MICROALGAE CULTIVATION UNDER HETEROTROPHIC AND PHOTOTROPHIC CONDITIONS FOR NUTRIENT RECOVERY FROM WASTEWATER

by

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

This PhD thesis was dedicated to my grandfather, Kadir Öz, who passed away by a car accident at the first year of my PhD study.

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Abstract

Nutrients such as nitrogen and phosphorus are the most important elements for living species, as they are used to synthesise the main components of cells; however, they are also considered amongst the main pollutants responsible for adverse effects on aquatic environments (e.g., eutrophication). Current nutrient control technologies at wastewater treatment works rely heavily on bacterial processes for nitrogen and phosphorus removal. Although these processes are effective at removing nutrients, microalgae cultivation has shown considerable promise in this regard with the further benefit of achieving more stringent discharge levels and increasing overall sustainable resource management. This PhD research aims to optimise both phototrophic and heterotrophic cultivation of microalgae as an alternative means for nutrient control and recovery in sewage treatment works. *Chlamydomonas reinhardtii* was used as a model species due to its ability to grow under both phototrophic and heterotrophic conditions and common presence in wastewater pond treatment systems. With regard to algal growth kinetics studies, higher specific growth rates were achieved under heterotrophic conditions than under phototrophic cultivation. Nitrate was found as the preferred nitrogen source under both heterotrophic and phototrophic cultivation. Higher specific growth rates for heterotrophic and phototrophic microalgae were obtained at lower carbon concentrations. With regard to the optimisation of a two-stage biological process combining phototrophic and heterotrophic cultivation under continuous flow with biomass recycling, the optimum operational conditions were determined as 48 h of hydraulic retention time (HRT) and 14 d of cell retention time (CRT) when a mix of ammonium and nitrate was used; recovery efficiencies for phosphorus and nitrogen were 40.0 and 93.2%, respectively. Nitrogen and phosphorus content in harvested *Chlamydomonas reinhardtii* varied from 5.3 to 9.6% and from 0.6 to 1.2% of dry biomass, respectively, and depending on operational conditions. Overall, the proposed two-stage biological process exhibited considerable potential for implementation in large wastewater treatment works to achieve simultaneous nutrient control and recovery. In order to assess its potential, from the perspective of its technical feasibility, a mass flow analysis of nutrients was conducted by using Yorkshire Water's Esholt Wastewater Treatment Works as a case study.

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List of Abbreviations

AD	Anaerobic Digestate
ADP	Adenosine Diphosphate
ANAMMOX	Anaerobic Ammonia Oxidation
ANOVA	Analysis of Variance
ASP	Activated Sludge Process
ATP	Adenosine Triphosphate
BBM	Bold's Basal Medium
BNR	Biological Nitrogen Removal
CCAP	Culture Collection of Algae and Protozoa
CFD	Computational Fluid Dynamics
CHP	Combine Heat and Power Engine
COD	Chemical Oxygen Demands
CRT	Cell Retention Time
DEFRA	Department for Environment, Food and Rural Affairs
DWD	Drinking Water Directive
EBPR	Enhanced Biological Phosphorus Removal
EPS	Extracellular Polymeric Substances
EU	European Union

FAO	Food and Agriculture Organization of the United Nations
FE	Final Effluent
FST	Final Settlement Tank
HTR	Heterotrophic reactor
HRAP	High Rate Algal Pond
HRT	Hydraulic Retention Time
IC	Ion Chromatography
LSD	Least Significant Differences
MPBR	Membrane Photobioreactor
OD	Optical Density
PAO	Phosphate Acculumatin Organisms
PBR	Photobioreactor
PST	Primary Settlement Tank
RABR	Rotating Algal Biofilm Reactor
SWW	Synthetic Wastewater
ST	Settlement Tank
TKN	Total Kjeldahl Nitrogen
TN	Total Nitrogen
TP	Total Phosphate
TSS	Total Suspended Solids
UK	United Kingdom

USEPA	United States Environmental Protection Agency
UWWTD	Urban Wastewater Treatment Directive
VSS	Volatile Suspended Solids
WSP	Waste Stabilisation Ponds
WWTP(s)	Wastewater Treatment Plant(s)

1. CHAPTER: INTRODUCTION

1.1. Background

Nitrogen and phosphorus are the most fundamental nutrients for living species. Nitrogen is used to synthesise proteins, amino acids and nucleic acids, while phosphorus is the main component of bones, teeth, DNA, RNA, ATP and phospholipids (Bougaran et al., 2010; Ashley et al., 2011). Despite the fact that reactive forms of nitrogen can be naturally produced by lightning and biological fixation of nitrogen gas (N_2) present in the atmosphere (Denk et al., 2017), reactive forms of phosphorus (phosphates) are very limited in the biosphere and thought to be a non-renewable resource for various reasons, including: (i) phosphates are only derived from phosphorus rock mines; (ii) over 70% of phosphorus rock reserves are located in only a few countries, mainly in China, Morocco and USA; and (iii) phosphorus cannot be synthesised artificially (Cooper and Carliell-Marquet, 2013; Kleemann et al., 2015; Cordell et al., 2009). This situation has led to the search for alternative means for the production of phosphorus fertilisers, including waste streams; phosphorus recovery from sewage is one of the alternatives currently being proposed, mainly because it is often lost in the effluent of wastewater treatment plants around the world, including those in the UK (Cordell et al., 2009; Cooper and Carliell-Marquet, 2013). Moreover, excessive amounts of phosphorus in aquatic environments generate undesirable consequences such as eutrophication, algal blooms and anoxia in water courses (Cooper and Carliell-Marquet, 2013).

Nutrient recovery at wastewater treatment plants has been presented as a sustainable solution for nutrient control and resource efficiency (Ashley et al., 2011). Current wastewater treatment plants are mainly based on bacterial processes for the biodegradation of organic materials and nutrient removal (DEFRA, 2012); nitrogen and phosphorus are removed by a nitrification-denitrification process and enhanced biological phosphorus removal, respectively, under different configurations with the combination of aerobic/anoxic and/or anaerobic tanks (Xu et al., 2014; Manyumba et al., 2009). Although these processes are efficient at the removal of nutrients, they require large amounts of energy and release substantial amounts of greenhouse gases such as CO_2 and N_2O (Wang et al., 2014a; Anbalagan et al., 2016; Kampschreur et al., 2009); as a consequence,

nutrient removal systems in existing wastewater treatment works are under close scrutiny due to the need to reduce their overall carbon footprint while coping with population growth; more stringent discharge consents, particularly for phosphorus (< 1 mg P L^{-1}) (UWWTD, 1991); and their contribution to the sustainable management of nutrient flows.

The use of microalgae cultivation in wastewater treatment systems has been demonstrated as being a promising option with the potential to simultaneously recover nitrogen and phosphorus in the resulting algal biomass, overcoming the deficiencies inherent to the current nutrient removal processes (Aslan and Kapdan, 2006). Microalgae has the capacity to take up nitrogen (as both ammonia and nitrate) and phosphorus species from wastewater (Wang et al., 2012). While the most common cultivation method for algal biomass production is based on phototrophic metabolism in the presence of light and CO_2 as energy and carbon sources, respectively, some microalga strains can grow under heterotrophic conditions, using organic carbon without the need for light (Perez-Garcia et al., 2011b). Higher biomass concentrations and higher lipid content are often achieved under heterotrophic cultivation conditions when compared to phototrophic conditions (Sakarika and Kornaros, 2016).

In conclusion, the increasing energy demand for aeration and the emission of greenhouse gases are considered to be drawbacks of the current nutrient control systems at wastewater treatment plants. Thus, the integration of microalgae cultivation systems into wastewater treatment plants could represent an alternative route for nutrient control, combining the benefits of N and P recovery with the production of algal biomass. In the published literature, there is still the need for further research on mixotrophic microalgae cultivation, particularly under conditions aimed at optimising both phototrophic and heterotrophic growth (using separate reactors for each process), defining conditions that separate hydraulic retention times from cell retention times in continuous flow operations, and maximising nitrogen and phosphorus uptake in wastewater treatment works.

1.2. Scope, Aim And Objectives

Current nitrogen and phosphorus removal technologies in existing wastewater treatment rely heavily on traditional and well-established bacterial processes, but their long-term sustainability is currently under scrutiny. New stringent discharge consents, mainly for dissolved phosphorus, will place further pressure on the need to develope alternative processes to reduce net energy consumption for highly efficient nutrient

control units. This research has investigated the potential for developing novel nutrient control and recovery processes based on phototrophic and heterotrophic cultivation of microalgae in existing large wastewater treatment works. Thus, this project aims to optimise both phototrophic and heterotrophic cultivation of microalgae as an alternative option for nutrient recovery from municipal wastewater, with the added benefit of enhancing bioenergy production by anaerobic co-digestion of sewage sludge and algal biomass. In order to achieve that the above, laboratory scale experiments were conducted to fully understand the kinetics controlling heterotrophic and phototrophic nutrient algal uptake. A continuous flow prototype was developed based on the improved understanding of process kinetics, with the potential to define design criteria and operational conditions for full-scale operation.

The objectives of this research are as follows:

1. To identify the kinetics controlling heterotrophic and phototrophic microalgal growth in wastewater and the potential for using microalgae for effective biological nutrient uptake and recovery.

2. To identify operational conditions for a two-stage biological process combining heterotrophic and phototropic microalgae cultivation for simultaneous nitrogen and phosphorus recovery via biological uptake under continuous flow conditions with biomass recycling.

3. To determine the potential to implement algal bioengineering processes for nutrient control and recovery in existing wastewater treatment works by developing a feasibility analysis of the optimised processes developed on the laboratory scale.

1.3. Background Context Supporting the Proposed Research at Leeds

The research conducted for over 40 years at the School of Civil Engineering, University of Leeds, has led to the development and consolidation of technologies for wastewater treatment based on the use of microalgae (Waste Stabilisation Ponds-WSP), in countries with both tropical and temperate climates (Mara, 2013). Over the past 10 years, their research activities have focussed on studying the use of novel algal-based technologies for nutrient control and resource efficiency in wastewater treatment systems

using alga ponds, rock filters, anaerobic digesters, duckweed ponds and photobioreactors (Johnson et al., 2007; Camargo-Valero, 2008; Camargo-Valero et al., 2009a; Usher, 2014; Adewale, 2015; Yulistyorini, 2016; Paterson, 2018).

The research work conducted in this PhD thesis builds upon the findings reported by Yulistyorini (2016) in order to assess the potential for implementing a novel nutrient control and recovery process based on the cultivation of microalgae under phototrophic and heterotrophic conditions with biomass recycling under continuous flow conditions, and further its relevance to existing wastewater treatment works.

Although Yulistyorini (2016) assessed the potential for enhanced phosphorus uptake via microalgae under controlled environmental conditions using a heterotrophic reactor followed by a phototrophic reactor in series, this project mainly focusses on the optimisation of a two-stage microalgal process combining phototrophic and heterotrophic cultivation, with biomass recycling under continuous flow conditions, to maximise algal biomass for both nitrogen and phosphorus uptake. Based on the findings reported by Yulistyorini (2016), this project tested a biological process using a phototrophic reactor followed by a heterotrophic reactor in series.

1.4. Proposed Research Methodology

The methodology followed in this research was proposed in order to answer the following research questions, which were further examined in detail in each of the thesis results and discussion chapters.

Research question linked to objective 1:

- How to scale-up microalgae cultivation using both heterotrophic and phototrophic conditions?
- What is the influence of various phosphorus concentrations on the kinetics controlling heterotrophic and phototrophic microalgae growth?
- How is heterotrophic and phototrophic microalgae growth affected by the change of other major nutrients (nitrogen and carbon sources)?

Research questions linked to objective 2:

- How can the efficiencies of biomass growth and nutrient uptake under mixotrophic microalgae cultivation be incremented?

- How do essential operational parameters for continuous flow systems (i.e., hydraulic retention time and cell retention time) influence algal biomass growth and nutrient uptake under mixotrophic conditions?

- What are the impacts of different nitrogen sources on algal biomass growth and nutrient uptake under mixotrophic conditions?

- What is the fate of nutrients in algal-based systems using mixotrophic conditions?

Research questions linked to objective 3:

- What is the fate of nutrients in current nutrient control systems at wastewater treatment works?

- How can the applicability of the proposed laboratory-scale microalgae system to large wastewater treatment plants be evalated?

Table 1-1 summarises the overall research approach and methodology. Research activities are listed and linked to the relevant research objective and thesis chapter in which detailed information regarding results and findings is provided.

Table 1-1 Research Methodology including research objectives and research activities

Research Objectives	Research Activities	Thesis Chapter

Objective 1:
To identify both heterotrophic and phototrophic microalgae growth kinetics by controlling nutrient concentrations and environmental conditions in a batch reactor
	• Culture propagation
	• Culture acclimatisation
	• Growth kinetics experiments
	  o Heterotrophic microalgae growth kinetics
	  o Phototrophic microalgae growth kinetics
		Chapter 4

Objective 2:
To identify optimum operational conditions for a two-stage microalgal process combining phototrophic and heterotrophic cultivation with biomass recycling under continuous flow conditions and optimising nutrient recovery via biological uptake
	• Culture propagation
	• Experiments to find optimum operational conditions
	  o Assessment of the effect of hydraulic retention time (HRT) on biomass growth and P uptake
	  o Evaluation of the impact of cell retention time (CRT) on biomass growth and P uptake
	  o Assessment of the influence of different nitrogen sources on biomass growth and P uptake
	• Mass flow analysis of nutrients in the two-stage microalgal process
		Chapter 5

Objective 3: To determine the potential to implement algal bioengineering processes in existing wastewater treatment works for simultaneous nutrient control and recovery using a microalgae process	• Water sampling on a monthly basis at Yorkshire Water's Esholt Wastewater Treatment Works to determine the fate of nutrients • Mass balance analysis of nutrients using research finding at Yorkshire Water's Esholt Wastewater Treatment Works as a case of study	Chapter 6

1.5. Thesis Structure

The motivations for this study and background context supporting the proposed research at the University of Leeds are explained in Chapter 1 along with the project's scope, aim and objectives. In addition, the research methodology and thesis structure are given.

Chapter 2 explains the research problem and research gaps identified with a deep and critical analysis of the published literature. Firstly, the current situation regarding global nutrient availability and supply challenges are discussed. Secondly, the situation with regard to wastewater treatment in the UK and current nutrient control and recovery systems are critically analysed. Thirdly, the possible advantages of using microalgae in wastewater treatment works are presented and the pathways by which microalgae take up nutrients are considered. Additionally, microalgae growth under different cultivation conditions is presented and contextualised with the use of wastewater as the culture medium. Lastly, the research problem and research gaps so identified are presented.

The materials and methods used for all experiments and the research methodology are described in detail in Chapter 3.

Chapters 4 to 6 contain an introduction to the rationale behind the work conducted, the research objectives, the research methodology for each research objective, the results obtained, data analysis and interpretation, and critical discussion and conclusions.

Growth kinetics of microalgae using phosphorus as the limiting nutrient under heterotrophic and phototrophic conditions are examined in Chapter 4. Furthermore, the effects of different nitrogen sources and different carbon concentrations on nutrient uptake is studied in order to determine the factors affecting the performance of microalgal-based processes on the environmental conditions potentially present in real wastewater treatment works.

Chapter 5 identifies the optimum operational conditions for a two-stage microalgae process combining phototrophic and heterotrophic cultivation conditions with biomass recycling under continuous flow conditions in order to maximise nutrient recovery via biological uptake.

The potential to implement nutrient control and recovery processes based on microalgae cultivation in current wastewater treatment works is evaluated in Chapter 6. As a case study, the mass flow analyses of nitrogen and phosphorus species, as performed at Yorkshire Water's Esholt Wastewater Treatment Works, are reported in order to assess the technical feasibility of implementing laboratory results on the real-world scale.

Chapter 7 aims to integrate the main findings of this thesis into the broader literature related to this topic. It argues how this thesis contributes to the current knowledge and literature. Lastly, recommendations for future studies are offered and discussed.

2. CHAPTER: LITERATURE REVIEW

In this chapter, the motivations for this study are discussed in detail. Firstly, the current limitations of nutrients availability on earth is explained. Secondly, the current nutrient control and recovery systems for sewage are analysed, with particular emphasis on existing practices at wastewater treatment works in the UK. Thirdly, possible advantages and disadvantages from using microalgae in wastewater treatment are discussed, along with the contributions that microalgae can provide in terms of improving nutrient recovery. Additionally, microalgae growth under different cultivation conditions is compared. Lastly, the identified research problem and research gaps are reported in detail.

2.1. Current Situation regarding Nitrogen and Phosphorus on Earth

Nutrients such as nitrogen and phosphorus are the most crucial nutrients supporting living species as they cannot be substituted during the synthesis of amino acids, enzymes, proteins, etc. (Denk et al., 2017; Ashley et al., 2011). Furthermore, they are essential for food production and therefore constitute the key components in any fertiliser used in agriculture (Cordell et al., 2009). The world demand for nitrogen and phosphorus fertilisers was estimated to increase by 1.3 and 2.0%, respectively between 2012 and 2016, along with the increase in world population (FAO, 2012).

The related literature shows that atmospheric nitrogen gas (N_2) cannot be utilised by most organisms, despite the fact that 78% of the atmosphere is made up of nitrogen. This situation limits access to nitrogen in aquatic and terrestrial ecosystems and reinforces the importance of the biogeochemical cycle of nitrogen on earth (Stüeken et al., 2016). Unlike nitrogen, phosphorus is considered a non-renewable resource because it is currently obtained from limited phosphate rock reserves as it cannot be synthesised and, therefore, its use cannot be replaced by any other element or manmade substance (Seyhan et al., 2012; Kleemann, 2015; Cordell et al., 2009).

The consumption of industrial phosphorus fertilisers has increased in the past six decades and, therefore, it is estimated that readily available phosphorus resources could be depleted within the next 50 to 100 years (Cordell et al., 2009; Cooper and Carliell-

Marquet, 2013; Jasinski, 2012). Moreover, over two-thirds of phosphate rock reserves in the world are located in only a few countries including China, Morocco and the USA. This imbalance in the distribution of phosphate rock mines across the world has created a monopolised global market in which many countries rely on imports to cope with their local demand for phosphorus fertilisers for food production (Nancharaiah et al., 2016). The importance of the biochemical cycle of phosphorus and the access to reliable sources of phosphate rock is now at the top of the international agenda in order to mitigate the possibility of a global phosphorus scarcity in the near future (e.g., UNEP's Global Partnership on Nutrient Management – GPNM).

Despite its important role in supporting living organisms, phosphorus is considered one of the main pollutants in freshwater ecosystems because it promotes eutrophication. In the UK, most phosphorus losses are accounted for from wastewater discharges at treatment works (Cooper and Carliell-Marquet, 2013). Phosphorus recovery from waste streams is considered a suitable option, providing access to secondary resources for the production of phosphorus fertilisers and acting as a viable alternative to phosphate rock itself (Nancharaiah et al., 2016). From the point of view of resource efficiency, the importance of nutrient recovery from waste streams is now considered a priority in supporting sustainable economic growth (Coppens et al., 2014).

2.2. Wastewater Treatment in the UK

Freshwater resources around the world are limited, and many are polluted by anthropogenic activities. With a dramatic increase in the world's population and the effects of climate change, the global water sector is under critical pressure (WATER-UK, 2006; DEFRA, 2016) and, therefore, the contribution towards sustainability from wastewater treatment works has become vital. In the UK, wastewater treatment works serve around 96% of the country's population. The remaining 4% corresponds to rural dwellers in remote areas far from the main sewers who are served by *in situ* treatment systems. More than 16 billion litres of wastewater are collected by 624,200 kilometres of sewers and transferred to around 9,000 wastewater treatment works (DEFRA, 2012; WATER-UK, n.d.-b).

2.2.1. Wastewater Treatment Works in the UK

Conventional wastewater treatment works are based on well-established bacterial processes aimed at removing organic material (carbon and nutrients) and that include four main steps: preliminary treatment, primary treatment, secondary treatment and tertiary treatment. At the preliminary treatment stage the sand, grits and large suspended solids present in raw wastewater are removed using manual or mechanised screens and grit chambers. After that, larger suspended organic solids are settled at the bottom of settlement tanks during the primary treatment stage. Secondary treatment involves the use of biological processes, mainly aerobic, with the Activated Sludge Process (ASP) being the most popular option followed by trickling filters. The Activated Sludge Process combines an aeration tank to stabilise and break down organic materials using aerobic bacteria, followed by a settlement tank to remove the resulting bacterial biomass. The sludge collected from primary and secondary settlement tanks is transferred to anaerobic digestion facilities for energy generation. Tertiary treatment processes are required for additional treatment including disinfection or nutrient removal before the final effluent is discharged into water bodies (DEFRA, 2012). A typical layout for modern wastewater treatment works is shown in Figure 2-1.

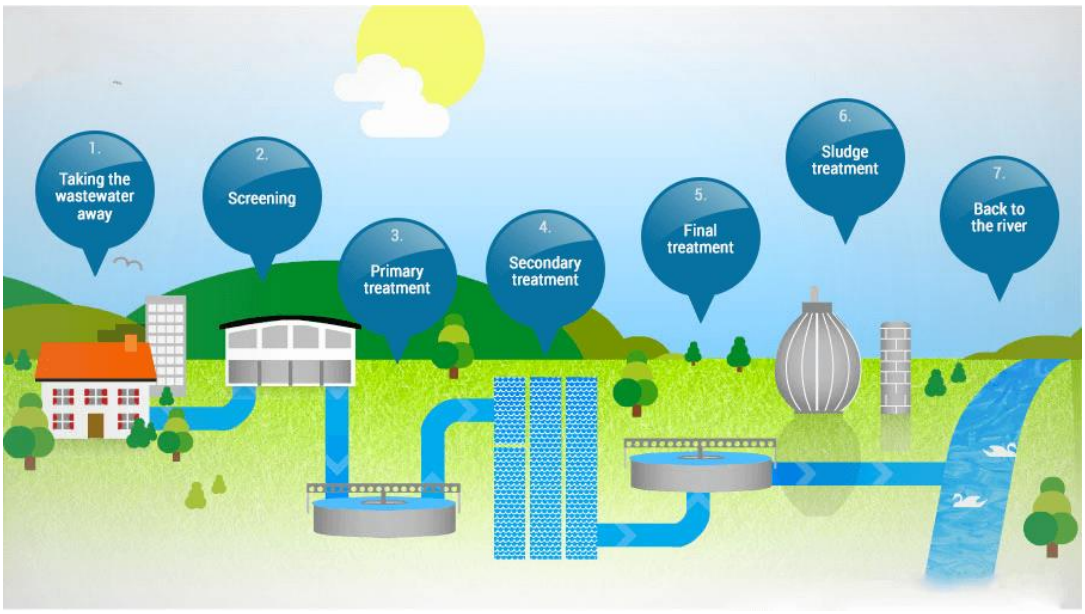


Figure 2-1 Wastewater treatment process flow diagram (WATER-UK)

2.2.2. Current Nutrient Removal in Wastewater Treatment Plants

The discharge of excessive amounts of nutrients coming from urban settlements, agricultural run-off and industrial emissions gives rise to serious environmental problems. Eutrophication is considered one of the most significant environmental problems caused by the discharge of nutrients into water bodies, which can lead to oxygen depletion in the water column, reduction of biodiversity and reduction of light penetration, affecting photosynthesis due to increased turbidity (Cai, 2013; Horan, 1990).

Moreover, the presence of ammonia in aquatic environments is highly regulated as it can have toxic effects on fish and aquatic species due to the fact that molecular (free) ammonia (NH_3) can permeate through most biological membranes, unlike ammonium ions (NH_4^+) (Randall and Tsui, 2002). Both molecular ammonia and ammonium ions coexist in an equilibrium controlled by the prevailing environmental conditions such as pH and temperature (Guštin and Marinšek-Logar, 2011). In aquatic environments, ammonia can be taken by fish across their gill epithelium and then converted to ammonium ions. Ammonium ions are more toxic to the fish than free ammonia (Levit, 2010). Ammonia is also a product of various metabolic routes and ends up excreted through the gills; however, ammonia can accumulate in fish until it reaches toxic levels because ammonia excretion can be suppressed at elevated ammonia concentrations in the aquatic environment (Oram, n.d.).

Ammonia can also poison fish by inducing detrimental effects on the central nervous systems. Fish may suffer from a loss of equilibrium, hyperexcitablity, increased respiratory activity and oxygen uptake as well as convulsions, coma and death *in extremis* (Oram, n.d.; Levit, 2010). Lower ammonia concentrations can result in decreased hatching success, reduction in growth rates and morphological development, as well as injury to gill tissues, kidneys and the liver (Levit, 2010; Oram, n.d.).

As a result, ammonium/ammonia should be removed from wastewaters to minimise their environmental impacts on aquatic ecosystems. Ammonium control at wastewater treatment works is achieved by oxydising ammonium to nitrate (biological nitrification process), while nitrogen control is performed by combining nitrification and denitrification process. Neither ammonium/ammonia control nor nitrogen control promotes nitrogen recovery.

2.2.2.1. Nitrogen Removal Processes

- Conventional nitrification/denitrification process

Nitrogen in water is found either as inorganic species such as nitrite (NO_2^-), nitrate (NO_3^-), ammonia (NH_3) or ammonium (NH_4^+) or as parts of organic molecules (proteins, amino acids, etc.), all typically found in raw wastewaters. Nitrogen species are converted into stabilised forms by biological processes throughout wastewater treatment works. Figure 2-2 depicts some potential biological nitrogen conversions.

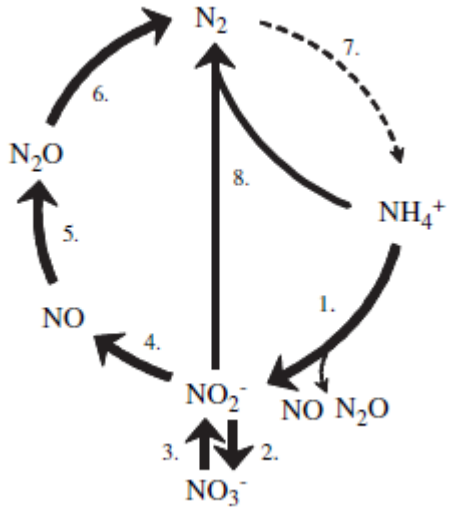


Figure 2-2 Biological nitrogen conversions in WwTW (Kampschreur et al., 2009)

Nitrification-denitrification is the main pathway used for the removal of nitrogen from wastewater via biological processes. During the nitrification process, ammonia/ammonium are first oxidised to nitrite (Route 1, Figure 2-2) and then to nitrate (Route 2, Figure 2-2) by *nitrosomonas* and *nitrobacter* bacteria, respectively. The autotrophic bacteria responsible for nitrification grow at a slower rate than heterotrophic bacteria (i.e., responsible for organic matter removal), and thus require longer hydraulic and sludge retention times. In addition, 4.57 g of oxygen is needed to oxidise 1 g of N-ammonium based on Equations 2.1 and 2.2, which represent the partial oxidation reactions for full nitrification.

$$2NH_4^+ + 3O_2 \xrightarrow{\text{Nitrosomonas}} 2NO_2^- + 4H^+ + 2H_2O \qquad \text{Eq. 2-1}$$

$$2NO_2^- + O_2 \xrightarrow{\text{Nitrobacter}} 2NO_3^- \qquad \text{Eq. 2-2}$$

Nitrate is converted into nitrogen gases under anoxic conditions with the need for several reduction steps, as shown in Equation 2.3; this is widely known as denitrification (Route 3 to 6, Figure 2-2). An additional organic carbon source is required as electron donor for the denitrification process.

$$NO_3^- \longrightarrow NO_2^- \longrightarrow NO \longrightarrow N_2O \longrightarrow N_2 \qquad \text{Eq. 2-3}$$

- Anaerobic ammonium oxidation – the ANAMMOX process

In addition to nitrification-denitrification, novel biological nitrogen removal processes have been developed over the last few decades. Anaerobic ammonia oxidation (ANAMMOX) (Route 8, Figure 2-2) can turn ammonium and nitrite into nitrogen gas under anaerobic conditions (Castro-Barros et al., 2017). ANAMMOX process does not require the additional organic compound and high energy for aeration as above, though ANAMMOX bacteria have an extremely slow growth rate and are highly sensitive to the presence of nitrite, oxygen, ammonia and other microorganisms (Georgiou et al., 2019).

- Physical and chemical processes

Biological ammonia removal processes are sensitive to shock and toxic loadings, and extreme weather conditions; they also require high retention times and hence, large reactor volumes (Yuan et al., 2016). Therefore, many physical and chemical processes including struvite precipitation (Huang et al., 2014), air stripping (Kinidi et al., 2018), ion exchange (Karadag et al., 2008) and membrane technologies (Hasanoğlu et al., 2010) are presently reported as representing alternative methods for N-recovery from wastewater. Table 2-1 summarises the removal efficiencies of physical and chemical methods for ammonium removal in the literature.

Table 2-1 Physical and chemical methods for ammonium removal and their removal efficiencies

Ammonia Removal Process		Wastewater Source	Removal Efficiency (%)	Reference
Struvite Precipitation		Anaerobically pre-treated domestic wastewater	71	Altınbaş et al. (2002)
		Sludge dewatering liquor	22	Remy (2014)
Air Stripping		Sludge liquor	61.5 – 84.6	Ellersdorfer (2018)
		Human urine	97	Başakçilardan-Kabakci et al. (2007)
		Artificial wastewater	99.5	Yuan et al. (2016)
			97	Quan et al. (2009)
Ion Exchange		Municipal wastewater	>96	Malovanyy et al. (2013)
		Sewage	>96	Booker et al. (1996)
		Digested sludge liquor	>95	Thornton et al. (2007)
Membrane Process	**Membrane Technologies**			
	Immersed membrane bioreactor	Municipal wastewater	68.6 – 79.9	Cote et al. (1998)
	Submerged hollow fibre membrane bioreactor	Municipal wastewater	100	Rosenberger et al. (2002)
	Membrane bioreactor	Municipal wastewater	79.2 – 85.5	Habib et al. (2017)
	Osmotic membrane bioreactor	Municipal wastewater	80 – 90	Qiu and Ting (2014)
	Bioelectrochemical system	Domestic wastewater	>95	Chen et al. (2017)
	Forward Osmosis-Anaerobic Membrane Bioreactor	Synthetic municipal wastewater	62.7 – 81.2	Chen et al. (2014)

Struvite (magnesium ammonium phosphate) is an insoluble mineral and is formed according to the reaction depicted in Equation 2-4, where the concentrations of Mg^{2+}, NH_4^+, and PO_4^{3-} should be higher than the solubility limit of struvite at alkaline pH (230 mg L^{-1}) (Huang et al., 2014). Despite its potential for nutrient reuse, the use of struvite as an agricultural fertiliser may be forbidden if co-precipitation with heavy metals and/or toxic substances in wastewater streams occurs (Yan et al., 2018). This route has been suggested as uncontrolled precipitation of struvite in pipes transporting wastewater is a recurrent problem at wastewater treatment works reducing internal diameters in pipes

transporting high P fluids and even causing complete blockages leading to costly pipe replacement (Song et al., 2018).

$$Mg^{2+} + NH_4^+ + PO_4^{3-} + 6H_2O \leftrightarrow MgNH_4PO_4.6H_2O\downarrow \quad \text{Eq. 2-4}$$

Ammonia stripping is a mass transfer process for removing ammonium nitrogen from a liquid by accumulating free ammonia in a gas current, most commonly compressed air bubbled through the liquid (Guštin and Marinšek-Logar, 2011). This process is generally performed in a double column; the first column is used for the stripping process while the second is for the absorption and concentration of ammonium in an acid solution for recovery (Cattaneo et al., 2019). In the stripping tower, the pH should be adjusted so as to be greater than 10 by adding lime or caustic soda to convert ammonium ions into ammonia gas (Equation 2-5) (Adam et al., 2019). This process is also dependent on air temperature and flow rate. High temperature and air flow rate in the column enhance the ammonia stripping rate (Guštin and Marinšek-Logar, 2011). In the absorption column, ammonia gas is absorbed from the air into a strongly acidic solution (e.g., sulphuric acid) to produce an ammonium salt (ammonium sulphate) and, therefore, ammonia can be recovered and reused (Cattaneo et al., 2019). From an economic perspective, the use of ammonia stripping could be limited since wastewaters contains low ammonia concentrations (Booker et al., 1996), but there is a potential for using this technology to recover ammonium from the digester liquor in anaerobic digesters processing sewage sludge.

$$NH_4^+ + OH^- \longleftrightarrow NH_3 + H_2O \quad \text{Eq. 2-5}$$

A reversible interchange of ions between the liquid and solid phases (e.g., polymeric or mineralic ion exchangers) is involved in ion exchange processes (Ding and Sartaj, 2016). Zeolite is one of the most commonly used ion exchangers for ammonium removal due to its high ion exchange capacity, comparatively high specific surface area and a relatively low price (Gupta et al., 2015). Ion exchange columns are operated in two working cycles: saturation and regeneration. The feed stream is delivered into the column and the effluent is discharged from the column in saturation cycle. In the regeneration cycle the ions absorbed by the ion exchanger are firstly replaced with a regenerant followed by re-equilibration, in which the regenerant is washed out and the column is restored to its initial condition (Bochenek et al., 2011). Although the ion exchange process allows nitrogen to be recovered from wastewater and exhausted regenerants to be used as

fertilisers, other ions such as Ca^{2+} and Mg^{2+} in wastewater are also transferred into the regenerant and could have a detrimental influence on nitrogen recovery (Malovanyy et al., 2013).

Membrane filtration is a simple physical process where the feed solution penetrates the membrane under pressure. It is classified according to the pore size and operational technologies such as ultrafiltration, nanofiltration, reverse osmosis, electrodialysis, etc. (Yan et al., 2018). The membrane concentrates ammonium ions in the reactor with no chemical and energy input and, therefore, it is considered a low-cost ammonia recovery process (Ye et al., 2018). Membrane fouling is the main disadvantage of membrane processes (Sari Erkan et al., 2018).

2.2.2.2. Phosphorus Removal Processes

Phosphorus is removed from domestic wastewater either by enhanced biological phosphorus removal (EBPR) or by chemical precipitation using iron or aluminium salts (Coppens et al., 2014). Regarding EBPR, anaerobic and aerobic conditions are needed to take up phosphorus from wastewaters by phosphate-accumulating organisms (PAOs). Under anaerobic conditions, volatile fatty acids (VFAs) are produced by fermentation which are subsequently assimilated by PAOs to synthesise poly 2-hydroxybutyrate (PHB). Orthophosphate is released into solution by breaking down stored polyphosphate in order to meet energy requirements for PHB synthesis. Under aerobic conditions, PHB is broken down, releasing energy that is used by PAOs for phosphate uptake. Then, phosphorus is converted into polyphosphate inside bacteria cells. Polyphosphate is stored in the sludge and the phosphate is removed with the waste sludge (USEPA, 2008; Manyumba et al., 2009).

Chemical precipitation is also commonly performed in wastewater treatment works in order to remove phosphorus. The main mechanism of phosphorus removal by chemical precipitation is coagulation-flocculation, where dissolved phosphate is converted into particulate form by adding the metal salts and, thereafter, the low-solubility chemical precipitates are separated from wastewater by sedimentation (Georgantas and Grigoropoulou, 2007; Caravelli et al., 2012). Metal salts such as aluminium sulphate, ferrous sulphate and ferric chloride are generally used as coagulants. Calcium salts can also be used, but in practice are rarely applied. Metal ions (Me) react with water molecules and form the corresponding hydrolysis products. One of the phosphorus precipitation

principles is the adsorption of phosphate ions in hydrolysis products (Aguilar et al., 2002). The reaction takes place as below (Equation 2-6);

$$Me^{3+} + (3\text{-}n)H_2O + nH_2PO_4^- \longleftrightarrow Me(OH)_{3-n}(H_2PO_4)_n + (3\text{-}n)H^+ \quad \text{Eq. 2-6}$$

The second scheme of chemical precipitation of phosphorus is the reaction between phosphate and metal hydroxides, as shown in Equation 2-7. Metal hydroxides are formed by the reaction between hydrolysis products with either water molecules or OH^- ions. Therefore, an alkaline pH is favourable for the formation of metal hydroxides (Thistleton et al., 2002).

$$Me(OH)_3 + nH_2PO_4^- \longleftrightarrow Me(OH)_{3-n}(H_2PO_4)_n + nOH^- \quad \text{Eq. 2-7}$$

Despite the fact that phosphorus is effectively removed by chemical precipitation from wastewater (Caravelli et al., 2012), the addition of chemicals increases operational costs (Pratt et al., 2012). Furthermore, struvite precipitation (Marti et al., 2017), ion exchange (Martin, 2010) and membrane processses (Qiu and Ting, 2014), as described previously, are available for P control and recovery from wastewaters.

2.3. Nutrient Uptake Metabolisms in Microalga Cells

Current biological wastewater treatment works are capable of meeting the discharge consents for organic matter, nitrogen and phosphorus (COD < 125 mg O_2 L^{-1}, TN < 10 mg N L^{-1}, TP < 1 mg P L^{-1}) (UWWTD, 1991); however, there are many associated limitations as the energy requirements for oxygen supply and mixing are particularly high and account for nearly 45-75% of the total energy consumption at wastewater treatment works (Anbalagan et al., 2016). Additional organic carbon is needed for denitrification and phosphorus uptake (Horan, 1990). Moreover, the Activated Sludge Process (ASP) could contribute to radiative forcing and stratospheric ozone depletion by emitting nitrous oxide (N_2O) as part of the nitrification-denitrification process (Kampschreur et al., 2009; Denk et al., 2017). Thus, the overall cost-effectiveness of existing wastewater treatment processes has been revised with regard to the increase in population, greenhouse gas emissions and more stringent discharge consents (Evans et al., 2017).

In this regard, biological nutrient uptake by microalgae has been presented as a promising opportunity for nutrient control and recovery from wastewater. Microalgae are photosynthetic species that use light and CO_2 as energy and carbon sources, respectively.

In addition, they metabolise and accumulate nitrogen and phosphorus in their cells, with the additional benefit of producing biomass with the potential to be used as feedstocks for the production of bio-fertilisers and biofuels due to their high nutrient value and lipid content (Brennan and Owende, 2010; Pittman, 2011; Ma et al., 2014).

2.3.1. Nitrogen Uptake by Microalgae

Nitrogen is the second-most essential nutrient for microalgae based on the stoichiometric relationship of 106:16:1 between carbon, nitrogen and phosphorus reported in microalgae cells, also known as the Redfield ratio (Redfield, 1934). Nitrogen is a constituent of organic molecules such as proteins, chlorophylls, and energy transfer molecules (ADP, ATP), which are synthesised from inorganic nitrogen species. Microalgae play a key role in the transformation of inorganic nitrogen species into organic forms as described in the nitrogen assimilation process by Cai et al. (2013) (Figure 2-3).

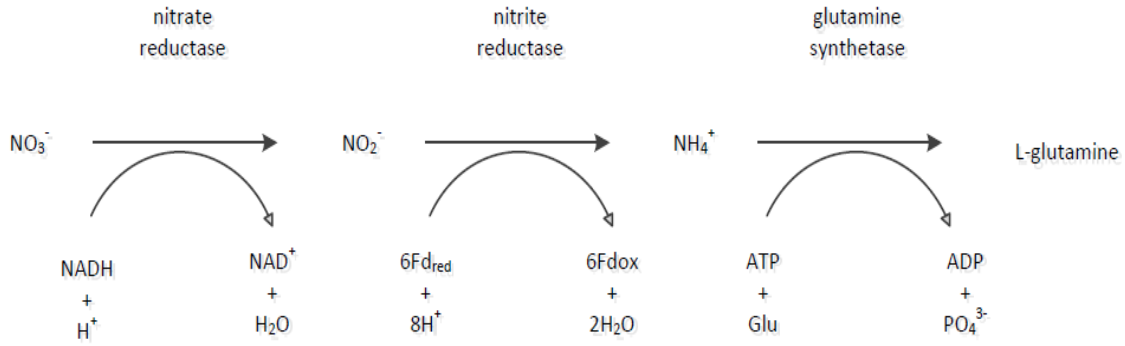


Figure 2-3 The assimilation of nitrogen by microalgae (Cai, 2013).

Ammonium, nitrate and nitrite can be used as nitrogen sources for biological algal uptake processes. Nitrate is first reduced to nitrite, then nitrite is reduced to ammonia using selective enzymes (nitrate and nitrite reductase, respectively). The reduced form of nicotinamide adenine dinucleotide (NADH) and ferredoxin (Fd) are used as electron transporters to transfer two and six electrons by nitrate and nitrite reductase, sequentially.

It can be clearly seen from Figure 2-3 that ammonium is preferred over nitrate because ammonium does not require any additional redox reactions before assimilation; therefore, it is considered the most energy efficient form of nitrogen for microalgae cultivation (Cai et al., 2013; Wilhelm et al., 2006; Collos and Berges, 2004). Glutamine

is produced by using glutamate (Glu) and energy within the members of the ammonium transporter family during ammonium assimilation (Cai et al., 2013; Wilhelm et al., 2006). This process is highly dependent on ammonium concentrations in solution. At high ammonium concentrations, ammonium is taken up with reductive amination of α-ketoglutarate and the enzyme glutamate dehydrogenase. At low concentrations, to begin with, glutamine is produced by the reaction of glutamate with ammonium; then, 2 moles of glutamate are formed with the transfer of the amino group of the glutamine to α-ketoglutarate (Collos and Berges, 2004).

Although ammonium is the most common form of nitrogen in raw wastewaters entering sewage treatment works, nitrate is the predominant form of nitrogen in aquatic environments due to the fact that nitrate is the most highly oxidised and stable form of nitrogen. When wastewaters containing nitrate are used for algal cultivation, it is possible that microalgae could also utilise nitrate as the nitrate reductase activity is triggered (Cai, 2013; Collos and Berges, 2004). In terms of nitrogen control at WwTP by algal uptake, it is important to assess the acceptable levels of aeration that allow the stabilisation of carbon compounds by heterotrophic bacteria, and the resulting nitrate conversion that can become the nitrogen removal target, along with any unoxidised ammonium nitrogen.

2.3.2. Phosphorus Uptake via Microalgae

Phosphorus is an essential constituent of nucleic acids and other metabolites, as well as being used in energy transfer, while still being considered as a pollutant responsible for eutrophication in freshwaters (Martinez et al., 1999; Correl, 1998). Phosphorus can be present in both organic and inorganic forms, and in soluble and insoluble forms in aquatic environments. Soluble inorganic phosphate (PO_4^{3-}), referred to as orthophosphate, is the preferred phosphorus species by microalgae (Griffiths, 2010). With the help of the phosphorylation process – i.e., ATP produced from ADP with energy input resulting from light in the case of photosynthesis and the oxidation of respiratory substrates or the electron transport system of mitochondria – phosphorus is assimilated into microalgae cells by converting phosphate ions into organic P forms (Cai, 2013). Besides inorganic phosphorus, microalgae can assimilate more complex phosphorus species by releasing extracellular polymeric substances, hydrolysing complex organic molecules containing phosphorus, and taking up phosphate into their cells (Kuenzler, 1965; Lin, 1977; Correl, 1998).

Phosphorus uptake by microalgae is highly dependent on phosphorus concentrations both in the feeding medium and alga cells, as well as on environmental conditions such as pH, temperature and light intensity (Martinez et al., 1999; Griffiths, 2010). Microalgae take up greater amounts of phosphorus when they have lower intracellular phosphorus concentrations (Griffiths, 2010). Some of the microalgae perform "luxury" phosphorus uptake, which leads to the storage of intracellular phosphorus reserves in the form of polyphosphate crystals under phosphorus-deficient conditions (Powell, 2009). Phosphatase enzymes, used to hydrolyse the ester bond of the phosphorylated amino acids to release inorganic phosphate and the unphosphorylated protein, are classified as acid- or alkaline-phosphatases, making phosphorus uptake pH-dependant (Kuenzler, 1965). On the other hand, phosphorus might be chemically precipitated at high pH, a situation commonly encountered when photosynthesis activity is at its maximum (Cai et al., 2013). It is expected that lower photosynthesis and P uptake rates might be attained at lower temperatures (Griffiths, 2010).

It has been confirmed that nitrogen and phosphorus are effectively taken up by microalgae and, therefore, microalgal cultivation in wastewater has been presented as a promising alternative to conventional nutrient control systems in wastewater treatment, with the benefit of overcoming the deficiencies currently faced by conventional activated sludge process with biological nitrogen and phosphorus removal, in which nutrient recovery is limited (Sriram and Seenivasan, 2012; Aslan and Kapdan, 2006; Wang et al., 2012).

2.4. Microalgae Growth under Different Trophic Conditions

Microalgae can be separately cultivated under phototrophic, heterotrophic and mixotrophic conditions, as well as under a combination of them (Zhou et al., 2012; Sakarika and Kornaros, 2016). The most common microalgae cultivation mode is phototrophic (Perez-Garcia et al., 2011b; Hu et al., 2017). Microalgae utilise CO_2 and/or HCO_3^- and light as carbon and energy sources; molecular oxygen (O_2) and new algal cells are produced as a result of the photosynthesis process. Phototrophic cultivation of microalgae in open ponds has various advantages including the use of naturally available carbon and energy sources from air and sunlight, low capital and operational costs, and low energy requirements; however, poor light penetration (self-shading effect in high cell density cultures), the risk of contamination/competition with other microorganisms, and

a strong dependence on environmental/weather conditions have limited phototrophic cultivation of microalgae at the commercial scale using open ponds (Perez-Garcia et al., 2011b; Brennan and Owende, 2010). Although closed photobioreactors (PBR) have overcome some of the drawbacks of open systems, the energy requirements for mixing have increased operational costs (Brennan and Owende, 2010; Hu et al., 2017). Thus, the potential implementation of mass cultivation of phototrophic microalgae is somewhat dubious due to the major limitations of phototrophic microalgae growth in both open ponds and closed PBRs.

Some strains of microalgae can grow under heterotrophic conditions. Organic carbon is consumed as an energy and carbon source by heterotrophic microalgae under fully dark conditions, using oxygen as a final electron acceptor (Chen and Johns, 1994). Heterotrophic cultivation can overcome the limitations of phototrophic cultivation of microalgae by eliminating the requirement for light and by using fermenters as reactors. In addition, higher cell densities for algal biomass cultivation, higher growth rates and higher lipid content can be achieved (Perez-Garcia et al., 2011b; Sakarika and Kornaros, 2016; Hu et al., 2017). Despite the many advantages attributed to heterotrophic cultivation of microalgae, the limited number of microalgae strains actually capable of growing in heterotrophic mode, the requirements of organic carbon and oxygen, higher operational costs, CO_2 emission and the high risk of contamination from bacteria are considered significant challenges that need to overcome for effective heterotrophic microalgae cultivation (Perez-Garcia et al., 2011b; Moon et al., 2013; Hu et al., 2017).

Mixotrophic cultivation means that microalgae are able to grow either phototrophically or heterotrophically or both, in which mixotrophic metabolism can occur either simultaneously or separately. Both organic and inorganic carbon are assimilated as carbon sources. Microalgae release CO_2 by heterotrophic assimilation which is subsequently used during photosynthesis (Chen et al., 2011). Light and organic materials are the energy sources for the fixation of inorganic carbon and aerobic respiration, respectively, and therefore microalgae growth does not solely depend on photosynthesis because organic carbon can support cell growth as well (Andrade and Costa, 2007; Brennan and Owende, 2010). Mixotrophic cultivation of microalgae can occur either in a single reactor or in separated, sequential reactors. With separated reactors, microalgae take up nutrients under phototrophic conditions and further nutrient uptake and lipid accumulation can take place in subsequent heterotrophic reactors (Zhou et al., 2012). Different growth regimes were investigated to identify which of them resulted in higher

algal biomass and biodiesel production rates (Moon et al., 2013). The results revealed that *Chlamydomonas reinhardtii* had the greatest growth rate and lipid accumulation under mixotrophic cultivation.

2.4.1. Nutrient Recovery by Phototrophic Microalgae

Microalgal biomass growth and nutrient recovery are strictly dependent on the cultivation conditions, such as selected microalgae species, nutrient concentrations in the growth medium, light intensity supply, photoperiod (light: dark ratio), temperature, etc. Nitrogen and phosphorus are required for microalgae growth due to the fact that the biomass composition of microalgae includes approximately 10% nitrogen and 1% phosphorus (Aslan and Kapdan, 2006; Redfield, 1934). The initial nutrient concentrations have to be sufficient to support microalgae growth without creating any issues that may inhibit microalgal metabolism (Ji et al., 2014). Light is the most critical factor influencing phototrophic growth of microalgae, simply because it is used as an energy source, i.e., eight photons of photosynthetically active radiation are required to fix one molecule of carbon dioxide (Brennan and Owende, 2010; Gonçalves et al., 2014). Despite this, light attenuation and self-shading within the culture appear as a result of achieving higher biomass concentrations and, hence, light penetration across the culture is also affected (Perez-Garcia et al., 2011b). On the other hand, the high light intensity can reduce photosynthetic activity, which highlights the importance of an optimum light: dark ratio as microalgae can repair any photo-induced damage in the cells in the absence of light (Gonçalves et al., 2014). Temperature is another essential parameter for microalgae cultivation (Akerstrom et al., 2014). Table 2-2 summarises the most important factors reported in the literature that influence the phototrophic growth of microalgae.

Table 2-2 Principal factors affecting microalgae growth

Microalgae Species	Nitrogen Concentration in Media (mg L^{-1})	Phosphorus Concentration in Media (mg L^{-1})	Light: Dark Ratio	Light Intensity	Temperature (°C)	Removal Efficiency (%)		References
						N	P	
Chlamydomonas reinhardtii	128.6 (TKN) 67.0 (NH_3)	120.6 (TP)	24h light	220 $\mu molm^{-2}s^{-1}$	25±1	83.0	14.5	Kong et al. (2010)
Chlorella sp.	14.73 19.01 22.09 28.52 29.45 38.03 (TN)	0.29 0.92 0.43 1.37 0.57 1.83 (TP)	12h: 12h	60 $\mu molm^{-2}s^{-1}$	25	17.0 27.4 42.7 51.5 42.9 58.9	82.5 92.9 89.6 95.3 91.9 97.1	Wang et al. (2012)
Chlorella sp.	10 40 100 200 300 400 (TN)	0.7 1.0 1.4 2.5 3.5 4.4 (TP)	24h light	100 $\mu Em^{-2}s^{-1}$	18 ± 2	93.5 98.1 74.0 72.0 61.9 58.0	56.0 56.5 54.6 57.9 51.9 52.1	Chen et al. (2012)
Chlorella zofingiensis	148 106 80 55 34 17 (TN)	156 111 85 58 36 18 (TP)	24h light	230 ± 20 $\mu molm^{-2}s^{-1}$	25 ± 1	78.7 81.0 82.7 77.8 70.9 69.0	85.0 89.2 98.2 98.6 99.4 100.0	Zhu et al. (2013)
Neochloris oleoabundans	65.4	2.5	24h light	200 $\mu molm^{-2}s^{-1}$	25 ± 2	100 100 100 100	96.4 97.3 97.0 96.9	Franchino et al. (2013)
Chlorella vulgaris	81.7 108.9 163.4 (NH_4^+-N)*	3.2 4.2 6.3 (PO_4^{3-}-P)*				100 100 100 100	95.7 96.9 96.0 96.0	
Scenedesmus obliquus						92.4 92.1 93.6	94.4 95.6 96.2	

						83.7	96.1	
Chlorella sp.	221 363 532 645 871 1210 (TN)	5 8 12 15 20 28 (TP)	24h light	115 $\mu molm^{-2}s^{-1}$	25.0 ± 0.5	47 84 47 34 25 37**	90 93 94 91 93 88**	Akerstrom et al. (2014)
Chlorella sp.	81-86 (TN)	4.1-4.3 (TP)	24h light	400 lux	25	41	100	Han et al. (2014)
Chlorella ellipsoidea						36	100	
Scenedesmus bijuga						28	100	
Scenedesmus quadricauda						37	100	
Desmodesmus sp.	20.6 41.2 82.5 (NH_4^+-N)*	1 2 4 (PO_4^{3-}-P)*	15h: 9h	120 ± 2 $\mu molm^{-2}s^{-1}$	24 ± 1	100 100 100	100 100 51.24	Ji et al. (2014)
Chlorella vulgaris	40 (NO_3^--N)	10 (PO_4^{3-}-P)	10h: 14h 14h:10h 24h light	36 60 120 180 $\mu Em^{-2}s^{-1}$	24 ± 1	42.3-99.0	16.9-67.6	Gonçalves et al. (2014)
Pseudokirchneriella subcapitata						43.5-100.0	17.5-51.2	
Synechocystis salina						46.9-100.0	9.8-37.9	
Microcystis aeruginosa						53.6-100	13.4-41.1	
Scenedesmus obliquus	17.45±0.78 (TN)	2.6±0.0 (TP)	14h: 10h 24h dark	250 $\mu molm^{-2}s^{-1}$	20 ± 1	90***	100***	Ruiz et al. (2014)
Chlamydomonas reinhardtii	15 (TN)	0.5 (TP)	12h: 12h	2000 lux	25 ± 1	55.0-80.8	92.5-100	Li et al. (2016)
Chlamydomonas reinhardtii	50 (NH_4^+-N) 50 (NO_3^--N)	100 (PO_4^{3-}-P)	16h: 8h	250 $\mu Em^{-2}s^{-1}$	20	100	34	Yulistyorini (2016)
Chlamyomonas reinhardtii	80±1 (TN) 20±1 (NH_3-N)	22±1 (TP)	24h light	60 $\mu molm^{-2}s^{-1}$	25-28	32.3-52.4 70-90	78-93	Qi et al. (2017)

* Nutrient concentrations were calculated based on the dilution rate of digestate samples in the studies.

** Removal efficiencies were calculated with the difference of nutrient concentration between the start and end of experiments.

***Removal efficiencies were calculated based on the data on the figure for nutrient removal.

A wide range of microalgae species have been used in recently published research for nutrient recovery from municipal and/or synthetic wastewater. The potential to recover nutrients from municipal wastewater via microalgae uptake (*Chlorella sp.*) was reported in the study conducted by Wang et al. (2012), with high total nitrogen (TN) and total phosphorus (TP) removal efficiencies in the range of 17.04-58.85% and 62.43-97.08%, respectively. They also analysed the optimum nutrient concentration to support maximum algal growth, finding that the greatest biomass production was achieved when mixing 50% influent with effluent in a wastewater treatment facility. *Chlamydomonas reinhardtii* performed satisfactorily with the removal of nutrients from the synthetic effluent from wastewater treatment plants and achieved removal efficiencies of total nitrogen, total phosphorus and organic materials as chemical oxygen demand in the range of 55.0-80.8%, 92.5-100.0% and 11.5-36.5%, respectively (Li et al., 2016). Han et al. (2014) cultivated four species of algae including *Chlorella sp.*, *Chlorella ellisoidea*, *Scenedesmus bijuga* and *Scenedesmus quadricauda* in synthetic wastewater, and concluded that all four species grew well and removed nearly 100% of total phosphorus present; however, nitrogen removal rates were low. The best performance with regard to nitrogen removal was reported for *S. quadricauda* with a 65% ammonium removal. *Chlamydomonas sp.*, *Scenedesmus sp.*, *Chlorella sp.* and *Euglena sp.* are the dominant algal genera in WwTP in the UK (Abis and Mara, 2005), which is one of the essential reasons why *Chlamydomonas reinhardtii* is preferred as the model organism in this study.

The cultivation of microalgae in digestate liquor also presents considerable potential to combine nutrient recovery and energy production. *Chlamydomonas reinhardtii* was cultivated in the centrate (i.e., liquid fraction from anaerobic digested) of a wastewater treatment system reporting 83.0 and 14.5% nitrogen and phosphorus removal efficiencies, respectively (Kong et al., 2010). Nutrient removal and biomass productivity from anaerobic digestate using *Chlorella* and *Scenedesmus* were studied by Chen et al. (2012); the study revealed that these non-filamentous green algae species could tolerate nitrogen concentrations as high as 200 mg TN/L with a net biomass productivity of 6.83 g/m^2day. Ji et al. (2014) investigated nutrient removal and biomass productivity by *Desmodesmus sp.* in diluted effluent (10%) from anaerobic digestion (AD) and found that average removal rates for TN, NH_4-N, TP and PO_4-P were 4.542, 5.284, 0.326 and 0.290 mg/L/day, respectively; the maximum biomass concentration reported was 0.412 g/L. Total nitrogen removal was lower than ammonia removal because ammonia is taken up by microalgae, and total nitrogen removal was calculated using unfiltered samples (alga

biomass included). Akerstrom et al. (2014) studied biomass production and nutrient removal from sewage sludge liquor (SL) using *Chlorella,* their results showed that the highest biomass production rate was in the range of 0.42 – 0.45 g dry weight/L/day at 40–50% SL, with the highest nitrogen removal rates of 33.6 – 42.6 mg TN/L/day being achieved at 40-70% SL, whereas the maximum phosphorus removal rates were between 3.06 and 4.10 mg TP/L/day at 50-100% SL. Unfortunately, this process can be limited by high solid concentrations and a dark colour in AD resulting in light attenuation, and therefore additional pre-treatment is necessary.

Furthermore, it has been reported that microalgae can effectively recover nutrients from animal manure. *Chlamydomonas reinhardtii* was cultured in piggery wastewater in order to maximise biomass growth and nutrient removal. In the range of 70-90%, 32.3-52.4% and 78-93% of ammonia, total nitrogen and phosphorus were removed, respectively (Qi et al., 2017). The work conducted by Chen et al. (2012) aimed to remove nutrients from anaerobically digested animal manure by using freshwater algal assemblages. They reported that *Chlorella sp.* and *Scenedesmus sp.* were the dominant algal genera at high nitrogen loadings. Zhu et al. (2013) investigated nutrient removal and biomass productivity in piggery wastewater using a tubular bubble column photobioreactor with *Chlorella zofingiensis*. The corresponding removal efficiencies of COD, TN and TP fluctuated with the following intervals: 65.81 to 79.84% for COD; 68.96 to 82.70% for TN; and 85 to 100% for TP. *Neochloris oleoabundans, Chlorella vulgaris* and *Scenedesmus obliquus* were cultivated in diluted agro-zootechnical digestate, the effluent of a pilot anaerobic digestor used to treat several mixes of cattle slurry and raw cheese whey (Franchino et al., 2013). The result revealed that all three strains were able to grow and achieved high removal rates of ammonium and phosphate, whilst *C. vulgaris* had the highest nutrient removal efficiencies. This situation has given a potential opportunity to apply microalgae cultivation in rural wastewater.

