Water neutral developments: How to successfully integrate micro-algae systems into wastewater management

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The Microbe

The Microbe is so very small
You cannot make him out at all,
But many sanguine people hope
To see him through a microscope.
His jointed tongue that lies beneath
A hundred curious rows of teeth;
His seven tufted tails with lots
Of lovely pink and purple spots,
On each of which a pattern stands,
Composed of forty separate bands;
His eyebrows of a tender green;
All these have never yet been seen –
But Scientists, who ought to know,
Assure us that they must be so…
Oh! Let us never, never doubt
What nobody is sure about

– Hilaire Belloc

Abstract

Treating municipal wastewater is necessary to limit the impact carbonaceous, nitrogenous and phosphorus matter present in spent water may have on receiving aquatic systems. Conventional wastewater treatment systems employing the activated sludge or biological nutrient removal process as the main phase of treatment, demonstrate a high proficiency at removing these contaminants. Despite this, these processes are described as problem shifting, simply causing secondary pollution because of high energy consumed, production of waste sludge and greenhouse gases. To improve the environmental impact of wastewater treatment, particularly in light of stricter effluent discharge standards, treatment processes that have low energy consumption without affecting performance are needed. A potential, more sustainable biological treatment process to remediate the contaminants from wastewater is by using microalgae. Although this concept has been extensively researched, limited commercial development has been achieved. A major hindrance to the implementation of microalgae to treat wastewater is the cultivation process, which is one of the main cost and energy burdens, and as such would not result in the much-desired reduction in overall energy consumption of wastewater treatment. This thesis evaluated the performance of a microalgae treatment process for primary settled municipal wastewater (PSW) in a laboratory setting under static culturing conditions, to examine the feasibility of a low energy treatment process. Initial experiments assessed three freshwater microalga to treat PSW under both optimal (aerated) and static (non-aerated) culture conditions. From these results, *Chlorella vulgaris* identified itself as the most promising species, exhibiting high inorganic nitrogen and phosphorus removal. The availability of a suitable carbon substrate was determined to be the main limiting-factor affecting the algal treatment performance under static cultivation. To investigate this, initial experiments of PSW enriched with glucose (<300 mg L^{-1}) as an organic carbon source to facilitate the bioremediation by *C. vulgaris* was performed. Characterisation of the wastewater revealed significant reductions in NH_3-N (from 28.9 to 0.1 mg L^{-1}) and PO_4-P (from 3.2 to 0.1 mg L^{-1}) in just 2 days. Additionally, the exogenous glucose appeared completely removed from the wastewater after the first day. These achieved levels of treatment in respect of both the NH_3-N and PO_4-P were much higher than those recorded without *C. vulgaris* treatment with or without glucose enrichment. The reliability of this process was evaluated across a further three independent batches of PSW with varying compositions and organic carbon sources. The efficiency of the microalgae treatment process at reducing NH_3-N and PO_4-P was consistent in PSW enriched with organic carbon, resulting in > 90% reduction of the inorganic compounds in each batch. Lastly, to overcome the material cost of applying commercial sources of organic carbon, experiments were conducted to evaluate the use of the carbohydrate rich by-product, pot ale, from the production of malt whiskey as a carbon substrate to promote microalgae growth and remediation in PSW. In batch experiments, repeated three times with wastewater collected and treated separately and sequentially, the efficiency of the microalgae in pot ale enriched PSW demonstrated a high variability at reducing NH_3-N and PO_4-P, between 99 to 58% and 94 to 58% respectively. When operated under semi-continuous mode the microalgae demonstrated to be reliable in treating pot ale enriched PSW however, the removal efficiency in NH_3-N, PO_4-P and COD declined slightly in each subsequent cycle following the replenishment of PSW. The results of the pot ale enriched experiments highlight future research needs, such as the optimisation of nutrient ratios in the PSW and control over pH, to ensure a consistent and reliable treatment performance. Overall the application of *C. vulgaris* to treat enriched PSW, without aeration, offers a key area to develop as an alternative low energy, biological wastewater treatment option.

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Dedication

I would like to dedicate this thesis to my family, in particular my parents John and Sandra Evans. Thank you both.

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List of Terminology

€ - Euro

°C - Degrees Celsius

µE m^{-2} s^{-1} - Microeinsteins per second per square metre

µm - Micrometre

cm - Centimetre

d^{-1} - per Day

e.g. - exempli gratia

g - Grams

i.e. - id est

kg - Kilogram

kWh - Kilowatt Hour

L/L^{-1} - Litre/per Litre

M - Mole per litre

m/m^2/m^3 - metre/metre squared/metre cubed

min - Minute

mL - Millilitre

mm - Millimetre

mM - Millimole per Litre

MWh - Megawatt Hour

nm - Nanometre

pH - Pondus Hydrogenium

pKa - Dissociation Constant

s/s^{-1} - second/per second

SB - Standard Deviations of the Blank

SD - Standard Deviation

SD_c - Standard Error of the Intercept

SD_m - Standard Error of the Slope

$SD_{y/x}$ - Standard Deviation of the Regression

S_{XO} - Error of the Calculated Concentration

t_{CAL} - Calculated t Value

t_{CRI} - Critical t Value

c - Intercept

m - Slope

y_B - Blank Signal

List of Acronyms

3-PGA - 3-phosphoglycerate

A^2O - Anaerobic-Anoxic-Oxic

ADP - Adenosine Diphosphate

ANAMMOX - Anaerobic ammonium oxidation

AOB - Ammonia Oxidising Bacteria

AS - Asparagine Synthetase

AspAT - Aspartate aminotransferase transfers

ATP - Adenosine Triphosphate

BBM - Bold Basal Medium

BNR - Biological Nutrient Removal

$BOD_{(5)}$ - Biological Oxygen $Demand_{(5)}$

CANON - Complete Autotrophic Nitrogen Removal over Nitrite

CCAP - Culture Collection of Algae and Protozoa

CI - Confidence Interval

CO_2 - Carbon Dioxide

CO2eq - Carbon Dioxide equivalent

COD - Chemical Oxygen Demand

COD_T - Theoretical Chemical Oxygen Demand

DIC - Dissolved Inorganic Carbon

DIN - Dissolved Inorganic Nitrogen

DL - Detection Limit

DNA - Deoxyribonucleic Acid

DO - Dissolved Oxygen

DOC - Dissolved Organic Carbon

DW - Dry Weight

EPS - Extracellular Polysaccharide

G3P - Glyceraldehyde-3-phosphate

GAO - Glycogen Accumulating Organisms

GDH - Glutamate Dehydrogenase

GOGAT - Glutamine 2-oxoglutarate amino transferase

GS - Glutamine Synthetase

HRAP - High Rate Algae Pond

HRT - Hydraulic Retention Time

IPCC - Intergovernmental Panel on Climate Change

LED - Light Emitting Diode

LOD - Limit of Detection

N - Nitrogen

N_2 - Nitrogen Gas

N_2O - Nitrous Oxide

NAD(H) - Nicotinamide Adenine Dinucleotide

NADP(H) - Nicotinamide Adenine Dinucleotide Phosphate

NED dihydrochloride - N-(1-Naphthyl)ethylenediamine dihydrochloride

NH_2OH - Hydroxylamine

NH_3-N - Ammonia-Nitrogen

NH_4^+ - Ammonium

NO - Nitric Oxide

NO_2-N - Nitrite-Nitrogen

NO_3-N - Nitrate-Nitrogen

NOB - Nitrite Oxidising Bacteria

P - Phosphorus

PAO - Polyphosphate Accumulating organisms

PBR - Photobioreactor

PE - Population Equivalence

PHA - Poly-β-hydroxyalkanoate

PO_4^{3-} - Phosphate

PO_4-P - Phosphate-Phosphorous

PPP - Pentose Phosphate Pathway

PSI/II - Photosystem I/II

PSW - Primary Settled Wastewater

RE - Removal Efficiency

RNA - Ribonucleic Acid

RuBisCo - Ribulose-1, 5-bisphosphatecarboxylase oxygenase

RuBP - Ribulose-1, 5-bisphosphate

SAG - Sammlung von Algenkulturen der Universität Göttingen

SHARON - Single Reactor Higher Activity Ammonia Removal over Nitrite

SRP - Soluble Reactive Orthophosphorus

STE - Secondary Treatment Effluent

TC - Total Carbohydrate

TCA - Tricarboxylic Acid Cycle

TDN - Total Dissolved Nitrogen

TDP - Total Dissolved Phosphorous

TN - Total Nitrogen

TP - Total Phosphorus

TSS - Total Suspended Solids

UWTD - Urban Wastewater Treatment Directive

v/v - Volume per Volume

V/Vm - Air Volume per Volume of Liquid per Minute

VFA - Volatile Fatty Acids

w/v - Weight per Volume

w/w - Weight per Weight

W_F - Final Weight

W_I - Initial Weight

WW+Air - Wastewater with Aeration

WW+Air+*C.v/A.o/H.r* - Wastewater with Aeration and either *C. vulgaris/H. riparia/A. obliquus*

