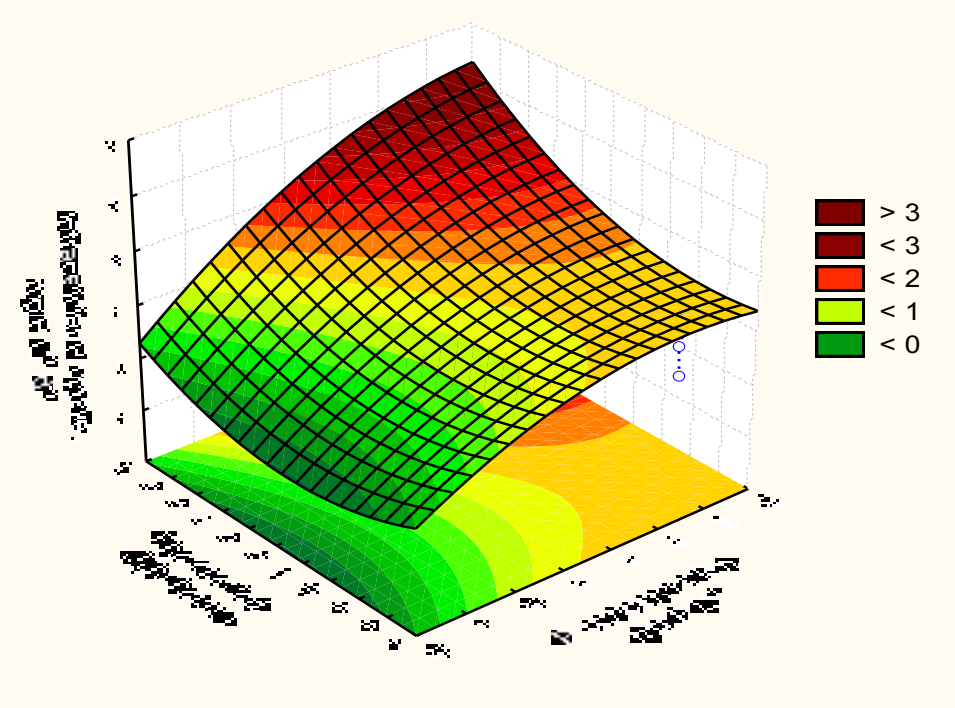
b

Figure 4-12 Response surface for optimising intracellular P uptake.

Key for axis titles: Z=intracellular P uptake, mg P L^{-1} d^{-1}; X=N concentration, mg N L^{-1}; in a), Y=P concentration, mg P L^{-1}; in b), Y=Photoperiod (light hours); in c), Y=Light intensity, $\mu Em^{-2}s^{-1}$; in d), X=P concentration, mg P L^{-1}, Y=Photoperiod (light hours); in e), X=Photoperiod (light hours), Y=Light intensity, $\mu Em^{-2}s^{-1}$



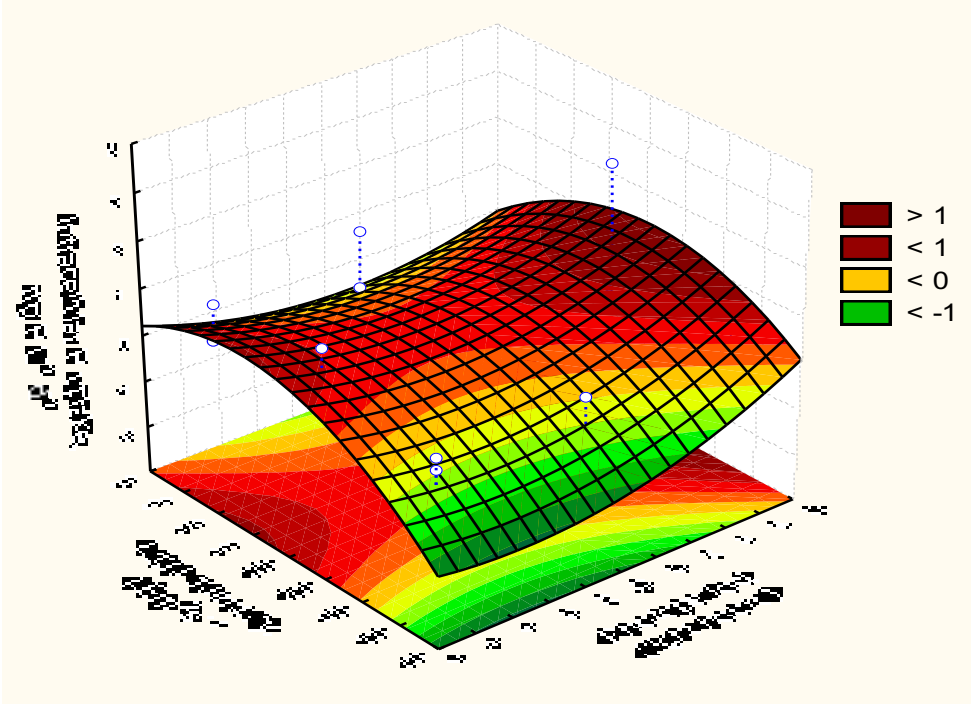
c



d

Figure 4-13 Response surface for optimising intracellular P uptake.

Key for axis titles: Z=intracellular P uptake, mg P L^{-1} d^{-1}; X=N concentration, mg N L^{-1}; in a), Y=P concentration, mg P L^{-1}; in b), Y=Photoperiod (light hours); in c), Y=Light intensity, $\mu Em^{-2}s^{-1}$; in d), X=P concentration, mg P L^{-1}, Y=Photoperiod (light hours); in e), X=Photoperiod (light hours), Y=Light intensity, $\mu Em^{-2}s^{-1}$



e

Figure 4-14 Response surface for optimising intracellular P uptake.

Key for axis titles: Z=intracellular P uptake, mg P L^{-1} d^{-1}; X=N concentration, mg N L^{-1}; in a), Y=P concentration, mg P L^{-1}; in b), Y=Photoperiod (light hours); in c), Y=Light intensity, $\mu Em^{-2}s^{-1}$; in d), X=P concentration, mg P L^{-1}, Y=Photoperiod (light hours); in e), X=Photoperiod (light hours), Y=Light intensity, $\mu Em^{-2}s^{-1}$

4.3.4.4 **Optimisation of algal biomass productivity**

An optimisation test was performed in order to find the optimum variables of the response for algal biomass productivity (Z). Once the optimisation model was fitted from the natural values then the predicted and the residual values were calculated. The following equation (eq. 4.2) represents a second-order response polynomial equation that calculate the predicted biomass productivity (Z) as a function of all the variables tested in previous sub-sections (see variables in Table 4-3).

The following equation elucidates the second-order response polynomial equation using tested environmental variables.

$$Z = -71.208 + 0.052X_1 - 2.510X_2 + 6.293X_3 - 0.739X_4 + 0.0013X_1X_2 + 0.0355X_1X_3 + 0.0015X_1X_4 + 0.058X_2X_3 + 0.0005X_2X_4 + 0.0003X_3X_4 - 0.0018{X_1}^2 + 0.0185{X_2}^2 - 0.314{X_3}^2 - 0.0019{X_4}^2$$

Eq. 4-2

The analysis of variance presented in Table 4.3 determined the regression coefficient of each variables and its correlation which affected the response. The lower *p*-value indicated that the parameter significantly influenced the response.

It can be seen that two of the linear coefficients (i.e., phosphorus and light intensity) were statistically significant at $p < 0.05$. This indicates that the increasing in P concentration and light intensity can stimulate algal growth productivity and generate high quantities of algal biomass. In contrast, N concentration and photoperiod alone did not affect algal biomass productivity. However, the interaction of N concentration and photoperiod with the other variables (i.e., N-P concentration, N-photoperiod, N-light intensity and P concentration-photoperiod) could enhance algal biomass production, which was statistically significant at a 95% confidence level.

Table 4-3 Regression coefficients for tested environmental variables and *p*-values determined by the fit of the response surface model

Variable	Regression Coefficient	t statistic	*p*-value
β_0	-71.2080	-1.9809	0.1187
X_1; N	0.0522	0.3548	0.7407
X_2; P	-2.5100	-4.5722	0.0102
X_3; Photo	6.2931	1.1020	0.3323
X_4; LI	0.7386	7.9591	0.0014
X_1X_2; *N P*	0.0013	3.3408	0.0288
X_1X_3; *N Photo*	0.0355	4.8039	0.0086
X_1X_4; *N LI*	0.0015	7.5780	0.0016
X_2X_3; *P Photo*	0.0577	4.8987	0.0085
X_2X_4; *P LI*	0.0005	1.4120	0.2308
X_3X_4; *Photo LI*	0.0003	0.0579	0.9566
X_1^2; *N N*	-0.0018	-17.6174	0.00006
X_2^2; *P P*	0.0185	3.5777	0.0232
X_3^2; *Photo Photo*	-0.3136	-1.3466	0.2494
X_4^2; *LI LI*	-0.0019	-11.8732	0.0003
R^2	0.99		
R^2 adj	0.96		

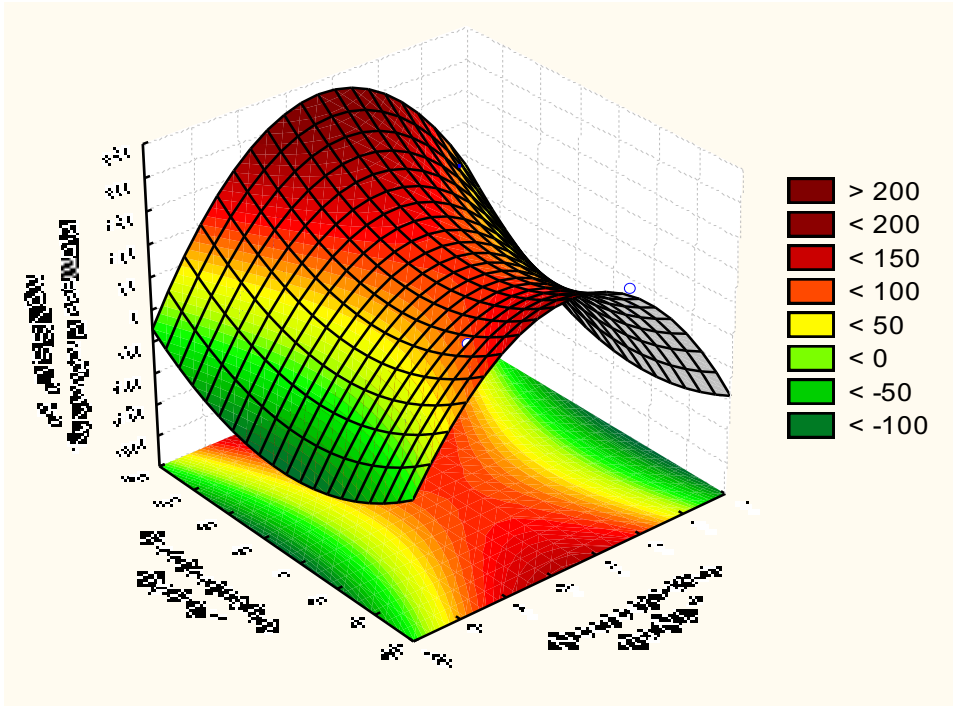
where, N: Nitrogen; P: Phosphorus; Photo: Photoperiod; LI: Light Intensity

Among these interactions, the interaction between N concentration and light intensity had the most significant effect to stimulate a higher algal biomass production. The interactions of *N-N, P-P, LI-LI* were not a real interaction, nevertheless the value of that interaction influence the quadratic effect on the response (Martinez et al., 2011).

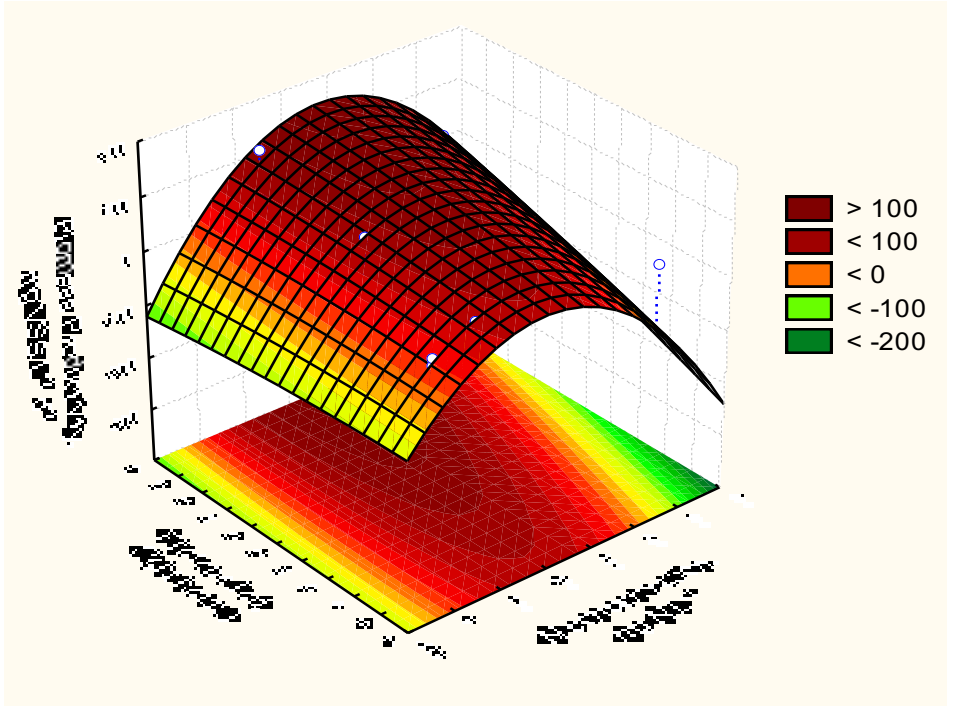
The response surface plots of algae biomass productivity are presented in Figure 4.13. The figures reported there represent a second order polynomial model (Equation 4.2).

The surface plots show the significant influence of the interaction between N and P concentrations (Figure 4.13a); N and photoperiod (Figure 4.13b); and N and light intensity (Figure 4.13c), on enhancing algal biomass productivity. The surface response plots described that the maximum biomass productivity would be achieved under the following environmental conditions: light duration longer than 16 hours, light intensity between 200 and 350 $\mu Em^{-2}s^{-1}$, P concentration higher than 100 mg P L^{-1} and N concentration between 200 and 300 mg N L^{-1}. In addition to these conditions, N concentration has the most important effect to shape the optimum point of algal

biomass productivity (Figure 4.13c). On the other hand, although P concentration shows a quadratic effect on the response, its interaction with photoperiod contributed a little bit towards the nature of the responses in which the highest biomass productivity would be reached at P concentration and photoperiod higher than 100 mg P L^{-1} and 18 hours respectively.



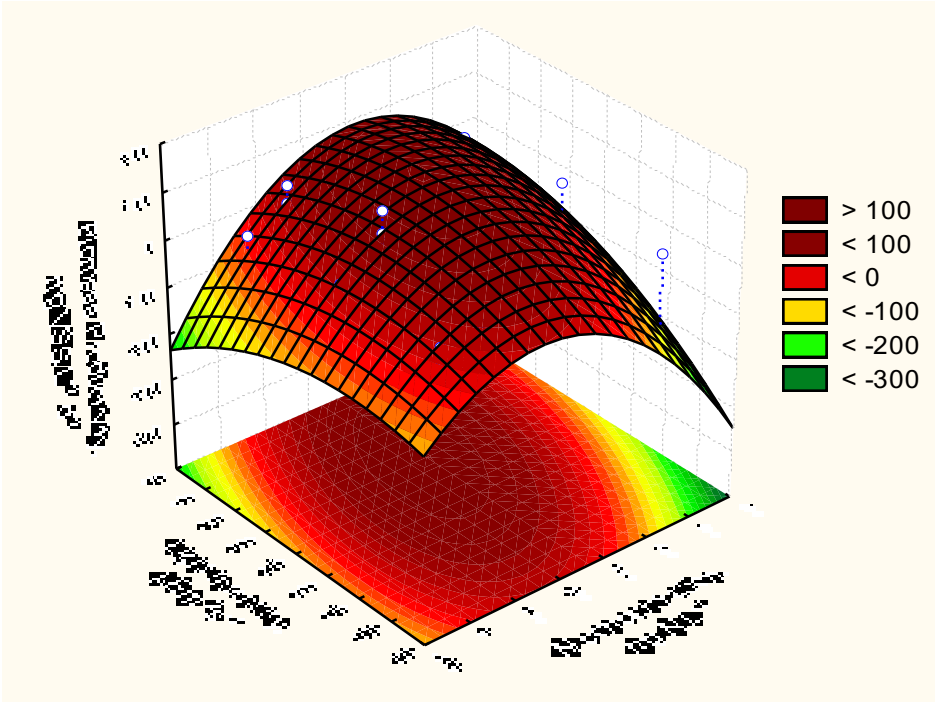
a



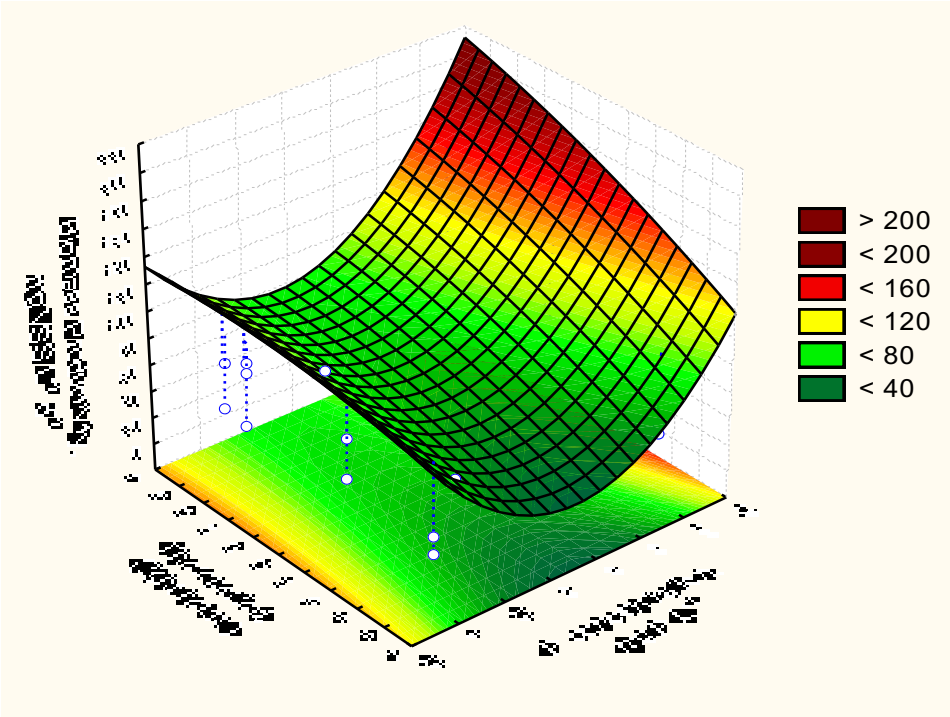
b

Figure 4-15 Response surface for optimisation algal biomass productivity.

Key for axis titles: Z=Algal biomass productivity, mg VSS L^{-1} d^{-1}; X=N concentration, mg N L^{-1}; in a), Y=P concentration, mg P L^{-1}; in b), Y=Photoperiod (light hours); in c), Y=Light intensity, µ$Em^{-2}s^{-1}$; in d), X=P concentration, mg P L^{-1}, Y=Photoperiod (light hours)



c



d

Figure 4-16 Response surface for optimisation algal biomass productivity.

Key for axis titles: Z=Algal biomass productivity, mg VSS L^{-1} d^{-1}; X=N concentration, mg N L^{-1}; in a), Y=P concentration, mg P L^{-1}; in b), Y=Photoperiod (light hours); in c), Y=Light intensity, $\mu Em^{-2}s^{-1}$; in d), X=P concentration, mg P L^{-1}, Y=Photoperiod (light hours)

4.3.4.5 Validation of optimum environmental factors for intracellular P uptake and algal biomass productivity

The predicted optimum intracellular P uptake and algal biomass productivity were verified by running some additional experiments in the lab in triplicate under the optimum conditions predicted from the RSM analysis. The optimum environmental conditions to maximise the two responses (i.e., intracellular P uptake and algal biomass production) in the algae cultures were conducted according to the following conditions: N concentration = 200 mg N L^{-1}; P concentration = 80, 100, 120 mg P L^{-1}; photoperiod= 16 hours light:8 hours dark; light intensity = 250 $\mu Em^{-2}s^{-1}$ (Table 4.4).

The validation of optimum conditions experiment was focus on the influence of P concentration in enhancing intracellular P uptake and biomass production and therefore, three different P concentration were chosen and only one concentration or condition for the other variables.

Table 4-4 Optimum conditions from Response Surface Methodology results

Variables	Intracellular P uptake	Biomass productivity	Selected conditions for validation experiment
N (mg N L^{-1})	200	200-300	200
P (mg P L^{-1})	120	>100	80, 100, 120
Photoperiod (light:dark)	16:8	>16:8	16:8
Light Intensity $\mu Em^{-2}s^{-1}$	100-400	200-300	250

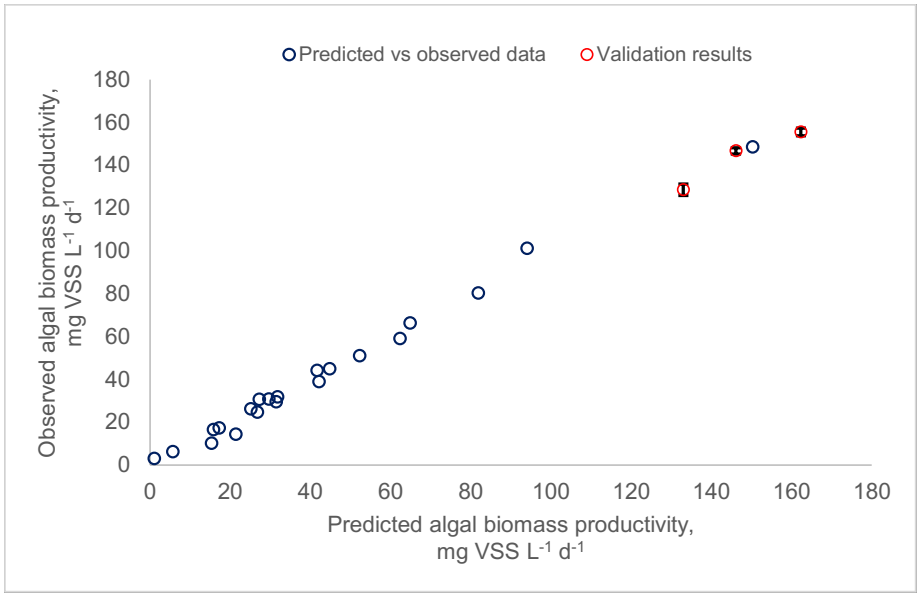
Results from the validation of the optimisation response for intracellular P uptake and algal biomass production are listed in Table 4.5.

Table 4-5 Validation results of intracellular P uptake and algal biomass productivity*

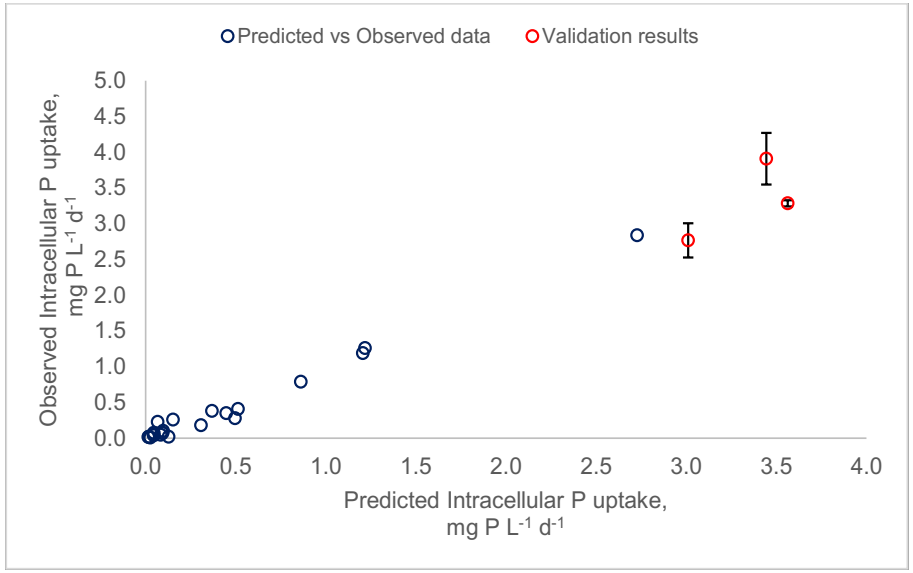
P concentration, mg P L^{-1}	Intracellular P uptake, mg P $L^{-1}d^{-1}$	Biomass productivity, mg VSS $L^{-1}d^{-1}$
80	3.29 ± 0.04	128.59 ± 2.66
100	3.91 ± 0.36	146.72 ± 1.50
120	2.77 ± 0.24	155.57 ± 1.81

* N concentration = 200 mg N L^{-1}; photoperiod= 16 hours light:8 hours dark; light intensity = 250 $\mu Em^{-2}s^{-1}$

The average figures for intracellular P uptake and algal biomass production rates obtained from the additional tests were 3.32 mg P $L^{-1}d^{-1}$ and 143.63 mg VSS $L^{-1}d^{-1}$ respectively. The results of the validation tests were plotted against the predicted and the observed results (Figure 4.14). That showed validation results to be close to the optimised figures predicted by the model, which were 2.84 mg P $L^{-1}d^{-1}$ and 148 mg VSS $L^{-1}d^{-1}$ respectively for intracellular P uptake and algal biomass production rates. The validation results were shown by red circle with the error bars. These conveyed that the model was very good to represent the optimisation experiment in order to obtain the best environmental conditions for algae cultivation in the nutrient-rich conditions.



a. Algal biomass productivity



b. Intracellular P uptake

Figure 4-17 Predicted vs observed results for optimum algal biomass production and intracellular P uptake

In addition, under the optimum conditions, the total phosphorus content of algae under the three tested P concentrations were 1.8, 2.0, and 1.6% for 80, 100, and 120 mg P L^{-1} respectively. The performance of luxury P uptake was confirmed by the presence of polyphosphate granules inside algae cells (Figure 4.15).

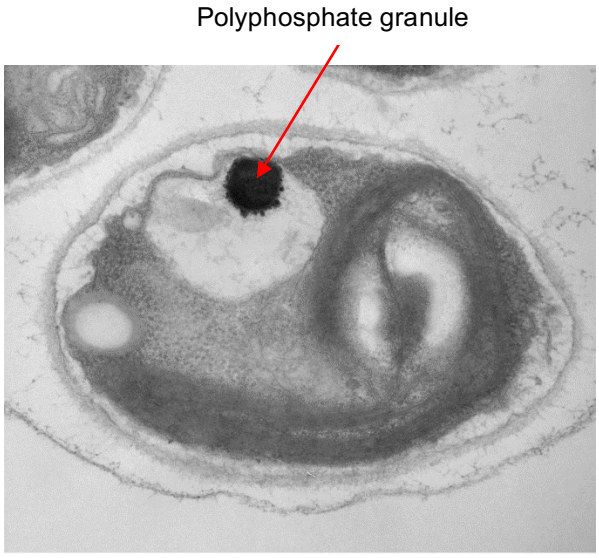


Figure 4-18 Polyphosphate granules in *C.reinhardtii* 11/32C cultivated under optimum environmental conditions

4.4 Discussion

4.4.1 Algal growth and nutrient uptake under favourable standard conditions

The aim of this study was to investigate the ability of *C.reinhardtii* 11/32C to grow and recover nutrients (i.e., N and P) in a standard nutrient media like BBM. Under tested conditions in tubular PBRs, *C.reinhardtii* 11/32C revealed its ability to recover N and P from a synthetic growth media. Under the favourable environment in which 83% of N-NO_3^- and 22% of P-PO_4^{3-} was removed from the media, *C.reinhardtii* 11/32C stored 4.7% and 1.3% for N and P respectively. This ability to remove nutrients was better than results reported also with *Chlamydomonas reinhardtii* grew in an Erlenmeyer flask using synthetic wastewater, which was reported to remove 42-55% N and 12-13% P (Kong et al., 2010a).

The use of NO_3^- as a sole nitrogen source in preliminary tests shown that *C.reinhardtii* 11/32C are able to assimilate nitrate and not only ammonium. The mechanisms for nitrate metabolisms include the reduction of NO_3^- into NH_4^+, which is assisted by nitrate and nitrite reductase (Cai et al., 2013). This means that *C.reinhardtii* 11/32C have enough energy to perform the reduction process in their cells. Furthermore, these findings also proved that *C.reinhardtii* 11/32C can uptake nutrients from its surrounding environment and to stored reserves at over 1% of P dry weight in their cells. This reserve of P is stored inside the cells as polyphosphate granules (Eixler et al., 2006); in this study, luxurious P uptake was confirmed to took place under non-stress environmental conditions (i.e., no limiting growth conditions).

4.4.2 Effect of different inorganic nitrogen sources on P uptake and algal growth

This study aimed to identify a favourable N sources for luxury P uptake and algal biomass production using *C.reinhardtii* 11/32C as a model organism. In photosynthetic process, the utilisation of HCO_3^- by microalgae will result in releasing hydroxyl ions ($HCO_3^- \rightarrow CO_2 + OH^-$) (Lohman et al., 2015). As a typical response to inorganic carbon assimilation by microalgae, the pH increases as photosynthetic activity progresses (Li et al., 2010b; Sawyer et al., 2003). Furthermore, in regard to the present of total ammonia in aqueous solution, the equilibrium between NH_4^+ and NH_3 is strongly pH dependent (Korner et al., 2001). The shift of NH_4^+ to NH_3 occurs due to changes in pH above 8, which displaces the equilibrium from NH_4^+ to NH_3 (Sawyer et al., 2003). The uncharged NH_3 is most toxic due to the fact that this form more soluble and readily transfer through the cell membranes than NH_4^+ (Korner et al., 2001). It is also well studied that NH_4^+ inhibits microalgal growth at concentration higher than 50 mg N L^{-1} (Ip et al., 1982) or even at 28 mg N L^{-1} (Tam and Wong, 1996), due to toxic effects caused by unionised ammonia (NH_3) (Kallqvist and Svenson, 2003).

Since NH_3 is a gas, it is potentially volatile and can be lost to the atmosphere. However, in general at the pH above 8 only 10% of total ammonia is present as a toxic forms of NH_3 and 90% as NH_4^+ (Collos and Harrison, 2014). This indicates that more N in forms of NH_4^+ can be assimilated by microalgae for their growth. The preference

of NH_4^+ over the other nitrogen sources also conveyed in Figure 4.5 that shows 97% of NH_4^+ utilised by *C.reinhardtii* 11/32C. Valero and Mara (2007) reported that NH_3 volatilisation was not the most important mechanism on N removal process, because the NH_3 and total N were removed mostly through microalgal biological uptake. They also confirmed that the removal rate of NH_4^+ is faster than the rate of NH_3 volatilisation (Valero, 2008).

Measurements of pH values during this study confirmed that the pH of the algae culture was as high as 9.6 when NH_4^+ was used as a sole nitrogen source. When other N sources were used, including a mix of NO_3^- and NH_4^+ and NO_3^-, the recorded pH was 9.8 and 9.7, respectively. As a consequence, the algal growth rate and biomass production of *C.reinhardtii* 11/32C in NH_4^+ was lower than in combination of NO_3^- and NH_4^+ or NO_3^-. Similar effects were also observed in *Chlorella sorokiniana* (Kim et al., 2013) and S*cenedesmus bijugatus* (Arumugam et al., 2013).

In addition, it was demonstrated that *C.reinhardtii* 11/32C preferred NH_4^+ over NO_3^- in which NH_4^+ removal was higher than NO_3^-. Consequently, this preference for ammonium at the concentration levels of the tests conducted, did not prove to have a major inhibitory effect on algal growth. In addition to different nitrogen sources, P uptake was better by microalgae when a combination of NO_3^- and NH_4^+ was used. It might be caused by the stress effect of changing N source from ammonia to nitrate from the media. In contrast, P content in the algae grown in NH_4^+ only was also higher than the figures found when the culture media contained only NO_3^-. It is because of the fact that the algae using NH_4^+ produced less biomass than when using NO_3^- and it may influence higher amounts of P content in algal cells.

Interestingly, P luxury uptake was also observed in *C.reinhardtii* 11/32C cultivated in the media containing both NO_3^- and NH_4^+. The resulting content of P found in the harvested algal biomass was 2.1% and it is higher than the typical amount of P reported in microalgae (≈ 1% P). Hence, the combination of NO_3^- and NH_4^+ has proved to be the best N source for enhancing algal growth and luxury P uptake in *C.reinhardtii* 11/32C.

4.4.3 Identification of optimum environmental conditions for intracellular P uptake

This study aimed to identify optimum environmental conditions for maximising intracellular P uptake and algal biomass production. The observed results demonstrated that intracellular P uptake was affected by the interaction between external P concentrations and other environmental factors (i.e., N, light intensity, photoperiod, etc.). An external P concentration of 100 mg L^{-1} was found to have an important influence on intracellular P uptake in *C.reinhardtii* 11/32C. The influence of increasing P media concentration on specific P uptake rates and in-cell polyphosphate accumulation in *Scenedesmus obliquu*s and on luxury P uptake in *Chlorella* sp., have been previously studied and concluded that increasing P media concentration stimulates algae to store more P reserves in their cells (Martinez et al., 1999) (Powell et al., 2008) (Zhu et al., 2015a). Additionally, low intracellular P uptake was observed under P-starving conditions (i.e., external P concentration of 1 mg P l^{-1}), which coincided with previous studies reporting that very low intracellular P concentrations (0.185% P) were obtained in microalgae cultures under P-limited conditions (Markou, 2012).

Considering the interaction with N, it was observed that low N concentrations (i.e., 10 mg N L^{-1}) triggered algal cells to store P reserves up to 3.6% in-cell P and that led to luxurious P uptake (Powell et al., 2009). It is predicted that an insufficient N concentration in the medium will lead to utilise more P in order to fulfil nutrient requirements and P storage; in fact, it is possible to reach P saturation when microalgae is cultured under N-limiting conditions (Zhu et al., 2015a). The influence of N availability for enhancing P removal from a synthetic medium has been reported to be significantly important (Beuckels et al., 2015).

In addition to nutrient availability, time exposure to light influence P removal rates. Results shown that by increasing the number of light hours, higher intracellular P uptake is stimulated in *C.reinhardtii* 11/32C. Moreover, (Goncalves et al., 2014) proved that P removal in *Chlorella vulgaris* may increase by increasing light exposure times.

4.4.4 Identification of optimum environmental conditions for algal biomass production

Results showed that algal biomass production was affected by N concentration and the resulting interactions with other variables (i.e., P concentration, light intensity and photoperiod). As an essential constituent for algal cell metabolisms and structure, N may represent up to 20% of dry algal cell weight (Juneja et al., 2013), which is firstly incorporated into cell tissue as amino acid glutamine (Cai et al., 2013). Nitrogen supplementation on algal biomass production has been reported for *Scenedesmus bijugatus* (Arumugam et al., 2013), *Chlorella pyrenoidosa* (Nigam et al., 2011), and *Monoraphidium* sp. SB2 (Wu et al., 2013). The results of the present study show that N is an important nutrient that promotes algal growth and help increasing biomass production. The results indicated that the medium level of N concentration tested (i.e., 100 mg N l^{-1}) created favourable conditions for maximising algal biomass production to a higher level when compared with 10 and 500 mg N L^{-1}.

N limitation may result in an unbalance of enzymes at cell level that can lead to discolouration of algal cells due to the decrease of chlorophyll synthesis and the increase carotenoid production (Grobbelaar, 2007; Juneja et al., 2013) and therefore, algal growth and biomass production may be reduced. The results of the study reported here are similar to those found in another study using different green microalgae species (*Chlorella vulgaris*) cultivated in culture media containing 10 mg N-NH_3 L^{-1}, where similar biomass production and P intake rates were reported (Tam and Wong, 1996).

Furthermore, a high N concentration of 500 mg L^{-1} (250 mg N-NH_4^+ L^{-1} and 250 mg N-NO_3 L^{-1}) inhibited algal growth (Kallqvist and Svenson, 2003; Larsdotter, 2006a) and may have resulted in cessation and toxicity in algal cells and therefore declining of cells growth (Li et al., 2008a). That may support the fact that N-rich waste streams like digested liquor are very likely to inhibit microalgae growth and hence, dilution with final effluent would be the most feasible option to implement nutrient recovery via biological algal uptake from that waste stream.

The influence of P concentration and its interaction with other variables (i.e., light intensity and photoperiod) on algal biomass production was found to be statistically significant at $p < 0.05$. The contribution of P on algal biomass productivity has been reported for *Chlorella* sp. (Zhu et al., 2015a) and *Scenedesmus obliquus* (Martinez et al., 1999) in which increasing P concentration stimulated the increase of algal biomass. This study observed so far that the highest intracellular P uptake rate was in Test 17 (Table 4.1) that helps to recover up to 35 mg P L^{-1} over a period of 14 days. This amount of intracellular P uptake rate generated from the different between the initial and on day 14 of P concentration at 100 mg P L^{-1} and approximately 65 mg P L^{-1} respectively. Under certain stress conditions, net phosphate uptake would be affected although intracellular P content will remain high and therefore, P content in algal biomass can range from 1 to 4%. This luxury P uptake was observed in tests 4, 10, 17 and 23 (Table 4.1), along with an increase in algal biomass production. These were achieved when the *C.reinhardtii* 11/32C cultured in the highest P concentration of 100 mg P L^{-1} and therefore, this outcome revealed that high P concentration contributes to enhance intracellular P uptake and algal biomass in *C.reinhardtii* 11/32C.

Light is an important energy source needed to support photosynthesis processes in microalgae. *Chlamydomonas reinhardtii* requires a light intensity regime from 200 to 400 $\mu Em^{-2}s^{-1}$ (Elizabeth, 2008). This study showed that light intensity makes a marked influence on algal biomass productivity and it was found that for the systems tested the most favourable light intensity was 250 $\mu Em^{-2}s^{-1}$.

4.4.5 Validation of predicted optimum intracellular P uptake and algal biomass productivity

Interesting results were observed when microalgae were cultivated under the predicted optimised environmental conditions (N concentration of 200 mg N L^{-1}, P concentration of 100 mg P L^{-1}, photoperiod of 16-hr light : 8-hr dark, and light intensity of 250 $\mu Em^{-2}s^{-1}$), which resulted in the highest intracellular P uptake and algal biomass production rates. That treatment conditions generated luxury P uptake with a percentage of in-cell P of about 3%. The validation results confirmed that the predicted

variable values, which were generated from optimum points for enhancing intracellular P uptake and algal biomass production, were correct and that the predicted models (equation 4.1 and 4.2) are a fair representation of the expected responses of independent variables under study.

4.5 Conclusions

In the present study, a combination of NO_3^- and NH_4^+ for algal growth was found to be the most appropriate nitrogen source, which produced higher intracellular P uptake and algal biomass productivity. Consequently, this nitrogen combination used as a sole nitrogen sources for further tests using *C.reinhardtii* 11/32C.