The environmental factors – i.e., nitrogen and phosphorus concentration in the culture medium, light intensity and photoperiod – influenced microalgal growth and nutrient recovery. Gonçalves et al. (2014) investigated how light irradiance and the light: dark ratio affected microalgae growth, CO_2 uptake and nutrient removal efficiencies using four different microalgae strains, *Chlorella vulgaris, Pseudokirchneriella subcapitata, Synechocystis salina* and *Microcystis aeruginosa*. They reported that higher biomass production, higher CO_2 capture rate and higher nutrient uptake rates were achieved when microalgae cultures were exposed to high light irradiance and a longer light to dark

period. By contrast with the study aiming to determine the effect of light on biomass concentration and nutrient uptake, Ruiz et al. (2014) concluded that biomass production was independent of light until the nutrient storage capacity in the biomass was maximal. In addition, they reported that *S. obliquus* was capable of nutrient uptake under dark conditions.

Yulistyorini (2016) investigated the effects of environmental factors on phototrophic cultivation under batch conditions and mixotrophic growth in a two-stage microalgal process that combined heterotrophic and phototrophic cultivation under continuous flow conditions with biomass recirculation in order to enhance P recovery by *Chlamydomonas reinhardtii*. The study found that the greatest phosphorus uptake rate was achieved at 100 mg N L^{-1}, 100 mg P L^{-1}, 250 $\mu Em^{-2}s^{-1}$ of light intensity and a 16h: 8h light: dark ratio, with the maximum biomass production rate of 149 mg VSS L^{-1} d^{-1}, and the recovery efficiencies of phosphorus and nitrate varied from 34 to 100% and from 9 to 100%, respectively whereas ammonium was fully recovered under all different environmental conditions tested. Moreover, phosphorus was released during phototrophic cultivation after heterotrophic growth under continuous flow conditions. Hence, the proposed system in this research was designed phototrophic cultivation followed by heterotrophic cultivation.

2.4.2. Nutrient Recovery via Heterotrophic Cultivation of Microalgae

Microalgae can be autotrophic or heterotrophic based on the metabolic pathway of carbon utilisation. The most common approach for microalgae cultivation is phototrophic due to the fact that microalgae inherently use natural and/or artificial light and inorganic carbon as energy and carbon sources, respectively. Microalgae can be cultivated in both open ponds and closed photobioreactors under phototrophic conditions. Open systems are vulnerable to extreme environmental conditions and microbial contamination. Although closed systems offer axenic cultures, high installation and operation costs can limit their application. In addition, effective light diffusion is considered the main deficiency in phototrophic microalgae cultivation (Hu et al., 2017; Perez-Garcia et al., 2011b). Heterotrophic cultivation of microalgae, i.e., utilising organic carbon as energy and carbon sources under conditions of dark has become more attractive due to avoiding the limitations of phototrophic cultivation, with the additional benefits of having higher cell density, a higher specific growth rate and higher lipid content (Sakarika and Kornaros, 2016).

There are various organic carbon sources that have already been tested such as acetate, glucose, glycerol, etc. (Bumbak et al., 2011; Perez-Garcia et al., 2011b). Perez-Garcia et al. (2011a) compared different carbon sources in terms of their ability to remove nutrients from wastewater under heterotrophic conditions using *Chlorella vulgaris*. They found that sodium acetate and glucose were the most efficient sources of organic carbon for both biomass production and ammonium removal. *Chlorella sorokiniana* and *Auxenchlorella protothecoides* were cultivated on three different organic carbon sources including acetate, butyrate and lactate under heterotrophic conditions to determine growth kinetics (Turon et al., 2014). It was concluded that both species could utilise acetate and butyrate; however, heterotrophic growth with lactate was not achieved.

Additional organic carbon could support both the microalgae growth and nutrient removal until the point at which their own concentrations limit further growth. The effect of acetate concentration on the growth of *Chlamydomonas reinhardtii* was examined by Chen and Johns (1994) who noted that 0.4 g/L of acetate was optimum to support growth, but biomass production was inhibited at higher concentrations. Perez-Garcia et al. (2011a) also reported that the rate of biomass growth and ammonium removal decreased beyond 0.12 M sodium acetate due to inhibition by excessive organic carbon.

On the other hand, nitrogen is one of the most important nutrients for heterotrophic metabolism. In fact, Zhang et al. (1999) reported that *Chlamydomonas reinhardtii* produced a higher biomass concentration and had a greater growth rate when cultivated with nitrate rather than with ammonium as a nitrogen source. Moreover, Shi et al. (2000) supported this argument by indicating that a much lower biomass concentration was obtained when using ammonium instead of nitrate in a culture medium under heterotrophic cultivation of *Chlorella protothecoides.* Although nitrate has to be reduced to ammonia via microalgal uptake, it can be concluded that nitrate is preferred nitrogen source for heterotrophic microalgae growth.

Despite the advantages of heterotrophic microalgae cultivation as mentioned, the major limitations of heterotrophic cultivation of microalgae are: (i) the limited number of microalgae species that can grow under heterotrophic conditions, as based on genetic characteristic such as *Chlamydomonas reinhardtii* and several *Chlorella sp.* (Chen and Johns, 1994; Kim et al., 2013b; Turon et al., 2014); (ii) being vulnerable to contamination and competition with other microorganisms, particularly bacteria due to the use of glucose, acetate, etc. as organic carbon sources; (iii) the emission of carbon dioxide from

respiration processses under dark, unlike phototrophic growth; and (iv) the increased cost resulting from the need for organic carbon sources and oxygen supply (Perez-Garcia et al., 2011b; Hu et al., 2017; Lowrey et al., 2014). The combination of microalgae cultivation wastewater treatment has contributed to decreasing costs because wastewater inherently contains organic carbon, i.e., acetate. Thus, in this study, *Chlamydomonas reinhardtii* was chosen as a model species to be cultivated under mixotrophic conditions to recover nutrients from wastewater in order to overcome the deficiencies of both phototrophic and heterotrophic cultivation with the additional benefit of reducing the cost of the carbon source.

Despite the fact that bacterial contamination is considered one of the major limitations of both phototrophic and heterotrophic cultivation of microalgae, the combination of algal and bacterial culture for wastewater treatment has been studied in order to improve energy efficiency and to develop sustainable technologies that reduce operational costs in wastewater treatment systems (Selvaratnam et al., 2014). There is a syntrophic relation between algae and bacteria. Algae produce oxygen supporting bacterial growth, while bacteria generate CO_2 to support algal biomass growth and a combined effect contributing to nutrient removal (Ma et al., 2014). Bacteria can biodegrade organic compounds to ammonium, phosphate and carbon dioxide, which can be easily metabolised by algae; meanwhile, algae take up nitrogen and phosphorus in their cells to support growth while using CO_2 and producing O_2 to support the growth of bacteria. Furthermore, algae supply various substrates for bacterial growth such as proteins, lipids, nucleic acids, etc. Bacteria utilise these molecules to synthesise products such as vitamin B_{12}. Bacteria also assimilate phosphorous using the O_2 produced during photosynthesis by algae (Ma et al., 2014; Liang et al., 2013). Organic matter and nutrients are removed from wastewater via aerobic carbon oxidation by bacteria and nutrient assimilation of algal biomass as a combination of microalgae and bacteria growth (de Godos et al., 2014).

Furthermore, the combination of bacterial and algal growth has promoted nitrogen and phosphorus uptake efficiencies. Liang et al. (2013) reported that greater removal efficiencies of ammonium and total phosphorus were obtained in a combined algae-bacteria system than in isolated algal or bacterial systems. The increment in nutrient uptake with the combination of microalgae and bacteria cultivation is generally attributed to the enhancement of the microalgae population; however, de-Bashan et al. (2005) pointed out that bacteria help to increase the capacity of microalgae cells with regard to

nutrient uptake. Perez-Garcia et al. (2010) revealed that high ammonium and phosphorus uptake by microalgae was achieved in the presence of bacteria using *Chlorella vulgaris* and *Azospirillum brasilence.*

2.4.3. Microalgae Harvesting

Microalgae harvesting is required for all industrial applications aimed at biomass production for biofuel, human and animal food, and high-value products (Chen et al., 2011; Singh and Patidar, 2018). Despite the fact that many techniques for microalgae harvesting have been tested, including coagulation-flocculation, gravity settlement, flotation, filtration, centrifugation, etc., harvesting microalgal biomass is one of the most serious barriers to the application of commercial microalgae cultivation due to the current high costs of existing harvesting techniques in operation, i.e., harvesting represents 20-30% of the total algal biomass production cost (Barros et al., 2015; Singh and Patidar, 2018). The combination of coagulation/flocculation followed by gravity settlement is considered the most cost-effective method for microalgae harvesting (Barros et al., 2015).

Gravity settlement is commonly applied for the separation of suspended solids in wastewater treatment systems. This process is highly dependent on the density and particle size of biomass flocs/alga cells and the induced sedimentation velocity (Chen et al., 2011). The diameter of microalgae cells is generally lower than 15 μm and their density is almost equal to water density; therefore, they have a sedimentation rate as low as 0.1-2.6 cm h^{-1} (Chatsungnoen and Chisti, 2016; Barros et al., 2015).

Coagulation/flocculation should be applied prior to gravity settlement in order to improve sedimentation rate. Flocculation is influenced by algal species; the properties of microalgae cell surfaces such as their cell size; net charge and hydrophobicity; the concentration of microalgae in suspension; flocculant type; polymer molecular weight; charge density of molecule and dosages; the presence of nutrients; pH; ionic strength of the culture and mixing conditions (Vandamme et al., 2013; Chatsungnoen and Chisti, 2016; Singh and Patidar, 2018; Chen et al., 2011; Barros et al., 2015). Due to the fact that many of these factors vary during the cultivation period, microalgae harvesting in the stationary phase of growth has advantages because of lower metabolic activity and zeta potential that together induce higher intracellular interactions and the formation of larger particles/flocs (Barros et al., 2015; Singh and Patidar, 2018).

Cationic flocculants can be categorised as belonging two types based on chemical composition, which include inorganic flocculants, such as multivalent cations, and organic flocculants (Singh and Patidar, 2018). Multivalent metal ions are widely used because of their cost-effectiveness; however, they are toxic and contaminate microalgae. Therefore, the applicability of this process can be hindered (Lee et al., 2008a). Organic flocculants – i.e., polymers – are more eco-friendly, but are much more expensive than metal salts and their action can be inhibited by high salinity (Chatsungnoen and Chisti, 2016; Lee et al., 2008a).

Cationic polymers are commercially used as flocculants in wastewater treatment works in order to harvest bacterial biomass; therefore, this process could be performed effectively for the algal biomass sedimentation and recycling (Granados et al., 2012). Cationic polymers are effectively applied to flocculate microalgae by neutralising the negative surface charge on microalgae cells and agglomerating then in larger flocs, which is referred to as the bridging process (Barros et al., 2015). Delrue et al. (2015) evaluated the flocculation potential of different cationic polymers, and reporting that cationic organic polymers was the best flocculants for Chlamydomonas reinhardtii in terms of flocculation efficiency.

Microalgae harvesting studies have also been conducted using bioflocculation due to the fact that the process can occur naturally and overcome the limitations of addition of both organic and inorganic chemical flocculants. Therefore, it is considered the most cost-effective and eco-friendly method for microalgae harvesting. The bioflocculation mechanism is achieved by adding the flocculating species in order to easily harvest non-flocculating microalgae (Singh and Patidar, 2018). Bioflocculation is the process whereby microorganisms such as bacteria or fungi produce bioflocculants, particularly extracellular polymeric substances (EPS), that induce algal sedimentation (Singh and Patidar, 2018). Extreme cultivation conditions are triggered to release EPS by microalgae and/or bacteria (Park et al., 2013).

The sedimentation rate is strictly dependent on the microalgae species due to the fact that some of them flocculate more readily than the others (Vandamme et al., 2013). Salim et al. (2012) confirmed that the sedimentation rate of non-flocculating microalgae was increased with the addition of flocculating microalgae as a bioflocculant. Moreover, microalgae and bacteria are aggregated, with cell adhesion occurring via EPS. Their sedimentation rate is faster than in cultures that contain only microalgae because the

resulting microalgae-bacteria flocs' size is larger (Barros et al., 2015). Microalgal and fungal flocs are caused by neutralising negative charges on the microalgae cell surface with the fungi's hyphae, which is positively charged (Vandamme et al., 2013). The induced autoflocculation process requires large amounts of an organic carbon source, for which wastewater seems ideal as it contains enough carbon substrate to support bioflocculation processes (Barros et al., 2015; Vandamme et al., 2013).

Su et al. (2011) studied sedimentation rates, biomass production and nutrient uptake in alga cultures. They achieved good sedimentation and attained high biomass concentrations and nutrient removal efficiencies. During sedimentation tests, the total suspended solids concentration decreased from 1.84 to 0.016 g/L after 20 minutes and nitrogen and phosphorus assimilation into biomass was calculated as being $44.9 \pm 0.4\%$ and $61.6 \pm 0.5\%$ of the total inlet nitrogen and phosphorus, respectively. It is worth noting that this study also showed that the main mechanism of nutrient removal was biomass uptake.

Park et al. (2011) reported that recycling harvested microalgae via gravity settling has increased the efficiency of microalgae harvesting with increasing microalgal particle/floc size and biovolume of readily settleable algae in the reactor at higher cell retention times. Furthermore, they proved that biomass recycling has contributed to biomass productivity via two main mechanisms: (i) the extended cell retention time has increased the duration of microalgal exposure to light; and, (ii) the enhancement of the net reproductive rate of the culture by increasing the proportion of larger algal colonies with a higher growth rate than the other life-cycle stages (Park et al., 2013).

2.5. Research Problem and Research Gaps

With the increased interest in developing sustainable resource management systems, the use of current nutrient control systems in existing wastewater treatment works is under scrutiny due to their major limitations, which are linked to the increasing energy demand for aeration, the emission of greenhouse gases and the poor recovery of nutrients. Furthermore, most of the current nutrient control system cannot consistently meet stringent nutrient discharge consents (Wang et al., 2012). Microalgae cultivation systems in wastewater treatment plants could be a promising option for nutrient control, with the additional benefits of N and P recovery and algal biomass production.

This review concludes that the current research gaps require further research to fully understand the factors controlling algal nutrient uptake and to elucidate the main design criteria and operational conditions of continuous flow systems using microalgae for nutrient control and recovery in wastewater treatment systems. In order to achieve this, it is important to systematically study the factors controlling the intended process and the use of a single algal strain is vital. *Chlamydomonas reinhardtii* 11/32C was chosen as the preferred model organism in this research, and a two-stage biological process was designed in this scheme due to the following four main reasons:

1) In the current literature, *Chlamydomonas reinhardtii*, a unicellular eukaryote organism, has been used in biochemical, biophysical and genomic approaches as well as in photosynthesis research for over 40 years, due to various advantageous features such as simple microbial lifestyle, rapid growth, short doubling time, strong adaptability, ease of cultivation, homogeneous physical and biochemical behaviour characteristics, as well as a controlled sexual cycle (Dent et al., 2001; Hippler et al., 1998; Harris, 2001; Grossman, 2000). This was the foundation for selecting *Chlamydomonas reinhardtii* in the research conducted in the BioResource Systems Research Group (School of Civil Engineering, University of Leeds) and has been selected for this particular study in order to allow direct comparisons with previous studies (i.e., Yulistyorini (2016); HyPAlgae project (BB/N016033/1).
2) *Chlamydomonas reinhardtii* has presented excellent potential for biomass growth, nutrient recovery and lipid accumulation under phototrophic conditions. (Li et al., 2016; Yulistyorini, 2016; Kong et al., 2010; Qi et al., 2017; Valledor et al., 2014; Yang et al., 2018). In order to overcome the deficiencies of phototrophic growth of microalgae, *Chlamydomonas reinhardtii* has been cultivated under heterotrophic conditions due to its mitochondrial respiratory capabilities by containing *cob* genes on the mitochondrial genome (Chen and Johns, 1994; Zhang et al., 1999; Hippler et al., 1998; Harris, 2001). Moreover, *Chlamydomonas reinhardtii* also grow under mixotrophic conditions in the presence of light using organic carbon (Moon et al., 2013). This characteristic is ideal for the proposed work, which includes microalgae cultivations under mixotrophic conditions.
3) *Chlamydomonas reinhardtii* has demonstrated its ability to potentially contribute as a practical solution as it has been reported to effectively settle by

gravity (Su et al., 2012). Furthermore, the additional cationic polymer and bacterial growth under heterotrophic conditions can help settle microalgae via a bridging process and biflocculation, respectively.

4) Mass cultivation of microalgae is generally based on batch systems. These cultivation systems could be limited for two main reasons: (i) the requirement of a long hydraulic retention time due to the slow growth rate of microalgae requiring a large reactor volume (Gao et al., 2016); and, (ii) high harvesting costs because of low cell productivity (Fernandes et al., 2015). Continuous flow systems have become more attractive by overcoming the disadvantages of batch cultures with reduction of land area and higher productivity rates. Furthermore, continuous flow systems provide steady-state conditions by maintaining constant nutrient concentrations or biomass densities (Fernandes et al., 2015) .

In summary, mixotrophic microalgae cultivation, including both phototrophic and heterotrophic microalgae growth (and the use of individual reactors for each process); under continuous flow conditions, is still poorly understood. Therefore, this PhD research aims to optimise such a system and maximise nitrogen and phosphorus uptake in wastewater treatment works.

3. CHAPTER: MATERIALS and METHODS

In this chapter, the materials, methods and research methodology used in this study are explained. They were selected in order to achieve the proposed aim and objectives. The purpose of this research is the optimisation of both heterotrophic and phototrophic cultivation of microalgae as an alternative option for simultaneous nutrient control and recovery in municipal wastewater treatment works. The proposed research activities were conducted in order to achieve the three main research objectives: (1) to identify the kinetics controlling heterotrophic and phototrophic microalgal growth; (2) to identify optimised operational conditions for a two-stage biological process combining phototrophic and heterotrophic microalgae cultivation; and (3) to determine the potential to implement algal bioengineering processes for nutrient control and recovery in existing wastewater treatment works.

3.1. Identification of Microalgae Growth Kinetics under Heterotrophic and Phototrophic Conditions

3.1.1. Culture Propagation Conditions

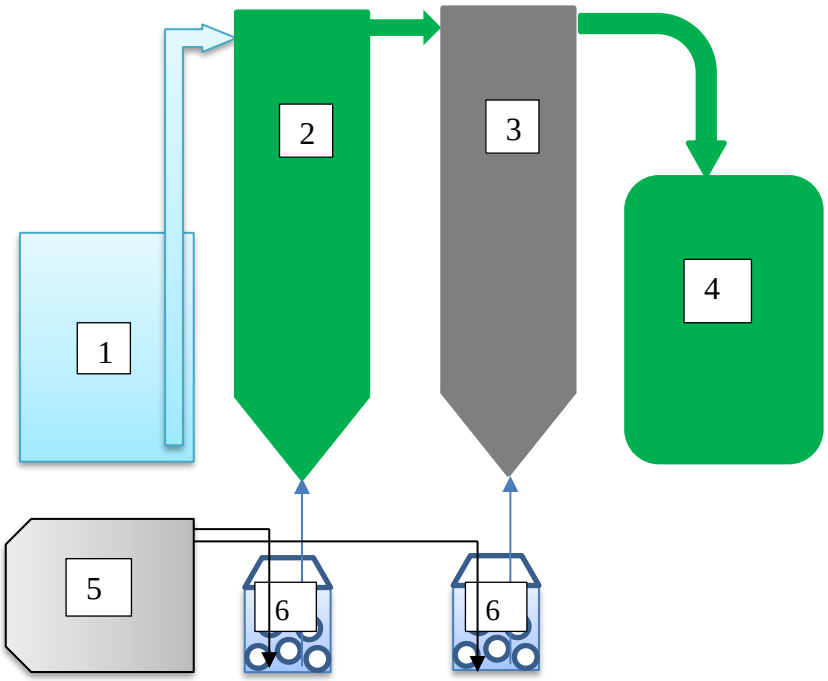
A pure culture of *Chlamydomonas reinhardtii* 11/32C was ordered from the Culture Collection of Algae and Protozoa, Scotland (CCAP), and propagated in 500 mL conical flasks containing 300 mL of Bold's Basal Media (BBM) (see Appendix A), using a shaking incubator (Infors Multitron), see Figure 3-1. Culture media was autoclaved at 121°C and 1 bar. Controlled environmental conditions for temperature, photoperiod and light intensity during cultivation (axenic microalgae culture) were set at 25°C, 24 h of light and 40 $\mu E/m^2S$, respectively.

Figure 3-1 Shaking incubator used for microalgae propagation and maintenance

3.1.2. Culture Acclimatisation Conditions

In order to adapt the microalgae strain (*Chlamydomonas reinhardtii* 11/32C) to phototrophic and heterotrophic cultivation conditions, the axenic microalga culture previously propagated (see Section 3.1.1) was added to the bioreactors (5–10% v/v) in a continuous flow system (Figure 3.2). This system comprises two tubular bioreactors with a working volume of 2.3 L each (diameter: 7 cm; height: 61cm), which were connected in series, with the effluent of the phototrophic reactor (PBR) feeding the heterotrophic reactor (HTR).

The system was fed continuously with synthetic wastewater (SWW) following a recipe reported by Yulistyorini (2016). The concentrations of macronutrients, including nitrogen, phosphorus and carbon, were adjusted to be within the range of concentrations typically found in the effluent of a conventional activated sludge process (Table 3-1). In addition, inorganic salts ($MgSO_4.7H_2O$, NaCl and $CaCl_2.2H_2O$), trace elements ($ZnSO_4.7H_2O$, $MnCl_2.4H_2O$, MoO_3, $CuSO_4.5H_2O$, $FeSO_4.7H_2O$ and $Co(NO_3)_2.6H_2O$) and pH conditioners (H_3BO_3, EDTA, KOH, and H_2SO_4) were added. In order to reduce any bacterial contamination, the SWW was autoclaved at 121°C and 1 bar before feeding the system.



(1) Feeding tank, (2) Phototrophic reactor (PBR), (3) Heterotrophic reactor (HTR), (4) Effluent, (5) Air pump, and (6) Humidifiers

Figure 3-2 Experimental set up for inoculum acclimatisation using a continuous flow system comprising a phototrophic reactor (PBR) followed by a heterotrophic reactor (HTR)

Table 3-1 Nutrient concentrations in synthetic wastewater (SWW)

Nutrient (source)	Concentration in SWW
Nitrate ($NaNO_3$)	25 mg N/L
Ammonium (CH_3COONH_4)	25 mg N/L
Phosphorus ($K_2HPO_4.3H_2O$)	15 mg P/L
Inorganic carbon ($NaHCO_3$)	400 mg C/L
Organic carbon ($CH_3COONa.3H_2O$)	100 mg C/L
$MgSO_4.7H_2O$	75 mg/L
NaCl	25 mg/L
$CaCl_2.2H_2O$	25 mg/L
$ZnSO_4.7H_2O$	8.82 mg/L
$MnCl_2.4H_2O$	1.44 mg/L
MoO_3	0.71 mg/L
$CuSO_4.5H_2O$	1.57 mg/L
$FeSO_4.7H_2O$	4.98 mg/L
$Co(NO_3)_2.6H_2O$	0.49 mg/L
H_3BO_3	11.42 mg/L
EDTA	50 mg/L
KOH	31 mg/L
H_2SO_4	0.001 mL

The PBR operated under continuous illumination (light intensity = 250 μE/m^{2}s), whilst the HTR was covered to avoid light penetration. Therefore, both reactors were operated under constant (24 h) light and dark conditions. The actual length of the photoperiod was controlled by the retention time in each reactor (hydraulic retention time); in other words, the flow rate defined the actual photoperiod. For this acclimation stage, the reactors were fed at a constant flow rate of 96 ml/h, which produced a photoperiod of 24 h light and 24 h dark. The PBR and HTR were fully mixed using an air pump coupled to a humidifier to saturate the air with water and reduce water losses in the reactors due to evaporation. The distilled water used in the humidifier was also autoclaved at 121°C and 1 bar to avoid any contamination. The effluent for the heterotrophic reactor was collected in a 10 L container.

3.1.3. Experimental Setup for Heterotrophic Microalgae Growth Kinetics

The microalgae culture previously acclimatised to heterotrophic growth conditions (Figure 3.2) was transferred to a 1 L bioreactor (Photobioreactor FMT 150, Photon Systems Instruments – PSI; see Figure 3.3).

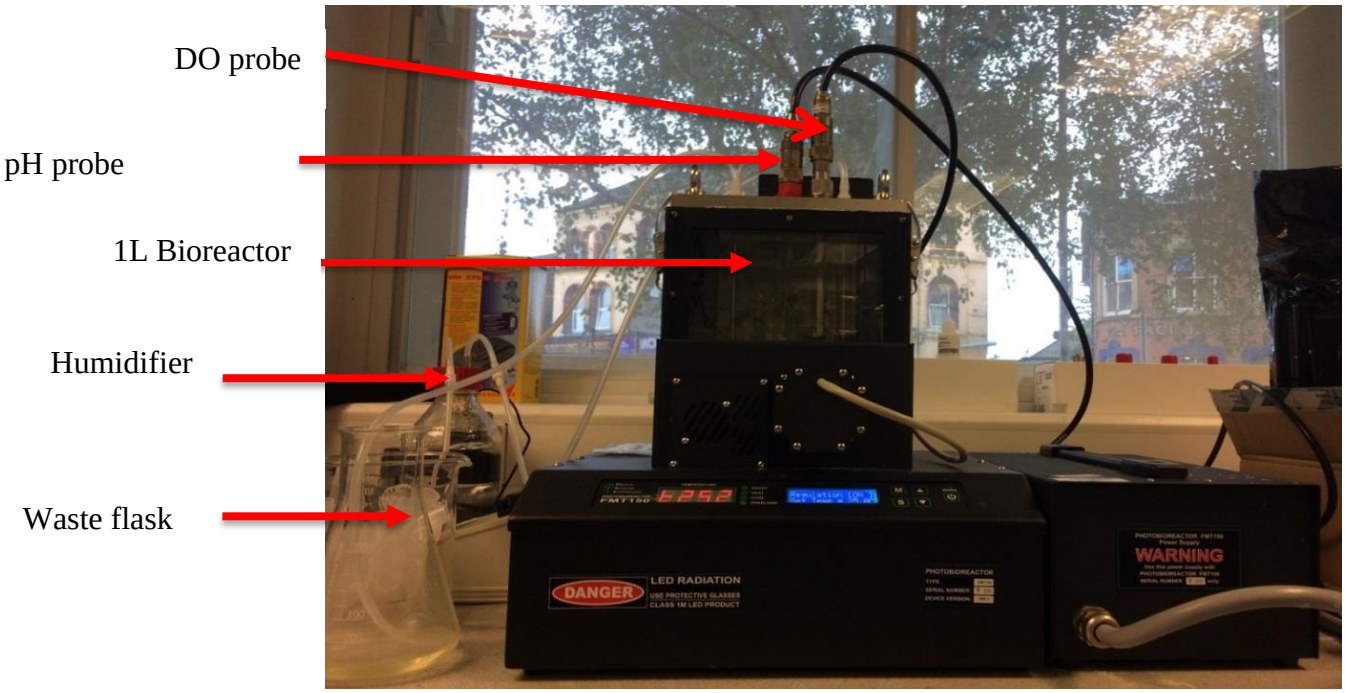


Figure 3-3 PSI bioreactor used for heterotrophic microalgae growth kinetic experiments

The bioreactor was operated for 72 h under fully dark conditions in order to quantify algal biomass growth. The temperature of the reactor was set at 25°C. Aeration and mixing were provided by using an air pump and magnetic stirrer to guarantee oxygen supply homogeneity. Air was saturated with water using a humidifier in order to reduce water losses due to evaporation. The distilled water used in the humidifier was also autoclaved at 121°C and 1 bar to avoid any contamination. In order to look for any possible microbial contamination, the cultures were also examined under the microscope.

In order to monitor environmental conditions inside the bioreactor, pH and temperature readings were recorded every minute using the pH/temperature probe, (InPro3253SG/120/PT1000, Mettler Toledo). Dissolved Oxygen (DO) was also recorded every minute using an O_2 probe (InPro6800/12/120, Mettler Toledo). Optical Density (OD) was measured at 680 nm and recorded every 2 hours. OD measurements were converted into biomass concentrations and reported as Volatile Suspended Solids (VSS) in mg L^{-1} using Equation 3.1, which was previously generated from the best linear fit (R^2 = 0.99) after generating a calibration plot from OD readings from a series of samples containing known concentrations of algal biomass grown heterotrophically and reported as VSS (mg L^{-1}). VSS analysis was conducted following standard methods (SM 2540-E; (APHA, 2012).

It is worth noting that although biomass growth monitoring often includes measurements of cell size and/or cell numbers, the quantification of biomass production using VSS preferred in this study allows for a better mass balance analysis when assessing the fate of the nutrients

$$VSS = 584.79*OD + 0.924 \qquad \text{Eq. 3-1}$$

where VSS is in mg L^{-1} and OD readings are reported at 680 nm.

In order to assess the influence of nutrient concentration (phosphorus, nitrogen and carbon) on algal biomass growth, a series of experiments were conducted in which the influence of each nutrient was tested independently. Table 3-2 lists the experimental plan to identify microalgae growth kinetics under heterotrophic conditions, considering the influence of the changes made to the initial concentration of nutrients. *Chlamydomonas reinhardtii* was cultivated in SWW using 1, 5, 10 and 15 mg P L^{-1} as the initial phosphorus concentration in order to identify the effect of phosphorus on heterotrophic microalgae growth kinetics. SWW was prepared with different nitrogen sources including nitrate,

ammonium, and a mix of both nitrogen sources (1:1 ratio) with a total initial nitrogen concentration of 50 mg N L^{-1} in order to determine the impact of nitrogen sources on the growth kinetics of heterotrophic microalgae. Initial organic carbon concentrations tested included 0.5, 1.0, 1.5 and 2.0 g C L^{-1} at 50 mg NH_4^+-N L^{-1} for the experiments conducted to assess the influence of organic carbon concentrations on heterotrophic microalgae growth kinetics. Initial P, N and organic C concentrations were selected based on data collected during the monthly water quality surveys run at the Esholt Wastewater Treatment Works (WWTW) (Chapter 6). Ammonium was preferred as the nitrogen source for the experiments using organic carbon due to the fact that it was the only inorganic nitrogen source apparent in the inlet of the Esholt WWTW, while nitrate only appears in the effluent of the Activated Sludge Process from the oxidation of ammonium.

Table 3-2 Experimental plan for heterotrophic microalgae growth kinetics

Number of Experiment	PO_4^{3-}-P (mg L^{-1})	NH_4^+-N (mg L^{-1})	NO_3^--N (mg L^{-1})	Organic C (mg L^{-1})	Inorganic C (mg L^{-1})
1	1	25	25	2000	400
2	5	25	25	2000	400
3	10	25	25	2000	400
4	15	25	25	2000	400
5	1	50	-	2000	400
6	5	50	-	2000	400
7	10	50	-	2000	400
8	15	50	-	2000	400
9	1	-	50	2000	400
10	5	-	50	2000	400
11	10	-	50	2000	400
12	15	-	50	2000	400
13	1	50	-	1500	400
14	5	50	-	1500	400
15	10	50	-	1500	400
16	15	50	-	1500	400
17	1	50	-	1000	400
18	5	50	-	1000	400
19	10	50	-	1000	400
20	15	50	-	1000	400
21	1	50	-	500	400
22	5	50	-	500	400
23	10	50	-	500	400
24	15	50	-	500	400

It is worth noting that there was not enough time to repeat the experiments in Table 3-2 because access to a new 1-L PSI photobioreactor (Figure 3-3) was only granted at the end of the PhD work reported herein. Meanwhile, preliminary experiments for heterotrophic microalgae growth kinetics were conducted using 1 L Duran bottles in

duplicate, the results for which are presented in Appendix B. However, persistent problems due to bacterial contamination resulted in considerable uncertainty over the data collected. Therefore, the data produced by using the 1 L PSI photobioreactor was reported due to the fact that the use of this more robust equipment with its very well controlled conditions allowed for much greater confidence in the data so recorded.

3.1.4. Experimental Setup for Phototrophic Microalgae Growth Kinetics

Microalgae from the PBR used for acclimation (Figure 3.2) were collected and added (5-10%, v/v) to inoculate 300 mL of SWW in 500 mL Erlenmeyer flasks placed in a shaking incubator (Infors Multitron), in operation under identical controlled environmental conditions as reported for culture propagation (see Section 3.1.1). The microalgae culture was kept in the incubator for a week until the stationary phase had been achieved. The OD was measured daily at a wavelength of 680 nm using a spectrophotometer (Thermo Scientific Biomate 3) in order to observe the growth of *Chlamydomonas reinhardtii* under phototrophic conditions. OD values were converted into VSS concentrations using Equation 3.2, which was obtained from the best linear fit (R^2 = 0.98) between the correlation of VSS concentrations of microalgae grown phototrophically and their corresponding OD readings at 680 nm. VSS was analysed following a standard method (Method 2540-E; (APHA, 2012)

$$VSS = 853.75*OD - 15.224 \quad \text{Eq. 3-2}$$

where VSS is in mg L^{-1} and OD readings are reported at 680 nm.

The same combinations of nutrient concentrations reported in Section 3.1.3 (Table 3-2) were used to develop a series of experiments designed to assess the influence of phosphorus, nitrogen and inorganic carbon concentration on phototrophic microalgae growth kinetics (Table 3-3). All experiments were conducted in duplicate. Phosphorus concentration varied between 1-15 mg P L^{-1}, nitrogen concentration was set at 50 mg N L^{-1} and initial inorganic carbon concentration was tested as being between 0.5 and 2.0 g C L^{-1} according to the changes in nutrient concentrations observed at the Esholt WWTW (Chapter 6). Organic carbon concentration had to be reduced in order to control bacterial growth contamination. The series of experiments summarised in Table 3-3 were conducted in duplicate.

Table 3-3 Experimental design to identify phototrophic microalgae growth kinetics

Number of Experiment	PO_4^{3-}-P (mg L^{-1})	NH_4^+-N (mg L^{-1})	NO_3^--N (mg L^{-1})	Organic C (mg L^{-1})	Inorganic C (mg L^{-1})
25	1	25	25	100	2000
26	5	25	25	100	2000
27	10	25	25	100	2000
28	15	25	25	100	2000
29	1	50	-	100	2000
30	5	50	-	100	2000
31	10	50	-	100	2000
32	15	50	-	100	2000
33	1	-	50	100	2000
34	5	-	50	100	2000
35	10	-	50	100	2000
36	15	-	50	100	2000
37	1	50	-	100	1500
38	5	50	-	100	1500
39	10	50	-	100	1500
40	15	50	-	100	1500
41	1	50	-	100	1000
42	5	50	-	100	1000
43	10	50	-	100	1000
44	15	50	-	100	1000
45	1	50	-	100	500
46	5	50	-	100	500
47	10	50	-	100	500
48	15	50	-	100	500

3.1.5. Data Processing

3.1.5.1. Net Biomass Productivity

Biomass productivity was identified by the changes in VSS concentration during the exponential growth phase (Equation 3-3)

$$\text{Biomass Productivity (mg VSS L}^{-1}\text{ d}^{-1}) = \frac{(X - Xi)}{(t - ti)} \qquad \text{Eq. 3-3}$$

where X_i and X are algal biomass concentrations at times corresponding with the beginning (t_i) and the end (t) of the exponential growth phase, respectively.

3.1.5.2. Growth Kinetics of Microalgae

Specific growth rates (μ) for heterotrophic and phototrophic growth of microalgae were determined from the gradient of graphs plotting the logarithm of volatile suspended solids (VSS) concentrations during the exponential growth phase against time under different initial phosphorus concentrations. Phosphorus was considered to represent the

limiting substrate to growth. The specific growth rates obtained for *Chlamydomonas reinhardtii* with different initial nitrogen sources and initial carbon concentrations were compared in order to identify the influence that these nutrients have on microalgae growth. The ratio of nutrients in wastewater (carbon, nitrogen and phosphorus) varies during the treatment process, and by understanding the effects of such changes on microalgal biomass growth so it is possible to select the best location within an existing wastewater treatment works to place a microalgal process.

The classical Monod's model (Equation 3-4) was selected to process growth kinetics data. Monod's model has been applied to calculate growth kinetics under conditions of limited substrates (Monod, 1949). Experimental data was processed using a non-linear regression analysis available through the Solver function in Microsoft Excel

$$\mu = \mu_{max}\frac{S}{Ks+S} \qquad \text{Eq. 3-4}$$

where:

μ = specific growth rate, d^{-1}

μ_{max} = maximum specific growth rate, d^{-1}

S = substrate (phosphorus) concentration, mg/L

K_s = half-saturation constant, mg/L

3.2. Operation of a Two-Stage Biological Process Combining Heterotrophic and Phototropic Microalgae Cultivation under Continuous Flow Conditions with Biomass Recycling

3.2.1. Culture Propagation

The same procedure was used for culture propagation as described in Section 3.1.1.

3.2.2. Experimental Setup for a Two-Stage Biological Process

The setup for this experimental phase is presented in Figure 3-4. The start-up of the process included the increment of algal biomass density in the reactors to rapidly reach steady-state conditions under continuous flow operation. Firstly, the 2.3 L photobioreactor (PBR) (diameter: 7 cm; height: 61 cm) was inoculated with *Chlamydomonas reinhardtii* 11/32C (5-10%, v/v in SWW), which was maintained under

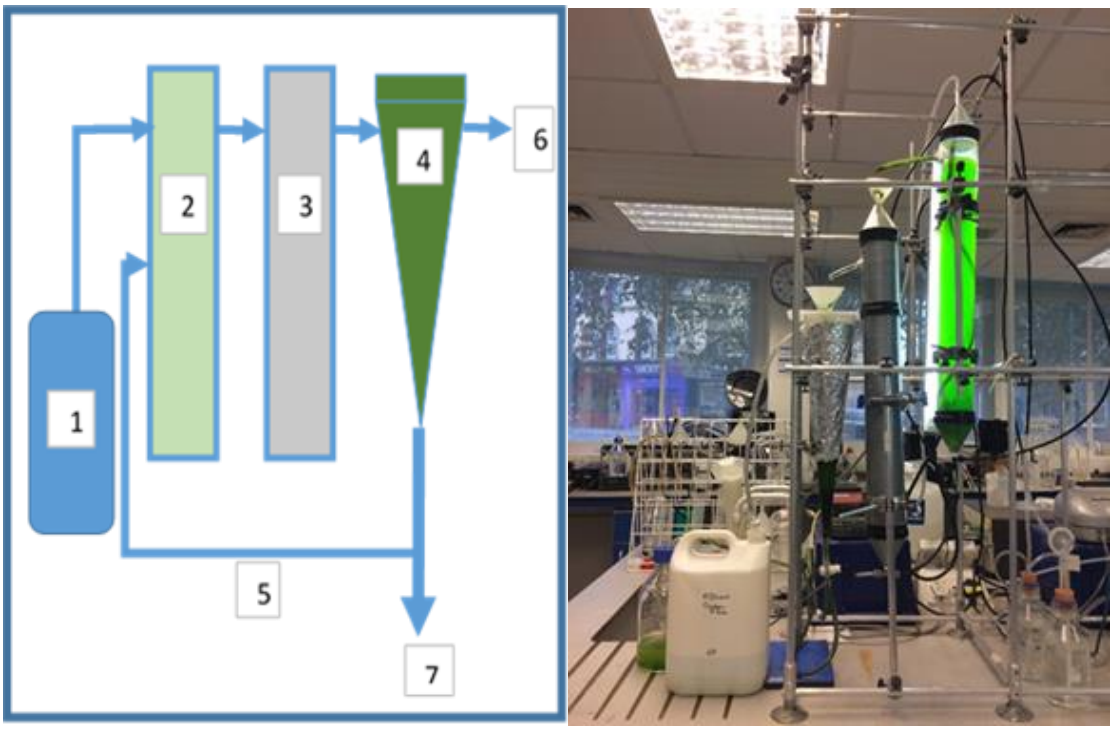
axenic conditions (see Section 3.1.1). The PBR was initially running under batch conditions with a 24 h photoperiod (Light intensity = 250 μE/m^2s) and at laboratory temperature (25°C) until stationary growth conditions were achieved. Biomass growth was monitored using OD readings. Thereafter, the entire system was operated under continuous flow conditions .

The two-stage microalgal process comprises a PBR that is continuously fed with SWW and connected in series to a heterotrophic reactor – HTR (effective volume: 2.3 L; diameter: 7 cm; height: 61 cm). The effluent from the HTR feeds by gravity a 1 L Imhoff cone that allows algal biomass sedimentation and recycling to the PBR by pumping, while the supernatant is collected for characterisation.

The system was continuously fed with SWW, mimicking the characteristics typically found in the effluent from a conventional activated sludge process (see Table 3-1), with the addition of 20 mg L^{-1} of cationic polymer (Zetag 50). The possible effect of the addition of cationic polymer in the feeding medium is to disperse it equally in the system and neutralise the negative surface charge on each microalgae cells. The use of cationic polymers as coagulation aids is common practice in WwTWs to facilitate the sedimentation of bacterial biomass and improve the quality of the secondary effluent; for these reasons, a similar approach was implemented to facilitate algal biomass sedimentation and recycling. Microalgae have a low sedimentation rate due to their small cell size and electronegatively charged cell surfaces. Engineered organic polymers can be used to stabilise such negative electrostatic charges, which in return help algal cells to agglomerate and form heavier flocs for more effective sedimentation (Gerardo et al., 2015).

Settled microalgal biomass was recycled into the PBR at a specific rate according to the predefined VSS target concentration (mixed liquor volatile suspended solids – MLVSS). A set amount of settled microalgal biomass (algal sludge) was discarded daily according to the predefined cell retention time – CRT (see Equation 3-7).

The two-stage biological process setup includes seven main components as presented in the process flow diagram and the picture depicted in Figure 3-4.a. and b, respectively.



a **b**

Figure 3-4 Process diagram (a) and picture of the experimental setup of the two-stage biological process (b)

(1) Feeding tank containing SWW; (2) PBR for phototrophic cultivation; (3) HTR for heterotrophic cultivation; (4) Imhoff cone for microalgae sedimentation; (5) Algal sludge recycling line; (6) Effluent tank; (7) Algal sludge purge.

Water samples were collected every other day until steady-state conditions were achieved from four sampling points including: (a) PBR effluent; (b) HTR effluent; (c) algal sludge recycling line; and, (d) Imhoff cone supernatant (final effluent) (see Figure 3-4.a). Collected data was processed to assess process performance and monitor biomass growth and nutrient uptake. Mass flow analyses of nutrients were conducted under steady-state conditions.

In order to monitor the stability of the entire system during operation, pH and DO were measured using a multimeter (Hach HQ 40d). Alkalinity was also analysed by titration using an autotitrator (Mettler Toledo T50) following a standard method (SM 2320-B; (APHA, 2012).

Microalgae growth was determined by monitoring changes in biomass concentration using gravimetric analysis for total suspended solids (TSS) and volatile suspended solids (VSS). Effective nutrient control (remaining concentration in solution)

and recovery (accumulation in algal biomass) was determined by monitoring the fate of nutrients (carbon, nitrogen and phosphorus), both soluble and suspended. The soluble and suspended forms of the nutrients were monitored following changes in the concentrations of phosphate (PO_4^{3-}-P), ammonium (NH_4^+-N), nitrate (NO_3^-), total chemical oxygen demand (COD), soluble COD, Total Kjedahl Nitrogen (TKN), soluble TKN, total phosphorus (TP) and soluble TP. All analytical tests were conducted following standard methods reported by APHA (2012). Furthermore, phosphorus content in algal biomass cells from both PBR and HTR was analysed following the method reported by Yao et al. (2011).

Mass flow analyses for phosphorus and nitrogen were performed with data collected from soluble and suspended forms of nutrients in samples collected from PBR and HTR.

3.2.3. Operational Conditions for a Two-Stage Biological Process

The two-stage biological process was operated under different operational conditions to optimise process performance based on operational parameters (HRT and CRT) and nitrogen source availability in the SWW. Microalgae is able to take up nitrogen as ammonium and, hence, there would not be any need for further nitrification in the activated sludge process; however, previous research conducted at Leeds has demonstrated that phosphorus uptake by microalgae depends on nitrogen source (i.e., ammonium to nitrate ratio) (Yulistyorini, 2016). The tested conditions are summarised in Table 3-4.

Table 3-4 Experimental plan for identifying optimum operational conditions

Number of Experiment	Variables			
	HRT (hours)	CRT (days)	Ammonium concentration in SWW (mg N L^{-1})	Nitrate concentration in SWW (mg N L^{-1})
1	36	14	25	25
2	48	14	25	25
3	72	14	25	25
4	48	7	25	25
5	48	21	25	25
6	48	14	50	-
7	48	14	-	50

Each experiment was operated for approximately one month, which gave the possibility of producing plenty of data to test the stability and performance of the process under the set conditions. Unlike tests with batch reactors that require several replicates to assess data reproducibility, the operation of continuous flow systems allows a similar assessment with the equivalent of a larger number of replicates, i.e., an experiment with an HRT of 72 h (3 d) run for 30 days is the equivalent of running 10 replicates in a batch reactor with a three-day residence time. The criteria selected to assess whether the system had reached steady-state conditions were biomass growth and P removal, which were calculated by monitoring the concentration of VSS in the reactors and soluble phosphorus in the final effluent (FE) after sedimentation. Generally speaking, it lasted around 15 days to reach steady-state conditions. Once steady-state conditions were reached, the system was operated for an additional 15 days and data collected for nutrient mass balance analysis. Biomass concentrations in each reactor with different operational conditions at steady-state conditions are reported as average ± one standard deviation.

3.2.3.1. The Effect of Hydraulic Retention Time (HTR)

Hydraulic Retention Time (HRT) is the average retention time of the feeding media in the reactor. HRT is one of the main parameters used in the design of continuous flow systems (Anbalagan et al., 2016). HRT is calculated dividing the volume of the reactor by the flowrate feeding the system (Equation 3-5). Flowrates for operating the two-stage biological process were calculated via Equation 3-5.

$$\text{HRT} = \frac{V}{Q} \qquad \text{Eq. 3-5}$$

$$D = \frac{Q}{V} \qquad \text{Eq. 3-6}$$

where:

HRT = hydraulic retention time, d

D = dilution rate, d^{-1}

V = working volume of each reactor, L

Q = flowrate of each reactor, Ld^{-1}

HRTs tested in the two-stage biological process were 36, 48 and 72 h, which corresponded to feeding flowrates of 2.5, 1.7 and 0.9 L d^{-1}, respectively, with a CRT of 14 days and a mix of 25 mg L^{-1} NH_4^+-N and 25 mg L^{-1} NO_3^--N in SWW.

3.2.3.2. The Impact of Cell Retention Time (CRT)

The system was tested at 7, 14 and 21 d of CRT by carefully controlling the amount of algal biomass daily purged from the system (algal sludge); the corresponding volume of algal sludge purged daily was 660, 330 and 220 mL, respectively. Other key operational conditions were set as follows: 48 h HRT and 50 mg N L^{-1} in SWW from a mix of 25 mg L^{-1} NH_4^+-N and 25 mg L^{-1} NO_3^--N. CRT conditions were calculated via Equation 3-7 (Metcalf&Eddy, 2003).

$$CRT = \frac{V\ (Xpbr+Xhtr)}{(Q-Qw)Xe+QwXr} \qquad \text{Eq. 3-7}$$

$$\mu = \frac{1}{CRT} \qquad \text{Eq.3-8}$$

$$Q_w = \frac{Vt}{CRT} \qquad \text{Eq. 3-9}$$

where:

CRT = cell retention time, d

μ = specific growth rate, d^{-1}

V = working volume of each reactor, L

V_t = volume of system, L

X_{PBR} = average biomass concentration in PBR, mg L^{-1}

X_{HTR} = average biomass concentration in HTR, mg L^{-1}

Q = flowrate of each reactor, L d^{-1}

Q_w = flowrate of wasted algae, L d^{-1}

X_e = biomass concentration in final effluent, mg L^{-1}

X_r = biomass concentration in the recycle line, mg L^{-1}

The net change in biomass concentration in the continuous flow systems was determined via Equation 3-10 (Becker, 1994), which is the difference between the specific biomass growth rate and the amount of biomass washed out of the system; it was also calculated via Equation 3-11. Under steady-state conditions, the specific growth rate should match the dilution rate (i.e., when $\frac{dX}{dt} = 0$ at steady-state, $\mu = D$) and biomass concentration should be constant (Herbert et al., 1956).

$$\frac{dX}{dt} = VX_i - VX_s \qquad \text{Eq. 3-10}$$

$$\frac{dX}{dt} = \mu X - DX \qquad \text{Eq. 3-11}$$

where:

V = working volume of each reactor, L

X_i = initial biomass concentration in each reactor, mg L^{-1}

X_s = biomass concentration at steady-state conditions, mg L^{-1}

μ = specific growth rate, d^{-1}

D = dilution rate, d^{-1}

3.2.3.3. The Influence of Different Nitrogen Sources

Nitrogen concentration in the SWW was set at 50 mg N L^{-1}, but the actual nitrogen source as modified to test its influence on the performance of the system. Tests were run solely using ammonium, nitrate, and a mix of ammonium and nitrate (1:1 ratio) at 48 h HRT and 14 d CRT.

Table 3-5 Summary of operational conditions tested in the two-stage biological process

Parameter	Value (unit)		
Total volume of the system	4.6 L (2*2.3L)		
Hydraulic Retention Time (HRT)	36 h, 48 h and 72 h (18 h, 24 h and 36 h for each reactor)		Equation 3.5
Flowrate of SWW (Q_f)	2.5 L d^{-1}	36 h HRT	
	1.7 L d^{-1}	48 h HRT	
	0.9 L d^{-1}	72 h HRT	
Dilution rate (D)	1.33 d^{-1}	36 h HRT	Equation 3.6
	1.00 d^{-1}	48 h HRT	
	0.66 d^{-1}	72 h HRT	
Cell Retention Time (CRT)	7 d, 14 d and 21 d		
Specific growth rate (μ)	0.14 d^{-1}	7 d CRT	Equation 3.8
	0.07 d^{-1}	14 d CRT	
	0.05 d^{-1}	21 d CRT	
Flowrate of harvested biomass (Q_w)	660 ml d^{-1}	7 d CRT	Equation 3.9
	330 ml d^{-1}	14 d CRT	
	220 ml d^{-1}	21 d CRT	
Average biomass concentration in effluent (X_e)	75 mg VSS L^{-1}		MLVSS concentration in PBR and HTR were assumed 1 and 1.5 g L^{-1}, respectively (Equation 3.7)
Flowrate of recycle biomass (Q_r)	600 ml d^{-1}		Biomass was recycled for 15 mins in each two-hour period.
Biomass concentration in recycle (X_r)	750 mg VSS L^{-1}		

3.2.3.4. Mass Flow Analysis of Nutrients

A mass balance analysis for each reactor and the entire process at steady-state conditions was conducted using the equations recommended by Metcalf&Eddy (2003). See equations 3-12 and 3-13.

Accumulation = inflow – outflow + generation (- consumption) Eq. 3-12

$$\frac{dC}{dt} V = Q C_0 - QC + r_c V \quad \text{Eq. 3-13}$$

where:

C = concentration of nutrient, mg L^{-1}

V = working volume of each reactor, L

Q = flowrate of each reactor, L d^{-1}

r_c = rate of concentration change

Reasonable assumptions were made, including: a) the inlet and outlet flowrates were taken to be constant in each reactor; b) the volume of water in each reactor was taken to be constant; c) the reactors were considered to operate under complete-mix conditions; and d) the chemical reactions that occur in the reactor follow first-order kinetics ($r_c = \pm kC$) (Metcalf&Eddy, 2003).

3.3. Implementation of Algal Bioengineering Processes for Nutrient Control and Recovery in Existing Sewage Treatment Facilities: Case Study at Esholt Wastewater Treatment Works

3.3.1. Esholt Wastewater Treatment Works

In order to assess the potential implementation of the proposed two-stage algal process, a comprehensive water quality survey was conducted at Esholt Wastewater Treatment Works (WwTW), Bradford, UK. Grab samples were collected on a monthly basis from selected sampling points between October 2014 and May 2017 (Figure 3-5) and analysed in the lab (Table 3-6). Collected data was used to calculate process performance and the fate of nutrients (carbon, nitrogen and phosphorus).

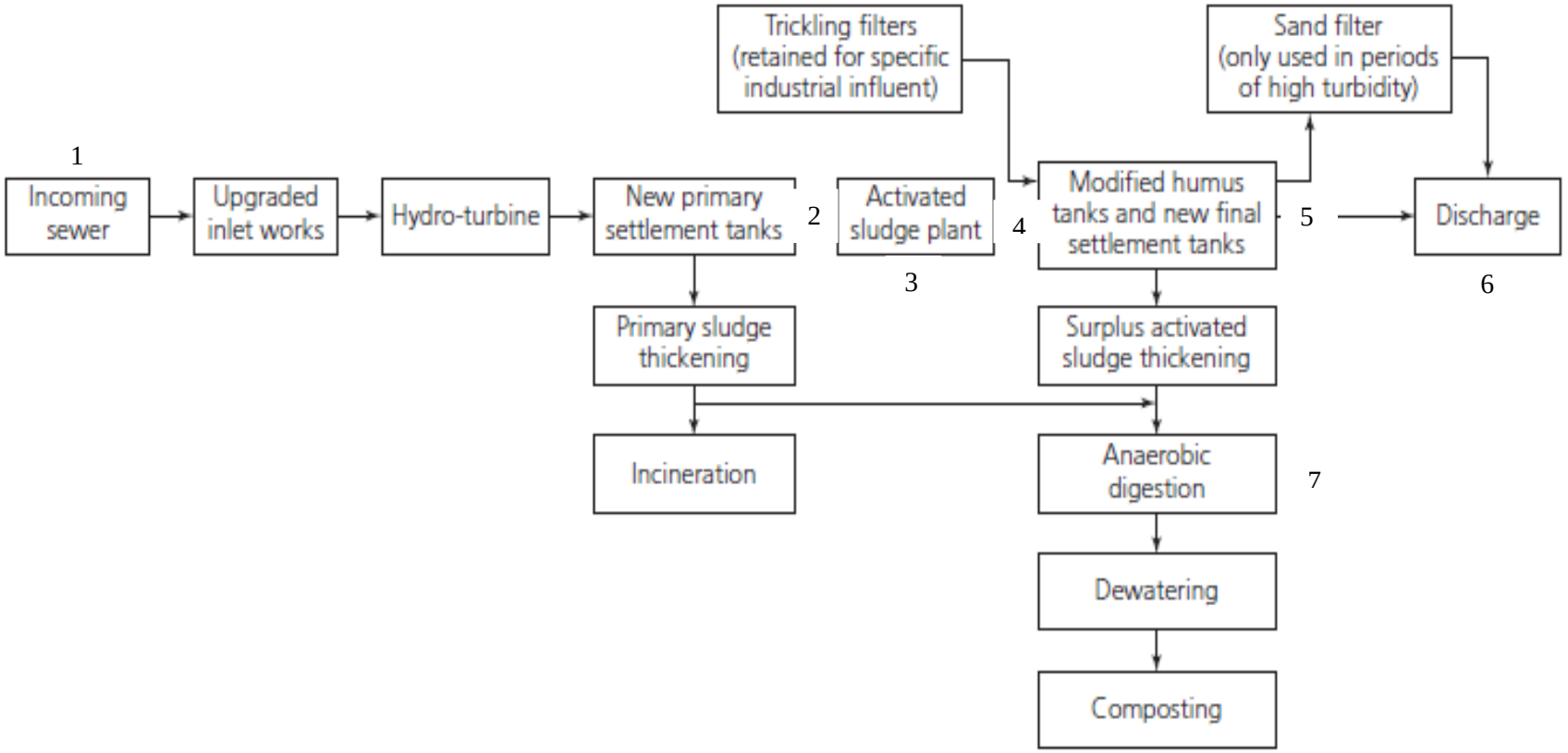


Figure 3-5 Process flow diagram for Esholt WWTW (Marsden et al., 2012, p.80)

1. Raw Wastewater (Inlet)

2. Effluent from PSTs (Primary Effluent)

3. Influent of Activated Sludge Units

4. Effluent from Activated Sludge Units

5. Effluent from FSTs (Secondary Effluent)

6. Final Effluent

7. Anaerobic Digestate (AD)

8. Digestate Liquor (Centrifuged AD)

Esholt WwTW is located in Bradford, UK, and receives an influent of 730,000 population equivalent (p.e.) of wastewater; the treated effluent is discharged into the River Aire (Marsden et al., 2012). This WwTW originally comprised primary sedimentation tanks (PST), followed by biological treatment in trickling filter units and corresponding humus tanks; sewage sludge used to be incinerated on site. Over the last decade, the entire system was upgraded to meet more stringent discharge standards (i.e., < 20 mg L^{-1} suspended solids, < 10 mg L^{-1} biological oxygen demand – BOD, and < 3 mg N L^{-1} ammonia). New assets at Esholt WwTW include an upgraded inlet works, a hydro turbine between the inlet works and PSTs (i.e., power generation resulting from the difference in hydraulic levels), six new PSTs to increase solid removal capacity, a new Activated Sludge Plant (ASP) with 12 aeration units for the removal of organic material and nutrients, eight modified and four new final sedimentation tanks (FST) for sludge removal, four new sludge thickening tanks, three new anaerobic digesters for sludge management and biogas production, and two new combined heat and power (CHP) engines for bioenergy production (Marsden et al., 2012).

Approximately 60 million tonnes of sewage sludge produced in waste water treatment works are converted to biogas via anaerobic digestion (AD) in the UK (Grasham et al., 2019). Combined heat and power (CHP) combustion is used for the biogas produced by AD for energy generation, which is used for onsite energy requirements. As a case study, Esholt WwTW demands 60,000 kWh of electricity per day, and the average daily energy generation is 40,000 kWh depending on the flowrate and operational conditions (Yulistyorini, 2016). Despite the fact that Esholt WwTW provides around one-third of its energy consumption from two CHPs, Yorkshire Water aims to make Esholt fully energy self-sufficient by developing the sludge treatment method (Rush J., 2012). In addition, a greenhouse gases equivalent of 4 million tonnes of carbon dioxide was released by water companies in 2011-2012, which represents 0.7% of total UK emissions (WATER-UK, n.d.-a). Carbon emission at wastewater treatment plants could be reduced around 60% by applying improved CHP processes such as molten carbonate fuel cells rather than the standard CHP process (Chacartegui et al., 2013). With two years of upgrade at Esholt WwTW between 2012-2014, a reduction in the carbon footprint of 9000 tonnes has brrn achieved, saving £1.3 million per year energy costs (Brockett J., 2014).