WW+*C.v/A.o/H.r* - Wastewater either with *C. vulgaris/H. riparia/A. obliquus*

WWC - Wastewater control

WWCO_2+*C.v* - Wastewater with carbon dioxide and *C. vulgaris*

WWG - Wastewater with glucose

WWG+*C.v* - Wastewater with glucose and *C. vulgaris*

WWGY+*C.v* - Wastewater with glycerol and *C. vulgaris*

WWPA - wastewater with pot ale

WWPA+*C.v* - wastewater with pot ale and *C. vulgaris*

Chapter 1 – Introduction

1.1 Background to the problem

The main aim of wastewater treatment is to significantly reduce the quantity of carbonaceous (organic; predominantly determined as biological oxygen demand (BOD)) materials and, where sensitive waters are involved, nitrogen (N) and phosphorus (P) compounds prior to being discharged into receiving systems [1, 2]. This is because the presence of these materials in large concentrations can have deleterious effects on dissolved oxygen (O_2) concentration levels, the trophic state and ultimately the well-being of the fauna and flora in the water [3–5]. Achieving improved ecological status of water sources is a growing focus for many developed and developing nations, in particular with reducing N and P in wastewater effluent [6–9]. Nitrogen and P are critical to the ecological health of aquatic ecosystems, but excess availability, particularly in inorganic forms can result in undesirable consequences such as eutrophication [3, 10]. Characterised by the increase in phytoplankton growth, blooms of toxic and non-toxic algae associated with eutrophication reduce water transparency resulting in attenuated light levels to submerged aquatic vegetation and hence reduced dissolved O_2 generation via photosynthesis [11–13]. The concentration of dissolved O_2 is further reduced during the decay of the formed biomass following nutrient deprivation, as heterotrophic bacteria digest the biodegradable organic matter (i.e. dead phytoplankton). It is estimated that the organic material of phytoplankton biomass produced from the discharge of 1 kg of P can exert 100 kg of O_2 demand, while that produced from the discharge of 1 kg of N can exert 14 kg of O_2 demand [2]. Consequently, hypoxic or anoxic conditions form and that can adversely affect the indigenous fauna and flora, causing loss of species diversity and ecosystem function in water bodies.

In Europe the Urban Wastewater Treatment Directive (UWTD) sets effluent discharge limits for chemical oxygen demand (COD) at 125 mg L^{-1} O_2, and for total phosphorus (TP) at 1 or 2 mg L^{-1} and total nitrogen (TN) at 10 or 15 mg L^{-1} for population equivalence (PE) of >100k or <100k, respectively [14]. Conventional wastewater treatment systems subject the wastewater to two main treatment phases: primary and secondary treatment. In brief, primary treatment aims to reduce the insoluble suspended solids concentration by facilitating the separation from the water, either by gravity settlement (i.e. sedimentation) or flotation. Thereafter, the wastewater flows into the secondary treatment stage. The objective of the secondary treatment stage is to reduce the residual organic and, to an extent also inorganic material from the water. This is achieved by indigenous, wastewater-borne microorganisms that are selectively cultivated and maintained in an aerobic environment. The microorganisms help to eliminate the O_2-demanding materials either by digesting them into an innocuous form such as carbon dioxide (CO_2) or nitrogen gas (N_2), and forming new biomass which is particulate in form and can be separated by further sedimentation, resulting in water free of polluting material [2]. In essence, the secondary stage of the treatment train condenses the self-purification process seen in nature, and expedited by the configurations of the reactor which maintains an optimal environment for microbial growth. The provision of O_2 is essential at this stage to enable the microorganisms to digest and

mineralise the materials into a form that is resistant to further biological activity. Biodegradable carbonaceous matter in wastewater is estimated to have an O_2 demand in the order of 2 kg O_2 kg^{-1} COD [2]. Maintaining this level of dissolved O_2 concentration during conventional secondary wastewater treatment is energy intensive and hence expensive. As for the nutrients, the quantity of N and P in wastewater is in excess of the microbial community's requirements, with only a small fraction sequestered for growth [1, 15]. To achieve TN and TP concentrations in wastewater effluent that is in compliance with the provisions of the UWTD, biological nutrient removal (BNR) systems are extensively used based on the processes of autotrophic nitrification, heterotrophic denitrification and enhanced biological phosphorus removal; performed in, for example, an anaerobic-anoxic-oxic reactor (A^2O), a Bardenpho sequence batch reactor or a DEPHANOX reactor configuration [1, 2]. Nitrification is also an aerobic process, and providing the required O_2 in conventional systems is equally expensive.

Despite these systems achieving significant reductions in carbonaceous, nitrogenous and phosphorus materials, there is growing concern that set discharge concentrations are not adequate enough to limit the effects of eutrophication, especially in small inland rivers. Wastewater effluent is estimated to hold N and/or P concentration three orders of magnitude or more than receiving systems [16–18]. For example, Andersen et al., (2004) [19] reported considerably higher nitrate (NO_3) and soluble reactive phosphorus (SRP) concentrations in a South Carolina stream downstream from the discharge point of two wastewater treatment facilities (NO_3-N: 50.5 mg L^{-1} and SRP: 3.7 mg L^{-1}) compared to the ambient concentrations measured upstream (NO_3-N: 1.6 mg L^{-1} and SRP: 0.3 mg L^{-1}). This is not surprising given that the two effluent discharges combined accounted for over 70% of the total measured flow at the downstream river location. Chambers et al., (2012) [20] evaluated the threshold of TN and TP concentration at which eutrophication in streams occurs to range between 0.21 to 1.2 mg L^{-1} and 0.01 and 0.1 mg L^{-1}, respectively. With regards to regulation concerning water quality, considerations are being put forward to lower the required TN and TP concentrations in the effluent before the water can be discharged, with P the main focus [21–24]. In most ecosystems, P is the rate limiting nutrient for phytoplankton growth; therefore, reducing inputs of P to receiving systems is considered key to reducing eutrophication [23, 25]. In the United Kingdom for example, revised effluent P concentrations based on site-specific standards are currently under consideration [26]. In this situation, a holistic approach is applied to determine effluent P concentrations that reflect the natural ecological P concentration of the water body, which include an account of the site's alkalinity and altitude. In other countries more stringent effluent P standards are set to all point source discharges regardless of population numbers served. For example, in Denmark a TP effluent concentration of 0.3 mg L^{-1} is applied to all municipal treatment facilities, whereas in Sweden a 90% reduction is required (compared to 80% reduction in relation to the load of the influent stated by the UWTD) [14, 26, 27].

In view of achieving more stringent effluent standards to improved water quality, concern has grown over the sustainability of conventional wastewater treatment systems in terms of economic feasibility and environmental impact. Energy consumption and greenhouse gas emissions from wastewater

treatment are among the aspects that have become key-factors concerning the overall performance of a wastewater treatment system [28, 29]. It is estimated that between 0.6 to 3% of the total electricity generated in developed nations is expended on treating wastewater, and depending on the source of energy, the associated carbon emissions can be substantial [30–32]. For example, the per annum CO_2 emission from electricity consumed for wastewater treatment in Germany was estimated to be 2.2 million tonnes, approximately 2.1 million tonnes in the United Kingdom, and approximately 11.5 million tonnes in the United States [33–36]. Of the energy consumed, it is estimated that 50% or more is expended on the O_2 transfer equipment in the biological secondary stage of the wastewater treatment train (Figure 1) [30, 31, 37, 38].