In addition, the optimisation tests proved that N concentration of 200 mg N L^{-1}, P concentration of 100 mg P L^{-1}, photoperiod of 16 hours light:8 hours dark, and light intensity=250 $\mu Em^{-2}s^{-1}$ generated the highest intracellular P uptake of 4 mg P $L^{-1}d^{-1}$ with average P biomass of 2% and algal biomass productivity of 147 mg VSS $L^{-1}d^{-1}$. It is shown that *C.reinhardtii* 11/32C were able to perform luxurious P uptake and can grow under nutrient-rich conditions. Based on the optimum conditions for *C.reinhardtii* 11/32C cultivation, the algae will feasible to grow in digested liquor and use the energy from the CHP for maintaining the light period and the intensity needed by the algae.

These results confirm that microalgae could be used for the integration of wastewater bioremediation and biomass feedstock production. Microalgae are able to take up high amounts of nutrients from wastewaters and also generate biomass with high content of P, which is suitable for the production of bio-fertiliser from waste streams.

Chapter 5 Exploring the presence of hyper P accumulators

5.1 Introduction

Phosphorus is an essential substance that play an important biological role in cellular metabolic process that produce functional components for cell growth and synthesis (Hu, 2007). Typically, P constitutes 1% dry-weight of algal biomass (Borchardt and Azad, 1968; Eixler et al., 2006), but under favourable growth conditions microalgae incorporate P into the cells above their typical level (>1% P), which is commonly known as luxury P uptake (Borchardt and Azad, 1968; Powell et al., 2008). The excess of P taken up by microalgae is stored inside their cells as polyphosphate granules (Powell et al., 2008; Eixler et al., 2006). The main purpose of polyphosphate is to store energy and phosphorus for vital algal cell functions (Kornberg, 1995).

The natural ability that microalgae have for storing P in their cells has the potential of being exploited for P recovery and remediation of wastewaters, which will bring additional opportunities for processing the resulting harvested algal biomass (Sivakumar et al., 2012). The ability of microalga to take up P and store it as polyphosphate has been previously identified and studied in *Chlamydomonas reinhardtii* at microcosms (Leitao et al., 1995; Ruiz et al., 2001a; Werner et al., 2007), and this study will further examine their ability to store P as polyphosphate granules in their cells by using *C.reinhardtii* 11/32C as a model organism.

Under the assumption that among a given algae population and regardless the environmental conditions used for cultivation, there would be some individuals able to accumulate higher portions of P and store it as polyphosphate granules in their cells (hyper P accumulating organisms), this Chapter explores the possibility to isolate such microorganisms from an alga culture. This approach is novel as there is no previous attempts reported in the literature aimed at isolating hyper P accumulators from *C.reinhardtii* 11/32C and exploring their potential use for P recovery from wastewater. In order to identify the presence of hyper P accumulators, the presence and location of polyphosphate granules were examined using fluorescence and electron microscopy techniques. Furthermore, these methods were followed by flow cytometry

and cell sorting analysis to isolate the identified hyper P accumulating organisms in a culture of *C.reinhardtii* 11/32C.

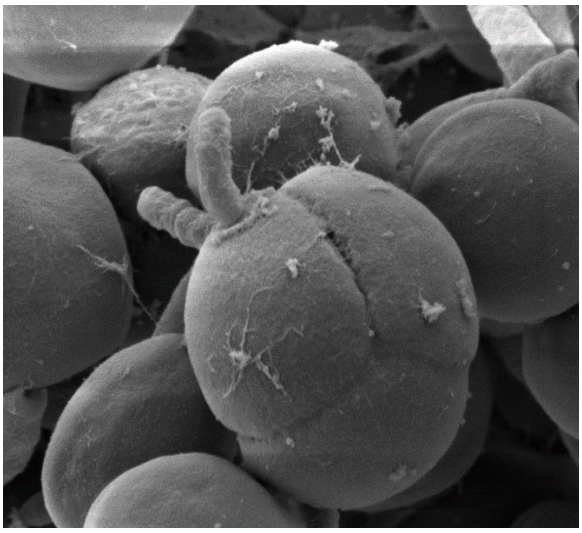
5.2 Methodology

This study is aimed to isolate the individual cells of *C.reinhardtii* 11/32C which have high content of P in the form of polyphosphate granules. It is assumed that hyper P accumulators naturally appear and therefore, this section describes the methods for identifying the presence of such organisms in alga cultures. In order to observe the cell surface morphology of *C.reinhardtii* 11/32C, scanning electron microscopy (SEM) was conducted at the School of Molecular and Cell Biology, Faculty of Biological Sciences, University of Leeds, followed by the developing and testing lab protocols for polyphosphate identification and cell sorting. For instance, fluorescence microscopy was used to verify the potential to use DAPI staining for identifying polyphosphate granules in algal cells; and transmission electron microscopy (TEM) was used to examine in detail cell organelles and to localise the presence of polyphosphate granules. As a result, fluorescence microscopy and TEM were used in conjunction in this study to confirm the presence of polyphosphate in the algal cells before conducting flow cytometry and cell sorting tests to isolate the identified hyper P accumulators in *C.reinhardtii* 11/32C.

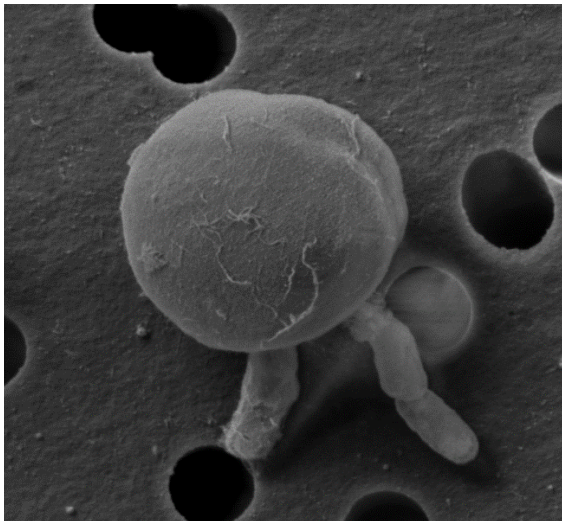
5.3 Results

5.3.1 Cell morphology of C.reinhardtii 11/32C

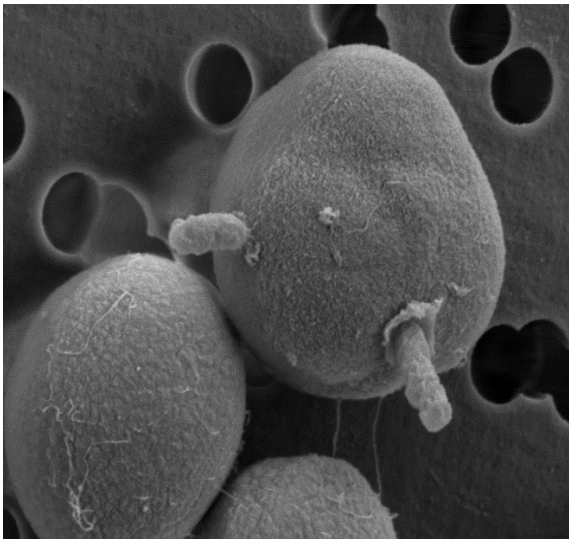
Figure 5.1 shows the morphology of *C.reinhardtii* 11/32C examined by SEM. In terms of their cell architecture, this particular alga strain has an ellipsoidal shape, two anterior flagella of equal length (10 - 12μm) (Harris, 2001; Nishikawa and Tominaga, 2001), and a thick cell wall (pointed by a red arrow in Figure 5.1 d). The flagella are located on the cell surface and stuck on the basal body (Figure 5.1 c). The cell uses the flagella for their motility and for cell-cell recognition during their mating process under nitrogen starvation (Silflow and Lefebvre, 2001).



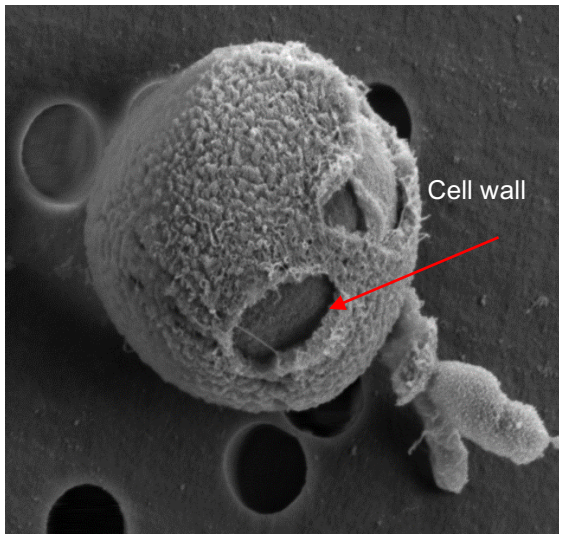
a. The cell will split up



b. A single cell



c. The flagella on the cell surface



d. The cell wall

Figure 4-19 Scanning electron microscope images of *C.reinhardtii* 11/32C

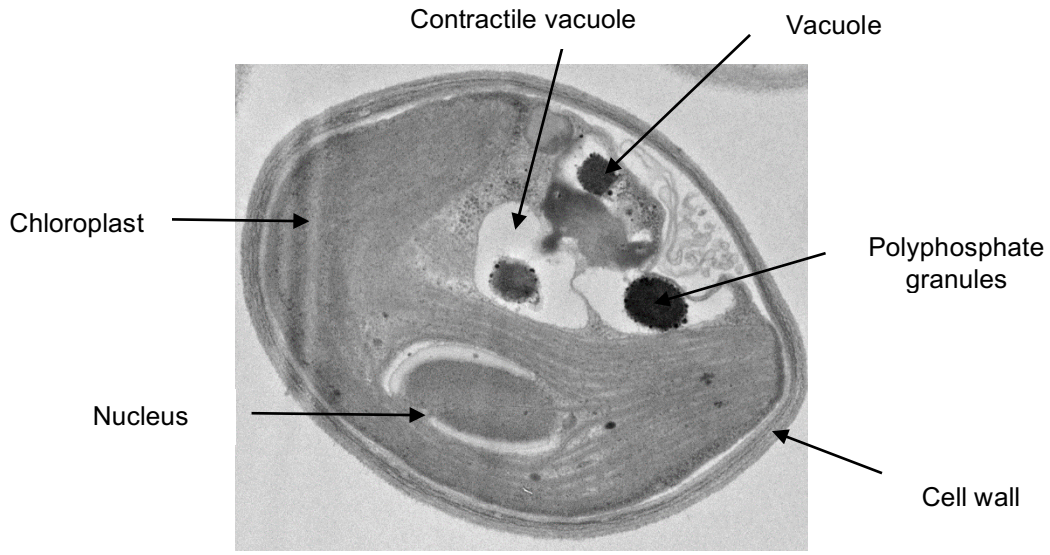


Figure 4-20 *C.reinhardtii* 11/32C cell geometry examined by using TEM microscopy

The maximum diameter of *C.reinhardtii* cells is about 10 μm, but its size varies through the cell cycle (Harris, 2001). The major cell wall component contains hydroxyproline-rich glycoprotein of high molecular mass (Harris, 2001; Komine et al., 2000). The cell wall in *C.reinhardtii* 11/32C is thick as it contains chains of proteins; in fact, Harris (2001) noted that their cell wall does not contain cellulose at all.

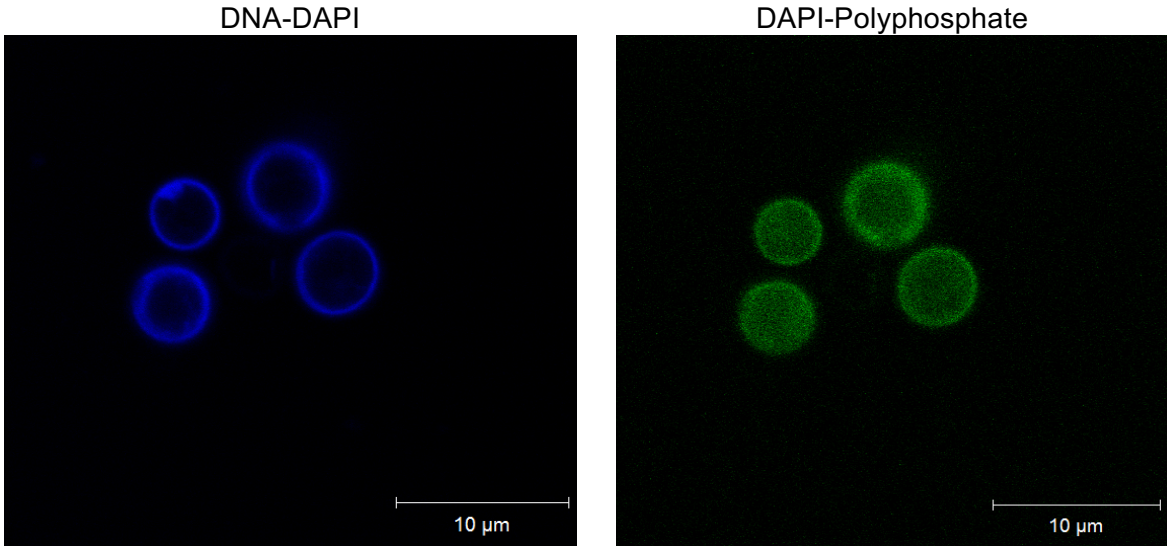
A single cup-shape chloroplast in *C.reinhardtii* occupies approximately two thirds of the total cellular volume and partially surrounds the nucleus (Figure 5.2) (Harris, 2001; Stauber and Hippler, 2004). The chloroplast play an important role on algal growth, because photosynthesis processes and the other essential biochemical reactions occurred in the chloroplast (Stauber and Hippler, 2004).

Most vacuoles are located near Golgi apparatus and contain electron-opaque material. The vacuole functions as a secretory vesicles which detect the extrusion of granules from the cells (Komine et al., 2000). Previous studies reported that in-cell production of volutin granules contained polyphosphate, which confirmed that other polyphosphate bodies are also located in the vacuole (Komine et al., 2000; Kornberg, 1995; Nishikawa et al., 2003; Ruiz et al., 2001a).

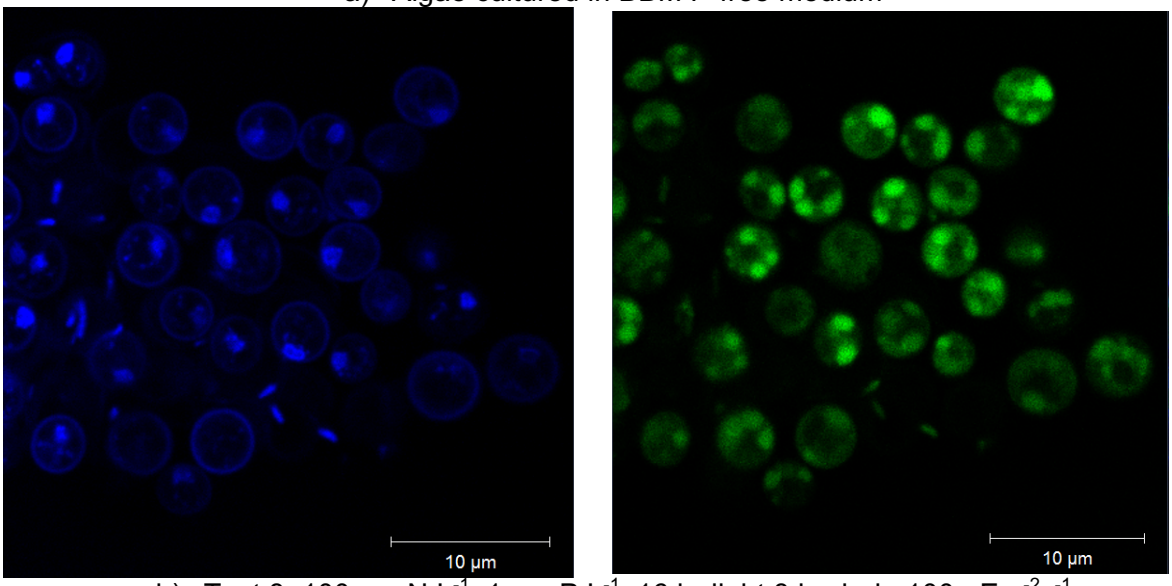
5.3.2 Observation of polyphosphate granules using fluorescence microscopy

Microalgae can accumulate phosphate and store it as polyphosphate granules under phosphorus-replete medium conditions (Qu et al., 2008). In order to observe the presence of polyphosphate granules in algal cells, a method was developed in the lab using DAPI staining techniques followed by visualisation with a confocal fluorescence microscope. DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) is a popular nuclear and chromosome counterstain, which emits blue fluorescence upon binding to AT regions of DNA; although the dye usually does not pass through cell walls, high concentrations will enter a live cell and can be used as a dye to detect polyphosphate granules (Ruiz et al., 2001a). In this study, DAPI was used to detect the presence of in-cell polyphosphate granules, but was necessary to correct all experiments by any interference caused by DNA-DAPI bounds. Therefore, DAPI used for DNA detection was excited at 405 nm and the emitted fluorescence was detected at 420-480 nm; DAPI for DNA detection emits their maximum fluorescence at 450 nm and shows as blue fluorescence (Zhu et al., 2015b). DAPI used for polyphosphate detection in *C.reinhardtii* 11/32C was excited at 405 nm and the emitted fluorescence was detected using a 535-590 nm band pass (BP) emission filter. DAPI-polyphosphate binding shows as green-yellow fluorescence (Gomes et al., 2013; Gomez-Garcia et al., 2013; Nishikawa et al., 2006).

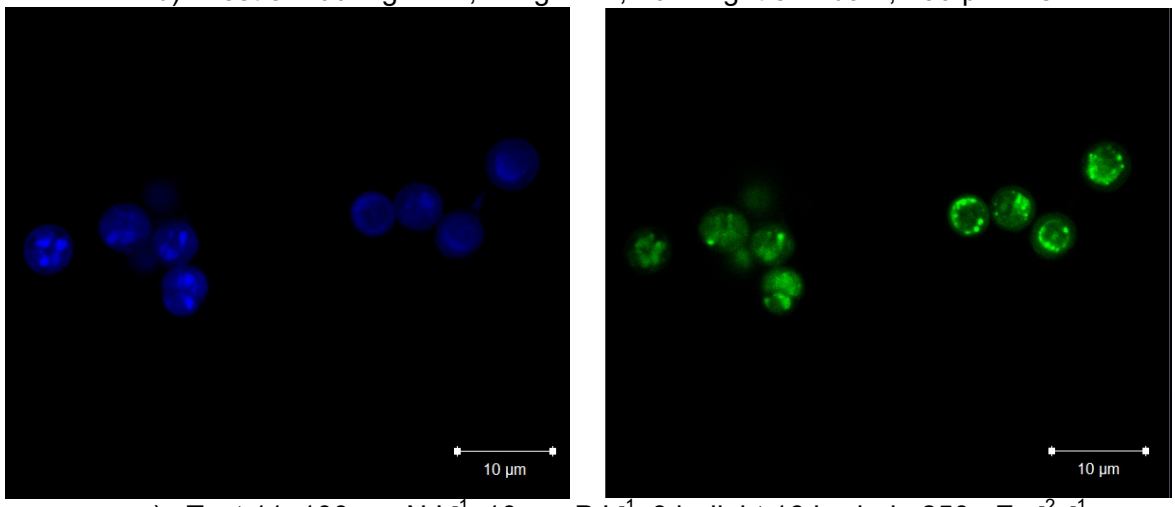
Fluorescence microscopic images detecting polyphosphate granules in *C.reinhardtii* 11/32C grown under a different culture conditions are shown in Figure 5.3. The five samples were selected randomly according to the lowest and the highest intracellular P uptake refer to the Table 4.1. These samples elucidated the presence of polyphosphate granules with the lowest up to the highest uptake of intracellular P. Furthermore, the control algae were grown in standard BBM (Section 3.1.2) without additional of P and the environmental growing conditions was following the CCAP protocol which describe in Section 3.1.1.



a) Algae cultured in BBM P-free medium

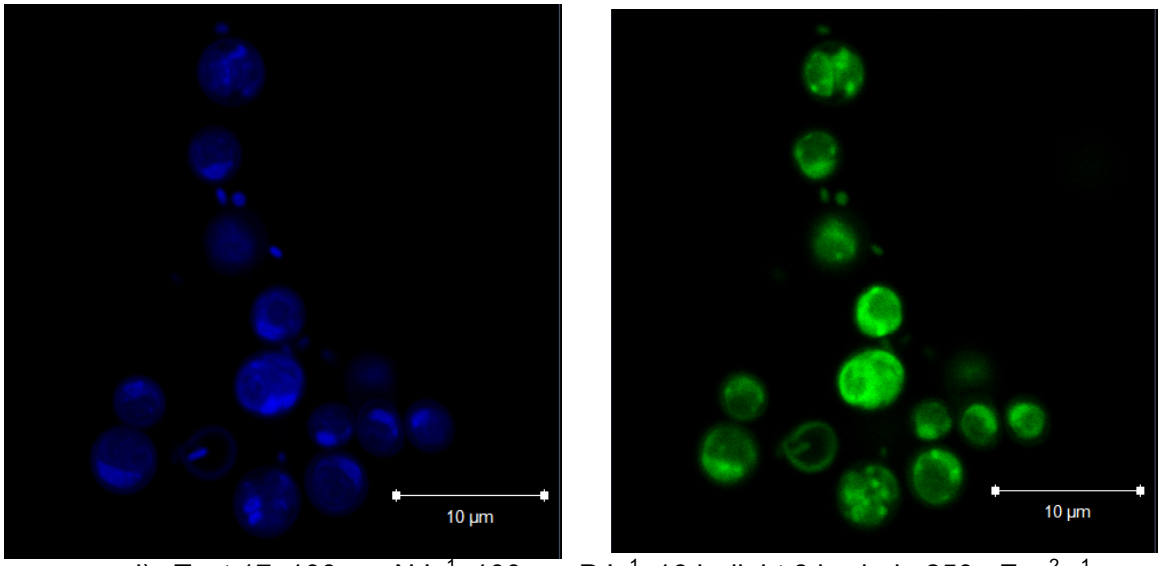


b) Test 8: 100 mg N L^{-1}; 1 mg P L^{-1}; 16 hr light:8 hr dark; 100 $\mu Em^{-2}s^{-1}$

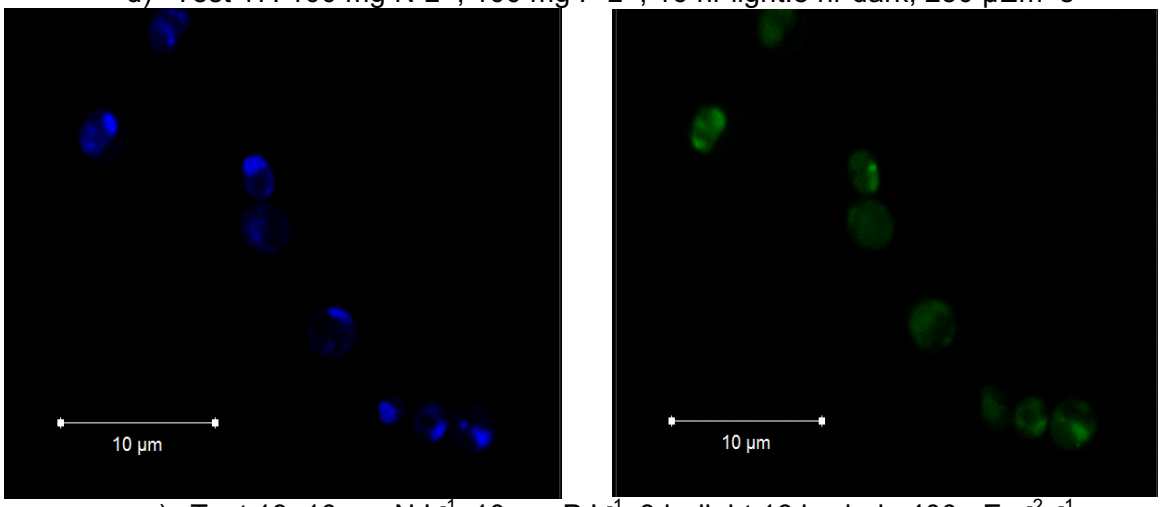


c) Test 11: 100 mg N L^{-1}; 10 mg P L^{-1}; 8 hr light:16 hr dark; 250 $\mu Em^{-2}s^{-1}$

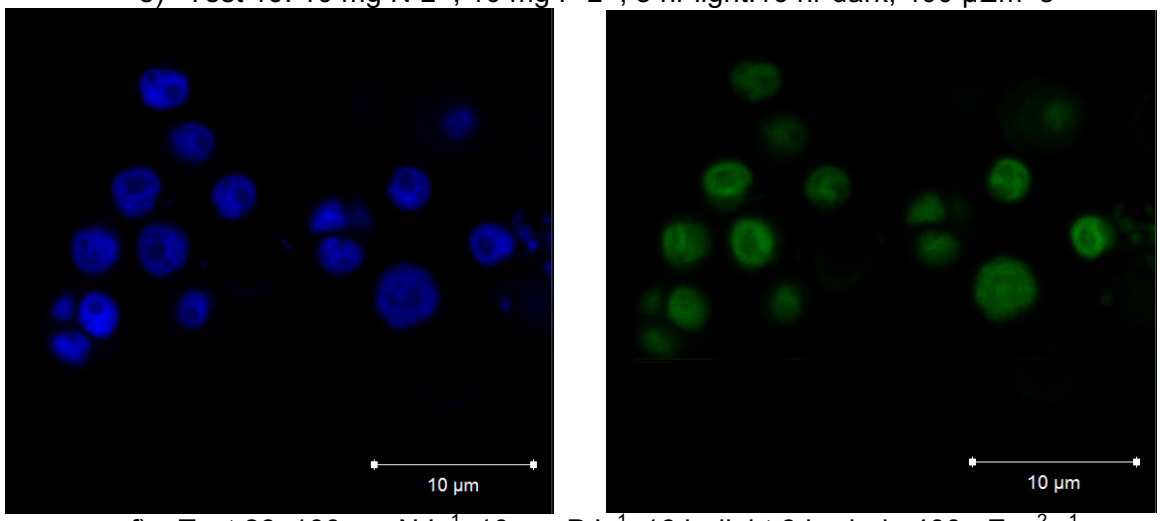
Figure 5-3 Observation of polyphosphate in *C.reinhardtii* 11/32C under different culture conditions (BBM (a) and Test 8, 11, 17, 19 and 26 (b – f) according to Table 4.1



d) Test 17: 100 mg N L^{-1}; 100 mg P L^{-1}; 16 hr light:8 hr dark; 250 µ$Em^{-2}s^{-1}$



e) Test 19: 10 mg N L^{-1}; 10 mg P L^{-1}; 8 hr light:16 hr dark; 400 µ$Em^{-2}s^{-1}$



f) Test 26: 100 mg N L^{-1}; 10 mg P L^{-1}; 16 hr light:8 hr dark; 400 µ$Em^{-2}s^{-1}$

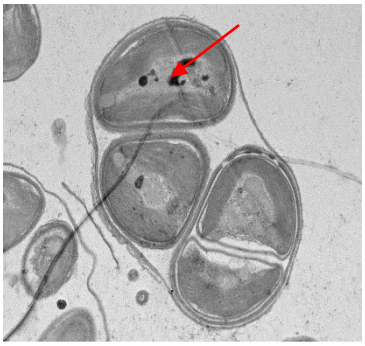
Figure 4-21 Observation of polyphosphate in *C.reinhardtii* 11/32C under different culture conditions (BBM (a) and Test 8, 11, 17, 19 and 26 (b – f) according to Table 4.1

Fluorescence microscopy images revealed that *C.reinhardtii* 11/32C assimilated the phosphate from the media and stored it as polyphosphate granules in their cells, as the control using P-free BBM media did not have in-cell DAPI-polyphosphate granules, but only adsorbed poly-P (Figure 5.3a). Figure 5.3b and c show sufficient polyphosphate spots in the cells, which are in line with the reported removal rate of phosphate from the medium (99%). However, the quantity of intracellular P in the biomass was only 0.3% and 0.8% for the microalgae shown in Figure 5.3b and c respectively (Test 8 and 11)

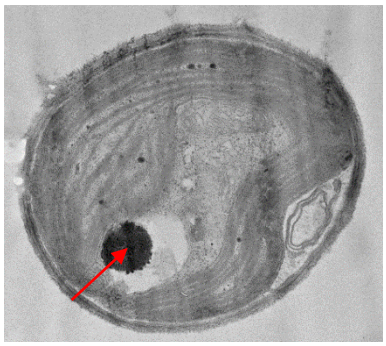
The algae grown in a P-replete medium (100 mg P L^{-1}) assimilated a higher amount of phosphate (remaining P concentration = 65 mg P L^{-1}) and accumulated it as polyphosphate granules (Figure 5.1d), which indicates the ability that *C.reinhardtii* 11/32C have to convert excess amounts of assimilated P into polyphosphate granules. Luxurious P uptake was observed and reported in Chapter 4 (in-cell P = 2%) and confirmed by using DAPI staining and fluorescence microscopy. Similar findings of luxurious P uptake has been reported for *Chlorella* (Zhu et al., 2015b). In contrast, algal cells shown in Figure 5.1e removed only 20% of soluble phosphate from the media and there were only able to accumulate very little in-cell P as shown from very few tiny spots of DAPI-polyphosphate detected inside their cells.

5.3.3 Localisation of polyphosphate granules

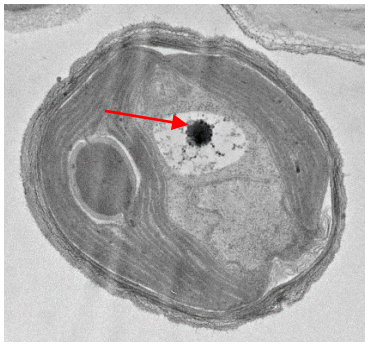
In *Chlamydomonas* species, the accumulation of phosphate in excess (i.e., luxury P uptake) is stored as polyphosphate and it is detected by electron-dense bodies together with calcium compounds (Siderius et al., 1996). The presence of in-cell polyphosphate granules was verified in this study by using electron microscopy (TEM). When electron microscopy is used, the polyphosphate bodies of microalgae are characterised by their high-dense electron density (Ruiz et al., 2001a). In this study, lead citrate was used to stain in-cell polyphosphate granules, in order to enhance scattering properties of biological components under the electron microscope. Lead citrate forms stable complexes as lead salts and it is also able to form bounds with biological tissue such as pyrophosphate groups (Reynolds, 1963).



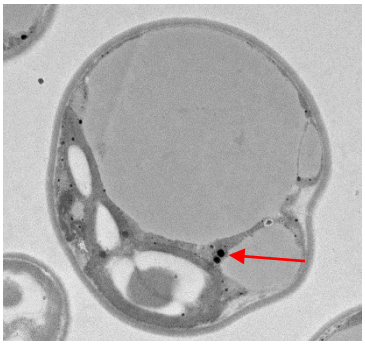
Test 8 (0.3% polyP)



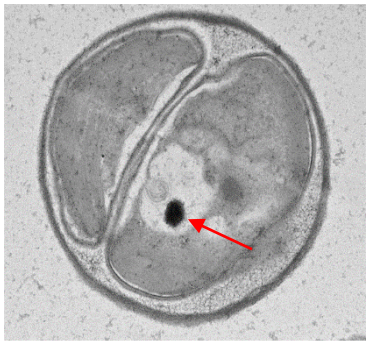
Test 17 (1.3% polyP)



Test 11 (0.83% polyP)



Test 19 (0.4% polyP)



Test 26 (0.68% polyP)

Figure 4-22 Location of polyphosphate granules in *C. reinhardtii* 11/32C by using electron microscopy (TEM)

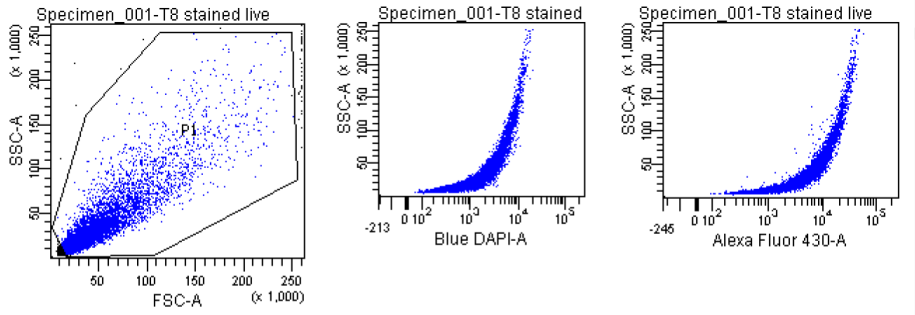
Figure 5.4 shows electron microscope images of polyphosphate granules identified in *C.reinhardtii* 11/32C, which was grown in different environmental conditions as described in Chapter 4. It can be seen from that figure the different sizes of polyphosphate granules. It was predicted that the size and number of polyphosphate granules depend on the total amount of intracellular P, which is dependent on P concentration in the medium (Acevedo et al., 2012; Docampo, 2006). Among the five different culture conditions reported in Figure 5.4, the minimum and the maximum intracellular P were 0.3% and 1.3% for tests 8 and 17, respectively. The lowest in-cell polyphosphate content was observed by very few tiny black dots (test 8); whilst the highest one was characterised by very large polyphosphate granules (test 17) (Figure

5.4). The largest polyphosphate granules confirms the ability that *C.reinhadrtii* 11/32C has to perform luxurious P uptake.

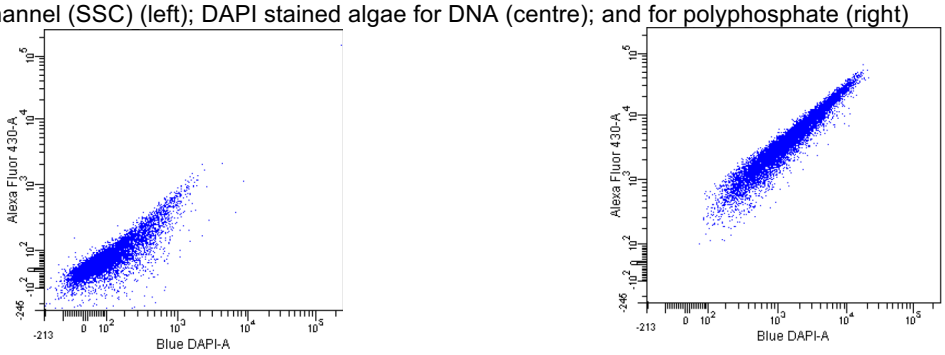
5.3.4 Flow cytometry and cell sorting analysis for hyper P accumulators

This experiment was conducted to isolate hyper P accumulating organisms present in a culture of *C.reinhardtii* 11/32C. The two different fluorescence of DNA and polyphosphate used 405 nm as the excitation. Because of the way the cytometer was set up it had to use two similar profiles to look at the small difference in emission. The Alexa 430 channel had a slightly a wider emission profile than the DAPI from which the green light (although still the blue along with it) can be seen more clearly. In this way a small shift in the Alexa-430 channel that indicates poly-P has identified. Due to the fluorescence emission were so close together this is only an indication and therefore, the flow cytometry and the confocal data have to be shown together to convincingly the presence of polyphosphate.

Alive algal cells from cultures conducted under two different environmental conditions were used for flow cytometry. The two selected alga samples coincided with the lowest (Test 8) and the highest (Test 17) content of intracellular P found in previous tests conducted in this study. The methods used for DAPI staining, flow cytometry and further cell sorting analysis were conducted as described in Section 3.2.3.