3.3.2. Mass Balance Analysis of Nutrients

The same procedure described in Section 3.2.3.4 was used for the mass flow analysis of nutrients at Esholt WWTW.

3.4. Analytical Procedures

All analyses performed in this research to identify algal biomass growth and nutrient recovery and uptake via microalgae at the lab- and bench-scale (Chapter 4 and 5), and to determine a mass flow of solids and nutrients at Esholt WWTW (Chapter 6), were conducted according to standard methods (see Table 3-5) (APHA, 2012).

Table 3-6 Analytical Methods used in this research

Analysis	Methods*
Total Suspended Solids (TSS)	2540 D
Volatile Suspended Solids (VSS)	2540 E
Reactive Phosphorus (RP)	4500-P.E
Intracellular Phosphorus	(Yao et al., 2011)
Total Phosphorus (TP)	4500-P.B
Ammonium (NH_4^+-N)	4500-NH_3 B and C
Nitrate (NO_3^-)	Ion Chromatography**
Total Kjeldahl Nitrogen (TKN)	4500-N_{org}
Chemical Oxygen Demand (COD)	5220 C
Bacterial Count	Serial Dilution

*Standard Methods for the Examination of Water and Wastewater 22th Edition (APHA, 2012), unless otherwise stated.
**Ion Chromatographer (Metrohm 850 Professional IC) using a with Metrosep A supp 5 column (length: 150 mm, diameter: 4 mm) and an eluent comprising 1 mM $NaHCO_3$ and 3.2 mM Na_2CO_3. Samples were passed through 0.45 μm Minisart syringe filters and diluted to adjust nitrate concentration to < 30 ppm.

3.5. Statistical Analysis

Data processing included statistical analyses for descriptive statistics and variable comparisons; all data were processed using IBM's SPSS Statistics 22 software suite. One-way analysis of variance (ANOVA) was used to evaluate the differences among the treatments. If ANOVA effects were significant, comparisons between the different means were made using *post hoc* least significant differences (LSD).

4. CHAPTER: MICROALGAE GROWTH KINETICS

4.1. Introduction

Microalgae have been studied for decades, particularly with regard to their capability to fix CO_2, nutrient uptake and lipid accumulation for biofuel production (Kasiri et al., 2015b; Xin et al., 2010; Sakarika and Kornaros, 2016). Wastewater has been proposed as a sustainable source of nutrients for algal biomass cultivation due to its high nitrogen and phosphorus concentrations (Lam et al., 2017). Therefore, microalgae cultivation in wastewater has been presented as a sustainable alternative that reduces the demand for freshwater and primary resources for nutrient production (i.e., phosphoric rocks, fossil fuels, etc.).

Microalgae can grow not only under phototrophic conditions but also under heterotrophic conditions. Heterotrophic cultivation of microalgae is highly cost-effective compared to phototrophic microalgae because the requirement for light is eliminated. Furthermore, issues related to self–shading effects experienced in photobioreactors with high biomass concentrations can be eliminated under heterotrophic culturing conditions. The most important advantage of heterotrophic microalgae growth is the possibility of achieving higher growth rates in comparison with phototrophic growth conditions (Perez-Garcia et al., 2011b; Sakarika and Kornaros, 2016).

However, there are still gaps in the published literature supporting the need for further research regarding the applicability of using microalgae for nutrient control and recovery in wastewater treatment works. In order to better understand algal-biomass productivity under operational conditions similar to those found in wastewater treatment processes, which could contribute to scale–up microalgae systems, it is important to determine growth kinetic models for microalgae under such circumstances (Kim et al., 2013a; Lee et al., 2015). To the best of our knowledge, this research is the first attempt to identify the growth kinetics of heterotrophic and phototrophic microalgae using phosphorus as limiting substrate using concentrations typically found in wastewater treatment processes. With this target in mind, microalgae growth kinetics were estimated under both heterotrophic and phototrophic conditions. The effect of initial phosphorus concentrations on heterotrophic and phototrophic microalgae growth kinetics was also

studied using different nitrogen sources and different initial organic and inorganic carbon concentrations.

4.2. Methodology

Chlamydomonas reinhardtii was selected due to its capability to grow under both phototrophic and heterotrophic conditions (Chen and Johns, 1994). Firstly, a pure culture of *Chlamydomonas reinhardtii* was propagated in 500 mL conical flasks containing 300 mL of Bold's Basal Media (BBM) (Section 3.1.1) before using it as inoculum to seed PBR and HTR bioreactors in the acclimation step (Section 3.1.2), which was immediately followed by the actual growth kinetics tests described in sections 3.1.3 and 3.1.4 (Figure 4-1). Growth kinetics experiments were conducted in order to investigate the effect of nitrogen source and phosphorus and carbon availability on algal biomass growth using *Chlamydomonas reinhardtii* as a model species (Table 3-2 and 3-3 show the conditions set for heterotrophic and phototrophic conditions, respectively). Initial P, N and C concentrations were determined based on data collected during the water quality surveys conducted at the Esholt Wastewater Treatment Works (WwTW) (See Section 3.3). Monod's model was used to calculate growth kinetics constants (Monod, 1949). Non-linear regression analysis was performed via the Solver function in Microsoft Excel for data processing.

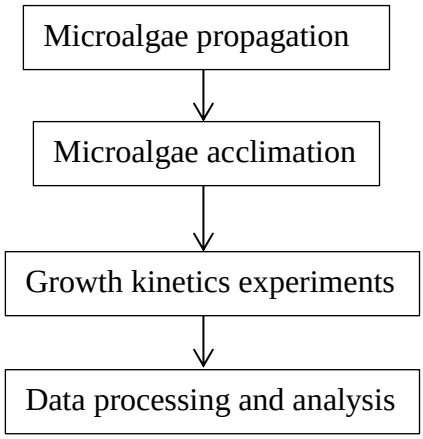


Figure 4-1 Methodology stages for determining growth kinetics of *Chlamydomonas reinhardtii* at heterotrophic and phototrophic cultivation

4.3. Results and Discussion

4.3.1. The Effects of Different Nitrogen Sources on Microalgae Growth under Heterotrophic Conditions using Phosphorus as the Limiting Substrate

Preliminary data to identify the heterotrophic growth kinetics of *Chlamydomonas reinhardtii* are presented in Appendix B. However, persistent bacterial contamination resulted in considerable uncertainty over the data collected. Therefore, a new 1-L PSI granted at the end of the PhD study was used to estimate the growth kinetics under heterotrophic conditions with the additional benefits of having a high degree of control over experimental conditions, thus giving more confidence in the data reported; however, there was not enough time to replicate the experiments.

Chlamydomonas reinhardtii was cultivated in a 1 L bioreactor using synthetic wastewater with different nitrogen sources including a mix of NH_4^{+}-N and NO_3^{-}-N, NH_4^{+}-N and NO_3^{-}-N with a range of initial phosphorus concentrations (1 to 15 mg P L^{-1}). Changes in algal biomass concentrations during the cultivation period as a response to the nitrogen source used are reported in Figure 4-2. Experiments were conducted until the microalgae culture reached a stationary growth phase (approximately for 72 h in total).

It can be seen in Figure 4-2.a that when a mix of NH_4^{+}-N and NO_3^{-}-N (1:1 N ratio) is used as nitrogen source, the final biomass concentration of *Chlamydomonas reinhardtii* significantly increased from 370 to 520 mg VSS L^{-1} with the increase of initial phosphorus concentration from 1 to 15 mg P L^{-1} (p = 0.022, one-way ANOVA). As regards biomass productivity, there was a sharp increase from 246 to 604 mg VSS L^{-1} d^{-1} with the increase in initial phosphorus concentration from 1 to 10 mg P L^{-1}, but biomass productivity decreased to 468 mg VSS L^{-1} d^{-1} at 15 mg P L^{-1}.

As seen in Figure 4-2.b, similar behaviour was observed when ammonium was used as the sole nitrogen source (p = 0.429 for a mix of ammonia and nitrate and solely ammonia, one-way ANOVA). The final biomass concentration of *Chlamydomonas reinhardtii* slightly increased from 370 to 420 mg L^{-1} VSS with the increase in initial phosphorus concentration from 1 to 10 mg L^{-1}, but there was a little decrease in the final biomass concentration (360 mg L^{-1} VSS) at 15 mg P L^{-1} initial phosphorus concentration (p values were greater than 0.05 for all tests). With regard to biomass productivity, a significant increase was observed from 377 to 580 mg L^{-1} VSS d^{-1} when P concentration

increased from 1 to 5 mg P L^{-1}. With a further increase in the initial phosphorus concentration, the resulting biomass productivity did not show significant changes.

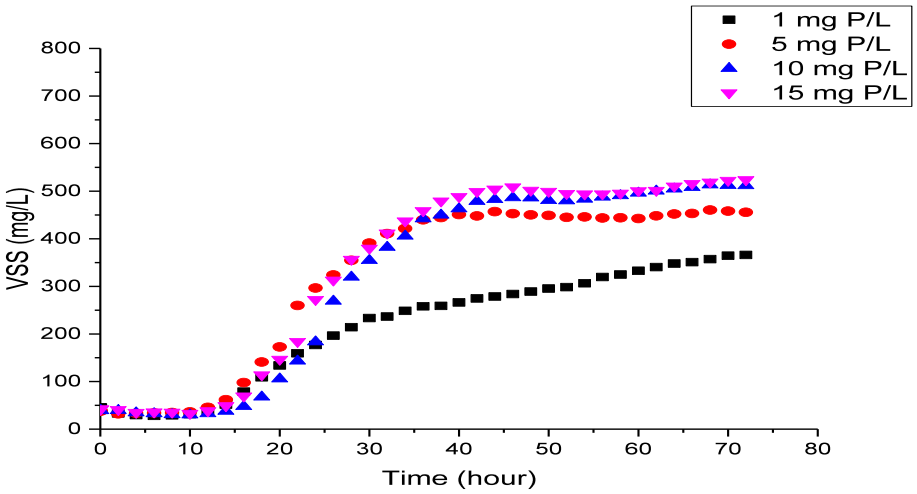
Chlamydomonas reinhardtii behaved differently when nitrate was used as the sole source of nitrogen (Figure 4-2.c) ($p < 0.05$ for nitrate and either a mix of ammonium and nitrate or ammonium, one-way ANOVA), in that the greatest biomass concentration and biomass productivity were 650 mg VSS L^{-1} and 861 mg VSS L^{-1} d^{-1}, respectively, at 5 mg P L^{-1}. At initial phosphorus concentrations higher than 5 mg P L^{-1}, lower biomass concentrations were obtained (around 610 mg VSS L^{-1}). Biomass productivity was found to be 622 and 757 mg VSS L^{-1} d^{-1} at 10 and 15 mg P L^{-1}. Statistical analysis (one-way ANOVA) shows that there were no significant changes in the final biomass concentrations (*p* values for all tests were greater than 0.05).

pH values were automatically recorded every minute by the probe connected to the PSI unit and with no buffer solution added to control changes in pH. All the experiments conducted with different nitrogen sources presented similar pH changes. The average initial pH was 9.04 ± 0.07, which slightly increased to 9.24 ± 0.02 until reaching the exponential growth phase. pH decreased to 9.06 ± 0.08 during the exponential growth phase. Thereafter, pH started to increase again and reached an average maximum value of 9.36 ± 0.03 at the end of the experiments (see Appendix C). It is worth noting that in the experiments using NH_4^+ as the nitrogen source, ionised ammonium was the main chemical species present as pH ranged between 9.04 ± 0.07 and 9.36 ± 0.03, so that any detrimental effects on algal growth could be attributed to the presence of unionised ammonia (NH_3) and should be discarded.

Overall, the effects of different initial phosphorus concentration and nitrogen sources on biomass growth were statistically significant (p = 0.011 and 0.000002, respectively, one-way ANOVA). The maximum biomass concentration achieved was 650 mg L^{-1} when nitrate was used as the nitrogen source, followed by the culture with a mixture of NH_4^+-N and NO_3^--N (520 mg L^{-1}). The culture with ammonia as the nitrogen source reported a maximum biomass concentration of 420 mg L^{-1}. Zhang et al. (1999) compared different nitrogen sources under heterotrophic conditions for *Chlamydomonas reinhardtii* and obtained higher biomass concentrations in culture media with nitrate (480 mg L^{-1}) than in the one with ammonium (440 mg L^{-1}). This could be attributed to the nitrate uptake metabolism. The nitrate assimilatory pathway involves a sequential reduction reaction from nitrate to nitrite, and from nitrite to ammonium with the high-

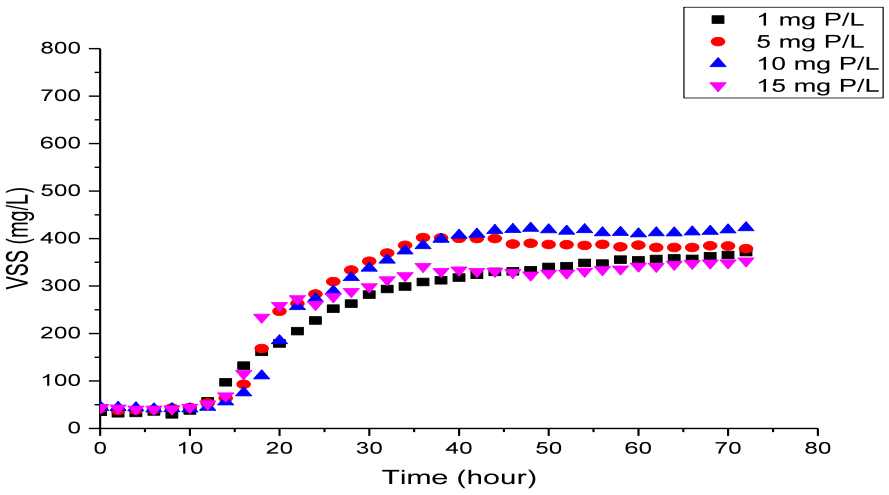
affinity nitrate and nitrite transporters encoded by a fourth member of the *Nrt2* gene family (Quesada et al., 1998; Navarro et al., 2000). These transporters depends on the carbon and nitrogen supply because their assimilatory metabolisms share organic carbon and energy (Navarro et al., 2000; Huppe and Turpin, 1994). Furthermore, adequate electron requirements to meet nitrate assimilation can be provided via respiration under dark conditions (Huppe and Turpin, 1994). Therefore, *Chlamydomonas reinhardtii* preferred nitrate to ammonia as the nitrogen source in the heterotrophic cultivation.

NH_4^+ and NO_3^-



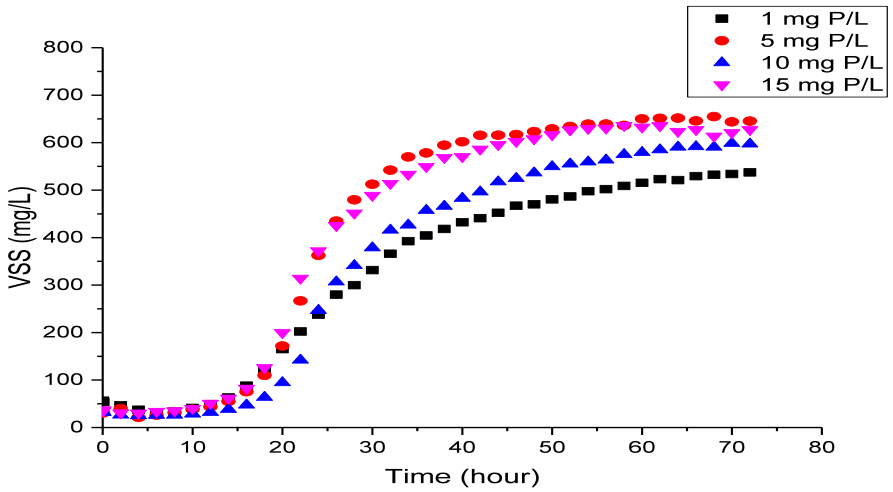
a

NH_4^+



b

NO_3^-



c

Figure 4-2 The variation in algal biomass concentrations during heterotrophic cultivation with P-limited concentrations using different nitrogen sources: a) a mix of NH_4^+-N and NO_3^--N (50:50); b) NH_4^+-N; and c) NO_3^--N.

4.3.2. The Effects of Different Nitrogen Sources on Heterotrophic Growth Kinetics of *Chlamydomonas reinhardtii* using Phosphorus as the Limiting Substrate

The specific growth rates of *Chlamydomonas reinhardtii* under heterotrophic conditions were determined using logarithmic regressions (plotting base ten logarithms of volatile suspended solids concentrations against time) for data collected from the exponential growth phase period (R^2 > 0.95 was achieved in all experiments, see Appendix D). Data was processed for nonlinear regression analysis (i.e., the Solver function in Microsoft Excel) using Equation 3-4 (Appendix E). Table 4-1 summarises the calculated heterotrophic growth kinetics under different initial phosphorus concentrations with different nitrogen sources.

Table 4-1 Growth kinetics of *Chlamydomonas reinhardtii* using different nitrogen sources under heterotrophic cultivation, using P as the limiting nutrient

Nitrogen Source	μ_{max} (d^{-1})	K_s (mg PL^{-1})
Mix of NH_4^+-N and NO_3^--N	1.77	0.17
NH_4^+-N	2.09	0.07
NO_3^--N	2.21	0.44

Specific growth rates of *Chlamydomonas reinhardtii* under heterotrophic conditions with different nitrogen sources and using phosphorus as the limiting substrate were significantly different (p = 0.044, one-way ANOVA). μ_{max} for ammonium was significantly higher than that found using a mix of ammonium and nitrate (p = 0.045, one-way ANOVA), while there was a slight increase between the ammonium and nitrate culture (p = 0.799, one-way ANOVA).

The greatest μ_{max} found was 2.21 d^{-1} when nitrate was used as the nitrogen source, followed by the ammonia culture (μ_{max} = 2.09 d^{-1}). The maximum specific growth rate in the mix of NH_4^+-N and NO_3^--N was the lowest (1.77 d^{-1}). Although similar results were attained in the study conducted by Zhang et al. (1999), which reported a higher specific growth rate of *Chlamydomonas reinhardtii* when using a nitrate culture media (μ_{max} = 1.488 d^{-1}) than when ammonium was used (μ_{max} = 1.392 d^{-1}), the difference between the maximum specific growth rates for nitrate and ammonium was greater in this study. Considering both the maximum algal biomass concentration and the maximum specific growth rate, nitrate is thus the favoured form of nitrogen source for heterotrophic cultivation of *Chlamydomonas reinhardtii.*

0.17, 0.07 and 0.44 mg P L^{-1} correspond to the half-saturation constants found when using a mix of NH_4^+-N and NO_3^--N, and NH_4^+-N and NO_3^--N, respectively.

4.3.3. The Effects of Different Initial Organic Carbon Concentrations on Microalgae Growth under Heterotrophic Conditions with Phosphorus as the Limiting Substrate

Chlamydomonas reinhardtii was cultivated under different initial organic carbon concentrations (0.5, 1.0 1.5 and 2.0 g org C L^{-1}, using acetate as carbon source) with 50 mg NH_4^+-N L^{-1} and variable P concentrations (limiting nutrient) in the absence of light to identify the influence of organic carbon concentration on the heterotrophic growth kinetics of microalgae. Although lower biomass concentrations and specific growth rates were obtained from the experiments with ammonium, it was chosen as the nitrogen source because it typically presents in the effluent of wastewater treatment works with no nitrogen control units.

Figure 4-3.a shows the results of the experiment using 2.0 g org C L^{-1} for various phosphorus concentrations when ammonium was used as the nitrogen source. The final biomass concentration of *Chlamydomonas reinhardtii* was slightly increased, from 370 to 420 mg L^{-1} VSS, with the increase in initial phosphorus concentration from 1 to 10 mg L^{-1} but there was also a small decrease in the final biomass concentration (360 mg L^{-1} VSS) at 15 mg P L^{-1} of initial phosphorus concentration (*p* values were greater than 0.05 for all tests). With regard to biomass productivity, a significant increase was observed from 377 to 580 mg L^{-1} VSS d^{-1} when the P concentration increased from 1 to 5 mg P L^{-1}. With a further increase in the initial phosphorus concentration, the resulting biomass productivity did not present any further significant changes.

Algal biomass growth with different initial P concentrations at 1.5 g org C L^{-1} is depicted in Figure 4-3.b. The highest final biomass concentration was 510 mg VSS L^{-1} at 1 mg L^{-1} initial P concentration. The maximum biomass concentration decreased to 300 mg VSS L^{-1} at 5 mg L^{-1} initial P concentration ($p = 0.261$ for 1 and 5 mg L^{-1} initial P concentrations, one-way ANOVA); while maximum algal biomass concentration increased to 465 mg L^{-1} at 10 mg L^{-1} initial P concentration ($p = 0.648$ for 5 and 10 mg L^{-1} initial P concentrations, one-way ANOVA). With regard to biomass productivity, there was a gradually increment from 488 to 652 mg VSS L^{-1} d^{-1} with incremental increases in initial P concentration from 1 to 10 mg L^{-1}. The lowest algae concentration (200 mg VSS L^{-1}) – which is statistically different from the ones obtained at lower initial phosphorus concentrations ($p < 0.05$ for 1 and 15 mg P L^{-1}, 5 and 15 mg P L^{-1} and, 10 and 15 mg P

L^{-1}, one-way ANOVA) – and biomass productivity (352 mg VSS L^{-1} d^{-1}) were found at 15 mg P L^{-1} concentration.

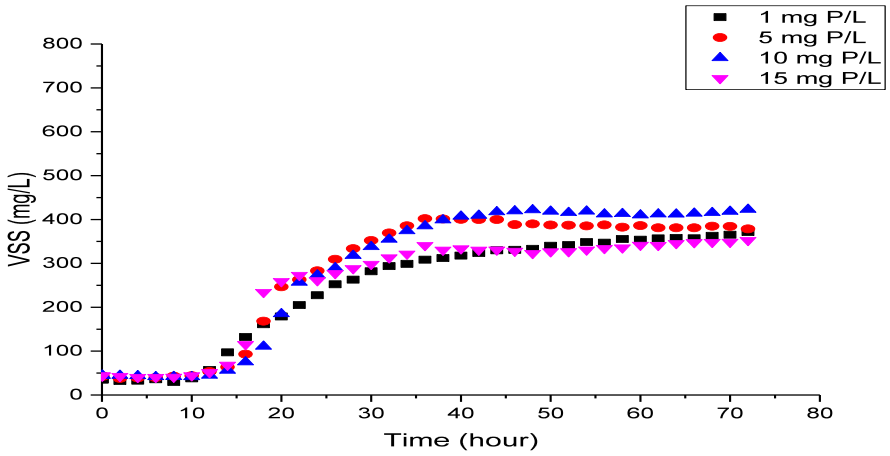
Figure 4-3.c shows the growth curves for *Chlamydomonas reinhardtii* in the experiment using different initial P concentrations and 1.0 g org C L^{-1} under conditions of heterotrophic cultivation. There was no apparent statistical difference in biomass concentration between the tests using 1, 5 and 10 mg L^{-1} initial phosphorus concentrations ($p > 0.05$ for 1 and 5 mg P L^{-1}, 1 and 10 mg P L^{-1} and, 5 and 10 mg P L^{-1}, one-way ANOVA). At 15 mg P L^{-1}, a significant decrease in algal biomass was observed (250 mg VSS L^{-1}) when compared with the other P concentrations tested with an average maximum biomass of 430 mg VSS L^{-1} ($p = 0.011$, 0.029 and 0.036 for 15 and 1, 5 and 10 mg P L^{-1}, respectively, one-way ANOVA). Regarding biomass productivity, there was a slight increase from 591 to 673 mg VSS L^{-1} d^{-1} between 1 and 5 mg P L^{-1}. At higher P concentrations, biomass productivity declined gradually from 518 mg VSS L^{-1} d^{-1} at 10 mg P L^{-1}, to 328 mg VSS L^{-1} d^{-1} at 15 mg P L^{-1}.

Figure 4-3.d shows the effects of initial P concentration using 500 mg org C L^{-1} on microalgae growth of *Chlamydomonas reinhardtii* along with heterotrophic cultivation period. The biomass growth trend was significantly different than those with different organic C carbon concentrations ($p < 0.05$, one-way ANOVA). It is worth noting that a death (declined) phase was observed under these cultivation conditions. Algal biomass concentration decreased sharply right after a short stationary phase. The increase in VSS concentration towards the end of the experiment could have been attributed to the fact that dead algae cells may be resuspended back into the culture due to their poor sedimentation capacity. The highest biomass concentration achieved at 1 mg P L^{-1} was 340 mg VSS L^{-1} at the end of the exponential phase, corresponding to 547 mg VSS L^{-1} d^{-1} biomass productivity. Although similar algal biomass concentrations were obtained (about 250 mg L^{-1} VSS) at higher initial P concentrations (*p* values were greater than 0.05 for 1 mg P L^{-1} and the other initial P concentrations, one-way ANOVA), biomass productivity steadily increased from 672 to 761 mg VSS L^{-1} d^{-1}.

The results demonstrate that the heterotrophic growth of *Chlamydomonas reinhardtii* concentration was significantly influenced by initial phosphorus and organic carbon concentrations ($p = 0.0026$ and $p < 0.05$, respectively, one-way ANOVA). Growing heterotrophic microalgae in higher organic carbon concentration is one of the vital requirements to attain higher biomass concentrations. Although Chen and Johns

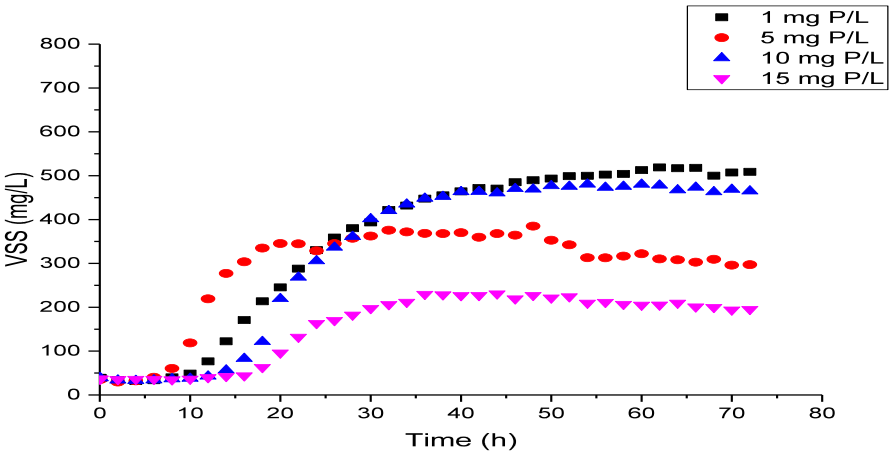
(1994) reported that at acetate concentrations greater than 0.4 g L^{-1} an inhibitory effect was found on the heterotrophic cultivation of *Chlamydomonas reinhardtii*, in this research, it was able to grow even when higher initial organic carbon concentrations were tested under heterotrophic conditions. This observation is also supported by the study published by Turon et al. (2014) using two different microalgae species (*Chlorella sorokiniana* and *Auxenochlorella protothecoides*) by using fermentative end-products (i.e., acetate, butyrate and lactate); they found that greater acetate concentrations did not inhibit microalgae growth and both species could grow under all acetate concentrations tested within a range between 0.1 and 1.0 g L^{-1} C.

2.0 g org C L^{-1}



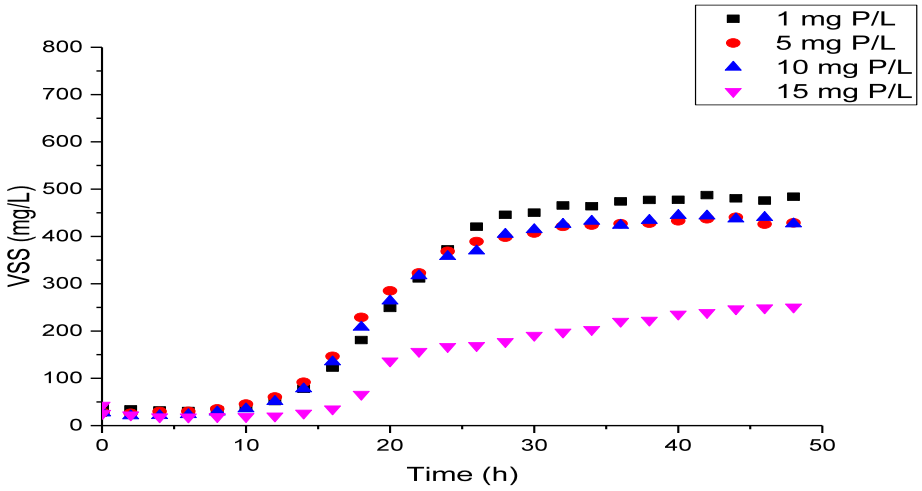
a

1.5 g org C L^{-1}



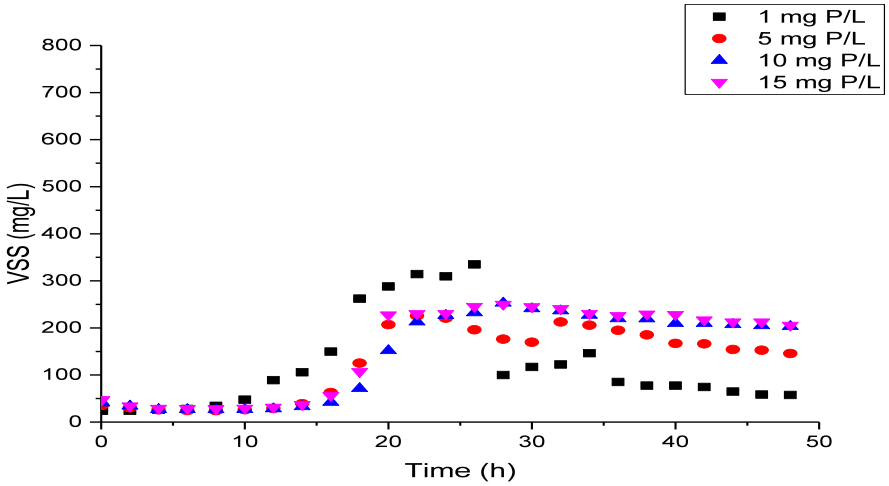
b

1.0 g org C L^{-1}



c

500 mg org C L^{-1}



d

Figure 4-3 Changes in algal biomass concentrations during heterotrophic cultivation with P-limited concentrations using different organic carbon concentrations: a) 2.0 g org C L^{-1}; b) 1.5 g org C L^{-1}; c) 1.0 g org C L^{-1}; and d) 500 mg org C L^{-1}

4.3.4. The Effects of Different Initial Organic Carbon Concentrations on Heterotrophic Growth Kinetics of *Chlamydomonas reinhardtii* with Phosphorus as the Limiting Substrate

The Monod model was used to identify the influence of different initial organic carbon concentrations on heterotrophic microalgae growth kinetics when using phosphorus as a limiting substrate. Specific growth rates of *Chlamydomonas reinhardtii* under heterotrophic conditions were determined using logarithmic regressions (plotting base ten logarithms of volatile suspended solids concentrations against time) for data collected from the exponential growth phase period (R^2 > 0.95 was achieved in all experiments, see Appendix D). Data were processed using nonlinear regression analysis with the Solver function in Microsoft Excel and Equation 3-4 (Appendix E). Table 4-2 lists the resulting data from the growth kinetics analysis using different organic carbon concentrations under heterotrophic conditions.

Table 4-2 Growth kinetics of *Chlamydomonas reinhardtii* under heterotrophic conditions at different initial organic carbon concentrations with P as the limiting substrate

Organic Carbon Concentration (g C L^{-1})	μ_{max} (d^{-1})	K_s (mg P L^{-1})
2.0	2.09	0.070
1.5	2.25	0.004
1.0	2.33	0.120
0.5	3.23	0.570

Table 4-2 demonstrates that there is a very strong negative correlation between the maximum specific growth rates (μ_{max}) and organic carbon concentration (Pearson correlation coefficient was -0.881, R^2 = 0.78), meaning that μ_{max} increased gradually from 2.09 to 3.23 d^{-1} by decreasing the organic carbon concentration from 2.0 to 0.5 g org C L^{-1}. The decrease in specific growth rate with increasing organic carbon concentration could be attributed to the fact that acetate assimilation could be saturated by adding it in excess (Bekirogullari et al., 2017). This situation also proved that higher organic carbon concentrations influenced the heterotrophic growth of *Chlamydomonas reinhardtii* (Chen and Johns, 1994).

The half-saturation constants (K_s) were found to be 0.07, 0.04, 0.12 and 0.57 mg P L^{-1} at 2.0, 1.5, 1.0 and 0.5 g L^{-1} organic carbon, respectively.

4.3.5. The Effects of Different Nitrogen Sources on Microalgae Concentration at Phototrophic Cultivation using Phosphorus as the Limiting Substrate

Chlamydomonas reinhardtii was cultivated in synthetic wastewater (SWW) with a mix of NH_4^+-N and NO_3^--N (50:50), NH_4^+-N and NO_3^--N with P as the limiting nutrient under conditions of continuous illumination (Figure 4-4). The variation in dry weight concentrations of microalgal biomass growing with a mix of NH_4^+-N and NO_3^--N is presented in Figure 4-4.a. It can be clearly seen that the growth trends are similar for all different initial phosphorus concentrations (p > 0.05 for all experiments, one-way ANOVA). A steady increment of algal biomass was observed with a maximum concentration at the end of the cultivation period ranging between 490 and 750 mg VSS L^{-1}, which corresponded to changes in initial P concentrations from 1 to 10 mg P L^{-1}, with biomass productivity determined to be 104 to 155 mg VSS L^{-1} d^{-1}. At 15 mg L^{-1} initial P concentration, there was a small decrease in biomass concentration and biomass productivity to 680 mg VSS L^{-1} and 132 mg VSS L^{-1} d^{-1}, respectively (p > 0.05 for all experiments, one-way ANOVA).

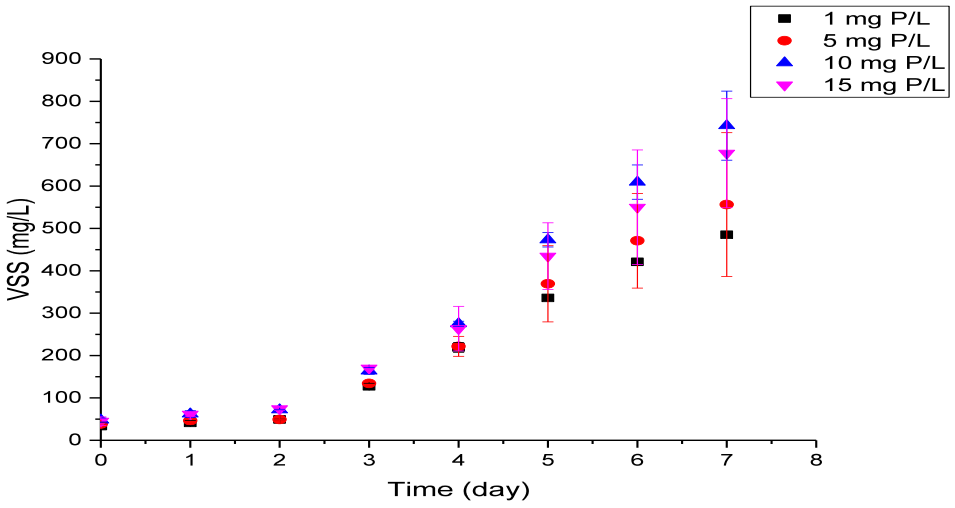
Figure 4-4.b depicts the variation in algal biomass concentration of *Chlamydomonas reinhardtii* during phototrophic cultivation using NH_4^+-N and different initial P concentrations. A similar trend was observed in growth curves from tests using 1 and 10 mg P L^{-1} as previously found when using a mix of nitrogen sources (Figure 4-4.a) (p = 0.870 for the mixture of nitrogen sources and ammonium, one-way ANOVA), with final biomass concentrations in the range of 430-550 mg VSS L^{-1}, which was consistent with the increment in biomass productivity from 118 to 124 mg VSS L^{-1} d^{-1}. The only difference between using ammonium and a mix of ammonium and nitrate as the nitrogen source was observed at 15 mg L^{-1} initial P concentration. Biomass concentration remained at similar values (560 mg VSS L^{-1}); however, biomass productivity decreased to 119 mg VSS L^{-1} d^{-1}. Biomass concentrations and biomass productivities were not significantly affected by different initial phosphorus concentrations (p values for biomass concentration and biomass productivities in all experiments were greater than 0.05, one-way ANOVA).

Algal biomass concentrations fluctuated depending on the initial phosphorus concentrations when nitrate was used as the nitrogen source (Figure 4-4.c), which was

statistically different from those using at a mixture of nitrogen sources and ammonium (p = 0.001 for nitrate and p = 0.005 for the mixture of nitrogen sources and ammonium, one-way ANOVA). The dry weight concentration of *Chlamydomonas reinhardtii* increased from 170 to 290 mg VSS L^{-1} at the end of the exponential growth phase with 1 to 5 mg P L^{-1}, which corresponded to the biomass productivity varying from 40 to 71 mg VSS L^{-1} d^{-1}. Algae biomass concentration and biomass productivity decreased slightly to 200 mg VSS L^{-1} and 65 mg VSS L^{-1} d^{-1}, respectively, at 10 mg P L^{-1}. The final dry weight concentration and biomass productivity rate increased nearly twofold at 15 mg P L^{-1} to 360 mg VSS L^{-1} and 119 mg VSS $L^{-1}d^{-1}$, respectively. Biomass productivity at 15 mg P L^{-1} was significantly different from the other initial phosphorus concentrations tested (p = 0.007, 0.038 and 0.027 for 1 and 15 mg P L^{-1}, 5 and 15 mg P L^{-1}, and 10 and 15 mg P L^{-1}, one-way ANOVA).

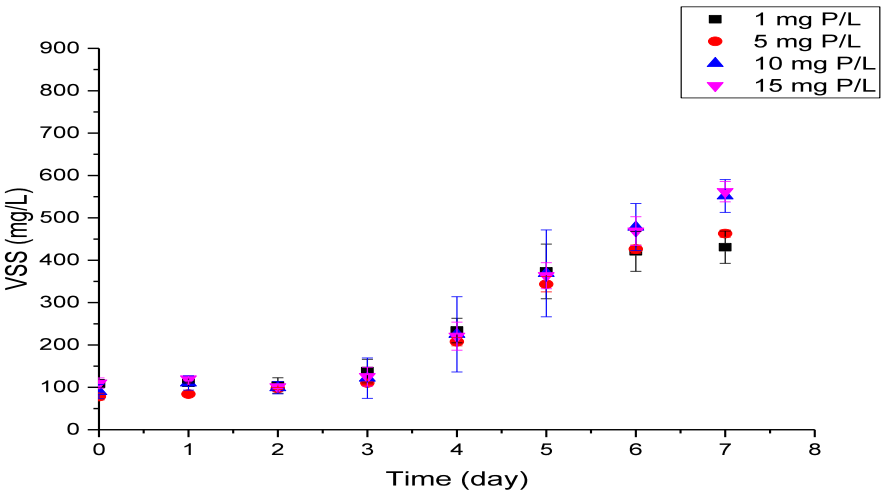
The effects of different nitrogen sources on *Chlamydomonas reinhardtii* growth were significant (p = 0.001, one-way ANOVA), whereas different initial phosphorus concentrations did not have a strong influence on biomass concentration (p = 0.417, one-way ANOVA). The highest biomass concentration was achieved when a mix of ammonium and nitrate was used as the nitrogen source (750 mg VSS L^{-1}), followed by the media with ammonium (560 mg VSS L^{-1}). The biomass concentration in the nitrate culture was the lowest as 360 mg VSS L^{-1}. Yulistyorini (2016) compared the effect of inorganic nitrogen sources (ammonium and nitrate as a mix and independently) on the phototrophic growth of *Chlamydomonas reinhardtii*, finding that the maximum cell concentration was greater when microalgae were cultivated with a mix of ammonium and nitrate in the culture media than the ones attained from the ammonium and nitrate cultures. Therefore, the mixture of ammonia and nitrate was preferred rather than solely ammonium and nitrate by *Chlamdomonas reinhardtii* under phototrophic conditions in this study due to the fact that they can consume either ammonium or nitrate as the nitrogen source (Harris, 2008).

NH_4^+ and NO_3^-



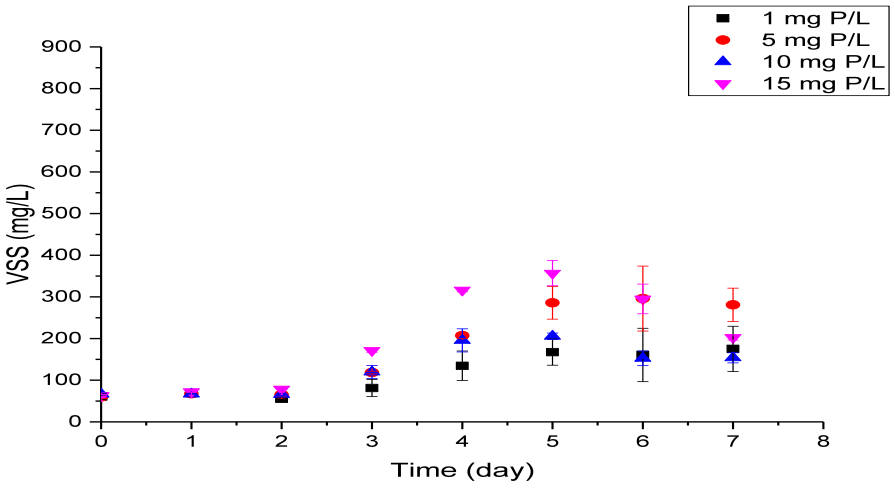
a

NH_4^+



b

NO_3^-



c

Figure 4-4 The variation in algal biomass concentrations during phototrophic cultivation with P-limited concentrations using different nitrogen sources: a) a mix of NH_4^+-N and NO_3^--N; b) NH_4^+-N; and c) NO_3^--N (These experiments were conducted in duplicate).

4.3.6. The Effects of Different Nitrogen Sources on Phototrophic Growth Kinetics of *Chlamydomonas reinhardtii* using Phosphorus as the Limiting Substrate

Specific growth rates for *Chlamydomonas reinhardtii* under phototrophic conditions were calculated from processed data using logarithmic regressions. Laboratory data was plotted in the form of base ten the logarithm ($\log_{10}$) of dry weight biomass concentrations collected during the exponential growth phase against time (R^2 > 0.95 was achieved in all experiments, see Appendix D). Phototrophic microalgae growth kinetics was calculated using a nonlinear regression analysis using the Solver function in Microsoft Excel and using Equation 3-4 (Appendix E). Monod's model was found to be a better fit for the determination of phototrophic microalgae growth kinetics in this study.

Table 4-3 presents the consolidated information regarding phototrophic microalgae growth kinetics for the experiments using different nitrogen sources. μ_{max} was found to be 0.22 and 0.24 d^{-1} for the experiments using a mix of NH_4^+-N and NO_3^--N and solely NH_4^+-N, respectively. μ_{max} increased slightly to 0.28 d^{-1} when NO_3^--N was used as the nitrogen source. The effect of different nitrogen sources on the specific growth rate was not significant (p = 0.239 between groups, one-way ANOVA).

Table 4-3 Growth kinetics of *Chlamydomonas reinhardtii* for phototrophic microalgae growth using different nitrogen sources with P as the limiting substrate

Nitrogen Source	μ_{max} (d^{-1})	K_s (mg L^{-1})
Mix of NH_4^+-N and NO_3^--N	0.22	0.04
NH_4^+-N	0.24	0.12
NO_3^--N	0.28	0.46

The availability of a nitrogen source was dependent on the photoperiod (Mezzari et al., 2013), and therefore the photoperiod influences phototrophic microalgae growth. Specific growth rates declined by 12% with a decrease in light supply by *Chlamydomonas reinhardtii* (Janssen et al., 2000). This was further confirmed by Tamburic et al. (2012), who found an increase in the specific growth rate of *Chlamydomonas reinhardtii* from 0.142 to 0.161 h^{-1} with increasing photoperiod from 12 h light: 12 h dark to continuous illumination. Thus, the increment in photoperiod to continuous illumination suggests considerable potential to achieve higher specific growth rates with phototrophic microalgae cultures.

The lowest half-saturation constant (K_s) was 0.04 mg P L^{-1} when a mix of NH_4^+-N and NO_3^--N was used as the nitrogen source, followed by the ammonia culture (0.12 mg P L^{-1}); the highest K_s (0.46 mg P L^{-1}) was achieved with nitrate.

4.3.7. The Effects of Different Initial Inorganic Carbon Concentrations on Microalgae Growth at Phototrophic Cultivation using Phosphorus as the Limiting Substrate

Chlamydomonas reinhardtii 11/32C was inoculated into flasks placed in a shaking incubator with initial inorganic carbon concentrations (from bicarbonate) of 0.5, 1.0, 1.5 and 2.0 g C L^{-1} at 50 mg NH_4^+-N L^{-1}, under P-limited conditions in the presence of light (24 h photoperiod), in order to identify the influence of phosphorus on phototrophic microalgae growth at different inorganic carbon concentrations (Figure 4-5).

Figure 4-5.a depicts the variation in algal biomass concentration by *Chlamydomonas reinhardtii* during phototrophic cultivation using 2.0 g L^{-1} initial inorganic C concentration. The final biomass concentration of *Chlamydomonas reinhardtii* increased from 430 to 550 mg VSS L^{-1} with an increment in initial phosphorus concentration from

1 to 10 mg P L^{-1}, which corresponded to an increase in biomass productivity from 118 to 124 mg VSS L^{-1} d^{-1}. Biomass concentration remained at similar values (560 mg VSS L^{-1}) at 15 m P L^{-1} initial phosphorus concentration, whereas biomass productivity decreased to 119 mg VSS L^{-1} d^{-1}. Biomass concentrations and biomass productivities were not significantly affected by different initial phosphorus concentrations (*p* values for biomass concentration and biomass productivities in all experiments were greater than 0.05, one-way ANOVA).

Figure 4-5.b presents the growth pattern of *Chlamydomonas reinhardtii* during phototrophic cultivation at 1.5 g L^{-1} inorganic C concentration. Algal biomass concentrations increased from 420 to 600 mg VSS L^{-1} with the corresponding increment in initial P concentrations from 1 to 10 mg L^{-1} and were consistent with the rise of biomass productivity from 87 to 150 mg VSS L^{-1} d^{-1}. There was a slight decrease in biomass concentration and biomass productivity to 540 mg VSS L^{-1} and 134 mg VSS L^{-1} d^{-1}, respectively, at 15 mg L^{-1} initial P concentration. The changes in biomass concentrations and biomass productivities, as depending on different initial phosphorus concentrations, were not significant (*p* values were greater than 0.05 for all tests, one-way ANOVA).

Dry weight concentrations of *Chlamydomonas reinhardtii* with various initial P concentrations at 1.0 g L^{-1} inorganic C concentration under phototrophic conditions are presented in Figure 4-5.c. Final biomass concentration increased from 310 to 400 mg VSS L^{-1} between 1 and 5 mg P L^{-1}, and a further increment in initial P concentration influenced, to some extent, the dry weight concentrations at the end of the growth period. Biomass productivity shows the same trend as biomass concentration, where there was a slight increase from 69 to 76 mg VSS L^{-1} d^{-1} with the increase in initial P concentration from 1 to 5 mg L^{-1}; further increases in initial phosphorus concentration did not translate into significant changes in biomass concentration or biomass productivity ($p > 0.05$, one-way ANOVA).

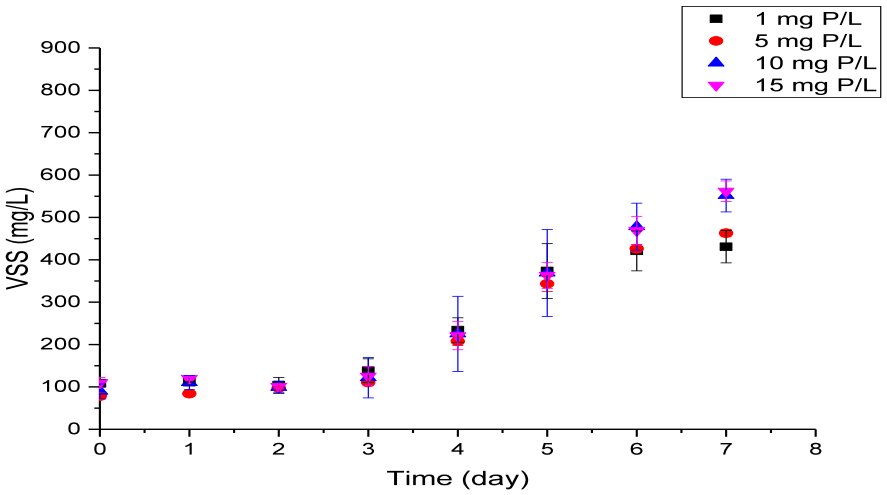
Figure 4-5.d shows the effects of different initial P concentrations on microalgae growth of *Chlamydomonas reinhardtii* at 500 mg L^{-1} inorganic C concentration under phototrophic cultivation conditions. The lowest final biomass concentration was found to be 200 mg VSS L^{-1} at 1 mg P L^{-1}, corresponding to 54 mg VSS L^{-1} d^{-1} biomass productivity. There was an approximately twofold increment in algae concentration to 380 mg VSS L^{-1}, consistent with the increase in biomass productivity to 61 mg VSS L^{-1} d^{-1}, at 5 mg L^{-1} initial phosphorus concentration. Biomass concentration and biomass

productivity decreased with increments in initial P concentration. The dry weight concentration of microalgae declined gradually to 350 and 300 mg VSS L^{-1} at 10 and 15 mg L^{-1} initial P concentration, respectively, corresponding to a constant fall in biomass productivity to 48 and 40 mg VSS L^{-1} d^{-1}. Overall, the growth trends at different initial phosphorus concentrations were similar (*p* values were greater than 0.05 for all tests, one-way ANOVA). As regards biomass productivity, this was significantly affected by initial phosphorus concentration ($p < 0.05$), except for 1 and 10 mg L^{-1} initial P concentrations ($p = 0.124$, one-way ANOVA).

From the experiments into the effects of different initial inorganic carbon and phosphorus concentrations on biomass concentrations, it was observed that although biomass concentration was significantly influenced by initial organic concentration ($p < 0.05$, one-way ANOVA), the initial phosphorus concentration did not have any particular influence on microalgae growth from a statistical perspective ($p = 0.704$, one-way ANOVA). From figure 4-5, it can be seen that higher biomass concentrations were obtained at higher initial inorganic carbon concentrations. This situation could be attributed to the increase in the rate of inorganic carbon uptake with increasing inorganic carbon concentration (Kasiri et al., 2015a). However, a further increase in inorganic carbon concentration could limit algal growth. Inorganic carbon uptake is associated with an increase in pH because H^+ is utilised while OH^- is released by photosynthetic metabolism (Eze et al., 2018). Furthermore, environmental parameters (i.e., light intensity, photoperiod and temperature) influence inorganic carbon uptake (Novak and Brune, 1985). Inorganic carbon uptake could be increased with increasing photoperiod up to continuous illumination and temperature (Jacob-Lopes et al., 2009; Hsueh et al., 2009).

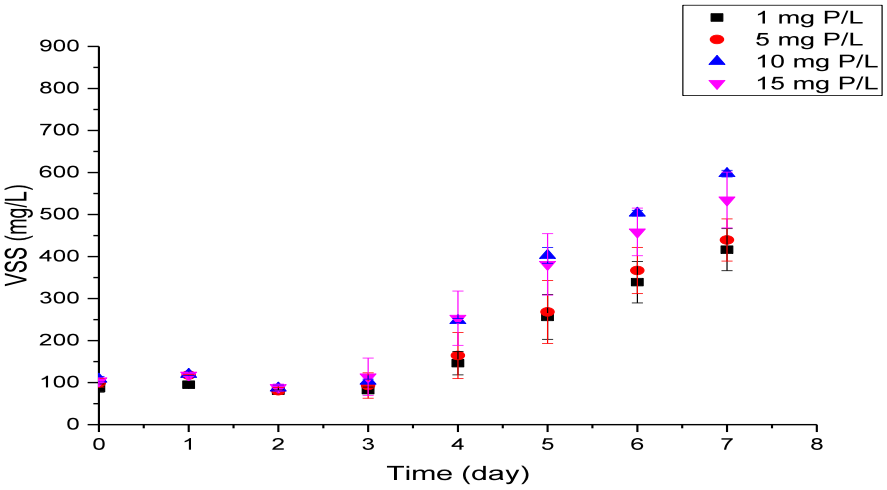
Overall, *Chlamydomonas reinhardtii* presented a similar trend for carbon uptake by both the heterotrophic and phototrophic forms of metabolism. The increase in initial carbon concentration increases algal biomass concentrations and biomass productivities.

2.0 g inorg C L^{-1}



a

1.5 g inorg C L^{-1}



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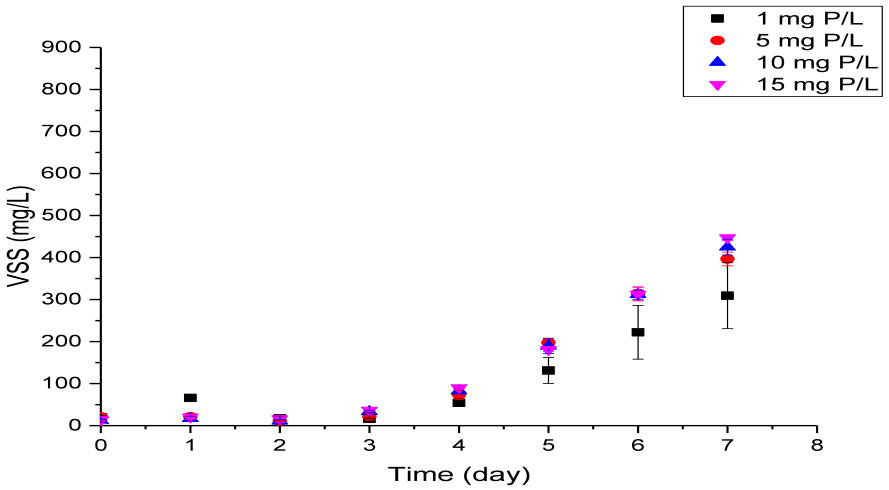
1.0 g inorg C L^{-1}



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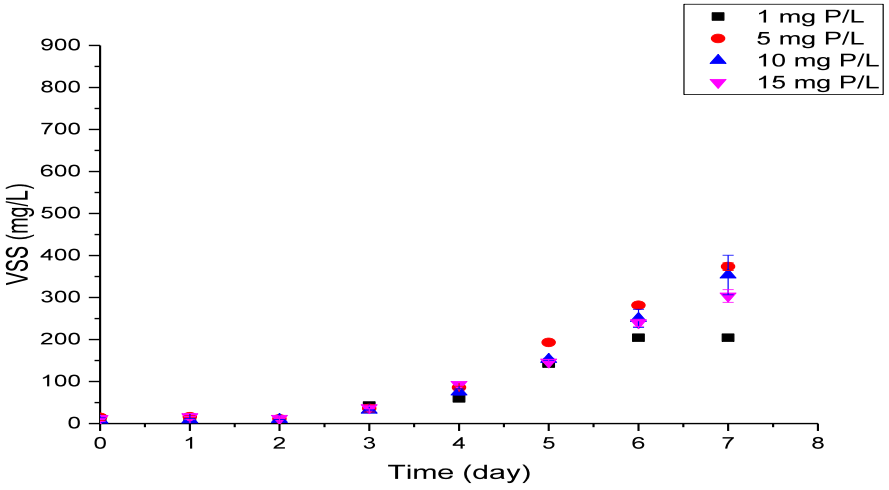
500 mg inorg C L^{-1}



d

Figure 4-5 Changes in algal biomass concentrations during phototrophic cultivation with P-limited concentrations and various initial inorganic carbon concentrations: a) 2.0 g C L^{-1}; b) 1.5 g C L^{-1}; c) 1.0 g C L^{-1}; and d) 500 mg C L^{-1} (These experiments were performed in duplicate).

4.3.8. The Effects of Different Initial Inorganic Carbon Concentrations on Phototrophic Growth Kinetics of *Chlamydomonas reinhardtii* using Phosphorus as the Limiting Substrate

Specific growth rates for *Chlamydomonas reinhardtii* under phototrophic conditions were calculated from processed data using logarithmic regressions. Laboratory data was plotted using the graphs of the base ten logarithms ($\log_{10}$) of dry weight biomass concentrations collected during the exponential growth phase against time ($R^2 > 0.95$ was achieved in all experiments, see Appendix D). Nonlinear regression analysis (Solver Function in Microsoft Excel) and Equation 3-4 were used to estimate the growth kinetics of *Chlamydomonas reinhardtii* under phototrophic conditions (Appendix E). Monod's model presented a better fit due to the fact that phosphorus did not show any inhibitory effect on microalgae growth under phototrophic conditions. The estimated growth kinetics at different initial inorganic carbon concentrations under phototrophic conditions are presented in Table 4-4.

Table 4-4 Growth kinetics of *Chlamydomonas reinhardtii* under phototrophic conditions at different initial inorganic carbon concentrations with P as the limiting substrate

Inorganic Carbon Concentration (g C L^{-1})	μ_{max} (d^{-1})	K_s (mg L^{-1})
2.0	0.24	0.12
1.5	0.27	0.10
1.0	0.37	0.01
0.5	0.46	0.87

Table 4-4 summarises a consolidated list of parameters describing the kinetics of phototrophic microalgae growth at different initial inorganic carbon concentrations. The maximum specific growth rate increased from 0.24 to 0.46 d^{-1} with the decrease in initial inorganic carbon concentration from 2.0 to 0.5 g inorganic C L^{-1}. This was consistent with the very strong negative correlation between inorganic carbon concentration and the maximum specific growth rate (Pearson correlation coefficient -0.98 and $R^2 = 0.96$). This could be related to the influence of pH on growth kinetics due to the fact that pH differs depending on alkalinity (Novak and Brune, 1985).