In regards to N and P, the complexity of the process through which removal is achieved increases the energy requirements substantially resulting in an increase in the overall cost of treatment. For example, in the A^2O system the wastewater transitions between anaerobic, anoxic and aerobic environments in sequence. Removal of N, P and carbonaceous materials is accomplished in the separate environments: inorganic N is removed by nitrifiers and denitrifiers in the aerobic and anoxic environments, respectively; inorganic P in the anaerobic and anoxic environments by phosphate-accumulating organisms; and carbonaceous material in the aerobic and anoxic environments by heterotrophic and denitrifying organisms respectively [2]. The separation of the different environments in space and time increases the complexity of the treatment process, while a higher quantity of O_2 is consumed for inorganic P and N removal by the respective organisms to facilitate assimilation or conversion. For example, based on the stoichiometric equation of the nitrification reaction, approximately 4.33 g O_2 is consumed per g N oxidised [1]. Maurer et al., (2003) [39]reported that the integration of nitrification alone into a conventional activated sludge system increases the energy consumption by approximately 60 to 80%. In a recent analysis by the Enerwater Research consortium, a more conclusive account of energy consumption for wastewater treatment is presented [40] [1]. Meta-analysis from 50 wastewater treatment plants based across Germany, Spain and Italy ranging between 1,000 and 100,000 PE capacity, reported average energy consumption of 0.49 kWh kg^{-1} COD, while the removal of TN and TP to permissible discharge concentrations amounted to 6.74 kWh kg^{-1} N and 8.26 kWh kg^{-1} P, respectively. While improving effluent quality is essential to safeguard water sources for future use, it is clear that lowering discharge standards drastically increases energy consumption, and unless sourced from renewable sources, a direct increase in carbon emissions. Based on an electricity generation carbon footprint of 0.421 kg CO_2eq kWh^{-1} (global OECD emission factor), the energy consumed to remove 1 kg N and P from the wastewater would generate 2.8 and 3.4 kg CO_2 equivalent respectively [41].

Other gases that are emitted from wastewater which contribute to the greenhouse effect are methane and hydrogen sulphide in the sewers and nitrous oxide (N_2O) in the treatment process [42–44]. The release of N_2O is of a particular concern as it has an approximate 320-fold stronger effect than CO_2, and therefore even low emission levels are undesirable [45]. Nitrogen oxides catalytically react with ozone

[1] Data from ENERWATER appendix data sheet D2.2

of the stratosphere, reducing the ozone layer by generating O_2 [46]. The Intergovernmental Panel on Climate Change (IPCC) reports that N_2O emissions from wastewater treatment account for approximately 2.8% of the total anthropogenic sources, and are expected to increase by approximately 13% between 2005 and 2020 [45, 47]. The emission of N_2O in the treatment process of wastewater is a consequence of the environmental conditions under which N-removal proceeds. During the biological nitrification reaction, ammonia (NH_3) is oxidised to nitrate and nitrite (NO_3 & NO_2) and in the denitrification reaction the formed NO_2 is reduced to N_2 [2, 48, 49]. Whilst during these biological reactions N_2O is formed as an intermediate, incomplete oxidation to NO_2 or reduction to N_2 is caused by non-optimal cultivation conditions (e.g. dissolved O_2 concentration, pH and temperature) that inhibit the completion of the reaction [47, 50]. Overall, it is estimated that conventional wastewater treatment systems contribute approximately 3% to the total global anthropogenic greenhouse gas emissions [45, 51].

A further drawback of conventional wastewater treatment systems, especially the activated sludge technology, is the high production of sludge. Between 2006 and 2007, the total quantity of sludge produced by 27 member states of the European Union was estimated at 10.1 million tonnes of dry solids, an amount which is expected to rise to 13 million tonnes by 2020 [52]. In the United States it is approximated that a total of 13.8 million tonnes of dry solids are generated annually from the estimated 15,000 public owned treatment works alone [53]. The handling and disposal of sewage sludge not only presents a significant challenge in wastewater management, but further adds to direct and indirect emissions of greenhouse gases and environmental problems. Although the disposal of sludge by direct application to land (agricultural use) is a feasible option, as the high N and P content serve as a fertiliser, the introduction of various regulations has made this an unacceptable operation in dealing with sludge (e.g. European Commission, 1986 [54]). High concentrations of toxic metals and persistent chemicals (e.g. polychlorinated biophenyls) that accumulate in the sludge can restrict the application on agriculture land, whilst to reduce the risks of contamination from residual pathogens, the sludge must be itself treated before being applied to soil in which crops are grown [55, 56]. At present, the most common disposal methods are either by incineration or landfill. Although sludge disposal by landfilling has been decreasing continuously in European member states (from 33% to 15% between 1992 and 2005), the method results in uncontrollable high methane and CO_2 emissions following decomposition of the organic material [57–59]. Furthermore, application of sewage sludge or ash (after incineration) to landfills can cause secondary pollution by the leaching of toxic metals and organic pollutants into surround soils and surface or groundwater systems [60].

Thus, although conventional wastewater treatment systems have been applied with relative success, their application has been described as problem shifting by way of leading to secondary pollution because of high-energy consumption and the production of waste sludge and greenhouse gases [29]. In order to reduce the environmental impact of wastewater treatment, it is therefore necessary to develop and adapt processes with a substantial reduction in energy consumption and sludge production. Key

criteria to achieving lower energy consumption are reducing aeration requirements and operation complexity without affecting performance with respect to meeting mandated effluent standards.

Microalgae, including eukaryotic algae and cyanobacteria, have demonstrated to be an environmentally friendly and sustainable alternative to energy-intensive and conventional biological treatment processes that are widely used today [61, 62]. The rationale behind the use of mixotrophic microalgae to treat wastewater lies in their ability to utilise organic and inorganic carbon, as well as inorganic N and P in wastewater for their growth, with the desired results of a reduction in the concentration of these substances in the water. The principal advantage of incorporating microalgae into wastewater treatment is the generation of O_2 through photosynthesis, necessary for heterotrophic bacteria to biodegrade carbonaceous materials. Under illuminated conditions, *in situ* photosynthetic aeration has the potential to reduce the requisite mechanical aeration and any associated costs and environmental impacts [63, 64]. Furthermore, wastewater treated by an algae-bacterial co-culture does not need to transition between different operating environments to facilitate inorganic N and P removal, requiring only a single-step treatment stage and thereby reducing the complexity and energy of the treatment process [65, 66]. This is because microalgae assimilate ammonia (NH_3) and phosphate (PO_4) directly and in concert for cell growth and metabolic function [67, 68]. As a result, microalgae treatment processes have a lower greenhouse gas emission rate; for instance, the majority of N is assimilated by the microalgae instead of being converted to nitrogen oxides. Various studies have reported on the emission of N_2O caused by microalgae in conjunction with associated microorganisms in wastewater treatment, although the quantities recorded were negligible [69, 70]. Based on the analysis of Alcántara et al., (2015) [71], a microalgae wastewater treatment process is estimated to have an emission factor of 0.0047% g N_2O-N g^{-1} N-input. Overall, furnishing wastewater with dissolved O_2 through microalgae photosynthesis without any energy consumption can therefore lead to reductions in terms of energy demand and associated greenhouse gas emissions.

Despite these advantages, several practical and economic challenges still hinder the implementation of microalgae to treat wastewater and would need to be addressed in order for it to reach industrial application. One such challenge relates to the energy consumed in the cultivation process. As with most conventional wastewater treatment operations, aeration and pumping systems are often used in microalgae culturing to generate turbulent flow that improve the exchange of O_2 and CO_2 to maintain an optimal environment for their performance. Life-cycle analyses by Stephenson et al., (2010) [72] and Jorquera et al., (2010) [73] on microalgae biomass production determined that the majority of the operational energy was consumed in the cultivation stage. The results suggest that mixing in photobioreactors (PBR) by means of pumping and/or aeration required approximately 10 times more energy than mixing by paddlewheels in high rate algae ponds (HRAP). In a case study carried out in Almeria, Spain, analysing the cost of operating a 30 m^3 PBR plant found that the use of recirculation pumps and aeration pumps to be, respectively, the first and second highest energy expenders in the operation [74]. The study also showed that the recorded power consumption of the recirculation

pumps and aeration pumps per unit were 24 and 96 kWh d^{-1} respectively; the reason the recirculation pumps accounted for the highest energy consumption is because ten units were employed and only one aeration pump. The overall rate of energy consumption was 15 kWh m^{-3}, which is a 100-fold higher in the energy consumption rate compared to mechanical and/or aerated mixing in conventional wastewater treatment systems (between 0.15 and 0.62 kWh m^{-3}; [31]. A similar conclusion was drawn by Gouveia et al., (2016) [66] when analysing the cost for microalgae wastewater treatment in a PBR. The authors estimated the cost to treat 1 m^3 of wastewater at approximately €95 under continuous operation (14 days), with the energy consumption (as electricity) the highest cost factor. This approximation does not compare favourably against the treatment cost by conventional wastewater treatment systems of between 0.1 and 0.2 € m^{-3} [75].