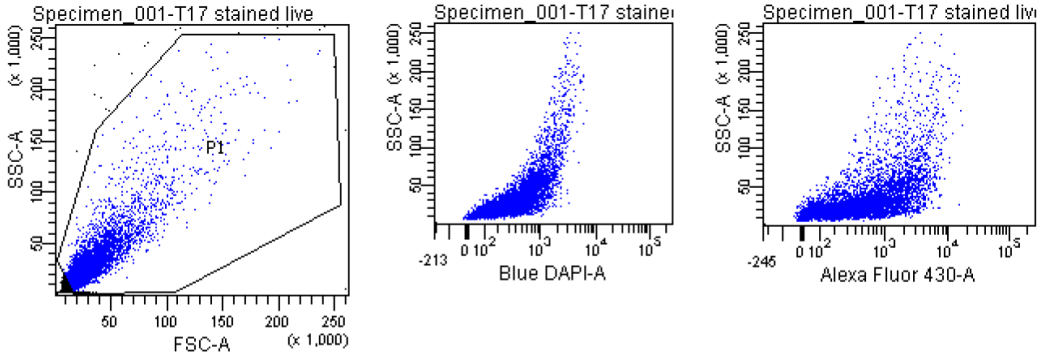


a. Algae (T_8) examined for the size by forward scatter channel (FSC) and the granular by side scatter channel (SSC) (left); DAPI stained algae for DNA (centre); and for polyphosphate (right)

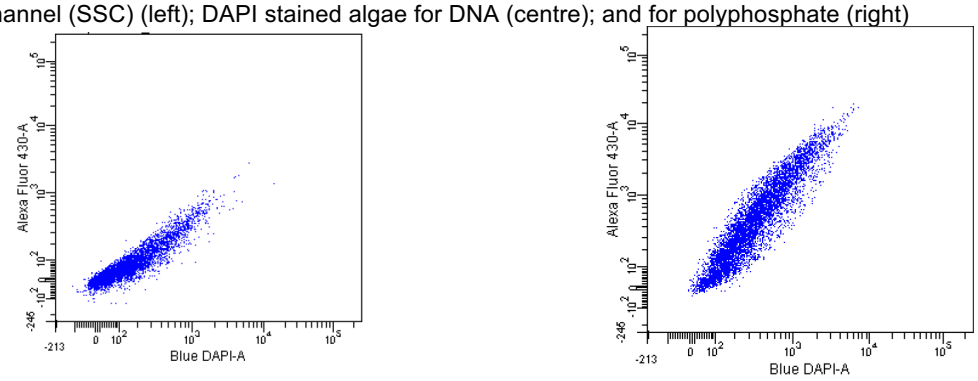


b. Unstained T_8 algae (left) and Stained T_8 algae (right)

Figure 4-23 Flow cytometry and cell sorting analysis for *C.reinhardtii* 11/32C cultivated in Tests 8



a. Algae (T_{17}) examined for the size by forward scatter channel (FSC) and the granular by side scatter channel (SSC) (left); DAPI stained algae for DNA (centre); and for polyphosphate (right)



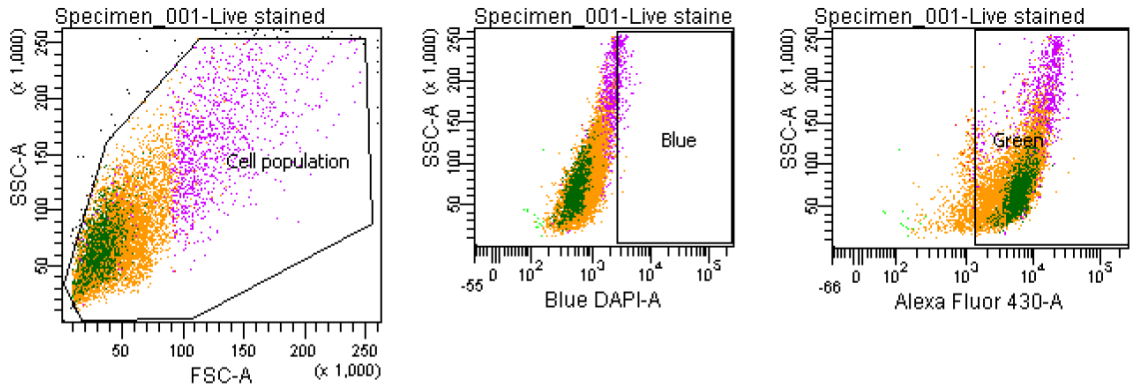
b. Unstained T_{17} algae (left) and Stained T_{17} algae (right)

Figure 4-24 Flow cytometry and cell sorting analysis for *C.reinhardtii* 11/32C cultivated in Tests 17

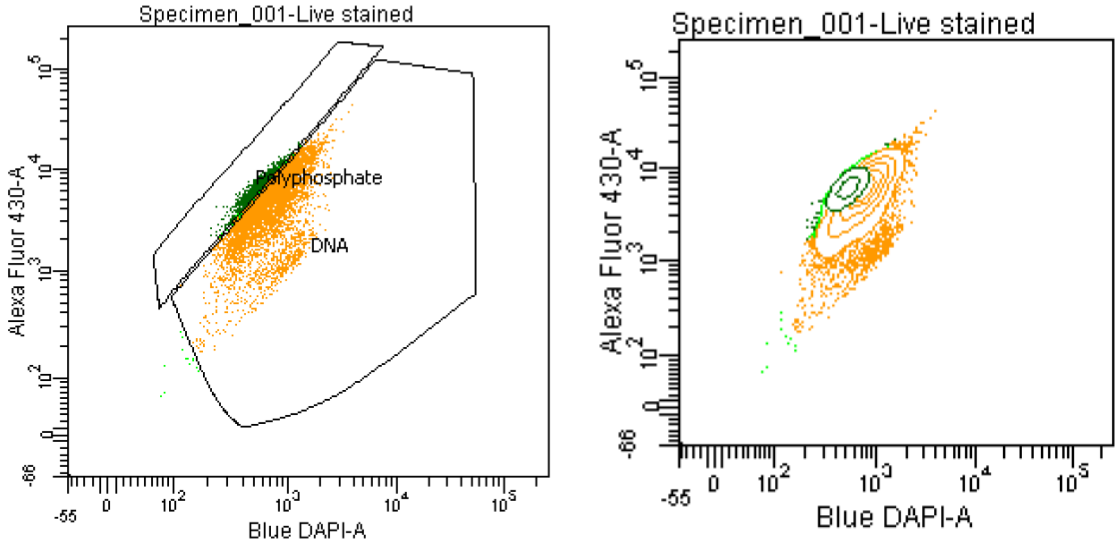
Flow cytometry analysis for two algae samples (i.e., Test 8 and Test 17) is reported in Figure 5.5 and 5.6. It was found that there was no major different between the sample containing DAPI stained algae for DNA-DAPI or DAPI-polyphosphate in Test 8 (Figure 5.5a). As a result, it was not possible to make a clear distinction between DAPI stained cells marked for DNA (Blue DAPI-A) and for polyphosphate (Alexa Fluor 430-A), as both groups appeared in the same scatter plot (Figure 5.5b). This scatter plot represented 88% of the total population of algae in the sample in which generated from histogram statistics. However, it was not possible to identify in which portion of that population the algae stained for DNA or polyphosphate were located, because they did not split significantly under the conditions set for the flow cytometry analysis.

Similar results were obtained from algae samples in Test 17, where no significant difference between the algae stained for DNA and polyphosphate was found; the 65% population of the algae in the scatter plot was representative of DNA and polyphosphate stained algae (Figure 5.6a). Although, there was a different pattern for this sample, as the algae stained for polyphosphate went further in the x-axis compared to the algae stained for DNA (Figure 5.6b).

Based on that, it was assumed that the individual samples may contain a very low number of hyper P accumulators (individual cells able to take up P at a higher rate than the average found in each sample) and for that reason, another experiment was conducted to explore the presence of hyper P accumulating organisms by mixing the two original samples. The algae cultured in Test 8 and Test 17 were mixed in one single sample, which were used for flow cytometry and cell sorting analysis. The results of that experiment are shown in Figure 5.7.



a. Mixed algae (T_{17} and T_8) examined for the size by forward scatter channel (FSC) and the granular by side scatter channel (SSC) (left); DAPI stained algae for DNA (centre); and for polyphosphate (right)



b. Cells gating analysis

DNA Polyphosphate Debris materials

Figure 4-25 Flow cytometry and cell sorting for a mixture of microalgae containing high and low levels of in-cell P

It is clearly presented in Figure 5.6a that mixed algae between the lowest and the highest intracellular P uptake show difference pattern for the algae stained-DNA and the algae stained-polyphosphate. The algae stained for DNA have fluorescence emitted between 10^2 and 10^3, whilst for polyphosphate was between 10^3 and 10^4.

When the individual results are overlaid (Figure 5.7b), it is possible to identify the benefits of this technique, considering that as a result 10.2% of the algae stained for polyphosphate identification were successfully isolated from the whole population which is clearly marked in green colour, Figure 5.7b.

5.4 Discussion

5.4.1 Observation of polyphosphate granules

The aim of this section was to observe the presence of polyphosphate granules in a culture of *C.reinhardtii* 11/32C. Polyphosphate is a polymer that plays an important biological role for energy storage and cell protection in both prokaryotes and eukaryotes (Aschar-Sobbi et al., 2008; Gomes et al., 2013; Nishikawa et al., 2003). Within alga cells, phosphate reacts with adenosine diphosphate (ADP) and absorbs energy in the form of adenosine triphosphate (ATP). In such conditions, the excess energy stored as ATP will be transferred into condensed phosphate granules in the form of polyphosphate (Borchardt and Azad, 1968; Cai et al., 2013; Kulaev and Kulakovskaya, 2000). This polyphosphate reserves is utilised by microalgae for their metabolism, cell growth and the formation of the cell wall (Cembella et al., 1984; Kulaev and Kulakovskaya, 2000). Moreover, the polyphosphate granules can also be used for cell protection from toxic compounds (Nishikawa et al., 2003).

To investigate the accumulation of polyphosphate granules in *C.reinhardtii* 11/32C, a DAPI staining method was developed in the laboratory. Polyphosphate granules were stained with DAPI and examined by using fluorescence confocal microscopy (Ruiz et al., 2001a). Figure 5.3 revealed the reliable distinction between DAPI-DNA and DAPI-polyphosphate from the same sample, which helps to identify the presence of poly-P granules with confidence. Similar findings have also been reported for *Corynebacterium glutamicum* (Klauth et al., 2006), but yet this technique has not been used for the identification of hyper P accumulating organisms. Considering that the blue fluorescence emitted by DAPI-DNA is distinctive from the greenish-yellow fluorescence emitted by DAPI-polyphosphate, it is expected that the intensity of the polyphosphate granules can be used as an indicator to estimate the content of polyphosphate storage in algal cells (Kawaharasaki et al., 1999; Mesquita et al., 2014; Nishikawa et al., 2006). This high intensity of polyphosphate granules was clearly

revealed by the analysis conducted in samples from algae cultured in tests 11 and 17, where samples have a total polyphosphate content of 0.8% and 1.3%, respectively.

In Test 8, the algae were cultured in a media containing 1 mg P L^{-1} with total P removal of 99% in 14 days. Under very low external P concentrations, the algae tend to remove almost 100% P from the media and very little P remains as stored polyphosphate (Qu et al., 2008). Although the stored polyphosphate granules in the algae grew in Test 8 were shown by very big green-yellow fluorescence (Figure 5.3b) during the exponential growth phase, it was assumed that due to the P limiting conditions in the media, any accumulated polyphosphate was rapidly consumed resulting in very low in-cell P concentrations at the end of the cultivation period (14th day).

In Test 11, microalgae were cultivated in a media containing 10 mg P L^{-1} and almost all of it was removed from the media (99% P removal in 14 days). Under such conditions, the microalgae grew better than in Test 8 (1 mg P L^{-1}) as confirmed by the specific growth rate (0.11 d^{-1}) in this experiment (Test 11). Therefore, the algae might not utilise high amounts of P for metabolism and will store P excess as polyphosphate granules in their cells; it was also noted that for Test 11, Nitrogen was supply in sufficient quantities (initial N concentration 100 mg N L^{-1}) and did not limit algal biomass growth. It was found that most of the P taken up in this tests was stored as polyphosphate, as at the end of the test the content of polyphosphate was equivalent to 83% of the total P found in the total algal biomass, which coincides with previous reports (Eixler et al., 2006; Zhu et al., 2015b).

Luxury P uptake was also evidenced in Test 17, where the algae were able to store 1.3% P as polyphosphate, which represented 82% of the total P in the algal biomass. Algae cultured in conditions with high concentrations of P would accumulate both acid soluble polyphosphate (ASP) and acid insoluble polyphosphate (AISP) (Powell et al., 2009). The ASP is employed for metabolism and production of DNA and proteins, whilst the AISP is involved in phosphorus storage which can act as P reserves and be assimilated later by the algae, when the external P concentration becomes a limit for growth (Miyachi et al., 1964).

As a consequence, the accumulation of polyphosphate granules in algal cells under the conditions set for tests 11 and 17 helped to obtained better results than test 8 regarding the DAPI staining technique for the identification of hyper P accumulating organisms (Figure 5.3 c and d).

On the other hand, the presence of polyphosphate granules in the algae cultured in test 19 (10 mg N L^{-1}; 10 mg P L^{-1}; 8 hr light :16 hr dark; 400 $\mu Em^{-2}s^{-1}$) was very small as compared with the other treatments (Figure 5.3e). As the size of polyphosphate granules in microalgae is 2μm in overall size (Bolier et al., 1992), probably the polyphosphate size in test 19 was less than 2μm. A small size of polyphosphate granules cannot be detected using microscopy techniques (Eixler et al., 2006). The tiny dots of polyphosphate were probably caused by the very small intracellular P uptake rates (0.05 mg P $L^{-1}d^{-1}$), resulting in a total polyphosphate content of 0.4%. In this experiment, the polyphosphate and total P in the algal biomass were similar at 0.4%, which indicated that no polyphosphate was stored in the cells. This is in agreement with Nishikawa et al. (2006) who reported that the amount of intracellular polyphosphate was in conjunction with total cellular P in a given culture.

Furthermore, the fate of P in the algae cultivated in Test 26 (100 mg N L^{-1}; 10 mg P L^{-1}; 16 hr light:8 hr dark; 400 $\mu Em^{-2}s^{-1}$) demonstrated that from the original amount of P initially set, the remaining P after 14 days of cultivation was found dissolved in the media (Figure 5.3f); that created interferences in the observations made for the identification of in-cell polyphosphate granules by confocal microscopy, as similarly reported by (Eixler et al., 2006). It was found that under the conditions set for Test 26, algae accumulated polyphosphate during the first two days only (up to ≈ 1%P) and then the in-cell polyphosphate content fluctuated through their growth period and continued to decrease from the 4th day as the intracellular P decreased to 0.68% P on day 14th. Similar fluctuation of polyphosphate inside cells of *Corynebacterium glutamicum* was also observed by Klauth et al. (2006). Consequently, polyphosphate granules in the algae cultivated in Test 26 could not be detected by confocal microscopy as there was no granule formation due to little P uptake and any in-cell polyphosphate is believe to be soluble in the vacuoles (Eixler et al., 2006).

5.4.2 Investigation on the location of in-cell polyphosphate storage

Chlamydomonas reinhardtii contains cytoplasmic vacuoles with plenty of electron-dense granules (Rao et al., 2009), which mainly consist of crystallised inorganic polyphosphate and commonly known as acidocalcisomes (Komine et al., 2000). Acidocalcisomes are acidic organelles with a high concentration of phosphorus present as pyrophosphate (PP_i) and polyphosphate (poly P) complexed with divalent cations, like Mg^{2+} and Ca^{2+}, and are distinctively recognised as electron-dense vacuoles when examined by TEM electron microscopy (Ruiz et al., 2001a).

This study confirmed the presence of polyphosphate granules and their location within the vacuoles of *C.reinhardtii* 11/32C (see region pointed with a red arrow in Figure 5.4), which coincides with the location of similar P storage organelles found in other strains of *Chlamydomonas reinhardtii* (Aksoy et al., 2014; Komine et al., 2000; Ruiz et al., 2001a); *Chlamydomonas acidophila* (Nishikawa and Tominaga, 2001; Nishikawa et al., 2003); green strains of *Dunaliella salina* (Bental et al., 1991); and *Saccharomyces cerevisiae* (Urech et al., 1978). Docampo (2006) reported that the polyphosphate found in *acidocalcisomes* is present in the form of microcrystals.

The accumulation of polyphosphate granules in microalgae varies depending on the environmental conditions used for their cultivation, such as osmotic stress, nutrient shift, N starvation and stationary phase adaptation (Kornberg et al., 1999). However, P concentration in the medium plays the most important biological role in enhancing polyphosphate accumulation in algal cells and this mechanism has been clearly identified and modelled (John and Flynn, 2000; Yao et al., 2011).

The size of polyphosphate granules found in tests 8 and 19 was very small as compared to those found in tests 11, 17 and 26 (Figure 5.4). It is speculated that such difference in granule size during the storage of polyphosphate in *C.reinhardtii* 11/32C is controlled by extracellular P concentrations as reported by Pick and Weiss (1991), who examined the fluctuation of polyphosphate storage in *Dunaleilla salina* caused by changes in P concentration in the culture media. In Test 8, P concentration in the

media was 1 mg P L^{-1} and it was assumed that most of the intracellular P (P uptake rate = 0.009 mg P L^{-1} d^{-1}) was used for cell maintenance and growth. Therefore, the electron microscopy analysis showed the formation of very small polyphosphate granules. In Test 19, in-cell polyphosphate was believed to be soluble in the vacuoles, which was confirmed by electron microscopy analysis and supported by the low in-cell P uptake and specific growth rates (0.05 mg P L^{-1} d^{-1} and 0.06 d^{-1} respectively). As reported by Powell et al. (2008) and Brown and Shilton (2014), P uptake is influenced by the external P concentration and luxury P uptake is expected to happened under conditions with high P environments. In fact, it was observed in a previous study also using *C.reinhardtii* that under conditions with low P concentrations extracellular phosphatase is secreted in order to assimilate any organic P available in the environment (Quisel et al., 1996). Moreover, the alkaline condition with pH around 9 and above was observed in Test 19 and this could trigger the hydrolysis of in-cell polyphosphate (Bental et al., 1991; Leitao et al., 1995; Nishikawa et al., 2006).

In microalgae, the polyphosphate kinase (PPK) enzyme is responsible for P transport from the medium into the cells (Docampo, 2006; Rao et al., 2009), but the actual intake of P takes place through plasma membranes that is controlled by the external P concentration (Meza et al., 2015). The external P concentration in tests 11 and 26 was 10 mg P L^{-1} and in Test 17 was 100 mg P L^{-1}. In Test 17, high amounts of available phosphate in solution may have led to acidification in the cytoplasm, which facilitated phosphate assimilation into the algal cell and the subsequent P accumulation at a higher rate when compared with tests 11 and 26 (Meza et al., 2015).

Inorganic phosphate stored in algal cells has a high density due to the molecular weight of inorganic polyphosphate (Meza et al., 2015; Pick and Weiss, 1991; Ruiz et al., 2001a). In this study, highly-dense polyphosphate storage was revealed as black granules by using a staining solution containing lead citrate and uranyl acetate (Reynolds, 1963), followed by TEM analysis. The polyphosphate granules found from electron microscopy (TEM) analysis (Figure 5.4) confirmed the presence of black granules, which indicates the very distinctive bounds between lead and polyphosphate confirming the location and abundance of in-cell P storage as polyphosphate granules.

In microalgae, polyphosphate is present as acid soluble polyphosphate (ASP) and acid insoluble polyphosphate (AISP). ASP is mostly utilised by the algae for generating DNA and proteins, which are used to support algal growth. Whereas AISP is mainly used by microalgae as polyphosphate storage. AISP can be utilised under stress conditions such as nutrient limiting environments (John and Flynn, 2000; Miyachi et al., 1964; Powell et al., 2009). For instance, the growth of algae in Test 17 significantly increased when the intracellular P uptake rate was almost 3 mg P L^{-1} d^{-1}, and the polyphosphate granules in their cells were still very dense. It means that luxury P uptake occurred in that algae culture and they stored the excess of assimilated P as polyphosphate (AISP). Due to the presence of phosphate in excess, most of the ASP was utilised to support algal biomass growth.

5.4.3 Isolation of hyper polyphosphate accumulating organisms

Flow cytometry methods have the potential to isolate individual cells to obtain axenic cultures or to sort individual cells with a desirable characteristic (Hyka et al., 2013). Following DAPI fluorescence and TEM experiments with *C.reinhardtii* 11/32C for the observation of in-cell polyphosphate granules, the same alga cultures were analysed by fluorescence activated cell sorter (FACS) to assess the possibility to isolate hyper polyphosphate accumulating individuals. According to the results from fluorescence microscopy (i.e., DAPI staining) and electron microscopy analyses, polyphosphate granules were found in algal cells and then, it was hypothesised that individual cells able to accumulate P at a higher rate than the average individuals could be isolated by using cell sorting techniques.

However, in the first analysis of flow cytometry (FCM), it was not possible to clearly distinguish algal cells containing higher content of in-cell polyphosphate, as there was no major difference from DAPI-DNA and DAPI-polyphosphate. Presumably, it was caused by the interference of chlorophyll fluorescence that intrinsically produced a noise factor that made an influence the fluorescence emitted due to polyphosphate content (Terashima et al., 2015). In fact, unstained *C.reinhardtii* 11/32C showed a red fluorescence when examined under the fluorescence confocal microscope, which indicated that chlorophyll indeed has a strong fluorescence effect even when the algae

were unstained. Similarly, the disruption caused by chlorophyll fluorescence noise was also identified when *Chlamydomonas* wild-type 137C were sorted for the presence of high lipid content and mutant cells stained with Nile Red (Terashima et al., 2015).

In *C.reinhardtii* cells, the chloroplast occupies approximately two thirds of the cellular volume (Harris, 2001; Stauber and Hippler, 2004), which also includes nucleoids containing DNA (Stauber and Hippler, 2004). The results from confocal and electron microscope shown that DNA containing nucleoids were adjacent to polyphosphate granules (Figure 5.3 and 5.4) and therefore, the algae cultivated under different environmental conditions and reporting low and high in-cell P content (i.e., tests 8 and 17) appeared in the same scatter plot during cell sorting analysis (Figure 5.5b and d). For that reason and in order to test the hypothesis that a low number of hyper P accumulating organisms cannot be isolated via cell sorting techniques, it was decided to increase the number of individuals performing luxury P uptake and the culture with low in-cell P (Test 8) was enriched with algae from the culture with high in-cell P content (Test 17) in a 50:50 ratio by volume. As a result, this method revealed that it was possible to sort 10.2% of the total cells, which contained individuals with high in-cell P (hyper P accumulating organisms) from a culture of *C.reinhardtii* 11/32C (Figure 5.6), that may lead to the possibility to isolate such individuals for further bioengineering applications.

However, the amount of polyphosphate in the algal cells are not stable and they depends on the environmental conditions such as P concentration in the media, light intensity and temperature (Powell et al., 2008). This study also revealed that the amount of the intracellular P uptake in *C.reinhardtii* 11/32C was influenced by the interaction of P with N concentration and the period of light (Chapter 4). Previous study elucidated that the amount of polyphosphate in bacteria depends on the P content in the growth media and the content of polyphosphate will reduce in P starvation media (Docampo, 2006). Furthermore, osmotic stress has reported to trigger hydrolyse and synthesise of polyphosphate content in algal cells. Polyphosphate granules will hydrolyse after hypoosmotic shock whereas the synthesise of polyphosphate will occur after hyperosmotic stress of the alga cells (Ruiz et al., 2001a). This indicate that

adaptation period in low or high nutrient concentration media influence the amount of polyphosphate store in algal cells. Moreover, Nishikawa et al. (2006) conveyed that polyphosphate content in algae are growth dependent. The ratio of polyphosphate and orthophosphate in vacuole increased from 2.4 to 12.5 when the cells grew on early exponential phase to stationary phase (Yang et al., 1993).

In order to maintain the hyper P accumulator in *C.reinhardtii* 11/32C, the algae must be constantly cultured in the lab as uni-algal or axenic strain using the optimum conditions: 100 mg N L^{-1}, 100 mg P L^{-1}, 16 hr light: 8 hr dark, 250 $\mu Em^{-2}s^{-1}$. The maintenance of this culture can be placed in incubator using semi-continuous growth system where the media can be replaced with the fresh one during the exponential phase. Alternatively, algae culturing in solid media (agar) might be the best choice for backup of the isolated algal culture for a period of six months (Day et al., 2015). In the future works, it is important to conduct a mutagenic process from isolated hyper P accumulator in *C.reinhardtii* 11/32C which able to store and lock the polyphosphate granules in their cells without release them to the environment. This attempt will help to enhance the biological role of polyphosphate storage accumulation for biological P removal in wastewater (Docampo, 2006). Previous studies provided a number of important information related to genetic engineering of *Chlamydomonas reinhardtii* for studying the genetic and molecular mechanisms of cellular process (Harris, 2001), cloning of flagella genes in *Chlamydomonas reinhardtii* (Tam and Lefebvre, 1993), and insertional mutagenesis for sensitivity to genotoxic stress (Plecenikova et al., 2014). Therefore, there will be an opportunity to conduct mutagenic strain of *C.reinhardtii* 11/32C able to enhance the polyphosphate accumulation.

5.5 Conclusion

This study proved that *C.reinhardtii* 11/32C was able to uptake P from the medium and store excess P as polyphosphate granules. The accumulation of polyphosphate granules depends on the external P concentration, which affects the actual intracellular P uptake rate. The presence of polyphosphate granules in algal cells appears as bright green-yellow fluorescence when examined by using DAPI staining and confocal microscopy. Electron microscopy was effective at identifying the location

of polyphosphate granules in *C.reinhardtii* 11/32C, which were the vacuoles. TEM results provided the evidence that luxurious P uptake took place in *C.reinhardtii* 11/32C under favourable environmental conditions.

Previous studies related to P recovery using microalgae reported that luxury P uptake occurred when the algae cells were starved in the environment with no or low P and then transferred into P-replete environment. However, this study found that luxury P uptake in *C.reinhadrii 11/32C* occurred without the need for a cell starvation process.

The ability of *C.reinhardtii* 11/32C to accumulate polyphosphate is an important biological mechanism that could be used to implement effective microalgae system for wastewater remediation and P recovery. In order to enhance the application of microalgae systems for P recovery from wastewater, hyper polyphosphate accumulating organisms can be isolated for a more effective engineered process.

Polyphosphate granules are not stable organelles in algal cells as they can be synthesised or hydrolysed to support cell metabolism and growth. Therefore, *Chlamydomonas reinhardtii* mutants able to permanently store polyphosphate granules inside their cells should be isolated for future study.

Chapter 6 Operation of a continuous flow mixotrophic system with recycle to enhance biomass production and luxurious P uptake

6.1 Introduction

Existing wastewater treatment plants remove P from wastewater through chemical precipitation and biological bacterial uptake. Chemical precipitation requires the addition of either metal salts (e.g., aluminium sulphate or iron chloride) or lime, but the resulting sludge contains P strongly bonded to the added metal (i.e., Al, Fe or Ca) that makes it very difficult to reuse as a fertiliser. Chemical precipitation of P can remove up to 90% phosphate from wastewater (de-Bashan and Bashan, 2004).

Biological P removal through enhanced bacterial phosphorus removal (EBPR) is generally considered to be a more sustainable pathway to remove P through modified activated sludge processes. Due to lower sludge production and less chemicals usage, it is economically affordable however, it has so far been applied at the large sites only (Martin et al., 2006; Toerien et al., 1990). However, biological processes for P removal require an additional carbon source (i.e., acetate) and due to the use of anaerobic digestion for energy generation from sewage sludge, most of the P is released again in the digested liquor and returned to the head of the works, which increases treatment costs. In order to achieve nutrient recovery from wastewaters, it has been suggested alternative chemical (struvite precipitation) and biological processes (microalgae systems).

Recently, the wastewater industry have made attempts to recover the nutrients from waste streams by producing struvite (a slow nutrient release fertiliser) (Jaffer et al., 2002). However, this practice requires the addition of magnesium and the adjustment of pH in a P rich-waste stream like the digested liquor coming from anaerobic digesters (Bhuiyan et al., 2008; Munch and Barr, 2001), but for those reasons it is not economically viable for small wastewater treatment plants or for large treatment works with no anaerobic digestion in place.

Microalgae are one of the promising future renewable energy resources which have no major impact on agriculture production (Brennan and Owende, 2010; Maity et al., 2014). Unlike terrestrial crops, photosynthetic microalgae utilise the energy from the sun and assimilate nutrients from their environment (e.g., wastewater). The algae can grow massively to produce a biomass that contains valuable products (i.e. protein, lipids and carbohydrates) (Mata et al., 2010; Spolaore et al., 2006). This versatile ability shown by microalgae can be used to produce biofuels, fine chemical products, food supplements and bio-fertilisers. Applications of algal biomass in agriculture have reported as seed conditioner, soil fertiliser and bio-spray for enhancing foliar during the growing season and flowering (Chojnacka et al., 2012). The use of blue-green algae (i.e. cyanobacteria) in rice filed can enhance soil fertility through biological nitrogen fixation (called Algalization) and this method has applied widely in some countries in Asia such as China, Philippines and India (Abdel-Raouf et al., 2012). Furthermore, dry biomass of green algae *Acutodesmus dimorphus* has applied as a seed primer, biofertiliser and foliar spray in order to enhance plant growth and fruit production of Italian tomato in Roma (Garcia-Gonzalez and Sommerfeld, 2016) and the results shown that the seed germination increase up to 50%. Dry biomass of *Chlorella vulgaris* has also reported to increase the fresh and dry weight and pigment content of *Lactuca sativa* (Faheed and Fattah, 2008).

In addition, their natural ability for recycling nutrients and capacity to grow even in wastewaters and produce biomass feedstock is an attractive aspect for developing algal based technologies for resource recovery. Microalgae systems can be used as part of an integrated approach for wastewater treatment not only for bioremediation, but also for biomass feedstock production for feeding existing anaerobic digesters (Dalrymple et al., 2013b; Pittman et al., 2011; Sivakumar et al., 2012). Considering the ability that microalgae have for assimilating high amounts of P and store it in their cells in the form of polyphosphate granules (Powell et al., 2008; Sivakumar et al., 2012), there is a great potential for developing alga-based systems for recovering P from the wastewaters.

In regard to the use of microalgae cultures for nutrient recovery, previous studies have focused on studying algal growth, biomass production and substrate utilisation under

mixotrophic batch conditions (Perez-Garcia et al., 2010), mixotrophic continuous flow systems (Chen and Johns, 1996; Ruiz et al., 2013) and photoautotrophic continuous flow bioreactors (Barbosa et al., 2003; Fernandes et al., 2015; Javanmardian and Palsson, 1991; Sforza et al., 2014). Other studies found in published literature have investigated the effect of recycling harvested algal biomass into the main photobioreactor under continuous flow conditions for nutrient recovery (Park et al., 2015; Xu et al., 2015a), but none has studied the used of separate bioreactors for controlling light (photoautotrophic) and dark (heterotrophic) processes.

Photoautotrophic cultivation of microalgae provides a potential contribution on CO_2 reduction as it is used as inorganic carbon source for their growth. In contrast, the production of the algal biomass highly depends on light availability, but increasing algal biomass content will create a self-shading effect that affects biomass growth and hence, the limitations of having highly dense alga cultures (Adesanya et al., 2014; Smith et al., 2015). Heterotrophic cultivation on one side reduces the requirement for light and promotes aerobic respiration on an organic carbon source. Algal biomass production will be maintained in dark conditions and will generate high cell density cultures (Mohan et al., 2015; Smith et al., 2015). However, heterotrophic cultivation is considered to be more costly than photoautotrophic due to the cost of an organic carbon source, which could add up to 50% of the total cost of algae cultivation (Cheng et al., 2009). Moreover, the risk of bacterial competition due to the utilisation of an organic carbon source is inevitable and may cause the reduction of nutrient assimilation and algal growth (Zhang et al., 2012).

In order to properly handle both the potentials and the obstacles of photoautotrophic and heterotrophic alga cultivation, the use of continuous flow mixotrophic culture has been identified as the best solution for nutrient recovery (particularly P) and production of algal biomass. But, the investigation of continuous flow mixotrophic algae cultivation in conjunction with algae recycle, to enhance algal biomass productivity and intracellular P uptake, requires more study as it is still poorly understood.

Enhancing algal biomass productivity, followed by increasing intracellular P uptake, has the potential to be implemented for P recovery and biomass production from

wastewater. Therefore, this study aims to investigate the potential use of microalgae processes to be implemented in wastewater treatment works as an alternative biological phosphorus recovery (BPR) system and to develop reliable processes for mixotrophic cultivation under continuous flow conditions.

6.2 Methodology

C.reinhardtii 11/32C were used as a model organism for this study and a preliminary study to assess growth kinetics and the influence of P concentrations was conducted before the actual operation of the continuous flow system. After that, microalgae was cultivated under continuous flow mixotrophic system using a feeding media that resembles wastewater (synthetic wastewater), using one 2L heterotrophic photobioreactor (HTR) and one 2L autotrophic photobioreactor (PBR) connected in series (Figure 3.7). Continuous flow mixotrophic cultivation was conducted under constant dark conditions in HTR and constant illumination in PBR (light intensity of 250 $\mu Em^{-2}s^{-1}$); therefore, the actual photoperiod was regulated by the flow rate feeding the system and the recycle flow of harvested biomass. The list of parameters monitored during the continuous flow operation of this algal-based system is as reported previously in Table 3.6. Culture samples were taken every two days over a period of 70 days and processed in the Public Health Engineering Laboratory, University of Leeds. Analytical results were processed for algal growth, biomass concentration and nutrient uptake as explained in Section 3.1.3. To validate the presence of polyphosphate granules in algal cells, TEM electron microscopy analysis was also conducted.

Microalgae cultivation in HTR and PBR was initially conducted by operating both reactors under batch conditions until the culture reached stationary phase. Then, the system was operated under continuous flow mixotrophic conditions. The continuous feeding of the reactors started in day 14 at a Hydraulic Retention Time (HRT) of 24 hours, where the fresh media was fed into the HTR. The continuous flow system was then operated at 36 and 48 hours HRT, when the P concentration in the final effluent reached steady state conditions. Details of the mixotrophic continuous flow system were explained in Section 3.3.2. The settled algal biomass collected from the bottom of an Imhoff sedimentation cone was recirculated into HTR. Moreover, the overflow

from the sedimentation tank was collected in the final effluent tank and analysed for the same parameters as water samples collected from HTR and PBR.

6.3 Results

6.3.1 Specific growth rate and kinetics in batch cultures

Algae cultivation in batch cultures was conducted in different environmental conditions with a wide range of N and P concentrations, light intensity and photoperiod. These experiments aimed to obtain kinetic growth characteristics of *C.reinhardtii* 11/32C. The kinetic characterisation of the system is not only useful to quantify specific growth rates in batch cultures, but can also be used to predict the growth rate in continuous flow operation (Ruiz et al., 2013).

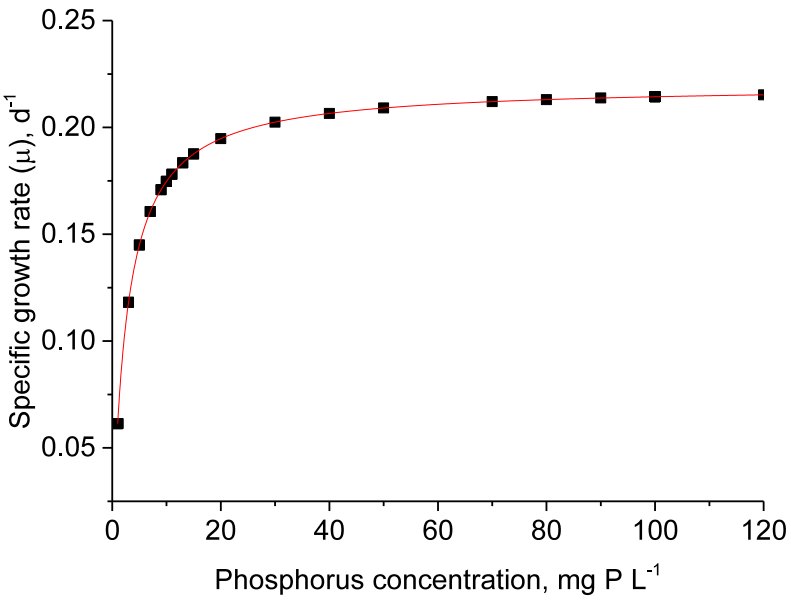


Figure 4-26 Kinetic growth rates for *C.reinhardtii* 11/32C as a function of P concentration

The specific growth rates of *C.reinhardtii* 11/32C cultivated in different environmental conditions of batch culture against a wide range of P concentration is illustrated in Figure 6.1. The calculation of the specific growth is referred as the increment of algal biomass concentration (mg VSS L^{-1}) over time. The model developed by (Monod, 1949) was used to calculate specific growth rates in limiting substrate conditions,

which can be represented by equation 3.1. From that is possible to obtain μ_{max} (maximum specific growth rate) and K_s (half-velocity constant or the value of P when $\mu/\mu_{max} = 0.5$). From Figure 6.1, the corresponding values for μ_{max} was 0.219 d^{-1} and 2.583 mg P L^{-1} for K_s were obtained with a regression coefficient R^2 of 1.0. These coefficients will be used to predict the specific growth rate of microalgae in the continuous flow culture.

6.3.2 Design parameters for a continuous flow culture

Nutrient concentrations listed in Table 3.6 were used for the cultivation of microalgae under continuous flow conditions. According to the equations explained in Section 3.3.1, the calculation of the selected design parameters are listed in Table 6.1.

Table 4-6 Design parameters used for operating a continuous flow system

Parameters	Value	Explanation
Specific growth rate, μ	0.2 d^{-1}	Kinetic growth (Figure 6.1)
Max. hydraulic retention time, HRT	5 d	Equation 3.2
Sludge (cells) retention time, SRT	5 d	Equation 3.7
Working volume, V	4,510 ml	This working volume is based on two reactors.
Media flowrate, Qin	3,910, 2,407, and 1,651 ml d^{-1}	Media flowrate is referred to the working volume of two reactors and different HRT (24, 36, 48hr).
Recycle flow rate, Qr	0.15 x 4,510 676.5 ml d^{-1}	The recycle algae is referred to Metcalf & Eddy (2004), p. 690
Harvested algae flowrate, Qw	460 ml	Equation 3.9
Concentration of algae in the recycle line, Xr	3,880 mg VSS L^{-1}	Based on the first trial experiment
Concentration of algae in the final effluent, Xe	70 mg VSS L^{-1}	Based on the first trial experiment

In order to obtain the expected algal biomass concentration in the system at 3 g VSS L^{-1} and the intracellular P content of 3%, this study was planned to be conducted for a

maximum of 5 days HRT for the entire process. However, the trend of the increasing biomass concentration and the accumulation of P in the algal cells are needed to be observed and therefore, the tested HRTs increased from 24h to 36h and ended with 48h. Consequently, the flowrate of fresh media feeding the HTR was set at 3,910, 2,407 and 1,651 mL d^{-1} for 24, 16 and 18 days respectively. The results for algal biomass, intracellular P uptake rate, the content of intracellular P and the quality of the final effluent are presented in the following section.

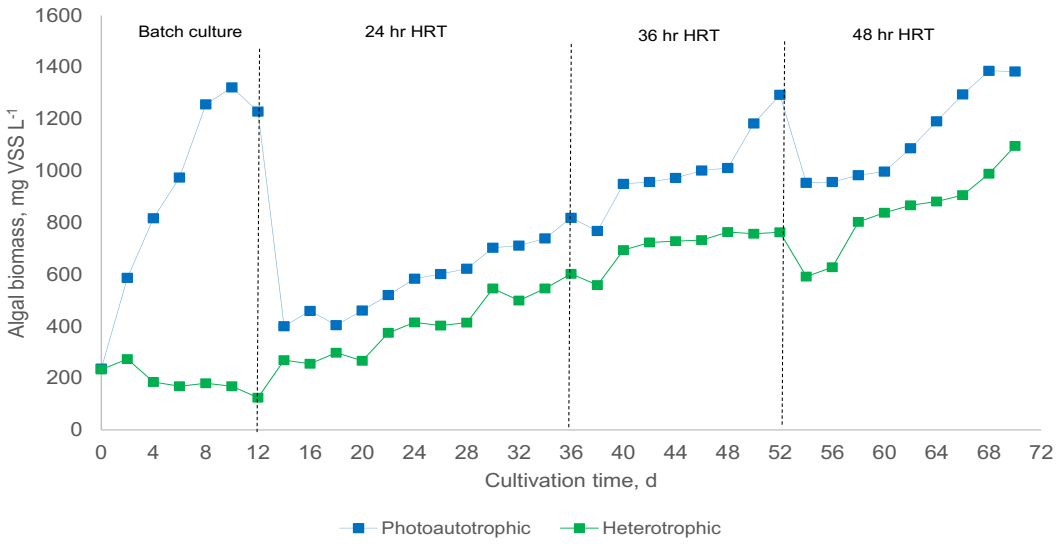
6.3.3 Continuous flow cultivation of microalgae for P recovery

6.3.3.1 Algal biomass concentration and productivity

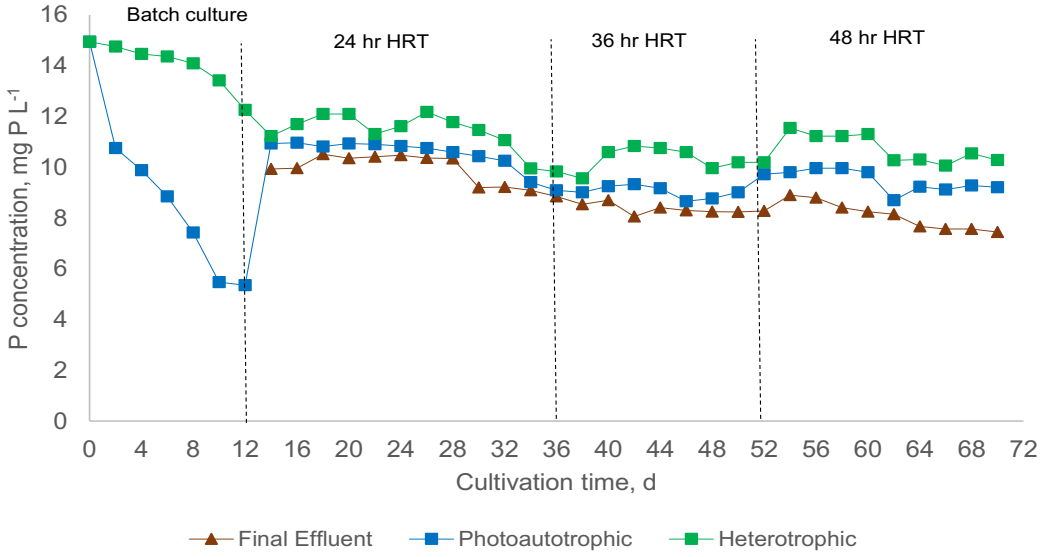
Algae inoculation in PBR and HTR were made with the same initial biomass concentration of 240 mg VSS L^{-1}. Algae cultivation under batch culture conditions for PBR and HTR was carried out for 12 days. After that period, the algal biomass and P concentration in the media was approximately constant at 1270 mg VSS L^{-1} and 5.4 mg P L^{-1} respectively, then the mixotrophic continuous flow cultivation of microalgae continued for the following 58 days. The system was operated at 12, 18 and 24 hours of HRT for each reactor (Figure 6.2a). Therefore, for the two reactors under operation (PBR and HTR) the total tested HRT of the system was 24 hr, 36 hr and 48 hr. In this study, HRT was changed when steady state conditions were reached based on the concentration of P in the final effluent (Figure 6.2b).