The half-saturation constants (K_s) were found to be 0.12, 0.10, 0.01 and 0.87 mg P L^{-1} at 2.0, 1.5, 1.0 and 0.5 g L^{-1} inorganic carbon, respectively. Half saturation constants were found to be very low under phototrophic conditions, which could be a result of the low phosphorus concentrations used as substrates (Kunikane and Kaneki, 1984).

4.3.9. Nutrient Recovery

The purpose of this chapter is to identify the growth kinetics of *Chlamydomonas reinhardtii* and nutrient recovery was analysed using a continuous flow system, as described in more detail in Chapter 5; however, nutrient concentrations were controlled at the beginning and end of the experiments. Table 4-5 reports phosphorus and nitrogen removal under heterotrophic and phototrophic conditions.

Table 4-5 Nutrient concentration at the beginning (t = 0) and the end of the cultivation period (t = 3 and 7 for heterotrophic and phototrophic growth, respectively)

Nutrient	Initial concentration (mg L^{-1})	Final concentration (mg L^{-1})	Recovery efficiency(%)
Phosphorus	1	ND*	100
	5	ND*	100
	10	5	50
	15	10	33
Ammonium	25	ND*	100
	50	ND*	100
Nitrate	25	ND*	100
	50	ND*	100

*ND : non-detectable

Chlamydomonas reinhardtii presented the same trends for phosphorus and nitrogen removal for all experiments regardless of cultivation conditions. At 1 and 5 mg L^{-1} initial phosphorus concentrations, *Chlamydomonas reinhardtii* utilised phosphorus in the culture completely. With a further increase in initial phosphorus concentration to 15 mg L^{-1}, the amount of phosphorus consumption by *Chlamydomonas reinhardtii* did not change (only 5 mg P L^{-1} was removed). As regards nitrogen removal, either ammonia or nitrate in the culture was fully removed by *Chlamydomonas reinhardtii.* This could be attributed to the molecular formula of microalgae cells as $C_{106}H_{118}O_{45}N_{16}P$ (Redfield, 1934). Therefore, microalgae cells generally contain approximately 1% and 10% of phosphorus and nitrogen, respectively (Powell, 2009; Camargo-Valero et al., 2009b), and

the optimum N/P ratio for microalgae growth is recommended to be between 6:1 and 10:1 (Wang et al., 2010). Nutrient removal obtained in this study is also in good agreement with the other published literature that used other microalgae strains such as *Chlorella vulgaris* (Kim et al., 2013a) and *Scenedesmus quadricauda* (Han et al., 2014).

4.3.10. Microbial Contamination

Growth kinetic studies were perfomed using pure single species under strictly controlled environmental conditions. Therefore, more precise data can be generated. However, being vulnerable to microbial contamination, particularly in open ponds, and due to the competitive and symbiotic relationship with other microorganisms, the applicability of growth kinetic models in real-world scenarios could be limited.

Chlamydomonas reinhardtii and culture-borne bacteria could grow (Figure 4-6), particularly when using heterotrophic cultivation. Previous research revealed that co-cultivating microalgae and bacteria results in each promoting the other's growth and increased nutrient removal efficiencies compared to single algal or bacterial cultures (Liang et al., 2013; Ma et al., 2014; de-Bashan et al., 2002). In spite of the mutual compound relationship between microalgae and bacteria, *Chlamydomonas reinhardtii* was the main species in the culture, which was likely due to two possible reasons: (i) algae can compete with bacteria for nutrients or produce substance-limiting bacterial growth; and (ii) the bacteria count can be reduced by higher algal inoculum concentrations (Ma et al., 2014). Moreover, the risk of bacterial contamination can be controlled or even avoided with pre-treatment methods such as autoclaving, adding antibiotics, filtration and/or pH adjustment (Ramsundar et al., 2017).

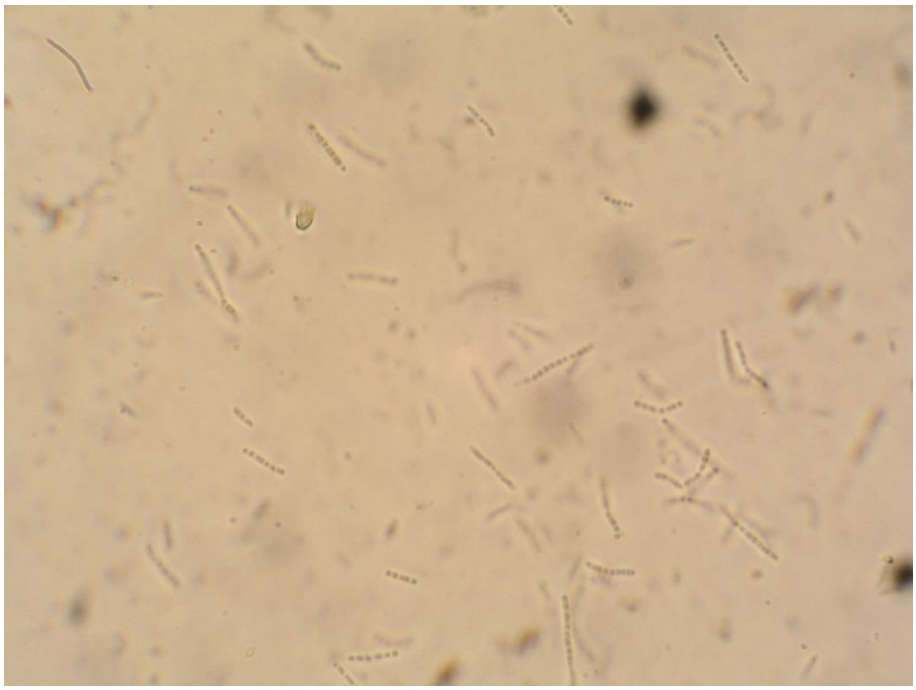


Figure 4-6 Microscopy photograph of the heterotrophic cultivation

It is important to highlight that it is sometimes challenging to make direct comparisons between growth kinetics conducted by different reserchers due to differences in microalgae strain and cultivation conditions. Considering the real-world scenario with the presence of microalgae consortium and other wastewater borne-microorganisms, *Chlamydomonas reinhardtii* can compete with other microorganisms and grow efficiently with a higher specific growth rate than found in the other previously published literature, particularly when using heterotrophic cultivation. Murwanashyaka et al. (2016) investigated algal growth characteristics under heterotrophic conditions by cultivating *Chlorella sorokiniana* in a modified bold 3N medium with 10 g/L glucose, finding the highest specific growth rate to be 1.4 d^{-1} at 26.8 mg L^{-1} initial P concentration. The effect of pH on the growth of *Chlorella vulgaris* in BG-11 medium under conditions of sulphur deprivation and in the absence of light was assessed by Sakarika and Kornaros (2016), and where the maximum specific growth rates were found to vary from 0.423 to 0.563 d^{-1} between pH 5.0-8.0. Kim et al. (2013b) compared the growth kinetics of *Chlorella sorokiniana* under different cultivation conditions and concluded that the highest specific growth rate achieved to be 0.53 d^{-1} using heterotrophic cultivation, followed by mixotrophic conditions of 0.44 d^{-1} and where the specific growth rate of phototrophic metabolism was the lowest as 0.24 d^{-1}. The effect of different nitrogen sources on heterotrophic growth of *Chlorella protothecoides* has also been investigated (Shi et al.,

2000) and revealed that the specific growth rates in the nitrate and urea cultures with a range of nitrogen concentrations between 0.06-0.12M were around 1.2 d^{-1}, whereas the specific growth rates in the ammonium culture were not detected due to the short period of the exponential growth phase.

With respect to phototrophic growth kinetics of *Chlamydomonas reinhardtii*, these were in a good agreement with those in previous publications that considered other strains such as *Chlorella vulgaris, Scenedesmus obliquus* etc. More or less identical specific growth rates of *Chlamydomonas reinhardtii* were obtained as 0.24 d^{-1} for *Chlorella sorokiniana, Chlorella vulgaris* and *Chlorella zofingiensis* (Kim et al., 2013b; Tam and Wong, 1996; Liu et al., 2011).

4.4. Conclusion

It is important to note that *Chlamydomonas reinhardtii* presented an extreme capacity to adapt and grow under all experimental conditions for both heterotrophic and phototrophic cultivation with different nitrogen and carbon sources for a wide range of concentrations that typify those found in wastewater treatment works. Higher specific growth rates and biomass productivity rates were attained under heterotrophic conditions than those at phototrophic cultivation. Nitrate was the preferred form of nitrogen for heterotrophic cultivation whereas no significant influence from any particular nitrogen source was found under phototrophic conditions.

From the perspective of implementing heterotrophic and phototrophic microalgae cultivation in current wastewater treatment works, *Chlamydomonas reinhardtii* could be cultivated in the effluent of activated sludge process, with extended aeration for nitrification, meaning that a bacterial process (activated sludge) can be used for organic carbon stabilisation, and the presence of nitrate and lower organic and inorganic carbon can be used to support both heterotrophic and phototrophic growth of *Chlamydomonas Reinhardti* for simultaneous nutrient control and recovery.

5. CHAPTER: OPERATIONAL CONDITIONS AND PERFORMANCE OF A TWO-STAGE BIOLOGICAL PROCESS

5.1. Introduction

Microalgae are considered as a promising solution for nutrient control and recovery at wastewater treatment works due to their capacity to uptake nutrients and high algal biomass production with the additional benefit of enhanced biofuel production (Hu et al., 2017). Although microalgae are phototrophic species that utilise inorganic carbon and sunlight as carbon and energy sources, they are also able to perform mixotrophic metabolism using organic carbon in the presence of light, or heterotrophic metabolism utilising organic carbon in dark conditions (Delgadillo-Mirquez et al., 2016). Despite the fact that heterotrophic microalgae growth overcomes the requirements for light to support phototrophic cultivation (Perez-Garcia et al., 2011b), it also has inherent disadvantages such as higher CO_2 emissions, the risk of bacterial and fungal contamination, and the need for an organic carbon source and a terminal electron acceptor (oxygen) (Lowrey et al., 2014).

Batch cultures have been used for the cultivation of algal biomass on the industrial scale; however, they have some limitations such as low biomass productivity, high harvesting costs and uncertain product quality (Fernandes et al., 2015). In the last few decades, continuous flow systems have caught the attention of the industry due to their ability to maintain growth rates at close to maximum values, reduce harvesting costs and stabilise the characteristics of any final products (Fernandes et al., 2015).

In the current literature, there is a limited number of research works investigating the use of continuous flow systems for the cultivation of algal biomass with the simultaneous benefit of nutrient control and recovery via microalgae uptake from wastewaters. In order to fill this gap, the work reported herein is aimed at identifying the optimum operational conditions for a two-stage biological process combining phototrophic and heterotrophic microalgae cultivation, and biomass recirculation under continuous flow conditions. The system was proposed in this scheme due to the following: (i) *Chlamydomonas reinhardtii* had been observed to grow well and assimilate nutrients under both heterotrophic and

phototrophic conditions (according to the results reported in Chapter 4); (ii) the heterotrophic reactor was connected to the photobioreactor in series because Yulistyorini (2016) reported that greater amounts of phosphorus accumulated in the cells of *Chlamydomonas reinhardtii* as polyphosphate under heterotrophic conditions compared to the ones obtained in the phototrophic cultivation; (iii) gravity settlement with the addition of cationic polymer was also considered after two-stage mixotrophic microalgae growth in order to help microalgae harvesting, which is considered one of the main issues with regard to commercial microalgae cultivation; and (iv) settled microalgal biomass was recycled into the inlet of the PBR at a specific rate to set predefined biomass concentrations in the system, and the HRT and CRT could be controlled independently. In order to assess the performance of the proposed system, a series of experiments were carried out under different operational conditions to test the effects of hydraulic retention time (HRT), cell retention time (CRT) and nitrogen source on microalgae growth, nutrient control and recovery.

5.2. Methodology

Chlamydomonas reinhardtii was selected as the model microalgae strain due to its ability to use both the phototrophic and heterotrophic forms of metabolism. Firstly, a pure culture was propagated using 300 mL samples of autoclaved BBM in 500 mL conical flasks placed in a shaking incubator under controlled environmental conditions (see Section 3.1.1). The propagated microalgae strain was used to inoculate a PBR (5-10% v/v) containing autoclaved BBM, which was operated under batch conditions until the microalgae reached a stationary growth phase. Next, the PBR was fed continuously with synthetic wastewater (see Table 3-1 for the associated recipe) at a specific flow rate to meet the set hydraulic retention time. The effluent from the PBR was used to feed a heterotrophic reactor (HTR) connected in series – i.e., HTR of a similar size and shape to the PBR but with opaque walls to prevent light penetration. The effluent from the HTR was settled in a 1 L Imhoff cone (ST) and the settled biomass recirculated into the PBR at a specific rate to meet the intended cell retention time. Finally, the effluent from the settlement tank (FE) was collected in a container and a certain amount of settled biomass was harvested daily (see Figure 3-5b).

The influence of HRT, CRT and nitrogen source on the performance of the two-stage microalgal process (PBR+HTR) was assessed by testing the following operational

conditions: (i) HRT tested at 36 h, 48 h and 72 h; (ii) CRT tested at 7 d, 14 d and 21 d; and (iii) nitrogen source tested using ammonium, nitrate and a mixture of ammonium and nitrate (1:1 ratio) (Table 3-4). These operational conditions were tested following the experimental design described in Table 3-5. Samples were collected from PBR, HTR, ST and FE (2, 3, 5 and 6 numbered sampling points in Figure 3-5.a, respectively) to monitor operational conditions and correlate these with microalgal biomass growth, as well as nitrogen and phosphorus uptake. Mass flow analyses of nutrients in both reactors were conducted when the system had reached steady-state conditions using equations 3-10 and 3-11.

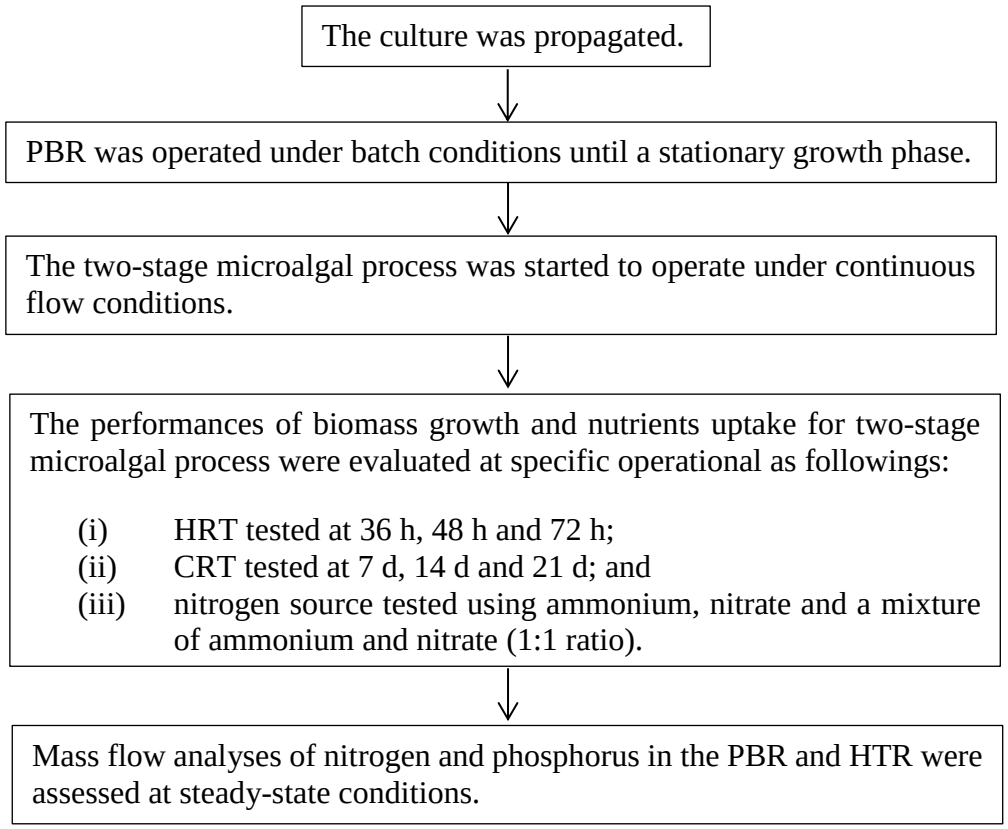


Figure 5-1 Steps to methodology used in Chapter 5

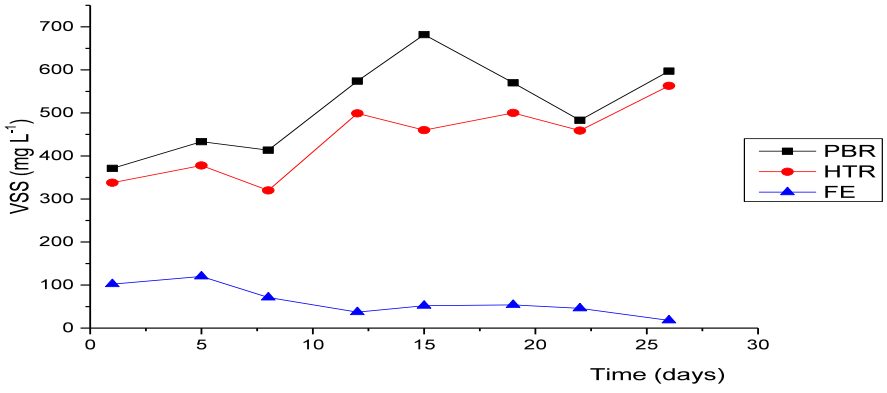
5.3. Results and Discussion

5.3.1. The Effects of Hydraulic Retention Time on Biomass Growth

Algal biomass concentrations in the effluent of the PBR, HTR and sedimentation unit (final effluent - FE) as a response to different initial feeding flowrates in the two-stage biological process are presented in Figure 5-2. It can be clearly seen that a slightly higher biomass concentration was achieved in the PBR than in the HTR for all HRTs

tested. Microalgae concentration in the PBR fluctuated between 370 and 680 mg VSS L^{-1}, 330 and 600 mg VSS L^{-1} and 380 and 570 mg VSS L^{-1} at 36 h, 48 h and 72 h HRT, respectively. Biomass concentrations in the HTR varied from 320 to 560 mg VSS L^{-1}, 340 to 560 mg VSS L^{-1} and 370 to 600 mg VSS L^{-1} at 36 h, 48 h and 72 h HRT, respectively. However, it was expected that a higher biomass concentration would be achieved in the heterotrophic reactor due to the fact that heterotrophic microalgae a have higher growth rate in comparison with the growth rate of phototrophic microalgae (see Chapter 4) (Morales-Sanchez et al., 2015). This can be attributed to the synthetic wastewater containing low organic carbon concentrations and bacterial contamination under heterotrophic conditions.

With regard to effluent quality, algal biomass in the final effluent showed different patterns with regard to the HRTs tested. At 48 h HRT, biomass concentration (dry weight) fluctuated between 90 and 160 mg VSS L^{-1}. While there was a constant decrease from 100 to 40 mg VSS L^{-1} at 36 h HRT, a continuous increase was observed from 80 to 150 mg VSS L^{-1} at 72 h HRT until steady-state conditions had been reached. Solids concentrations were quite high, even though a cationic polymer was added to the feeding media to increase the settling ability of the microalgae. Harvesting is considered the main limitation to the large-scale cultivation of microalgae due to its limited settlement capacity. Sedimentation, centrifugation and filtration are the most common methods to separate solid and liquid phases. All harvesting techniques have some deficiencies such as the land area requirement for sedimentation, and high energy consumption for the others (Gerardo et al., 2015).



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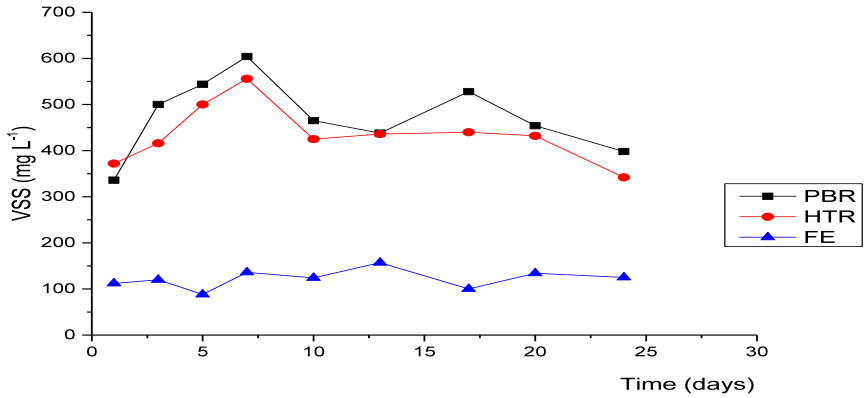

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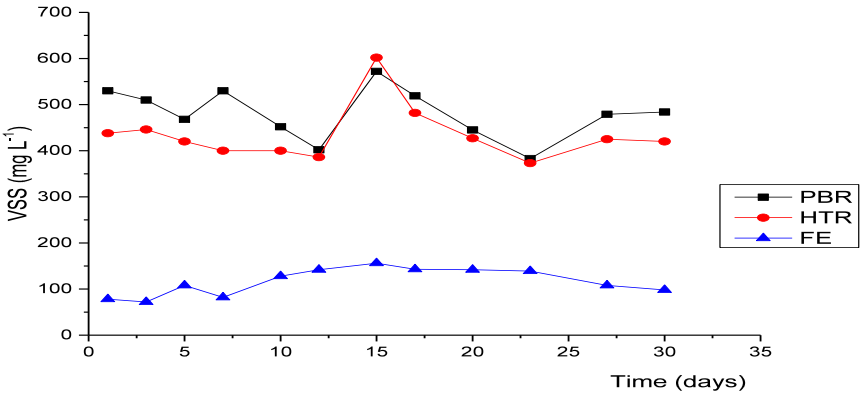

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Figure 5-2 Microalgae concentration under different hydraulic retention times: a) 36 h HRT; b) 48 h HRT; and c) 72 h HRT.

The cell concentration in the system should be constant under steady-state conditions, meaning that the specific growth rate (μ) should match the dilution rate (*D*). However, it is very difficult to achieve true steady-state conditions in a biological system so there are commonly referred to as "pseudo" steady-state conditions with a reasonable margin of change in the controlled variable. VSS concentrations varied by up to 20% between any two given samplings, which is acceptable due to one of the main difficulties operating continuous culture systems being that microalgae tends to attach to reactor walls during long cultivation periods (Fernandes et al., 2015). Thus, it was determined whether the system had reached steady-state according to the phosphorus concentration in the effluent.

Average microalgae concentrations at different hydraulic retention times under steady-state conditions are reported in Table 5-1. There was a similar trend in the change of biomass concentration with HRTs under both phototrophic and heterotrophic conditions. The average dry weight concentration of microalgae declined from 581 ± 71 to 457 ± 81 mg VSS L^{-1} for PBR, from 496 ±4 2 to 415 ± 63 mg VSS L^{-1} for HTR with increasing HRTs from 36 h to 48 h. This could be attributed to the reduced nutrient supply at higher HRT due to the decrease in flowrate (Gao et al., 2018). A further increment in HRT to 72 h did not result in any significant difference in algal biomass concentration for both the PBR and HTR ($p = 0.988$ for PBR and $p = 0.915$ for HTR, one-way ANOVA).

Table 5-1 Biomass concentrations in each reactor with different HRTs under steady-state conditions (Average ± standard deviation)

Sampling Point	36 h HRT (mg VSS L^{-1})	48 h HRT (mg VSS L^{-1})	72 h HRT (mg VSS L^{-1})
PBR	581 ± 71	457 ± 81	462 ± 51
HTR	496 ± 42	415 ± 63	425 ± 39
ST	651 ± 114	734 ± 36	714 ± 95
FE	41 ± 15	128 ± 20	126 ± 21

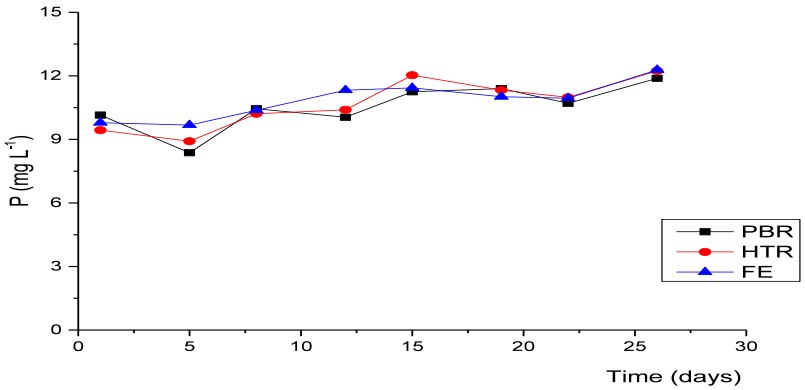
Conversely, average biomass concentrations in the algal recycling line (referred to as the settlement tank (ST)) was incremented from 651 ± 114 to 734 ± 236 mg VSS L^{-1} with an increase of HRT from 36 h to 48 h. Higher biomass concentration in the ST and poor sedimentation of microalgae caused an increment in algae concentration in the final effluent (FE). For the FE, the biomass concentration was found to be 41 ± 15 mg VSS L^{-1} at 36 h HRT and was increased threefold to 128 ± 20 mg VSS L^{-1} at 48 h HRT; after that, dry weight concentrations of biomass in the ST and FE remained stable at 72 h of HRT. Although a lower solids concentration in the effluent was the intention, 48 h HRT was chosen as the optimum dilution rate due to the FE containing a lower phosphorus concentration (see Section 5.3.2). Furthermore, the discharge consent for solids is set to be 35 mg L^{-1} TSS in the European Union Urban Wastewater Treatment Directive (UWWTD, 1991). Therefore, the additional process was required to improve the settlement quality of the algae and reduce solids concentration in the effluent for the implementation of a two-stage biological process in the wastewater treatment plant.

5.3.2. The Effects of HRT on Nutrient Uptake

The uptake of nutrients from the two-stage biological process was determined by analysing phosphate, ammonium and nitrate in the PBR, HTR and FE samples. Phosphorus concentrations in each reactor at 36 h, 48 h and 72 h HRT are presented in Figure 5-3. At 36vh HRT, phosphorus concentrations increased from 9.8 to 11.3 mg P L^{-1} but then remained stable under steady-state conditions. It can be clearly seen from the adaptation period that there was a constant decline in phosphorus concentration from 15.0 to 9.0 mg P L^{-1} at 48 h HRT. At 72 h HRT, the phosphorus concentration was found to fluctuate around 9.0 mg P L^{-1}. Phosphorus uptake efficiency incremented from 24.6% by 40% with the increase of HRT from 36 h to 48 h due to the increased time, providing more time to assimilate phosphorus (Gao et al., 2018; Iman Shayan et al., 2016); it then remained invariant (40%) at 72 h HRT. Therefore, 48 h HRT was chosen as the optimum because prolonged HRT requires a larger reactor volume.

P in the effluent at 36 h was significantly different than the ones at 48 h and 72 h ($p = 0.000028$ for 36 h and 48 h HRT; $p = 0.000004$ for 36 h and 72 h HRT, one-way ANOVA), whereas P in the effluent did not change significantly between 48 h and 72 h ($p = 0.348$ for 48 h and 72 h HRT, one-way ANOVA).

Ammonium recovery rates in the PBR were found to be 90.1, 88.4 and 89.4% at 36 h, 48 h and 72 h HRT, respectively, while nitrate was fully consumed under phototrophic conditions. There were not any changes in ammonium concentration under heterotrophic conditions. (Data is not presented here but is fully explained in Section 5.3.3 with mass flow analysis of nitrogen).



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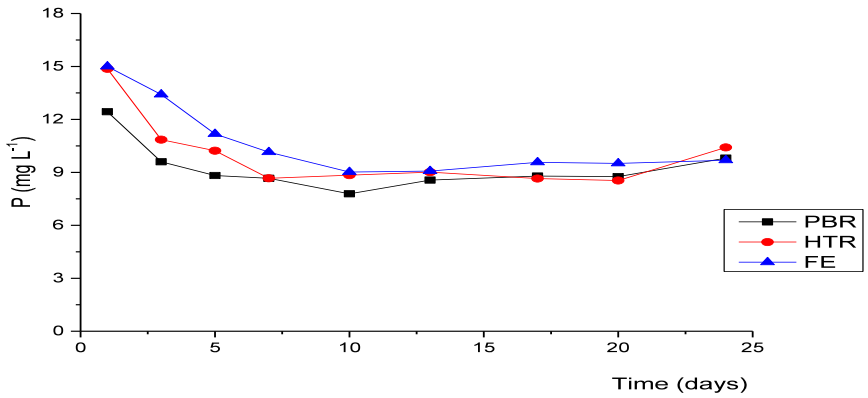

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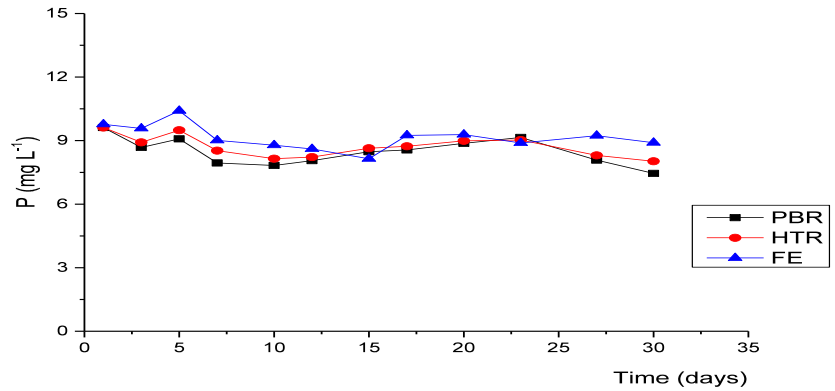

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Figure 5-3 Changes in P concentration during the cultivation period for different HRTs: a) 36h HRT; b) 48h HRT; and c) 72h HRT.

There is a two-stage kinetic process for phosphate uptake by algae. Firstly, algae cell surfaces adsorb the phosphate substrate until there is an equilibrium between adsorption and desorption. Then, surface-adsorbed phosphate is transferred into algae cells by passing through the cell membrane. This metabolism controlled by the intracellular phosphorus pool size in which algae growth is dependent on intracellular phosphorus (Yao et al., 2011).

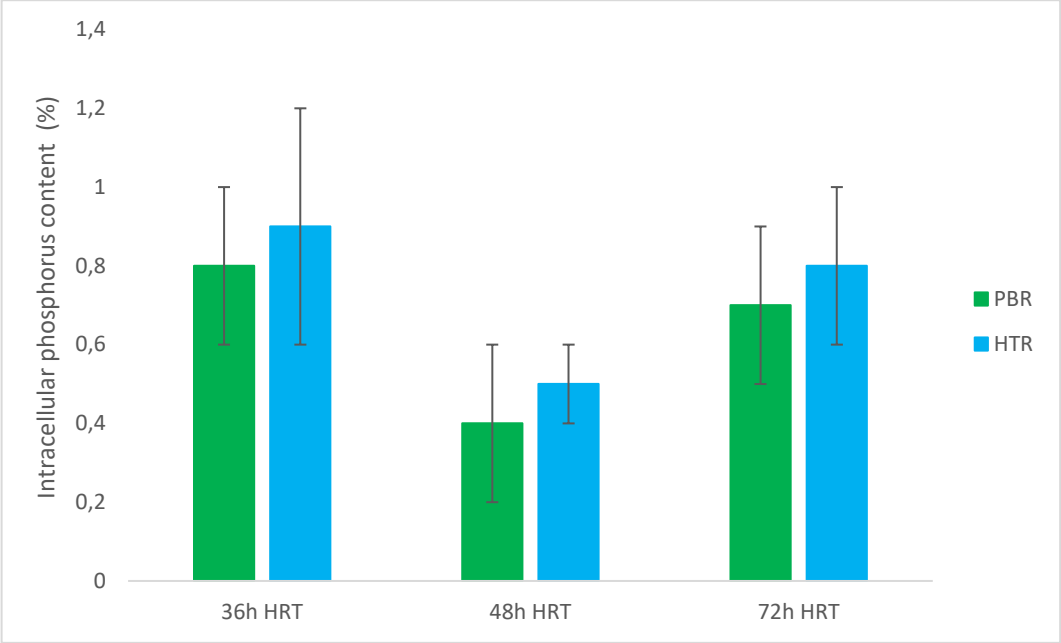


Figure 5-4 Intracellular phosphorus content in PBR and HTR at different HRTs at steady-state.

Intracellular phosphorus concentration was analysed for both phototrophic and heterotrophic cultivation during steady-state conditions in order to identify the amount of phosphorus taken up via microalgae cells (Figure 5-4). *Chlamydomonas reinhardtii* recovered higher amounts of phosphorus under heterotrophic conditions compared with the phototrophic reactor in all HRTs. This contradicted the ideas that phosphorus uptake is increased in the presence of light (Jansson, 1988). Heterotrophic conditions could be found to be more robust than phototrophic cultivation, triggering the maintenance of phosphorus in the algal cell. The highest intracellular P content obtained at 36 h HRT with the highest biomass concentrations found in HRT experiments. Although the greatest phosphorus uptake was obtained at 48 h HRT at 40%, there was a significant decrease in intracellular P concentration with the increment in HRT from 36 h to 48 h due to higher bacterial contamination at 48h HRT. Bacterial contamination was determined by bacterial count in the heterotrophic reactor, which was found as 255,400 and 306,000 CFU at 36 h and 48 h HRT, respectively. It was concluded that the presence of bacteria helps to increase the phosphorus uptake. The combination of microalgal and bacterial systems having higher nutrient recovery efficiency than pure microalgal system has already been reported (Liang et al., 2013). With a further increase in HRT to 72 h, intracellular P content showed a sharp increase with the same P recovery efficiency of 40% because of

the decline in bacterial cell counts to 264,000 CFU. This situation be attributed to feeding the system with the sterilised SWW and cleaning the pipes regularly.

The same trend was obtained for intracellular P in both the PBR and HTR ($p = 0.425$ for PBR and HTR), for which fluctuations were observed with the increase of HRT. There was a significant decrease in intracellular P content from $0.8 \pm 0.2\%$ to $0.4 \pm 0.2\%$ in the PBR and from $0.9 \pm 0.3\%$ to $0.5 \pm 0.1\%$ in the HTR between 36 h and 48 h HRT, corresponding to $p = 0.022$ for 36 h and 48 h HRT in the PBR, and $p = 0.042$ for 36 h and 48 h HRT in the HTR (one-way ANOVA.) Intracellular P content increased sharply up to $0.7 \pm 0.2\%$ and $0.8 \pm 0.2\%$ both in the PBR and HTR, respectively at 72 h HRT ($p = 0.076$ for 48 h and 72 h HRT in the PBR, and $p = 0.115$ for 48 h and 72 h HRT in the HTR, one-way ANOVA). For all HRTs tested, the microalgae assimilated higher phosphorus concentrations into their cells under heterotrophic conditions than under phototrophic conditions. This could be attributed to the fact that lower microalgae concentrations and similar phosphorus recovery efficiencies were reported in the HTR when compared to the performance in the PBR.

5.3.3. Mass Flow Analysis of Nutrients in HRT Experiments

Figure 5-5 presents the percentage of phosphorus species related to total phosphorus in the continuous flow system at different hydraulic retention times (36 h, 48 h and 72 h). The inlet of the PBR at 36 h of HRT included 9.7% suspended organic phosphorus coming from biomass recycling and 90.3% phosphate. There was a slight increase in suspended organic phosphorus entering the PBR at 48 h HRT from 9.7% to 14.7%, which corresponded to the decrease in phosphate from 90.3% to 85.3%. The inlet of the PBR at 72 h of HRT contained 16.7% and 83.3% suspended organic phosphorus and phosphate, respectively. The increment in the percentage of suspended organic phosphorus at the inlet of the PBR was due to the dilution caused by the increase of flowrate required to set the respective HRT tested.

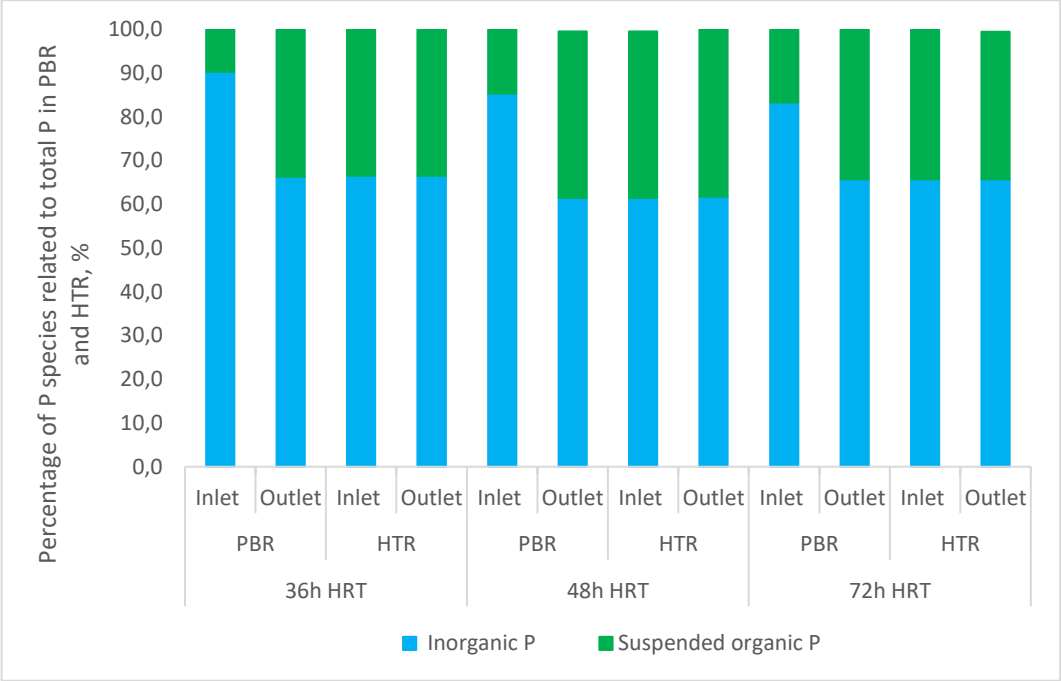


Figure 5-5 Distribution of phosphorus species through the system in different HRTs

Phosphorus uptake increased from 9.7% to 33.7% in the PBR and phosphate decreased from 90.3% to 66.3% in the effluent of the PBR at 36 h HRT. On increasing HRT from 36 h to 48 h, phosphorus uptake incremented to some extent from 33.7% to 38.1% at the outlet of the PBR corresponding to the decrease in phosphate from 66.3% to 61.5%. Only a small-scale decline was observed in phosphorus uptake from 38.1% to 34.3% consistent with increasing phosphate from 61.5% to 65.7% at 72 h HRT.

Interestingly, the percentage of phosphate species did not show any differences from phototrophic cultivation under heterotrophic conditions in all HRT experiments.

The percentage of nitrogen species related to total nitrogen in the PBR and HTR at different HRTs are exhibited in Figure 5-6 (36 h, 48 h and 72 h of HRT). Three main nitrogen species, ammonium, nitrate and suspended organic nitrogen, were calculated in order to identify the nitrogen mass balance in the two-stage biological process. Unfortunately, 100% of the nitrogen uptake was not considered via microalgae because of bacterial growth in the system. Therefore, the unaccounted percentage of nitrogen was assumed to be denitrification, which can occur in the anoxic micro-zone of biomass flocs showing low amounts of dissolved oxygen (Gonzalez-Fernandez et al., 2011).

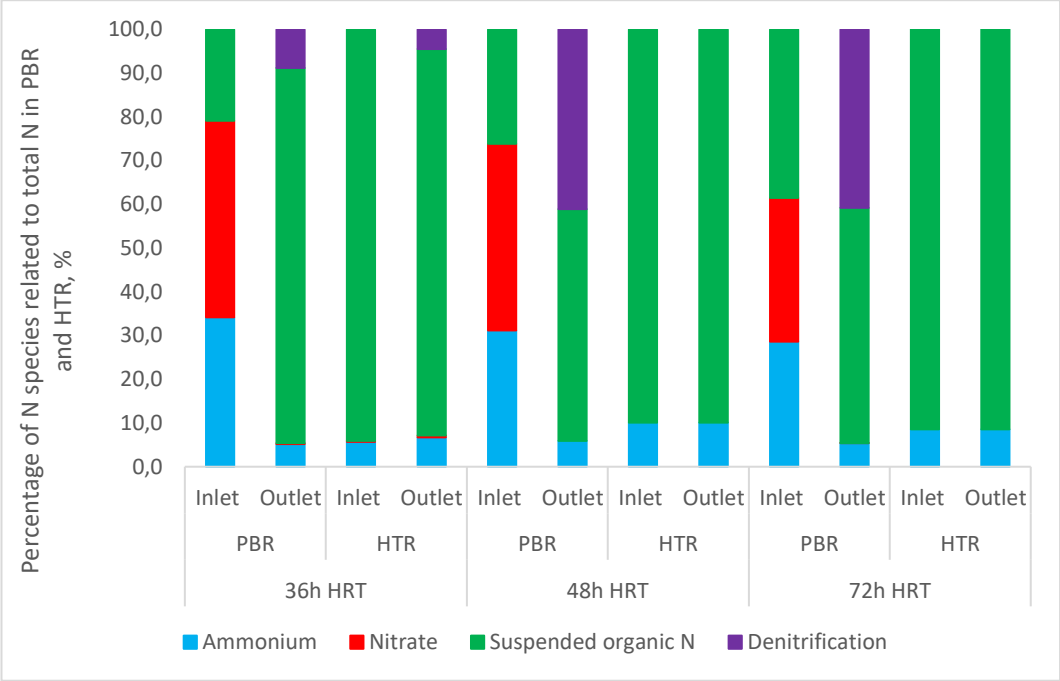


Figure 5-6 Distribution of nitrogen species throughout the system with differing HRTs

Moreover, pH varied from 9.67 to 10.15, from 9.65 to 10.45 and from 9.68 to 10.17 in PBR and from 9.59 to 9.82, from 9.45 to 10.18 and from 9.50 to 9.85 in HTR at 36 h, 48 h and 72 h of HRT, respectively. Ammonium ions can be turned into ammonia gas and volatilised at high pH (pH>10.5) (Kinidi et al., 2018). Therefore, ammonia stripping was not considered for the nitrogen mass balance.

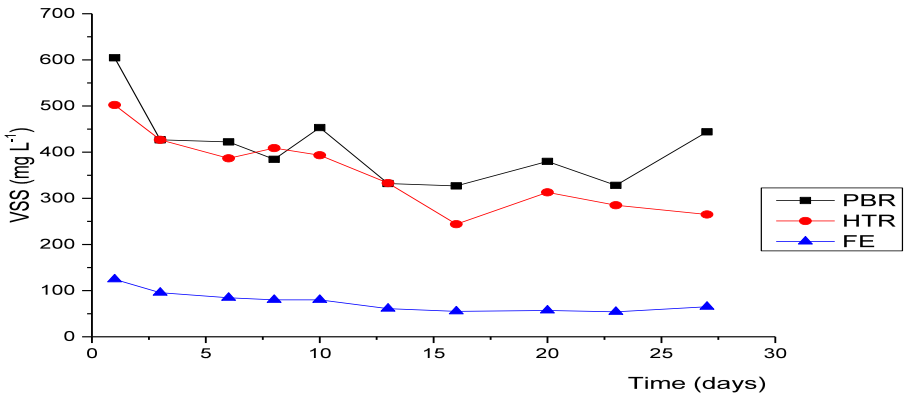
The inlet of the PBR included ammonium, nitrate and suspended organic nitrogen at 34.2%, 44.9% and 20.9% for 36 h HRT, 31.2%, 42.7% and 26.1% for 48 h HRT, and 28.6%, 32.9% and 38.5% for 72h HRT, respectively. The decrease in ammonium and nitrate and the increase in suspended organic nitrogen in the inlet of the PBR were attributed to the reduction of the flow rate of inlet.

The same percentages of ammonium were attained in the PBR with a sharp decrease to 5.2%, 5.9% and 5.4% at 36 h, 48 h and 72h HRT, respectively. Suspended organic nitrogen increased by greater than fourfold, up to 85.9%, over twofold to 53.0% and to 53.8% at 36 h, 48 h and 72h HRT, respectively. Nitrate was fully consumed throughout the PBR, while only 8.7% of nitrogen removal was calculated to be denitrification at 36 h HRT, where around 41% of nitrogen was removed by denitrification at both 48 h and 72 h HRT.

Suspended organic nitrogen and ammonium at the inlet of the HTR was detected to be 94.1% and 5.7% for 36 h HRT, 89.9% and 10.1% for 48 h HRT and 91.6% and 8.4% for 72 h HRT, respectively. Suspended organic nitrogen decreased to 88.4% and there was a marginal increase in ammonium to 6.7% at 36 h HRT, whereas the percentage of nitrogen species' mass remained stable under heterotrophic conditions at both 48 h and 72 h HRT. Regarding denitrification, 4.5% of nitrogen was removed by denitrification at 36 h HRT, while denitrification was not calculated for both 48 h and 72 h HRT.

5.3.4. The Impact of Cell Retention Time on Microalgae Growth

In order to identify the effect of cell retention time on biomass growth, the continuous flow system was operated at different CRTs of 7 d, 14 d and 21d at 48h HRT using a mix of ammonium and nitrate as the nitrogen source (Figure 5-7). The same growth trend was observed in the PBR and HTR throughout the experiments for cell retention times ($p = 0.103$ for PBR and HTR, one-way ANOVA). A slightly higher microalgae concentration was obtained in the PBR than in the HTR. Dry weight concentrations fluctuated from 330 to 600 mg VSS L^{-1}, from 330 to 600 mg VSS L^{-1} and from 260 to 810 mg VSS L^{-1} for the PBR and from 250 to 500 mg VSS L^{-1}, from 340 to 560 mg VSS L^{-1} and from 230 to 860 mg VSS L^{-1} for the HTR at 7 d, 14 d and 21 d CRT. Biomass concentration in the final effluent varied from 50 to 120 mg VSS L^{-1}, from 90 to 160 mg VSS L^{-1} and from 40 to 80 mg VSS L^{-1} at 7 d, 14 d and 21d CRT.



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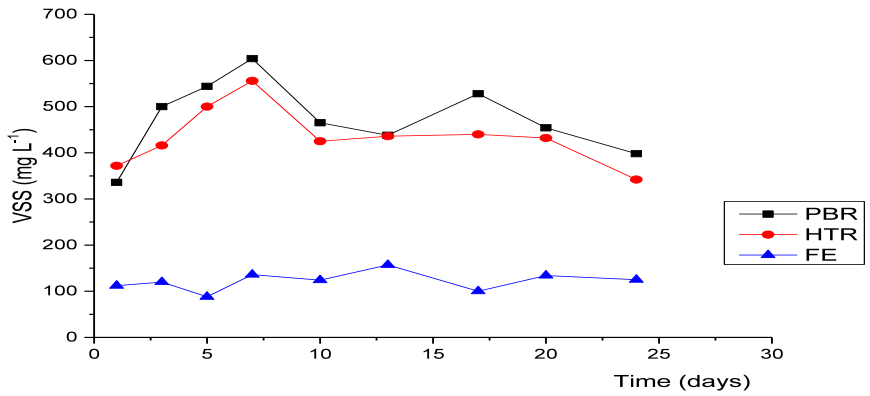

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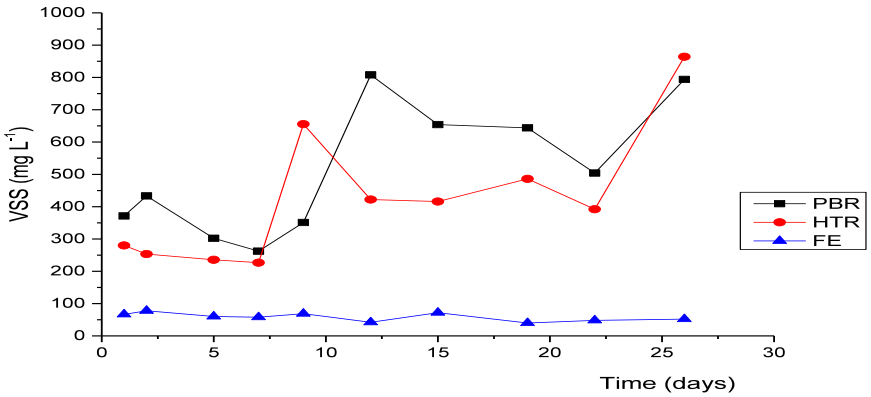

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Figure 5-7 Algal biomass concentration variation with different CRTs: a) 7 d CRT; b) 14 d CRT; and c) 21 d CRT.

It can be clearly anticipated that biomass concentration increased when cultivated under phototrophic and heterotrophic conditions with incrementing cell retention times at fixed hydraulic retention times. This was also observed by Gardner-Dale et al. (2017), who reported that lower biomass concentrations were obtained at lower CRTs with *Chlamydomonas reinhardtii*. Table 5-2 summarises microalgae concentrations in each reactor of the continuous flow system under steady-state conditions at different CRTs. Firstly, between 7 d and 14 d CRT, the increase of algal biomass concentrations of 362 ± 51 to 457 ± 81 mg VSS L^{-1} and from 288 ± 36 to 415 ± 63 mg VSS L^{-1} in the PBR and the HTR, respectively ($p = 0.208$ for 7 d and 14 d CRT in the PBR, and $p = 0.246$ for 7 d and 14 d CRT in the HTR, one-way ANOVA). At 21 d CRT, algal biomass concentrations in the PBR significantly increased to 681 ± 125 mg VSS L^{-1}; however, a slight increase

was observed in HTR up to 516 ± 198 mg VSS L^{-1}, corresponding to p = 0.003 for 14 d and 21 d CRT in the PBR, and p = 0.397 for 14 d and 21 d CRT in the HTR (one-way ANOVA).

In the settlement tank, *Chlamydomonas reinhardtii* presented greater settlement efficiency when incrementing cell retention time from 7 d to 14 d corresponding to a twofold increase in microalgae concentration in ST from 390 ± 148 to 734 ± 236 mg VSS L^{-1} (p = 0.030 for 7 d and 14 d CRT, one-way ANOVA). It remained stable at 738 ± 154 mg VSS L^{-1} at 21 d CRT (p = 0.999 for 14 d and 21 d CRT, one-way ANOVA). Surprisingly, effluent quality varied with regard to solids concentration at different CRTs. FE included an approximate two-fold dry weight concentration at 14 d HRT (128 ± 20 mg VSS L^{-1}) which was significantly different from figures obtained at 7 d and 21 d CRT (58 ± 5 and 51 ± 13 mg VSS L^{-1}, respectively) (p = 0.000014 for 7 d and 14 d, and p = 0.000005 for 14 d and 21 d CRT, respectively).

Table 5-2 Microalgae concentrations in the different stages at different CRTs under steady-state conditions throughout the two-stage biological process (Average ± standard deviation)

Sampling Point	7d CRT (mg VSS L^{-1})	14d CRT (mg VSS L^{-1})	21d CRT (mg VSS L^{-1})
PBR	362 ± 51	457 ± 81	681 ± 125
HTR	288 ± 36	415 ± 63	516 ± 198
ST	390 ± 148	734 ± 236	738 ± 54
FE	58 ± 5	128 ± 20	51 ± 13

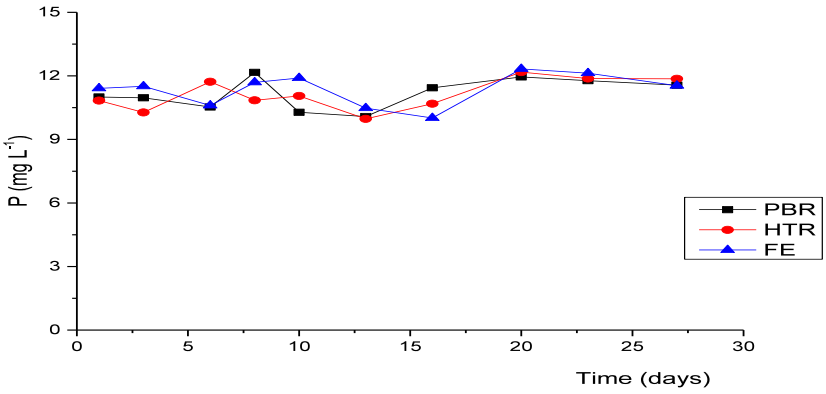
5.3.5. The Impact of Cell Retention on Nutrient Uptake

Phosphorus concentrations over the two-stage biological process at the tested CRTs at 7 d, 14 d and 21d are presented in Figure 5-8. Overall, there was no significant difference in P concentration throughout the continuous flow system with varying CRT, meaning that the P in each reactor showed a similar trend (p = 0.958, p = 0.909 and p = 0.989 for PBR and HTR, for PBR and FE, and for HTR and FE, respectively, one-way ANOVA). Average P concentrations under steady-state conditions at 7 d and 21d CRT were 11.3 and 11.9 mg P L^{-1}, consistent with lower P recovery efficiencies via *Chlamydomonas reinhardtii* which were obtained as 24.7% and 20.7%, respectively (p = 0.408 for 7 d and 21 d CRT), and which were significantly different in comparison with P concentrations in the final effluent and recovery efficiency of P uptake achieved 9.0 mg

L^{-1} P and 40%, sequentially at 14d CRT ($p = 0.004$ for 7 d and 14 d CRT, and $p = 000441$ for 14 d and 21 d CRT, one-way ANOVA).

Greater phosphorus uptake at 14 d CRT could be attributed to higher biomass concentration at this time compared to that at 7 d CRT. Although biomass concentration continued to increase when further increasing CRT to 21 d, there was a decrease in P uptake which could be the result of biomass decay and excretion of phosphorus from algae cells into the media (Gardner-Dale et al., 2017). Hence, 14 d CRT was considered the optimum producing the lowest content in the final effluent.

The ammonium recovery rates achieved in the PBR were 100.0%, 88.4% and 87.7% at 7 d, 14 d and 21 d CRT, respectively, and nitrate was fully consumed by microalgae under phototrophic conditions. Heterotrophic conditions offered only limited contribution to nitrogen recovery. (Data is not presented here but is fully explained in Section 5.3.6 with the nitrogen mass flow analysis.)



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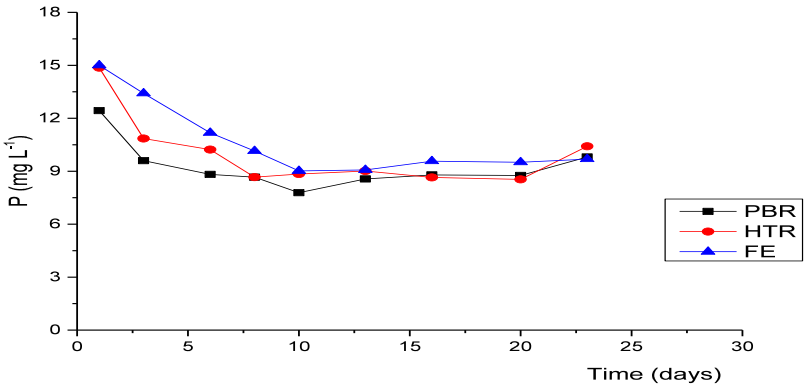

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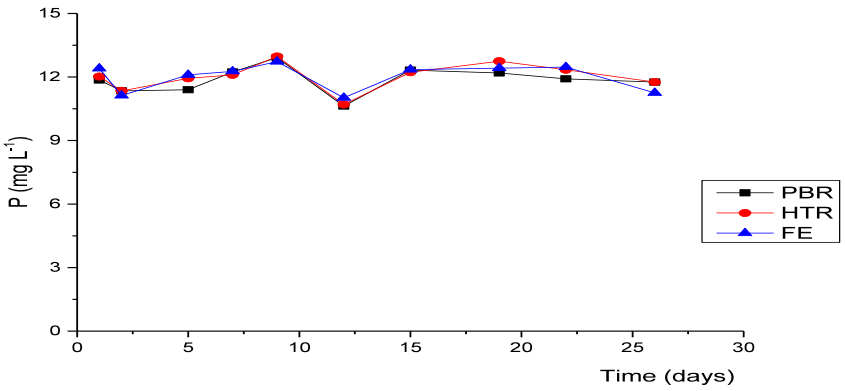

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Figure 5-8 The effect of CRT on phosphate uptake and recovery: a) 7 d CRT; b) 14 d CRT; and c) 21 d CRT.

Figure 5-9 shows intracellular P content in *Chlamydomonas reinhardtii* cultivated under phototrophic and heterotrophic conditions at various cell retention times. There was a dramatic decrease in intracellullar P content in both PBR and HTR from 0.8 ± 0.1% to 0.4 ± 0.2% and from 0.9 ± 0.1% to 0.5 ± 0.1%, respectively, on increasing CRT from 7 d to 14 d ($p = 0.009$ for 7 d and 14 d CRT in the PBR, and $p = 0.005$ for 7 d and 14 d CRT in the HTR, one-way ANOVA). While intracellular P incremented to 0.6 ± 0.1% at 21 d CRT ($p = 0.509$ for 14 d and 21 d CRT, one-way ANOVA) under phototrophic cultivation, it remained steady at 0.5 ± 0.2% under heterotrophic condition ($p = 0.981$ for 14 d and 21 d CRT, one-way ANOVA).

Interestingly, at only 21 d CRT, higher intracellular P content was observed in the PBR than was achieved in the HTR, which corresponded to phototrophic microalgae having a lower growth rate and thus requiring more time to take up P, and with a shorter cultivation time needed for heterotrophic microalgae growth (Kim et al., 2013b).