The principal reason for aeration in the cultivation of microalgae is to supply carbon in the form of CO_2 to the algae, an important nutrient required for growth and to facilitate the assimilation of inorganic N and P [67]. However, the energy required to compress the air (enriched or not with CO_2) is an energy-intensive process and is one of the main factors that account for the high operation cost [76–78]. A life cycle assessment conducted by Kadam (2002) [79] calculated the electrical consumption of CO_2 injection required in a 1000 hectare sized HRAP to be 22.2 kWh t^{-1} CO_2. In this scenario 680 tonnes of CO_2 were injected into the system per day to ensure a microalgae productivity rate of 45 g m^{-2} d^{-1} consuming 15.1 MWh of electricity at an estimate expense of 1760[2] € d^{-1}. Furthermore, aeration inevitably results in CO_2 loss from the suspension to the atmosphere by outgassing and is a major constraint in ensuring a sufficient concentration of carbon for microalgae use [80, 81]. Approximately 51 to 60% of the CO_2 injected into microalgae cultivation is lost to the atmosphere, translating to a significant efficiency and monetary loss [82, 83]. An alternative approach to overcome the operational cost and inefficiencies associated with carbon supply via aeration is to supplement the medium directly with dissolved carbon, such as inorganic carbon salts (i.e. bicarbonate) or organic substrates (i.e. glucose) [84–86]. The premise of this approach theoretically ensures the complete utilisation of the added carbon by the microalgae. Additionally, by incorporating waste streams rich in bioavailable carbon to augment the supply, the treatment of wastewater by microalgae would have wider environmental benefits through resource recovery and reduced material costs, and in so doing align to the concept of a circular economy model.

A further influence on the economic feasibility of implementing microalgae to treat wastewater is the stage of the treatment train the process is introduced. The application of microalgae in wastewater has customarily been applied to polishing secondary treatment effluent – i.e. in tertiary treatment after the energy intensive secondary treatment stage, to further reduce the inorganic N and P concentrations (see Figure 1.1). Consequently, the introduction of microalgae at this stage of the treatment train would not result in the much-desired reduction in overall energy demands of wastewater treatment. As described above, this is largely a direct result of additional mixing and aeration provided. A more

[2] Based on the average 2016 electricity price of 0.1668 € kWh^{-1} for industrial consumers (Eurostat). Prices are from the first half of the year (January to June) and exclude VAT and other recoverable taxes and levies.

effective treatment process would be to integrate the microalga into the treatment train as the secondary biological treatment phase, applied to treat primary settled wastewater directly.

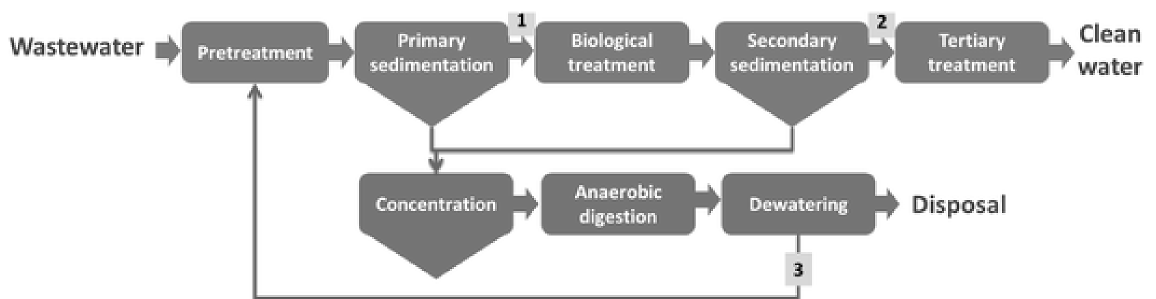


Figure 1.1 – Simplified scheme depicting the different treatment stages of municipal wastewater [87]. 1, primary settled wastewater effluent; 2, secondary treatment effluent; and 3, centrate following sludge dewatering.

The sustainable development of a wastewater treatment system needs to be technologically feasible, environmentally friendly and economical. At present, integrating microalgae as an alternative biological wastewater treatment option is technologically and environmentally feasible, but not economically competitive and therefore unsustainable. To be competitive with conventional wastewater treatment systems, a drastic reduction in the energy consumption of microalgae-based wastewater treatment process is necessary. An additional aspect which has to be taken into consideration when implementing microalgae as the biological treatment stage for wastewater is the efficiency and reliability of the processes performance. Multiple studies have evaluated different microalgae species in treating wastewater; however, these were mostly performed independent of one another under varying environmental and cultivation conditions. As such a direct comparison in the treatment performance of a particular microalgal strain to a wastewater source cannot be made definitively. Furthermore, there is a lack of replication among studies, which take into consideration the variable composition of wastewater.

1.2 Aim and objectives

The aim of this research was to carry out a thorough evaluation, using a set of carefully implemented laboratory experiments, of the performance of a static microalgae cultivation strategy for remediating COD, N and P in settled municipal wastewater. The specific objectives of the study were:

- To carry out laboratory experiments to investigate COD, N and P removal capacity of the microalga *C. vulgaris*, *H. riparia* and *A. obliquus* from settled municipal wastewater under defined culture conditions for the purpose of selecting a species with high efficiency and tolerance to a specific wastewater site effluent;

- Identify the factor(s) that either limit or improve the performance efficiency of the microalgae under a static culturing system;
- Experimentally assess the performance of the static microalgae treatment process in response to wastewater enrichment with different exogenous organic carbon sources, including an industrial waste source
- Empirically verify the reliability of the developed static microalgae treatment process under varying wastewater influent compositions;
- Investigate the performance of the microalgae static wastewater treatment process under semi-continuous operation

1.3 Thesis layout

This thesis contains six chapters, including a literature review (Chapter 2) and a Materials and Methods (Chapter 3). Experimental work is divided between three chapters (Chapters 4 to 6), each intended to scale up in the complexity of developing and evaluating a static microalgae wastewater treatment process as a sustainable alternative to conventional wastewater treatment. Chapter 4 presents experimental work conducted to assess the performance of three microalga species for treating the PSW, with the findings from this enabling the selection of a suitable species depending on COD, N and P removal efficiency. Moreover, the culture conditions, particular light intensity and exposure cycle, temperature and initial microalgae inoculation concentration, were evaluated to examine whether the conditions were appropriate in facilitating microalga growth and treatment of the wastewater. Chapter 5 presents an investigation to determine the biotic factors influencing the nutrient removal capacity and biomass productivity of the selected microalga under static cultivation conditions. Chapter 6 expands on the findings from the previous chapters by evaluating the performance under semi-continuous operation. Finally, Chapter 7 summarises the key conclusions and provides recommendations for future work with a view to further develop the static microalgae treatment process towards large scale application.

Chapter 2 – Wastewater treatment and microalgae

2.1 – Structure and function of conventional wastewater treatment processes

Wastewater treatment systems are engineered to facilitate the transformation of contaminants and to purify the water in the most efficient and economical way. This is achieved through a combination of biological, physical and chemical methods controlled through the design of specific operational units that are built to form a process train. In a conventional wastewater treatment system, several steps are employed to achieve the agreeable levels of pollutant removal, with the primary, secondary and tertiary stages of the process train designed to reduce specific fractions of the pollutants from the water. Figure 2.1 presents a typical process flow in a conventional wastewater treatment system, highlighting the particular fractions removed at the different stages.

2.1.1 Preliminary treatment

When wastewater is received by a treatment plant via the sewer system, it is initially treated to remove the larger objects and suspended solids matter. This preliminary treatment phase consists of the wastewater passing through a screen to remove gross solids (e.g. rags, cans, leaves, wood fractions, plastics objects etc.) that are carried by the water. If these materials are not removed they could cause damage to pumps, valves and other downstream equipment leading to blockages and overflow problems [1, 88]. Finer screens are then employed to remove detritus material (e.g. grit, sand, stones etc.) that originate from road surface runoff; otherwise the abrasive action of these materials can further damage pumps and other mechanical equipment. The retained materials are commonly washed to reduce the presence of faecal matter before being compressed for disposal, either by incineration or landfill. Other operational processes, such as dissolved air floatation, shredding or pre-chlorination can be applied to condition the influent wastewater for improved efficiencies in the subsequent treatment stages [2].