Results showed that algal biomass concentrations were always higher in PBR, than those found in HTR for the duration of all tests (Figure 6.2a). In the batch culture phase, the algal biomass reached stationary growth phase at approximately 1,270 mg VSS L^{-1} in PBR and 172 mg VSS L^{-1} in HTR. The algal biomass concentration in both reactors decreased once the system started to be fed continuously due to dilution and algal biomass being washed out. Moreover, decreasing of algal biomass from batch to continuous flow was caused by the reduction of flowrate and the adaptation of algae with the continuous system. Within the three different HRT the algal biomass concentration in PBR ranged from 400 to 819 mg VSS L^{-1} for 12h HRT, from 768 to 1294 mg VSS L^{-1} for 18h HRT, and from 954 to 1384 mg VSS L^{-1} for 24h HRT.

Whereas in HTR, algal biomass ranged from 269 to 603; 559 to 763; and 592 to 1096 mg VSS L^{-1}, respectively for each HRT tested. In addition, the algal biomass concentration in both reactors slightly increased when P concentration in the final effluent achieved steady state at the end of every HRT (Table 6.2). On the other hand, the algal biomass concentration in HTR was lower than PBR. Presumably it was caused by lower organic carbon concentration than inorganic carbon.



a. Algal biomass concentration of *C.reinhardtii* 11/32C in continuous system



b. P concentration in the media over period of 70 days

Figure 4-27 Changes in algal biomass and P concentration during the operation of the continuous flow mixotrophic system

Table 4-7 Comparison of algal biomass and P concentration in steady state condition

Time (d)	P concentration in the final effluent (mg P L^{-1})	Algal biomass in PBR (mg VSS L^{-1})	Algal biomass in HTR (mg VSS L^{-1})
32 - 36	9.1 – 8.9	711,739,819	499,546,603
48 - 52	8.25 – 8.28	1011,1183,1294	764,757,763
68 - 70	7.7 – 7.4	1386,1384	989,1096

Interestingly, the biomass productivity in the continuous flow mixotrophic culture was bigger than in batch culture conditions. In the continuous flow culture, the total biomass productivity rates were 299, 222, and 223 mg VSS L^{-1} d^{-1} respectively for 24, 36 and 48 hours of HRT for the entire system. These values were approximately double than the maximum algae biomass productivity found for *C.reinhardtii* 11/32C in batch culture conditions. Moreover, *C.reinhardtii* 11/32C cultivated in the continuous mixotrophic system able to produce high harvested biomass on average at 2.6 g VSS L^{-1} daily. Results from the tested continuous flow systems appears to indicate that biomass productivity for *C.reinhardtii* 11/32C under such conditions was better than others reported for similar continuous flow systems using *Chlorella minuttisima* and *Dunaleilla tertiolecta* (Tang et al., 2012), *Pavlova lutheri* (Carvalho and Malcata, 2005), and *Chlorella pyrenoidosa* (Wen et al., 2014).

Nevertheless, the biomass productivity in each HRT under continuous flow culture decreased when the HRT changed from 24 to 36 hr HRT and then remained relatively constant at 36 and 48 hours HRT (Figure 6.3). In this case, changing of HRT also influenced a decline in performance regarding nutrient removal, as average P uptake rates were 15, 10, and 7.5 mg P L^{-1} d^{-1} for HRT 24, 36 and 48 hours respectively. For the same HRT, average N uptake rates were 50, 33, and 25 mg N L^{-1} d^{-1}, respectively.

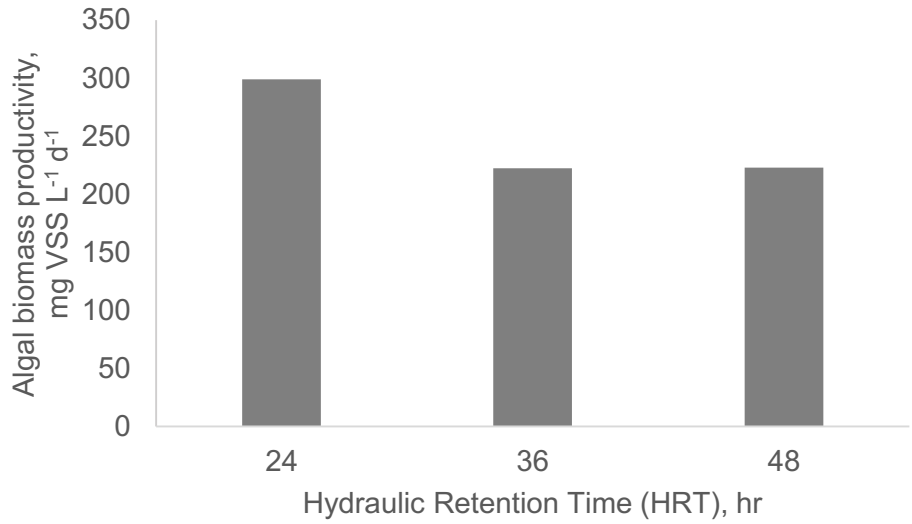


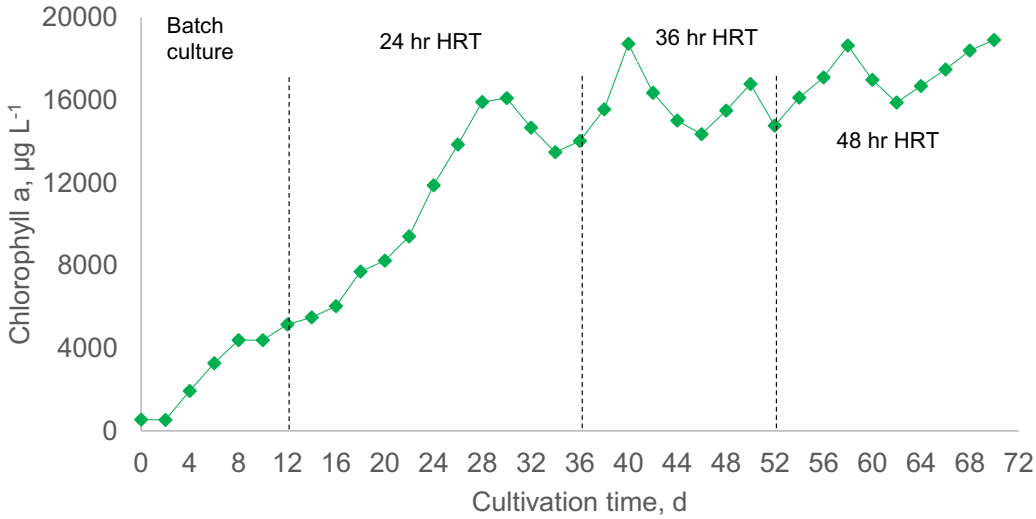
Figure 4-28 Algal biomass productivity for mixotrophic continuous flow conditions as a function of HRT for the entire system

6.3.3.2 Chlorophyll-a content and bacterial population

Chlorophyll-a content was measured from samples collected from the PBR to monitor the presence of algae. This measurement proved that increasing algal biomass correlated very well with chlorophyll-a content (Sharma et al., 2012). As the algae cultivated in PBR was illuminated continuously at 250 $\mu Em^{-2}s^{-1}$, they utilised sufficient energy for photosynthesis and increased chlorophyll-a content as a result of increments in algal biomass (Oncel and Sukan, 2011). It can be seen from Figure 6.4a that the chlorophyll-a content increased over the period for batch until middle of 24 hr HRT, afterwards the chlorophyll-a gradually increase until the end of 48 hr HRT. The increase of the chlorophyll-a content was in line with the increase of the algal biomass. These results indicated that microalgae grew constantly in the exponential growth phase. It was expected after each change of HRT, the chlorophyll-a content of algae grew in PBR increased and this trend was similar to the algal biomass concentration.

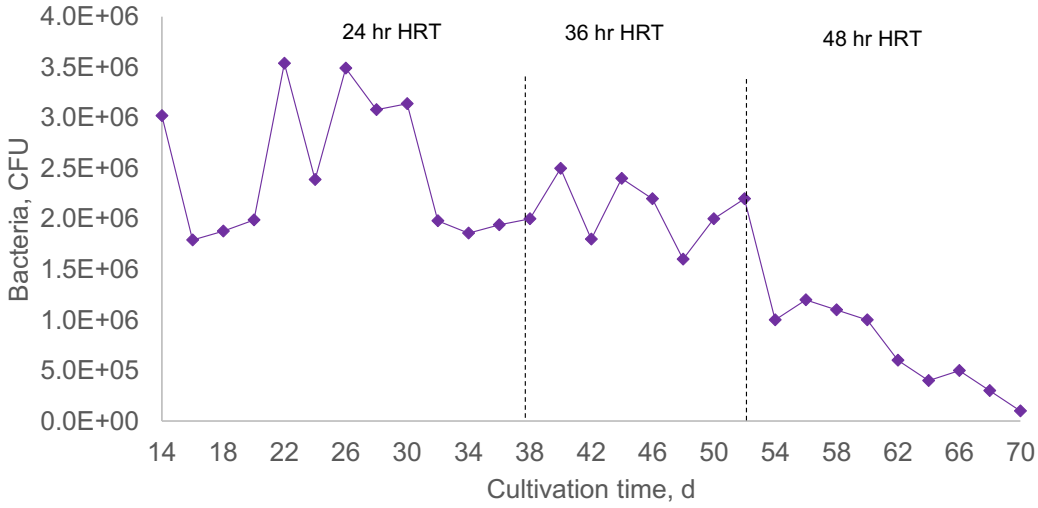
Furthermore, bacterial population was also measured from samples collected in the HTR to monitor the level of the bacterial growth. The concentration of bacteria in the microalgae culture is shown in Figure 6.4b. Fortunately, due to regular maintenance and cleaning of all the tubing and the use of autoclaved feeding media, the presence

of bacteria decreased over the period when the continuous flow culture was tested. However, at the end of day 14 bacteria growth still high as they acclimatised with exponential growth phase in batch culture. Afterwards, the growth of bacteria gradually decreased over time of continuous culture.



a. Chlorophyll-a in photoautotrophic culture

Figure 6-4 Chlorophyll-a content in the PBR and heterotrophic bacteria counts in the HTR in the mixotrophic continuous flow system



b. Bacteria population in heterotrophic culture

Figure 4-29 Chlorophyll-a content in the PBR and heterotrophic bacteria counts in the HTR in the mixotrophic continuous flow system

6.3.3.3 Intracellular P uptake

The intracellular P uptake in *C.reinhardtii* 11/32C was determined in both the heterotrophic and photoautotrophic cultivation reactors (PBR and HTR) with the three different HRTs (Figure 6.5).

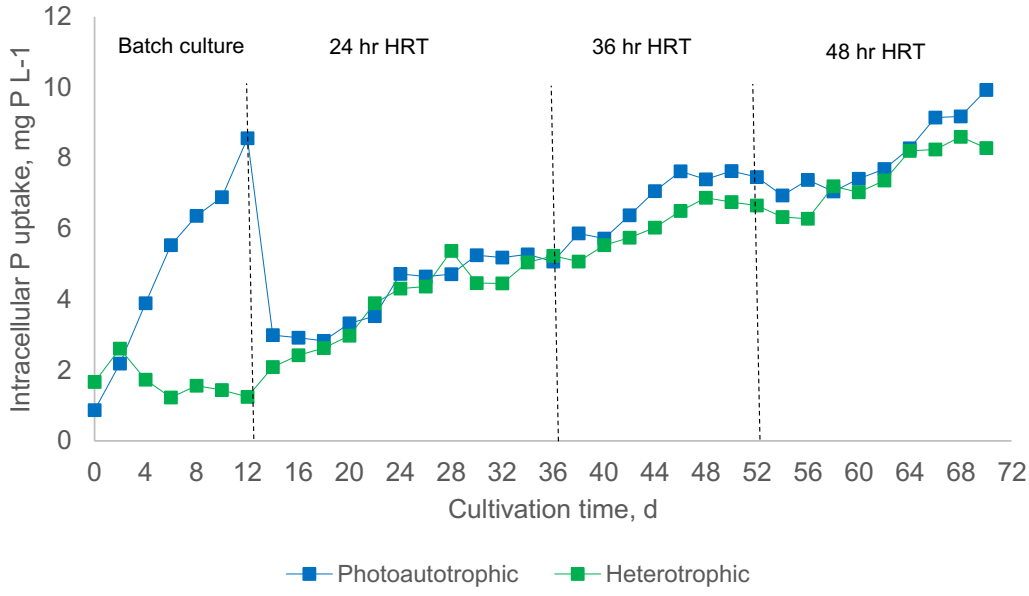


Figure 4-30 Intracellular P uptake rates for a series of (a) photoautotrophic and (b) heterotrophic reactors under different HRTs

Intracellular P uptake rates increased over the three different HRTs tested during the total 70 days of testing. This result is consistent with the increase of algal biomass concentration (Figure 6.2). That indicates intracellular P influenced algal growth as reported by Rhee (1973).

However, intracellular P uptake rate declined at 36 hrs HRT and remained relatively unchanged at 48 hrs HRT (Figure 6.6). This result is consistent with the pattern for biomass productivity (Figure 6.3). The mean P uptake rates were 2.62, 1.73 and 1.73 mg P L^{-1} d^{-1} for 24, 36 and 48 hours HRT respectively. On average, these values were higher than P uptake rates reported for *Scenedesmus obliquus* (1.4 mg P L^{-1} d^{-1}) cultivated in a continuous flow flat panel photobioreactor (Ruiz et al., 2013) and very close to P uptake rates found for *Scenedesmus sp.* (McGinn et al., 2012). The trend of the decrease of intracellular P uptake over different HRT is in agreement with data

reported by Ruiz et al. (2013), who also found that P uptake rates declined with the increasing of HRT.

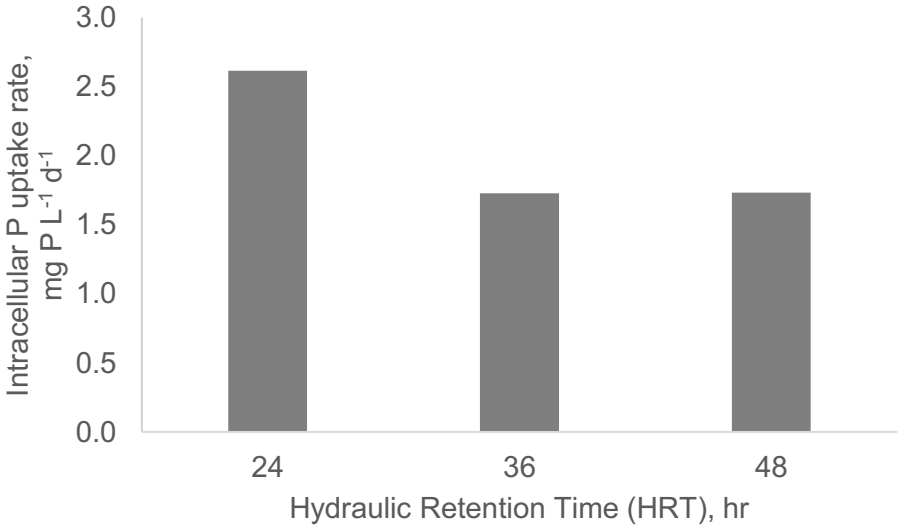


Figure 4-31 Intracellular P uptake rates for *C.reinhardtii* 11/32C cultivated under three different HRTs

The intracellular P uptake rate for *C.reinhardtii* 11/32C cultivated in a mixotrophic continuous flow system was in line with previous findings obtained from batch cultures using a wide range of P concentration between 1 and 120 mg P L^{-1} (Figure 6.7), which confirms the reproducibility of the experiments. A Michaelis-Menten model was employed to characterise intracellular P uptake rates for *C.reinhardtii* 11/32C cultivated in batch conditions using a wide range P concentration (Spijkerman and Coesel, 1996). From that model, the value of the maximum specific intracellular P uptake rate (Vmax) was 3.2 mg P L^{-1} d^{-1} and the half saturation constant for the system (Km) was 10 mg P L^{-1}.

Moreover, the P concentration used in the continuous culture was 15 mg P L^{-1} as this value was represented the average P concentrations in effluent of activated sludge process which is introduced into the model. As the mixotrophic microalgae system was planned to place after ASP or even replace the function of the secondary treatment in the future, P concentration of 15 mg P L^{-1} was used in the experiment. In addition, according to Figure 6.7 the predicted uptake rate of intracellular P would be 2 mg P L^{-1} d^{-1}.

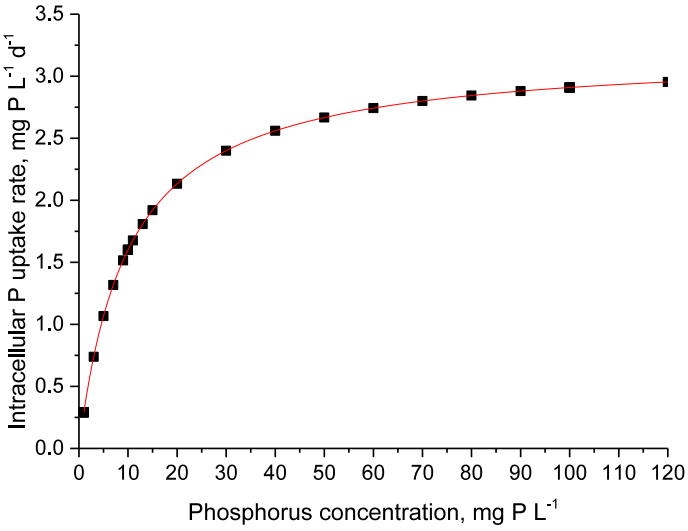


Figure 4-32 Intracellular P uptake rate for *C.reinhardtii* 11/32C in a batch culture under a wide range of P concentrations

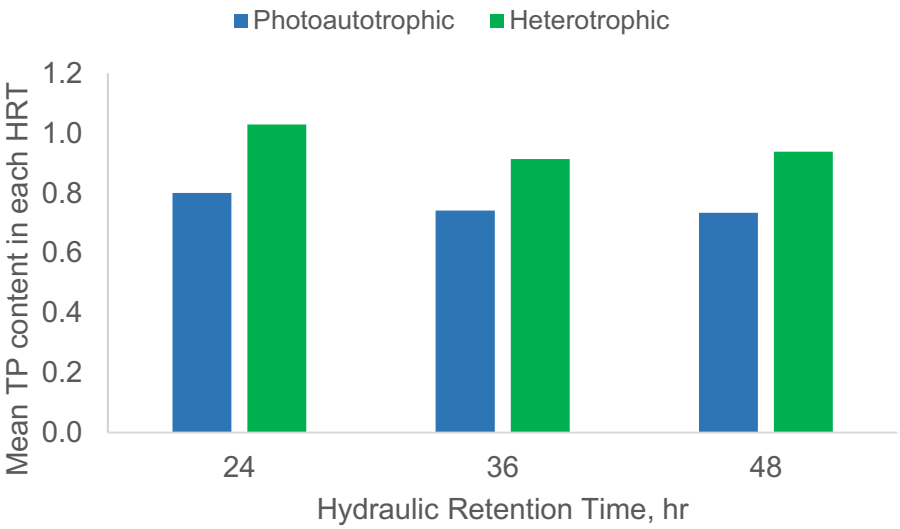


Figure 4-33 Ratio of total phosphorus accumulation in *C.reinhardtii* 11/32C grown in heterotrophic and photoautotrophic condition

In Figure 6.8 the mean between algal P content in the heterotrophic and photoautotrophic reactors in the three different HRT is presented. The mean were 0.8%, 0.74% and 0.73% in PBR and 1.03%, 0.91% and 0.94% in HTR respectively. These results indicate that most of the P accumulated in algal biomass occurred the

in heterotrophic reactor rather than in the photoautotrophic culture, with higher content at low HRTs.

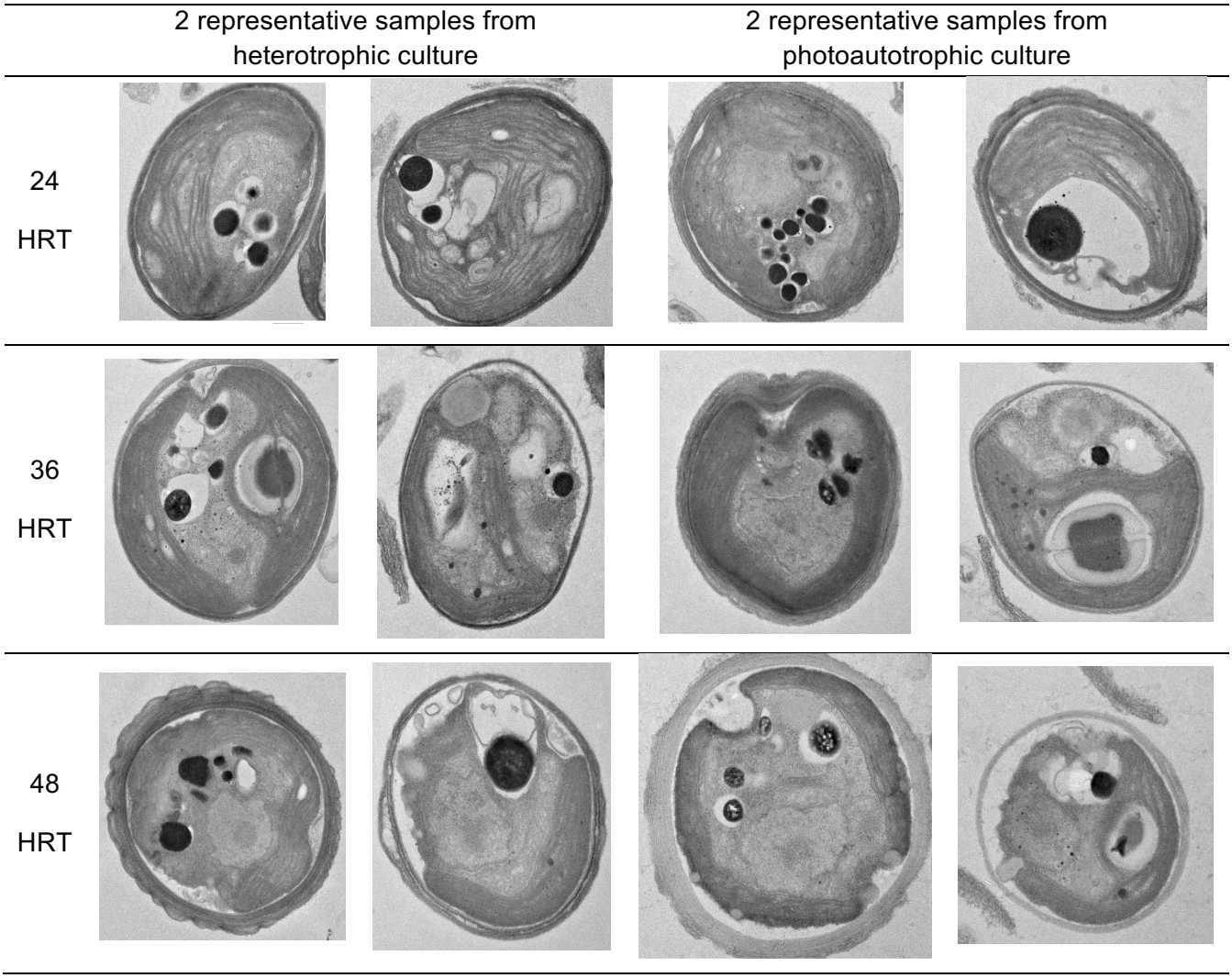


Figure 4-34 Transmission electron microscope (TEM) analysis of polyphosphate accumulation in *C.reinhardtii* 11/32C cells

The presence of P accumulation in the form of polyphosphate granules was also monitored during continuous flow operations. Two examples of polyphosphate granules in algae samples per each set HRT are shown in Figure 6.9. The images revealed that algae growing under heterotrophic conditions accumulate greater amounts of polyphosphate shown by darkest spots than under photoautotrophic conditions. This can be seen from the figures that polyphosphate granules in HTR were bigger than in PBR. Additionally, the algal cells cultivated at 24 hours HRT seems to store more polyphosphate than under others HRTs tested. The size of

polyphosphate granules was reduced along with the increment in HRT, which could correlate with the reduction in intracellular P ratio found between HTR and PBR (Figures 6.5 and 6.8).

6.3.3.4 Water quality in the final effluent

The water quality in the final effluent of the entire system fed with synthetic wastewater is shown in Figure 6.2b and 6.10. In the tested system, the concentration of P in form of soluble reactive phosphorus (SRP), ammonium (NH_4^+), nitrate (NO_3^-) and total suspended solids (TSS) were monitored every two days over a period of 70 days. To measure N and P concentration in the final effluent, filtered sample were processed according to the method described in Table 3.2.

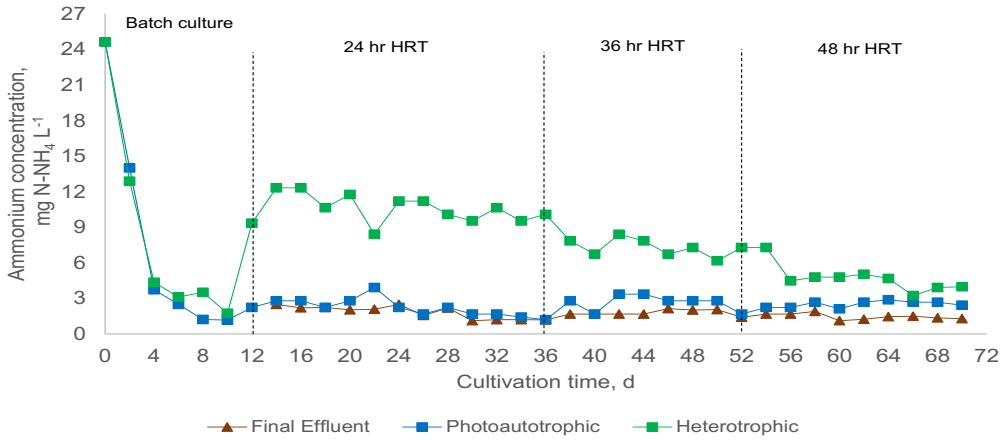
The initial P concentration in the media feeding the system was 15 mg P L^{-1}. This P concentration decreased over period of the continuous culture with range of value 9.1 to 8.9, 8.27 to 8.25, and 7.7 to 7.4 at the end of HRT 24, 36 and 48 hr respectively (Table 6.2). As a result an average of P recovery from the synthetic wastewater increased from 34 to 50 % under the three HRTs tested. The recovery significantly increased at 10% when moving from 24 to 36hrs HRT and only 1% when HRT was changed from 36 to 48 hrs (Figure 6.2b). Whereas, in batch culture conditions, *C.reinhardtii* 11/32C recovered P from the media with efficiency ranging from 22 to 70% in media containing P concentrations between 50 and 120 mg P L^{-1} (*data not shown*). Previous studies using *C.reinhardtii* have also revealed that this strain could remove approximately 15% of P from anaerobic digestate in a batch culture with the initial P concentration was 121 (mg P-TP L^{-1}) using a BIOCOL reactor (Kong et al., 2010b). Thus, these results confirm the benefits of using a continuous flow cultivation system, which is effective at recovering P via biological algal uptake.

With regard to nitrogen recovery and control, the initial ammonia concentration was 25 mg N-NH_4^+ L^{-1}, which was significantly reduced in the final effluent. As *C.reinhardtii* 11/32C prefer ammonium as nitrogen source, the final concentration of NH_4^+ was 1.7 mg N-NH_4^+ L^{-1} (Figure 6.10a). This result is consistent with the previous experiment describe in Section 4.3.2 and also with research works conducted with *C.vulgaris* in a continuous flow culture (de-Bashan et al., 2002), and *Scenedesmus obliquus* in a

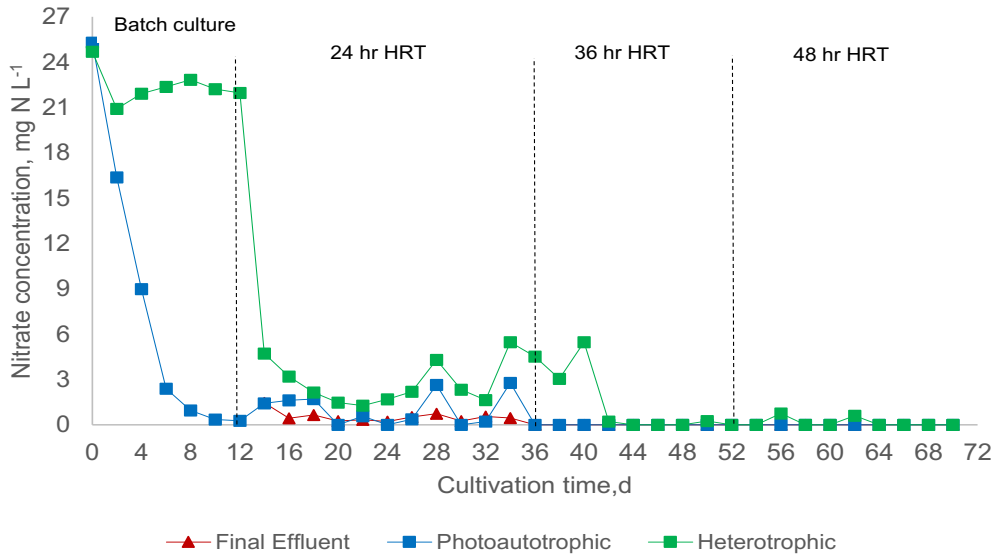
semi-continuous flow culture (Voltolina et al., 2005). The final concentration obtained in the final effluent would meet the discharge consent for NH_4^+ in a large wastewater treatment works in the UK (< 3 mg N-NH_4^+ L^{-1}) (Marsden et al., 2012).

Surprisingly, NO_3^- concentration was completely removed from the culture media in the final effluent (Figure 6.10b) with an approximately 100% removal by day 36 and remained minimal or zero for the rest of the experiment. The removal of both N sources have resulted in the assimilation of N into algal biomass, with N content in algal biomass between 3 and 13% dry weight. The maximum content of N in *C.reinhardtii* 11/32C was above the typical figures reported for green microalgae, which is 10% (Camargo-Valero et al., 2010; Hu, 2007). With the initial NH_4^+ and NO_3^- concentration was 25 mg N L^{-1} for each nitrogen source, on average 96% of nitrogen (i.e. NH_4^+ and NO_3^-) was recovered via biological algal uptake (Figure 6.10a and 6.10b).

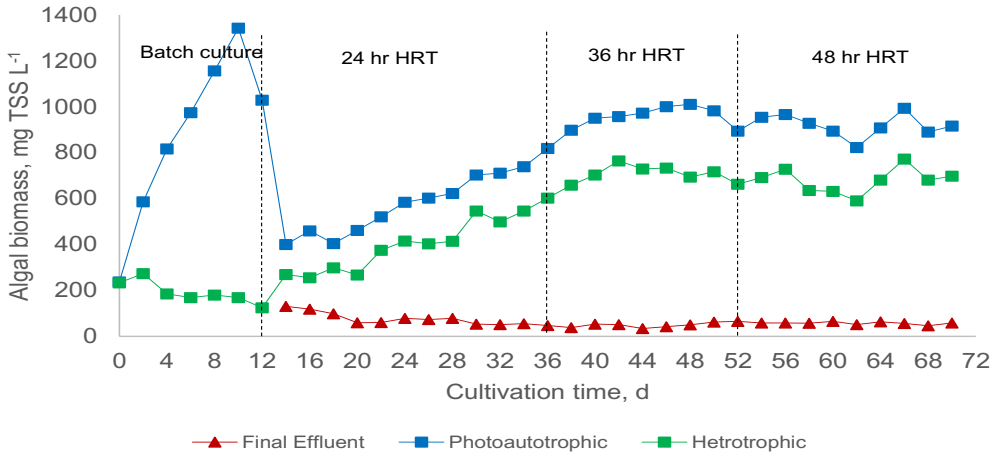
Algal biomass concentration in the final effluent reached 62 mg TSS L^{-1} (Figure 6.10c). This algae biomass concentration is still higher than the discharge consent quality for TSS from treated sewage which should be < 20 mg TSS L^{-1} for a large wastewater treatment works (Marsden et al., 2012). Water samples from each treatment unit (i.e., HTR, PBR and final effluent tank) can be visualised from Figure 6.11, where it is possible to observe the remaining algal biomass concentration in the final effluent (Figure 6.11 c) and the need to optimise biomass recovery after achieving nutrient control via biological uptake. Thus, further work is needed to reduce the algal biomass (TSS) in the final effluent (< 20 mg TSS L^{-1}).



a. Ammonium concentration in the media over period of 70 days

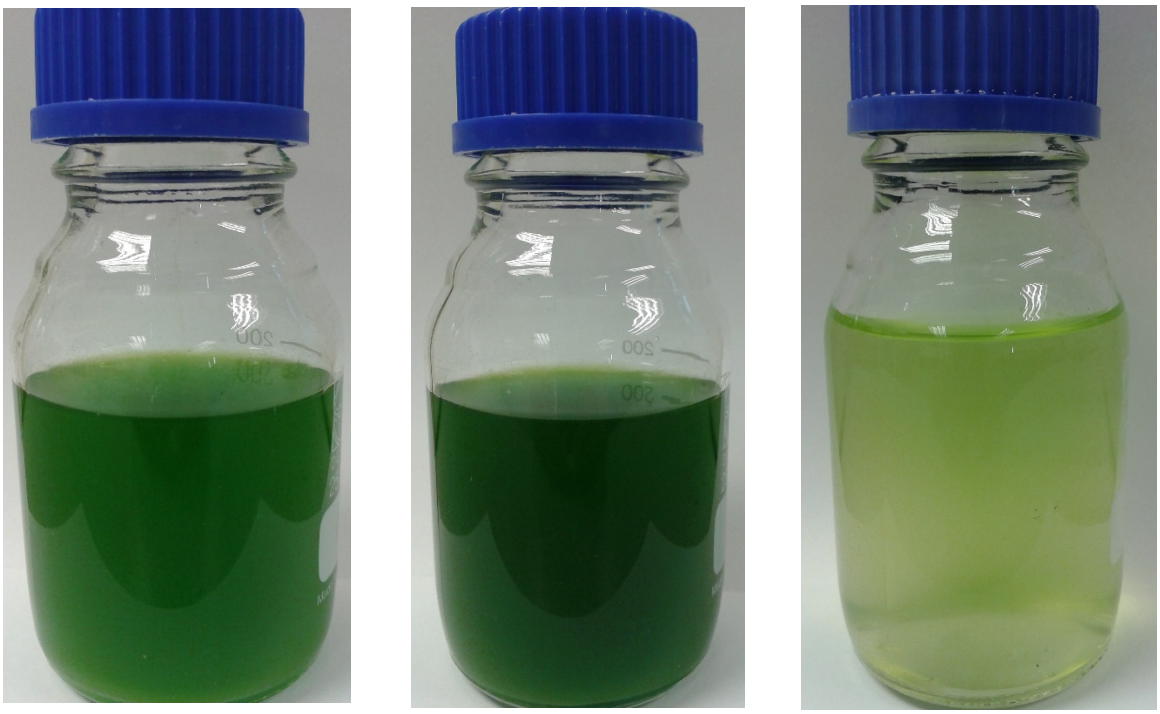


b. Nitrate concentration in the media over period of 70 days



c. Algal biomass as total suspended solids in continuous culture system

Figure 4-35 Water quality characteristics in the reactor and final effluent



a b c

Figure 4-36 Algal biomass in (a) heterotrophic reactor, (b) photoautotrophic reactor, and (c) final effluent

6.4 Discussion

6.4.1 Algal growth in the continuous flow mixotrophic culture

This study aimed to investigate algal biomass production and P recovery from synthetic wastewater via biological algal uptake in a continuous flow system combining heterotrophic and photoautotrophic growth conditions (mixotrophic mode). Previous studies have reported that algal biomass production under heterotrophic growth conditions can achieve high-density cultures and overcome the limitations regarding poor light penetration when high-density cultures are kept under photoautotrophic conditions (Bumbak et al., 2011; Mohan et al., 2015; Perez-Garcia et al., 2010). In this study, and perhaps due to the arrangements made for the treatment units (i.e., HTR followed by PBR), the resulting cell density in the heterotrophic reactor was lower than in the photoautotrophic reactor (Figure 6.2 and 6.11). That distinctive characteristic in the tested system could be attributed to bacterial infection in HTR and the progressive increment of HRT as illustrated in Figure 6.4. In this condition, algae and bacteria competed for nutrients, which may affect algal growth (Bumbak et al., 2011; Zhang et

al., 2012). It could be the case that in order to reduce bacterial growth, the photoautotrophic stage could be used as the first treatment unit, which could help to increase algal biomass production in the heterotrophic unit and to achieve higher in-cell P content in harvested algal biomass.

The combination of autotrophic and heterotrophic cultivation of microalgae has the potential to be implemented in existing wastewater treatment works with the added benefit of producing additional feedstock for energy production via anaerobic digestion. Under limited light conditions, some alga strains can adjust their metabolism from autotrophy to heterotrophy and therefore, they would be able to utilize both inorganic and organic compounds from the external media (Becker, 1994). Mixotrophic culture promotes maximum biomass productivity as a result of the combination of autotrophic and heterotrophic conditions and particularly when it is possible to use low-cost sources of carbon to support algae growth (i.e., flue gas from CHP units as inorganic carbon source and remaining organic carbon from conventional activated sludge units or acetate obtained from anaerobic digesters) (Bjornsson et al., 2013; Smith et al., 2015).