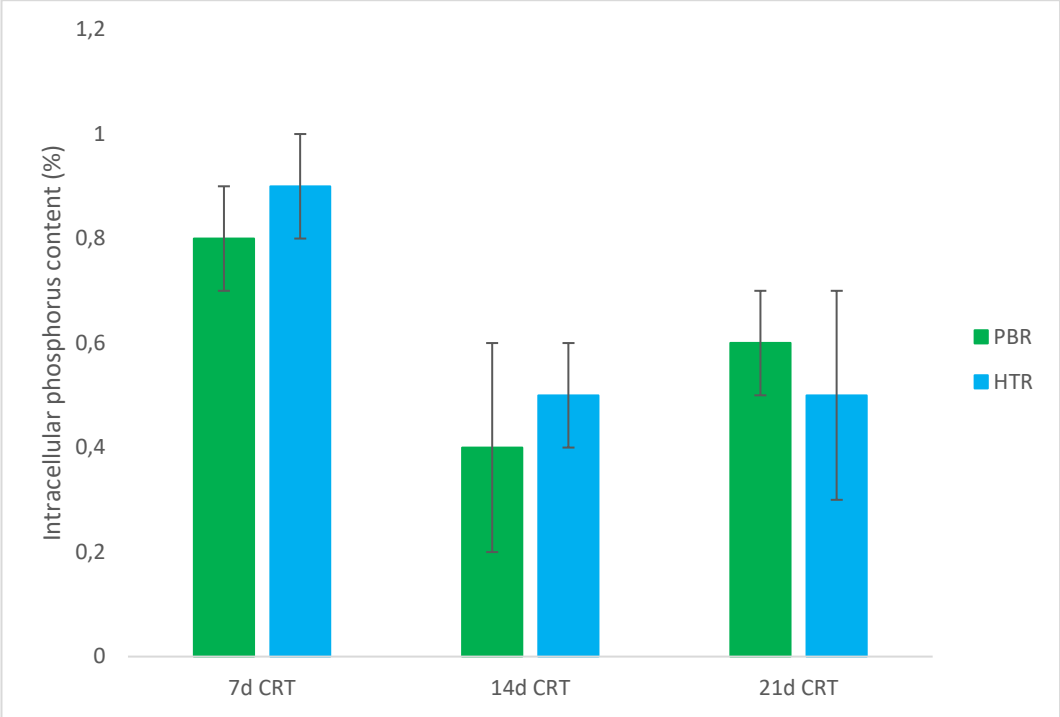


Figure 5-9 Changes in intracellular phosphorus content in PBR and HTR at various CRTs

5.3.6. Mass Flow Analysis of Nutrients for CRT Experiments

The ratio of phosphate and suspended organic phosphorus at various CRTs is depicted in Figure 5-10. While only 9.0% of suspended organic P was obtained influent to the PBR at both 7 d and 21 d CRT, it increased by over a half to reach 14.7% at 14 d CRT. This was consistent with the decrease in phosphate inlet of PBR, down from 91.0% to 85.3% from 7 d and 21 d to 14 d CRT. The difference could be caused by larger amounts of suspended organic P and lower phosphate concentrations in ST found at 14 d CRT in comparison with the figures obtained at 7 d and 21 d. In the PBR, the highest increase in suspended organic P under phototrophic cultivation achieved was 23.4% (from 14.7% to 38.1%) at 14 d CRT. It was lower at 7 d and 21 d CRT at 16% (from 9.0% to 25.0%) and 19.5% (from 9.0% to 28.5%), respectively. The percentage of phosphate was reduced to 75.0%, 61.9% and 71.5% at 7 d, 14 d and 21 d CRT.

There was a little contribution to phosphorus uptake via *Chlamydomonas reinhardtii* under heterotrophic conditions for the experiments with different CRTs.

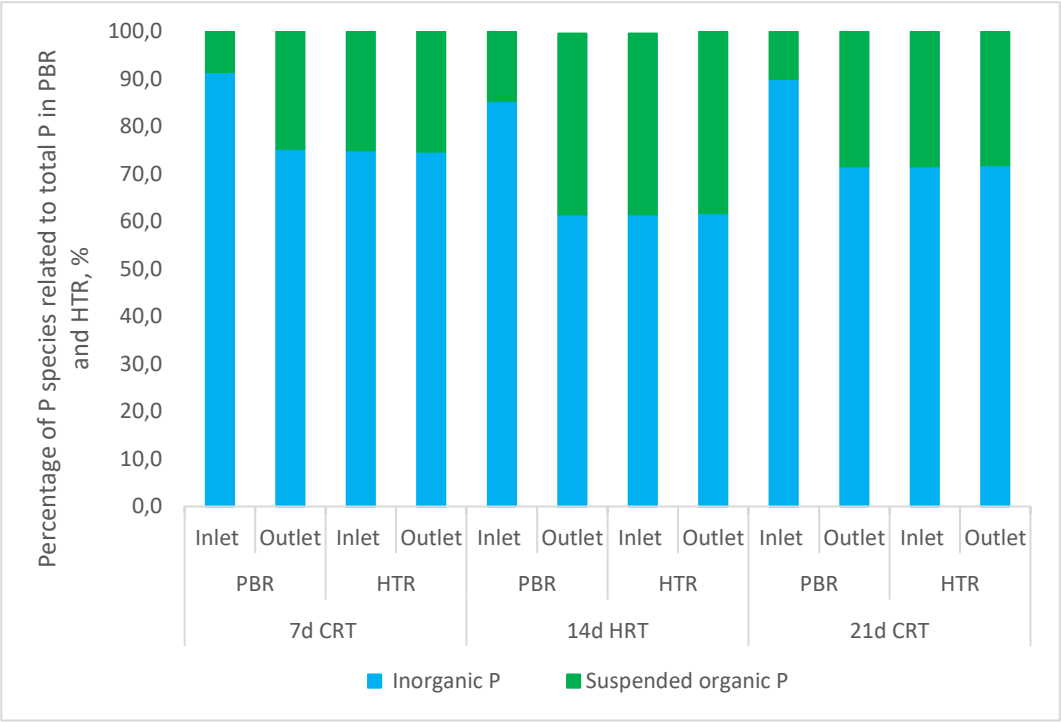


Figure 5-10 Distribution of P species with varying CRTs

Figure 5-11 presents the distribution of N species for *Chlamydomonas reinhardtii* with different CRTs. Regardless of cell retention times, the influent to the PBR contained the same amount of ammonium, nitrate and suspended organic N at 31.9%, 43.3% and 24.8%, respectively. Ammonium uptake under conditions of phototrophic cultivation decreased gradually from 29.2% to 25.2% and to 24.7% with the increase in CRT from 7 d to 14 d and to 21 d. Nitrate was fully consumed in the PBR in all CRTs. It was observed that there was a larger increase in suspended organic N in the PBR at 7 d and 21d CRT (58.9% and 55.7%, respectively), compared to the figure obtained at 14 d, which was 26.9%. In terms of denitrification in the PBR, 13.2% of nitrogen removal by denitrification was obtained at 7 d and 21 d CRT, whereas there was an over three-fold increase as 41.1% at 14 d CRT.

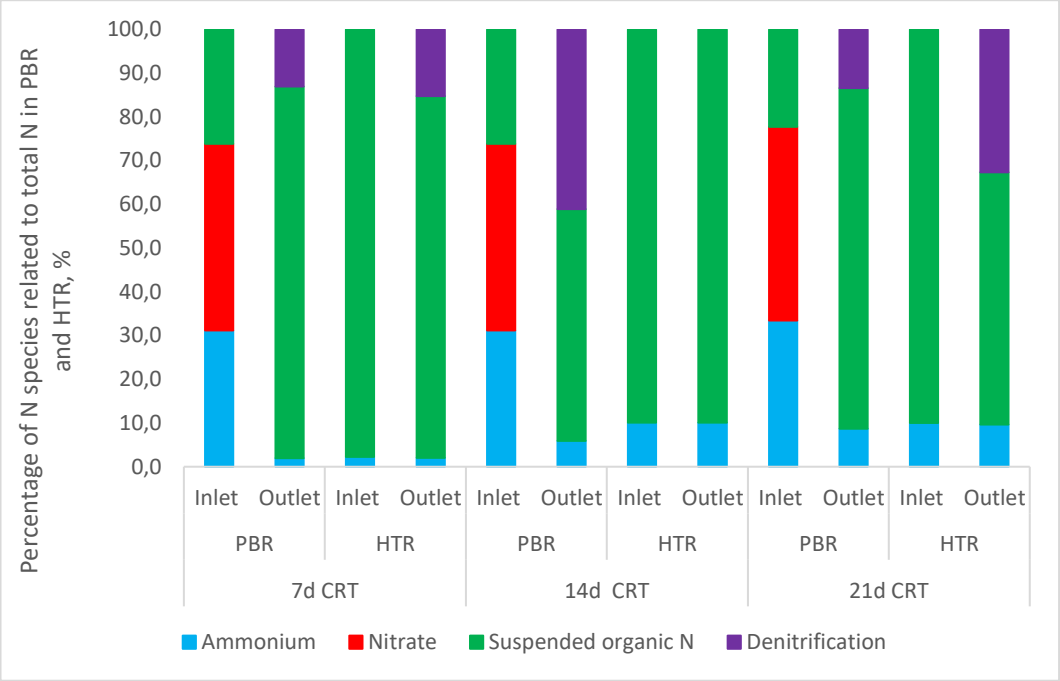


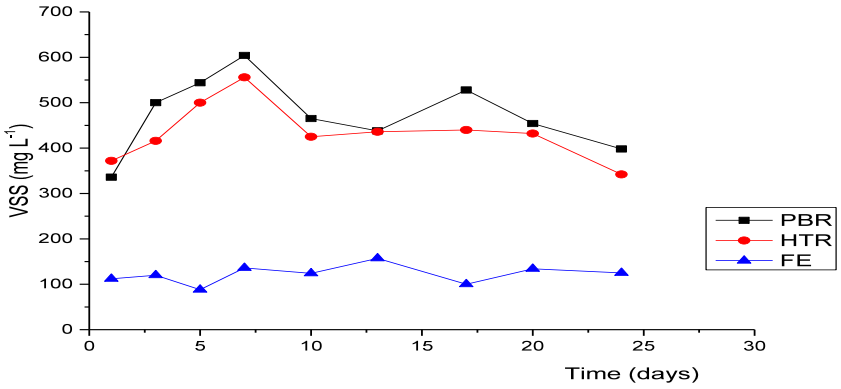
Figure 5-11 Distribution of N species at various CRTs

Regarding the inlet of the HTR, higher suspended organic N was observed at 7 d CRT of 97.7% than at both 14 d and 21 d CRT (90%). The remainder in each of the above instances consisted solely of ammonium. There were not any differences in ammonium effluent from the HTR in any of the CRTs, whereas the amount of suspended organic N remained steady at 90.0% at 14 d CRT, whilst 15.2% and 32.6% decreases in suspended organic N were attained at 7 d and 21 d CRT, respectively, which were calculated to be denitrification.

5.3.7. The Influence of Different Nitrogen Sources on Biomass Growth

Chlamydomonas reinhardtii was cultivated in a feeding medium including different nitrogen sources as a mix of ammonium and nitrate, ammonium alone, and nitrate alone (Figure 5-12). Similar microalgae concentrations were achieved in the PBR and HTR. Biomass growth fluctuated between 330 and 600 mg VSS L^{-1}, between 230 and 540 mg VSS L^{-1}, and between 300 and 490 mg VSS L^{-1} for the PBR, and from 340 to 560 mg VSS L^{-1}, from 270 to 550 mg VSS L^{-1}, and from 290 to 470 mg VSS L^{-1} for the HTR when a mix of NH_4^+-N and NO_3^--N, NH_4^+-N and NO_3^--N were used as the nitrogen sources, respectively. The dry weight of biomass in the final effluent differed from 90 to 160 mg VSS L^{-1}, from 50 to 200 mg VSS L^{-1} and from 30 to 80 mg VSS L^{-1} with different

nitrogen sources as a mix of NH_4^+-N and NO_3^--N, and solely NH_4^+-N and NO_3^--N, respectively.



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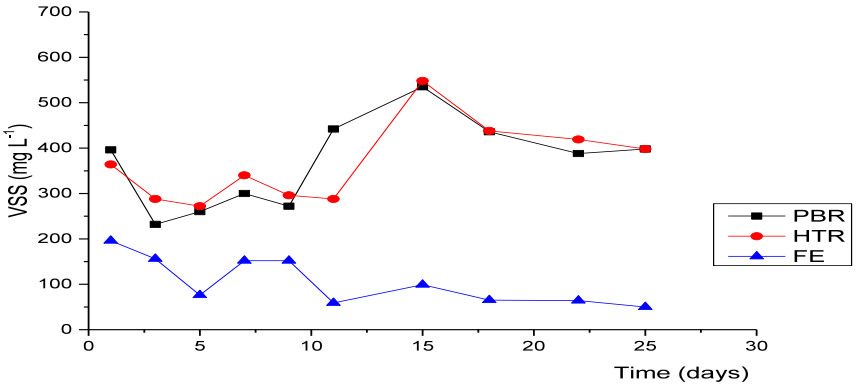

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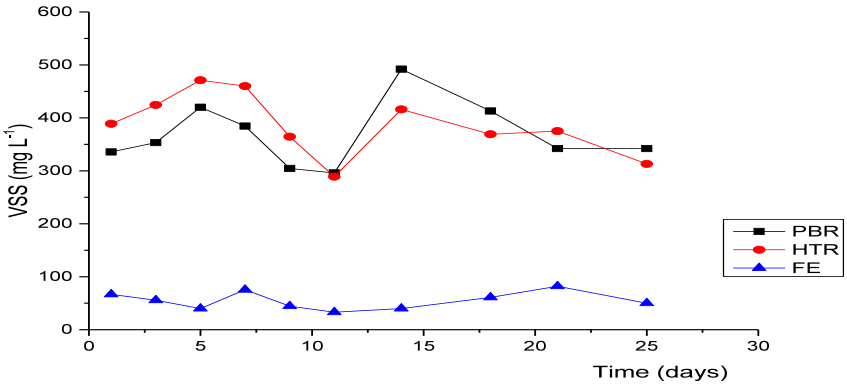

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Figure 5-12 Biomass growth during cultivation with different nitrogen sources: a) a mix of NH_4^+-N and NO_3^--N (50:50); b) NH_4^+-N; and c) NO_3^--N.

Table 5-3 presents the biomass concentration found in each reactor with the synthetic wastewater containing different nitrogen sources. Nitrogen sources did not show any significant effect on algal biomass concentration for the two-stage biological process ($p = 0.605$ for PBR and $p = 0.243$ for HTR, one-way ANOVA). While average microalgae concentrations obtained using a mix of NH_4^+-N and NO_3^--N and solely NH_4^+-N were similar (457 ± 81 and 440 ± 58 mg VSS L^{-1} for the PBR, 415 ± 63 and 418 ± 93 mg VSSL^{-1} for the HTR), there was a slight decrease to 407 ± 114 and 352 ± 51 mg VSS L^{-1} in the PBR and HTR, respectively, when nitrate was used as the nitrogen source.

Table 5-3 Algal biomass concentration in the continuous flow system at steady-state conditions with various nitrogen sources (average ± standard deviation)

Sampling point	Mix of NH_4^+-N and NO_3^--N (mg VSS L^{-1})	NH_4^+-N (mg VSS L^{-1})	NO_3^--N (mg VSS L^{-1})
PBR	457 ± 81	440 ± 58	407 ± 114
HTR	415 ± 63	418 ± 93	352 ± 51
ST	734 ± 236	1493 ± 115	449 ± 95
FE	128 ± 20	67 ± 19	53 ± 19

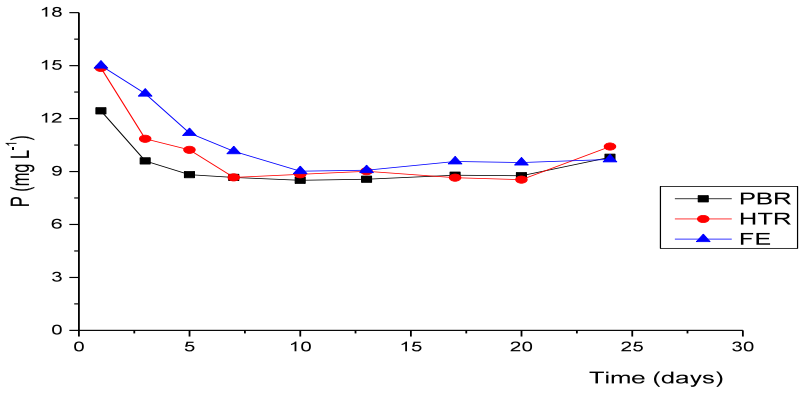
The settlement efficiency of algal biomass presented significantly different trends with the various nitrogen sources ($p = 0.000198$, one-way ANOVA). The highest and the lowest dry weight concentrations were achieved with solely ammonium and solely nitrate (1493 ± 115 and 449 ± 95 mg VSS L^{-1}, respectively); 734 ± 236 mg VSS L^{-1} was obtained when a mix of ammonium and nitrate was used. Surprisingly, the lowest effluent quality, as based on solid concentration was obtained with a mix of NH_4^+-N and NO_3^--N, including the greatest biomass concentration (128 ± 20 mg VSS L^{-1}) ($p = 0.001$ for with ammonium alone and the mixture of ammonium and nitrate, and $p = 0.000153$ for with a mixture of ammonium and nitrate and solely nitrate, one-way ANOVA). With the use of solely NH_4^+-N and NO_3^--N in the synthetic wastewater, average microalgae concentration was decreased by half to $67 \pm 19$and 53 ± 19 mg VSS L^{-1} VSS, respectively ($p = 0.502$ when cultivated with ammonium alone and nitrate alone, one-way ANOVA).

5.3.8. The Influence of Different Nitrogen Sources on Nutrients Uptake

Figure 5-13 exhibits the effect of different nitrogen sources on P concentration in the continuous flow system. It can be clearly seen that there was no significant difference in phosphorus uptake by *Chlamydomonas reinhardtii* in each reactor when cultivating with different nitrogen sources ($p = 0.764$, one-way ANOVA). Under steady-state

conditions, the lowest P concentration in the effluent achieved was 9.0 mg P L^{-1} using a mixture of ammonium and nitrate, correspond to 40% P uptake efficiency (Figure 5-13.a). This was significantly different from when ammonium alone and nitrate alone were used as the nitrogen source ($p = 0.001$ for when cultivating with a mixture of ammonium and nitrate and solely ammonium and solely nitrate, one-way ANOVA). Similar P concentrations in the FE were obtained as 11.3 mg P L^{-1} when *Chlamydomonas reinhardtii* was cultivated in ammonia culture and nitrate culture (see Figure 5-13.b and Figure 5-12.c respectively), consistent with the P uptake efficiencies attained being 24.7% ($p = 0.924$ for ammonium alone and nitrate alone, one-way ANOVA). Thus, a mix of ammonium and nitrate was the favoured nitrogen source for the two-stage biological process.

An ammonium recovery rate of 88.4% was achieved and nitrate was taken up via *Chlamydomonas reinhardtii* at phototrophic cultivation when a mixture of ammonium and nitrate were used as the nitrogen source. Ammonium uptake efficiency was reduced to 66.4% when ammonium alone was used. Nitrate was fully consumed via microalgae uptake under phototrophic conditions in nitrate alone culture. There was a small contribution to nitrogen recovery in the HTR. (Data is not presented here, but is fully explained in Section 5.3.9 with the nitrogen mass flow analysis).



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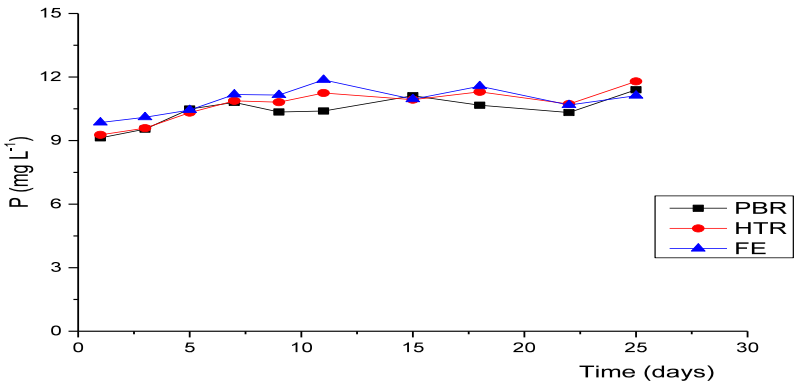

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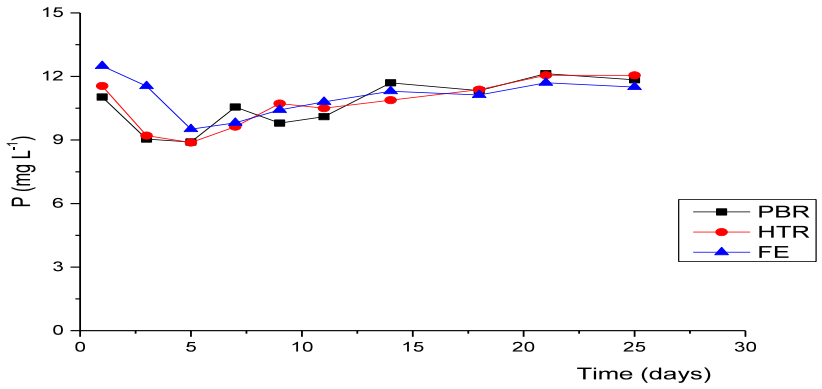

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Figure 5-13 P concentration throughout the cultivation period with different nitrogen sources: a) a mix of NH_4^+-N and NO_3^--N (50:50); b) NH_4^+-N; and c) NO_3^-- N.

Intracellular P content of *Chlamydomonas reinhardtii* under conditions of phototrophic and heterotrophic cultivation is depicted in Figure 5-14. While there was a slight difference in the percentage of intracellullar P content between the PBR and HTR, this was observed only when using a mix of ammonium and nitrate as the nitrogen source; with the use of solely ammonium and solely nitrate, intracellular P content was the same in both the PBR and HTR ($p = 0.856$ for PBR and HTR, one-way ANOVA). In addition, the variance in intracellular P content in the PBR and HTR with different nitrogen sources showed a similar trend as intracellular P content was increased gradually from $0.4 \pm 0.2\%$ to $0.6 \pm 0.1\%$ and then to $0.7 \pm 0.2\%$ for PBR and from 0.5 ± 0.1 to $0.6 \pm 0.1\%$ and then to $0.7 \pm 0.2\%$ for HTR when the nitrogen source varied from a mix of NH_4^+-N and NO_3^-

-N to solely NH_4^+-N and then to NO_3^--N, respectively (p-values for different nitrogen sources in both PBR and HTR are greater than 0.05, one-way ANOVA).

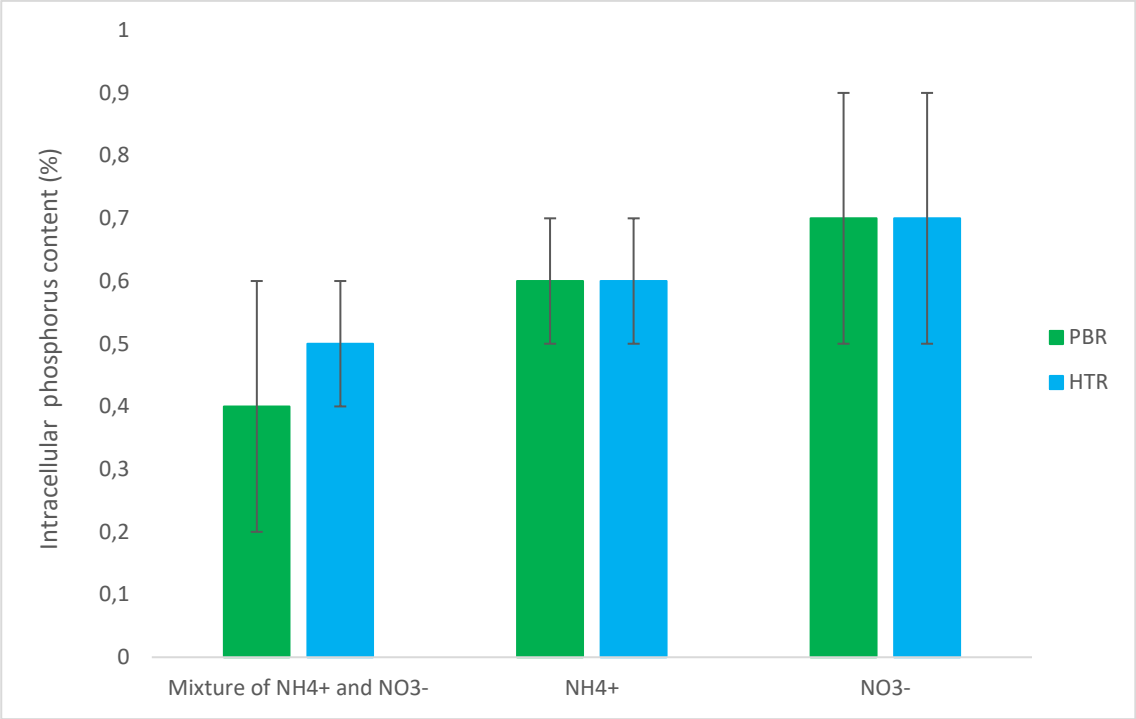


Figure 5-14 The percentage of intracellular P content in PBR and HTR when using different nitrogen sources

The relationship between phosphorus uptake by *Chlamydomonas reinhardtii* and different nitrogen sources showed an upwards pattern from a mix of NH_4^+-N and NO_3^--N to ammonium alone and then to nitrate alone (Figure 5-14). This could be attributed to nitrate being found as the favoured form of nitrogen for *Chlamydomonas reinhardtii* under both heterotrophic and phototrophic conditions (Section 4.3.2 and 4.3.6, respectively). The aim of the experiments with different nitrogen sources was to identify which treatment unit would be better for the implementation of the proposed system in the wastewater treatment works, which is the final objective of this research, because different nitrogen sources are presented in each treatment step; for instance, the inlet of the wastewater treatmen works contains ammonia alone, whereas nitrate appears after the Activated Sludge Process with its extended aeration.

5.3.9. Mass Flow Analysis of Nutrients for Different Nitrogen Species Experiments

The distribution of P species related to total phosphorus in the PBR and HTR with different nitrogen sources is shown in Figure 5-15. The inlet of the PBR contained phosphate at 86.4%, 85.3% and 94.4% and suspended organic phosphorus at 13.6%, 14.7% and 5.6% when *Chlamydomonas reinhardtii* was cultivated in media that included solely ammonium, a mix of ammonium and nitrate, and solely nitrate, respectively. The difference in suspended organic P with the use of NO_3^--N was consistent with the lowest biomass concentration in the settlement tank being obtained.

Overall, suspended organic phosphorus incremented in the effluent of the PBR regardless of the nitrogen source used. When a mix of NH_4^+-N and NO_3^--N, and solely NH_4^+-N or NO_3^--N was used as the nitrogen source, the increases in suspended organic P under phototrophic conditions achieved were 23.4%, 7.5% and 13.0%, respectively. P species remained stable under heterotrophic cultivation conditions.

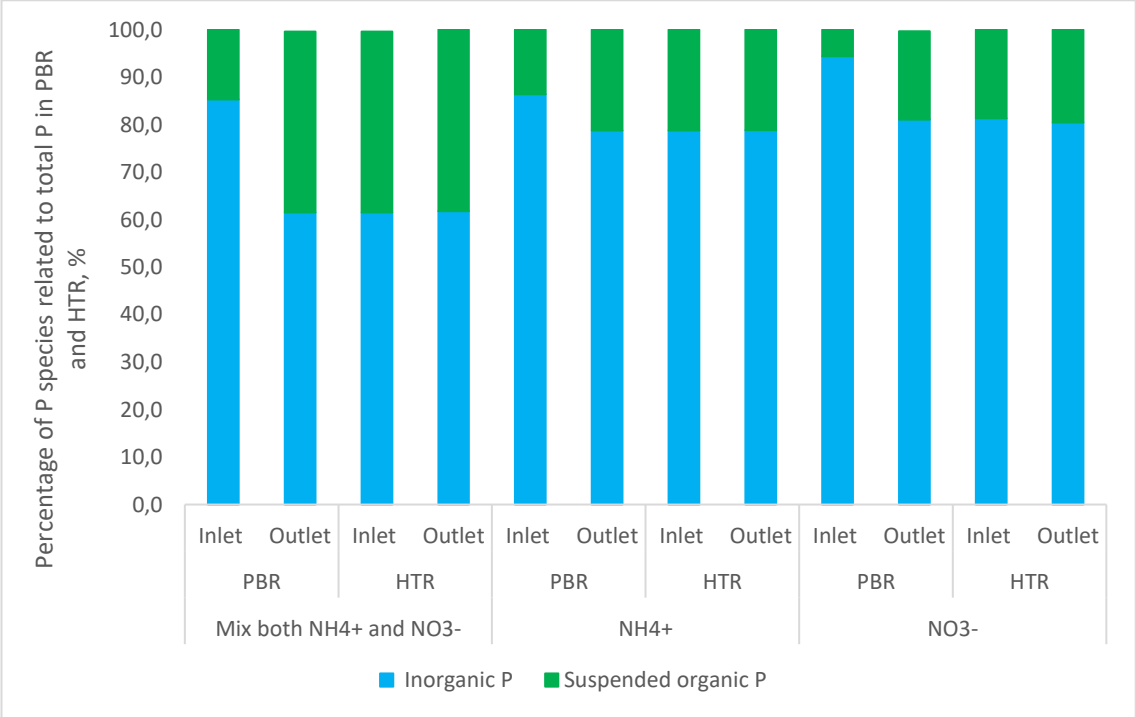


Figure 5-15 Distribution of P species in the PBR and HTR using different nitrogen sources

In regard to the division of phosphorus species related to total phosphorus, the increment in suspended organic P was ranged between 7.5% and 24% under phototrophic cultivation whilst heterotrophic growth made only a small contribution to phosphorus uptake in all experiments. The optimum operational conditions were chosen as 48 h HRT, 14 d CRT and a mix of nitrogen sources with 23.4% suspended organic P content.

The distribution of N species related to total nitrogen in the PBR and HTR with different nitrogen sources is presented in Figure 5-16. When *Chlamydomonas reinhardtii* was cultivated with a mix of NH_4^+-N and NO_3^--N, the inlet of the PBR included ammonium, nitrate and suspended organic nitrogen levels of 31.2%, 42.7% and 26.1%, respectively. A significant decline in ammonium to 5.9% was observed, and nitrate was thoroughly removed in the PBR corresponding to the increment in suspended organic N to 53.0%. Nitrogen removal by denitrification, calculated to be 41.1%. With regard to the distribution of N species under heterotrophic conditions, 10.1% and 89.9% of ammonium and suspended organic nitrogen were achieved in the inlet of the HTR and which remained steady in the HTR itself.

For the experiments where NH_4^+-N was the sole nitrogen source, levels of 54.9% and 45.1% ammonium and suspended organic nitrogen were observed in the inlet of the PBR. There was a sharp decrease in ammonium by over half to 22.0%, and suspended organic N was similarly obtained as 47.4% in the effluent of the PBR. The remainder, at 30.6%, was determined to be result of denitrification. The inlet of the HTR included 31.7% and 68.3% ammonium and suspended organic N, respectively. While heterotrophic reactor gave a small contribution to ammonium, suspended organic N declined to 59.4% in the effluent of the HTR. 10.4% of the nitrogen was removed by denitrification.

When using NO_3^--N as the sole nitrogen source, the percentage of nitrate and suspended organic N were calculated to be 78.6% and 21.4% in the inlet of the PBR, respectively. Nitrate was entirely consumed, so that the levels of suspended organic N jumped threefold to 59.8% in the outlet of the PBR. 37.2% of the total nitrogen was removed by denitrification. No nitrogen uptake/removal was achieved via either the microalgal or denitrification process under heterotrophic conditions.

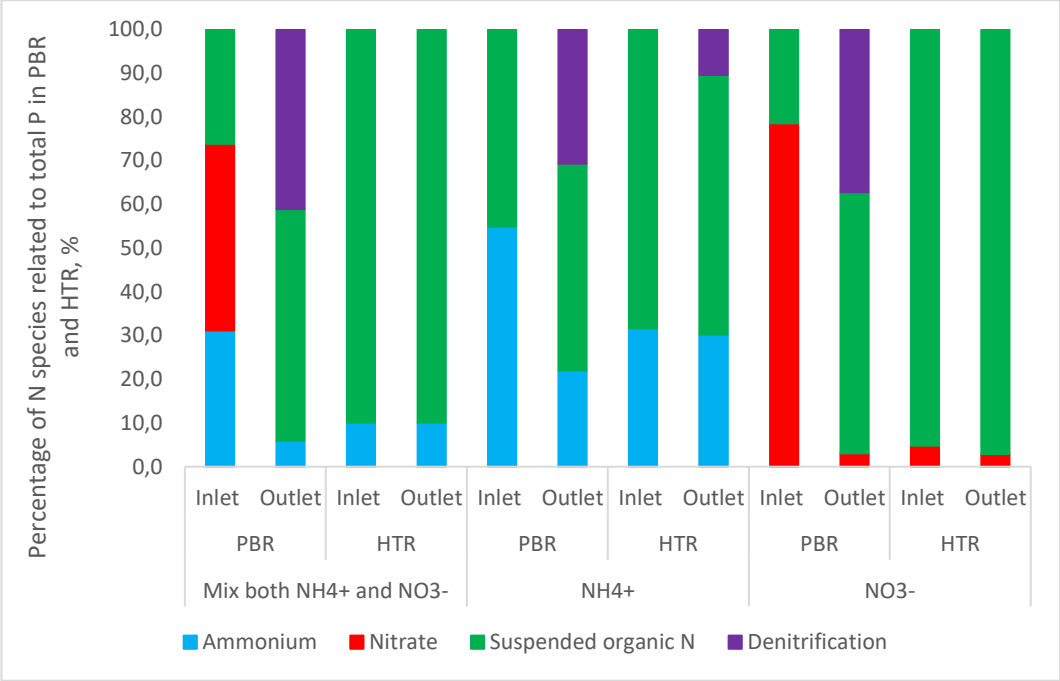


Figure 5-16 Distribution of N species in PBR and HTR using different nitrogen sources

The theoretical value for phosphorus and nitrogen in microalgae is about 1.3% and 9.2% of the dry weight based on the molecular formula of microalgae cells, i.e., $C_{106}H_{118}O_{45}N_{16}P$ (Redfield, 1934). The P content in *Chlamydomonas reinhardti* varied between 0.5%-1.2% of the dry weight in the PBR and 0.7%-1.3% of the dry weight in the HTR. Furthermore, Powell (2009) found that the average percentage of phosphorus in algal biomass was 1.2% of dry weight, but could increase up to 3.16% with luxury P uptake. *Chlamydomonas reinhardtii* contained nitrogen varying from 5.1%-9.3% of the dry weight in the PBR and 4.8%-9.9% of the dry weight in the HTR. These results were consistent with those achieved by Camargo-Valero (2008), in which the maximum nitrogen content in microalgae achieved was 10%.

5.3.10. Organic Material Removal

Organic carbon is essential for the heterotrophic metabolism of microalgae, and therefore was monitored as COD. In all experiments, over 80% of the organic carbon was consumed under phototrophic conditions and remained steady at 80% under heterotrophic conditions (data not presented). It was considered one of the possible reasons for the small contribution to phosphorus uptake in the heterotrophic reactor. This was attributed to several possible reasons, as follows:

- Low organic carbon could limit the heterotrophic growth of microalgae in accordance with its molecular formula of.
- Phosphorus is assimilated via active transport requiring ATP because microalgae cells have a negative surface charge and orthophosphate is the preferable form of phosphorus ($H_2PO_4^-$ or HPO_4^{2-}) (Cembella et al., 1982). ATP for P uptake could be obtained by mitochondrial oxidative phosphorylation in the dark, where phosphorylation requires some form of energy input to produce ATP from ADP (Cembella et al., 1982; Cai et al., 2013). This energy input could be provided by the electron transport system of the mitochondria such as the carboxylic acid cycle (TCA) (Cai et al., 2013; Perez-Garcia et al., 2011b). The TCA cycle is the oxidation pathway of acetyl coA in the mitochondria. Acetyl coA is produced after acetate is taken up by the monocarboxylic acid/proton transporter protein (Perez-Garcia et al., 2011b; Hu et al., 2017).

5.3.11. The Comparison with Alternative Approaches

The comparison with the alternative approaches in terms of phosphorus uptake by microalgae in the previously published literature was summarised in Table 5-4. Zhou et al. (2012) developed a hetero-photoautotrophic mode of microalgae growth, an integrated sequential two-stage algae culture processes, for nutrient recovery and lipid accumulation. *Auxenochlorella protothecoides* was first cultivated in heterotrophic culture to improve nutrient recovery and maximise cell density, and then solid and liquid phases were separated by self-sedimentation; secondly, harvested biomass was cultivated into the supernatant enriched with CO_2 under phototrophic conditions in order to further nutrient recovery and accumulate higher lipid content in the nitrogen-deficient culture. The total phosphorus removal efficiency achieved was 98.5%. Although concentrated municipal wastewater that included a higher phosphorus concentration (212 ± 7.2 mg PO_4^{3-} L^{-1}) was used as the culture medium, further investigation of this system under continuous culture conditions was recommended. *Scenedesmus obliquus* was cultivated in a flat-panel reactor in continuous culture by feeding the system with secondary effluent at a light intensity of 250 $\mu molm^{-2}s^{-1}$ and with a photoperiod that employed 14 h: 10 h light:dark cycle. Phosphorus recovery efficiencies ranged between 90%-98% in different HRTs from 1.1 d to 3.4 d (Ruiz et al., 2013). Sutherland et al. (2014) investigated the effect of depth of high rate algal ponds (HRAPs) on microalgae growth and nutrient recovery in different seasons. The primary effluent of domestic wastewater with

additional CO_2 was used and wastewater algae (algal consortium dominated by *Mucidosphaerium pulchellum*) was inoculated. HRAPs were operated with 9 d, 6 d and 4 d retention times in the winter, spring and summer, respectively. The highest removal efficiency for phosphorus was achieved in the winter (34.3 ± 25.7% – 26.7 ± 25.6%), followed by the summer (23.4 ± 17.6% - 19.8 ± 16.2%), whilst phosphorus recovery efficiency in the spring (19.2 ± 13.5% - 11.6 ± 10.4%) was the lowest of all the HRAPs depths tested (200 mm, 300 mm and 400 mm). Phosphorus recovery decreased with increasing depth of the HRAPs. *Chlorella vulgaris* was preferred as the model microalgae strain and cultivated in the membrane photobioreactor (MBPR) in the continuous culture. Aquaculture wastewater was used as the culture medium and the MBPR was operated at HRT of 1 d, a light intensity of 9000 lux and temperature of 25 ± 2°C. 82.7% phosphorus recovery was obtained (Gao et al., 2016). Iman Shayan et al. (2016) studied the influence of HRT on nutrient removal and bioproduct production using a rotating algal biofilm reactor (RABR) in a fed-batch culture. The RABR was fed by municipal wastewater and sludge with which the mixture of algal strains was cultivated. The RABR was operated using 2 d and 6 d HRTs and 8 h: 16 h dark: light period and a light intensity of 200 ± 20 $\mu molm^{-2}s^{-1}$. The maximum phosphorus recovery efficiency achieved was 99.5% at 6 d HRT. Although higher P recovery efficiencies were achieved in their research using alternative cultivation systems, lower phosphorus concentrations were used to feed the systems compared to the ones used in this study. In addition, other nutrients did not present any limitation for P uptake, unlike this study.

Similar phosphorus uptake efficiency (50%) was obtained by Yulistyorini (2016) in which *Chlamydomonas reinhardtii* was cultivated under mixotrophic conditions combining heterotrophic and phototrophic microalgae growth with biomass recirculation under continuous flow conditions. It was recommended to cultivate mixotrophic microalgae in the continuous culture using a phototrophic reactor followed by a heterotrophic reactor arranged in series in order to retain the polyphosphate accumulation. This approach was used in this study to identify the optimum operation conditions for the two-stage microalgal process in order to maximise biomass production for nutrient uptake. Even though slightly lower biomass concentrations and phosphorus uptake efficiencies were obtained in this thesis, one might nevertheless anticipate that both microalgae growth and nutrient recovery will be increased when the operational conditions are optimised because the system demonstrated considerable potential for

implementation in conventional wastewater treatment works with regard to its capacity for nutrient recovery and control.

Table 5-4 Comparison phosphorus uptake efficiency with the previous literature

Microorganism	Cultivation system	Phosphorus recovery efficiency (%)	Reference
Auxenochlorella protothecoides	Sequential hetero-photoautotrophic two-stage	98.5	Zhou et al. (2012)
Scenedesmus obliquus	Flat-panel reactor in the continuous culture	90-98	Ruiz et al. (2013)
Microalgal consortium (the most dominant specie is *Mucidosphaerium puchellum*)	High rate algal ponds	11.6±10.4-34.3±25.7	Sutherland et al. (2014)
Chlorella vulgaris and *Scenedesmus obliquus*	Membrane photobioreactor in the continuous culture	82.7	Gao et al. (2016)
Sludge including mixture of algae	Rotating algal biofilm reactor in the fed-batch culture	99.5	Iman Shayan et al. (2016)
Chlamydomonas reinhardtii	Mixotrophic culture under continuous flow conditions	50	Yulistyorini (2016)
Chlamydomonas reinhardtii	Two-stage biological process comprising phototrophic and heterotrophic microalgae growth under continuous flow conditions with biomass recycle	40	This study

5.4.Conclusion

The aim of this chapter was to identify the optimum operational conditions for the two-stage biological process combining phototrophic and heterotrophic microalgae cultivation with biomass recirculation under continuous flow conditions with regard to nutrient control and recovery via biological uptake. *Chlamydomonas reinhardtii* recovered phosphorus and nitrogen at 40% and 93.2%, respectively, under optimum operational conditions of 48 h HRT, 14 d CRT and a mix of nitrogen sources. The contribution from the HTR to nutrient recovery was limited under all experimental conditions. Regarding the mass flow analyses of nutrient, the nitrogen and phosphorus content in *Chlamydomonas reinhardtii* varied from 5.3% to 9.6% of the dry weight and from 0.6% to 1.2% of the dry weight throughout the system under the various operational conditions tested. Although nutrient recovery could not be accounted for by only microalgal uptake due to bacterial contamination, the two-stage biological process combining phototrophic and heterotrophic microalgae cultivation with biomass recycling under continuous flow conditions demonstrated excellent potential in terms of its implementation in conventional wastewater treatment works with respect to its capacity for nutrient recovery and control by achieving high nitrogen and phosphorus recovery. Furthermore, algal biomass concentration and P uptake via microalgae could be improved if *Chlamydomonas reinhardtii* is cultivated in a media including greater carbon and nitrogen concentrations.

6. CHAPTER: THE POTENTIAL TO IMPLEMENT MICROALGAE SYSTEMS IN WASTEWATER TREATMENT PLANTS

6.1. Introduction

With the increase in urbanisation and global population, the amount of wastewater produced by anthropogenic activities has increased considerably in the last few decades (Cai et al., 2013; Gonçalves et al., 2017). The release of an excessive amount of wastewater containing nitrogen and phosphorus, limiting nutrients for plankton, into water courses results in serious environmental problems such as eutrophication by algal blooms (Evans et al., 2017; Bougaran et al., 2010). In this context, it is important to remove sufficient amounts of nutrients from wastewater sufficiently prior to discharge.

Current wastewater treatment works use a well-established bacterial process in order to remove organic materials, namely the activated sludge process (ASP). Biological nutrient removal (BNR) processes are used to remove nitrogen and phosphorus from wastewater. BNR combines anaerobic, anoxic and aerobic phases. Nitrogen is removed by a nitrification and denitrification process with autotrophic and heterotrophic bacteria under anaerobic and anoxic conditions, respectively. Phosphorus is recovered by enhanced biological phosphorus removal (EBPR) with phosphorus accumulating organisms (PAOs) under serial anaerobic and aerobic or anoxic conditions (Xu et al., 2014; Vargas et al., 2016). In addition to BNR, chemical and physical processes can be applied to remove nutrients from wastewater, such as through ammonia stripping, phosphorus precipitation, etc.

Despite the fact that the BNR process is effective at removing nutrients, it has various drawbacks such as high cost, high energy consumption due to the need for aeration, the need for additional chemical reagents and the emission of greenhouse gases such as CO_2 and N_2O from the degradation of organic matter and incomplete denitrification (Anbalagan et al., 2016; Iman Shayan et al., 2016; Shen et al., 2017; Evans et al., 2017; Gao et al., 2018). Clearly, there is a need to develop an alternative process that allows for a reduction in net energy consumption for highly efficient nutrient recovery from

wastewater due to more stringent discharge consents, mainly with regard to dissolved phosphorus.

Wastewater has been proposed as a sustainable feedstock for microalgae growth due to the fact that it contains large amount of the nitrogen and phosphorus that microalgae require in water for their growth (Shi, 2009; Wang et al., 2010). In order to identify which treatment location is better for microalgae cultivation in wastewater, many studies have been undertaken that considered different treatment steps such as the primary effluent, secondary effluent, and digested liquor because of each treatment step includes different nitrogen and phosphorus concentrations (Cho et al., 2013; Su et al., 2012; Lv et al., 2016; Cho et al., 2011; Wang et al., 2010; Kong et al., 2010).

Primary effluent is thought to be more appropriate for heterotrophic and/or mixotrophic microalgae cultivation because it contains higher organic carbon than any of the other treatment steps; however, primary effluent may not be practical for large-scale cultivation due to the competition with other microbial consortia, particularly in the presence of large amounts of bacteria (Guldhe et al., 2017). Wang et al. (2010) compared four different wastewater samples, namely raw wastewater, primary effluent, secondary effluent and centrate (centrifuged sludge), in order to evaluate the feasibility of microalgae growth by *Chlorella sp.*, noting that centrate is the most appropriate location for microalgae growth, due to higher nitrogen and phosphorus concentrations than the other three locations. Despite this finding, the main challenge to microalgae cultivation in liquor is a high suspended solids content, inducing shortened light transmittance (Akerstrom et al., 2014). As suggested by Arias et al. (2018), recycling digestate liquor after the secondary effluent increases the availability of nutrients for microalgae cultivation because secondary effluent contains limited soluble nitrogen and phosphorus. In addition, a mix of digestate liquor and secondary effluent offers an excellent solution to decrease the colouration of the liquor and to reduce the amount of potential contaminants.

Microalgae have also been attractive for the food, pharmaceutical and energy sectors because microalgae biomass includes valuable bioactive metabolites such as lipids and fatty acids, pigments (astaxanthin, β-carotene), protein (amino acids), carbohydrates and vitamins (Matos, 2017). However, commercial cultivation of microalgae is not cost-effective (Sharma et al., 2017). Despite the fact that the combination of microalgae production and wastewater treatment can reduce costs due to the high nutrient content in

wastewater that is required for microalgae growth, there might be certain limitations with regard to food and pharmaceutical applications due to the possibility of biologically and/or chemically toxic substances being present in the harvested algal biomass due to the septic conditions in wastewater treatment works (Matos, 2017; Mostafa, 2012). The Regulation on Food Safety and the Regulation on Novel Food and Novel Food Ingredients, Food and Drug Administration, Food Standards Australia New Zealand and Agencia Nacional de Vigilancia Sanitaria regulate for the commercial production of microalgae-based food in the EU, the USA, Australia and New Zealand and Brazil, respectively (Garcia et al., 2017; Matos, 2017). Thus, microalgae can only be cultivated in wastewater treatment works in order to produce low-value products such as biofuel, biodiesel, etc.

Microalgae cultivation in wastewater treatment works can thus overcome the disadvantages of the BNR processes with the additional benefit of producing biofuels (Monfet and Unc, 2017; Ramsundar et al., 2017; Gonçalves et al., 2017). In this regard, this chapter assesses the potential to implement a two-stage biological process that combines phototrophic and heterotrophic microalgae cultivation under continuous flow conditions with biomass recycling in currently existing wastewater treatment plants. A mass balance of nutrients was determined using a lab-scale algal bioengineering process (Chapter 5), whilst the nitrogen and phosphorus mass balances were analysed at Esholt Wastewater Treatment Works.

6.2. Methodology

This study investigated the potential for the implementation of a two-stage mixotrophic microalgae cultivation under continuous flow conditions with biomass recycling within an existing nutrient recovery and control system. The removal of nutrients at Esholt WwTW was analysed as a case study. Wastewater samples were collected on a monthly basis from October 2014 to May 2017 at different treatment stages such as raw wastewater, primary effluent, inlet and outlet activated sludge process, anaerobic digestion, secondary effluent and the final effluent (sampling points are illustrated in Figure 3-6, numbered 1, 2, 3 and 4, 7, 5 and 6, respectively). Solids and nutrients were determined by TSS, VSS, TKN, NH_4^+-N, NO_3^--N, TP, PO_4^{3-}-P and COD analyses according to that appropriate standard methods (see Table 3-5) (APHA, 2012). Mass flow analyses of nitrogen and phosphorus throughout Esholt WwTW and a two-

stage mixotrophic microalgal process were conducted. A model was designed in order to scale-up the proposed system for implementation in large-scale wastewater treatment plants.

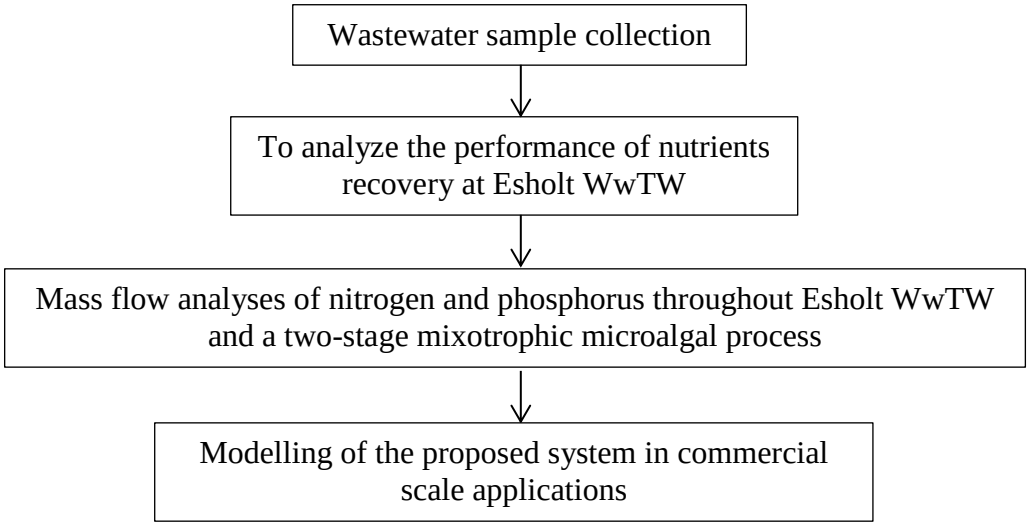


Figure 6-1 Stages of methodology used in Chapter 6

6.3. Results and Discussion

6.3.1. Annual Wastewater Characterisation at Esholt WwTW

Annual wastewater characterisation was performed with samples from the inlet, and primary and secondary effluent at Esholt WwTW. The variations in concentrations of phosphorus, ammonium, nitrate and organic materials during the sample collection period are presented in Figure 6-2 (some data could be missing because of the maintenance of equipment in the laboratory).

Overall, nutrients (except nitrate) showed the same trends for the inlet in the summer and winter seasons. Higher concentrations of nutrients were obtained in the summer than in the winter. This could be attributed to total rainfall continuously increasing from summer to winter, resulting in the dilution of the nutrients present in the raw wastewater (MetOffice). It can be clearly seen that an unforeseen loading occurred in December 2016.

Similar patterns were observed between the inlet and PST for all nutrients. Where they were increased/decreased at the inlet, there was an increment/decline in the primary effluent. PST is a physical treatment for settling larger suspended organics. Besides solids

removal in primary effluent, average PO_4^{3-}-P, NH_4^+-N and COD removals of 68.2%, 27.7% and 65.6% were achieved, respectively.

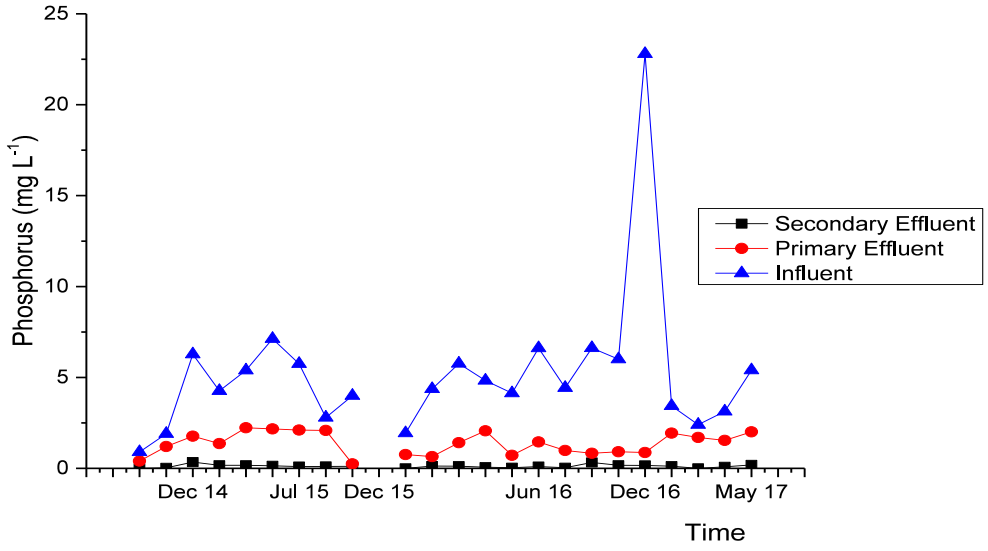
There was a further removal of phosphorus, ammonium and organic materials in the secondary effluent after the Activated Sludge Process. While slight increases in removal efficiencies of PO_4^{3-}-P and COD of 29.5% and 28% were observed, a sharp increment was observed for NH_4^+-N removal (68.2%) because the nitrification process was completed in the ASP. Total removal efficiencies of PO_4^{3-}-P, NH_4^+-N and COD were achieved of 97.7%, 95.9% and 93.6% ,corresponding to average PO_4^{3-}-P, NH_4^+-N and COD concentrations of 0.1, 1.1 and 56.5 mg L^{-1}, respectively.

Nitrate is showed a somewhat different behaviour pattern in wastewater treatment plants. Nitrate concentration in the inlet fluctuated between 0.1 to 2.0 mg NO_3^--N L^{-1} along with the sample collection period due to the fact that nitrate is an oxidised form of nitrogen and, therefore, raw municipal wastewater cannot contain nitrate inherently. Whereas there was no difference in nitrate concentration between raw wastewater and primary effluent, a significant increment was observed in the secondary effluent due to the nitrification process and incompleted denitrification in the ASP due to high ammonia loading from the digested liquor into the ASP. The average nitrate concentration was found to be 6.3 mg NO_3^--N L^{-1} in the FST.

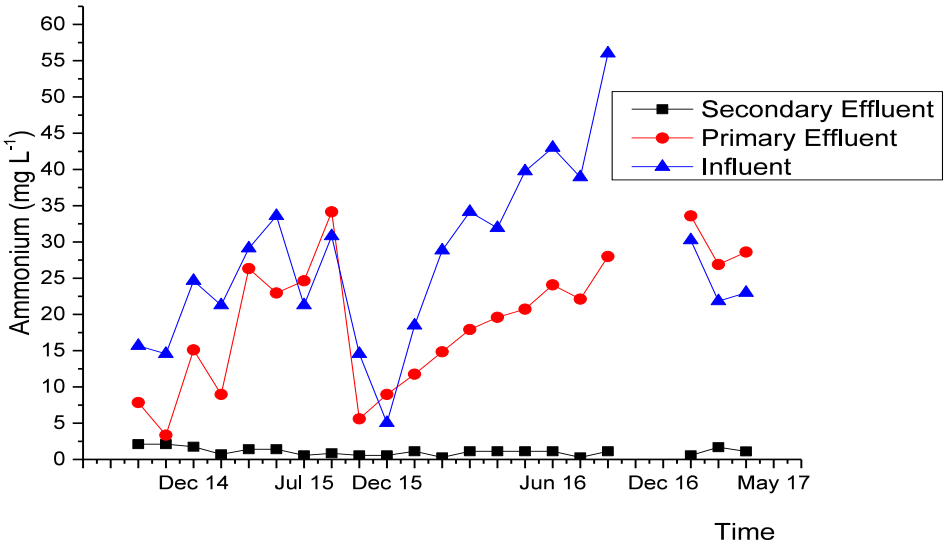
Meanwhile, higher phosphorus concentrations were attained in the final effluent (1.5 mg PO_4^{3-}-P L^{-1}) due to sand filters between FST and FE. This caused a reduction in phosphorus recovery of 65.9%. Sand filters do not have any influence on ammonium, nitrate and organic matter concentrations. The average TP, TN and COD concentrations in the FE were obtained as 1.7, 10.6 and 59.9 mg L^{-1}, respectively.

Although the discharge consents more than 100,000 p.e. set by the European Union Urban Wastewater Treatment Directive were successfully met for COD and TN (125 mg O_2 L^{-1} and 10 mg N L^{-1}, respectively), 1.7 mg L^{-1} of TP concentration in effluent at Esholt WwTW was slightly higher than the discharge consent of 1 mg P L^{-1} (UWWTD, 1991). This could be attributed to additional P loading from the anaerobic digester to the ASP. There is no discharge consent for nitrates; however, the maximum value set for nitrates is <50 mg NO_3 L^{-1} in the European Union Drinking Water Directive (DWD, 1998) to reduce any possible adverse impact of nitrates on human health.

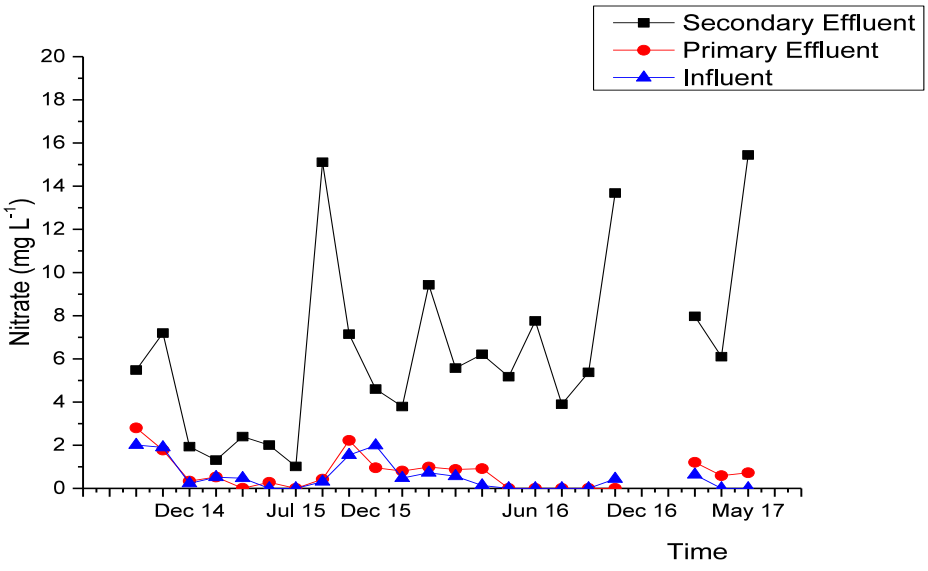
Incompleted denitrification resultes in the emission of nitrous oxide (N_2O), which has an approximately 300-fold stronger greenhouse effect than carbon dioxide (IPCC, 2001). Typically, full-scale wastewater treatment plants release between 0-14% of their total nitrogen loads as N_2O into the atmosphere (Kampschreur et al., 2009). Nitrous oxide is produced as a side-product of nitrification and intermediates in the catabolic respiratory pathway in denitrification (Kampschreur et al., 2008; Kim et al., 2010). The main parameters influencing nitrous oxide emission in wastewater treatment plants are dissolved oxygen levels, nitrite concentrations and the COD/N ratio. The release of N_2O is incremented under limited COD/N ratio conditions at the denitrification stage and, therefore, an additional organic carbon source is required to augment the denitrification rate, which in turn increases the operational cost of wastewater treatment plants (Fernandez-Nava et al., 2010). Thus, an increase in denitrification rate would not represent a rational solution at Esholt WwTW with regard to meeting the discharge consents for TN.