2.1.2 Primary treatment

The primary treatment stage is designed to remove the smaller fractions of insoluble inorganic and organic material, which is achieved because of the difference in density of the materials relative to the water [1, 2]. This is accomplished in a large sedimentation basin in which the wastewater is detained for a designated period of time, usually 2 hours. During this time, denser solids with adequate settling velocities settle to the floor of the tank, while materials less dense (e.g. oil, grease etc.) rise to the surface. The settled solids (i.e. sludge) are collected into a hopper by the continuous action of mechanical scrapers, while the floating materials (i.e. scum) are skimmed from the surface. Under optimal operation, the efficiency of separation at this stage can reduce the suspended solids concentration by 50 to 70%, and since a proportion of the solid is composed of organic matter, a 25 to 40% reduction in BOD is achieved [1, 89]. This process is the most practical and economical method for reducing the carbonaceous material, directly reducing the strength of the wastewater and thus making

it more tractable to the secondary treatment stage. After settlement has taken place, the wastewater is known as "primary settled wastewater" (PSW) and is displaced from the sedimentation basin to the secondary treatment stage by the incoming raw wastewater. The effluent still contains a high amount of soluble material – organic and inorganic in nature – as well as insoluble colloidal material, which collectively needs to be removed through further treatment.

2.1.3 Secondary treatment

The objective of the secondary treatment stage is to reduce the O_2-demanding materials (i.e. BOD) in the wastewater, and in certain process train configurations the removal of inorganic material, such as ammonium (NH_4^+) and phosphate (PO_4^{3-}), is also achieved. The process promotes the growth of microorganisms (e.g. heterotrophic bacteria, protozoa and fungi) which take advantage of these materials as a source of food and energy, concomitantly digesting and eliminating them from the water [1, 90]. The two main cultivation techniques adopted as the biological treatment process are either suspended growth systems in which the microorganisms are suspended in the water column, or biofilms in which they naturally become fixed to a surface medium [1, 2].

In attached growth systems, the wastewater passes over and, to an extent, also through a biological film fixed to an immobile medium held in a tank. As the pollutants in the water come into contact with the biological film composed of a consortium of microorganisms that have been conditioned to the wastewater environment, they feed on and metabolise the contaminants [1, 89]. Oxygen in this process is supplied by natural ventilation as air flows through the interstices created by the medium and diffuses into the biological film. The three common attached growth systems are rotating biological contractors, trickle filters and fluidized bed biological reactors [1, 2].

In suspended growth systems the microbial community is maintained in suspension in an aerobic environment. Oxygen is provided through compressed air systems, mechanical agitation or injection of relatively pure O_2 [1, 2]. Various reactor configurations have been designed and are currently operated, such as step feed, sequence batch, oxidation ditch, plug-flow and complete mixed reactors; all of these are loosely based on the principles of the activated sludge process [2]. The typical activated sludge process consists of a main aeration basin and a secondary settling basin. In the aeration basin, the sludge is composed of microbial aggregates and colloidal matter contained in flocs, and is maintained in suspension by the aeration or mixing equipment, which facilitates the contact between the microorganisms and oxidisable contaminants in the water. In a continuous system, the treated water that is mixed with the sludge is displaced by the incoming wastewater to the settling basin following an appropriate hydraulic retention time. A proportion of the settled sludge is recycled to the aeration basin in order to maintain the concentration of adapted (activated) microorganisms necessary to maintain a high treatment performance [1, 2]. This process achieves an approximately 70 to 85% removal of the BOD from the water [89].

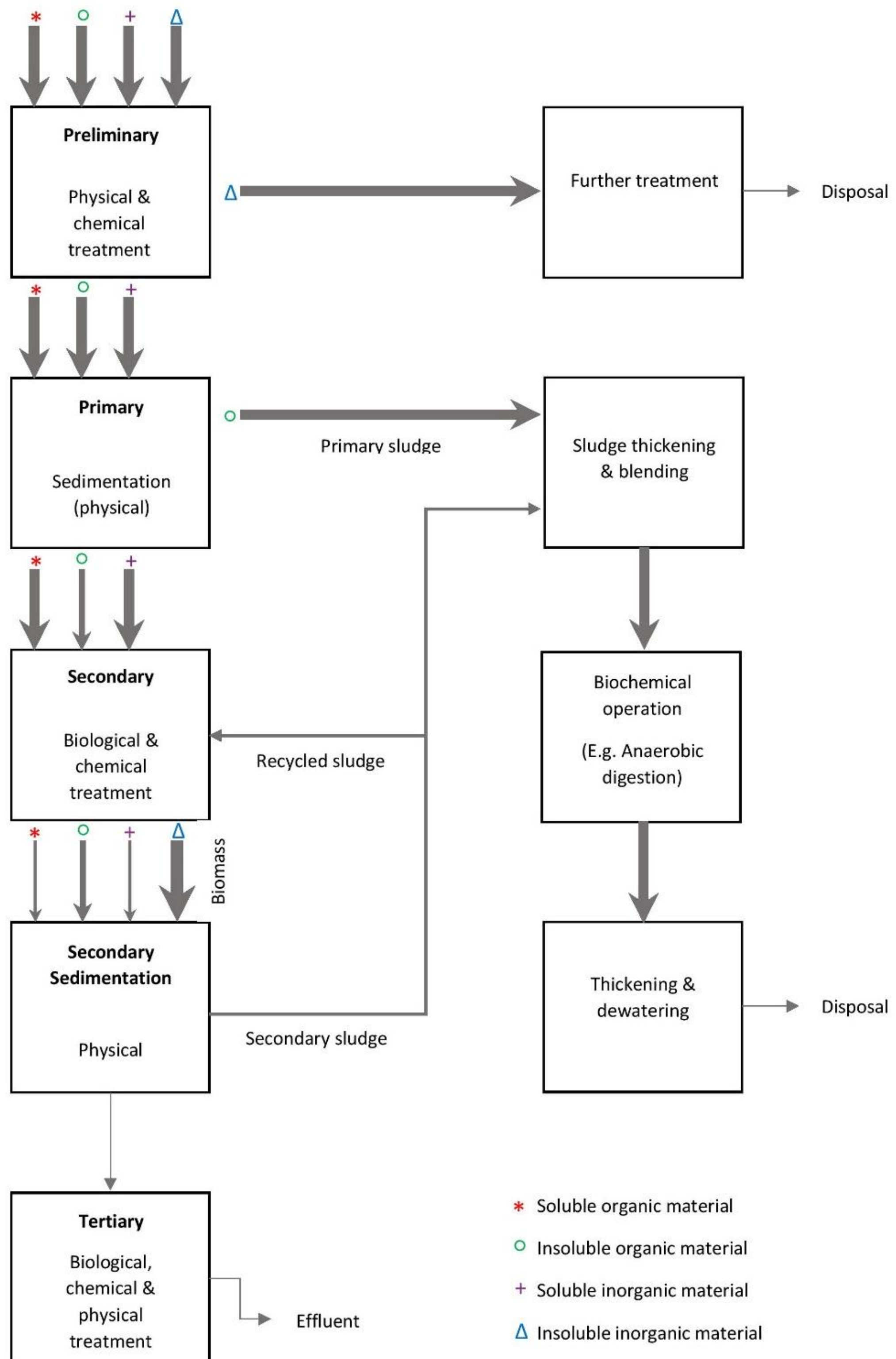


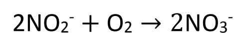
Figure 2.1 – Schematic illustrating the role of the treatment operations effect on the different pollutant fractions in the course of a conventional wastewater treatment plant (adapted from [2]).

When discharging wastewater into sensitive waters, it is necessary to remove much of the N and P to limit the effects of eutrophication. In general, the quantity of N and P is in excess of the activated sludge microbial community's requirement, and only a small fraction is sequestered for growth. Various treatment processes have been developed that target the removal of N and P, and these are predominantly based on the biological process of autotrophic nitrification, heterotrophic denitrification and enhanced biological phosphorus removal [1, 2]. In practice, these processes are a modification of the conventional activated sludge process, but which cycles the water through defined anaerobic, anoxic or aerobic environments to encourage the growth and function of specific microorganisms that are adapted at removing these inorganics.