In addition, this study demonstrates that algae biomass concentration increases at longer HRT. These results demonstrated that applications at a larger scale should balance biomass production and the effects on nutrient depletion along the process, particularly when using a series of reactors, Therefore, the decrease in biomass productivity in the continuous mixotrophic culture would have be predicted due to the reduction of the nutrient loading rate (Liu et al., 2016; Xu et al., 2014) and for that reason, the recycle of harvested algal biomass from the sedimentation unit into the first treatment vessel (HTR) is important to increase algae biomass concentration and productivity. This approach is particularly useful in algae culture with low growth rates – i.e., low temperature conditions (Lee and Shen, 2004).

6.4.2 Intracellular P accumulation

The results of intracellular P uptake demonstrated that HRT influences the amount of P metabolised into algal cells and hence, higher HRT would benefit higher in-cell P content. That benefits P recovery from wastewater and promotes the accumulation of

polyphosphate. This characteristic, follows by an increment of algal biomass concentration with longer HRT, may suggest that intracellular P uptake controls algal growth (Rhee, 1973).

The concentration of P in the algal biomass was affected by the availability of N and as a result, the recovery of P by microalgae is also determined by N concentration in the media (Beuckels et al., 2015). Similar finding were previously described in Chapter 4.

Intracellular P uptake in PBR was slightly higher than in HTR. Presumably, it was caused by the introduction of continuous illumination that drives phosphate uptake in algal cells (Jansson, 1988). In the thylakoid membrane, which is located inside the chloroplast, the incoming light powered the movement of electrons through the electron chain to produce a biochemical reductant (NADP). Simultaneously, protons that split from the water are transformed from the stroma into the thylakoid membrane and create a positive charge, which drives the synthesis of adenosine triphosphate (ATP). At the same time, adenosine diphosphate (ADP) also reacts with phosphate to absorb energy and to form ATP in the cells and forms energy-rich bonds. Therefore, algae take up more P in the presence of light to incorporate it in the production of ATP (Borchardt and Azad, 1968; Jansson, 1988; Masojidek et al., 2007).

Although the assimilation of external P increased over the cultivation period, intracellular P uptake rates slightly dropped with the increment of HRT. That is assumed as a result of the influence that the increased number of algal cells may have over P concentrations in the media due to the natural competition for nutrients (Jansson, 1988). That also can be explained due to the induced dilution generated as a consequence of increasing HRT, thus the nutrient loading rate is reduced and that affects algal growth and nutrient uptake rates. This finding was similar to others reported for bacteria (Xu et al., 2014) and *Scenedesmus obliquus* (Ruiz et al., 2013).

6.4.3 Water quality in the final effluent

In regard to P removal from the aqueous phase, the ability of *C.reinhardtii* 11/32C to recover P from the synthetic wastewater was quite high (50%). The P recovery was gradually improving with longer HRTs, which indicates that algal growth and nutrient

uptake are correlated, as the main mechanism for P removal is biological algal uptake. In this study, the initial P concentration of 15 mg P L^{-1} was used to feed the algae under continuous flow of the mixotrophic microalgae cultivation. This concentration was referred to the kinetics for P uptake in which half saturation constant was closed to 15 mg P L^{-1}. Moreover, according to the wastewater characterisation the average concentration of P in the effluent of activated sludge was 13 mg P L^{-1} and therefore, a concentration of 15 mg P L^{-1} was chosen for the design of the continuous mixotrophic microalgae system.

With regard to nitrogen removal, biological nutrient uptake was also identified as the main mechanism for ammonium and nitrate control. According to the experiment reported previously in a batch culture, *C.reinhardtii* 11/32C preferred NH_4^+ over NO3$^-$, creating a simultaneous effect that triggers an increment in intracellular P uptake rates. The maximum content of N found in the culture of *C.reinhardtii* 11/32C was higher than the typical values commonly reported for green microalgae and therefore, the removal of NH_4^+ in the continuous flow system was particularly efficient with a final concentration that could meet the currently set discharge consent for large sewage treatment works (< 3 mg N-NH_4^+ L^{-1}) (Marsden et al., 2012). The results also confirmed that the continuous flow mixotrophic culture could completely remove NO_3^- from the liquid, which will contribute to effective nitrogen recovery from wastewater.

The average TSS concentration in the final effluent was quite high (62 mg TSS L^{-1}), despite the fact that a polymer (i.e., Zetag 50) was added to facilitate algal biomass sedimentation. High content of solids in the final effluent was influenced by the poor settling capacity of the algae, which has been identified as one of the major limiting algal biomass recovery (Choi et al., 2010). This is particularly difficult to achieve with *C.reinhardtii* 11/32C as *Chlamydomonas reinhardtii* strain have flagella and mobile within the media and therefore difficult to form flocs. The flagellated algae can avoid the flocculation and swim out from the flocs (Gerardo et al., 2015). That can interrupt the separation process solid-liquid even under the presence of polymer aids (Gerardo et al., 2015).

Several microalgae harvesting techniques such as sedimentation, centrifugation, flotation and filtration have applied to remove algae from the solution. Algal sedimentation is the simplest method for algae harvesting, but the velocity of the settling depends on the algae strain and their size, therefore the actual velocity is vary between 0.4 to 2.2 m d^{-1} (Gerardo et al., 2015; Milledge and Heaven, 2013; Peperzak et al., 2003). This method requires low capital cost and operational, however land requirement for the construction of a pond is very high (Singh et al., 2011).

Centrifugation is the most rapid method to remove algae from the liquid which based on the cell size and the cell density between biomass and media (Christenson and Sims, 2011). It provides high recovery rate because the algae can concentrated very easily (Gerardo et al., 2015), but at a large scale this method is considered as an energy intensive due to very high energy consumption. Similarly, high energy requirement is also needed by dissolved air flotation method for about 7.6 kWh m^{-3} due to high pressure required to supersaturate flotation with air (Wiley et al., 2009).

Filtration is also considered as an effective to harvest algae that may gain up to 100% cell retention and recovery (Gerardo et al., 2014). In addition, there is no addition of chemical which lead to integrate this method with microalgae cultivation for bioenergy production as it has no requirement for chemical extraction from the algal biomass (Gerardo et al., 2015). In order to gain high algal biomass concentration and low energy requirement for the algae harvesting, further research is needed to investigate eco harvesting for microalgae cultivation. As an example the use of *Moringa oleifera* can be considered as bio-flocculants for microalgae harvesting (Yang et al., 2016; Hamid et al., 2016).

The presence of algal cells in the final effluent can also increase chemical oxygen demand (COD) due to the content of organic matter in algal cells. A substantial proportion of COD in the effluent of aerated lagoon due to the presence of algae also reported by Gronlund et al. (2004) and Khorsandi et al. (2014).
Thus, further research should be done to study about the key properties of algae and their motile characteristics, particularly *C.reinhardtii* 11/32C and a favourable settling method for harvesting microalgae.

6.5 Conclusions

The continuous flow cultivation of microalgae under mixotrophic conditions confirmed that such system has the potential to be implemented in existing wastewater treatment works with the aim to contribute to nutrient recovery and control and algal biomass production. The intracellular P content in algal biomass samples collected from the heterotrophic reactor was higher than in the photoautotrophic reactor. In order to hold the polyphosphate inside algal cells for a longer period, it is suggested to place the heterotrophic culture after the photoautotrophic reactor.

In regard to the quality of the final effluent, the concentrations of NH_4^+ and NO_3^- would meet the discharge consent for a large sewage treatment works. Interestingly, N content in algal biomass was higher than reported values for green microalgae and that give great confidence in using this systems for simultaneous nitrogen and phosphorus removal. However, further studies are needed to find the optimum organic carbon concentration for heterotrophic growth; to reduce bacterial infection (or to proper study the effect of bacteria-algae symbiosis), to find the most suitable HRT for algal growth and intracellular P uptake; and to improve the final effluent quality regarding P content.

Chapter 7 Assessing the potential for implementing P recovery via biological algal uptake at a large sewage treatment works

7.1 Introduction

The increase pressure for delivering “good water quality status” in all rivers has driven the UK water industry to continue reducing the impact from sewage discharges, mainly from nitrogen and phosphorus. These substances should meet a maximum concentration in the final discharge, but a near future bringing more stringent discharge consents is demanding sustainable solutions not only for nutrient control, but recovery as well. This research was conducted to study the potential use of algal-based technology for P control and recovery, by assessing the consequences of implementing such technology in an existing sewage treatment works. In order to do so, a case study was conducted at Yorkshire Water’s Esholt Wastewater Treatment Works (WWTW). Initially, the current treatment process was evaluated by conducting a nutrient mass balance analysis and then, assessing how a microalgae system could be implemented to retrofit the existing wastewater treatment works there.

According to the results found in this work, the water quality in the final effluent at Esholt WWTW met the required discharge consents according to the current criteria set by the Environment Agency (EA), the Fresh Water Directive (FWD) and the EU Urban Waste Water Directive (UWWD). However, and despite the new process in place for bioenergy generation via anaerobic digestion, the site still utilise over 50% of the total energy required for the entire treatment process in keeping running the activated sludge process for the removal of ammonia, carbonaceous organic matter and phosphorus with very little opportunities for resource recovery, apart from carbon for methane production. Nevertheless, current heat and power production on site is not enough and imported energy from the grid is required for the treatment, which will inevitably keep increasing due to population growth in the catchment area and new regulations expected to be implemented ($P<0.1$mg P L^{-1}).

In order to help with the development of more efficient nutrient control systems and to reduce the burden regarding energy consumption, the potential to implement

microalgae systems for nutrient recovery from wastewater (mainly P via biological algal uptake), coupled with algal biomass production (as feedstock for AD) was studied in this work. According to the previous chapter, the continuous mixotrophic microalgae system could generate minimum of 2.6 g L^{-1} and maximum of 3.6 g L^{-1} daily of harvested algal biomass which is fed with 4.5 L d^{-1} of the synthetic wastewater. This amount of biomass can be co-digested with sludge to enhance biomethane production in which this co-digestion can increase bio-methane production up to 61% (Menger-Krug et al., 2012).

The results reported in this chapter are aimed to reveal the potential of implementing a continuous flow microalgae system in an existing wastewater treatment works. This proposed microalgae system would be able to help meeting the discharge consents set for NH_4^+ and PO_4^{3-}, giving realistic alternatives for N and P recovery and increasing bioenergy production to achieve a carbon and energy neutral wastewater treatment.

7.2 Methodology

This study investigated the potential of introducing a continuous flow microalgae system in an existing sewage treatment works. A case study was conducted at Esholt WWTW, which initially characterises the current performance of the actual treatment systems by conducting a nutrient mass flow analysis on site. This analysis was mainly focused on N and P species by interpreting lab results from wastewater samples collected at different sampling points. In addition to that analysis, previous findings from the study conducted in a continuous flow mixotrophic microalgae system (Chapter 6) were used to prove the hypothesis that P recovery via biological algal uptake from wastewater can be a sustainable alternative to traditional nutrient control strategies. A process model was also conducted to determine the capacity for nutrient removal that a continuous flow microalgae system may have if used to replace the existing nutrient control process at Esholt WWTW.

7.3 Results

7.3.1 Case of study: nutrient control at Esholt Wastewater Treatment Works

The Yorkshire Water's Esholt Wastewater Treatment Works (WWTW) is located in Bradford, West Yorkshire, UK. It treats a mix of domestic and industrial wastewater coming from a population equivalent (p.e.) of 730,000; the treated effluent is discharged into the River Aire (Marsden et al., 2012). The original treatment works (i.e., trickling filters) started operations 100 year ago, but it was upgraded with a modern activated sludge system to meet more stringent discharge consents in the final effluent 7 years ago (i.e., 20 mg L^{-1} suspended solids; 10 mg L^{-1} BOD; and 3 mg L^{-1} ammonia).

In order to meet these new requirements, Esholt uses up to 85,563 kWh/d to treat the sewage, but this energy consumption was flow dependant and on average would be nearer to 63,000 kWh/d. In response to the EU Urban Wastewater Directive, the UK water industry consumes a total of 9,016 GWh per year (Water-UK, 2011), from which 50% is used for wastewater processes (Water-UK, 2016). In addition, the total energy consumption in 2011 had a net greenhouse gas emissions of 5.01 Mt CO_2 (Water-UK, 2011). Esholt upgraded the old treatment works and in September 2009, several treatment processes were commissioned for wastewater treatment and onsite renewable energy generation, including a new inlet works, a hydro turbine, six primary settling tanks, a brand new activated sludge process, four new secondary settling tanks, three anaerobic digesters and two CHP units (Marsden et al., 2012).

The current process layout was implemented by the Esholt WWTW in order to meet the required consents and to improve efficiency in the process. For instance, a hydro-turbine was installed between the inlet works and the primary settling tank (PST) to generate electricity; new PSTs were constructed to improve solids removal; 12 multi-stage activated sludge reactors (ASP) were also constructed to control organic matter, ammonium and phosphorus; reuse of bio-solids from the humus tank and final settling tank (FST); and an upgraded anaerobic digestion (AD) process for bioenergy generation.

To investigate the current nutrient flow on site, wastewater samples were collected from different sampling points at Esholt WWTW and analysed at the labs of the Institute for Public Health and Environmental Engineering (iPHEE), School of Civil Engineering, University of Leeds. The samples were collected on a monthly basis over a period of 15 months from May 2014 to February 2016; mean lab results (n=15) are presented in Table 7.1.

Table 4-8 Wastewater characterisation and removal efficiency at Esholt WWTW

Parameters	Unit	Inlet	After Primary Settling Tank	After Final Settling tank	Final Effluent	Removal efficiency (%)
TKN	mg N L-1	60.4	25.5	3.0	1.8	97
NH_4^+	mg $N\text{-}NH_4^+$ L^{-1}	45.0	18.2	1.3	0.7	98
NO_3^-	mg $N\text{-}NO_3^-$ L^{-1}	7.7	5.5	9.0	9.0	-
PO_4^{3-}	mg $P\text{-}PO_4^{3-}$ L^{-1}	5.3	1.9	0.9	1.0	81
TP	mg P L^{-1}	6.4	2.2	1.1	1.0	84
COD	mg COD L^{-1}	704.4	272.5	54.3	46.4	93
VSS	mg VSS L^{-1}	289	82	29	21	93

7.3.1.1 Overall nutrient and organic matter removals

It can be seen from Table 7.1 that the process is able to remove 97% and 98% for TKN and NH_4^+, respectively. The concentration of total nitrogen and NH_4^+ decreased from 60 to 2 mg N L^{-1} and from 45 to 0.7 mg $N\text{-}NH_4^+$ L^{-1}, respectively. The total nitrogen and NH_4^+ concentration in the final effluent have fulfilled the discharge consent set by the Water Framework Directive (WFD, 2000). In contrast, NO_3^- concentration in the final effluent increased 1.2 times in comparison with the raw wastewater. Although there is no discharge consent for nitrates, the nitrate concentration of 9 mg $N\text{-}NO_3^-$ L^{-1} (40 mg NO_3^- L^{-1}) in the final effluent is still below the maximum value set for nitrates in the EU Drinking Water Directive (ND, 2010; DWD, 1998) (<50 mg NO_3^- L^{-1}) – i.e., nitrates are not removed in a conventional drinking water treatment works and downstream of the discharge, the presence of high concentrations of nitrates in the

raw water could be problematic. For a modern multi-stage activated sludge process, the NO_3^- concentration of about 9 mg NO_3^--N L^{-1} falls within the range of values expected, particularly considering the COD:N ratio in the raw wastewater (>5) (Lin et al., 2016).

In terms of phosphorus, the removal of SRP and TP were 81% and 84% respectively and the concentration of SRP in the final effluent was 1 mg P L^{-1}, meeting the current target for SRP set in the Urban Wastewater Treatment Directive (UWWTD, 1991). Considering that an additional load of P coming from the anaerobic digester (digested liquor) is added to the activated sludge process at Esholt WWTW, the current system is constantly re-processing P at higher operational costs. There was no other particular P removal process applied on the site, apart from the activated sludge process. The quality of the final effluent (including SRP) improved through tertiary treatment by using slow sand filter.

Organic matter reported in the form of COD and volatile suspended solids (VSS) was efficiently removed (up to 93%), with a final concentration of 46 mg COD L^{-1} and 21 mg VSS L^{-1}, both in line with the EU Urban Wastewater Treatment Directive (UWWTD, 1991).

In general, the quality of the final effluent at Esholt WWTW fulfils the current regulation framework for wastewater treatment. However, further analysis is required to investigate the fate of C, N and P that have been converted into gas or bond within the waste sludge. This analysis will be described in the following sections. Moreover, the incoming wastewater flow rate and the flows within the process were also gathered from the site and they are listed in Table 7.2. These flow rates are used to support mass balance analysis.

Table 4-9 Typical Wastewater flow rates at Esholt WWTW

Treatment Unit	Flow rate (Q), $m^3 d^{-1}$
Inlet	
▪ After storm overflow	280,022
Primary Settling Tank	
▪ 5 tanks operated	279,936
▪ 6 tanks operated	279,936
Activated Sludge Process	
▪ Inlet work	280,022
▪ Digested liquor	1,598
▪ Filtrate return	1,440
▪ Return activated sludge (RAS)	223,949
Secondary Settling Tank	
▪ 11 tanks operated	280,368
▪ 12 tanks operated	279,936
Tertiary Tank	280,022

Source: Esholt Wastewater Treatment Works

According to Esholt WWTW flowrate, the inlet works before storm overflow was a maximum of 6,879 L s^{-1} and the overflow after the storm was 3,241 L s^{-1}. Each primary tank takes a maximum of 648 L s^{-1} with 1 tank out of service or 540 L s^{-1} with all six tanks in service. Furthermore, the selector has 3,241 L s^{-1} passed forward from inlet works, the centrifuge liquor at 55.5 L s^{-1} for 20 minutes out of every hour. The filtrate return of 50 L s^{-1} for 20 minutes out of every hour and return activated sludge (RAS) was 2,592 L s^{-1} with 100 L s^{-1} of this going to the front end of the selector. Afterwards, each secondary tank takes a maximum of 270 L s^{-1} with all tanks in service or 295 L s^{-1} with eleven tanks in service. Finally, the tertiary treatment takes a maximum of flowrate 3,241 L s^{-1}. All flowrate then multiply by 86,400 second day^{-1} in order to get the value that listed in Table 7.2.

7.3.1.2 Seasonal changes in nutrient concentration at Esholt Wastewater Treatment Works

- *Nitrogen species*

The seasonal changes in concentration for nitrogen species (NO_3^-, NH_4^+ and organic nitrogen) are reported in Figure 7.1. The NO_3^- concentration in all treatment units was higher in summer than in winter. In raw wastewater, the average concentration of

NO_3^- in the summer was 9.5 mg NO_3^-- N L^{-1} and in winter was 3 mg NO_3^-- N L^{-1}; a similar trend occurred for NH_4^+ and organic N concentration. On average, the NH_4^+ and organic N concentration in the final effluent were less than 2 mg NH_4^+- N L^{-1} and 1.9 mg N L^{-1}, respectively.

An organic nitrogen refer to all N that present in organic matter in which in domestic wastewater it presents in the form of proteins or degradation products such as polypeptides and amino acids (Sawyer et al., 2003). A sum of NO_3^-, NH_4^+ and organic nitrogen is represent as total nitrogen (TN). According to the UWWTD, the requirement for discharge limit of TN from urban wastewater treatment works with a population more than 100,000 p.e. is 10 mg N L^{-1} (UWWTD, 1991). In addition, the fraction of organic nitrogen can account for 40% to 85% of TN (Qin et al., 2015) which indicate the stringent discharge limit for TN between 4 and 8.5 mg N L^{-1}. In this study the concentration of organic nitrogen from wastewater was 1.9 mg N L^{-1} and therefore, it still below the discharge limit.

- *Phosphorus species*

The fluctuations of phosphorus species (PO_4^{3-} and organic phosphorus) captured in this study are shown in Figure 7.2. The PO_4^{3-} concentration in raw wastewater fluctuated within the 15-month sampling period. On average, the PO_4^{3-} concentration in summer 2014 and summer 2015 was almost the same (6 mg P L^{-1}) and in winter 2014 - 2015 the average PO_4^{3-} level decreased to 4.9 mg P L^{-1}, while in winter 2015 - 2016 dropped to 3.3 mg P L^{-1}.

On the other hand, the average PO_4^{3-} level in the final effluent was higher in winter 2015 - 2016 compared to the previous seasons. Whereas in June 2014, PO_4^{3-} concentration in the final effluent reach the peak of 2 mg P L^{-1}, afterwards the concentration decreased gradually until July 2015 to approximately 1 mg P L^{-1}.

Similarly, the same trend also occurred for the organic P concentration in the final effluent in which the mean concentration in summer was higher than in winter. Even though the organic phosphorus concentration in the final effluent increased more than

1 mg P L^{-1} in January and February 2016, but on average the mean concentration was 0.64 mg P L^{-1}.

P is delivered into the receiving water as a mixture between dissolved and particulates and it also biologically active element. The particulates (organic phosphorus) can be released into the water column by enzymatic hydrolysed into orthophosphate which is bioavailable for algae (Correll, 1998).

As stated in UWWTD regulation, the discharge limit for total phosphorus (TP) in the final effluent of urban wastewater is 1 mg P L^{-1} (UWWTD, 1991) and the fraction of organic phosphorus account for 26% to 81% of TP (Qin et al., 2015) and therefore, the mean concentration of organic phosphorus in the wastewater final effluent (i.e. 0.64 mg P L^{-1}). Furthermore, on average the concentration of orthophosphate (P-PO_4^{3-}) in the final effluent was 0.98 mg P L^{-1} and sum of both elements are 1.6 mg P L^{-1}. The average of TP concentration was a bit higher than the discharge consent, but the P concentration in the final effluent was not a stringent consent for Esholt WWTW and hence still consider as a very good treatment.

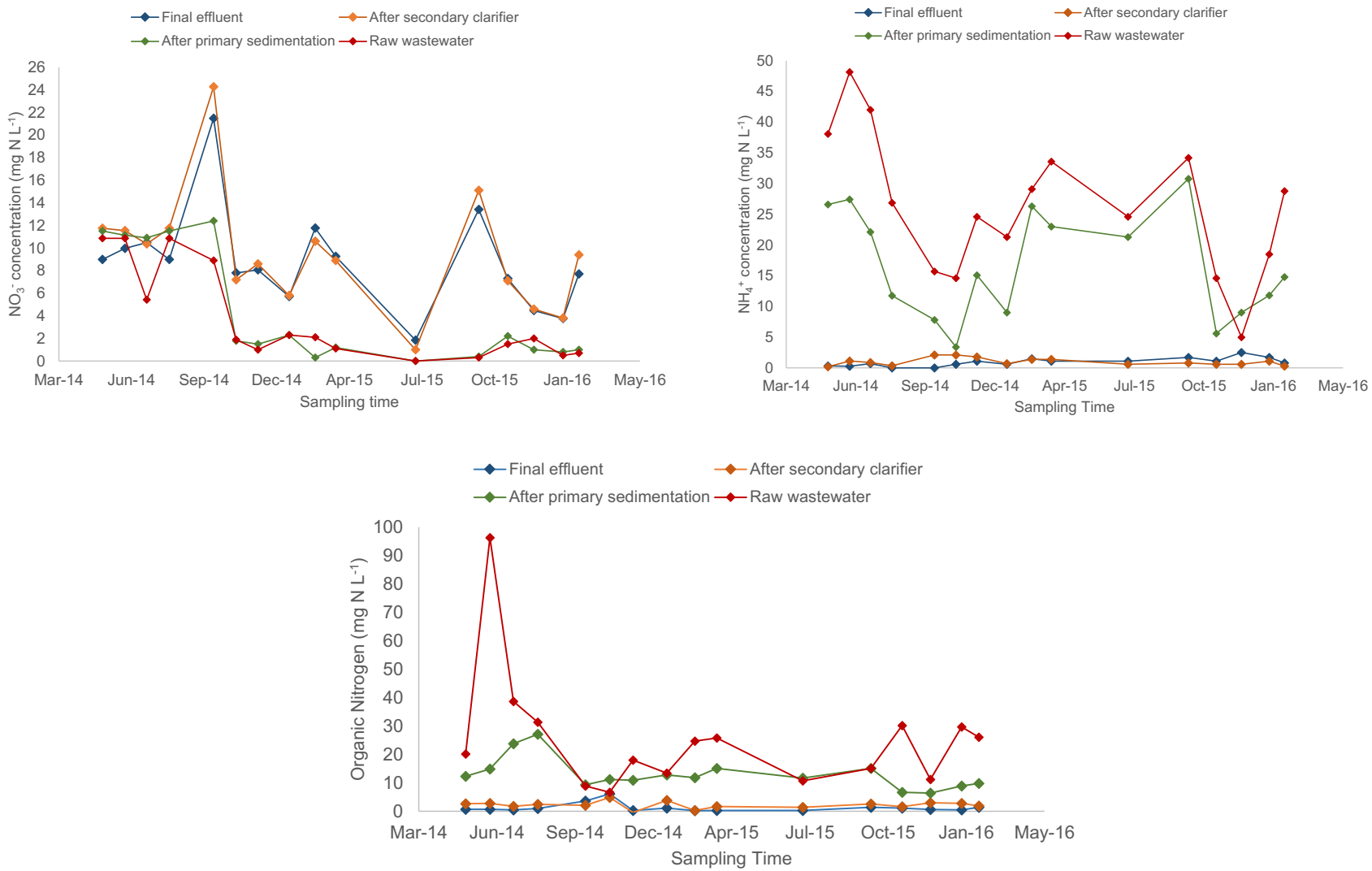


Figure 4-37 Seasonal changes in Nitrogen species at Esholt Wastewater Treatment Works

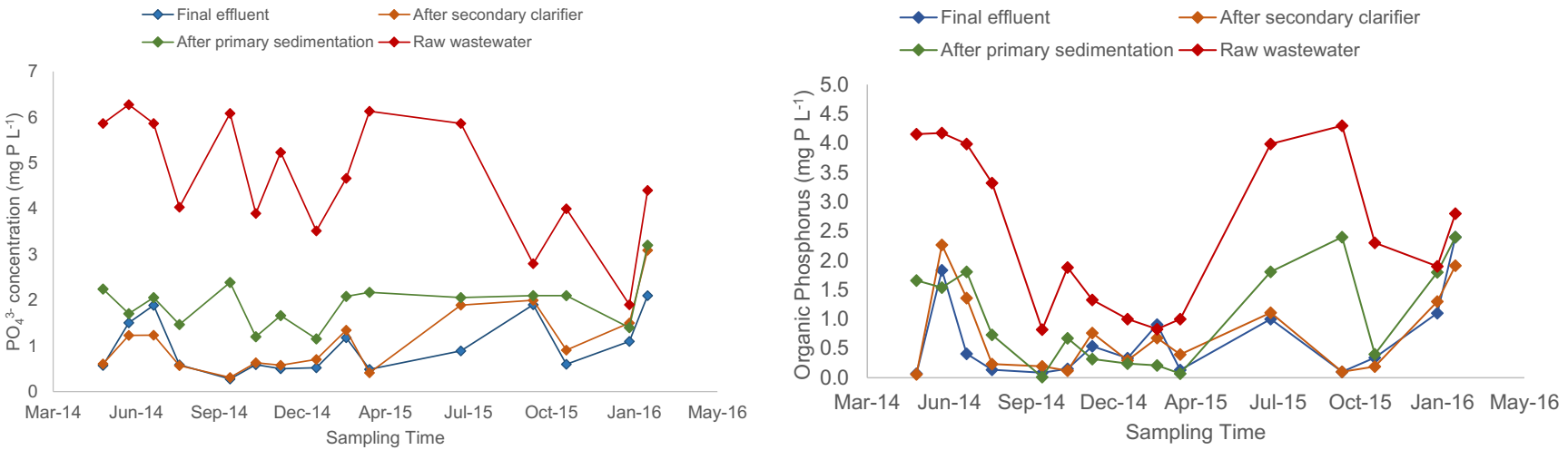


Figure 4-38 Seasonal changes of Phosphorus species at Esholt Wastewater Treatment Works

7.3.2 Mass balance analysis at Esholt Wastewater Treatment Works

The study of mass balance in municipal wastewater treatment works is important to estimate the fluxes of substances and to compare operational condition (Puig et al., 2008). In this study a mass balance of the N and P over the plant at Esholt WWTW was conducted in order to understand the performance and to comprehend the nutrient flow of the wastewater treatment work. The N and P mass balance of Esholt WWTW are described in the following section.

7.3.2.1 Nitrogen balance

Nitrogen in wastewater treatment is eliminated through nitrification and denitrification, which converts NH_4^+ into NO_3^- and NO_3^- into N_2 and N_2O (Dhamole et al., 2007; Mekinia et al., 2009). This elimination pathway is considered as an open system (Nowak et al., 1999; Puig et al., 2008). The nitrogen balance of Esholt WWTW is illustrated in Figure 7.3. It is considered that nitrogen entering the activated sludge system is equal to nitrogen leaving the system.

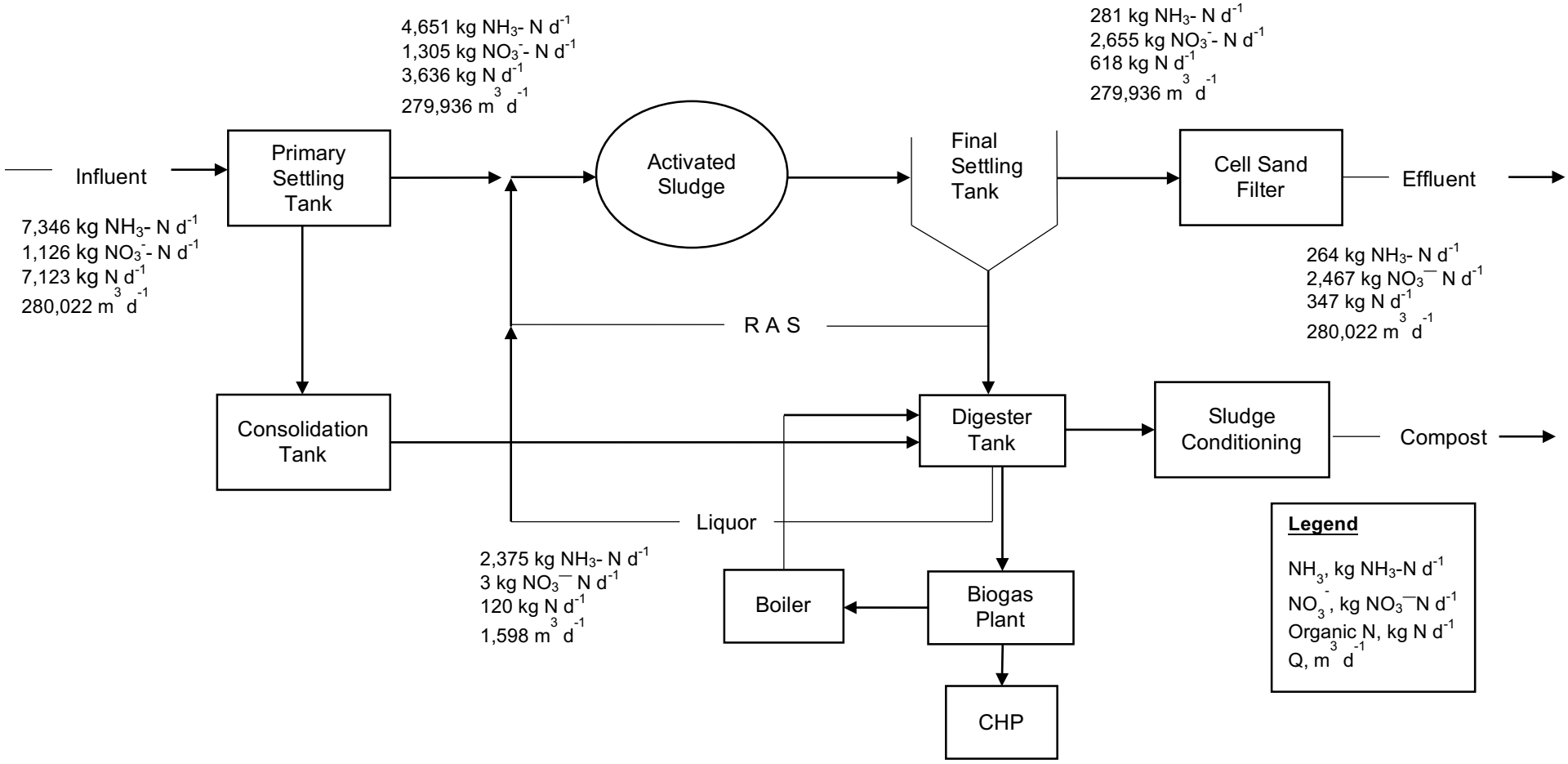
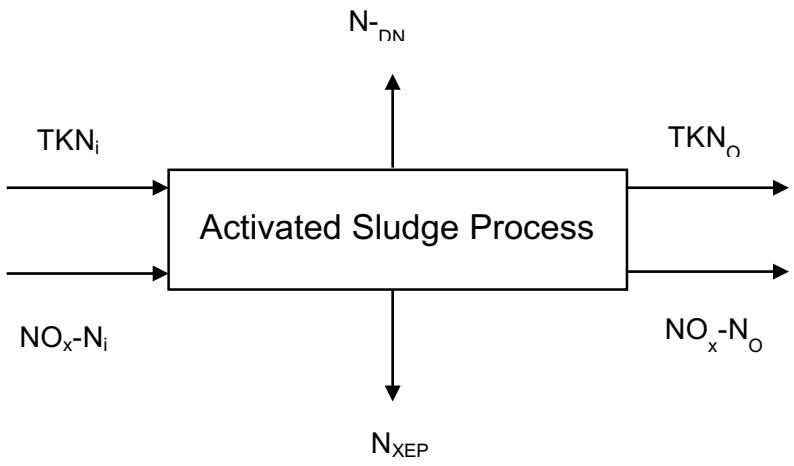


Figure 4-39 Nitrogen mass balance at Esholt Wastewater Treatment Works

According to Nowak et al. (1999) the nitrogen mass balance of the wastewater treatment using an activated sludge system is illustrated in Figure 7.4.



Legend:

TKN_i	TKN in the influent	NO_x-N_O	Oxidised nitrogen in the effluent
NO_x-N_i	Oxidised nitrogen in the influent	N_{-DN}	Nitrogen denitrified
TKN_O	TKN in the effluent	N_{-XEP}	Nitrogen in waste activated sludge

Figure 4-40 Nitrogen mass balance for wastewater treatment using activated sludge system

In order to support the nitrogen balance, the required input data are calculated based on data describe in Figure 7.3 and 7.4. The nitrogen removed via denitrification process and the nitrogen present in waste activated sludge were calculated according to equation 7.1 (Nowak et al., 1999) and equation 7.2. Furthermore, the summary of results are listed in Table 7.3.

$$N_{DN} = TKN_i + NO_{X\_}N_i - TKN_O - NO_{X\_}N_O - N_{XEP} \quad \text{Eq. 4-3}$$

$$N_{XEP} = Org.N_{influent} - Org.N_{effluent} \quad \text{Eq. 4-4}$$

Table 4-10 Data required for nitrogen balance calculations

No	Data required	Unit, Kg d^{-1}
1	TKN in the influent (NH_4-N + org. N)	14,468
2	Oxidised nitrogen in the influent (NO_3^{-*})	1,126
3	Nitrogen in waste activated sludge	3,018
4	TKN in the effluent (NH_4-N + org. N)	612
5	Oxidised nitrogen in the effluent (NO_3^{-*})	2,467
6	Nitrogen denitrified	9,497

*Nitrite concentration was not detected and therefore, oxidised forms of nitrogen are reported as nitrates

Based on the calculation above, the transformation of nitrogen at Esholt WWTW is illustrated in the following figure.

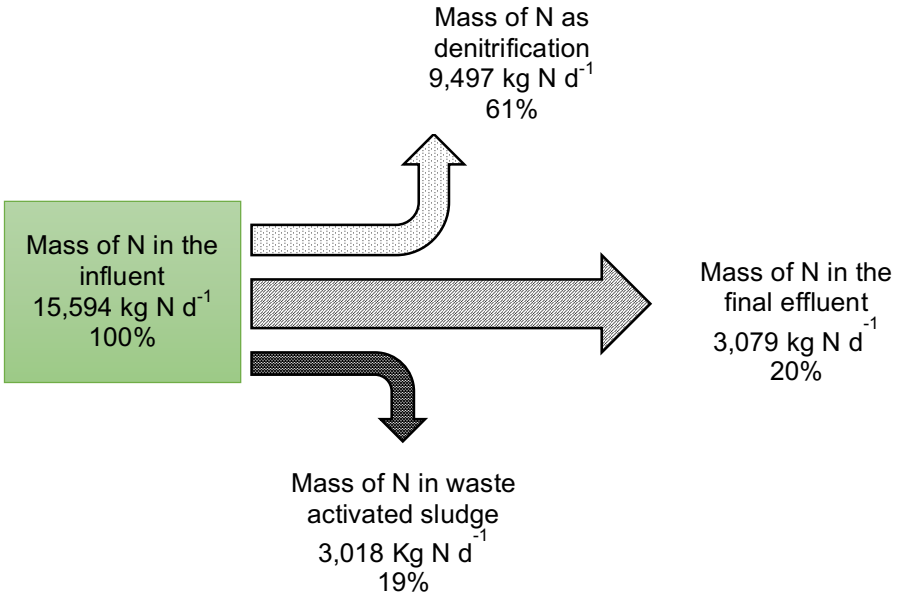


Figure 4-41 Nitrogen transformation in the activated sludge process

The N inflow at Esholt is transformed and transported into several fluxes leaving the multi-stage activated sludge unit, including gaseous emissions (i.e., N_2 and N_2O) and liquid streams (i.e., nitrified effluent and N-rich activated sludge surplus). In simultaneous nitrification-denitrification processes occurring in a multi-stage activated sludge process, ammonium is firstly oxidised to nitrite, then to nitrate and following

anoxic conditions, the nitrate is finally converted mainly into N_2 gas, but with some emissions of nitrous oxide (≈14% of all nitrogen removed by nitrification-denitrification is emitted to the atmosphere as N_2O) (Fernandez-Nava et al., 2010; Mekinia et al., 2009; K.Bernat et al., 2003). The denitrification rate reported at Esholt was higher than the one reported for the Gdansk and Gdynia wastewater treatment works in Poland (temperate climate country), with 25% and 30% of N removal via denitrification, respectively (Mekinia et al., 2009). As a part of denitrification, N_2O is a crucial greenhouse gases and it impacts to the atmosphere is 300 stronger than CO_2 emission. Moreover, decreasing of the N_2O emission rate require high energy for aeration in activated sludge process (Kampschreur et al., 2009; Wu et al., 2014).

The amount of nitrogen bound into the surplus activated sludge and the total of nitrogen present in the final effluent were 19% and 20% respectively, which confirms that the nitrification-denitrification process is very effective at controlling ammonium in the final effluent, but very inefficient at recovering nitrogen or reducing N loads into receiving waters. The percentage of N leaving the system within the surplus activated sludge was lower than the published research conducted in a WWTW with a similar process layout (41.3% to 49.7% of N in the sludge); whereas the N present in the final effluent ranged from 33.3% to 37.4% (Lee et al., 2008).