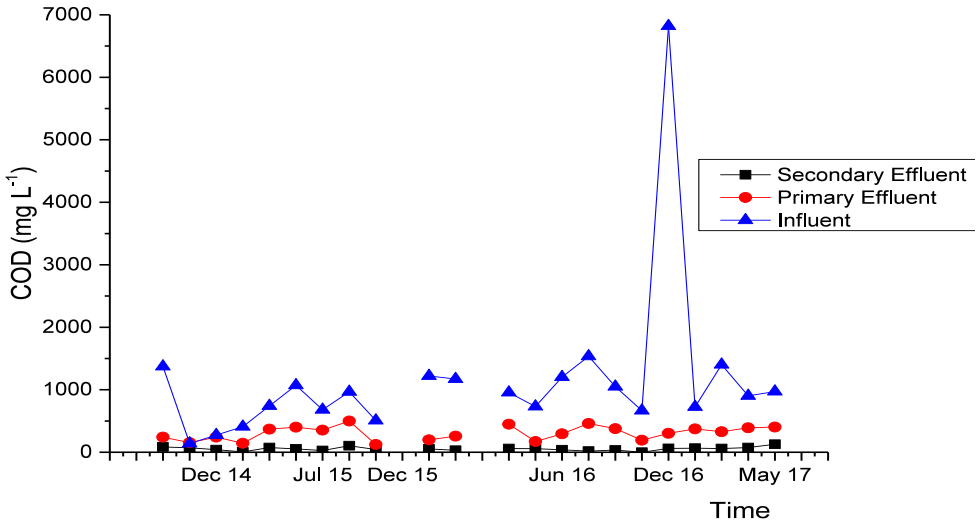
a


Ammonium (mg L-1)
60
55
50
45
40
35
30
25
20
15
10
5
0
Dec 14
Jul 15
Dec 15
Jun 16
Dec 16
May 17
Time
Secondary Effluent
Primary Effluent
Influent

b


Nitrate (mg L-1)
20
18
16
14
12
10
8
6
4
2
0
Dec 14
Jul 15
Dec 15
Jun 16
Dec 16
May 17
Time
Secondary Effluent
Primary Effluent
Influent

c



d

Figure 6-2 Annual variations of nutrients at Esholt WwTW: a) Phosphorus (P); b) Ammonium (NH_4^+-N); c) Nitrate (NO_3^--N); and d) Organic materials (COD).

6.3.2. Mass Flow Analyses of Nutrients at Esholt WwTW

It is important to analyse the mass balance of nutrients in wastewater treatment plants in order to identify the nutrient fluxes and to compare operational data under different conditions (Nowak et al., 1999; Puig et al., 2008). In this research, the mass flow analysis of nutrients was performed throughout Esholt WwTW to understand the fate of N and P during the treatment process. Table 6-1 lists the flowrates in each tank at Esholt WwTW (the data were obtained from Esholt WwTW).

Table 6-1 Flowrates for each treatment step at Esholt WwTW

Treatment Unit	Flowrate (Q) ($m^3\ d^{-1}$)
Inlet	280022
PST	280022
ASP;	
➢ Inlet	280022
➢ Digested Liquor	1598
➢ Recycled Filtrated Digested (RFD)	1440
➢ Recycled Activated Sludge (RAS)	223949
FST	280022
FE	280022

6.3.2.1. Mass Balance Analysis of Nitrogen

Various forms of nitrogen (NH_4^+-N, NO_3^--N, N_2O, N_2 and organic N) were found due to the fact that nitrogen was removed via a nitrification-denitrification process by the bacteria in wastewater treatment plants (Metcalf&Eddy, 2003). The nitrogen mass balance throughout Esholt WwTW is presented in Figure 6-3.

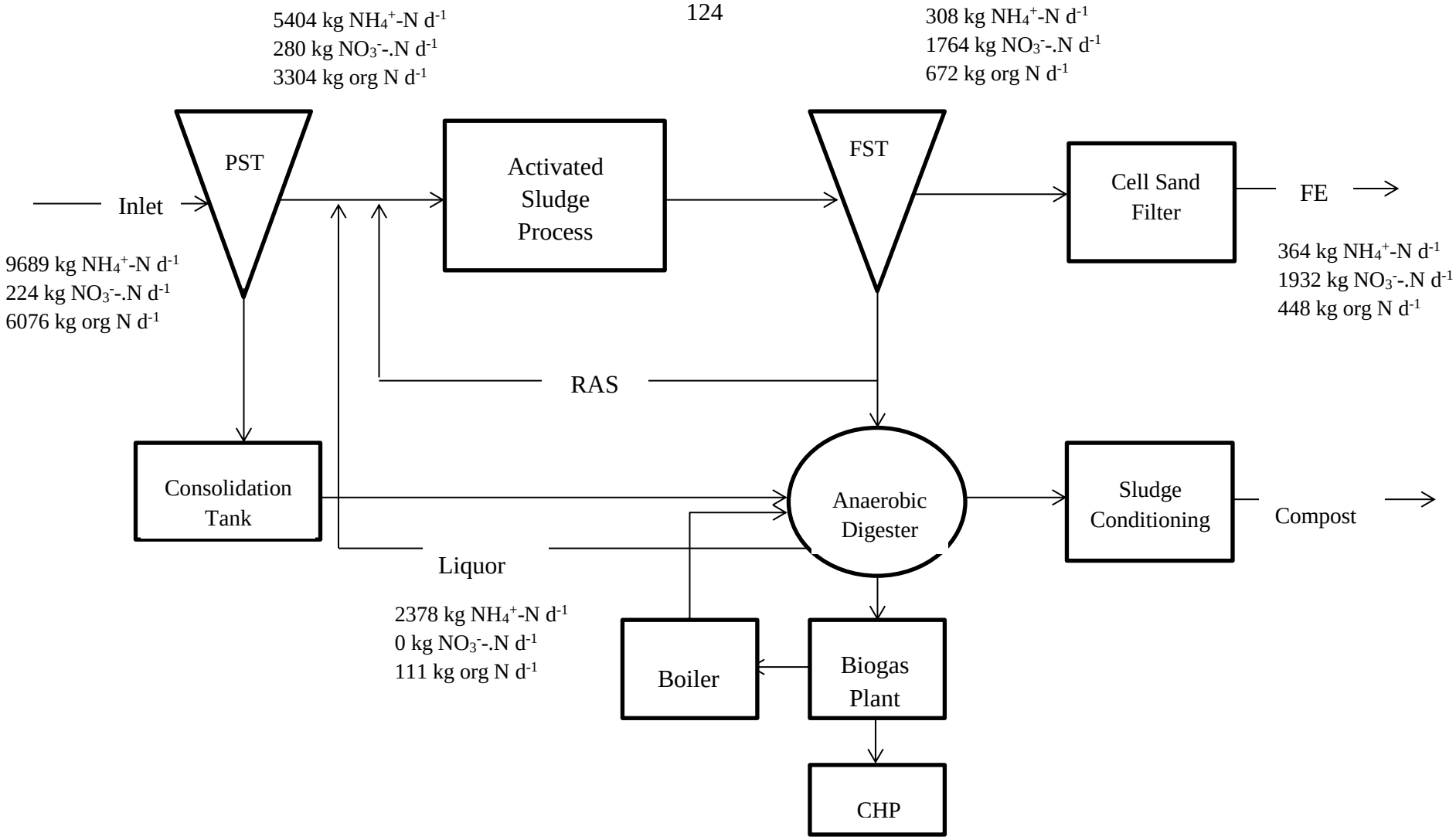


Figure 6-3 The nitrogen mass balance at Esholt WwTW

The boundaries of the system should be defined precisely in order to perform an accurate mass flow analysis. In this study, the inlet and outlet of the system were determined as being the inlet of activated sludge and the outlet of the final settlement tank, respectively (as shown in Figure 6-4). Nitrogen species coming from primary effluent and digested liquor were considered a resource for ASP, and nitrogen forms in the secondary effluent were taken into account in the outlets of the system.

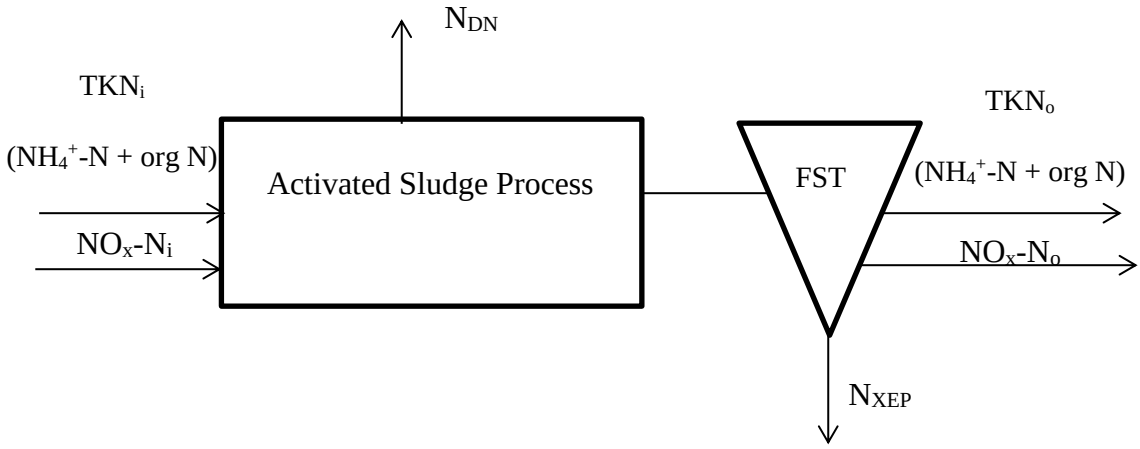


Figure 6-4 Nitrogen species at the system boundary

The masses of nitrogen remove from ASP by the denitrification process (N_{DN}) and wasted biomass (N_{XEP}) were calculated using equations 6-1 and 6-2, respectively, as recommended by Nowak et al. (1999).

$$N_{DN} = TKN_i + NO_x\text{-}N_i - TKN_o - NO_x\text{-}N_o - N_{XEP} \quad \text{Eq. 6-1}$$

$$N_{XEP} = \text{Org } N_i - \text{Org } N_o \quad \text{Eq. 6-2}$$

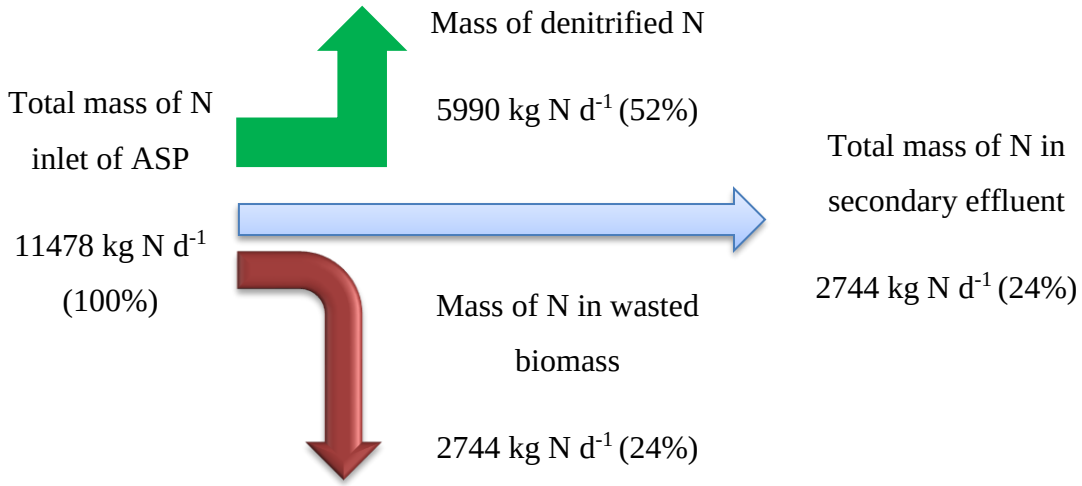


Figure 6-5 Mass balance of nitrogen in the ASP

Figure 6-5 depicts the mass balance of nitrogen during the activated sludge process. Whereas the greatest nitrogen removal achieved was 52% by nitrification-denitrification process, around half of this ratio (24%) was recovered by transferring the sludge into an anaerobic digester. Secondary effluent contained 24% of the nitrogen as well. Nitrification-denitrification was thought to be the main nitrogen removal process in Esholt WwTW due to the mass of denitrified nitrogen. While 64% of the nitrogen's mass in the effluent was the form of nitrate, ammonia and organic nitrogen forms constituted only 11 and 25%, respectively due to higher nitrification rate.

Lee et al. (2008b) obtained 33.3% and 49.7% of the nitrogen removal by denitrification and bacterial uptake, respectively, with a further 14.9% of the nitrogen being discharged. To compare with the nitrogen balance at Esholt WwTW, the denitrification rate was lower and greater amount nitrogen was removed by waste activated sludge. In addition, a lower denitrification rate achieved was 25% and 30% in two different full-scale wastewater treatment plants than that at Esholt WwTW (Mekinia et al., 2009).

6.3.2.2. Mass Balance of Phosphorus

Phosphorus is presented in both organic form and in phosphate form in wastewater treatment plants. The mass balance of phosphorus species in Esholt WwTW is presented in Figure 6-6.

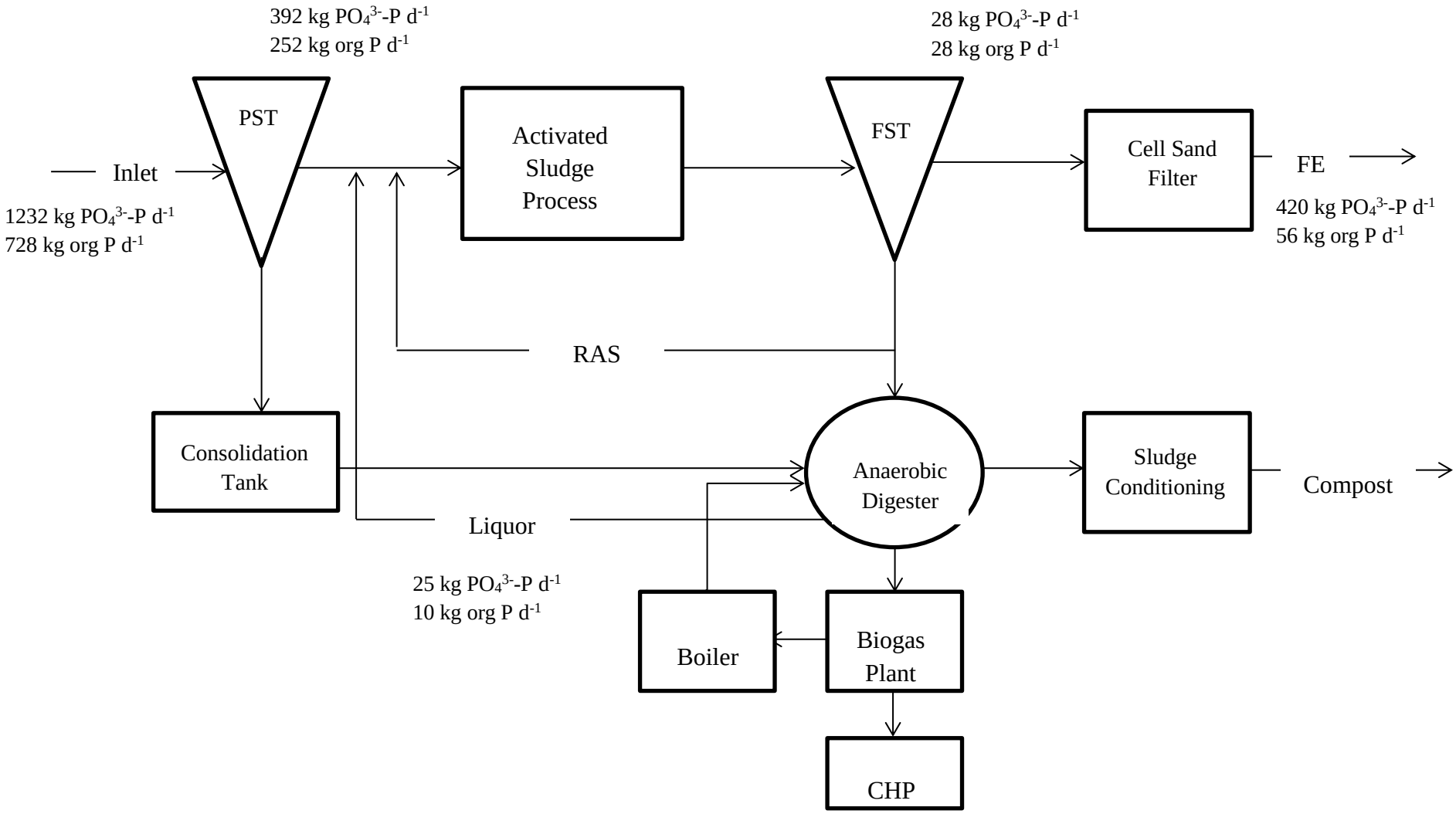


Figure 6-6 The phosphorus mass balance at Esholt WwTW

The same assumptions for the nitrogen mass balance with regard to the boundaries of the system and resource for the inlet of the ASP were used in order to perform a mass flow analysis of phosphorus species in the ASP. Phosphorus is recovered from wastewater treatment plants by wasted activated sludge as, shown in Figure 6-7. The mass balance of phosphorus was identified via equation 6-3, as suggested by Nowak et al. (1999).

$$TP_i = TP_o + P_{XEP} \qquad \text{Eq. 6-3}$$

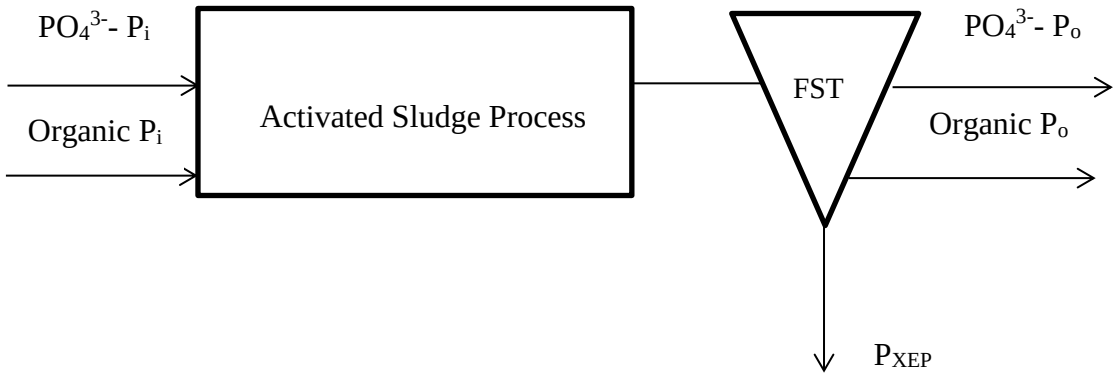


Figure 6-7 P species at the system boundary

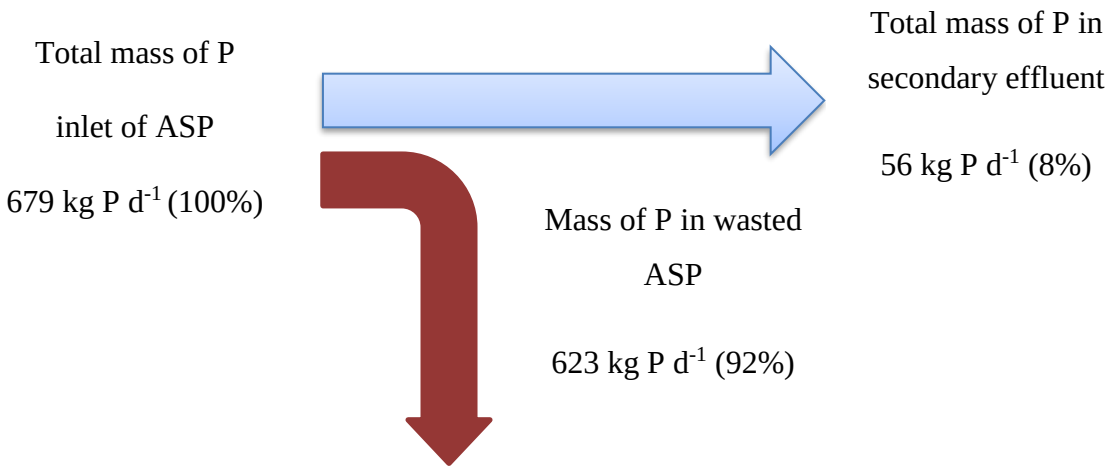


Figure 6-8 Mass balance of phosphorus in the ASP

The percentage mass of total phosphorus in the activated sludge process is shown in Figure 6-8. 92% of the mass of phosphorus was recovered by waste activated sludge, with the remaining 8% transferred to the tertiary treatment step, proving that the essential mechanism for phosphorus removal is bacterial uptake because EBPR is used for phosphorus removal at Esholt WwTW. Similar results have been reported in the literature.

Cornel and Schaum (2009) found that 90% of phosphorus was recovered by sludge in a typical German municipal wastewater treatment plant. Phosphorus uptake via sludge ranged from 83.38% to 91.24% of the total phosphorus loading in the A^2O process for a municipal wastewater treatment plant in China (Rui-Xia et al., 2011).

Enhanced phosphorus biological removal is achieved by recovery through activated sludge under anaerobic and aerobic/anoxic conditions (Semerci and Hasılcı, 2016). EBPR has shown that there is an almost twofold increment in phosphorus removal, i.e., from 40-50% to 90%, compared to conventional wastewater treatment plants (Venkatesan et al., 2016). A lack of organic carbon can limit EBPR and, therefore, additional carbon sources are required (Zou and Wang, 2016).

In conclusion, the increased cost due to the requirement for external organic carbon sources for denitrification and enhanced biological phosphorus removal, and the emission of nitrous oxide during denitrification are considered the main deficiencies of existing wastewater treatment plants with regard to nitrogen and phosphorus recovery. Although Esholt WwTW demonstrably met the discharge consents for COD and TN, higher TP than allowed by the associated discharge consent (< 1 mg P L^{-1}) was observed in the final effluent. Furthermore, with regard to nitrogen removal, higher nitrification rates resulted in an increased nitrate concentration in the final effluent and where nitrogen is removed by denitrification rather than by bacterial uptake. Thus, more environmentally friendly and cost-effective treatment methods, such as microalgae cultivation in wastewater treatment plants, could represent potential solutions to overcome these problems.

6.3.3. Mass Balance Analyses of Nutrients in the Two-Stage Biological Process

Mass flow analyses of nutrients in a two-stage biological process combining phototrophic and heterotrophic microalgae (under continuous flow conditions with biomass recycling) were performed in Chapter 5 for different operational conditions. Table 6-2 and 6-3 summarise the mass balance of nitrogen and phosphorus, respectively, under all experimental conditions tested.

Table 6-2 Nitrogen balance in two-stage biological process

Number of Experiment	HRT (h)	CRT (d)	NH_4^+-N in SWW (g m^{-3})	NO_3^--N in SWW (g m^{-3})	Sus Org N in PBR (mg d^{-1})	Sus Org N in HTR (mg d^{-1})	Sus Org N in the system (mg d^{-1})	N in biomass in PBR (% DW)	N in biomass in HTR (% DW)	Total N in biomass (% DW)
1	36	14	25	25	124	116	240	7.0	7.7	7.3
2	48	14	25	25	60	60	120	5.7	6.3	6.0
3	72	14	25	25	38	41	79	5.4	6.3	5.8
4	48	7	25	25	78	67	145	9.3	9.9	9.6
5	48	21	25	25	90	58	148	5.7	4.8	5.3
6	48	14	50	-	83	74	157	8.2	7.5	7.9
7	48	14	-	50	48	53	101	5.1	6.5	5.8

Table 6-3 Phosphorus balance in two-stage biological process

Number of Experiment	HRT (h)	CRT (d)	PO_4^{3-}-P in SWW (g m^{-3})	Sus Org P in PBR (mg d^{-1})	Sus Org P in HTR (mg d^{-1})	Sus Org P in the system (mg d^{-1})	P in biomass in PBR (% DW)	P in biomass in HTR (% DW)	Total P in biomass (% DW)
1	36	14	15	18	18	36	1.0	1.2	1.1
2	48	14	15	11	13	24	1.1	1.3	1.2
3	72	14	15	7	7	14	0.9	1.0	1.0
4	48	7	15	10	8	18	1.2	1.2	1.2
5	48	21	15	14	12	26	0.9	0.9	0.9
6	48	14	15	7	7	14	0.5	0.7	0.6
7	48	14	15	7	6	13	0.7	0.8	0.8

With regard to the mass balance of nitrogen (Table 6-2), the mass of suspended organic nitrogen varied between 38 and 124 mg d^{-1} in the PBR, and from 41 to 116 mg d^{-1} in the HTR under different operational conditions. While the highest total mass of suspended organic nitrogen in the two-stage biological process was achieved at 36 h HRT, the lowest was found at 72 h HRT. This was attributed to higher nutrient load at a higher flowrate of the feeding medium.

Generally, suspended organic nitrogen in the PBR was higher than that in the HTR; however, the percentage of nitrogen in the biomass in the PBR was mostly lower than in the HTR. Total nitrogen in the two-stage biological varied from 5.3% to 9.6% of the dry weight. This fluctuation could be explained by various different microalgae concentrations that were obtained under different operational conditions, even though nitrogen recovery efficiencies remained similar.

With regard to the mass balance of phosphorus (Table 6-3), overall no significant differences were observed in the mass of suspended organic phosphorus in each reactor. Suspended organic phosphorus incremented by around two-and-a-half-fold with the decrease of HRT from 72 h to 36 h as suspended organic nitrogen due to the increased nutrient load. In addition, there was a sharp increase in the mass of suspended organic phosphorus from 18 to 24 mg d^{-1} between 7 d and 14 d CRT, which then remained stable on further increase of CRT to 21 d. Moreover, the greatest mass of suspended organic phosphorus was achieved when a mix of ammonium and nitrate were used. The use of single nitrogen sources did not appear to have any significant influence.

The percentage of phosphorus in the biomass in the HTR was equal to or greater than that in the PBR under different operational conditions. This could be attributed to lower biomass concentrations in the HTR compared to in the PBR, although similar suspended organic phosphorus concentrations were attained in each reactor for all experiments. The total percentage of phosphorus in the biomass in the two-stage biological process varied from 0.6 to 1.2% of the dry weight. The maximum P content in the biomass was achieved at 48 h HRT, 14 d CRT, and using a mix of ammonium and nitrate; therefore, these conditions were identified as the optimum operational conditions with regard to phosphorus recovery via *Chlamydomonas reinhardtii* for the two-stage biological process in order to implement a lab-scale system in existing wastewater treatment works.

Nitrogen and phosphorus content in *Chlamydomonas reinhardtii* differed from 5.3 to 9.6% of the dry weight and from 0.6 to 1.2% of the dry weight, respectively, throughout a two-stage biological process in this research. The typical nitrogen and phosphorus contents in algae cells were found to be 10% and 1%, in sequence (Camargo-Valero et al., 2009b; Larsdotter, 2006; Tarayre et al., 2016; Brown and Shilton, 2014). Phosphorus uptake by microalgae is incremented by up to 3% of the dry weight by luxury uptake mechanisms (Shilton et al., 2012; Tarayre et al., 2016; Camargo-Valero et al., 2010).

With regard to ammonium recovery efficiency in the two-stage biological process in Chapter 5, 88.4% of NH_4^+-N was recovered by *Chlamydomonas reinhardtii* under optimum operational conditions, namely 48 h HRT, 14 d CRT and a mix of nitrogen sources. A similar N recovery efficiency was obtained in the literature. Li et al. (2011) revealed more than 93% and 89% NH_4^+-N and TN removal with a mixture of microalgae consortium in municipal wastewater. Ammonium recovery of 97% and 90% was achieved in both artificial and urban wastewater by *Scenedesmus obliquus* under semi-continuous culture (Ruiz-Marin et al., 2010) Moreover, ammonium was fully consumed by microalgae and bacteria, *Chlorella vulgaris* and *Azospirillum brasilence*, under semi-continuous culture (de-Bashan et al., 2002). It has been shown that high ammonium recovery efficiency is achieved by microalgae assimilation by overcoming the drawback of nitrification, namely the need for aeration.

Besides an effective ammonium recovery, nitrate was fully removed in this research (Chapter 5). However, as stated in Chapter 5, it is important to note that denitrification also occurred in the two-stage biological process and, therefore, 100% nitrate assimilation was not possible by *Chlamydomonas reinhardtii.* This was monitored by measuring nitrous gas during the experimental period. The nitrate uptake ability of *Chlamydomonas reinhardtii* was confirmed by Su et al. (2012), achieving a nitrate recovery efficiency of 92.7%.

To summarise, 93.2% of total nitrogen recovery was achieved in the two-stage biological process. The nitrogen recovery trend for both nitrogen species in this study was similar to those obtained by Yulistyorini (2016). In comparison with the literature on the use of other microalgae species, a higher TN recovery efficiency was attained. Ruiz et al. (2013) found that TN removal varied from 81% to 91% with different HRTs ranging from 1.1 d to 3.4 d under continuous culture by *Scenedesmus obliquus* being cultivated in secondary effluent from a wastewater treatment works in Spain. *Chlorella vulgaris* has

been reported to show 86.1% TN removal in a membrane photobioreactor by continuous feed with aquaculture wastewater (Gao et al., 2016).

With regard to phosphorus recovery, 40% of phosphorus was assimilated into *Chlamydomonas reinhardtii* under the optimum operational conditions in this research. Unfortunately, P recovery efficiency was lower compared that reported in the literature (Yulistyorini, 2016; Ruiz et al., 2013; Li et al., 2011; Gao et al., 2016; de-Bashan et al., 2002), which ranged from 50% to 98%. This could be attributed to different microalgae species such as *Chlorella, Scenedesmus,* etc., environmental and operational conditions such as the use of various types of PBRs, and the cultivation of microalgae in different feeding media etc.

6.3.4. Modelling of Phosphorus Recovery in the Two-Stage Biological Process with regard to the Potential for Implementation in Existing Wastewater Treatment Works

Although the phosphorus content in biomass achieved was 1.2% of the dry weight, the total phosphorus concentration in the final effluent of mixotrophic microalgae cultivation at continuous culture was 9.0 mg P L^{-1}, which is quite high according to the discharge consents set by the Urban Wastewater Treatment Directive (UWWTD, 1991). In order to resolve this issue, a kinetic-based model for phosphorus recovery was proposed to reduce total phosphorus in the final effluent to meet the discharge consent (< 1 mg P L^{-1}).

6.3.4.1. Design Parameter for Phosphorus Uptake by Chlamydomonas reinhardtii in the Two-Stage Biological Process

First-order kinetics was used to identify the coefficient for phosphorus uptake by *Chlamydomonas reinhardtii* in a two-stage biological process combining phototrophic and heterotrophic microalgae cultivation with biomass recirculation under continuous flow conditions. Under steady-state conditions, $\frac{dC}{dt}$ = 0 and replacing the first-order reaction ($r_c = \pm kC$), Eq 6-4 can be obtained as follows:

$$C = \frac{Co}{1+(k\theta)} \qquad \text{Eq 6-4}$$

where:

C, C_o = Phosphorus concentration in the effluent and at time t and time 0, respectively, mg P L^{-1}

k = Kinetic coefficient for phosphorus uptake, d^{-1}

θ = Hydraulic retention time, d

The kinetic coefficients for phosphorus uptake obtained for the various operational conditions of the two-stage biological process are listed in Table 6-4. k values varied from 0.25 to 0.67 d^{-1} under different operational conditions. The highest phosphorus uptake coefficient, 0.67 d^{-1}, was used for the model as it was achieved at the optimum operational conditions.

Table 6-4 P uptake coefficients for *Chlamydomonas reinhardtii* under phototrophic and heterotrophic conditions

Number of Experiment	PO_4^{3-}-P in SWW (g m^{-3})	P in PBR (g m^{-3})	P in HTR (g m^{-3})	k in PBR (d^{-1})	k in HTR (d^{-1})
1	15	11	11	0.48	-
2	15	9	9	0.67	-
3	15	9	9	0.67	-
4	15	11	11	0.36	-
5	15	12	12	0.25	-
6	15	11	11	0.36	-
7	15	11	11	0.36	-

In order to meet the discharge consent for phosphorus (<1 mg P L^{-1}), a serial reactor setup was required. The number of reactors required was determined by Equation 6-5 by adding n times the kθ term in equation 6-4.

$$C = \frac{Co}{1+(k\theta)^n} \qquad \text{Eq 6-5}$$

A series of six phototrophic and heterotrophic reactors and one settlement tank were required under the optimum operational conditions (24 h HRT for each reactor, 14 d CRT and a mix of nitrogen sources) which could achieve a less than 1 mg P L^{-1} effluent

concentration. The number of reactors required decreased with increasing HRT; however, longer HRT required an increase in volume of the reactors.

6.3.4.2. Design Parameters for Large-Scale Cultivation of the Two-Stage Biological Process in Terms of Phosphorus Uptake

In the previous section, the optimum operational conditions were identified as 24 h HRT for each reactor, 14 d CRT and a mix of nitrogen sources because the maximum coefficient for phosphorus uptake was achieved under these conditions (Table 6-4). Thus, 1 d HRT was used for the model of the implementation of mixotrophic microalgae growth under continuous flow conditions. In addition, it can be clearly seen from Table 6-4 that there was not any contribution to P uptake by *Chlamydomonas reinhardtii* under heterotrophic conditions. However, the heterotrophic reactor was taken into account when modelling the large-scale cultivation of mixotrophic microalgae growth under continuous culture conditions due to higher lipid content in microalgae at heterotrophic cultivation than for phototrophic growth (Perez-Garcia et al., 2011b).

The average water consumption per person has been estimated to be 150 L per day (ccwater, n.d.). A small-scale wastewater treatment works serving 2,000 p.e. suggested by UWWTD (1991) was designed in this study. Thus, flowrate was calculated as 300 $m^3 d^{-1}$ by multiplying water consumption per person per day and person equivalent. Equation 3-5 was used in order to identify that total volume for a wastewater treatment plant to sustain a 300 $m^3 d^{-1}$ flowrate in 1 d HRT.

There are many types of photobioreactor for mass cultivation of microalgae such as shallow open ponds of circular or raceway types, tubular closed PBR (arranged vertically or horizontally) and flat-panel PBR. In this model, horizontal tubular closed PBR was preferred due to their potential for mass cultivation of microalgae with regard to a large illuminated area (Huang et al., 2017). The same diameter PBR used for laboratory experiments in this research was chosen, 7 cm, for use in modelling the light path in the PBR, which is slightly longer than typical values for the diameter for tubular PBRs, which are reported to vary between 10-60 mm (Huang et al., 2017). The length of a tubular PBR can extend several hundred metres (Huang et al., 2017); however, it is suggested that they do not exceed 160 m in length in order to provide sufficient linear velocity in the PBR (Richmond et al., 1993) and, therefore, the optimum length of reactor was deemed to be 10 m. The number of reactors required was calculated to be 7920 by dividing the total volume of the inlet by the volume of the reactor. Taking into account

these values, design parameters for the large-scale cultivation of a two-stage biological process combining phototrophic and heterotrophic microalgae cultivation with biomass recirculation under continuous flow conditions are summarised in Table 6-5. The front and side view of the proposed wastewater treatment plant with a two-stage biological process are illustrated in Figure 6-9 and 6-10, respectively.

Table 6-5 Design parameters to implement the microalgae process into a small wastewater treatment plant

Parameter	Value (Unit)
Hydraulic Retention Time	1 (d)
Flowrate	300 (m^3d^{-1})
Total Volume	300 (m^3)
Diameter of reactor	0.07 (m)
Length of reactor	10 (m)
Volume of reactor	0.038 (m^3)
Number of reactors required	7920
Number of modules needed	55
Number of the reactor in a module	144
Land area	688 (21.5*32) (m^2)
Height of the module	20 (m)

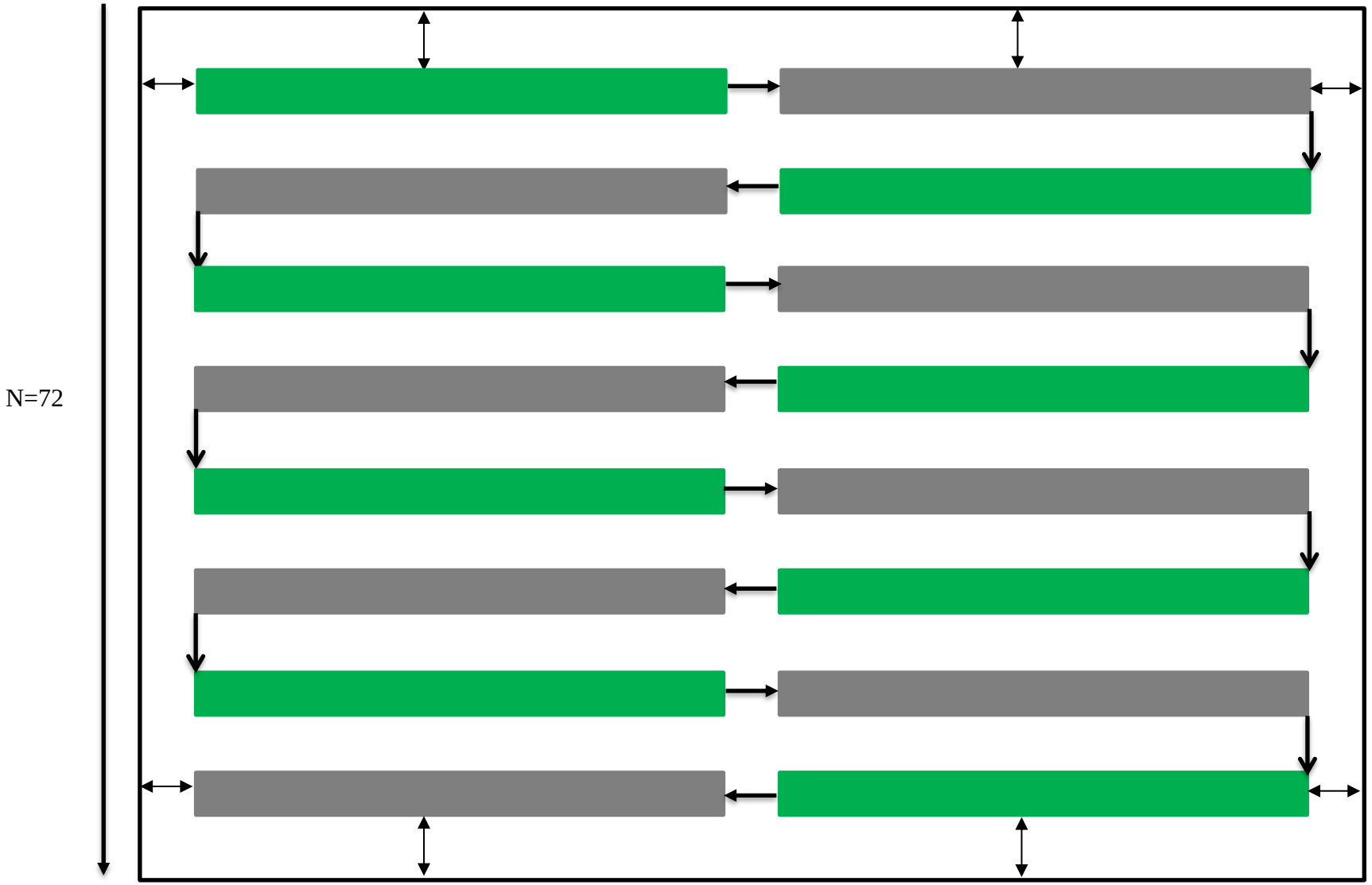


Figure 6-7 The front view of one module in the modelled small scale wastewater treatment work with mixotrophic microalgae cultivation under continuous flow conditions, where green and grey bars represent PBR and HTR, respectively.

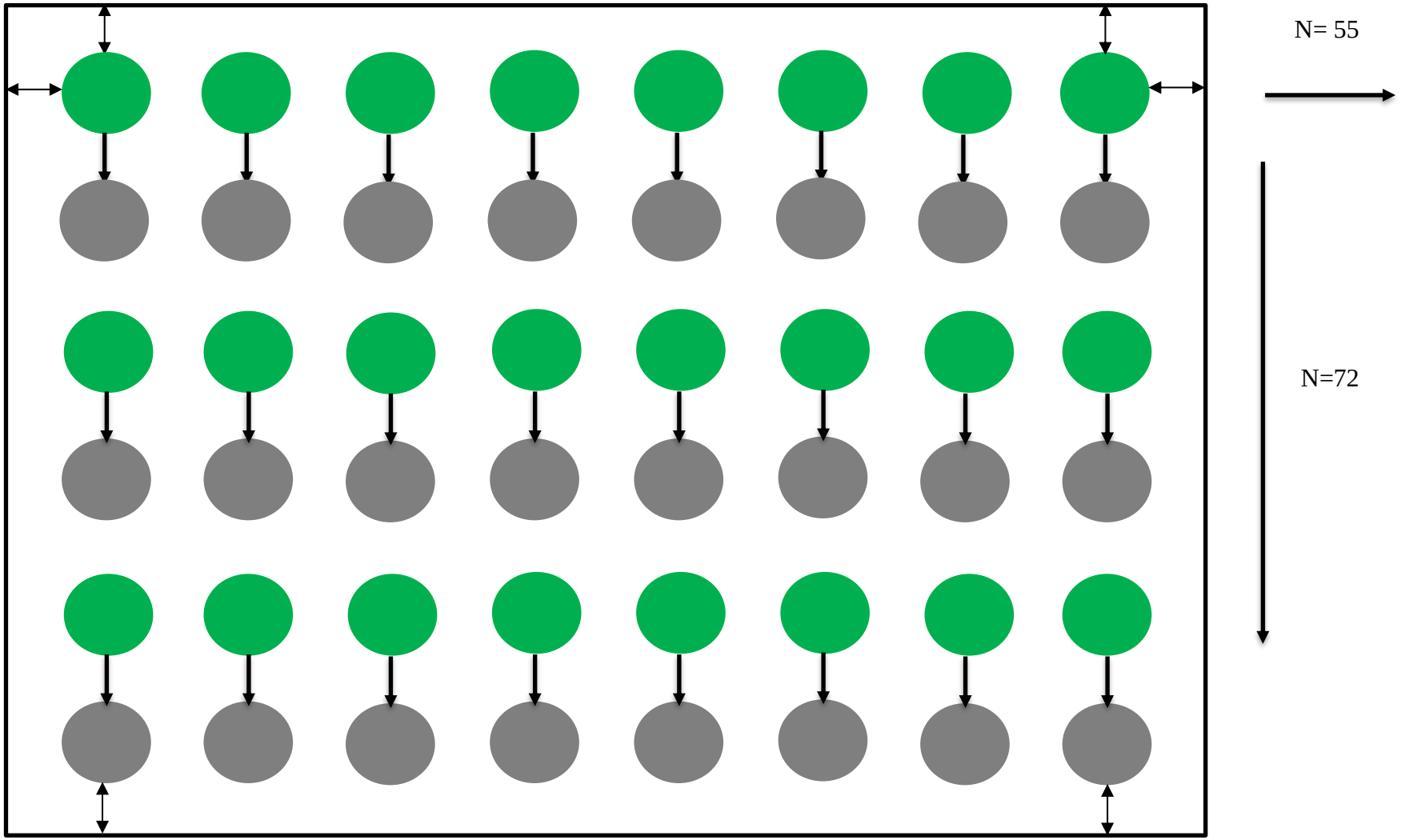


Figure 6-8 The side view of modelled small scale wastewater treatment work with mixotrophic microalgae cultivation under continuous flow conditions, where green and grey bars represent PBR and HTR, respectively.

Figure 6-9 shows that one module of the final proposed design contains a total of 144 reactors, half of them for phototrophic cultivation and the other half for heterotrophic growth. There are 72 rows from top to bottom of the module. A total of two reactors, one PBR and one HTR, are placed 50 cm from the beginning and the end of the module in a row. The distance between each reactor and row are proposed to be 50 cm and 20 cm, respectively. Furthermore, wastewater flows from PBR to HTR in the odd-numbered rows and, thereafter, is transferred to the next row (from HTR to PBR in the even-numbered rows). When wastewater flow is complete in one module, it is transferred to the top of the next one. It can be clearly seen in Figure 6-10 that the proposed design for the small-scale wastewater treatment plant requires 55 modules in series, with a distance of 50 cm between each module. In conclusion, the proposed design requires 688 m^2 (21.5m*32m) and 20 m of land area and height, respectively.

A 730,000 population equivalent of wastewater is delivered into Esholt WwTW with a flowrate of 280,022 m^3d^{-1}. It was calculated that 933 proposed small-scale wastewater treatment plants would be required in order to implement a two-stage biological process combining phototrophic and heterotrophic microalgae cultivation under continuous flow conditions with biomass recycling in Esholt WwTW as based on the design parameters reported in Table 6-5, which would correspond to a large land area of 642,184 m^2. It is worth noting that land area is one of the most important criteria for the design of algal wastewater treatment and the land area requirement should be reduced by optimising the design parameters.

6.3.4.3. The Potential for Biogas Production at the Proposed System

25-40% of operational costs result from the energy consumption of different operational units such as collection, pumping, aeration, etc. in conventional wastewater treatment works (Gu et al., 2017). Primary treatment is less energy intensive than secondary treatment; however, pumping in the primary treatment and aeration in the secondary treatment are the two highest energy-consuming components of the process (Plappally and Lienhard V, 2012). Secondary treatment, particularly aeration, consumes about 50-60% of the total energy requirements, whereas 15-20% of the energy is used for sludge treatment (Gu et al., 2017). Moreover, tertiary treatment for nutrient removal such a nitrification-denitrification is, even more, energy-intensive with increasing the energy consumption due to the extended aeration (Wakeel et al., 2016; Plappally and Lienhard V, 2012). The average energy consumption for wastewater treatment in most countries is nearly the same (ranging from 0.25 to 0.67 kWh/m^3), with the UK one of the most energy

intensive at 0.64 kWh/m^3 (Hernandez-Sancho et al., 2011). Although wastewater treatment plants consume a huge amount of energy, they commonly generate biogas and heat and electricity by anaerobic digestion and combine heat and power technology, respectively (Gu et al., 2017). This has made energy self-sufficient wastewater treatment works more attractive in recent years.

As a case study of Esholt WwTW, it was reported that an average energy of 63,000 kW is consumed while electrical energy equivalent to 45,000 kW is generated daily, depending on the flowrate (Yulistyorini, 2016). Additional energy is needed for approximately 30% of the total energy requirement to operate Esholt WwTW.

Microalgae are considered third-generation renewable biofuels due to the organic components of the biomass containing 6.7-68.4%, 14.9-84.0% and 0.8-63.2% carbohydrates, proteins and lipids, respectively (Xia et al., 2015). Anaerobic digestion of microalgae is one of the processes by which biogas is produced with an associated methane yield in the range of 24-800 mL CH_4 g^{-1} VS, depending on the microalgae species (Ward et al., 2014). However, the C/N for the microalgae biomass (lower than 10) can limit mono digestion of microalgae in the long term because ammonia is produced by anaerobic digestion of proteins which results in the inhibition of methanogens, the accumulation of volatile fatty acids and digestion failure (Ganesh Saratale et al., 2018). Co-digestion of microalgae with carbon-rich substrates such as waste activated sludge (WAS) represent a promising approach to WWTPs becoming energy self-sufficient with an increasing the C/N ratio (Wang et al., 2013). Yuan et al. (2012) assessed the effects of waste activated sludge on anaerobic digestion of *Chlorella sp.* and achieved almost twofold the biogas yield with the co-digestion of microalgae and WAS. A similar outcome was also reported by Wang et al. (2013). 587 mL g^{-1} VS of the methane was produced from anaerobic digestion of *Chlamydomonas reinhardtii* (Mussgnug et al., 2010). Moreover, anaerobic digestion of mixotrophic microalgae results in higher methane production than phototrophic cultivation (Alcántara et al., 2013). In conclusion, the implementation of the proposed system, mixotrophic cultivation of *Chlamydomonas reinhardtii*, at Esholt WwTW could well increase the amount biogas and heat and electricity generate by anaerobic co-digestion with WAS.

In terms of the proposed system, a 24 h/24 h:light/dark photoperiod was applied for the mixotrophic cultivation of *Chlamydomonas reinhardtii*. In outdoor conditions, the application of a 24 h light duration was not feasible because of the day/night period in a

day. In addition, the operation cost would be increased due to the requirement for artificial light. Therefore, a decrease in final biomass concentration would presumably be observed when the proposed system, a two-stage mixotrophic microalgae cultivation under continuous flow conditions with biomass recycling, was implemented for a large-scale outdoor cultivation system.

Janssen et al. (2000) reported that the specific growth rate of *Chlamydomonas reinhardtii* was reduced by 12% when the photoperiod decreased by 33% and the average day/night ratio in a year is assumed to be 12 h/12 h; therefore, around 0.7 g VSS L^{-1} of biomass concentration was predicted. A 210 kg total biomass would thus be produced daily at a flowrate of 300 m^3 d^{-1}. Nearly 85% of solid content was harvested by gravity settlement with additional cationic polymer (See in Chapter 5) and 20% of the settled microalgae would be transferred in anaerobic digestion to produce methane, which was calculated to be 36 kg VSS L^{-1}. Mussgnug et al. (2010) reported that 587 mL methane was produced per g of volatile solids by *Chlamydomonas reinhardtii* so that 21,132 mL methane would be generated – theoretically – by using the proposed system. Furthermore, microalgae could produce an energy of between 18-21 MJ kg^{-1} (Stephens et al., 2010) and the energy production was predicted to be approximately 200 kWh (1 kWh=3.6 MJ).

6.3.4.4. The Potential for Biomass Productivity and Phosphorus Uptake in the Proposed System

The proposed lab-scale system, a two-stage mixotrophic microalgae cultivation under continuous flow conditions with biomass recycling, produced a total biomass concentration of *Chlamydomonas reinhardtii* of 0.9 g VSS L^{-1}, as composed of 43.9% carbon, 6.0% nitrogen and 1.2% phosphorus based on the mass percentage of volatile solids. Lower biomass concentration and biomass content were found in this study than those attained by Yulistyorini (2016), which were 2.5 mg VSS L^{-1} of biomass comprising 11.4% nitrogen and 1.7% phosphorus at 48 d HRT under mixotrophic cultivation conditions in the continuous culture.

Cell densities in the tubular reactors ranged between 2 and 6 g L^{-1} and it is reported that a 10 g DW L^{-1} cell density can be achieved when the closed reactors are well-designed; however, the self-shading effect can limit microalgae growth at higher cell densities than 4 g L^{-1}. Cell densities in the range of 0.25-1 g L^{-1} can be obtained in open ponds (Rawat et al., 2013; Stephens et al., 2010). This can be attributed to average

biomass productivities in the open ponds and closed reactors (in the variation of 10-20 and 20-45 $gm^{-2}d^{-1}$, respectively) (Christenson and Sims, 2011).

In comparison with some of the literature with other microalgae species, similar biomass concentrations have been reported to the one attained in this study. Lam and Lee (2012) cultivated *Chlorella vulgaris* under different conditions with continuous illumination and up to 0.8 g L^{-1} of microalgae biomass was obtained. Moreover, they pointed out that microalgal concentration was decreased to around 0.5 g L^{-1} in the outdoor cultivation. The final biomass concentration of *Chlorella ellipsoidea* was reported to be 0.77 g L^{-1} for indoor cultivation with a light/dark ratio of 16 h/8 h. The same was also observed in that around one-third of the reduction was seen in the final biomass concentration, 0.53 g L^{-1}, when the system was scaled up 100 times under the outdoor conditions (Wang et al., 2014b). Pereira et al. (2018) compared the size of the large-scale outdoor microalgae cultivation systems by *Tetraselmis sp.* under semi-continuous flow in tubular PBRs, and reported average biomass concentrations of 1.0 and 0.8 g L^{-1} in 100 and 35 m^3 systems, respectively. It was concluded that biomass concentration could be reduced in the scaled-up outdoor microalgae cultivation due to uncontrolled outdoor conditions, the variation in the day/night ratio and the temperature, and the high likelihood of contamination by other microorganisms, particularly bacteria.

The current implementation of microalgae cultivation in the large-scale wastewater treatment works shows high biomass production and nutrient recovery efficiency. The pilot-scale microalgae cultivation was implemented in the Upper Blackstone Water Pollution Abatement District in Millbury, Massachusetts, to polish the effluent. The system includes three main stages; firstly, the wastewater is mixed with CO_2 and a mixture of algae and other microorganisms, then the mixture flows through a mile of 2-inch diameter pipes, looped in six rows in a small lighted greenhouse. Finally, the mixture is separated by microfiltration into the treated water with the biomass. Nitrogen and phosphorus recovery efficiencies of 36.0% and 94.3% were reported, corresponding to average nitrogen and phosphorus concentrations in the effluent of 3.23 and 0.015 mg L^{-1} (UBWPAD, 2014; Spencer, 2014; CLEARAS, n.d.).

Gordon (2017) reported an advanced biological nutrient recovery (ABNR) system implemented in the South Davis Sewer District South Plant in Missoula, Utah, with a flowrate of 4 million gallons per day. The ABNR contained tubular reactors in a closed system. The biomass productivity was anticipated to be around 8 lbs per day, with the

efficiencies of the nitrogen and phosphorus uptake at 62.5% and 95.7%, respectively, which was consistent with 3.0 and 0.03 mg L^{-1} average nitrogen and phosphorus concentrations in the effluent (Gordon, 2017; CLEARAS, n.d.).

Europe's largest microalgae cultivation in closed reactors is located in Klotze, Germany, with the land area of 10,000 m^2. These tubular reactors were designed to adopt a fence-like construction in order to distribute light over a large surface area. Around 130-150 tonnes of algal biomass in the form of *Chlorella vulgaris* is produced annually, corresponding to a range of 35-41 g m^{-2} d^{-1} biomass productivity (Schenk et al., 2008; Pulz, 2001). Higher biomass productivity and nutrient uptake efficiencies were reported compared with the proposed system due to the limitations of the lab-scale two-stage mixotrophic microalgae cultivation and lower initial nitrogen and phosphorus concentrations than those in the feeding medium cultivating *Chlamydomonas reinhardtii* in this study.

6.3.4.5. Limitations of the Proposed System

Despite the potential of the proposed system, as mentioned earlier, there are still limitations for mass cultivation of microalgae to overcome to allow for the implementation of a two-stage mixotrophic microalgae cultivation within the current wastewater treatment works, such as the need for organic carbon to allow for heterotrophic/mixotrophic growth, the limited number of strains that grow under heterotrophic/mixotrophic conditions, contamination with other microorganisms, the harvest of microalgae, and high the land area requirements (Chen and Johns, 1996; Hu et al., 2017; Fernandes et al., 2015; Gerardo et al., 2015).

Organic carbon is required for both energy and carbon metabolism of the heterotrophic and/or mixotrophic microalgae growth. Many forms of organic carbon substrate have been investigated for microalgae production, the most commonly used of which are glucose and acetate. Additional glucose and acetate in the culture media increases the operational cost for the commercial production of microalgae, however. In order to reduce costs, wastewater could well present an alternative organic carbon source because it includes acetate (Lowrey et al., 2014); however, additional acetate may still be needed. Furthermore, high concentrations of organic carbon substrates can limit heterotrophic growth. As an example of this, concentrations above 20 gL^{-1} of glucose and 0.4 gL^{-1} of acetate inhibited the growth of *Chlorella sorokiniana* and *Chlamydomonas reinhardtii*, respectively (Chen, 1996).

In addition, the number of microalgae strains capable of growing heterotrophically is very limited. This is attributed to the fact that most microalgae are obligate photoautotrophs. They cannot utilise sugar due to incomplete metabolic pathways such as the tricarboxylic acid cycle and the absence of an enzymatic reaction such as oxoglutarate dehydrogenase (Morales-Sanchez et al., 2015; Hu et al., 2017).

Moreover, it is difficult to maintain an outdoor microalgae system anexic culture due to the risk of contamination (Hu et al., 2017). Microalgae have to compete with other microorganisms, particularly bacteria, under heterotrophic and/or mixotrophic conditions because high organic carbon concentrations favour bacterial growth (Stephens et al., 2010). Despite the fact that open ponds are more vulnerable to microbial contamination than closed reactors, long operation periods can increase the risk of contamination (Wang et al., 2014b; Stephens et al., 2010). Unfortunately, bacterial contamination was observed in a two-stage mixotrophic microalgae cultivation in this study.

One of the main disadvantages of large-scale microalgae cultivation is how to harvest them. Microalgae generally have a cell diameter of less than 15 μm, which is challenging to microalgae harvesting (Chatsungnoen and Chisti, 2016; Singh and Patidar, 2018). Currently, microalgae harvesting processes such as coagulation-flocculation, sedimentation, centrifugation, etc., are not cost effective and represent 20-30% of the biomass production costs (Barros et al., 2015; Gerardo et al., 2015). In addition, microalgae can attach to the reactor walls which makes harvesting more difficult (Fernandes et al., 2015). In this study, the discharge limit for total solids (35 mg L^{-1} TSS) could not be met due to harvesting problems (UWWTD, 1991).

Another limitation of the proposed two-stage mixotrophic microalgae cultivation system is the need for a large number of reactors and land area. 7920 reactors would be required for a large-scale wastewater treatment works with a flowrate of 300 m^3d^{-1} based on the design parameters reported in Section 6.3.4.2. The land area needed for the proposed system and the implementation of the proposed system at Esholt WwTW were estimated to be 688 m^2 and 642,184 m^2.