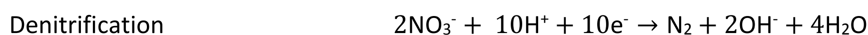
2.1.3.1 Biological nitrogen removal

Inorganic N abatement from wastewater begins with nitrification in which NH_3 is converted to NO_2 and further to NO_3 [1, 2] (Figure 2.2). This is performed by two functionally defined groups of autotrophic bacteria involving a series of complex enzymatic oxidation reactions that are tightly regulated in response to environmental cues and substrate concentration [49, 91]. In the first step of nitrification, ammonia-oxidising bacteria (AOB; e.g. *Nitrosomonas* sp.) oxidise NH_3 to hydroxylamine (NH_2OH), an intermediate compound in the reaction, catalysed by ammonia monooxygenase [50]. Subsequently, NH_2OH is oxidised to NO_2 by hydroxylamine oxidoreductase. Following this step, nitrite-oxidising bacteria (NOB; e.g. *Nitrobacter* sp.) oxidise the formed NO_2 to NO_3 catalysed in a single step reaction [49, 91]. Both groups of bacteria sequester O_2 as the electron acceptor, deriving their energy and reducing power from the reaction to fix inorganic carbon and N for growth [92]. The nitrification reaction is merely responsible for increasing the oxidation state of N and has no substantial effect at reducing the N load in the water. Denitrification is the process in which the formed NO_3 (and NO_2) is converted to N_2 by a range of heterotrophic bacteria (e.g. *Rhodanobacter*, *Paracoccus*, *Thauera* and *Asoarcus* genera etc.) [49, 93] (Figure 2.2). The reaction proceeds under anoxic conditions in which NO_3 is used as the terminal electron acceptor by the bacteria as they oxidise organic material [2]. The stoichiometric reactions are as follows:

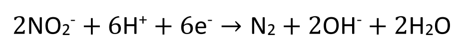
Nitrification $NH_3 + 1.5O_2 \rightarrow NO_2^- + H^+ + H_2O$



$2NO_2^- + O_2 \rightarrow 2NO_3^-$



Denitrification $2NO_3^- + 10H^+ + 10e^- \rightarrow N_2 + 2OH^- + 4H_2O$



$2NO_2^- + 6H^+ + 6e^- \rightarrow N_2 + 2OH^- + 2H_2O$

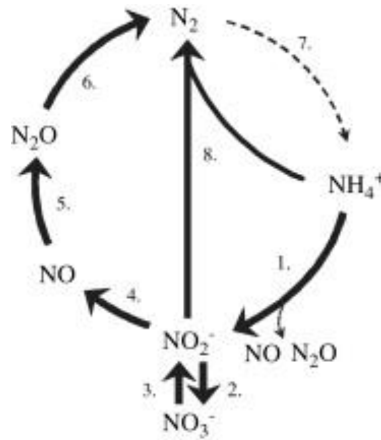


Figure 2.2 – Schematic depicting the nitrogen cycle [49]. 1, NH_3 oxidation; 2, NO_2 oxidation; 3, NO_3 reduction; 4, NO_2 reduction; 5, NO reduction; 6, N_2O reduction; 7, N_2 fixing (not relevant in most wastewater treatment systems); and 8, ANAMMOX reaction involving the sequential reaction of NH_3 oxidation and NO_2 reduction. Reactions 1 and 2 are completed by AOBs and NOBs respectively, and reaction 3 to 6 by heterotrophic denitrifiers.

In combination, the above biochemical reactions convert the inorganic N in wastewater to N_2 which dissipates to the atmosphere, thereby eliminating N from the water. Despite the significant reductions of inorganic N achieved, significant limitations still affect the performance. A major limitation is the susceptibility of nitrifiers and denitrifiers to sudden pH and temperature changes, as well as the susceptibility of these organisms to toxic compounds [94, 95]. The operation and design of the reactor is critical to the process and must accommodate the particular physiological requirements of the different bacteria. Since nitrifiers have a slow generation time, a sufficiently long solid retention time is necessary to retain an adequate population of nitrifiers in the water. To exemplify, a significant effect was reported in the abundance of nitrifiers when the solid retention time was reduced from 15 to 5 days in an anaerobic-anoxic reactor [96]. The percentage of AOBs decreased from 0.67 to 0.35%, and concomitantly the efficiency of NH_4^+-N removal was reported to decrease from 90 to 26%. It is estimate that the minimum doubling time of AOBs is between 7 to 8 hours, while that of NOBs is between 10 to 13 hours [97]. Furthermore, the two processes need to be separated in time and space, with the autotrophic bacteria requiring O_2, while an anoxic environment must be established to encourage denitrifiers to use the N bound O_2 as the electron acceptor [1]. In addition to the high operational costs involved for meeting the O_2 demand of nitrifiers, incorrect dissolved O_2 regulation between the two environments can result in N_2O formation.

In the denitrification reaction, NO_3 is catalysed in series by different reductase enzymes that yield nitric oxide (NO) and nitrous oxide (N_2O) as intermediates in the overall reaction ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) (Figure 2.2; Skiba, 2008). In the presence of low dissolved O_2 concentrations, the nitrous oxide reductase enzyme becomes inhibited resulting in the incomplete reduction with a partial formation of N_2O [99–101]. The opposite effect is observed during nitrification, with insufficient dissolved O_2 concentrations (<1 mg L^{-1} O_2) resulting in NH_2OH not being completely oxidised and consequently

forming N_2O [50, 100]. The main source of emissions during nitrification is from AOBs that have been shown to possess the necessary genes for the reductive enzymes to reduce NO_2 to NO and then to N_2O [102, 103].

Alternative inorganic N removal systems have been developed to overcome the limitations of high aeration cost, requirement for exogenous carbon and to reduce sludge production in comparison to the above nitrification and denitrification processes. The anaerobic ammonium oxidation (ANAMMOX) process involves the oxidation of NH_3 directly to N_2 using NO_2 as the oxidant instead of O_2 – a reaction that corresponds more closely to a denitrification reaction rather than to a nitrification reaction [104] (Figure 2.2). A preceding partial nitrification reaction is necessary in which part of the NH_3 in the wastewater is oxidised to NO_2 (approximately 50%) and the subsequent conversion of NO_2 to NO_3 is prevented. This can be accomplished by the single reactor higher activity ammonia removal over nitrite (SHARON) reaction in which NH_3 is converted to NO_2 [105, 106]. SHARON is accomplished by AOBs under aerobic conditions and at sufficiently high temperature – between 26 and 35°C – with operational parameters favouring the growth of AOB over NOB, such as O_2 concentration and hydraulic retention time [105, 107].

Alternatively, complete autotrophic nitrogen removal over nitrite (CANON) is a process design in which the reactor configuration facilitates the partial nitrification and Anammox in a single process unit, allowing the two different groups of bacteria to operate in tandem [108, 109]. These processes are, however, not without their disadvantages. At present, the ANAMMOX reaction is known only to be performed by bacteria belonging to the phylum *Planctomycetes* [108]. These bacteria have a slow generation time, with doubling times for particular strains occurring every 5 to 11 days [110, 111]. Furthermore, the biochemical reactions are inhibited in the presence of high O_2 concentrations, and by NO_2 and NH_3 concentrations exceeding 70 and 45 mg L^{-1} N respectively [112–115]. Overall high investment costs, dilute concentrations of NH_4^+ in municipal wastewater, and complex operation conditions limit the application of these processes mainly to industrial effluents.

2.1.3.2 Biological phosphorus removal

Biological P removal is achieved by polyphosphate accumulating organisms (PAOs; e.g. *Acinetobacter*), which have the unique capability of accumulating polyphosphate from their environment in excess of their metabolic requirements [2]. These bacteria store carbon products under anaerobic conditions driven by adenosine triphosphate (ATP) hydrolysis, and store PO_4^{3-} under aerobic conditions at the expense of carbon metabolism via respiration. Under anaerobic conditions, PAOs assimilate fermented products, such as volatile fatty acids (VFA), and metabolise the carbon to the storage molecule poly-β-hydroxyalkanoate (PHA). In concert, ATP and reducing compounds nicotinamide adenine dinucleotide (NADH), which drive the biochemical reaction, are generated from the hydrolysis of the stored polyphosphate and catabolism of glycogen respectively. The hydrolysed PO_4^{3-} group is expelled from the cell in conjunction with a metal cation to help maintain a balanced intracellular charge [2].

Subsequently, when the wastewater enters the following aerobic condition, it is low in carbonaceous material but rich in PO_4^{3-}. PAOs are stimulated to grow with the provision of O_2, and the accumulated PHA is catabolised to supply their energy and carbon needs. Polyphosphate synthesis is also stimulated to regenerate the cellular reserves with the PO_4^{3-} substrate taken from the water. As a result of PAO growth and their polyphosphate storage capacity, the quantity of PO_4^{3-} assimilation exceeds the amount released in the anaerobic condition. Net P removal from the water is achieved by wasting a portion of the sludge, thereby directly eliminating the PAOs with the captured polyphosphate [2].