7.3.2.2 Phosphorus mass balance

An overall phosphorus mass balance was also conducted at Esholt WWTW as described in Figure 7.6. The P commonly follows the wastewater flows and does not leave the system through gaseous emissions (Nowak et al., 1999; Puig et al., 2008). According to Nowak et al. (1999), for the wastewater treatment works that operate an activated sludge system, the phosphorus in the influent is equal to the sum of the phosphorus in the final effluent and in waste activated sludge (equation 7.3).

$$P_i = P_{XEP} + P_O \qquad \text{Eq. 4-5}$$

Based on equation 7.3, the P mass balance in the wastewater treatment works using an activated sludge system is illustrated in Figure 7.7

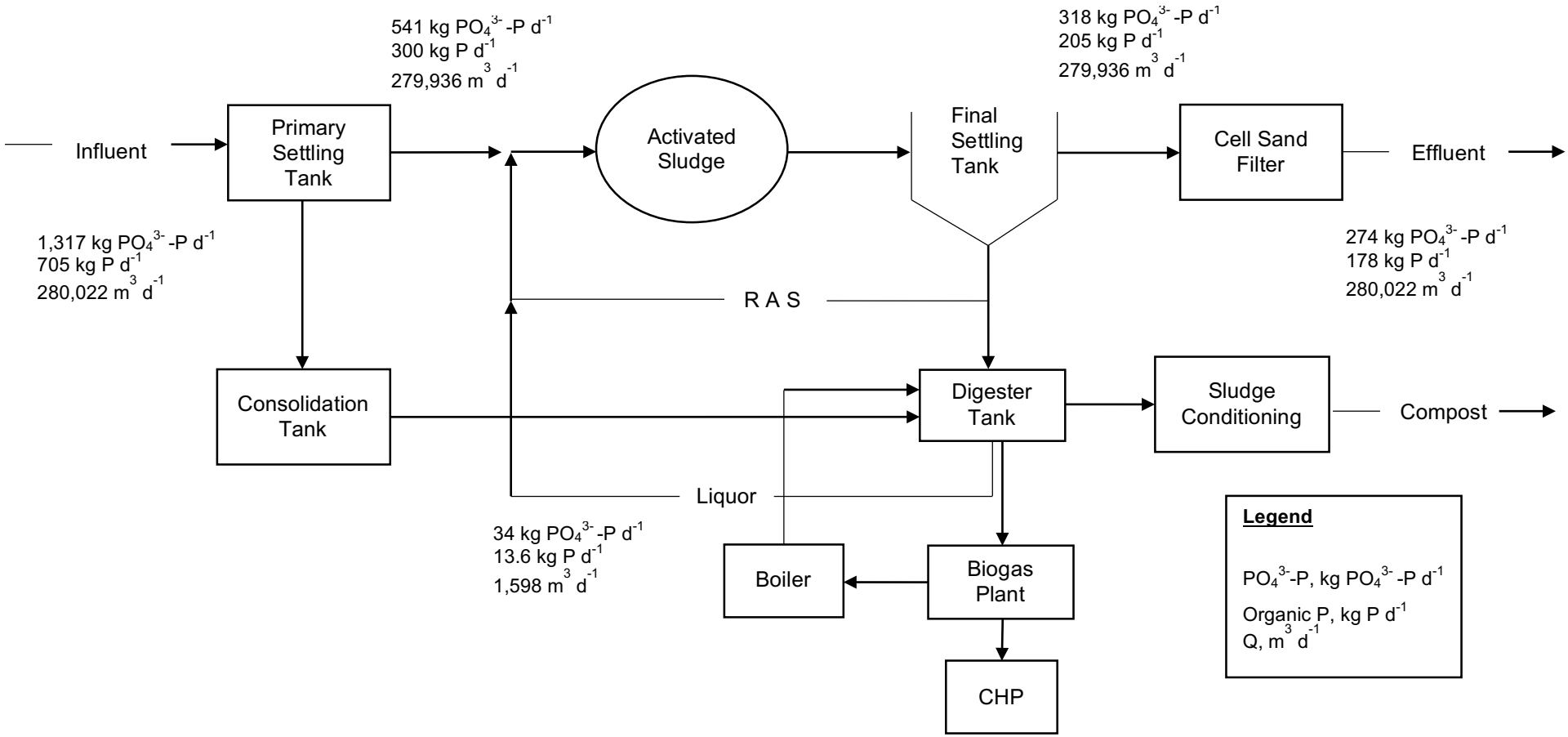
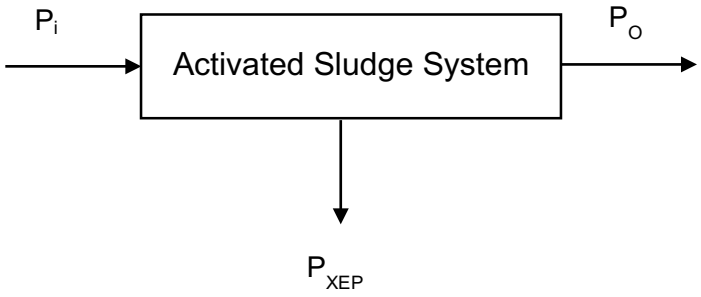


Figure 4-42 Phosphorus mass balance at Esholt Wastewater Treatment Works



Legend:

P_i	Total phosphorus in the influent	P_{XEP}	Phosphorus in waste activated sludge
P_O	Total phosphorus in the effluent		

Figure 4-43 Phosphorus mass balance for wastewater treatment using activated sludge system

The P mass balance revealed that 78% of P in the wastewater was removed through bacterial uptake and incorporated into the surplus activated sludge stream. Whereas the remaining 22% will be further removed during tertiary treatment at Esholt (Figure 7.6). This tertiary treatment is particularly used in period of high turbidity for the final cleaning process to improve wastewater effluent standard before it is discharged into River Aire. This treatment can remove the remaining inorganic compounds such as nitrogen and phosphorus. Furthermore, the total amount of P incorporated into bacterial biomass was 1570 Kg P d^{-1} (78%) (Figure 7.8).

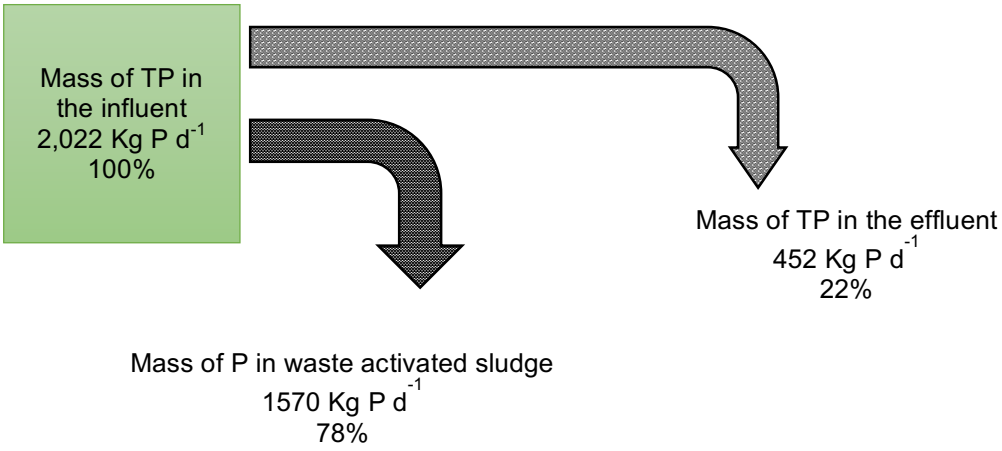


Figure 4-44 Phosphorus transformations in the activated sludge process

7.3.3 Mass balance in a continuous flow mixotrophic microalgae system

A mass balance analysis was conducted to quantify the N and P balance in microalgae under mixotrophic growing conditions which refer to the results in Chapter 6. The N and P mass balances from the continuous flow system tested at lab-scale using *C.reinhardtii* 11/32C are shown in Table 7.4. The recovery of N and P via biological algal uptake were in the range of 95% to 97% for nitrogen and 34% to 46% for phosphorus. During microalgae cultivation in the continuous flow system, the initial nitrogen source was a mix of NH_4^+ and NO_3^-. The ammonium concentration dropped from 25 mg N-NH_4^+ L^{-1} to 1.83, 1.79 and 1.47 mg N-NH_4^+ L^{-1} for 24, 36 and 48 hours of HRT, respectively. Interestingly, the concentration of NO_3^- decreased to nearly zero (i.e., below detection limit), which indicates that almost 100% of NO_3^- was assimilated into algal cells. This biological assimilation contributed to an N content in the algal biomass between 11% and 14% (g N g VSS^{-1}). These values were higher when compared to typical N content reported for *Scenedesmus* sp. (3.1% and 5.9%) in a similar continuous flow system using secondary effluent (Ruiz et al., 2013).

Table 4-11 N and P mass balance from a lab-scale, continuous flow mixotrophic microalgal system

Initial nutrient concentration			Nutrient in the biomass*		Recovery*	Final nutrient* concentration	
Nitrogen mass balance							
HRT	$N\text{-}NH_4^+$ (g m^{-3})	$N\text{-}NO_3^-$ (g m^{-3})	Susp. org. N (g N m^{-3})	$N_{biomass}$ (g N g VSS^{-1})	$N_{recovery}$ (%)	$N\text{-}NH_4^+$ (g m^{-3})	$N\text{-}NO_3^-$ (g m^{-3})
24	25	25	68.78	13.92	95.36	1.83	0.49
36	25	25	110.46	13.18	96.42	1.79	0.00
48	25	25	106.96	11.37	97.06	1.47	0.00
Phosphorus mass balance							
HRT	$P\text{-}PO_4^{3-}$ (g m^{-3})		Susp. org. P (g P m^{-3})	$P_{biomass}$ (g P g VSS^{-1})	$P_{recovery}$ (%)	$P\text{-}PO_4^{3-}$ (g m^{-3})	
24	15		8.72	1.83	34.07	9.89	
36	15		14.00	1.66	44.33	8.35	
48	15		16.09	1.67	46.07	8.09	

* Presented as an average value.

In addition and depending on the HRT, 34% to 46% of the initial P was removed also via biological algal uptake as these amounts correspond to intracellular P found in the effluent of the PBR (9 to 16 mg P L^{-1}), which implied an empirical microalgal P content of about 2%. The amount of P incorporated into the cells was higher than the typical 1% P reported in green microalgae and indicates that luxurious P uptake took place in the continuous flow mixotrophic culture. However, the removal rate of P was lower than 80% and generated a final effluent with a concentration higher than the discharge consent for a large sewage treatment works (<1 mg P L^{-1}). In order to overcome this obstacle, a simple model based on the kinetic study conducted in Chapter 4 was used to assess P recovery in order to determine design parameters to meet a final P concentration <1 mg P L^{-1}. This model is described in the following section.

7.3.4 Modelling phosphorus recovery in a continuous flow mixotrophic microalgal system

A first-order kinetics is commonly used for predicting the removal of organic matter and nutrients in wastewater treatment systems (Horan, 1991). This kinetics is very commonly used for BOD removal in activated sludge processes. In this study, a first-order kinetics is also used to describe removal kinetics for P in continuous flow mixotrophic cultures. Due to continuous mixing occurs in a complete mixed reactor, the effluent concentration is equal to the reactor contents (Metcalf and Eddy, 2004; Horan, 1991). The general form of the mass balance for a complete mixed reactor according to Metcalf and Eddy (2004) is written in the following equation.

$$Accumulation = inflow - outflow + generation$$

$$\frac{dC}{dt}V = QC_0 - QC + r_cV \qquad \text{Eq. 4-6}$$

Under steady-state condition, the solution of the equation 7.4 is written in the equation below.

$$C_e = \frac{C_0}{(1 + k\theta)} \qquad \text{Eq. 4-7}$$

Where C_e is the corresponding effluent P concentration (mg P L^{-1}), C_0 is the initial P concentration (mg P L^{-1}) in any given reactor (either the heterotrophic or the photoautotrophic reactor), k is P uptake kinetic coefficient (d^{-1}), and θ is the hydraulic retention time (d).

The equation 7.5 was used to calculate the kinetics of P removal in each of the three different HRT. The kinetics values are listed in Table 7.5.

Table 4-12 P uptake rate coefficients using *C.reinhardtii* 11/32C

HRT, d	Start-end of each different HRT	P conc. in HTR mg P L^{-1}	P conc. in PBR mg P L^{-1}	P conc. in the FE mg P L^{-1}	k HTR d^{-1}	k PBR d^{-1}	k FE d^{-1}
0.50	C_{d0}	15.00	9.83	9.09	1.05	0.16	0.02
0.50	C_{d36}	9.83	9.09	9.00			
0.75	C_{d36}	15.00	10.12	9.17	0.64	0.14	0.15
0.75	C_{d52}	10.12	9.17	8.26			
1.00	C_{d52}	15.00	10.30	9.21	0.46	0.12	0.22
1.00	C_{d70}	10.30	9.21	7.56			
Average of P removal kinetics					0.72	0.14	0.13

HTR = Heterotrophic reactor, PBR = Photoautotrophic reactor, FE = Final Effluent

The kinetics coefficient (average values) listed in Table 7.5 were used to predict P removal vial algal uptake at the corresponding hydraulic retention time (HRT), in order to meet a discharge consent of 1 mg P L^{-1}. A series of 6 complete mixed reactors equation was used to predict the hydraulic retention time to meet the P discharge consent (i.e. ≤ 1 mg P L^{-1}). This equation is explained below.

$$C_e = \frac{C_0}{(1 + k\theta)^n} \qquad \text{Eq. 4-8}$$

Where n is the number of reactor used in series.

According to the kinetics coefficient (average values) in Table 7.5 and using Equation 7.6, the P consent of ≤ 1 mg P L^{-1} in the final effluent can be achieved within HRT of 1 days (Table 7.6).

Table 4-13 A series of complete mixed reaction to achieved the P consent

HRT	k_{HTR}	C_0	C_{HTR}	k_{PBR}	$C_{0\text{-}PBR}$	C_{PBR}	k_{FE}	$C_{0\text{-}FE}$	C_{FE}
d	d^{-1}	mg P L^{-1}	mg P L^{-1}	d^{-1}	mg P L^{-1}	mg P L^{-1}	d^{-1}	mg P L^{-1}	mg P L^{-1}
0.25	0.72	15	5.56	0.14	5.56	4.52	0.13	4.52	4.38
0.5	0.72	15	4.38	0.14	4.38	2.92	0.13	2.92	2.74
0.75	0.72	15	2.67	0.14	2.67	1.46	0.13	1.46	1.33
1	0.72	15	1.71	0.14	1.71	0.78	0.13	0.78	0.69

In addition, the HRT of 1 day was used to calculate the working volume requirement for HTR and PBR, these calculations were referred to 6 series of heterotrophic and photoautotrophic reactor and one of the settling tank. The calculation of these working volume of the reactors in the algae system (lab scale) and the real algae-based wastewater treatment are explained in the following section.

7.3.5 Design criteria for a continuous flow microalgae system for Phosphorus recovery in an existing wastewater treatment works

According to the results explained in Table 7.6, the HRT of 1 day used to determine the typical working volume of the heterotrophic and photoautotrophic reactor of the mixotrophic microalgae system. The equation 3.3 used to calculate the working volume requirement for heterotrophic and photoautotrophic reactor and the results of a design criteria for a continuous flow microalgae system are described in Table 7.7.

A typical design (Table 7.7) was designed for a small scale of wastewater treatment with a service covering of 2,000 population equivalent (UWWTD, 1991). In the UK approximately of 150 L of water a day is required by a person for a daily consumption (Waterwise, 2012) and therefore, an approximate of maximum 300 m^3 wastewater will be produced daily from that covering service area. According to the typical design that present in Table 7.7, the application of the mixotrophic microalgae system is illustrated in Figure 7.9 to 7.11.

Table 4-14 Typical design of P recovery in a small WWTW using continuous flow microalgae system

Parameter	Value	Unit
Total HRT (HTR and PBR), θ	1	d
Overflow rate, Q	300	$m^3 d^{-1}$
Total working volume	300	m^3
Diameter of bioreactor	0.2	m
Height of bioreactor	3	m
Volume of each bioreactor	0.0942	m^3
Total number of bioreactor in one module	3,185	-
Number of storey in one module	8	
Number of bioreactor in one storey	398	
Land area needed for one module	152	m^2
Height of one module (8 storey)	40	m

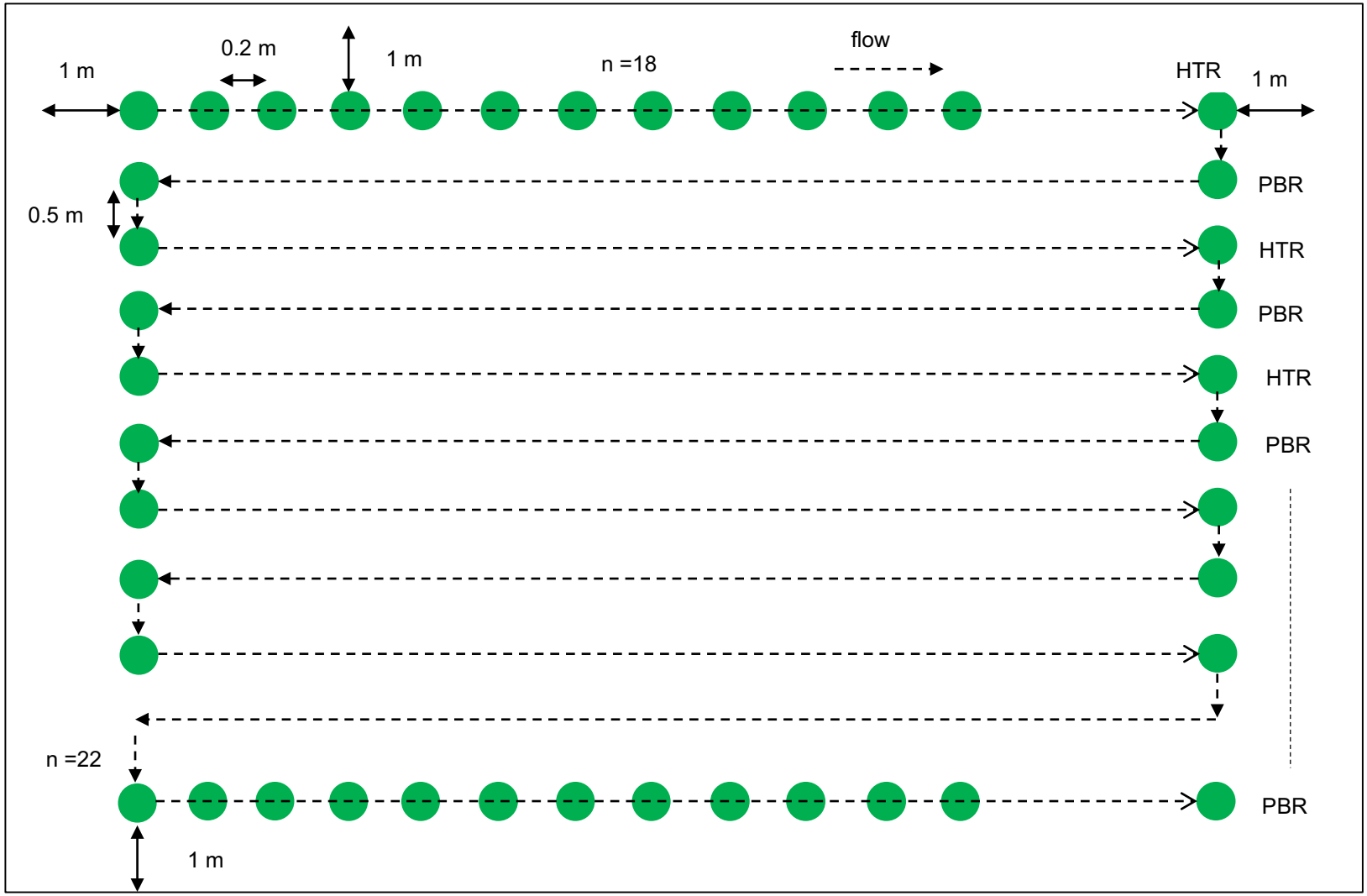


Figure 4-45 Top view of the mixotrophic microalgae system in a small scale WWTW

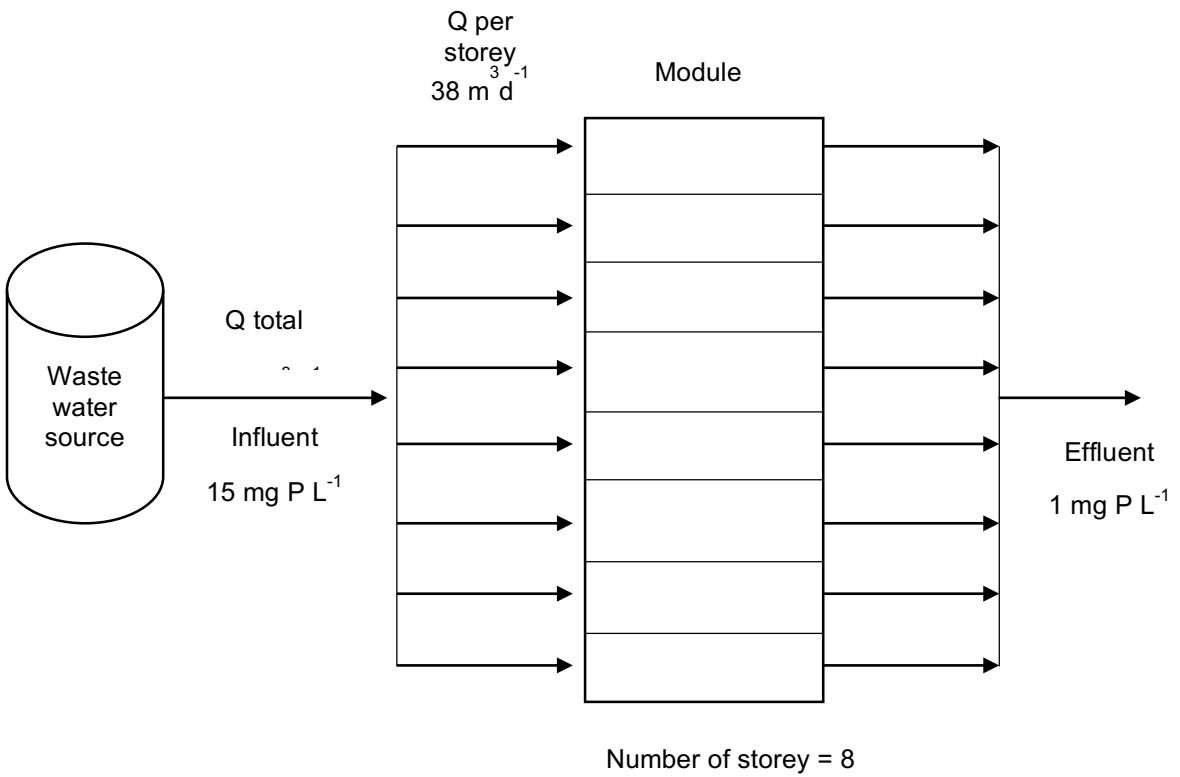


Figure 4-46 The overview of wastewater flow into the storey of the mixotrophic microalgae system

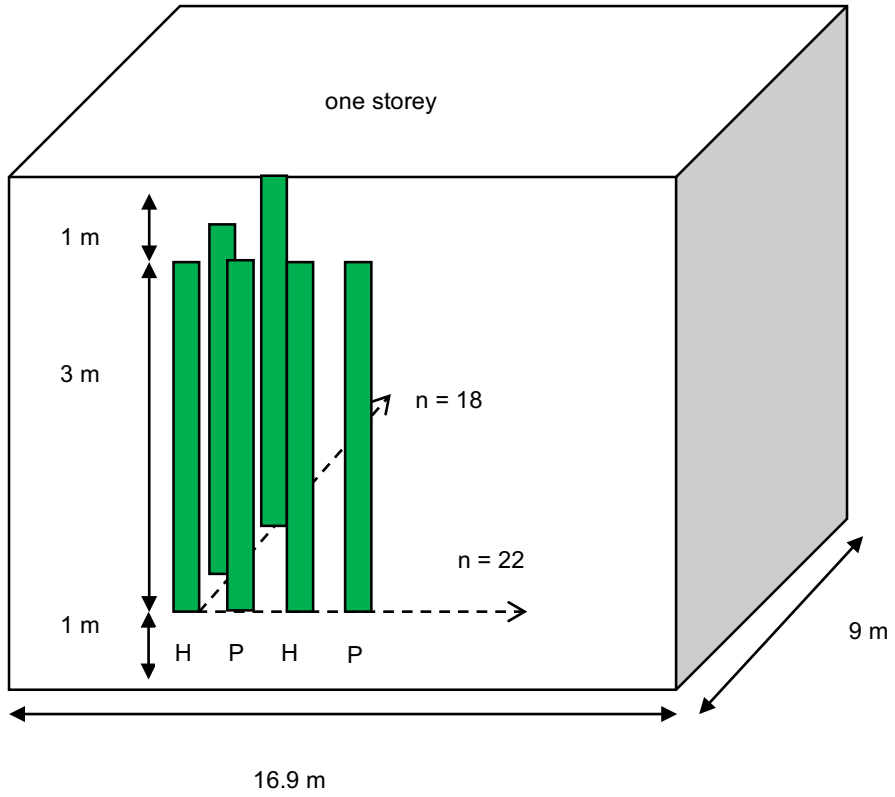


Figure 4-47 Typical design of the mixotrophic microalgae system in one storey. The number of the photobioreactor row is 22 and each row contains of 18 bioreactors. H = heterotrophic; P = photoautotrophic

In order to implement the mixotrophic microalgae system in Esholt WWTW, the flow rate of 280,020 m^3 d^{-1} which represent the wastewater produced from 730,000 population equivalent at Esholt WWTW (Marsden et al., 2012) was used to calculate the working volume of the microalgae reactor for P recovery. Based on the typical design (Table 7.7) and the amount of wastewater flowrate, Esholt WWTW requires 933 modules of the mixotrophic microalgae system and 141,971 m^2 of land area to build the system. Algae system requires a huge covering area for construction both closed or open system and the land area is considered as a crucial factor for the implementation of the microalgae system for wastewater treatment (Chiu et al., 2015; Williams and Laurens, 2010). However, the land requirement, size and cost of the closed microalgae system can be reduced by implementing the design optimisation study (Slegers et al., 2013; Norsker et al., 2011) and therefore, further study is needed in order to enhance algal growth and nutrient recovery and to improve design of the algae reactor.

A suggestion is considered to retrofit the microalgae system at the current Esholt WWTW in which the algae system is placed after activated sludge process (Figure 7.12 and 7.13). The algae fed with the nutrient from the effluent of activated sludge process in which still contain high N and P concentration (i.e. >100 mg N-TKN L^{-1} and >10 mg P-PO_4^{3-} L^{-1}) and also the liquid decanted biosolids. The harvested algae can be co-digested with waste activated sludge (WAS) to enhance bio-methane production. Yuan et al. (2012) have reported that algae biomass digested under thermophilic condition generated higher biogas production than mesophilic. Algal biomass digestion will reach the optimum biogas yield for energy recovery when the content of lipid in algal cells is below 40% (Sialve et al., 2009). In this study *C.reinhardtii* 11/32C have the potential for enhancing biomethane production as the algae contain of approximately 21% of lipid (*data not shown*). Furthermore, the harvested P enriched algal biomass can be processed as biofertiliser (Solovchenko et al., 2016) because *C.reinhardtii* 11/32C contain important elements of N and P at 14% and 2% respectively. Moreover, the energy from co-digested of WAS and algal biomass can be used as an electricity to support microalgae cultivation and harvesting.

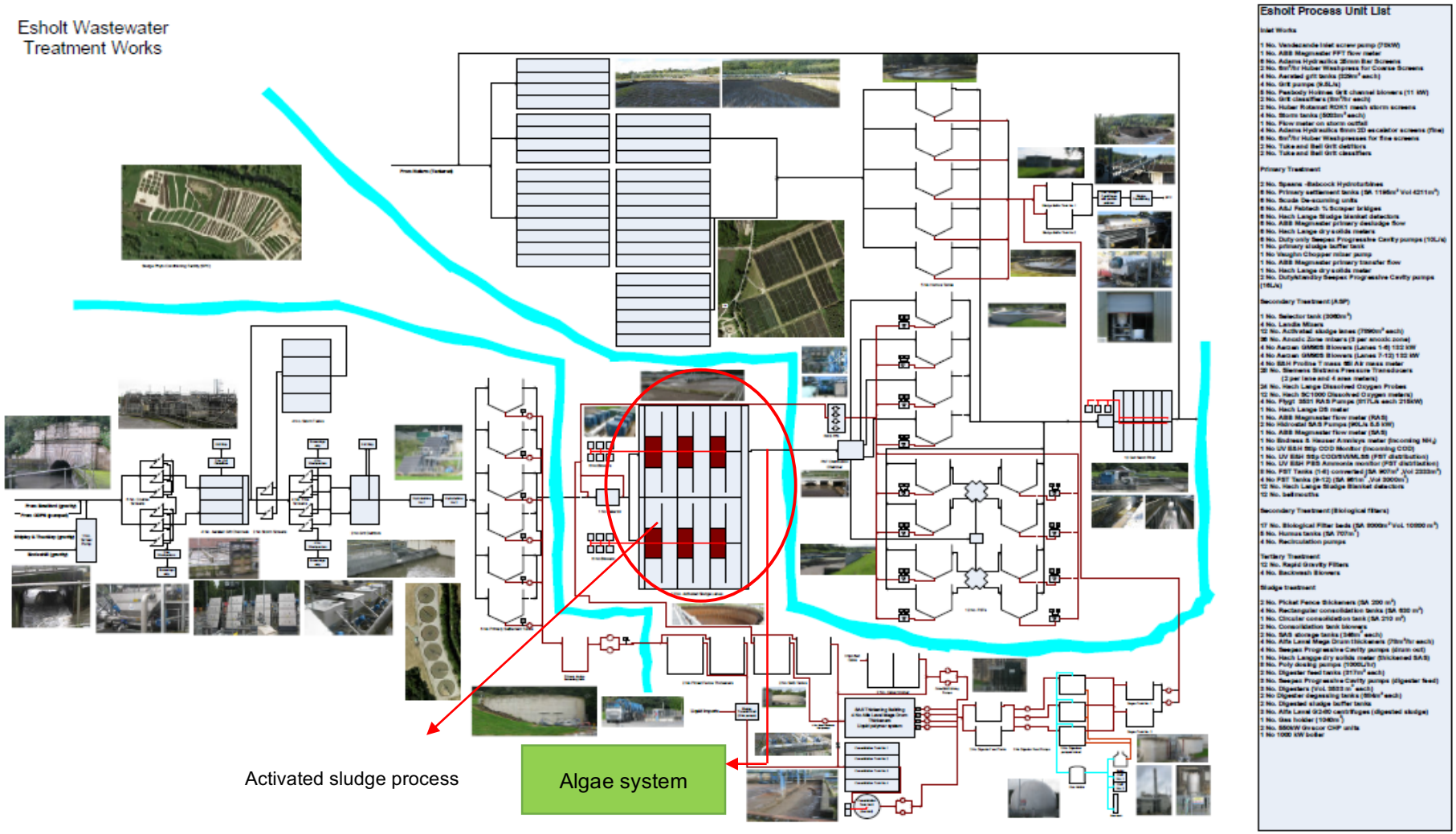


Figure 4-48 Microalgae system in the existing Esholt wastewater treatment works

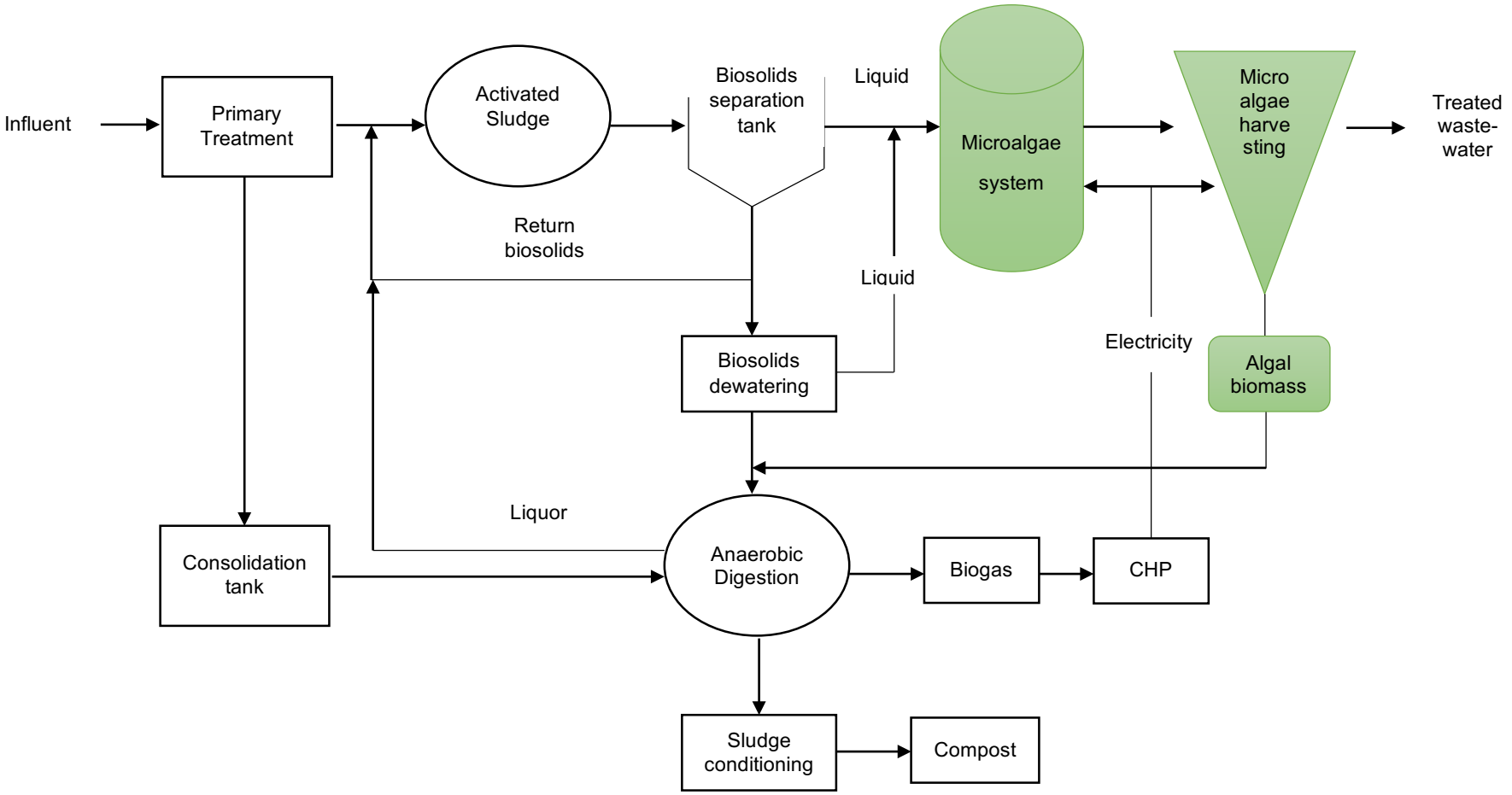


Figure 4-49 Integration of a continuous flow mixotrophic microalgae system in an existing wastewater treatment plant

7.3.6 Energy balance at Esholt Wastewater Treatment Works and the energy requirement for microalgae cultivation

Esholt WWTW uses up to 85,563 kW d^{-1} to treat the sewage, but on average would be nearer to 63,000 kW d^{-1}. The site can generate electricity up to 62,785 kW d^{-1}, but this depends on the quantity and quality (methane content) of the gas available. On average the site generates electricity of 45,000 kW d^{-1} (Baker, 2016). The figures indicate, if the plant can reduce the electricity consumption on site a bit, then the site is capable of going energy neutral. However, this depends on flow fluctuation that come into the site as high flows result in more energy use due to increased pumping around the site.

In order to observe the amount of the electricity used for each stage on the site, the typical energy uses in wastewater treatment works was adopted from Metcalf and Eddy (2004). The energy consumption to treat the sewage in percentage and kW d^{-1} are listed in Table 7.8. The average value is used in this calculation due to electricity consumption and generation were fluctuated.

Table 4-15 Electricity consumption and production at the Esholt Wastewater Treatment

Stage	Energy need (%)	Energy used (kW/d)
Inlet pumping and headworks	4.9	3087
Primary clarifier and sludge pumps	10.3	6489
Activtaed sludge aeration	55.6	35028
Secondary clarifier and RAS	3.7	2331
Thickener and sludge pump	1.6	1008
Effluent filters and process water	4.5	2835
Solids dewatering	7	4410
Tertiary treatment	3.1	1953
Heating	7.1	4473
Lighting	2.2	1386
Total energy needed per day		63000

It is obvious from the table that the secondary treatment accounts for 56% of the total power followed by the primary sedimentation and sludge pumping for 10.3%. Activated sludge process is the highest for energy consumption in the wastewater treatment works. Jenicek et al. (2013) studied the energy self-sufficient in the sewage treatment plant and they found that over 40% energy in the sewage treatment used for the oxidation of organic pollutants and nitrogen compounds.

Moreover, the biogas from the digester can generate 71% of the electricity and that amount used on the site. It is assumed that there was 18,000 kW d^{-1} of the external electricity needed to treat the wastewater.

In addition to the energy requirement for microalgae cultivation, the energy output from microalgae cultivation is generated from the digestion process of the algal biomass to produce biomethane. Previous study investigated biogas production from several microalgae including *Chlamydomonas reinhardtii* and they found that *Chlamydomonas reinhardtii* generate biomethane of 587±8.8 ml CH_4 g VSS^{-1} (Mussgnug et al., 2010). Whereas *Chlorella vulgaris* generated biomethane of 3,333 kJ kg^{-1} VSS (Adewale, 2015). Furthermore, dry algal biomass of *C.reinhardtii* 11/32C grown in mixotrophic system consist of 14% N and 2% P which offer potential benefit to use as fertiliser. This benefit can help a wastewater company to obtain an additional income from fertiliser production.

In regard to energy requirement for *C.reinhardtii* 11/32C cultivation in photobioreactor, this closed system needs energy for lighting, pumping of medium and algae culture, and aeration. The energy uses for these purposes is obtained from biomethane production from co-digested WAS and algal biomass which converted into electricity.

7.4 Discussion

7.4.1 Wastewater characteristics at Esholt Wastewater Treatment Works

Currently the water industry in the UK is facing new challenges to improve the quality of their final discharges and to meet more stringent discharge consents in the near future, particularly for N and P as a result of the pressure to deliver “good status” in

surface waters by implementing the EU Water Framework Directive (Priestley, 2015). For instance, the Urban Wastewater Treatment Directive and Freshwater Fish Directive (UWWTD, 1991; FFD, 2006) request a discharge consent of 1 mg L^{-1} for N-NH_4^+ and for P-PO_4^{3-}. These legislations implied that the removal efficiency for nutrient must achieved over 90%.