6.4. CONCLUSION

In order to access the potential to implement a two-stage biological process combining phototrophic and heterotrophic microalgae cultivation under continuous flow

conditions with biomass recirculation in current wastewater treatment works, mass flow analyses of nutrients for both a two-stage biological process and Esholt WwTW were performed.

Esholt WwTW achieved 65.9 and 95.9% phosphorus and ammonium recovery efficiencies by EBPR and the nitrification-denitrification process. Although the discharge consent for TN was met, Esholt WwTW could not meet its consent for TP. Regarding the mass balance analysis, 52% and 24% of the total nitrogen were removed by the nitrification-denitrification process and wasted biomass, respectively. 92% of the total phosphorus was recovered by waste activated sludge.

40 and 93.2% of the total phosphorus and total nitrogen were recovered by *Chlamydomonas reinhardtii* using mixotrophic microalgae cultivation under continuous culture conditions, respectively. 1.2% and 6.0% of phosphorus and nitrogen were assimilated into algae cells, in sequence under the optimum operational conditions of 48 h HRT, 14 d CRT and a mix of nitrogen sources. The P uptake coefficient was calculated to be 0.67 d^{-1}. In order to meet the more stringent discharge consent for phosphorus (< 1 mg P L^{-1}), a large number of reactors and large land area were required based on the proposed model.

Retrofitting of mixotrophic microalgae cultivation under continuous flow conditions in existing wastewater treatment works indicated the potential for sustainability in terms of nutrient control and recovery. Further studies are required in order to reduce the limitation for microalgae cultivation in wastewater treatment work.

7. CHAPTER: OVERALL CONCLUSIONS AND FUTURE RECOMMENDATIONS

7.1. Overall Conclusions

The aim of the proposed research project was to develop a novel algal-based bioengineering process for nutrient control and recovery; that also had the potential to be implemented to improve current wastewater treatment works using a two-stage biological processes combining phototrophic and heterotrophic cultivation with biomass recycling under continuous flow conditions. The main contributions towards each research objective can be summarised as follows:

As a result of the laboratory experiments conducted for research objective 1, heterotrophic and phototrophic microalgae growth kinetics were studied by controlling nutrient concentrations and environmental conditions in batch reactors. Specific growth rates and biomass productivity rates for *Chlamydomonas reinhardtii* under heterotrophic conditions were greater than those obtained in phototrophic cultivation.

In addition, nitrate was the preferred form of nitrogen in heterotrophic cultivation, whereas there was no significant influence of nitrogen sources under phototrophic conditions. With increasing carbon concentration under both heterotrophic and phototrophic conditions, lower specific growth rates of microalgae were observed.

With regard to the results from research activities conducted to achieve research objective 2, from the optimum operational conditions for a two-stage biological process combining phototrophic and heterotrophic cultivation under continuous flow conditions with biomass recycling, and with the aim to optimise nutrient recovery via biological uptake, it can be concluded that there was only a limited contribution from the heterotrophic reactor on nutrient recovery under any of the operational conditions tested.

The optimum operational conditions were identified as 48 h HRT, 14 d CRT and a mix of nitrogen sources considering the nutrient recovery efficiencies obtained under these conditions. The highest recovery efficiencies for phosphorus and nitrogen were 40% and 93.2%, respectively, which corresponded to 9.0 mg PO_4^{3-}-P L^{-1} and 3.4 mg NH_4^+-N

L^{-1} in the final effluent. The quality of the final effluent met the discharge consent for nitrogen but not for phosphorus. In order to further reduce phosphorus concentrations to the levels expected according to the corresponding discharge consents (< 1.0 mg P L^{-1} as phosphate), it is suggested that the PBR should make a partial contribution to nitrogen removal, so the remaining nitrogen entering the HTR could support algal growth for phosphorus removal. Moreover, nitrogen and phosphorus content in *Chlamydomonas reinhardtii* varied from 5.3% to 9.6% of the dry weight and from 0.6% to 1.2%of the dry weight under different operational conditions.

In terms of the third research objective, the potential implementation of algal bioengineering process in existing wastewater treatment works, to contribute to nutrient control and recovery was assessed. Removal efficiencies for phosphorus and ammonium in Esholt WwTW were found to be 65.9% and 95.9% from sampling surveys, and which corresponded to meeting the discharge consent for TN; however, larger amounts of phosphorus than allowed for presented in the final effluent. The main processes of nitrogen and phosphorus removal were considered nitrification-denitrification and waste activated sludge, as confirmed by mass balance analyses of nitrogen and phosphorus in the activated sludge process.

In summary, a two-stage microalgae process combining phototrophic and heterotrophic cultivation with biomass recycle under continuous flow conditions was found to be a potential option for nutrient recovery via microalgae assimilation by overcoming the limitations that current nitrification-denitrification and enhanced biological phosphorus removal processes have with regard to nutrient control removal in existing wastewater treatment works.

7.2. Future Recommendations

Despite the advantages of implementing mixotrophic microalgae cultivation within wastewater treatment works, further studies are required in order to reduce the limitations to such systems as the requirement for organic carbon and the limited number of strains that can grow under heterotrophic/mixotrophic conditions, high land area requirements, contamination with other microorganisms and the harvest of microalgae must still be addressed. Future research is thus recommended in the following areas;

- The system configuration can be modified so that each reactor can have different HRTs (different reactor volumes) in order to increase the contribution of the heterotrophic culture to nutrient uptake, as based on the difference between specific growth rates under heterotrophic and phototrophic conditions.

- The system could be investigated under higher organic carbon loading in order to enhance the efficiency of heterotrophic microalgae cultivation.

- A constant scraper could be used in the settlement tank to overcome the limitations of harvesting microalgae. In addition, the recirculation ratio could be varied in order to increase the settled biomass concentration.

- Process modelling based on computational fluid dynamics (CFD) tools could model what would be required to better understand how flow conditions change in the bioreactors and improve the design of the system for implementation in wastewater treatment works (WwtW).

- The optimum treatment location within WwTW would be determined based on nutrient preferences for microalgae and bacteria in order to enhance process efficiency from the perspective of nutrient recovery and energy consumption.

- A comprehensive energy balance should be conducted in order to compare energy efficiencies in the activated sludge process, microalgae cultivation and the combination of activated sludge and microalgae processses.

- Finally, the proposed model needs to be scaled up to the level of a pilot-scale wastewater treatment works.

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APPENDIX A

culturecollection of algae and protozoa

media recipes

Bold's Basal Medium (BB)

Freshwater algae

Stocks		**per 400 ml**
(1)	$NaNO_3$	10.0 g
(2)	$MgSO_4.7H_2O$	3.0 g
(3)	NaCl	1.0 g
(4)	K_2HPO_4	3.0 g
(5)	KH_2PO_4	7.0 g
(6)	$CaCl_2.2H_2O$	1.0 g
		per litre
(7)	Trace elements solution (autoclave to dissolve):	
	$ZnSO_4.7H_2O$	8.82 g
	$MnCl_2.4H_2O$	1.44 g
	MoO_3	0.71 g
	$CuSO_4.5H_2O$	1.57 g
	$Co(NO_3)_2.6H_2O$	0.49 g
(8)	H_3BO_3	11.42 g
(9)	EDTA	50.0 g
	KOH	31.0 g
(10)	$FeSO_4.7H_2O$	4.98 g
	H_2SO_4 (conc)	1.0 ml

Medium	**per litre**
Stock solutions 1 - 6	10.0 ml each
Stock solutions 7 - 10	1.0 ml each

Make up to 1 litre with glass distilled or deionised water.

CCAP (Culture Collection of Algae and Protozoa), Dunstaffnage Marine Laboratory,
Oban, Argyll, PA37 1QA, UK
Tel: +44 (0)1631 559000 Fax: +44 (0)1631 559001 Email: ccap@sams.ac.uk Web: www.ccap.ac.uk

Figure A-1 Bold's Basal Medium Recipe (CCAP)

APPENDIX B

Preliminary experiments to estimate heterotrophic growth kinetics of *Chlamydomonas reinhardtii* were conducted in 1L Duran bottles in duplicate. Sterilized SWW described in Table 3-1 was used as feeding media however initial organic carbon concentration was increased up to 2 g C L^{-1} in order to supply adequate organic carbon concentration for heterotrophic microalgae growth. Initial P concentration was set to be 1, 3, 5, 8, 10, 13 and 15 mg P L^{-1} in order to identify the influence of phosphorus on heteotrophic microalge growth. Acclimated culture for heterotrophic growth conditions was inoculated in Duran bottles (The culture acclimatization procedure in Section 3.1.2 was performed).

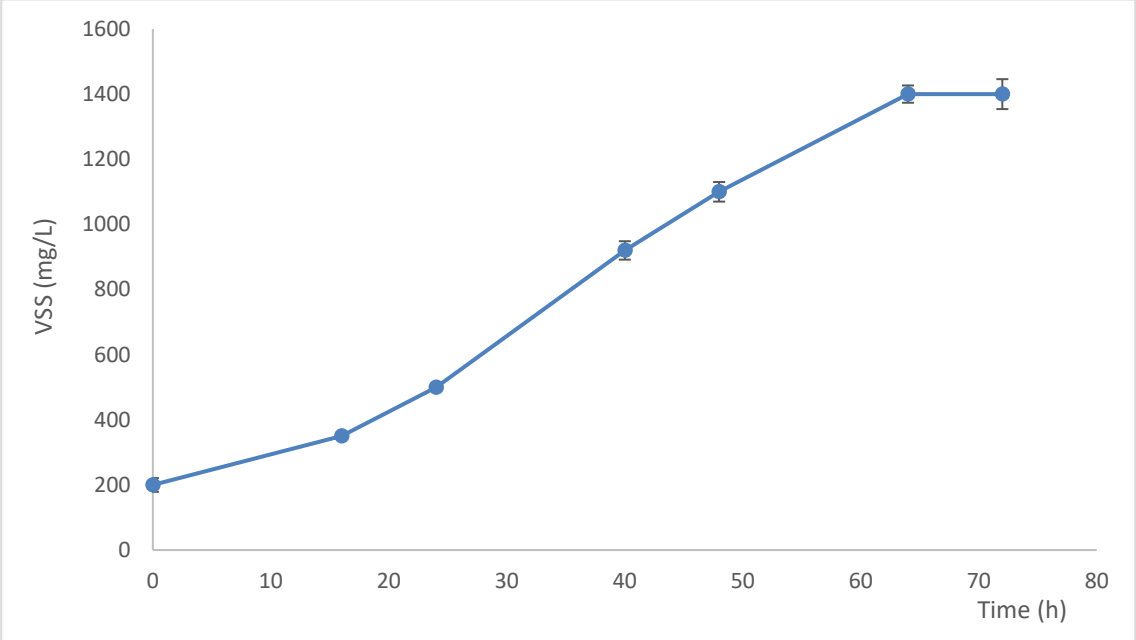


Figure 01 Heterotrophic microalgae growth at 1 mg P L^{-1}

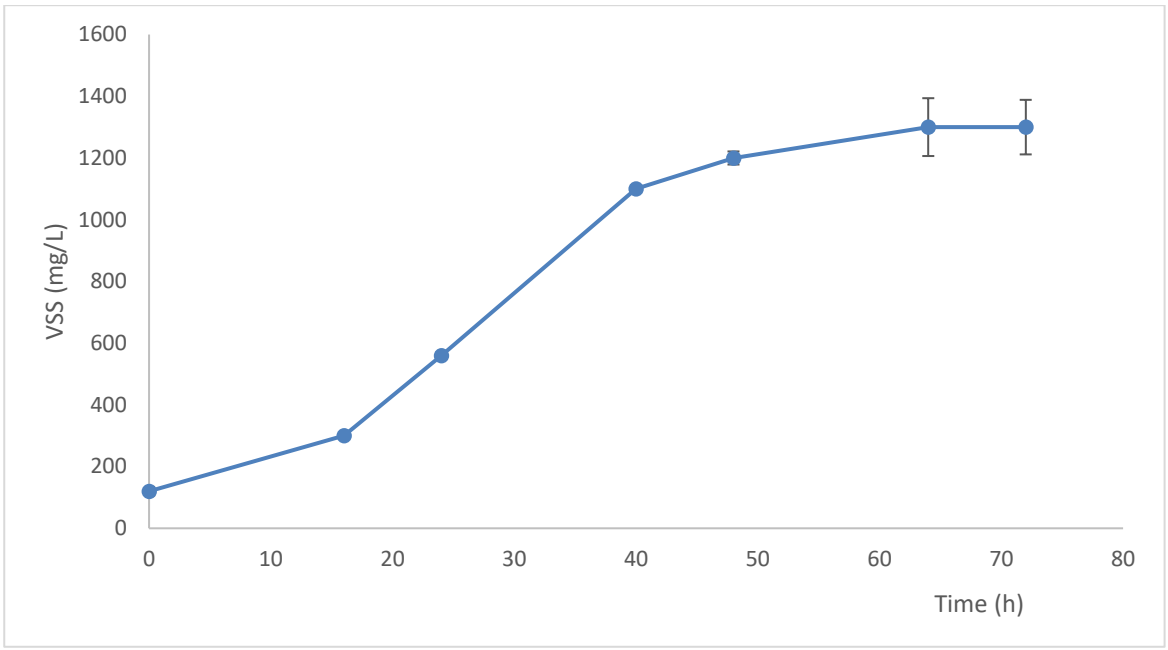


Figure B-2 Heterotrophic microalgae growth at 3 mg P L^{-1}

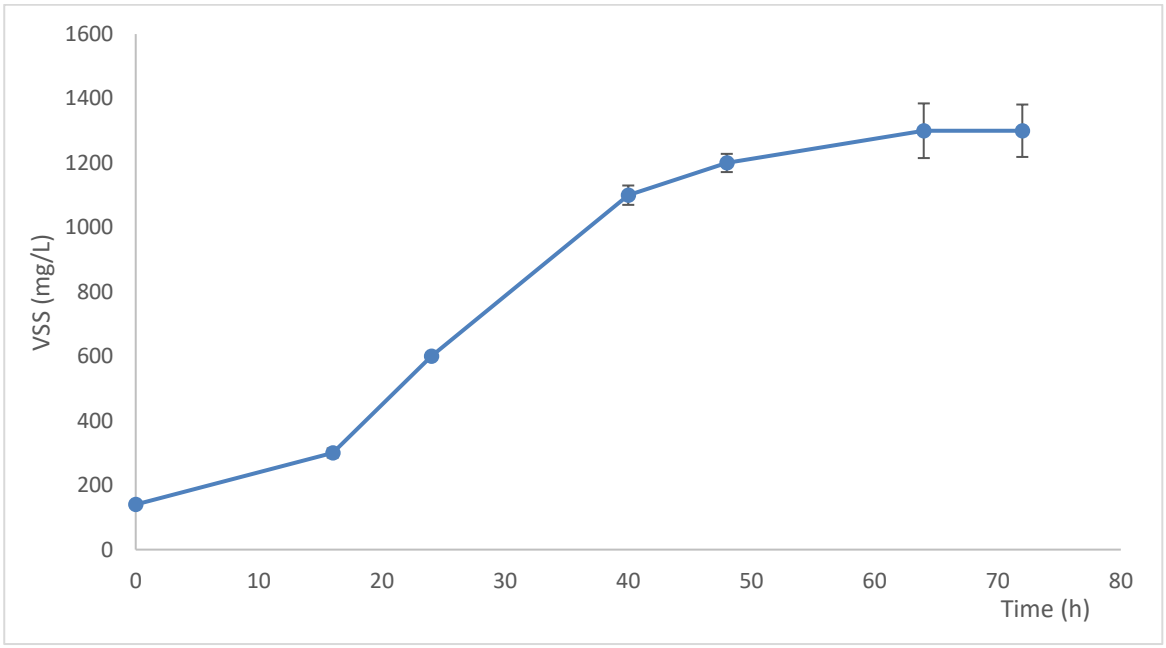


Figure B-03 Heterotrophic microalgae growth at 5 mg P L^{-1}

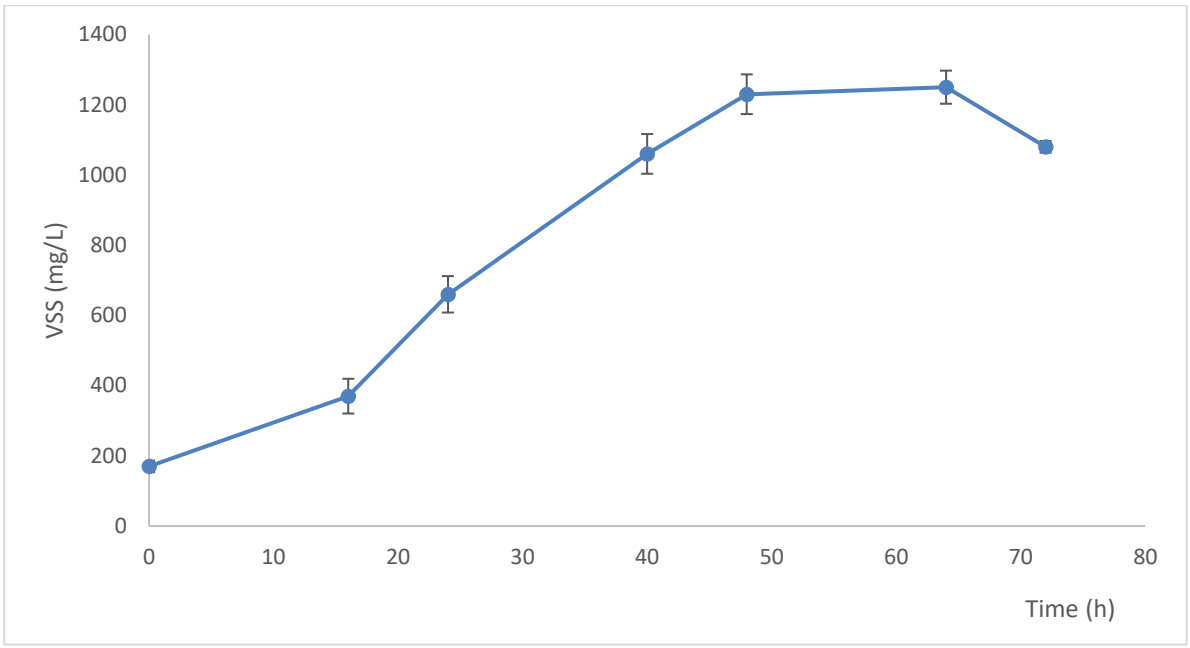


Figure 004 Heterotrophic microalgae growth at 8 mg P L^{-1}

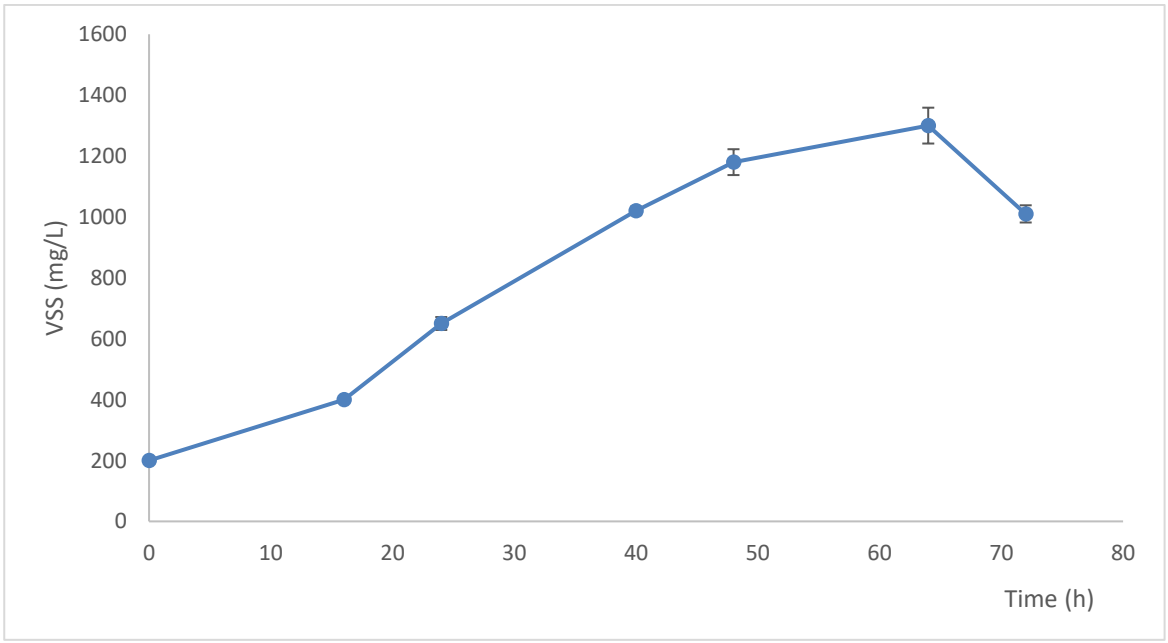


Figure 05 Heterotrophic microalgae growth at 10 mg P L^{-1}

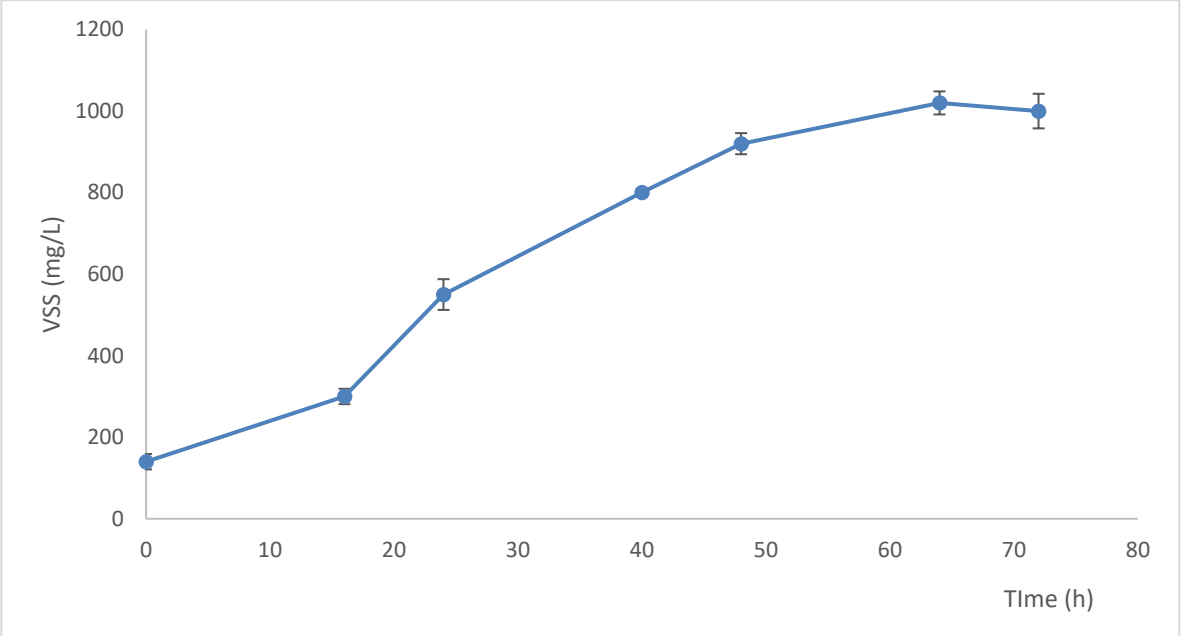


Figure B-06 Heterotrophic microalgae growth at 13 mg P L^{-1}

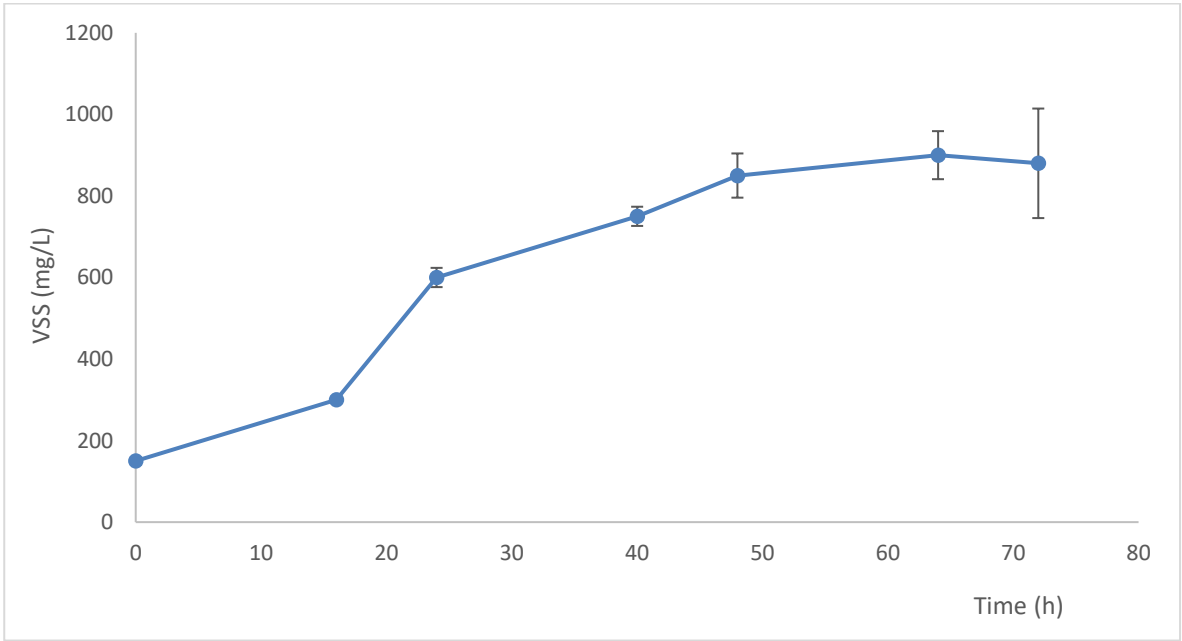


Figure 07 Heterotrophic microalgae growth at 15 mg P L^{-1}

APPENDIX C

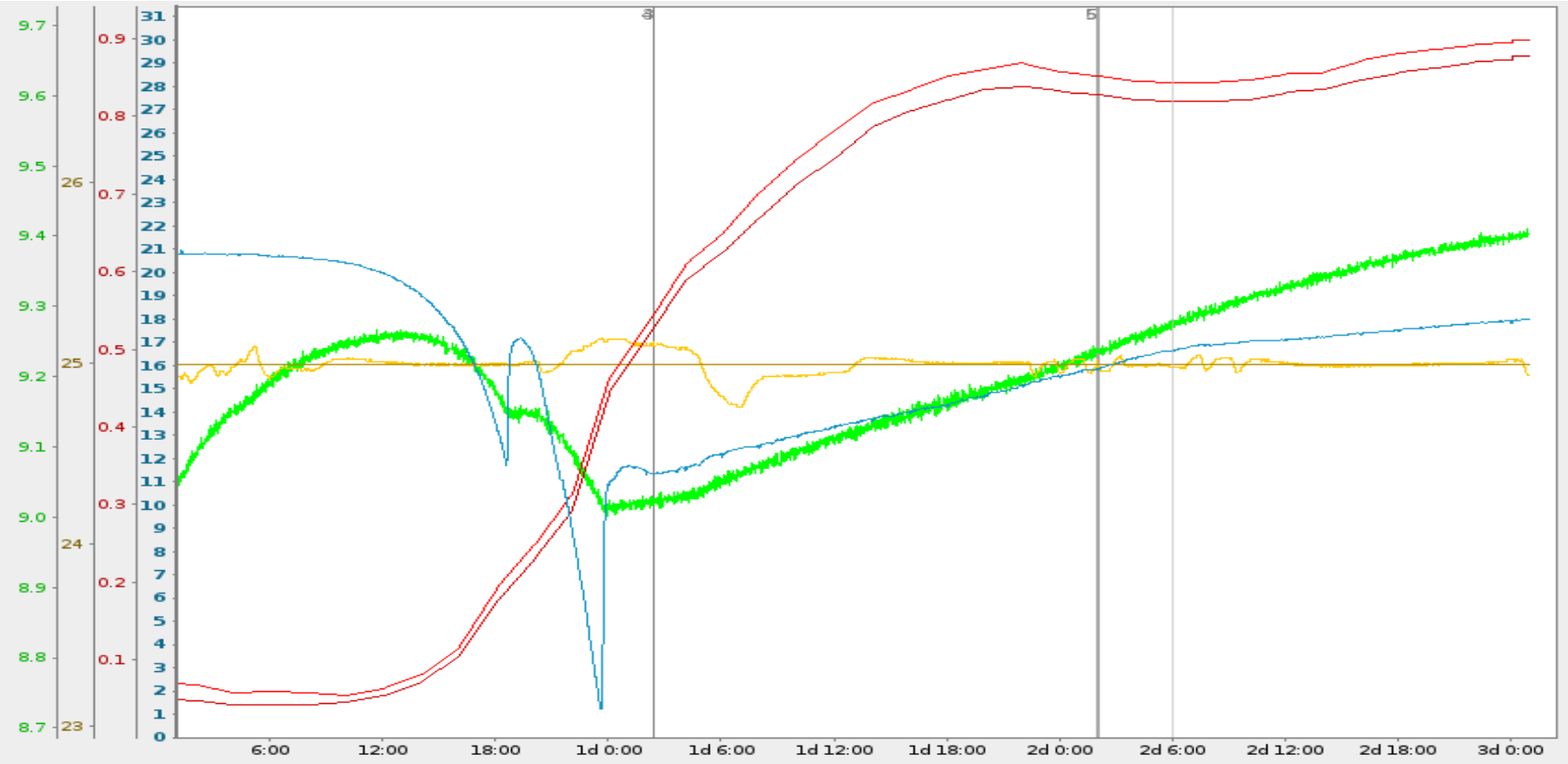


Figure 01 Screenshot picture of result for a heterotrophic growth kinetic experiment at PSI

***Green line presents pH data**

APPENDIX D

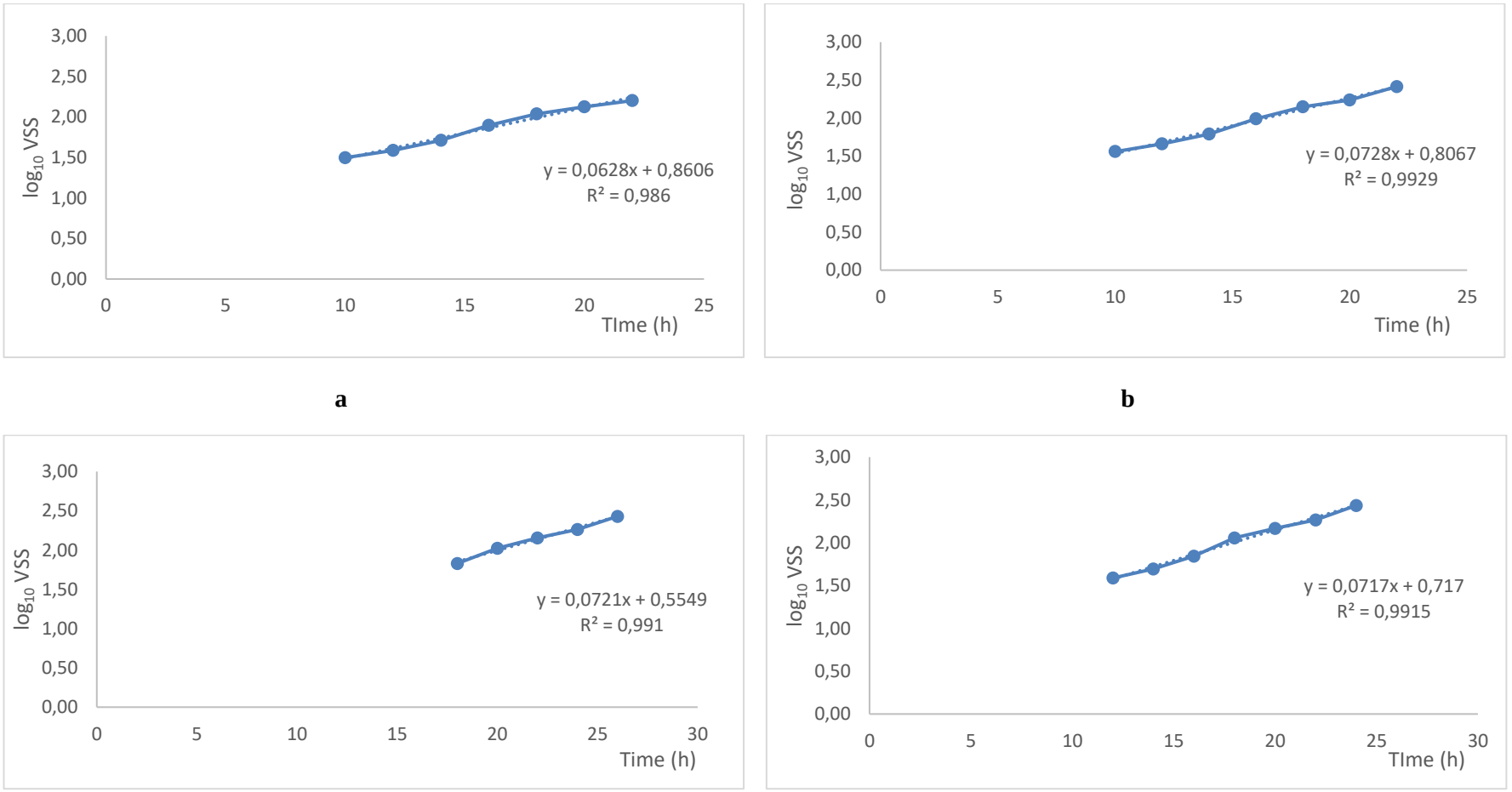
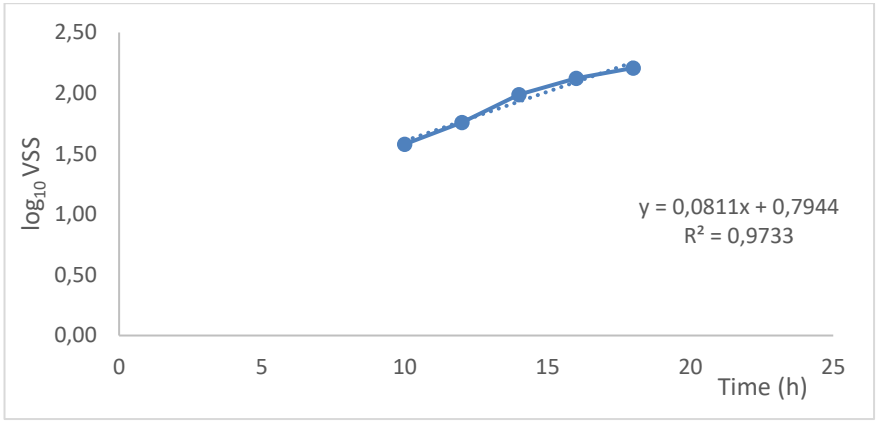


Figure D-1 Specific growth rates for *Chlamydomonas reinhardtii* using nitrogen source as a mix NH_4^+-N and NO_3^--N (50:50) under heterotrophic conditions with different initial P concentrations as; a) 1 mg L^{-1}; b) 5 mg L^{-1}; c) 10 mg L^{-1}; and d) 15 mg L^{-1}.



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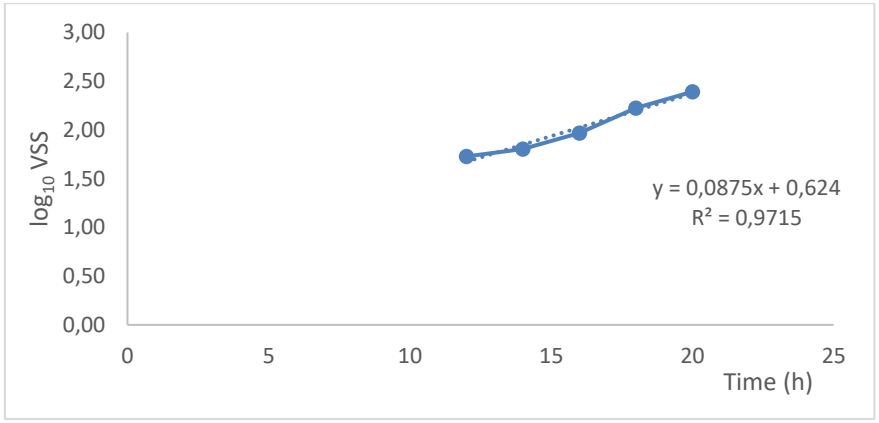

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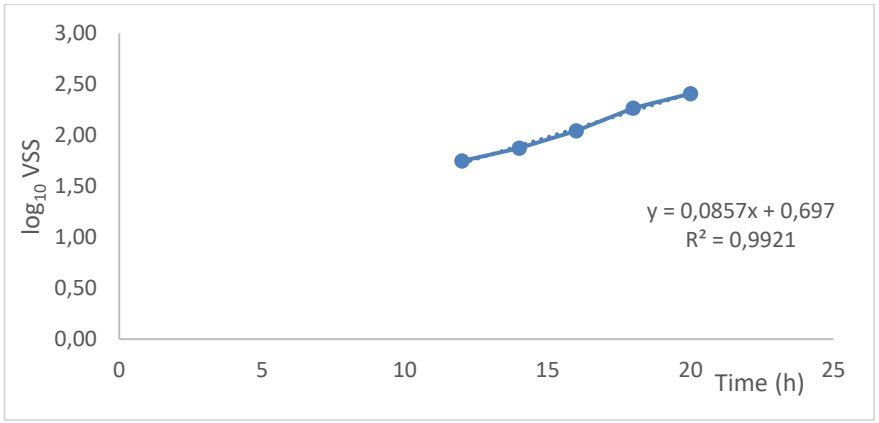

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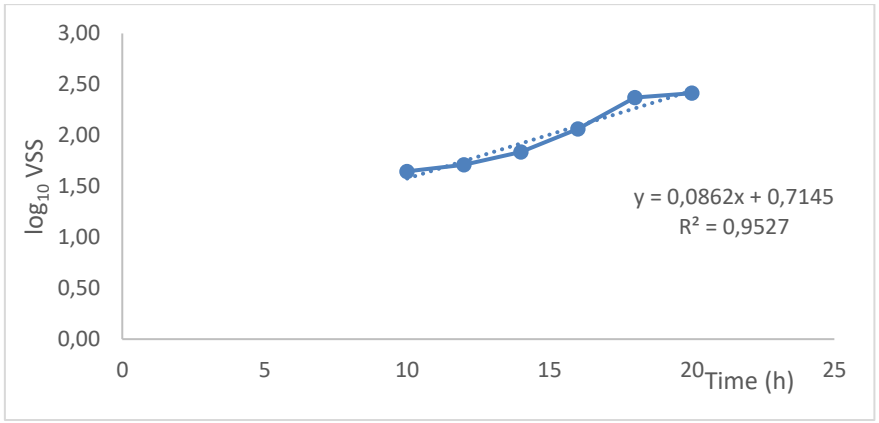

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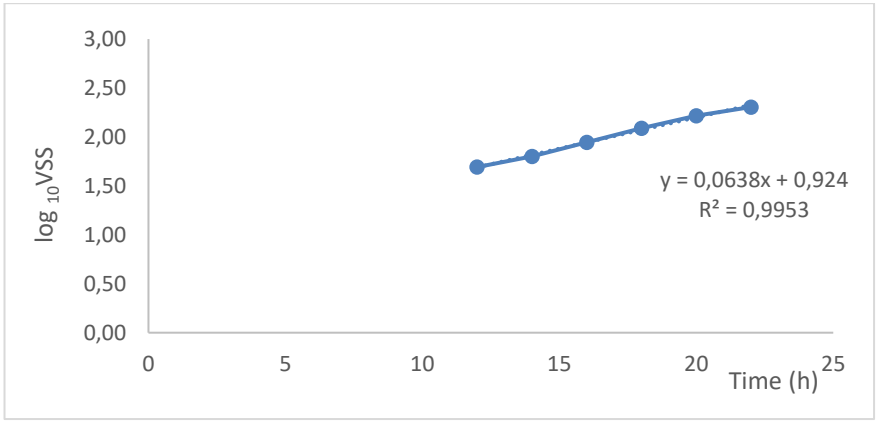
Figure D-2 Specific growth rates for *Chlamydomonas reinhardtii* using nitrogen source as NH_4^+-N under heterotrophic conditions with different initial P concentrations as; a) 1 mg L^{-1}; b) 5 mg L^{-1}; c) 10 mg L^{-1}; and d) 15 mg L^{-1}.



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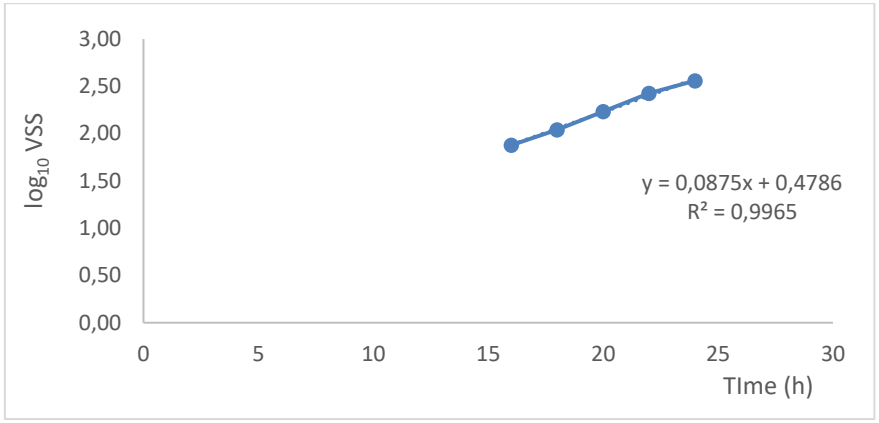

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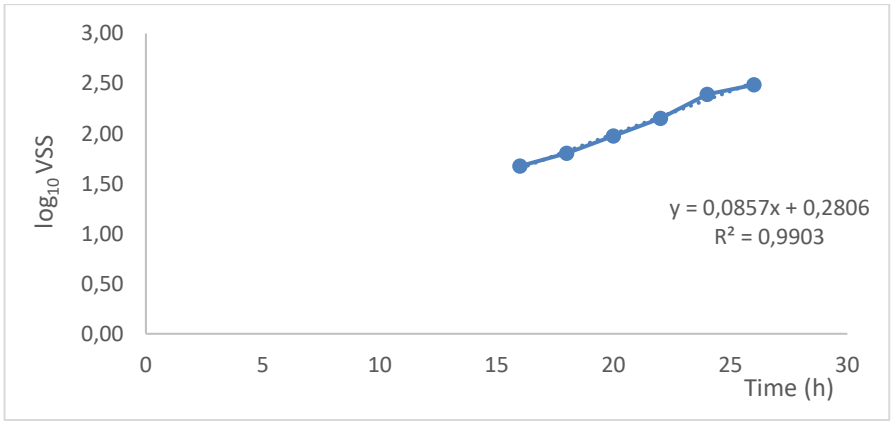

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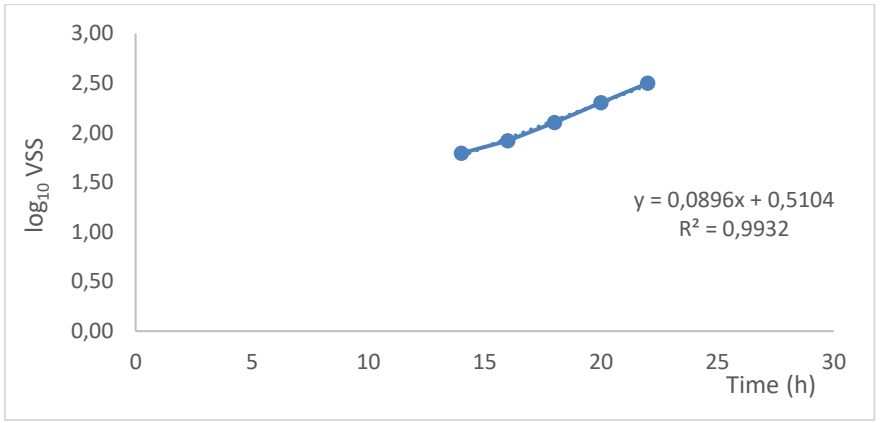

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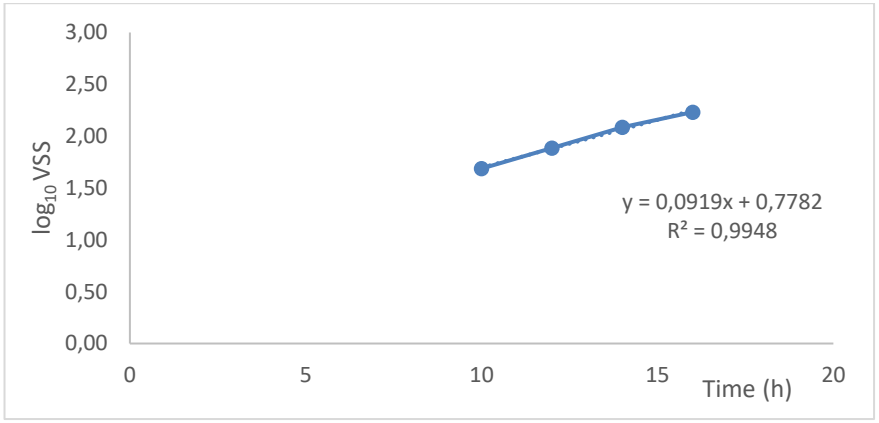
Figure D-3 Specific growth rates for *Chlamydomonas reinhardtii* using nitrogen source as NO_3^--N under heterotrophic conditions with different initial P concentrations as; a) 1 mg L^{-1}; b) 5 mg L^{-1}; c) 10 mg L^{-1}; and d) 15 mg L^{-1}.



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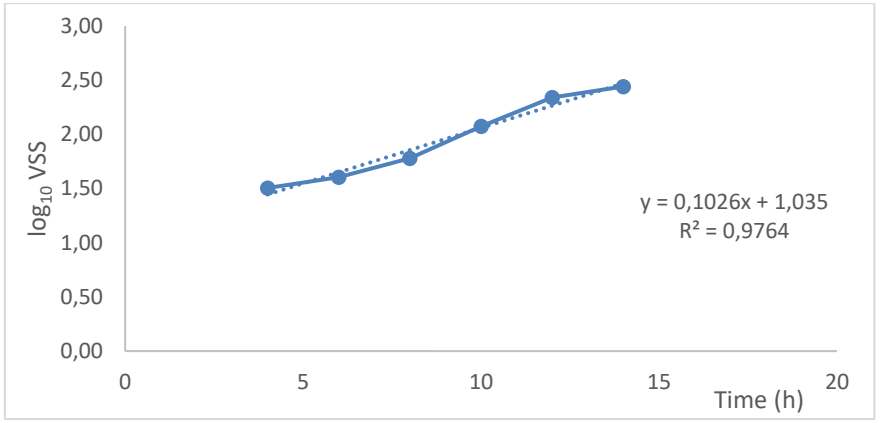

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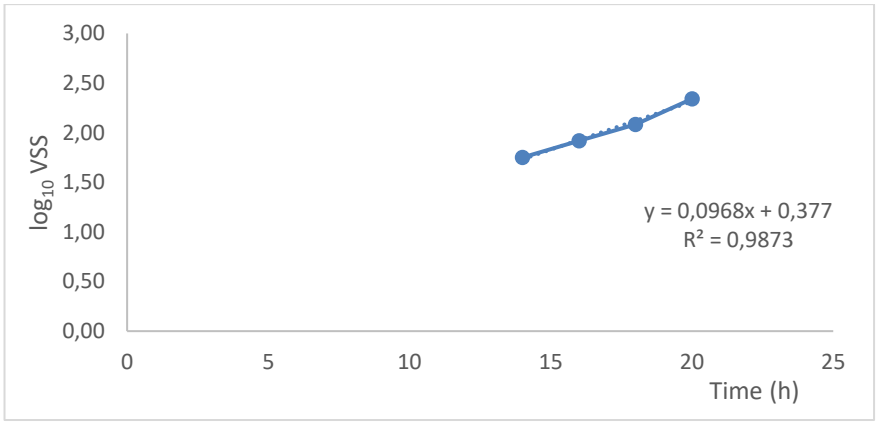

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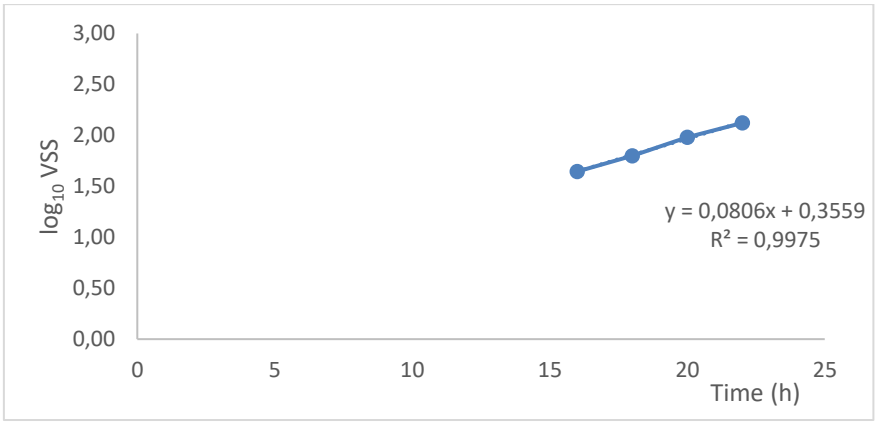


d

Figure D-4 Specific growth rates for *Chlamydomonas reinhardtii* at 1.5 g organic C L^{-1} under heterotrophic conditions with different initial P concentrations as; a) 1 mg L^{-1}; b) 5 mg L^{-1}; c) 10 mg L^{-1}; and d) 15 mg L^{-1}.

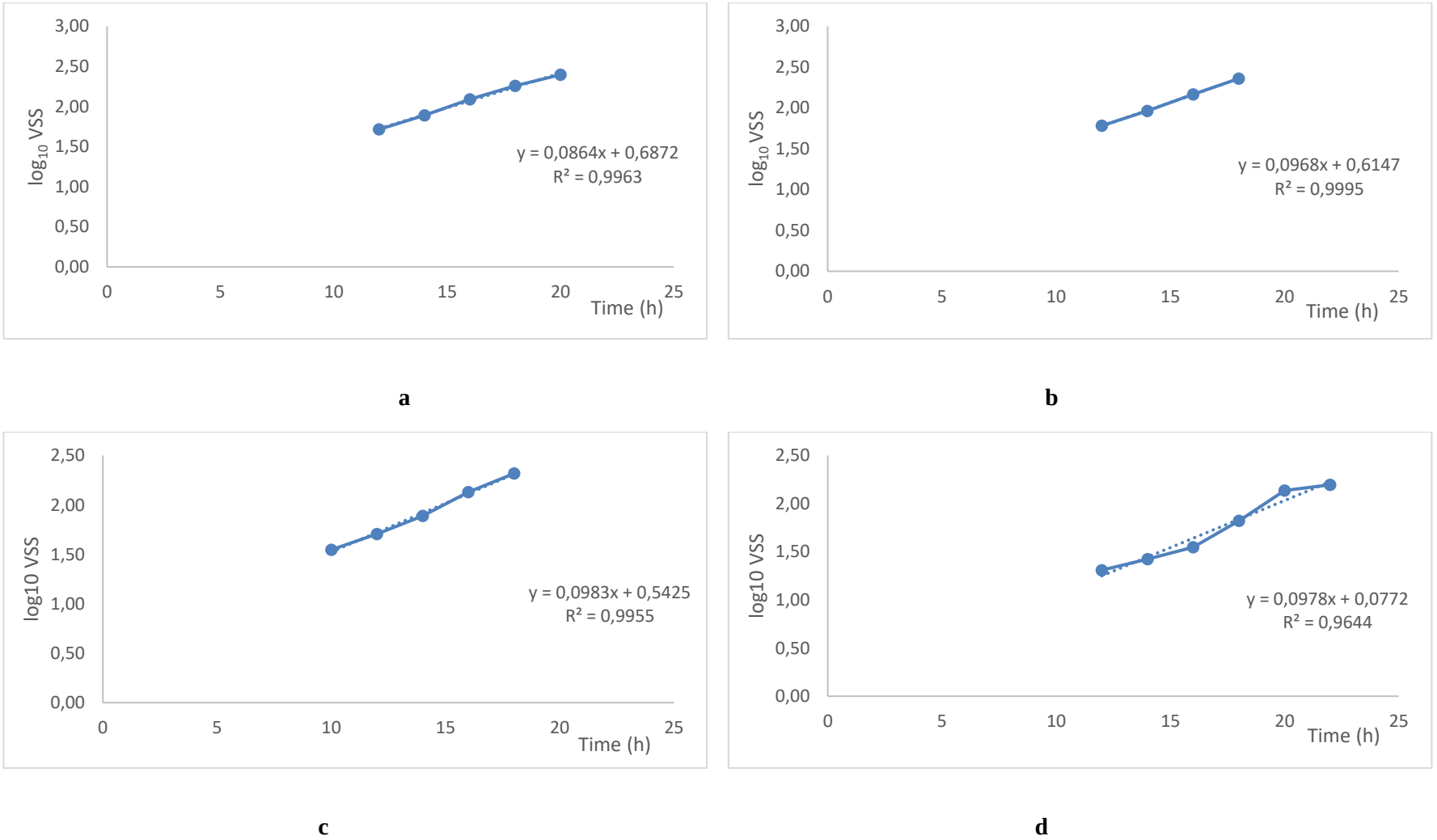
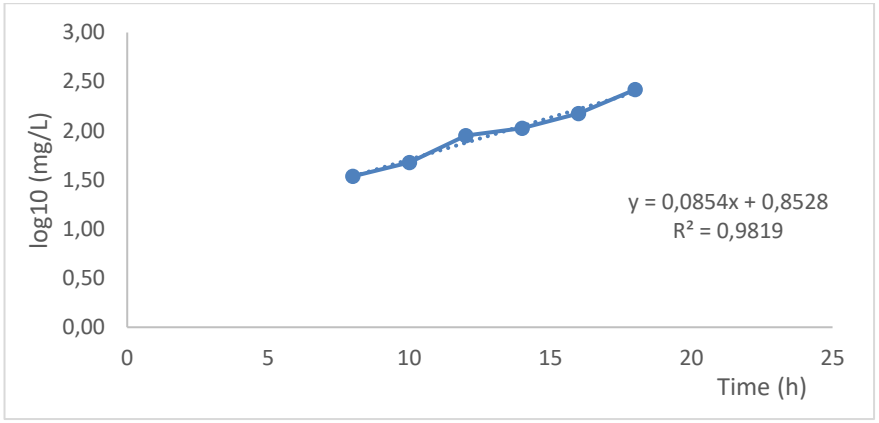


Figure 0-5 Specific growth rates for *Chlamydomonas reinhardtii* at 1 g organic C L^{-1} under heterotrophic conditions with different initial P concentrations as; a) 1 mg L^{-1}; b) 5 mg L^{-1}; c) 10 mg L^{-1}; and d) 15 mg L^{-1}.



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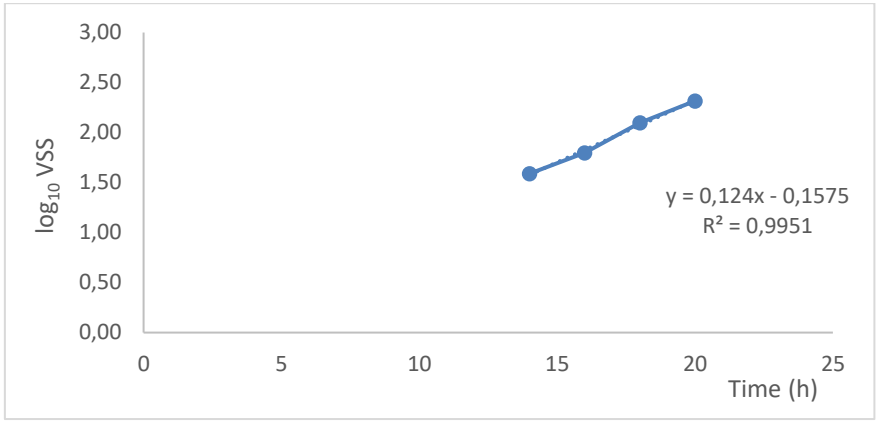

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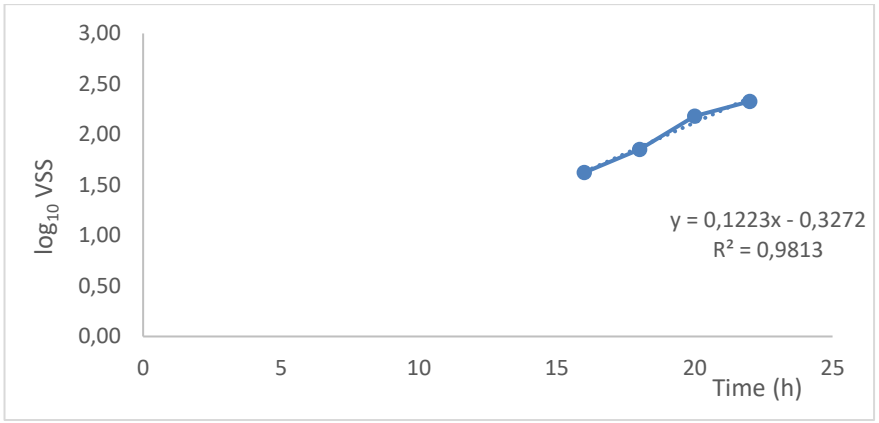

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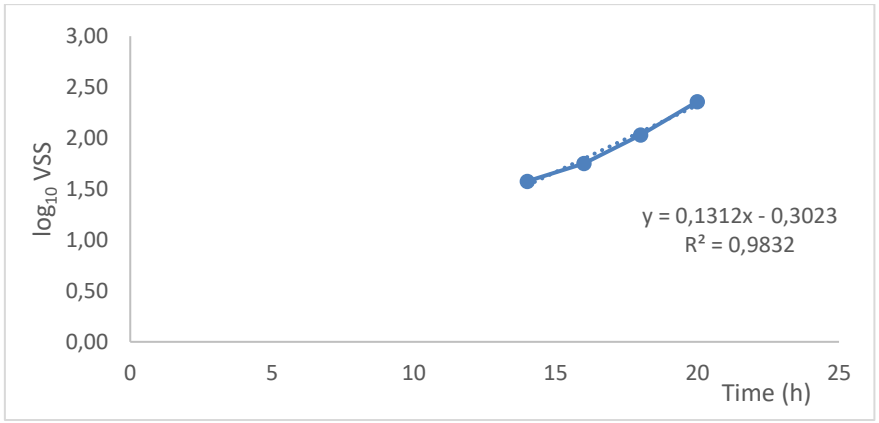

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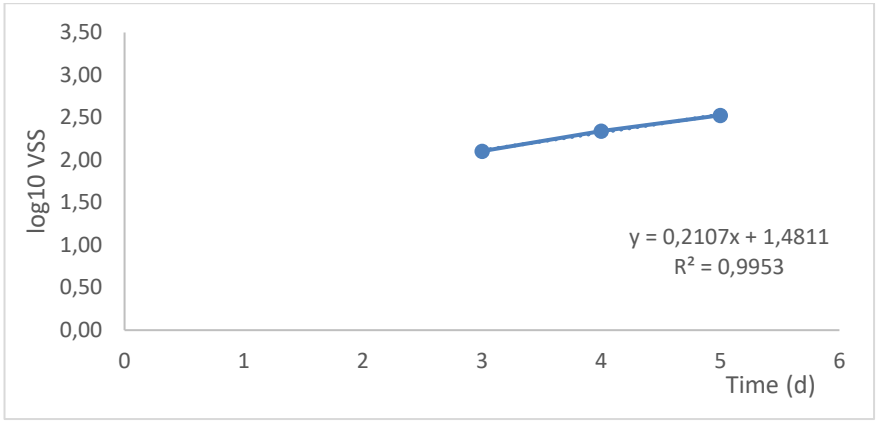
Figure D-6 Specific growth rates for *Chlamydomonas reinhardtii* at 0.5 g organic C L^{-1} under heterotrophic conditions with different initial P concentrations as; a) 1 mg L^{-1}; b) 5 mg L^{-1}; c) 10 mg L^{-1}; and d) 15 mg L^{-1}.