Although this process is capable of significantly reducing the P concentration in wastewater, PAO abundance and activity are vulnerable to changes in environmental conditions, and thus efficiencies in P reduction can be highly variable. In addition to the complexity of having to intermittently circulate the water between an aerobic and anaerobic environment, the temperature, pH and sudden inflow of water following rainfall can substantial affect the efficiency of the bacteria to accumulate and remove P [116]. In a batch experiments conducted at a pH of 6.5, significantly lower rates of P uptake, PAO growth and PHA utilisation have been reported compared to pH conditions between 7 and 7.5 [117]. A different study reported that the deterioration in P removal efficiency at a lower pH was in part a result of a shift in the microbial community with an increase in bacterial groups that compete with PAOs for carbon substrates [118]. Glycogen accumulating organisms (GAO) are known competitors, and like PAOs will proliferate under the same conditions [116]. GAOs are understood to metabolise glycogen under anaerobic conditions, enabling them to assimilate and store VFAs as PHA, which can be oxidised under aerobic conditions for simultaneous growth and glycogen storage [119, 120]. GAOs do not contribute to P removal and therefore are undesirable in that they negatively affect the performance of P removal. It is suggested that low temperatures favour the growth of PAOs over GAOs, with reported P uptake rates decreasing, and carbon utilisation increasing as temperatures change from 20 to 35°C, which correlate with a decrease in the fraction of PAOs and increase in GAOs [121]. As such, the operation of biological P removal may be more challenging in warm climates or summer periods. However, while low temperatures favour PAO growth, markedly lower temperatures (e.g. 5°C) can significantly reduce the rate kinetics of the reaction and require higher sludge age causing a knock-on effect on other aspects of wastewater treatment [2, 122].

Inorganic P removal can also be achieved by chemical means through addition of a coagulant. Common chemicals used are lime or salts of aluminium or iron that react with PO_4^{3-} to produce an insoluble metal phosphate salt [89, 123, 124]. This precipitates out from the water and can be separated in the settling phase of either the primary or secondary treatment, or in a separate settling phase if added to a post-secondary treatment. The addition of a coagulant can remove between 70 and 90% of all PO_4^{3-}, with efficiencies of >90% reported with longer hydraulic retention times and repeated dosing [125–127]. However, chemical-induced precipitation of P will increase the material cost for treatment, as well as sludge volume leading to higher quantities of sludge that commonly is either buried at landfills or incinerated [1, 126]. Furthermore, the formation of these precipitate salts, and presence of residual

ions (e.g. Mg^{2+}) and resultant high pH from these reactions can cause a serious problem in the downstream process of anaerobic digestion [1, 128].

2.1.4 Tertiary treatment

In circumstances where a higher quality of effluent is required, a third (tertiary) stage of treatment is applied. Methods employed during this stage attempt to further reduce the load of total suspended solids, organic matter, and inorganic N and P beyond what is achievable by primary and secondary treatment. This is achieved either by filtration processes (ultra or sand), carbon adsorption, further coagulation, or reverse osmosis. Disinfection is commonly applied at this stage.

2.2 Microalgae wastewater treatment

The investigation into the biological removal of carbonaceous, nitrogenous and phosphorus material via microalgae in wastewater effluents has been evaluated by several studies. This has been performed with various microalgal species on a range wastewater types, including municipal, agricultural, brewery, refinery, and industrial effluents with varying efficiencies in treatment performance and microalgae growth [129–132]. The strain *Scendesmus obliquus* has been demonstrated to successfully remove nutrients (carbon, N and P) from piggery wastewater [133, 134], while *Chlorella pyrenoidosa* successfully grew in dairy production effluent [135]. Other *Chlorella* species, including *Chlorella vulgaris*, have been reported to be suitable candidates in the remediation of N and P from municipal wastewater effluent at the primary stage (PO_4^{3-}-P: 8 to 3 mg L^{-1}; NH_4^+: 119 to 37 mg L^{-1}), secondary stage (PO_4^{3-}-P: 6.1 to 0.5 mg L^{-1}; NH_4^+-N: 6.9 to 0.8 mg L^{-1}) and from centrate (TP: 215 to 40 mg L^{-1}; TN: 116 to 12 mg L^{-1}) [66, 136, 137]. Choi (2016) [138] reported 88% BOD, 82% TN and 54% TP removal from initial concentrations in brewery effluent by *C. vulgaris*. Other microalgae species examined for their bioremediation potential include *Chlamydomonas* sp., *Nanochloropsis* sp., *Dunaliella* sp., *Spirulina* sp. and *Botryococcu* sp. [139, 140].

2.2.1 Carbon, N and P ratios in different waste streams

A significant influence to the microalgal treatment performance is the composition of the wastewater. In order to grow and function, microalgae require three primary nutrients: carbon, N and P [67]. The assimilation of these nutrients is strongly affected by the overall composition of nutrients that are available in the cultivation medium [141]. Nutrient utilisation rates by microalgae are closely associated with their growth, and a limited supply of a primary nutrient can significantly reduce their growth rate [142–144]. In this context, to ensure optimal nutrient removal efficiency from the cultivation medium, an optimal ratio of nutrients that is reflective of the microalgal elemental stoichiometry needs must be present. The average elemental composition of freshwater microalgae, normalised on carbon, was determined to be $C_1N_{0.15}P_{0.0094}$ [145]. Further to this, trace amounts of micronutrients, such as calcium, magnesium, potassium, manganese, silica, zinc, iron, etc., are essential and generally abundantly available in wastewater [67, 146, 147].

Within a conventional municipal wastewater treatment train, two different wastewater streams are identified as potential points at which to integrate a microalgae treatment process; either to treat PSW or secondary treatment effluent (STE). As stated earlier in Chapter 1, *a more economical and environmental sustainable treatment process would be to integrate microalgae as the secondary treatment phase, directly treating PSW to effluent standards*. In addition, PSW exhibits a more optimum nutrient ratio and hospitable microflora to support microalgae growth compared to STE (detailed below). When comparing the carbon, N and P quantity in PSW and STE, it can be concluded that they are relatively similar in nutrient composition, but differences exist in their concentrations. Table 1 and 2 summarise the N, P and carbon concentrations of municipal wastewater from PSW and STE respectively, as reported in recent studies on microalgae cultivation[3].

Table 1 – Carbon, N and P ratios in PSW used in microalgal-based wastewater treatment studies

Microalgae species	N	P	C	Ratio (C:N:P)	Reference
Chlorella sp.	38.9[b]	6.9[e]	224	100/17/3	[148]
Alga consortium & bacteria	45[a]	6.5[e]	400	100/11/1.6	[149]
Chlorella vulgaris & bacteria	48.4[a]	3.9[e]	158	100/30/2.5	[150]
Algae consortium & bacteria	93[c]	33[f]	176[g]	100/53/18	[151]
Desmodesmus communis & bacteria	33.6[c]	1.54[e]		-	[152]
Scenedesmus sp. ZTY1 & bacteria	41[b]	8.4[e]	235	100/17/3.5	[153]
Microalgae screening	36.1[bS]	4[eS]	93[S]	100/39/4	[154]
Desmodesmus communis & bacteria	32.4[c]	2.4[f]		-	[155]
Chlorella protothecoides	37.4[b]	2.6[e]		-	[156]
Chlorella vulgaris	43.3[d]	0.6[f]	256	100/17/1	[157]
Neochloris oleoabundas	40.8[d]	10[e]	242	100/17/4	[158]
Chaetomorpha linum	24.5[d]	2.4[e]	307	100/8/1	[159]
Microalgae screening	23[b]	8.6[e]	270	100/8.5/3	[160]
Chlorella vulgaris	36.3[d]	4.2[f]	317	100/11/1.3	[161]
Microalgae screening	41[b]	4.7[e]	70	100/58/7	[162]
Chlorella pyrenoidosa	27.1[d]	10.1[f]	240	100/11/4	[163]
Chlorella vulgaris (WWTP 1)	84[bS]	6[eS]	150[S]	100/56/4	[164]
Chlorella vulgaris (WWTP 2)	42[bS]	5.9[eS]	180[S]	100/23/3	[164]
Chlorella protothecoides & bacteria	44.4[d]	8[f]	130	100/34/6	[165]
Algal-bacterial consortium	18.9[d]	3.8[e]	140	100/20/3.5	[166]
Average	**31**	**5.6**	**158**	**100/19/3**	

[a]Total Kjeldahl Nitrogen; [b]TN; [c]NH_3-N; [d]NH_4^+-N; [e]TP; [f]PO_4-P; [g]Total organic carbon; [S]Soluble fraction; Unless otherwise stated, carbon is measured as COD

[3] The studies cited for this analysis were sourced from Web of Knowledge, published between 1990 and 2017 with the key words "microalga*, municipal*" and either "+primary*wastewater" or "+secondary*wastewater".