According to the results from the wastewater characterisation that was conducted from May 2014 to February 2016, it was found that the quality of the final discharge from Esholt WWTW meets the discharge consent set by the Urban Wastewater Treatment Directive and Freshwater Fish Directive (UWWTD, 1991; FFD, 2006); the removal of nitrogen and phosphorus species at Esholt WWTW were over 90% and 80% respectively. High removal efficiencies (> 90%) were also obtained for total suspended solids and COD.

However, the average of NO_3^- concentration was slightly higher in the effluent of the secondary sedimentation compare to raw wastewater. That was due to incomplete denitrification in the anoxic zones of the multi-stage activated sludge reactor (Dhamole et al., 2009; Mohan et al., 2016), which indicates a higher nitrification rate than the actual denitrification rate. The increase of NO_3^- might be caused by the input of high concentration of NH_3-N from the digested liquor, which contains 1,458 mg NH_3-N L^{-1}. Therefore, the addition of carbon is needed to enhance biological denitrification in the activated sludge process (Fernandez-Nava et al., 2010) and to decrease NO_3^- concentration in the final effluent.

On the other hand, the regular wastewater characterisation showed that the concentration of nutrients in the treated wastewater at Esholt WWTW was higher in summer than in winter. This is because a large seasonal variation in flow rates arriving at WWTW and high precipitation during the winter season that may result in increased water flows entering the treatment works and therefore, those flows create a dilution effect on the nutrient concentration arriving on site (Power et al., 2014).

7.4.2 Implementation of a continuous flow microalgal system at Esholt

The mass balance analysis in this study was conducted to investigate the fate of nutrients entering within the raw wastewater at Esholt WWTW. A mass balance is an important tool to conduct a mass flow analysis of key substances such as C, N and P (Nowak et al., 1999). In this study, mass balances for N and P were analysed to explore the prospect of implementing a continuous flow microalgal system in an existing wastewater treatment works.

According to the results for the wastewater characterisation described previously, the incoming N sources were removed from the wastewater through two different pathways: denitrification (61%) and bacterial uptake (19%), the remaining N was washed out the system within the final effluent (20%), mainly as nitrate. That produces a treated effluent with 0.7 mg L^{-1} of NH_4^+-N. Interestingly, the extent of nitrification was higher than denitrification, which resulted in high NO_3^- concentrations and very little nitrogen recovery.

On the other hand, if full denitrification is required an additional (external) carbon source would be needed and may increase up to 50% the total costs for wastewater treatment (Fernandez-Nava et al., 2010; Macdonald, 1990). Another operational problem that may appear if full denitrification is conducted, it would be sludge flotation in the final clarifier due to a higher consumption of NO_3^- by denitrifying bacteria and the release of N_2 gas (Richard et al., 2003). Hence, it is likely that Esholt deliberately reduces the level of denitrification as they comfortably meet the ammonium discharge consent and there is no need for additional total nitrogen removal.

The percentage of P incorporated into surplus activated sludge was considered to be 78% of the total P entering the treatment works (22% was found to leave in the final effluent). It was reported that P in wastewater at Esholt WWTW remove through activated sludge process without the addition of the chemical dosing for P precipitation (Baker, 2016), but although the use of chemical dosing facilities is widely applied in wastewater treatment works in the UK, for instance Deephams Sewage Treatment Works (Harte, 2012) and Dwr Cymru Welsh Water (Walding, 2015), they contribute very little to P recovery.

In this study, the amount of P incorporated into secondary sewage sludge was 78% and it is lower than the typical efficiencies found in municipal wastewater treatment systems in Germany, where they typically take up to 90% of total P (Cornel and Schaum, 2009). This indicates that waste activated sludge (WAS) is one of most important by-products in wastewater treatment works that can be used as a source for P recovery and reuse (He et al., 2016). However, the presence of pathogens at this stage may reduce their direct reuse in agriculture and then further treatment would be required. Other studies have reported that approximately 40% of the incoming P ends up bound to bacterial biomass, while 50% appears precipitated as inorganic P in the WAS (Petzet et al., 2012; Xu et al., 2015b). This study also found that 24% of P (kg P kg VSS^{-1}) was bound into WAS, and previous studies have reported P content in the region between 12.4% and 36.2% (mg P g VSS^{-1}) (Xie et al., 2011; Xu et al., 2015b).

In regard to the ability that microalgae have for nutrient assimilation into algal cells, the continuous flow mixotrophic algal system proved to be able to remove between 95% and 97% of nitrogen entering the treatment units. As illustrated from EDX analysis (Section 4.3.1), N content in harvested algal biomass (*C.reinhardtii* 11/32C) was 13.5%. Average N content in algal biomass is typically reported to range between 7% and 20% dry weight (Hu, 2007; Juneja et al., 2013) and consequently, microalgae have the potential to be used for nitrogen recovery via biological algal uptake with the capacity to remove over 90% N, regardless of the original nitrogen species (either ammonium, nitrate or both) but depending on algae strain and growth conditions (Becker, 1994; Juneja et al., 2013). Boyle and Morgan (2009) reported that *Chlamydomonas reinhardtii* was able to store N in their cells up to 6% and 11% under autotrophic and heterotrophic culture conditions respectively.

Ammonium is the preferred nitrogen specie for microalgae growth as an additional redox reaction is not required as it is for nitrate and therefore, ammonium is readily taken up by microalgae (Cai et al., 2013; Larsdotter, 2006a; Tam and Wong, 2000; Wang et al., 2015a). Ammonium is abundant in wastewater and conventionally remove through nitrification-denitrification processes that increase operational costs due to the need for extended aeration (Wang et al., 2015a). To overcome this problem and reduce energy costs, microalgae based systems have been widely identified as a

feasible alternative biological process for nutrient removal in wastewater treatment works, without the need of nitrification (Ji et al., 2013; Menger-Krug et al., 2012; Ogbonna et al., 2000).

Previous studies have reported that *Chlamydomonas reinhardtii* is able to assimilate NO_3^- for their growth by converting nitrate into ammonia in order to facilitate transport through the cell wall and to produce amino acids (Fernandez and Galvan, 2007; Fernandez and Galvan, 2008; J. et al., 1983). This ability has been proven in this study where *C.reinhardtii* 11/32C removed NO_3^- from synthetic wastewater with 98% efficiency. Similarly, another *Chlamydomonas reinhardtii* strains have been reported to utilised NO_3^- with removal efficiencies of 93% (Su et al., 2012), as well as other microalgae species like *Chlorella* sp. (62%; (Wang et al., 2010), and *Scenedesmus accutus pvuw12* (100%; (Doria et al., 2012). These results indicate that *C.reinhardtii* 11/32C is a suitable photosynthetic microorganism for NO_3^- recovery which can be employed in wastewater treatment.

Phosphorus is the second most important macronutrient for microalgae growth. Microalgae are able to store P reserves in the form of polyphosphate (Tarayre et al., 2016; Powell et al., 2008). In this study, *C.reinhardtii* 11/32C was used to demonstrate the ability that green microalgae have to store polyphosphate, in agreement with the work published by Werner et al. (2007). Tests conducted in a continuous flow mixotrophic system showed that the maximum P recovery achieved with *C.reinhardtii* 11/32C was 50%, which resulted in an intracellular P content of 2%. The results of the intracellular P uptake described in Section 6.3.3 showed a trend of increasing intracellular P uptake with the increase of HRT and according to the model for P uptake described in Section 7.3.4, a HRT of 11 days (i.e., in total for both autotrophic and heterotrophic processes) would be required for a continuous flow mixotrophic algae culture in order to achieve a discharge consent of 1 mg P L^{-1}, with a predicted total P recovery of 93%.

Microalgae have proven to be effective at improving wastewater quality and also for P recovery, as has been found to efficiently take up P and store it inside algal cells (up to 3% P dry weight) (Camargo-Valero et al., 2010; Tarayre et al., 2016). Such ability

has been confirmed by this study and several other studies that have investigated the potential of using microalgae for nutrient recovery from wastewater. For instance, *Chlamydomonas reinhardtii* removed up to 99% P from the effluent of a secondary clarifier with a removal rate of 0.85±0.05 mg P L^{-1} d^{-1} (Su et al., 2012).

Furthermore, the uptake rates of P were always higher than N, even though microalgae require more N than P which indicate a luxury consumption of P (Redfield, 1958). Microalgae proved to be able to retain the excess of P in their cells longer than bacteria (J.Currie and Kalff, 1984) and this superiority offer another benefit for P recovery in wastewater treatment as the algal biomass could be stored for longer before any further process if required.

Hence, microalgae system may provide a new process for biological nutrient recovery that could be used to retrofit existing wastewater treatment works, with the benefit of achieving both N and P removal and biomass production for nutrient recovery. The algae system potentially may substitute nitrification-denitrification in multi-stage activated sludge units and conventional bacterial-based P removal processes.

7.4.3 Where algal-based nutrient recovery should be placed within existing wastewater treatment works?

The implementation of P recovery along with wastewater treatment provides an opportunity for combining environmental protection and resource recovery (Cornel and Schaum, 2009). Recently, various studies with algae were conducted to investigate the possibility of including microalgae cultivation in conventional wastewater treatment systems using digested liquor from anaerobic digesters (Kong et al., 2010a; Rusten and Sahu, 2011; Wang et al., 2010; Yuan et al., 2012). This P-rich source typically contain over 50 mg P L^{-1} (Cornel and Schaum, 2009); digested liquor samples collected at Esholt WWTW reported 25 mg P L^{-1}.

Generally, N- and P-rich wastewater streams are re-circulated back to the head of the works (Yuan et al., 2012) or to the activated sludge selector, as it happens at Esholt WWTW. This recirculation increases nutrient loading and upsets the nitrification-denitrification process (Yuan et al., 2012). Hence, the use of digested liquor as a

nutrient source for microalgae cultivation may reduce the nutrient load of the wastewater treatment and produce pathogen-free biosolids (i.e., thermal hydrolysis is now a common practice before mesophilic anaerobic digestion of sewage sludge).

However, several drawbacks are impeding the implementation of microalgae cultivation using digested liquors. Firstly, the colour of the digested liquor is often pitch black, contains a lot of solid particles, and has extremely low light transmittance (Rusten and Sahu, 2011). The dark black colour is due to the presence of metallic sulphide salts (i.e., FeS) as a result of sulphate reduction under anaerobic conditions (Suschka and Grubel, 2014). In order to address this problem, pre-treatment for improving light penetration for microalgae growth is required. However, this pre-treatment needs the addition of coagulant aids and solid removal units (Rusten and Sahu, 2011) and inevitably, that would result in the demand for more energy and chemicals for treatment.

Alternatively, such nutrient-rich wastewater streams can be fed into a precipitation or crystallisation tank for struvite production and to reduce N and P concentrations before feed into a microalgae system (Cornel and Schaum, 2009; Desmidt et al., 2015; He et al., 2016). The digested liquor also contains toxic substances that may inhibit microalgae growth (Munoz and Guieysse, 2006b); for instance, ammonia was found to be a toxic form for microalgae (Kallqvist and Svenson, 2003).

Microalgae has demonstrated their great potential as a sustainable biological P removal process in wastewater treatment (Ruiz et al., 2013; Tarayre et al., 2016), which could be coupled to a secondary treatment unit for nutrient recovery. This biological nutrient removal (BNR) provides several benefits to improve existing and new wastewater treatment systems. The process would reduce energy costs for aeration (Manser et al., 2016; Mennaa et al., 2015); would eliminate the need for nitrification and denitrification processes (Tarayre et al., 2016); would contribute to simultaneous environmental control and nutrient recovery (Sawayama et al., 1998; Su et al., 2012; Boelee et al., 2012; Mennaa et al., 2015); and would reduce the net CO_2 footprint of sewage treatment works (Dalrymple et al., 2013b; Menger-Krug et al., 2012). Additionally, harvested microalgal biomass has a great potential as could be a

source feeding into the production of high-value products, biofuels and other bioproducts (Tarayre et al., 2016; Mennaa et al., 2015; Mallick, 2002; Mata et al., 2010; Spolaore et al., 2006).

It is predicted that in the future, the use of microalgae systems for secondary treatment will contribute to a more sustainable wastewater treatment processes (Figure 7.10). Apart from the benefits mentioned above, the harvested algal biomass can be delivered into anaerobic digesters and be co-digested with a blend of thermally hydrolysed primary and secondary sludge for bio-methane production (Dalrymple et al., 2013b; Yuan et al., 2012). The co-digestion of harvested algal biomass with sewage sludge may increase bio-methane production up to 61% (Menger-Krug et al., 2012). Eventually, this extra bio-methane would generate enough heat and power to reduce the overall fossil carbon footprint and to generate additional renewable energy (Horan and Camargo-Valero, 2013).

However, in order to implement an effective microalgal process, several stages need further development. Future research is required for mathematical modelling based on lab results from continuous flow microalgae systems to better determine design criteria (i.e., Computational Fluid Dynamics (CFD) modelling). The CFD modelling can be used to stimulate and to analyse the fluid flow in a photobioreactor in order to produce quantitative and qualitative data for enhancing microalga growth (Seo et al., 2014). Afterwards, further experiment should be done to validate the analyse results produced by the CFD modelling. Moreover, CFD can be used as a tool for scale-up of microalgae cultivation reactor (Bitog et al., 2011) (i.e., bench-scale of mixotrophic microalgae cultivation). Finally, a small pilot-scale system would help to test the limitations and benefits of such system.

7.4.4 Opportunities and limitations of the application of mixotrophic microalgae cultivation for wastewater treatment

Microalgae are able to grow in nutrient-rich environment such as wastewater and to efficiently remove N and P from the wastewater. This make microalgae-based wastewater treatment as an attractive method for sustainable wastewater treatment and it was reported to effectively remove P from the wastewater without the addition

of chemical substances (Hoffmann, 1998; Mallick, 2002). Hence, this method offer the benefits of resource recovery and recycling (Hoffmann, 1998).

Microalgae have been cultivated in several modes such as photoautotrophic, heterotrophic and mixotrophic. The photoautotrophic mode requires light as a source of energy and inorganic carbon for performing photosynthetic process, whereas the heterotrophic mode depends on organic carbon and organic substances without the addition of light. Moreover, the mixotrophic mode is the combination between those culture conditions (Brennan and Owende, 2010; Perez-Garcia and Bashan, 2015). Photoautotrophic cultivation is a common method applied in the field for algae-based wastewater treatment which accomplish either in open pond or photobioreactor (Wang et al., 2014). However, since photoautotrophic algae are light dependent, the increase of algae turbidity will impede the light penetration into the algae culture and thus prohibit algal biomass production (Wang et al., 2014). Whereas, the mixotrophic algae culture can utilise organic carbon for their growth, thus light energy is not limiting factor for their biomass production (Chang et al., 2011; Mohan et al., 2015). The following section describes the potential to retrofit the mixotrophic microalgae system at Esholt and wastewater treatment works in general.

7.4.4.1 Opportunities

Mixotrophic microalgae culture have reported by several studies which able to generate higher algal biomass production for biomass feedstock and biofuel (Abinandan and Shanthakumar, 2015; Li et al., 2014; Wang et al., 2014). Li et al. (2014) reported that specific growth rate, biomass production and lipid content of green microalgae *Chodatella* sp. were much greater when the algae grown in mixotrophic than autotrophic culture. Moreover, this study coincided with Li et al. (2014) shown that the algal biomass production of *C.reinhardtii* 11/32C were almost double in mixotrophic than in photoautotrophic mode. Mixotrophic cultivation method can also maintain longer period in exponential growth phase (Perez-Garcia and Bashan, 2015), therefore the algal biomass production is became greater. In this study, *C.reihardtii* 11/32C produced on average harvested biomass at 2.7 g VSS L^{-1} and maximum at 3.6 g VSS L^{-1}. This amount of biomass can be co-digested with activated waste sludge in wastewater treatment works in order to enhance bio

methane production. Mussgnug et al. (2010) investigated biomethane production in *Chlaydomonas reinhardtii* and his work proved that *C.reinhardtii* generate 587 ml biogas g VSS^{-1} with an increase of biogas production up to 123%. Currently, Esholt WWTW uses up to 85,563 kW d^{-1} to treat the sewage, but this is flow dependant and an average would be nearer to 63,000 kW d^{-1}. The site can generate electricity up to 62,785 kW d^{-1}, but this is depends on the quantity and quality (methane content) of the gas available. On average the site generates electricity of 45,000 kW d^{-1} which represent 69% of the total energy requirement. Hence, co-digested of the algal biomass with waste activated sludge is considerable to enhance biogas production for renewable energy in the wastewater treatment works.

In spite of its great potential for sustainable and renewable biomass feedstock, the implementation of mixotrophic algae in large scale of commercial production is expensive for its production (Wang et al., 2014). However, since microalgae cultivation require water, carbon and nutrient for their biomass growth, this barrier can be reduced by culturing of mixotrophic algae using wastewater as a source media for their growth. This is because wastewater rich in biodegradable organic compounds (i.e. organic N, P and C) (Zhou et al., 2012b). According to wastewater characterisation results in which the wastewater sample collected from Esholt, the concentration of TKN, Total Phosphorus (TP) and COD were 52 mg N L^{-1} , 7.2 mg P L^{-1} and 778 mg L^{-1} respectively. In that concentration, organic N and P were represent 45% and 35%, and the remaining fraction were inorganic nutrient. These amount of nutrient provide a potential growth media for microalgae and especially *C.reinhardtii* 11/32C to be implemented at Esholt WWTW. Moreover, many of microalgae especially *Chlamydomonas, Scenedesmus* and *Chlorella* can utilise partially degraded organic compounds under mixotrophy condition and able to growth under heterotrophic condition using simple organic carbon such as acetate and glucose (Tam and Wong, 1996).

In addition, the elimination of the light requirement in heterotrophic culture side reduce cost of the energy supply and minimise the contamination of photosynthetic microorganisms. Heterotrophic culture also minimise the growth reduction of algae due to cells self- shading effect in the inner part of the bioreactor and loss of biomass

during respiration period (Perez-Garcia and Bashan, 2015). Accordingly, since organic carbon can be utilised under mixotrophic mode, the algae growth is not depends on the photosynthetic process, thus it reduce a photo inhibition effect (Wang et al., 2014). As a result of the greater algal biomass growth under mixotrophic than photoautotrophic cultivation, the higher algal biomass production offer the benefits to increase efficiency of N and P recovery (Gupta et al., 2016; Perez-Garcia and Bashan, 2015). In this study, photoautotrophic and mixotrophic algae cultivation reach N recovery for over 95%. Whereas P recovery increased from maximum 35% in photoautotrophic culture to 50% in mixotrophic culture when the algae exposed to high concentration of P in the media (i.e. > 50 mg P L^{-1}).

In general municipal wastewater contain high concentration of ammonia (Tam and Wong, 1996) and at Esholt the maximum of ammonia concentration was 58 mg NH_4^+-N L^{-1}. In order to remove an organic matter, N and P from wastewater, the WWTW require up to 50% of energy to support activated sludge process (Metcalf and Eddy, 2004). It is known that ammonium as a nitrogen preference for microalgae growth and this study revealed that *C.reinhardtii* 11/32C able to assimilate over 90% of ammonium and 50% of PO_4^{3-} from the media. The ability of *C.reinhardtii* 11/32C to utilise N and P under this mixotrophic growth condition offer a novel green technology to replace the secondary wastewater treatment and reduce the energy consumption. The majority of 90% ammonia is removed through algal cell assimilation and therefore, the implementation of the microalgae system in the wastewater treatment works can mitigate ammonia and CO_2 gas emission in wastewater (Kang and Wen, 2015).

7.4.4.2 Limitations

Mixotrophic microalgae cultivation is depends on organic compounds (i.e. carbon) to maintain their biomass production (Chandra et al., 2014; Perez-Garcia and Bashan, 2015). Various of organic carbon sources can be assimilated by microalga and among them glucose and acetate are the most common of organic carbon for microalgal growth medium (Wang et al., 2014). In spite of the benefits of organic carbon to enhance algal biomass production, the cost of carbon source for growth medium represent 50% of the total operational cost for microalgae cultivation (Cheng et al., 2009). For long term operation, the cost of organic compounds supply in mixotrophic

growth system will be higher than photoautotrophic, although the illumination requirement for photosynthetic process is reduced (Tabernero et al., 2012).

In addition, the mixotrophic microalgae cultivation require a large number and high volume of bioreactor to meet the target of algal biomass production (Tabernero et al., 2012), especially for a large scale cultivation. It is because the mixotrophic growth system involve synergetic culture for photoautotrophic and heterotrophic. Moreover, most of the green microalgae are autotrophs and therefore, not all of them can be cultivated as heterotrophs or mixotrophs and this algal strain limitation can be hindered commercialisation of the mixorophic microalgae system (Perez-Garcia and Bashan, 2015).

Contamination by the other microorganisms (especially bacteria) is also important consideration in mixotrophic culture as many of them grow faster than microalgae (Perez-Garcia and Bashan, 2015; Deschenes et al., 2015). This is caused by the addition of organic carbon in the culture to increase the biomass yield of microalgae (Deschenes et al., 2015). Therefore, it is important to achieve an axenic microalgae culture which able to grow under mixotrophic conditions but it will turn to increase the cost of microalgae production (Wang et al., 2014)

Another disadvantages of the mixotrophic microalgae cultivation is the downstream process related to the algal biomass harvesting. Current harvesting process by using centrifugation technique can contribute up to 30% of the total cultivation cost (Wang et al., 2014). The difficulty of microalgae harvesting is due to the cells size of the microalgae which influence on the drag of the cells in the fluid (Gerardo et al., 2015). As a consequent of inadequate of the algae harvesting, the algae still present in the liquid. Hence, lower capacity of algae harvesting influence the efficiency of N and P recovery. This occurred in this study where the algal concentration in the final effluent still 62 mg L^{-1} as total suspended solids.

7.4.4.3 Feasibility of the implementation of mixotrophic microalgae cultivation at Esholt Wastewater Treatment works

Integration of the mixotrophic microalgae culture with wastewater treatment serve important benefit to combine of the algal biomass feedstock production and nutrient removal in wastewater. In comparison with the conventional wastewater treatment, microalgae-based wastewater treatment is more efficient to capture the energy potential of the dissolved organic component and nutrient contained in wastewater (McCarty et al., 2011; Zhang et al., 2016). The mixotrophic microalgae cultivation was estimated to use the energy requirement between 50 and 110 kWh ML^{-1} of wastewater and this is much lower than the 230 – 960 kWh ML^{-1} that required by activated sludge process (Craggs et al., 2011). This will offer a potential benefit for WWTW as less energy is required for microalgae-based wastewater treatment. Moreover, co-digested waste activated sludge and the algal biomass provide an additional energy to the treatment plant.

Furthermore, harvested algal biomass can be further processed for biofuel (Brennan and Owende, 2010; Chisti, 2008; Demirbas and Demirbas, 2011) and biofertiliser (Wang et al., 2015b). Algae can be used as biofertiliser in agriculture field not only to improve the soil fertile but also as important nutrient to enhance agriculture production and quality (Abdel-Raouf et al., 2012; Faheed and Fattah, 2008; Garcia-Gonzalez and Sommerfeld, 2016). In this study, *C.reinhardtii* 11/32C served an important potential to accumulate large quantities of P as polyphosphate through luxury P uptake. This P enriched algal biomass offer sustainable resource to crop plants as biofertiliser (Solovchenko et al., 2016). Therefore, the implementation of the mixotrophic microalgae cultivation will help Esholt WWTW to achieve zero waste regulation as there is no waste will be generated on the site.

On the other hand, the mixotrophic microalgae system require enormous volume of reactor and large land area to be applied in a large scale of algae cultivation for wastewater treatment. In order to address this problem, further study is needed to optimise design of bioreactor and to enhance nutrient uptake and biomass production and thus, reduce the hydraulic retention time and volume of the reactor. Moreover, further study is require to investigate for mass production of mixotrophic algae

cultivation in conjunction with wastewater treatment (Perez-Garcia and Bashan, 2015). In regards to Eshlolt WWTW, closed microalgae cultivation system is recommended as this type of cultivation require less land area than open pond system.

In addition to the risk of contamination, it is recommended to isolate the wild type of microalgae which able to assimilate a wide range of organic and inorganic compounds from wastewater (Zhang et al., 2016). Further research is required to isolate *Chlamydomonas reinhardtii* strain which able to adapt and to survive in wastewater and can compete with bacteria.

7.5 Conclusions

The study presented in this Chapter explored the potential for implementing a continuous flow microalgae system in an existing wastewater treatment works. A case of study was conducted at Esholt WWTW to evaluate its performance regarding nutrient removal and the response of the current process to fluctuations in the quality of the works influent. A nutrient mass balance was also conducted. At Esholt, it was found that all nitrogen species were removed from the wastewater with high efficiency (95%), while phosphorus removal was above 80%. The efficiency of the system generated a final effluent of good quality that meets the corresponding discharge consents.

On the other hand, NO_3^- concentration in the final effluent increased due to a higher rate of nitrification than denitrification. This route requires high-energy costs for aeration in order to remove ammonia from wastewater. Additionally, the level of the incoming nutrient and organic matter in the raw wastewater was higher in summer than winter, which leads to high loads.

Regarding the nutrient mass balance analysis, it was found that 19% and 78% of N and P was bound into the waste activated sludge, whereas 20% and 22% of them remained in the final effluent. A portion of 20% of N in the final effluent was caused by the increase of nitrates concentration as a consequence of a higher rate of nitrification. These results revealed that waste activated sludge is the most importance source for nutrient recovery, especially for P. However, the extraction of nutrients from the sludge

would require further treatment for pathogen inactivation. Nutrient recovery from the secondary treatment without previous nitrification-denitrification could create an alternative for implementing a microalgal based system, which would effectively recover N and P via biological uptake and potentially could contribute to pathogen removal as already found in waste stabilisation ponds systems.

This study has proven that *C. reinhardtii* 11/32C could recover nutrients at 95% and 50% for N and P respectively. However, the P recovery rate was lower than expected; in fact the initial P concentration was higher than the one expected to be in the effluent of a conventional activated sludge process (not nitrification-denitrification or bacterial P removal) and the tested system would require very long HRTs to meet the targeted P recovery. But series of bioreactor can reduce the HRTs of the mixotrophic operation and volume of the bioreactor.

In order to meet the discharge consent of 1 mg P L^{-1}, future research is required for mathematical modelling based on lab results from continuous flow microalgae systems to better determine design criteria (i.e., CFD modelling), followed by bench-scale test to validate the model and scale up.

Chapter 8 General Discussion

The study of a microalgae system integrated in a conventional bacterial based wastewater treatment have included several laboratory observations and experiments in four areas: (a) identification of important environmental factors influencing optimum P uptake and biomass production in *Chlamydomonas reinhardtii* 11/32C.This experiment was conducted through design of experiment (DoE) using fractional factorial design and the results were analysed using RSM, (b) exploring the presence of hyper P accumulating organisms through confocal microscopy and TEM analysis. The hyper P accumulator were isolated through flow cytometry and cell sorting analysis, (c) operation of a continuous flow mixotrophic system with recycle to enhance biomass production and luxurious P uptake. This experiment investigated the potential to implement the mixotrophic microalgae cultivation for wastewater treatment, (d) assessing the potential for implementing P recovery via biological uptake at a large sewage treatment works. This chapter summaries the new findings found as part of the research work in these four areas, which will contribute to improve our knowledge about nutrient removal (particularly P) in a biological process for wastewater treatment using microalgae.

8.1 Influence of environmental conditions on luxurious P uptake and biomass production in Chlamydomonas reinhardtii 11/32C

Microalgae cultivation requires numerous consideration to optimise nutrient uptake and algal biomass production. In particular of P uptake, naturally microalgae can accumulate P in their cells up to 1% of dry weight (Borchardt and Azad, 1968). But in particular conditions, microalgae can assimilate more P and attain the cells P higher than 1%. In addition to P uptake, algal biomass production is also influenced by many important environmental factors. However, in order to focus on the outcome for P uptake and algal biomass production, four important environmental factors (N and P concentration, photoperiod, and light intensity) used in this study. The important effect of these four environmental factors in enhancing P uptake and biomass production in *C.reinhardtii* 11/32C are described in the following sub section.

8.1.1 Growth and nutrient removal in Chlamydomonas reinhardtii 11/32C under different nitrogen sources

The elemental nutrient composition of phytoplankton (i.e., microalgae in this case) could be represented with the C, N and P ration of 106:16:1, which was early reported by Redfield (1958). The ratio indicates that C is the main element of the algae which generally exceed the requirement in an ecological system, while N and P are limiting factors for cell growing (Hao et al., 2012). In addition, Borchardt and Azad (1968) observed three different phosphate ranges for microalgae growth (and in cell P content, %): 0 - 1.5 mg P L^{-1} (0-3% PO_4^{3-}) as a P-dependent growing zone; 1.5 - 4.5 mg P L^{-1} (3-9% PO_4^{3-}) as a zone of P storage and above 4.5 mg P L^{-1} (9% PO_4^{3-}) as a P saturation zone. It has also been found that a P concentration below 1.5 mg P L^{-1} causes a decrease in algal growth rates and hence, this concentration was reported as a critical level. As a consequence, luxury P uptake will occur when the P incorporated in the algal cells is found above the critical level (3% as PO_4^{3-} or 1% as P dry weight).

However, algal P uptake and growth depend on algae species and growing conditions (Patel et al., 2012). Furthermore, the ability of microalgae to take up P in their cells and to enhance their growth and biomass productivity, are also influenced by a wide range of environmental factors such as N and P concentration, light intensity, temperature, and photoperiod (Blair et al., 2013; Li et al., 2010a; Markou et al., 2012; Powell et al., 2008; Roleda et al., 2013; Janssen et al., 1999). In regard to how such environmental conditions would lead to luxury intracellular P uptake coupled with enhanced algal biomass production, it is important to examine how environmental factors can stimulate algal growth and in-cell P storage.

In order to enhance intracellular P uptake and algal biomass production, different N sources were tested in this study. Arumugam et al. (2013) examined six different N sources (i.e., KNO_3, $NaNO_3$, Urea, Ca $(NO_3)_2$, NH_4NO_3, and NH_4Cl) on *Scenedesmus bijugatus* biomass production. They found that KNO_3 was the best nitrogen source to maximise algal biomass in *Scenedesmus bijugatus.* The preference of microalgae to uptake ammonia as N source has also been observed by Tam and Wong (1996). In a simulated settled domestic wastewater, *Chlorella vulgaris* completely removed NH_4^+-

N within 24 hours a in photobioreactor, proving that NH_4^+-N is the preferred compound of N source for microalgae as a redox reaction is not incorporated in its nutrient assimilation pathway (Cai et al., 2013; Larsdotter, 2006a). Although, microalgae prefer to assimilate NH_4^+-N, the increase of pH above 8 will cause dissociation and convert NH_4^+-N to NH_3. This NH_3 is a toxic compound to microalgae (Kallqvist and Svenson, 2003), the concentration of NH_4^+ above 500 mg L^{-1} (Li et al., 2008b; Tam and Wong, 1996) cause algal growth to decline due to cell growth inhibition.

Previous studies examined the growth and nutrient removal properties of *Scenedesmus* sp. LX1 using different N sources (i.e., $NaNO_3$, NH_4Cl, urea) (Li et al., 2010b). Instead of NH_4^+, the algae grew better in $NaNO_3$ or urea and efficiently removed N and P. In comparison with $NaNO_3$ or urea the algal growth and nutrient removal were lower when compared with NH_4Cl. This work reported that the higher the algal growth was, the higher the nutrient removal (or uptake). It also shown that N preference is species dependent and N source as for *Scenedesmus* sp. LX1 and *C.reinhardtii* 11/32C.

As wastewater may contain different forms of N sources (e.g., organic nitrogen, NO_3^-, NH_4^+), it is important to understand the effect of different N sources on microalgae growth and nutrient uptake rates. In the current research, the effect of different N sources (i.e., NO_3^- and $NH_4^+{}_{(1)}$, $NO_3^-{}_{(2)}$, $NH_4^+{}_{(3)}$,) on *C.reinhardtii* 11/32C biomass growth and intracellular P uptake were tested. The results (section 4.3.2) showed that *C.reinhardtii* 11/32C prefer to assimilate NH_4^+ and consequently its removal was higher than the other N sources tested. However, the algal biomass and intracellular P uptake were higher in combination tests using a mix of NO_3^- and NH_4^+ than the test using NO_3^- or NH_4^+ only. It was predicted that once the concentration of NH_4^+ depleted in the media, the algae carried on using NO_3^- for their growth until reach stationary growth phase. Due to the fact that NH_4^+ was the prefered nitrogen compound for *C.reinhardtii* 11/32C rather than NO_3^-, this change in the N source could affect N uptake conditions; in fact, the transition between NH_4^+ and NO_3^- triggered *C.reinhardtii* 11/32C to assimilate more N and P from the media for their growth. As a result, the algal biomass growth rate was higher than the rates obtained when algae were

cultivated in NO_3^- or NH_4^+ only. Moreover, this change in N source influenced the algae to assimilate more P from the media and stored it in their cells.

The interactive effect of NH_4^+ and NO_3^- uptake by *Nannochloropsis* sp. on the algal growth was studied by Hii et al. (2011). They found that *Nannochloropsis* sp. also preferred to take up NH_4^+ rather than NO_3^- when these N compounds were available in the same media. Also, they reported the algal growth was higher when *Nannochloropsis* sp. was cultivated in both nitrogen sources (NH_4^+ and NO_3^-) than NH_4^+ or NO_3^- only. But Hii et al. (2011) did not examine the effect of different N compounds on algal growth, which enhance intracellular P uptake.

Therefore, the findings in this study revealed that *C.reinhardtii* 11/32C could be used in wastewater treatment, as this alga prefers to assimilate NH_4^+ over NO_3^- for their growth and it can be used to replace biological nitrification. The algae can be fed by using the effluent of conventional activated sludge process as a nutrient source. Moreover, the transition effect of N assimilation between NH_4^+ and NO_3^- triggered higher uptake of intracellular P in the algae cells.

8.1.2 Optimisation of environmental factors enhancing intracellular P uptake and algal biomass

C.reinhardtii 11/32C has shown to be able to take up nutrient (particularly intracellular P) for their growth and utilised more NO_3^- after have efficiently removed NH_4^+ from the culture media. This study also attempted to induce luxurious intracellular P uptake coupled with enhancing biomass production using *C.reinhardtii* 11/32C.

Powell et al. (2008) observed luxury P uptake in *Scenedesmus* spp., which grew in a continuous culture inoculum reactor. They examined the effects of phosphate concentration, light intensity and temperature on luxury uptake of P in a continuous culture, which mimicked the treatment process happening in waste stabilisation ponds. Powell and co-workers found that the average of P in *Scenedesmus* spp. was on average 1.27%, with a maximum of 3.16% and a minimum of 0.41%. The highest content of P was obtained at the highest temperature tested (25^{o}C) and low light

intensity conditions (60 $\mu Em^{-2}s^{-1}$). Interestingly, the highest P concentration at 15 mg P L^{-1} did not affect the P content in the algal biomass in *Scenedesmus* spp. Powell et al. (2008) did not study the enhancement of algal biomass production along with the achievement of luxury P uptake in their work.

Additionally, Zhu et al. (2015a) investigated influence of P supply on starch and lipid accumulation in *Chlorella* sp. under N depletion conditions. The results conveyed that above 3% of the biomass P was attained at high P concentration (10mM = 310 mg P L^{-1}). This P accumulation has shown marginal effect on the cell density, but a positive effect on biomass growth. This indicates that the algae effectively removed high concentration of P from the media and stored it as polyphosphate. The P storage used to increase biomass growth without following by the increase of cell density.

On the other hand, this study attempted to assess the effect of four different environmental factors: N and P concentration, light intensity and photoperiod to simultaneously enhance algal biomass production and intracellular P uptake. The results elucidated that algal biomass productivity was ranging from 3 to 149 mg VSS L^{-1} d^{-1} and these have been achieved through a wide range of interaction between environmental factors (section 4.3.2). Furthermore, a Response Surface Methodology (RSM) was used to identify the optimum environmental conditions that control the increase of algal biomass production. The response surface plot showed that the optimum conditions found were 200-300 mg N L^{-1}, 100 mg P L^{-1}, 16 hours light period and 200-350 μEm^{-2} s^{-1} light intensity. According to the results, interactions between N concentration and light intensity showed the most significant influence on biomass productivity followed by P concentration, light intensity and interaction between N and P.

The result on biomass productivity in this study agreed with Xiao et al. (2015), who reported that N concentration and light intensity are the most important environmental factors enhancing algal biomass productivity. Xiao and co-workers used 400 mg KNO_3 L^{-1}, 50 mg $NaH_2PO_4.2H_2O$ L^{-1} and continuous illumination in the cultivation of *Nannochloropsis oceanica* IMET1 in a batch annular glass column. They obtained algal biomass dry weight at 34.14 mg L^{-1} h^{-1} at light intensity of 331.2 μEm^{-2} s^{-1}.

However, they only applied NO_3^- as sole of N source and there was no simultaneously of N assimilation for algal growth. Additionally, NO_3^- is not abundant N source in wastewater but it is mainly plentiful in natural soil (Fernandez and Galvan, 2008). The effect of NO_3^- concentration and light spectral compositions on biomass production of the red algae *Palmaria palmata* also studied by Parjikolaei et al. (2013). They also reported high NO_3^- concentration and white light exposure stimulate the highest algal biomass production.

In regard to algal biomass growth, Li et al. (2010a) elucidated that N:P ratio ranged from 5:1 to 12:1 stimulate NO_3^- and PO_4^{3-} removal from 83% to 99%. This removal reach maximum algal growth of *Scenedesmus* sp. LX1 at $2.21.10^6$ cells ml^{-1}. This implies that they need extremely high NO_3^- over PO_4^{3-} concentration to achieve maximum algal growth rate. Whilst in this study, the algal biomass productivity require N:P ratio at 3:1. The growth of the biomass was caused by the simultaneous effect of interaction between NH_4^+ and NO_3^-.

Furthermore, the effect of the N and P concentration, light intensity and photoperiod on intracellular P uptake were also tested. The results showed that the minimum of intracellular P uptake was 0.08 mg P L^{-1} d^{-1} and the maximum was 2.84 mg P L^{-1} d^{-1} which occurred at P concentration of 1 mg P L^{-1} and 100 mg P L^{-1} respectively. As a result, these uptake incorporated P in algal biomass ranging from 0.3% to 4.0%. However, according to the response surface plot, the P concentration only did not show an important effect on the intracellular P uptake (section 4.3.3). This result in line with the previous work conducted by Powell et al. (2008) but opposite with Zhu et al. (2015a).

The optimisation of the environmental conditions for intracellular P uptake in *Chlamydomonas reinhardtii* 11/32C observed at 200 mg N L^{-1}, 120 mg P L^{-1}, 16 hours light period and 100-400 μEm^{-2} s^{-1} light intensity by applying RSM. Three linear parameters (i.e. N, light and photoperiod) were statistically significant on intracellular P uptake. As mentioned before that N concentration triggers the algal biomass production, this implies that the higher algal biomass produced, the more P can be uptake by *Chlamydomonas reinhardtii* 11/32C. Beuckels et al. (2015) evaluated the

influence of N concentration on P removal by *Scenedesmus* and *Chlorella,* and they conveyed sufficiently high N concentration was needed to enhance P removal from wastewater. In regard to the effect of light intensity, similar results were observed by Sancho et al. (1999) in S*cenedesmus obliquus* and Jansson (1988) in algae and bacteria. As microalgae obtain the energy through photosynthetic process which is light dependent and therefore, light intensity drives P uptake in microalgae. Intracellular P uptake in this study not only influence by the light intensity but light dark cycle also stimulate microalgae to utilise more P in their cells. This similar outcome was found by Shatwell et al. (2014) who study in *Nitzschia acicularis*.