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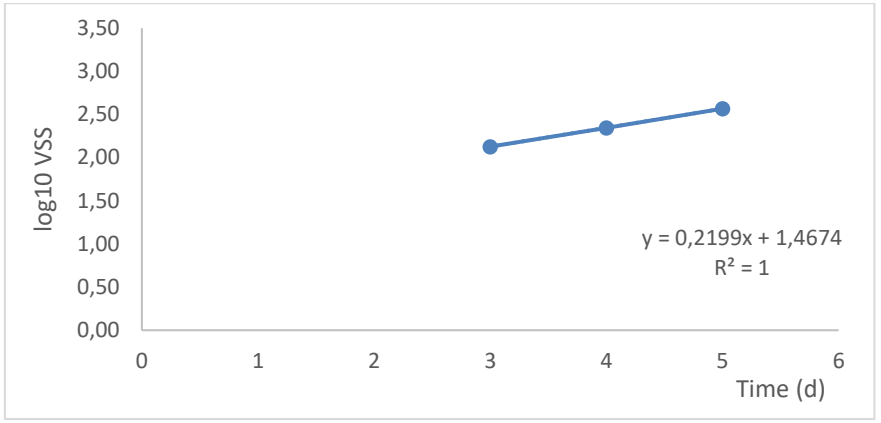

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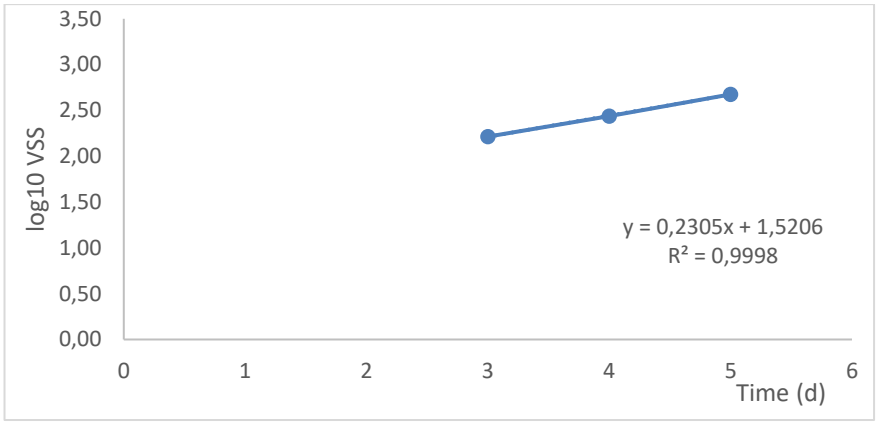

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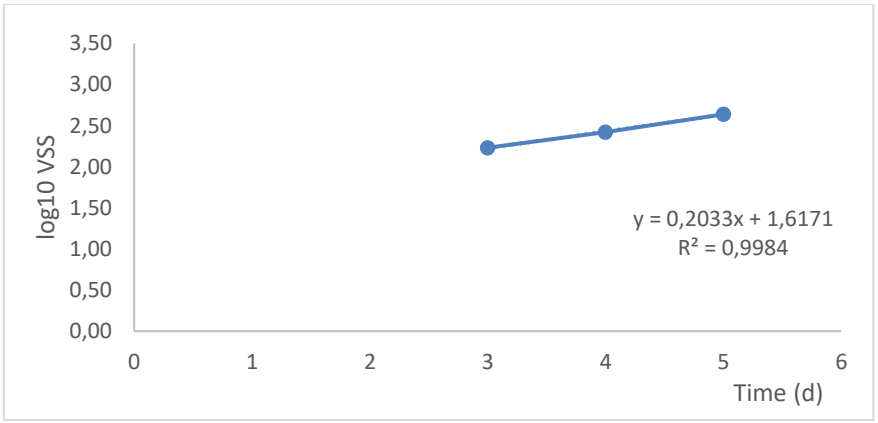
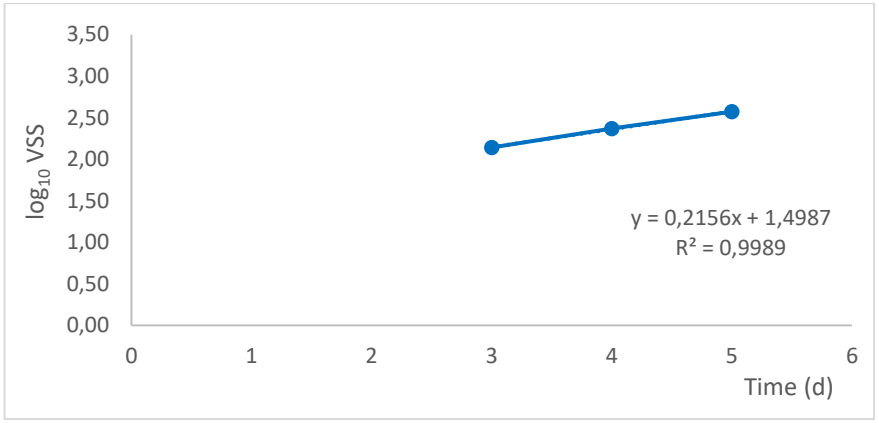


d

Figure D-7 Specific growth rates for *Chlamydomonas reinhardtii* using nitrogen source as a mix NH_4^+-N and NO_3^--N (50:50) at phototrophic cultivation with different initial P concentrations as; a) 1 mg L^{-1}; b) 5 mg L^{-1}; c) 10 mg L^{-1}; and d) 15 mg L^{-1}.



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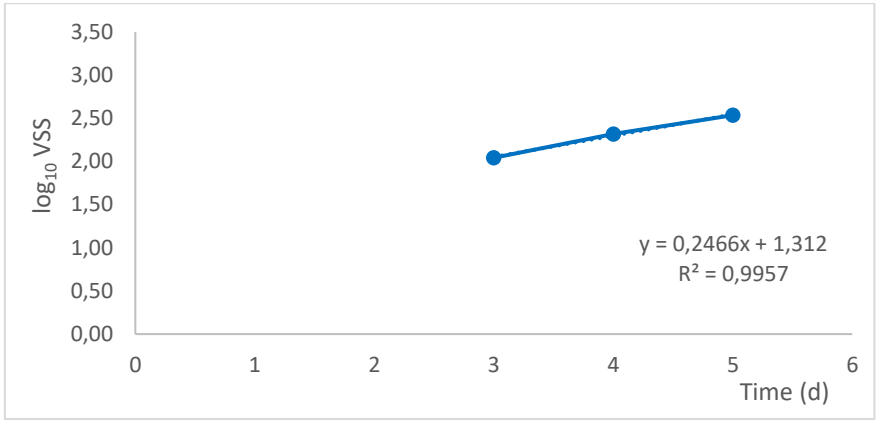

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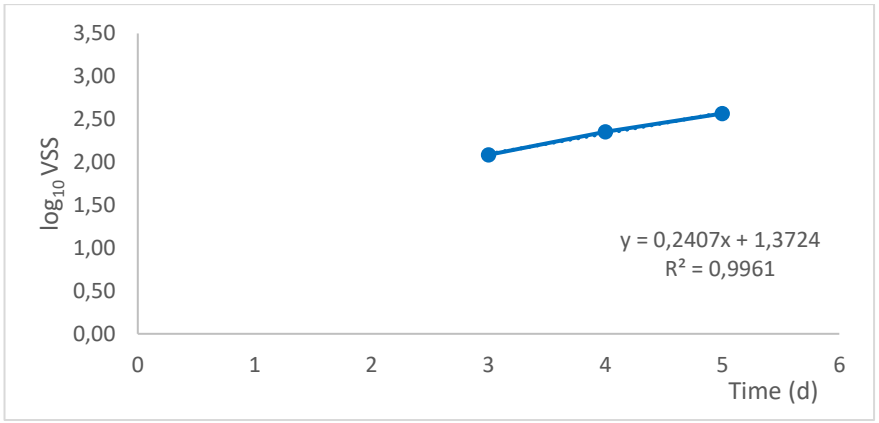

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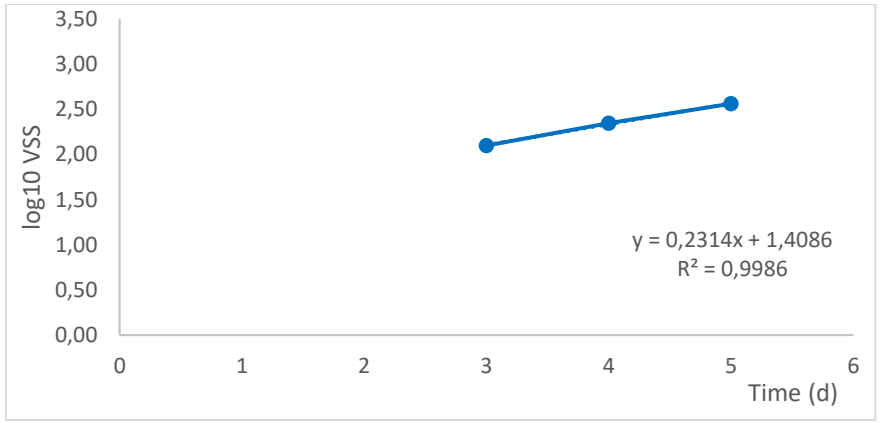

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Figure D-8 Specific growth rates for *Chlamydomonas reinhardtii* using nitrogen source as NH_4^+-N at phototrophic cultivation with different initial P concentrations as; a) 1 mg L^{-1}; b) 5 mg L^{-1}; c) 10 mg L^{-1}; and d) 15 mg L^{-1}.

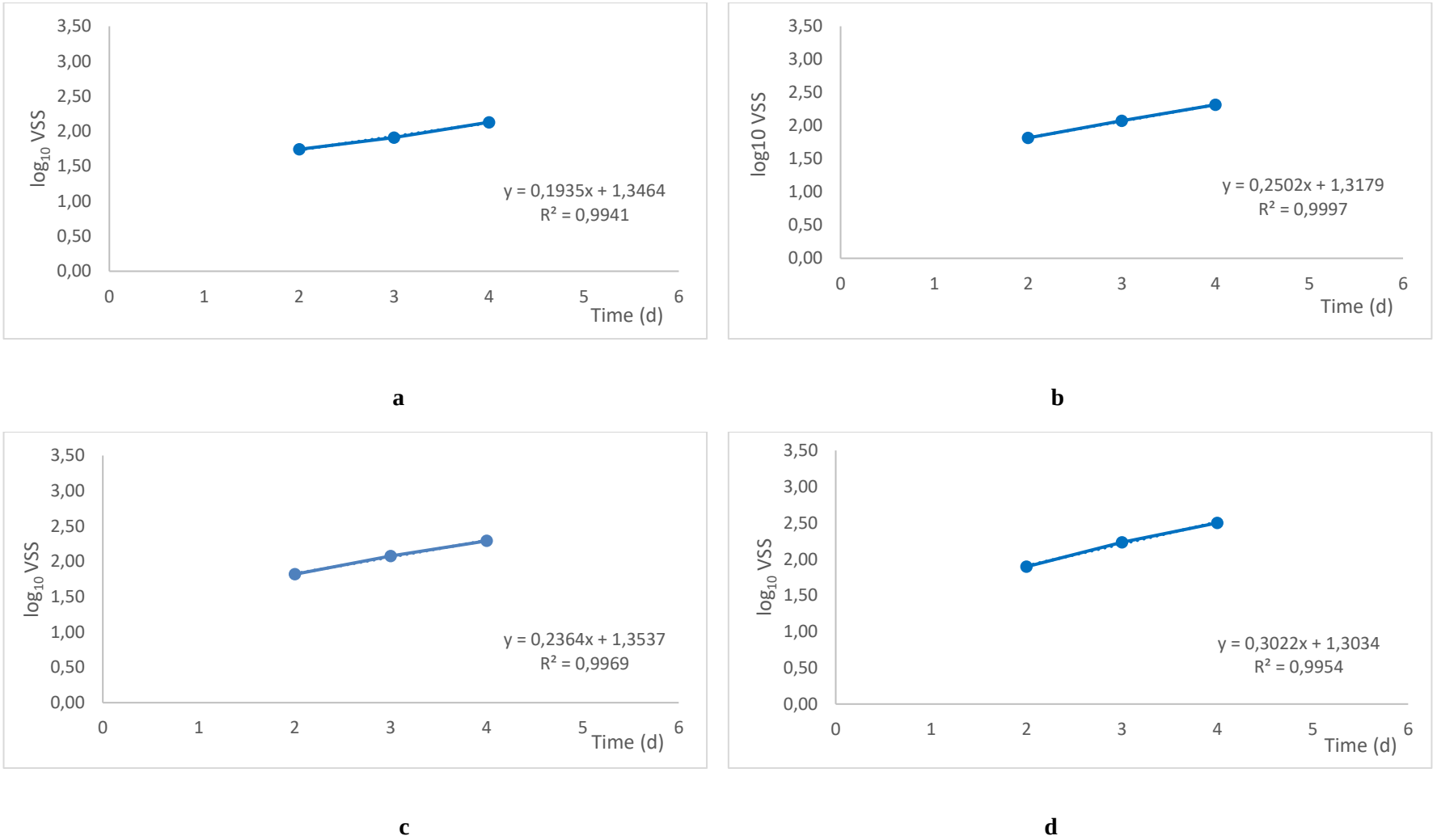
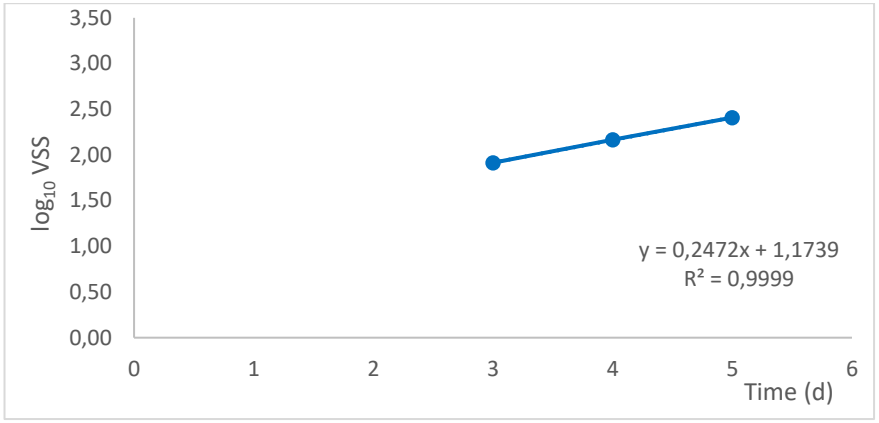


Figure 0-9 Specific growth rates for *Chlamydomonas reinhardtii* using nitrogen source as NO_3^--N at phototrophic cultivation with different initial P concentrations as; a) 1 mg L^{-1}; b) 5 mg L^{-1}; c) 10 mg L^{-1}; and d) 15 mg L^{-1}.



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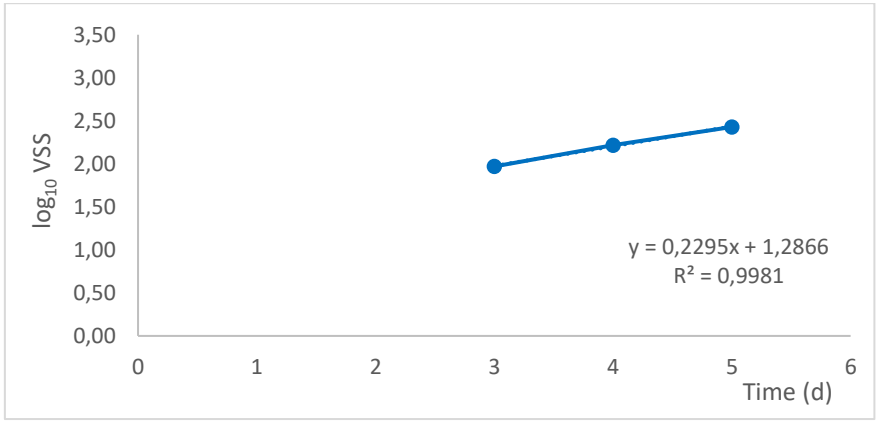

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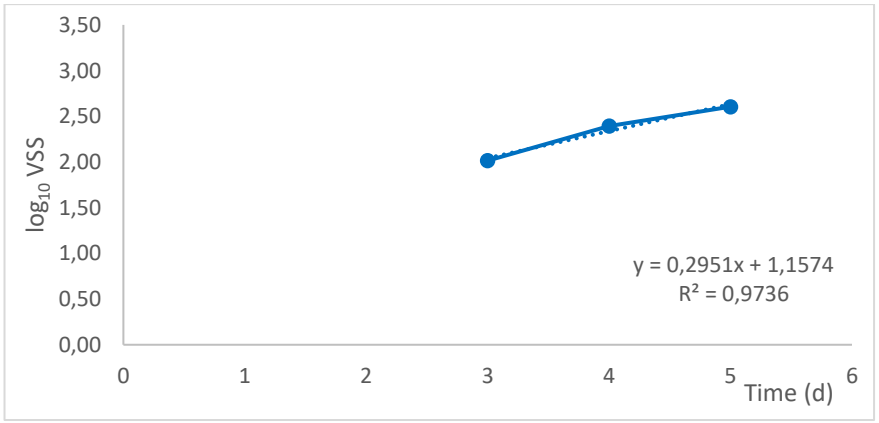

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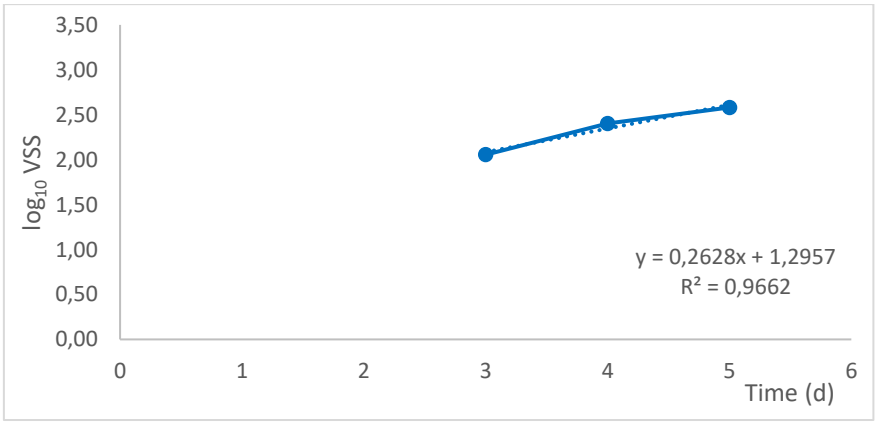

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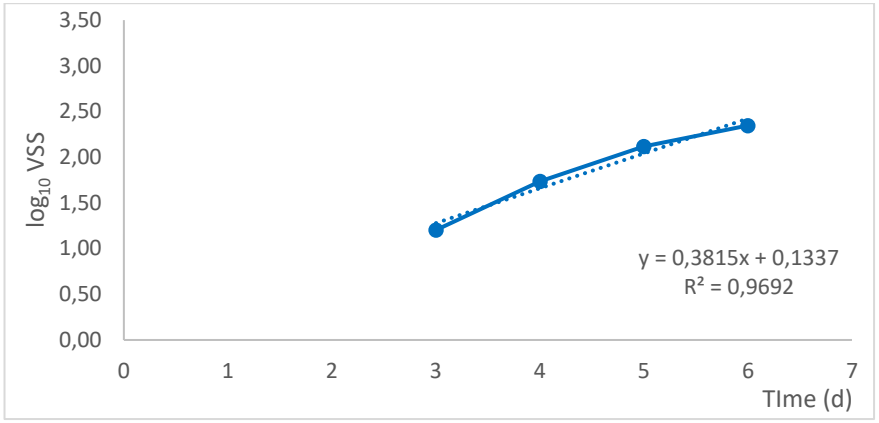
Figure 0-10 Specific growth rates for *Chlamydomonas reinhardtii* at 1.5 g inorganic C L^{-1} at phototrophic cultivation with different initial P concentrations as; a) 1 mg L^{-1}; b) 5 mg L^{-1}; c) 10 mg L^{-1}; and d) 15 mg L^{-1}.



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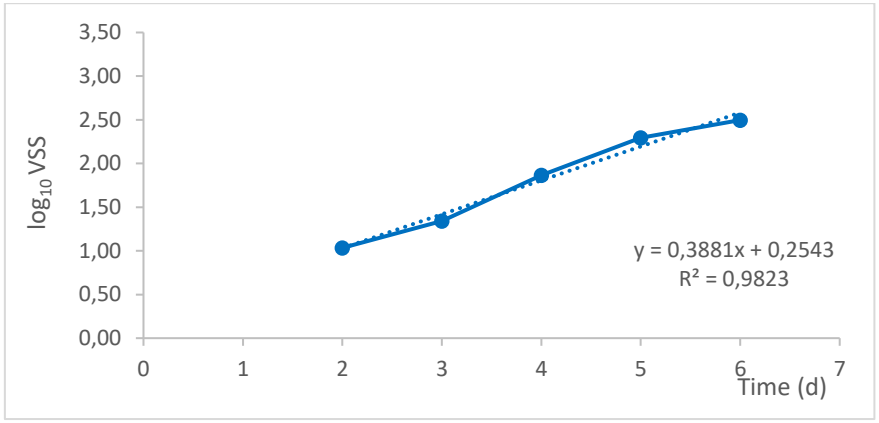

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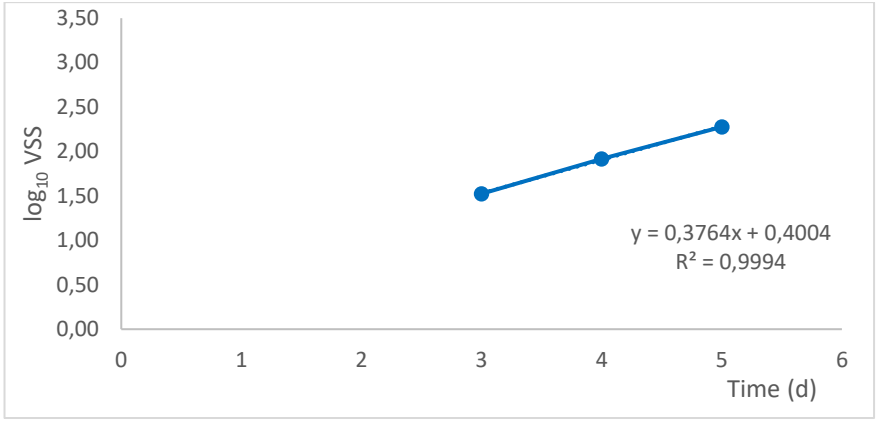

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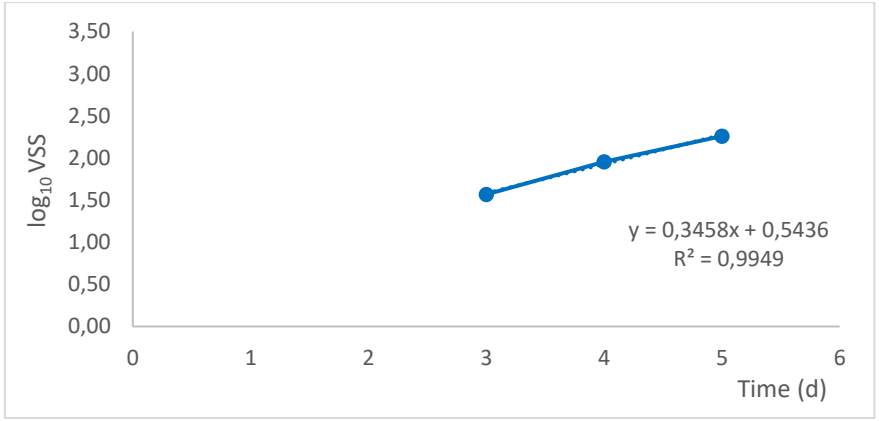

d

Figure 0-11 Specific growth rates for *Chlamydomonas reinhardtii* at 1 g inorganic C L^{-1} at phototrophic cultivation with different initial P concentrations as; a) 1 mg L^{-1}; b) 5 mg L^{-1}; c) 10 mg L^{-1}; and d) 15 mg L^{-1}.

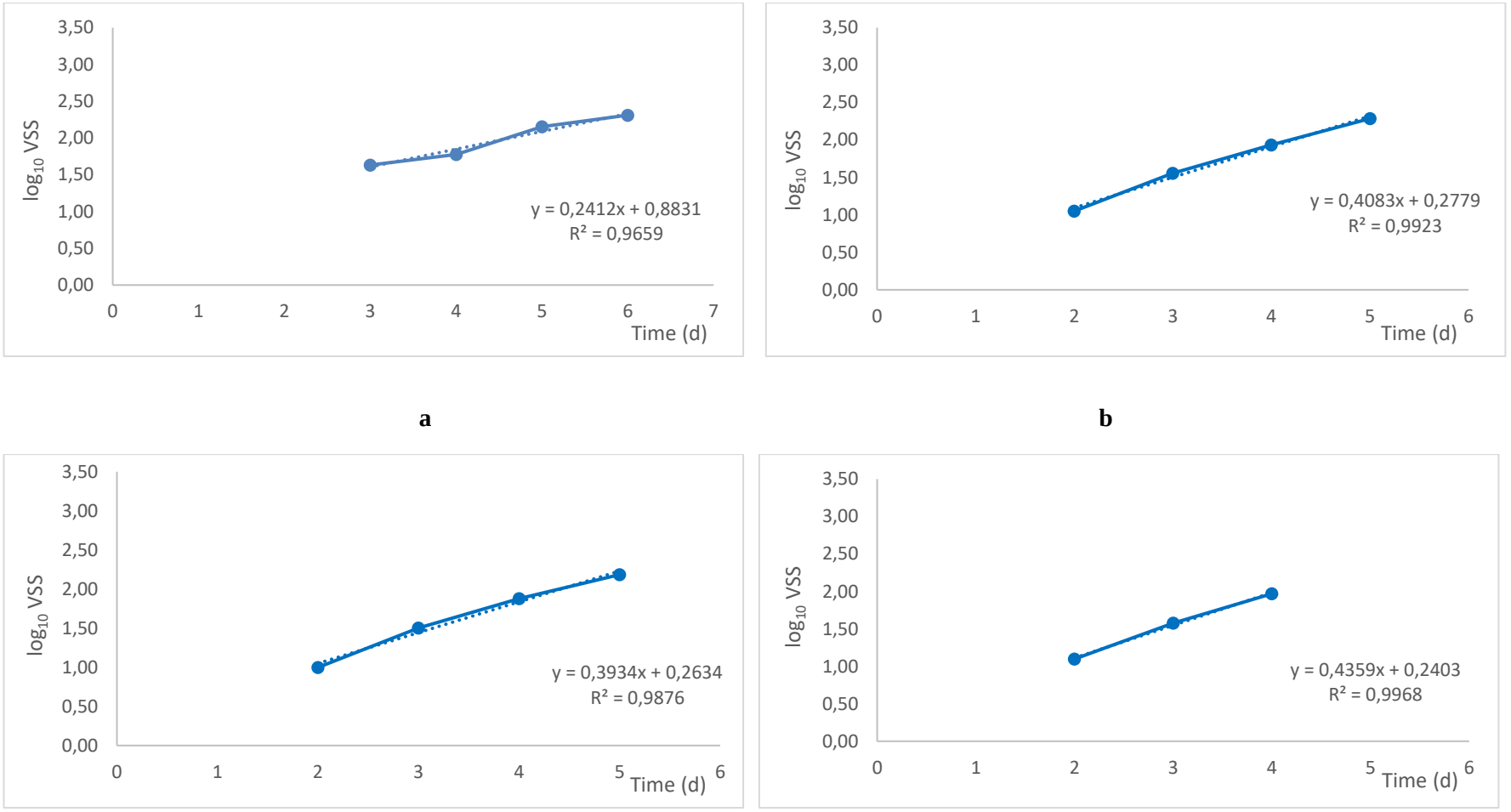

Figure 0-12 Specific growth rates for *Chlamydomonas reinhardtii* at 0.5 g inorganic C L^{-1} at phototrophic cultivation with different initial P concentrations as; a) 1 mg L^{-1}; b) 5 mg L^{-1}; c) 10 mg L^{-1}; and d) 15 mg L^{-1}.

APPENDIX E

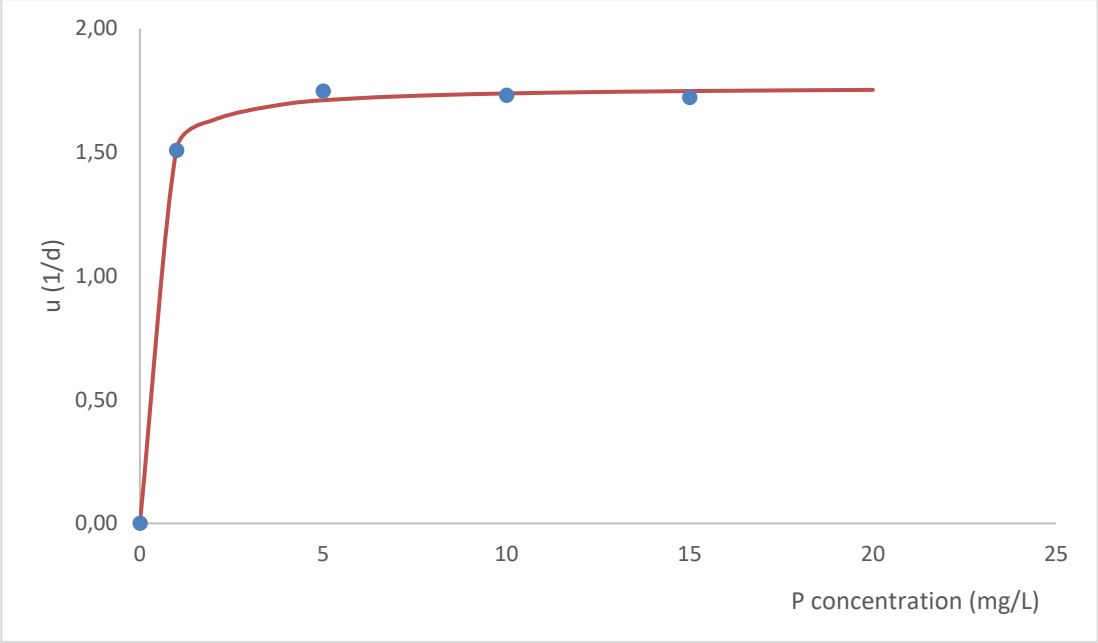

Figure 0E-1 Growth kinetics for *Chlamydomonas reinhardtii* as a function of P concentration using nitrogen source as a mix NH_4^+-N and NO_3^--N (50:50) under heterotrophic conditions

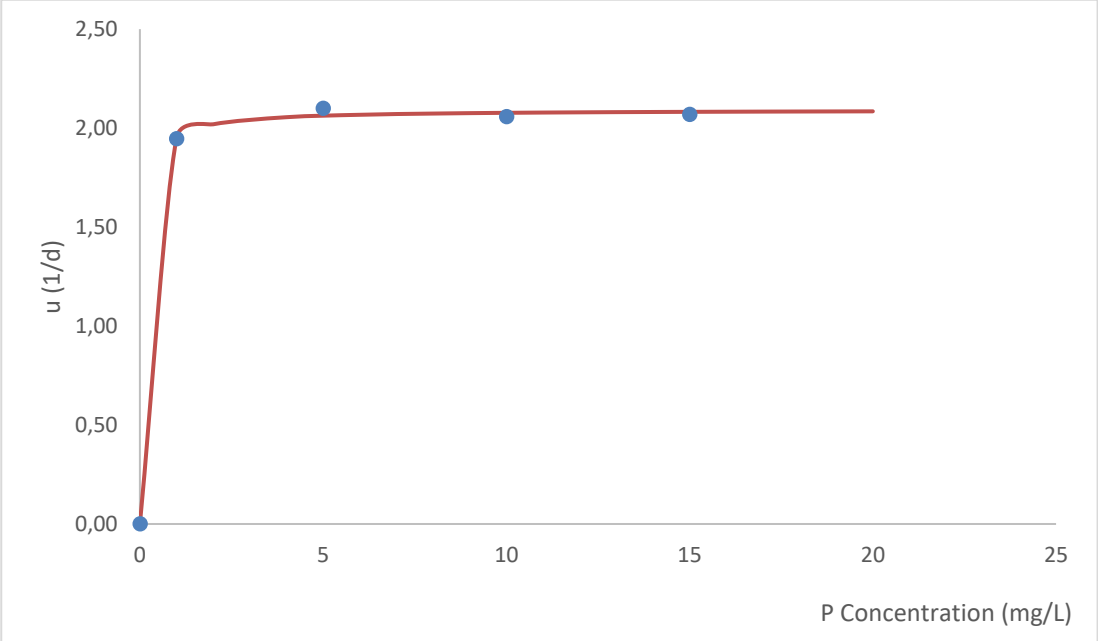

Figure 0E-2 Growth kinetics for *Chlamydomonas reinhardtii* as a function of P concentration using nitrogen source as NH_4^+-N under heterotrophic conditions

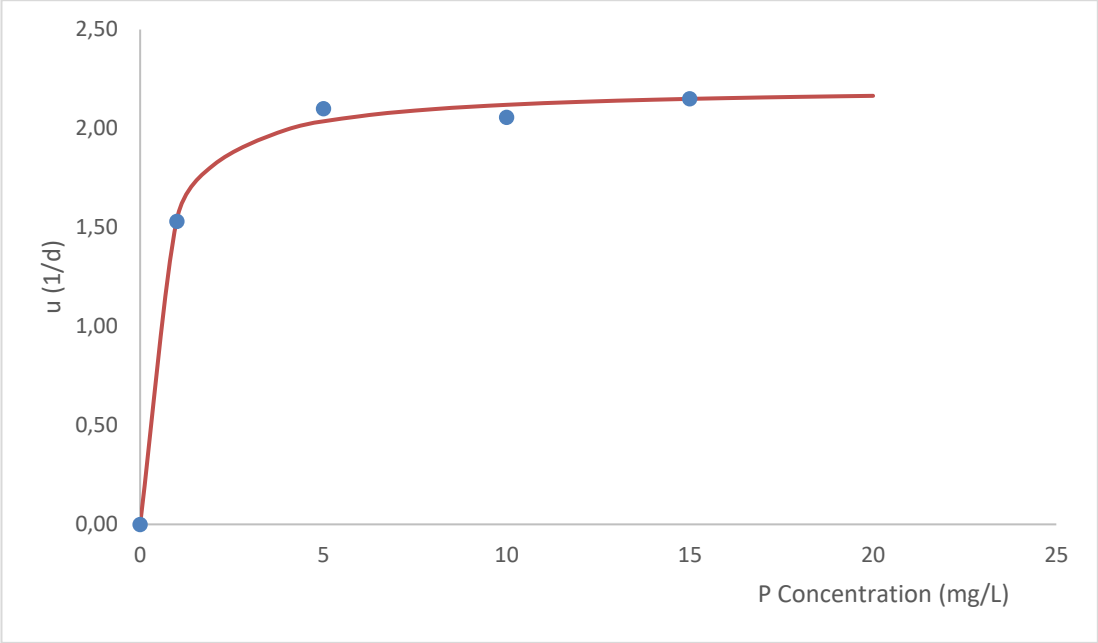

Figure E-3 Growth kinetics for *Chlamydomonas reinhardtii* as a function of P concentration using nitrogen source as NO_3^--N under heterotrophic conditions

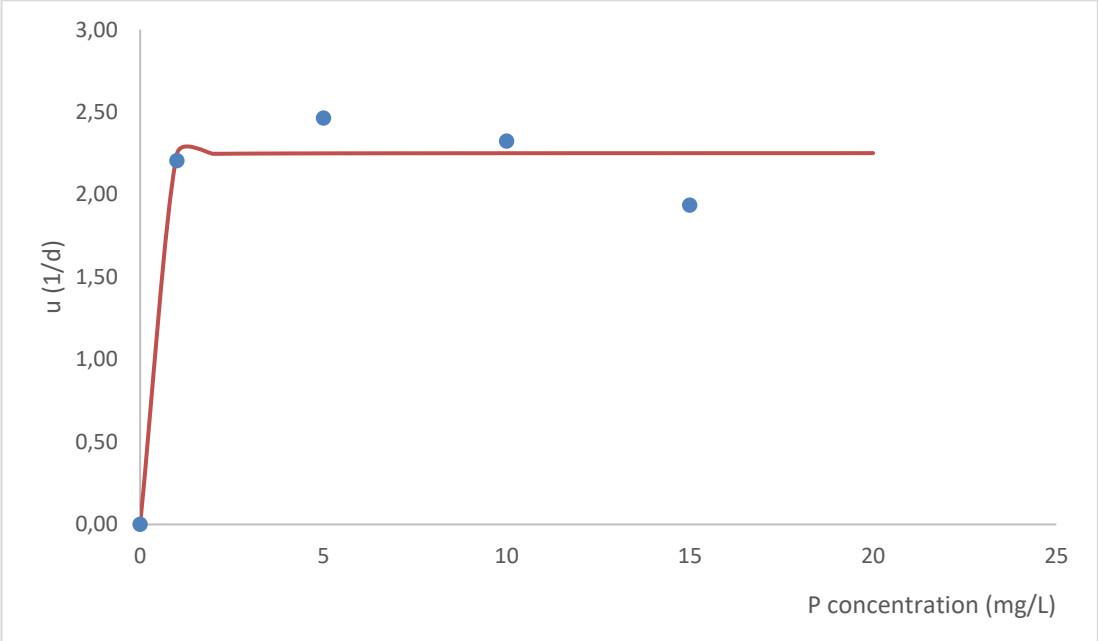

Figure E-4 Growth kinetics for *Chlamydomonas reinhardtii* as a function of P concentration at 1.5 g organic C L^{-1} under heterotrophic conditions

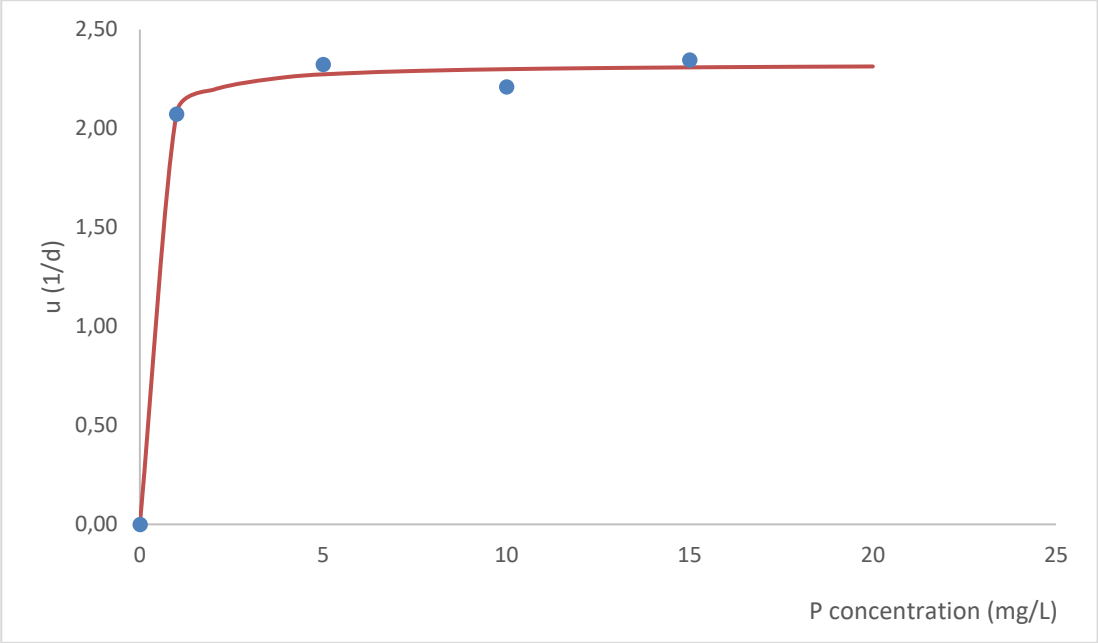

Figure E-5 Growth kinetics for *Chlamydomonas reinhardtii* as a function of P concentration at 1 g organic C L^{-1} under heterotrophic conditions

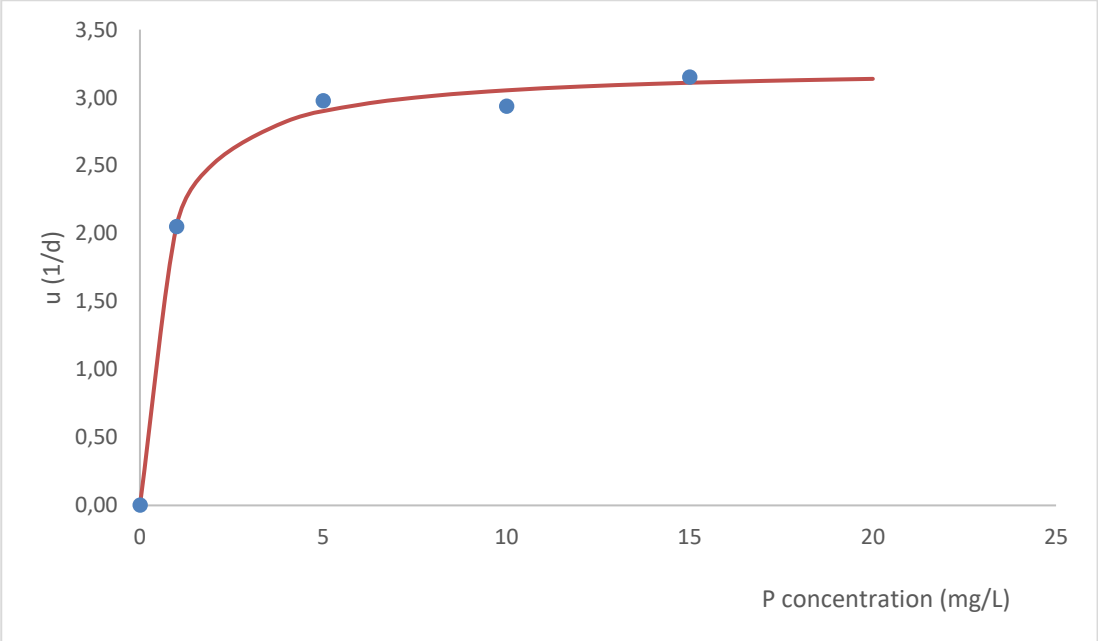

Figure E-6 Growth kinetics for *Chlamydomonas reinhardtii* as a function of P concentration at 0.5 g organic C L^{-1} under heterotrophic conditions

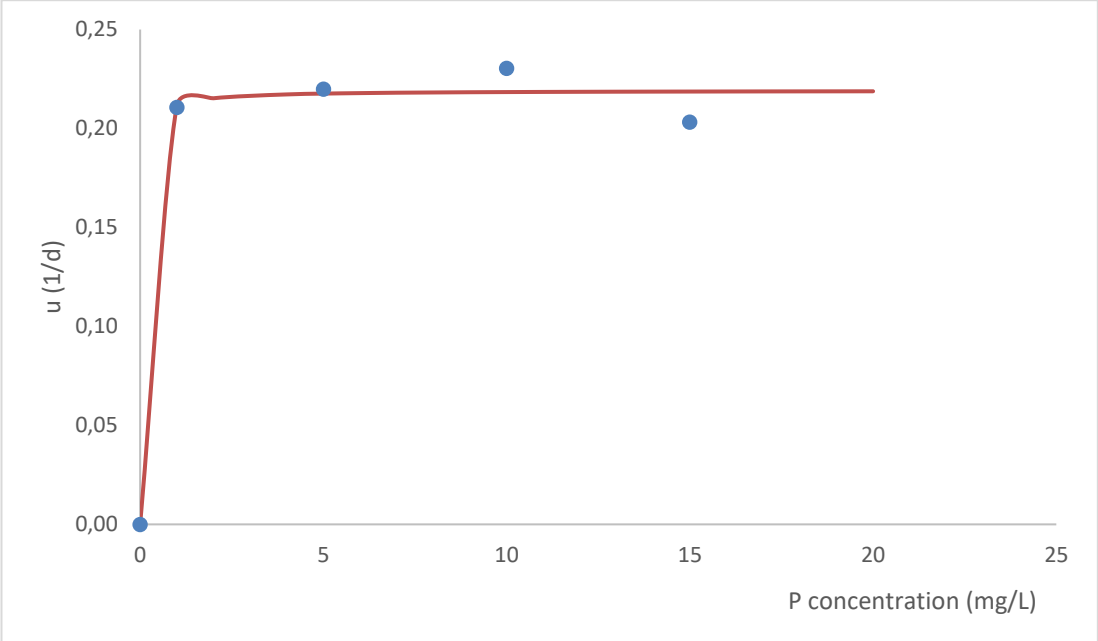

Figure E-7 Growth kinetics for *Chlamydomonas reinhardtii* as a function of P concentration using nitrogen source as a mix NH_4^+-N and NO_3^--N (50:50) at phototrophic cultivation

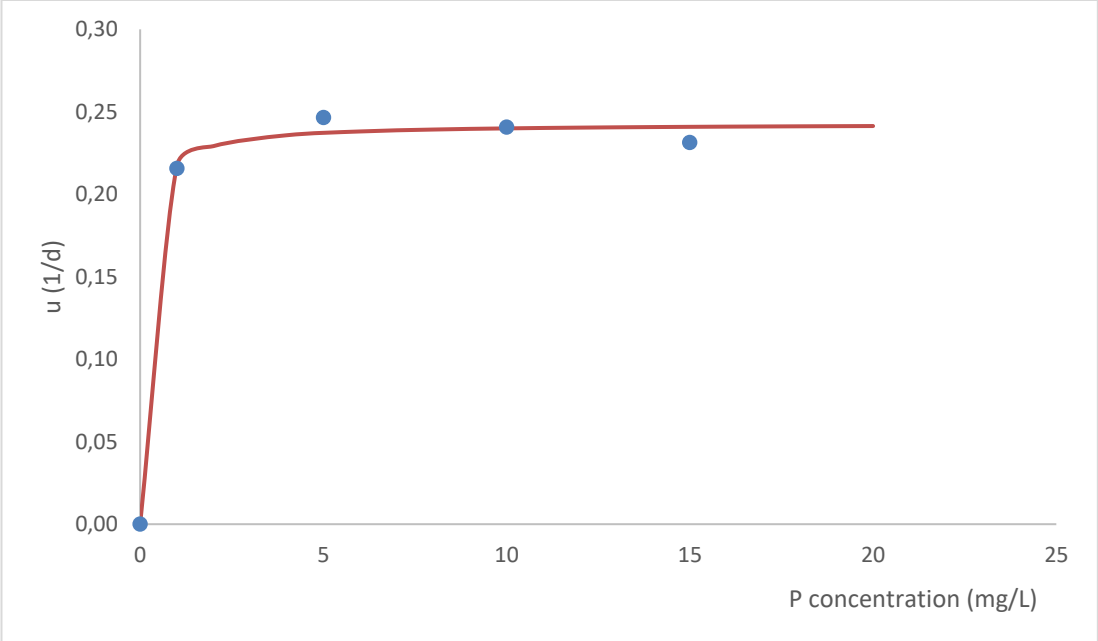

Figure E-8 Growth kinetics for *Chlamydomonas reinhardtii* as a function of P concentration using nitrogen source as NH_4^+-N at phototrophic cultivation

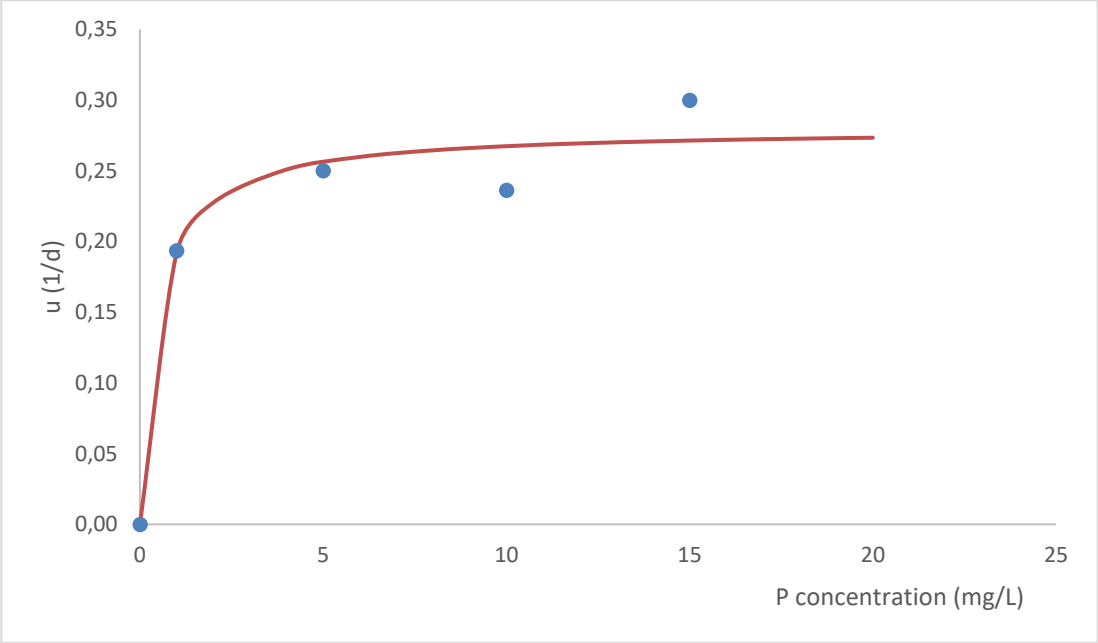

Figure E-9 Growth kinetics for *Chlamydomonas reinhardtii* as a function of P concentration using nitrogen source as NO_3^--N at phototrophic cultivation

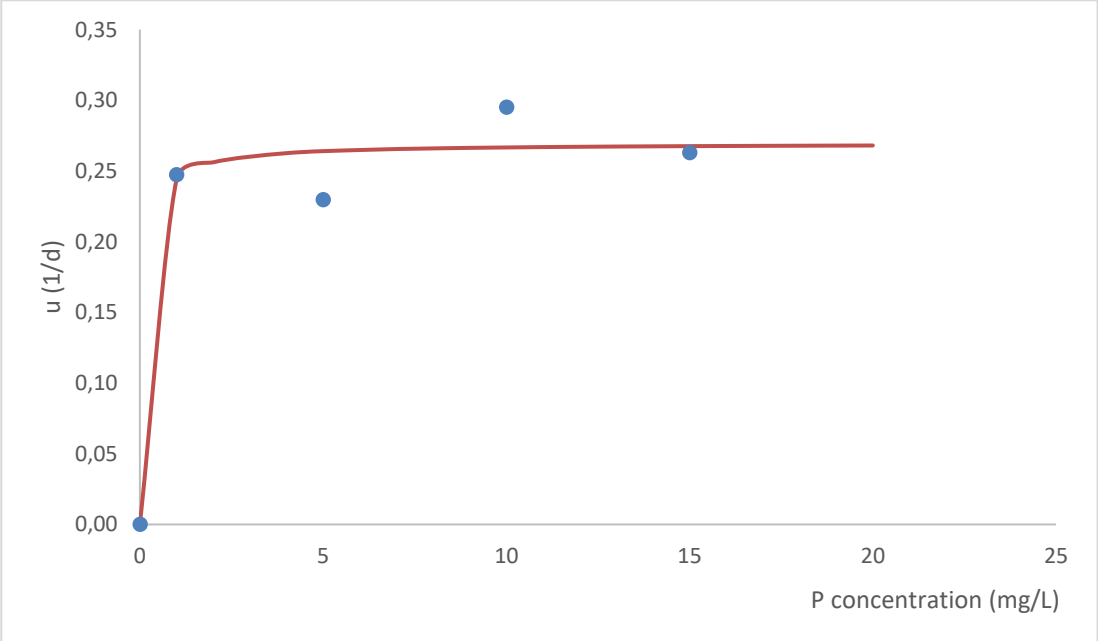

Figure E-10 Growth kinetics for *Chlamydomonas reinhardtii* as a function of P concentration at 1.5 g inorganic C L^{-1} at phototrophic cultivation

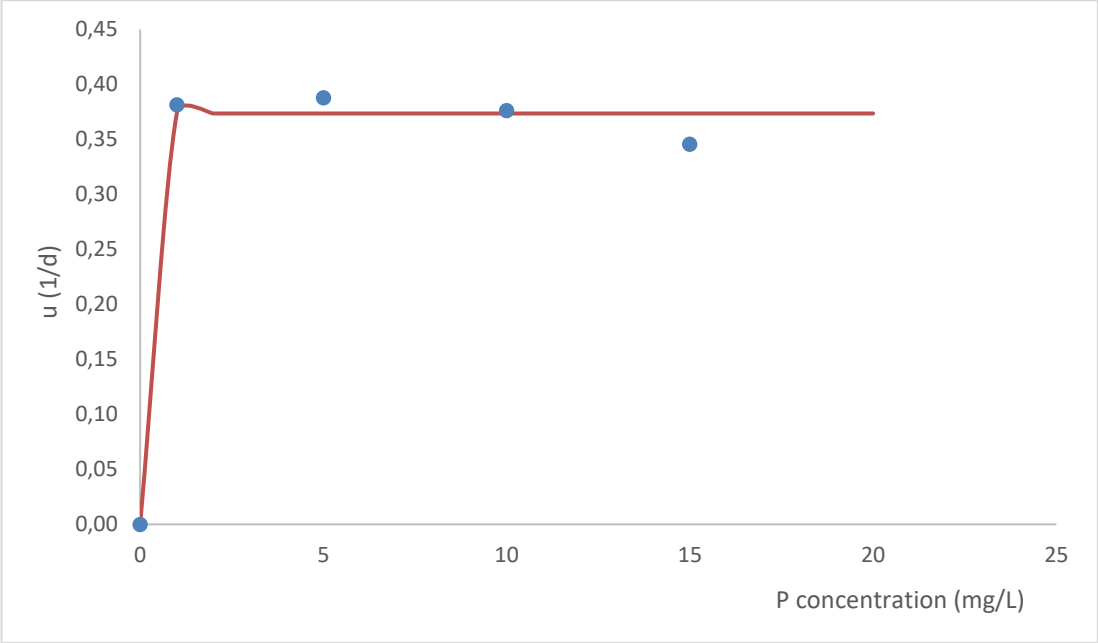

Figure E-11 Growth kinetics for *Chlamydomonas reinhardtii* as a function of P concentration at 1 g inorganic C L^{-1} at phototrophic cultivation

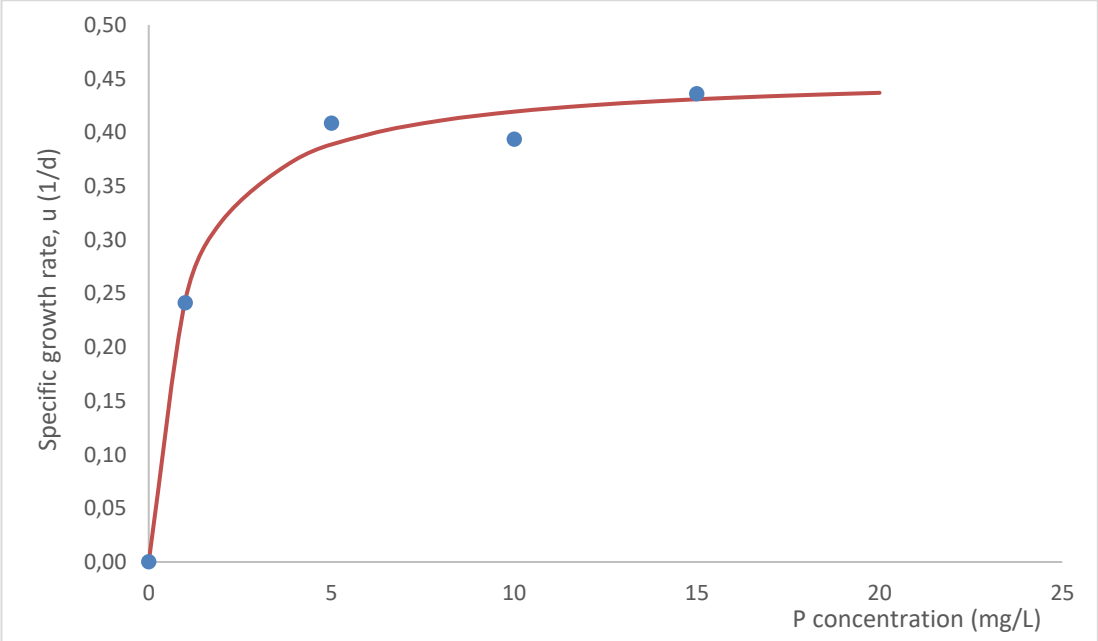

Figure E-12 Growth kinetics for *Chlamydomonas reinhardtii* as a function of P concentration at 0.5 g inorganic C L^{-1} at phototrophic cultivation