Eventually, a validation test was conducted to prove the optimisation environmental factors influence on algal biomass production coupled with intracellular P uptake in *Chlamydomonas reinhardtii* 11/32C. The results concluded that environmental conditions at 200 mg N L^{-1}, 100 mg P L^{-1}, 16 hr light:8 hr dark period and 250 μEm^{-2} s^{-1} light intensity were the optimum conditions for *C.reinhardtii* 11/32C to attained high intracellular P uptake and algal biomass productivity at 4±0.4 mg P L^{-1} d^{-1} and 147±1.5 mg VSS L^{-1} d^{-1} respectively.

These findings are important to prove the consideration that *C.reinhardtii* 11/32C can be employed in wastewater treatment as biological P removal and shown the potential to cultivate in nutrient-rich condition. This alga strain has ability to assimilate nutrient from low (N = 10 mg N L^{-1}; P = 1 mg P L^{-1}) to high (N = 500 mg N L^{-1}; P = 100 mg P L^{-1}) concentration to support their growth and intracellular P accumulation.

8.2 Exploring the presence of hyper P accumulating organisms

This study was conducted to explore the presence of the hyper P accumulator in *C.reinhardtii* 11/32C culture where the results are described in Chapter 5. The algae were cultivated in batch culture using photobioreactors. This hyper P accumulator were isolated from five different environmental conditions which are ranging from the lowest to the highest intracellular P uptake. Combination of these environmental factors in enhancing intracellular P uptake are described in Chapter 5.

Chlamydomonas reinhardtii 11/32C in this study have been shown to accumulate polyphosphate. This polyphosphate has a function as an energy reserve (Kulaev and Kulakovskaya, 2000; Harold, 1966) in numerous microorganisms and algae (Aitchison and Butt, 1972). However, the level of the polyphosphate accumulation within algal cells are varies and depends on certain environmental conditions.

Aitchison and Butt (1972) reported that external phosphate concentration reveals the most important effect on polyphosphate content in microalgae (*Chlorella vulgaris*). The starved algae uptake the phosphate rapidly when they expose into replete-phosphate medium until the phosphate content in their cells is exceed. The effect of rapid change of the external phosphate to the level of polyphosphate content in *Chlamydomonas acidophila* KT-1 (acidophilic algae) and *Chlamydomonas reinhardtii* C-9 (neutrophilic algae) also studied by Nishikawa et al. (2006). They concluded that the external phosphate stress influences the polyphosphate content in the algae cells. But, in spite of the approximate similar growth rate, the content of polyphosphate in neutrophilic algae was 3 times higher than acidophilic algae. The different of this polyphosphate accumulation ability is caused by the specific property of the cell membrane (Nishikawa et al., 2006).

In regard to polyphosphate accumulation, Powell et al. (2009) observed the accumulation of acid soluble (ASP) and acid insoluble polyphosphate (AISP) in *Scenedesmus* cells. They examined the soluble and insoluble polyphosphate accumulation of *Scenedesmus* in three different external P concentration (i.e. 5, 15, and 30 mg P L^{-1}). The results showed that the algae grew in 5 mg P L^{-1} accumulate AISP but not ASP, while in 15 and 30 mg P L^{-1} the algae accumulated ASP and AISP. But in 5 and 15 mg P L^{-1} the accumulate polyphosphate used for their growth, whereas in 30 mg P L^{-1} only partially stored polyphosphate were consumed. These results indicated that high level of external phosphate stimulates microalgae to consume more P for their growth and store the excess as polyphosphate which known as luxury P uptake.

This study investigated the ability of *C.reinhardtii* 11/32C to uptake P and to accumulate it as polyphosphate. The uptake of intracellular P in *C.reinhardtii* 11/32C

showed to increase and this was followed by the increase of the algal biomass. The results from confocal microscopy and TEM analysis showed that the algae store more polyphosphate when they culture in high P concentration media (i.e. 100 mg P L^{-1}) (section 5.3.2). The DAPI-polyphosphate granules were shown by greenish yellow fluorescence in which the intensity reveal the amount of polyphosphate content in the algal cells (Gomez-Garcia et al., 2013; Mesquita et al., 2014; Nishikawa et al., 2006).

Furthermore, similar results observed by Qu et al. (2008) who examined *Chlorella pyrenoidosa*. They found the assimilation of cellular P increase gradually with the cells growth although the algal biomass achieved stationary phase. Additionally, the presence of luxury P uptake in *C.reinhardtii* 11/32C also shown by the size of polyphosphate bodies which is recognised by black lead precipitation in TEM (Ruiz et al., 2001a; Harold, 1966). The TEM results proved that the polyphosphate granule in *C.reinhardtii* 11/32C was located in the vacuole and these polyphosphate bodies also recognised by the other studies (Aksoy et al., 2014; Komine et al., 2000; Ruiz et al., 2001a).

In comparison with the other studies related to luxury P uptake and the storage of polyphosphate in the algal cells, this study found that *C.reinhardtii* 11/32C can play an important biological role on luxurious P uptake without cells starvation process. The findings also demonstrated that *C.reinhardtii* 11/32C are able to accumulate more P and stored it as polyphosphate bodies in the cells. This biological mechanism is shown important for *C.reinhardtii* 11/32C because they have ability to be implemented in bioremediation of the wastewater nutrient especially for P recovery in wastewater treatment.

8.3 Algal biomass production and P recovery from wastewater using a continuous flow mixotrophic system

The production of algal biomass and the potential of P recovery in *C.reinhardtii* 11/32C under continuous flow of mixotrophic microalgae culture were observed in this study (Chapter 6). Additionally, the potential use of this mixotrophic microalgae cultivation in wastewater treatment works for biological phosphorus removal was also investigated.

Results on the algal biomass production and the ability of *C.reinhardtii* 11/32C to assimilate and to accumulate intracellular P in their cells are explained in the following section.

8.3.1 Algal biomass production

This study used integration of heterotrophic and phototrophic microalgae cultivation equipped with algae recycle to enhance algal biomass production and simultaneously to recover of nutrient (mainly P) from the synthetic wastewater. This mixotrophic culture contributes the integration mechanism of both photosynthetic and heterotrophic components in the algal growth cycle (Kong et al., 2012). As photoautotrophic alga is light dependent, its growth decreases during the night due to respiration of carbohydrates. Thus, simultaneous growth of heterotrophic algae can reduce the loss growth in day light and sustain the algal growth with the addition of organic carbon (Mohan et al., 2015).

A two-stage heterotrophic and phototrophic cultivation using *Chlorella sorokiniana* were studied in a closed PBR (Zheng et al., 2012). They compared the algal growth in heterotrophic and phototrophic cultivation and reported that algae productivity (cells ml^{-1} d^{-1}) in heterotrophic was 7 times greater than phototrophic culture. Kong et al. (2012) compared the enhancement of algae biomass in *Botrycoccus braunii* using several organic carbon sources (i.e. sodium acetate, sodium citrate, glucose and sucrose), inorganic carbon and brewery wastewater. Kong and co-workers cultured the algae in mixotrophic and phototrophic mode. They found that organic carbon can stimulate the increase of algal biomass and the algal cells density were promoted significantly in mixotrophic culture than phototrophic. Among the organic carbon, glucose showed the best soorce of carbon for *B.braunii*. Additionally, in untreated brewery wastewater, the algae achieved 1 g L^{-1} of biomass when the algae grow in 20 days batch culture. These results indicated that microalgae can enhance their growth and biomass in the absence of light and use organic carbon as both energy and carbon source (Bumbak et al., 2011; Perez-Garcia et al., 2011; Wang et al., 2014).

In contrast with the previous studies, the results in this study showed that the algal biomass concentration in heterotrophic was lower than in photoautotrophic culture.

Presumably it was caused by insufficient organic carbon supply for *C.reinhardtii* 11/32C in heterotrophic culture because the concentration of organic carbon (i.e.sodium acetate) was only a quarter of total organic and inorganic carbon. Although the concentration of organic carbon in the media was lower than inorganic, but the bacterial growth considerably high. It is because bacterial growth limited by the supply and the concentration of organic carbon (Eiler et al., 2003). Therefore, it is suggested to change the arrangement of the reactor series to be phototrophic followed by heterotrophic in order to enhance biomass production.

Furthermore, ratio of C/N can also affect the algae growth and nutrient utilisation in microalgae grew in mixotrophic mode. Pagnanelli et al. (2014) examined the ideal ratio of glucose and nitrate (C/N) to enhance algal biomass in *Chlorella* and they conveyed that C/N ratio at 35 is the best for *Chlorella* and C/N ratio at 87 is suitable for *Chlorella protothecoides* (Wang et al., 2013b). Kong et al. (2012) revealed that the concentration of organic carbon (i.e. glucose) above 20 g L^{-1} can inhibit microalgae growth.

However, during 70 days cultivation, the alga biomass production in this mixotrophic continuous study increased and it was accompanied by the increase of hydraulic retention time. It was elucidated because the algae have enough time to utilise nutrient from the media and therefore, enhance the algae biomass. The algal biomass production in *C.reinhardtii* 11/32C revealed greater than the other previous continuous cultures (Carvalho and Malcata, 2005; Tang et al., 2012; Wen et al., 2014).

In addition, this study also observed simultaneously growth of *C.reinhardtii* 11/32C under heterotrophic and phototrophic mode in which there was a combination growth metabolism. Microalgae can continue grow photosynthetically while assimilating and metabolising organic carbon (Smith et al., 2015). Hence, this simultaneous mode can enhance algal biomass production in whole system. Moreover, recycle of algae is likely to contribute in rising of the algal growth in continuous mixotrophic culture and eventually escalating the harvested algae at approximately 2.6 g L^{-1} d^{-1}. Similar results of the effect of algae recycle on the biomass and settling performance of *Pediastrum boryanum* was also observed by Park and Craggs (2014). They found that the recycle

of 10% daily biomass production can enhance total biomass concentration of the algae cultivated in high rate algal pond.

The results in our study elucidated that the continuous mixotrophic culture has a potential to generate sufficient algal biomass as a result of simultaneous growth between heterotrophic and phototrophic culture. Presumably the stress mode of the algae when grew in the sufficient and in the absence of light while assimilating combination of organic and inorganic carbon can trigger the algae to grow faster than in the conventional one, for instance phototrophic or heterotrophic only.

8.3.2 Intracellular P uptake and recovery

C.reinhardtii 11/32C has shown their ability to uptake intracellular P under mixotrophic growth condition. The uptake was greater in phototrophic than heterotrophic culture. It was predicted due to the continuous illumination in phototrophic growth. This result was similar with the previous work conducted by Qu et al. (2008). Furthermore, Qu et al. (2008) examined P assimilation in *Chlorella pyrenoidosa* which cultivate in three different conditions (i.e. heterotrophic, autotrophic and mixotrophic). They found maximum cellular P assimilation in the biomass were 8.1, 16.1 and 30 mg P-PO_4^{3-}/mg dry weight for heterotrophic, mixotrophic and autotrophic respectively. These results indicated that illumination and photoautotrophic mode can enhance P metabolism and assimilation in the algae cells.

On the other hand, the content of total phosphorus of *C.reinhardtii* 11/32C cultured in heterotrophic was greater than in phototrophic reactor. It was likely due to the algae biomass concentration in heterotrophic lower than phototrophic culture and this elucidated that the algal growth in heterotrophic also less than in phototrophic culture. Therefore, the stored P in the heterotrophic algae cells still higher than in the phototrophic algae. This is also confirmed by the bigger size of polyphosphate granules of algae cultured in heterotrophic than phototrophic (section 6.3.3.3).

In the mixotrophic continuous culture of this study, a maximum of 50% P removed from the synthetic wastewater. This removal attained maximum at 1.3% and 0.81% of

intracellular P in algal biomass for algae cultured in heterotrophic and phototrophic mode respectively. Whereas, *Scenedesmus obliquus* stored maximum at 0.72% of P when culture in continuous flow system (Sancho et al., 1999). These results indicated that *C.reinhardtii* 11/32C capable to accumulate P in their cells higher than another green microalgae.

Interestingly, the intracellular P content of *C.reinhardtii* 11/32C grown in batch culture was 2.8 times higher than in the continuous culture. The maximum intracellular P attained in the algal biomass of batch culture was 3.6% and this achievement was higher than the percentage of P in *Scenedesmus* sp (3.16%) when cultured in rectangular reactor vessel with continuous feeding at 10 days retention time (Powell et al., 2008). The intracellular P content (polyphosphate) was lower in the continuous mode due to the algae were maintained to grow exponentially and as a consequence, the stored polyphosphate will be utilised faster for the algal growth. Whereas, in batch culture, the stored polyphosphate will be increased in the late of exponential phase due to cell aging and less algae growth (Harold, 1966). However, the trend of the intracellular uptake in the continuous mixotrophic culture in this study shown that the uptake increased with the increase the hydraulic retention time.

Overall, cultivation of *C.reinhardtii* 11/32C in the continuous mixotrophic culture confirmed that the algae generated high harvested algal biomass daily. This outcome coupled with sufficient P recovery from the media and stored as polyphosphate in their cells with the content over 2%. This P accumulation can be increased with the extent of hydraulic retention time. These features revealed that this microalgae system offer important mechanism on biological P removal and algal biomass production which have benefit for wastewater treatment especially for P recovery and generation of renewable energy in wastewater treatment works.

8.4 Investigating the potential of implementing a continuous flow mixotrophic microalgae systems in an existing wastewater treatment works

Chapter 7 case study was based on Esholt WWTW serving 760,000 population equivalent from Bradford and the west part of Leeds (Bense, 2013; Marsden et al.,

2012). According to the wastewater characterisation, Esholt WWTW has achieved high efficiency of nutrient removal at 49% and 39% for N forms and 64% and 54% for P forms respectively for primary settling tank and aeration tank. As a result, the wastewater treatment site can deliver the final effluent quality which meet the discharge regulation based on UWWTD and FFD (FFD, 2006; UWWTD, 1991).

On the other hand, the current main nutrient elimination at Esholt WWTW still rely on activated sludge process. This aeration process has main drawback due to high energy consumption which represents 60% of the total energy requirement for the conventional wastewater treatment plant (Shi, 2011). Furthermore, the primary sludge and surplus activated sludge thickening are loaded into anaerobic digestion facility (Marsden et al., 2012) for renewable energy production. After digestion process, the effluent (digested liquor) which contains high nutrient concentration particularly NH_4^+ (1600 mg NH_4^+-N L^{-1}) and PO_4^{3-} (24 mg PO_4^{3-}-P L^{-1}) loaded back into the aeration tank. This conventional method has contributed a considerable amount of high nutrient level into activated sludge process and increase the energy consumption for aeration (Menger-Krug et al., 2012).

In order to contribute an improving wastewater treatment in a sustainable way, this study attempts to provide a sustainable method for biological wastewater treatment in particular for P recovery. As a consequence to achieve high standard of sewage discharge regulation and to reduce the impact of GHG emissions, the UK water industry must have a target to achieve of 20% renewable energy generation by 2020 (Howe, 2009; Trumper, 2007). In regard to reduce the GHG emissions and generate renewable energy on site, the investigated microalgae system in this study may assist the wastewater treatment target for biological nutrient recovery, mainly for P.

The research results showed that a continuous mixotrophic culture can remove nutrient from the media in forms of N (97%) and P (50%) (Chapter 6) and *C.reinhardtii* 11/32C can store carbon in their cells at 50% (Chapter 4). This microalgae system achieved N content in the algal biomass at 13% and NH_4^+ concentration in the final effluent at 1.7 mg NH_4^+-N L^{-1}. Even though the P concentration in the final effluent remained at 7 mg P L^{-1} and it was higher than the discharge consent (i.e. 1 mg P L^{-1}),

the continuous mixotrophic algae system attained the maximum intracellular P in their cells at 2.1%. However, the extension of hydraulic retention time may enhance the uptake of intracellular P. A model of P utilisation in complete mixed reactors with recycle has been done to obtain the best hydraulic retention time to meet P level at approximately 1 mg P L^{-1} in the final effluent (see Chapter 7). In order to meet this target, the series of 4 HTR and 2 PBR are suggested with the total hydraulic retention time of 1 days.

The performance of the continuous mixotrophic alga culture in this study revealed that the microalgae system has a potential to be accomplished in the conventional wastewater treatment works. As all nutrient resources including CO_2 or carbon source require for algal growth is available on site at wastewater treatment works (Menger-Krug et al., 2012). As mentioned previously in Chapter 7 that in Esholt WWTW, 78% and 22% of P and 19% and 20% of N were incorporated into the waste activated sludge and remained in the secondary effluent respectively. These sources can be used as nutrient growth for microalgae as an integrated biological nutrient removal and algal biomass production for bio-products and also co-digested to enhance biomethane production. Previous study reported that co-digested microalgae enhances biomethane production up to 60% (Menger-Krug et al., 2012).

In order to capture the nutrient resources leaving from the ASP, the continuous mixotrophic microalgae system can be placed after aeration tank. However, before feed it into microalgae system, the effluent of ASP should be decanted to remove the solids particle. Additionally, the supernatant after the sludge dewatering can also be added into the system. The reason to place the continous mixotrophic microalgae system after ASP is because *C.reinhardtii* 11/32C require sufficient light sources to process photosynthetic and to assimilate high portion of P for their growth (Borchardt and Azad, 1968; Qu et al., 2008). Hence, it will be unattainable if the system placed after anaerobic digestion and used digested liquor for their nutrient growth because this nutrient source is very dark, contains a lot of amount of solids particles and low light transmittance (Rusten and Sahu, 2011; Suschka and Grubel, 2014). Moreover, considerable concentration of ammonia from digested liquor can inhibit microalgae grow due to the toxic effect (Kallqvist and Svenson, 2003).

The evaluation results from this chapter have convinced that the continuous microalgae system is appropriately to be placed after aeration tank. However, this option is shifted from the previous results mentioned in Chapter 4 in which the optimisation results revealed the algae cultivate in batch culture was suitable to grow in nutrient-rich environment (digested liquor). As mentioned previously, it seems that the microalgae system is difficult to cultivate after anaerobic digestion particularly for enhancing intracellular P uptake.

The performance of *C.reinhardtii* 11/32 cultivated in the continuous mixotrophic mode revealed the ability of the algae to recover nutrient and to generate significant algal biomass. Co-digested WAS and microalgae can contribute in enhancing biomethane production and renewable energy. This energy can support microalgae based wastewater treatment for cultivation and harvesting. In addition, this microalgae system can assimilate C and N and therefore, they can eliminate GHG emission such as CO_2 and N_2O which are contributing to the climate change. As a consequence, the microalgae based wastewater treatment offer possibility to replace the activated sludge process.

The overall results shown that in the future there will be a possibility to re-route the conventional biological nutrient removal for nitrification-denitrification and P removal using the continuous microalgae system which provide more sustainable method for nutrient recovery without significant energy consumption. Therefore, this system may reduce the external energy requirement but can generate renewable energy on site.

8.4.1 Microalgae harvesting

Microalgae harvesting is a major challenge to the implementation of an integrated microalgae system in wastewater treatment. The harvesting technology is depend on many considerations including cell size, density, settling velocity and the value of end products (Brennan and Owende, 2010; Cheah et al., 2016; Christenson and Sims, 2011; Gerardo et al., 2015). Recovery of algal biomass can be a serious consideration because of the small cell size which is ranging from 3 to 30 μm (Grima et al., 2003), settling velocity of the green microalgae approximately at 0.1 m d^{-1} (Cheah et al., 2016; Milledge and Heaven, 2013), and primary sedimentation with no flocculation can only

reach 7% of algal biomass in forms of total suspended solids as density of microalgae is closed to water density at 1,025 kg m^{3-} (Gerardo et al., 2015; Millero and Lepple, 1973). As a consequence, microalgae harvesting is estimated to contribute up to 30% cost of the total algal biomass production (Grima et al., 2003).

In order to reduce the total operational cost for algal biomass production, a low cost of microalgae harvesting is important to consider as a first step to reduce the energy consumption (Milledge and Heaven, 2013) and therefore, sedimentation is the best solution for low energy algae harvesting. In this study, sedimentation technique was used to separate *C.reinhardtii* 11/32C from the liquid with the harvested algae up to 3.6 g L^{-1} daily in forms of volatile suspended solids. A 20 mg L^{-1} of Zetag 50 (polymer) was added into the feeding media as flocculants because flocculants can increase the density of algae, the size of algal flocs and the settling velocity of algae (Brennan and Owende, 2010). Polymeric flocculants have been used extensively for algal biomass recovery (Grima et al., 2003). Flocculation using polyelectrolytes is quite effective for freshwater microalgae (Sukenik et al., 1988). This method can effectively recover algal biomass for more than 80% when use Zetag 63 and Zetag 92 (Bilanovic et al., 1988; Grima et al., 2003). Moreover, as a result of inorganic carbon consumption during photosynthesis process, the pH increase and result in enhancing precipitation of algae and this has reported in *Scenedesmus dimorphus* (Sukenik and Shelef, 1983). The settling of algae under alkaline condition with pH range between 10 and 10.6 also reported by Knuckey et al. (2006). In this study, the pH between 8 and 10 during *C.reinhardtii* 11/32C cultivation under mixotrophic condition proved to help the algae settling.

In regards to the implementation of mixotrophic microalgae in the existing WWTW, gravity sedimentation followed by filtration is recommended. Gravity sedimentation operation for microalgae harvesting is considered similar to secondary clarifier in the existing wastewater treatment plant. In order to achieve optimum algal biomass recovery higher than 99%, the settling algal flocs should be reach 1 cm min^{-1} (Gerardo et al., 2015). The sedimentation tank is equipped with a conical settling tank to enhance the algae harvesting. It is recommended that the conical settling tank is also equipped with algae scraper in order to collect the algae which is stick on the wall of

the settling tank. The velocity of the algae scraper is supposed to be 0.1 rpm to ensure the concentrated algae is homogeneous and flowing into the outlet at the bottom of the cone (Gerardo et al., 2015). The algae biomass that concentrated at the bottom of the cone should be decanted periodically and automatically. This system is similar with the operational microalgae system during laboratory experiment. Moreover, decanted algae are directed into porous gravity filtration for algae dewatering. Filtration provides high efficiency of algal biomass recovery and allow separation of the shear microalgae species (Barros et al., 2015). However, this method requires regular membrane replacement due to the possibility of filter clogging and pumps the concentrated algae at the bottom of the filter. In order to reduce the energy requirement for pumping of the concentrated algae, the algae can be collected manually. The overall energy requirement for the combination of algal settling and filtration is 0.01 kWh m^{3-} (Gerardo et al., 2015).

8.5 Global implication of microalgal biomass production

Globally, water industry is facing a problematic challenge to its operating cost including climate change mitigation, reducing of carbon emission and achieving the discharge consent of sewage effluent (Palmer, 2010). In the UK, these challenges lead to contribute 10% of the total water company operating cost for supporting the energy consumption (Palmer, 2010). Currently, in the era of climate change wastewater treatment works with low emission of GHG and high efficiency in recycling nutrient is highly demand (Wang et al., 2016).

Worldwide WWTW employ activated sludge process (ASP) for removing organic matter (i.e. BOD) and nitrogen from wastewater and generate high quality of effluent but ASP also has some drawbacks. For instance, nitrification-denitrification process require a lot of aeration which lead to high energy consumption, ASP process contributes GHG emission such as CO_2 and N_2O to the atmosphere (Wang et al., 2016). These GHG are produced mainly during nitrification-denitrification process (Kampschreur et al., 2009; Wang et al., 2016). In particular to N_2O emission, ASP contributes up to 90% and the volume of this gas will increase with the quantity of aeration flow. Moreover, the impact of N_2O emission to the atmosphere is reported 300-fold stronger than CO_2 (Kampschreur et al., 2009). In addition to P removal,

current commercial process for P removal from wastewater is chemical precipitation by using metal salt (de-Bashan and Bashan, 2004). Nevertheless this method offer a little chance for P recovery from wastewater treatment.

In order to achieve sustainable wastewater treatment, it is very important to maximise nutrient recovery and to eliminate GHG emission from wastewater (Mahdy et al., 2015). This may help WWTW to meet the effluent discharge consent and the environmental regulation. Microalgae based wastewater treatment offers several advantages over the conventional treatment. These advantages are including low energy requirement, reduction of the sludge formation, elimination of GHG and production of the useful biomass (Oilgae, 2009). In this microalgae system, the nutrient transfer into algae cells for the production of biomass is became promising technology (Mahdy et al., 2015).

Worldwide, algal biomass production has driven important attention in providing raw material for biofuels and non biofules. Many companies have involved in the development of microalgae biomass production, those are in the US 78%, in Europe 13% and others 9% (Singh and Cu, 2010). In 2016, The Chinese Microalgae Industry Alliance (CMIA) estimated that its industry has supplied about two-third of global microalgae biomass production which is used for nutraceutical, animal feed, fuels and fertiliser (Chen et al., 2016). In India, the first India's Algenol algae production platform has developed by the Algenol and Reliance Industries which produce oil refinery from microalgae with a capacity of 668,000 barrels d^{-1} at a 7,500 acre. This plant recycles CO_2 from industrial process for ethanol, gasoline and jet fuels (Lane, 2015). Whereas in the UK, a £26 million has been funded by UK Carbon Trust to build algae farm in Northern Africa and a £8 million to Algae Biofuels Challenge (ABC) to develop algae research program (Singh and Cu, 2010). Moreover, microalgae have been developed for treating various wastewater for secondary, tertiary and digested treatment (Mahdy et al., 2015; Menger-Krug et al., 2012; Wang et al., 2016). In this context, microalgae promote their ability not only for bioremediation but also as a renewable energy source (Rawat et al., 2011) and as a commercial bioproduct (Spolaore et al., 2006).
This study proved that *C.reinhardtii* 11/32C capable to recover nutrient from wastewater and accumulate the N and P in their cells up to 14% N and 2% P under

mixotrophic continuous flow cultivation, and up to 9% N and 3.6% of P under batch culture. In addition to the nutrient, this algae able to accumulate C at 52% and have lipid content at 21%. Under mixotrophic growing condition, *C.reinhardtii* 11/32C produced maximum 3.6 g VSS L^{-1} of biomass. This algal biomass is profitable to further process as fertiliser or co-digested with activated sludge to produce biomethane.

Wastewater can be used as a nutrient source for microalgae because it contains N, P and C which is suitable for microalgae cultivation (Pittman et al., 2011). According to mass balance analysis of Esholt WWTW, 78% of P in wastewater bound to WAS and 61% of N (i.e. N_2, N_2O) released to the atmosphere as a result of nitrification-denitrification process. These portion shown that the amount of non-recycle nutrient in wastewater are relatively high. Supposing that amount feed into microalgae system as nutrient source, the algae can assimilate it and convert it into biomass. Cultivation of *C.reinhardtii* 11/32C under mixotrophic growing condition revealed that the algae able to store polyphosphate at 2% and the recovered P has potential to re-use as a substitute of mined P fertiliser (Cornel and Schaum, 2009). Algal biomass can also be co-digested with sludge to enhance 60% of biomethane production and therefore, generate a renewable energy on site (Menger-Krug et al., 2012). This potential promotes the mixotrophic microalgae system to replace activated sludge process and reduce high amount of an energy consumption.

Currently this mixotrophic microalgae-based wastewater treatment is very challenging to implement in the large scale of WWTW like Esholt because this system requires a considerable of the land area to build bioreactor. However, at present this system is applicable for small scale of WWTW which treat wastewater from less than 10,000 population. In the future, there is real possibility to develop a novel sustainable microalgae system to recover nutrient from wastewater and generate renewable energy. This includes enhancing the algal biomass production and reducing the bioreactor volume.

On the other hand, the mixotrophic microalgae system may offer feasibility to be implemented in developing country like Indonesia in which the discussion of the

application of mixotrophic microalgae system focus on the wastewater treatment facility and the fertiliser supply. Indonesia is the fourth most populated country in the world with population of 245 million in which almost half of the population lives in urban areas (WB and AusAID, 2013). In 2011, the average of the urban growth rate was 3.3% and the requirement of wastewater facility is growing rapidly (WB and AusAID, 2013). Currently, Indonesia still faces a crucial problem particularly of poor sanitation facility for domestic wastewater and its services. The access to the wastewater service facilities in 2000 only covered up to 37.5% in urban areas and 36.5% in rural areas, and on average increased to 55% in 2005 (Roosmini and Rahmawati, 2005).

The existing wastewater treatment facilities in Indonesia was studied by ESP (2006) and they reported that three types of wastewater treatment such as aeration ponds, aerated lagoon, ASP and rotating biological contactor (RBC) are the common wastewater treatment facility in Indonesia. Aerated lagoon is widely applied in Indonesia following by aeration ponds. ASP was applied in Balikpapan but this technology only run for several months due to lack of monitoring and knowledge skill to treat wastewater using activated sludge process. On average, those facilities still low in control and monitoring, and there is no opportunity for resource recovery.

Furthermore, in regards to the availability of fertiliser, Indonesia still import the key chemical materials for fertiliser from Jordan which distributed through Aceh, Kalimantan and Surabaya (Utami, 2016). There are 83% of fertiliser plants produce urea and the remaining of the plants produce triple superphosphate (TSP), superphosphate (SP-36) and ammonium sulphate (AS). Nonetheless, the raw materials for the fertiliser compounds especially phosphate and potassium chloride have to be imported (FAO, 2011). Moreover, some problems related to the failure of supply network fertiliser cause delay in delivering the fertiliser to the farmers and this lead to the serious impact on agriculture product (Utami, 2016). As a consequence of these problems, the price of fertiliser in Indonesia is very expensive.

In order elucidate these problem, mixotrophic microalgae system offer the potential to be implemented in Indonesia. Algae-based wastewater is suitable to apply in tropical country (i.e. Indonesia) as the tropical country provides warmer temperature and

constant sunlight (Oilgae, 2009). The land area requirement to build the microalgae-based wastewater system is also in relatively low cost compare to UK price. This microalgae-based wastewater system may offer low cost wastewater treatment for treating domestic wastewater without high skill operator is needed.

In regards to wastewater, Cordell et al. (2011) reported that human excreta produce of 0.35% P by dry weight with magnitude of 0.3 and 0.6 kg P $year^{-1}$ for vegetarian and meat-based diet respectively (Cordell et al., 2009) . In addition 56% of the Indonesian population had an access to the improved sanitation facility in 2011 (WSP, 2015). Therefore, an approximately 41.2 and 82.3 kT P $year^{-1}$ will be generated from human waste annually in Indonesia.

Suppose the microalgae-based wastewater is implemented in Indonesia, it will assist the acceleration of the providing wastewater treatment facility for domestic or non-domestic purposes. Moreover, the harvested algae which contain P in forms of polyphosphate can be used as bio-fertiliser. This bio-fertiliser may help Indonesian farmer to support their fertiliser requirement for the agricultural activity.

Chapter 9 Overall Conclusions and Recommendations

9.1 Research conclusions

This research work investigated the potential for integrating microalgae systems into existing wastewater treatment processes, in particular as a biological phosphorus recovery and algal biomass production system. This study has achieved the aim and objectives presented in Chapter 1. The following section summaries the main conclusions from the experiment studies.

Under laboratory controlled conditions *C.reinhardtii* 11/32C revealed to be able to uptake nutrient from the media and they incorporated the nutrient elements into their biomass at 5.8% N and 1.6% P. In particular of P content in the algal biomass, this percentage of P was higher than the typical biomass P in natural conditions (i.e.1%). The experiments on the effect of different N sources (i.e NO_3^- and NH_4^+, NO_3^- or NH_4^+) in enhancing intracellular P uptake and biomass production revealed that *C.reinhardtii* 11/32C was preferred to uptake NH_4^+ rather than NO_3^-. Additionally, combination between NO_3^- and NH_4^+ as a sole of nitrogen for *C.reinhardtii* 11/32C has created stress transition effect which trigger higher algal biomass productivity and intracellular P uptake compare to the algae cultivated in NO_3^- or NH_4^+ only.

In addition, the optimisation test and response surface analysis conveyed that the environmental conditions: N (200 mg N L^{-1}), P (100 mg P L^{-1}), photoperiod (6 hr light:8 hr dark), and light intensity (250 $\mu Em^{-2}s^{-1}$) generate the optimum intracellular P uptake at 4 mg P L^{-1} d^{-1} and the algal biomass productivity of 147 mg VSS L^{-1} d^{-1}. From the optimisation test results, it was presented that *C.reinhardtii* 11/32C accumulate intracellular P ranging from 0.3% to 4%. These outcome elucidated that the algae are able to perform luxurious P uptake and to grow in nutrient-rich conditions.

The potential of *C.reinhardtii* 11/32C to perform luxurious P uptake was conveyed by the storage of the excess of P as polyphosphate granules in their cells. The size of the stored of polyphosphate granules depends on the amount of intracellular P uptake. These granules were confirmed by cells examination using confocal microscopy and

transmission electron microscope. The presence of polyphosphate granules in the algal cells appeared as a bright green yellow fluorescence and these polyphosphate bodies were located in the vacuoles. This study found that luxurious P uptake in *C.reinhardtii* 11/32C occur without cells starvation process. In addition, these hyper P accumulators were sorted through flow cytometry analysis and 10.2% of hyper polyphosphate accumulators in *C.reinhardtii* 11/32C were isolated. However, the stored polyphosphate granules in the algal cells are not stable and they depends on the growth cycle, and therefore the mutants which are able to lock the polyphosphate bodies in their cells is required to isolate for future study.

The continuous mixotrophic microalgae system revealed that the algae can remove the nutrient at 97% N and 50% P from the synthetic wastewater. The continuous microalgae culture could meet the discharge sewage effluent regulation at 1.7 mg NH_4^+-N L^{-1}, however the P concentration in the final effluent still 7.4 mg PO_4^{3-}P L^{-1}. In order to meet the target consent at approximately 1 mg PO_4^{3-} P L^{-1}, modelling P recovery was conducted for HTR and PBR. This treatment required of hydraulic retention time at 2 days in total for heterotrophic and phototrophic culture.

Moreover, the algae in the continuous culture produced on average 248 mg VSS L^{-1} d^{-1} of biomass productivity and 2.03 mg P L^{-1} d^{-1} of intracellular P uptake with the total 2.1% of P incorporated into the *C.reinhardtii* 11/32C biomass. The algal biomass production in the continuous system was almost double than in batch culture but the P biomass content was lower. This indicated that *C.reinhardtii* 11/32C can grow in different conditions either heterotrophic or phototrophic and the simultaneous cultivation between heterotrophic and phototrophic. Furthermore, the algae recycle enhanced the algal growth and biomass production in the system.

Intracellular P uptake and the amount of P incorporated in the algal cells in continuous mixotrophic culture were lower than in batch culture. These results elucidated that the algae grow faster in the steady state conditions and consume more polyphosphate storage to support their cells metabolism, and as a consequence the polyphosphate content in the algae cells decreased. Whereas in batch culture, exponential growth phase of the algae occurred in the short period and the cells will reach stationary

phase, and therefore can accumulate more polyphosphate. These results confirmed that the intracellular P uptake contributes to microalgae growth not only in the heterotrophic but also in phototrophic culture.

The mass balance analysis confirmed that the Esholt WWTW remove over 95% of N forms and 80% of P forms. These removal level generated final effluent quality that meet discharge consent legislation according to Freshwater Fish Directive and Urban Wastewater Directive. Regarding to the wastewater mass balance analysis, we found that 78% and 22% of P from the Esholt wastewater incorporated into the waste activated sludge and in the organic particles of the secondary clarifier effluent. Whereas 19%, 20% and 61% of N were distributed in the waste activated sludge, secondary clarifier effluent and converted into N_2 through denitrification process. These results revealed that the effluent of activated sludge process becomes a considerable source for nutrient recovery. However, the extraction of nutrient from the sludge requires further treatment that trigger higher energy and chemical consumption.

This study proved that *C. reinhardtii* 11/32C are able to grow in different culture as heterotrophic and phototrophic algae and showed as an excellent nutrient remover microorganism. This ability offer the prospect of microalgae system to be implemented as a biological nutrient removal in the existing wastewater treatment works. In particular of P recovery, *C. reinhardtii* 11/32C showed their ability to accumulate high amount of polyphosphate and stored the P in the cells up to 3.6% as luxurious P uptake. This alga conveyed the potential to substitute the chemical precipitation method with biological P removal by using microalgae in wastewater treatment. Also this alga could completely remove N forms from the growth media, and therefore demonstrated their ability to substitute nitrification-denitrification process. Moreover, *C.reinhardtii* 11/32C able to produce high harvested biomass on average at 2.6 g VSS L^{-1} and maximum at 3.6 g VSS L^{-1} daily. These important competency on nutrient recovery coupled with high algal biomass production convince that the continuous mixotrophic microalgae system can be retrofitted into existing wastewater treatment works.

9.2 Further Research

Further research is needed to improve our understanding on P recovery from wastewater, using microalgae as nutrient accumulating microorganisms. The following recommendations can be suggested for future works:

- Modelling external phosphorus that transport through microalgae cells in order to understand how the algae accumulate the polyphosphate and when will they utilised it. This model will be useful to enhance intracellular P uptake and accumulation of polyphosphate in microalgae cells. Practically, this model can be applied on enhanced biological phosphorus removal using microalgae.
- Modelling important parameters to improve the performance of the continuous culture reactor system. This may help to develop mass transfer, nutrient uptake and microalgae growth to be able to implement in the existing wastewater treatment works.
- The study on the algae settling mechanisms is also important to consider especially when the experiment use motile algae like *Chlamydomonas reinhardtii* which have flagella for moving activity. This will help the algae settling performance and also enhance the amount of harvested algae.
- The use of mutant *Chlamydomonas* as an experiment organisms which have ability to store polyphosphate without release it to the environment also important to consider for P recovery from wastewater. In addition to this point, further experiment using mixed organisms such other microalgae strains and bacteria might be more realistic approach to integrate microalgae system into the existing wastewater treatment works as in activated sludge process there are many type of microorganisms present and involve in wastewater treatment.
- Further energy balance analysis is required to complete the life cycle analysis for the current wastewater treatment works and the continuous mixotrophic microalgae system.
- In order to retain of the polyphosphate accumulation in *C.reinhardtii* 11/32C, it is necessary to run the mixotrophic microalgae cultivation under continuous flow which set the photoautotrophic before the heterotrophic reactor.
- In order to investigate the potential of the mixotrophic microalgae cultivation under continuous flow in the existing conventional WWTW, scaling-up of that system to the pilot scale in WWTW must be considered.

- The investigation to find the optimum organic carbon concentration in heterotrophic cultivation is important as the outcome can be used to study the effect of bacteria-algae symbiosis and the most appropriate HRT for enhancing algal biomass production and intracellular P uptake in algal cells.

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