In STE the concentration of N, P and carbon (represented as the COD) were in the range of 0.63 and 50 mg L^{-1} N, 0.1 and 26 mg L^{-1} P, 11 to 340 mg L^{-1} O_2, respectively. In PSW the concentrations were higher, with N in the range of 23 to 93 mg L^{-1}, P in the range of 1.5 and 33 mg L^{-1}, and COD in the range of 93 and 400 mg L^{-1} O_2. By comparing the average C/N/P ratio of the different wastewater effluents with the proximate composition of freshwater microalgae, it can be observed that PSW more closely matches the stoichiometric ratio. With an average C/N/P ratio of 100/34/7, STE contains either an excess ratio of N to P or, conversely, is limited in carbon (Table 2). In STE, nearly all of the pollutants that could be a source of bioavailable carbon are degraded in the biological treatment stage with the remaining carbon material being composed of complex polymers that are either recalcitrant or only partially digestible [1, 167]. In STE the ratio of biodegradable dissolved organic carbon to dissolved organic carbon (DOC) has been reported to range between 0.21:1 and 0.28:1, with a concentration of DOC as low as 7.8 mg L^{-1} [168].

Table 2 – Nutrient ratios in STE used in microalgal-based wastewater treatment studies

Microalgae species	N	P	C	Ratio (C:N:P)	Reference
Chlorella sp.	19.1^a	0.3^d	42	100/45/0.7	[148]
Haematococcus pluvialis	42.4^c	2.6^e	22	100/193/12	[169]
Scenedesmus sp. ZTY1 & bacteria	11^a	1.9^d	41	100/27/4	[153]
Desmodesmus communis & bacteria	1.47^a	0.1^d		-	[155]
Chlorella vulgaris	0.63^b	0.6^e	96	100/0.6/0.6	[157]
Neochloris oleoabundas	44^b	26^d	59	100/75/44	[158]
Chaetomorpha linum	17.9^b	0.5^d	30	100/60/2	[159]
Microalgae screening	7^a	1.6^d	38	100/18/4.2	[160]
Microalgae consortium	50^a	15^d	63	100/79/24	[170]
Microalgae consortium	17^a	1.9^e	34	100/50/5	[171]
Scenedesmus dimorphus	15.8^a	0.8^d	32	100/49/2.5	[172]
Microalgae consortium	16.5^a	1.5^d	11	100/150/13	[173]
Chlorella sp.	18.9^a	1.7^d	11	100/171/15	[174]
Neochloris oleoabundans	12.3^b	3^e	340	100/3.6/1	[175]
Botryococcus braunii	11.9^a	11.5^e	50	100/24/23	[176]
Chlorella vulgaris (WWTP 2)	65.6^{aS}	7.5^{dS}	90^S	100/73/8	[164]
Chlorella vulgaris (WWTP 1)	36^{aS}	2.4^{dS}	90^S	100/40/3	[164]
Average	**22.8**	**4.6**	**66**	**100/34/7**	

^{a}TN; ^{b}NH_4^+-N; ^{c}NO_3-N; ^{d}TP; ^{e}PO_4-P; ^{S}Soluble fraction; Unless otherwise stated, carbon is measured as COD

The discrepancy in carbon, N and P concentrations between the different wastewater streams has been demonstrated to affect microalgal removal efficiencies. In a comparative study, Wang et al., (2010) [148] found that a *Chlorella* sp. had a higher average specific growth rate with a concomitant improved efficiency in inorganic N and P removal from PSW, compared to STE. The removal capacity of the microalga from PSW was 68.5% TN and 90.6% TP, and from STE 50.8% TN and 4.96% TP. Moreover, a

56.5% decline in COD was recorded from the PSW, while in the STE an increase of 22.7% was registered, indicating that oxidisable carbon matter was being excreted by the microalgae. In a study by Cabanelas et al., (2013) [164], a similar effect in treatment efficiency with the microalga *C. vulgaris* strain SAG211-12 was observed across the two types of wastewater streams. Higher TN, TP and COD removal rates were recorded when cultured in PSW compared to STE, with experiments for each wastewater stream conducted on samples from two independent wastewater treatment plants. Higher *C. vulgaris* growth rates were recorded in the PSW samples, varying from 111 to 125 mg L^{-1} d^{-1} compared to 63 to 68 mg L^{-1} d^{-1} in the STE samples.

In respect to the ratio of bioavailable N and P, various studies have demonstrated the ability of microalgae to grow and effectively treat wastewater under ratios that deviate from the canonical N and P stoichiometry of freshwater microalgae [68, 142, 177]. Kapdan and Aslan (2008) [141], for example, reported a lower residual NH_4-N concentration when treating synthetic wastewater with *C. vulgaris* after an optimum N:P ratio was established for the species. In this study, effluent NH_4-N concentrations decreased from 5.1 to 2 mg L^{-1} when the N:P ratio was increased from 4:1 to 8:1, with a significant decline in removal efficiency occurring with increasing ratios. Alketife et al., (2017) [144] reported a slightly higher optimal N:P ratio for a different *C. vulgaris* strain, with complete N and P removal achieved at a ratio of 10:1. Arbib et al., (2013) [178] examined the removal efficiency of *S. obliquus* under varying N:P ratios, and concluded that for an efficient simultaneous nutrient removal the ratio should be between 9:1 and 13:1. Complete removal of N and P is achievable outside the optimal N:P ratio as long as the ratio lies between the minimum and maximum cellular N:P demands [179]. In general, an N:P ratio of 30:1 suggests a deficit in P availability and a 5:1 ratio a deficit in N availability for microalgae [180].

Numerous studies employing different culturing techniques have demonstrated the success of microalgae in treating PSW, albeit with varying degrees of efficiency (Table 3 and references therein). For example, from unsterilized PSW using *C. vulgaris* cultured in a microalgal membrane bioreactor, up to 96.6% of TN and 92.7% of TP was removed in addition to 96.9% of COD [181]. In a different study, the microalga *Chlorella protothecoides* was capable of removing NH_3-N and PO_4-P from PSW with an efficiency of 94% and 62%, respectively [165]. However, the authors state that the organic matter concentration in PSW remained constant, a possible result of CO_2 sparging which promoted autotrophic metabolism over heterotrophic metabolism (described below). AlMomani and Örmeci (2016) [158] demonstrated removal efficiencies of 63.2% NH_4^+-N, 32.4% total dissolved P and 64.9% COD from PSW employing a native microalgal consortium isolated form the secondary wastewater basin of a treatment plant. Although the depuration of the nutrients from the wastewater sample mediated by the microalgae consortium is far lower than in the other two studies, it must be noted that the cultures were treated under near static conditions, which would have lowered the mass transfer rates of substances (e.g. O_2 and CO_2) and optimal growth conditions. The difference in the autochthonous flora of the wastewater between PSW and STE is shown to have an effect on microalgal growth and

treatment performance. Ramos Tercero et al., (2014) [165] reported that aerobic bacteria from the activated sludge that were present in the final effluent had strongly competed with algal growth, indicating that sterilization of the STE was necessary. By comparison, *C. protothecoides* seemed to be resistant to competition with the autochthonous microbial community of PSW. In a study by Sforza et al., (2014) [156], no difference in *C. protothecoides* growth was detected between unsterilized and sterilised PSW, corroborating the reported observation that the autochthonous microbial community of the PSW may not negatively affect algal growth. Thus, in a proposed microalgal wastewater treatment process, to ensure efficient treatment and minimise the potential negative effects of bacteria competing with microalgae, it would be more appropriate to integrate the microalgae after the primary settling stage.

2.2.2 Carbon, N and P removal mechanism by microalgae

2.2.2.1 Carbon

In photoautotrophic mode, microalgae can utilise inorganic carbon, predominantly CO_2, as their primary carbon source [67]. In aqueous solutions, gaseous CO_2 dissociates into bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions depending on the pH, with the precise equilibrium subject to the temperature of the environment, cation concentration and salinity [182]. In freshwater environments at 25°C, HCO_3^- forms the dominant inorganic carbon species between pH 7 and 8, while CO_3^{2-} forms at pH >10.3, and H_2CO_3 at pH <6.35 (Figure 2.3) [183, 184]. As a result of the non-polar nature of CO_2, it can easily diffuse across the plasma membrane of microalgal cells, whereas HCO_3^- requires active transport mechanisms (Figure 2.4) [67, 68, 185]. In the chloroplast, HCO_3^- is rapidly catalysed to CO_2 through the enzymatic action of carbonic anhydrase to facilitate the fixing of inorganic carbon [67, 186]. Most microalgae have adapted carbon concentration mechanisms to minimise the loss of photosynthetic activity in order to improve CO_2 accumulation rate within the chloroplast because of the low CO_2 concentration in aquatic environments [187].

Microalgae convert inorganic carbon to organic carbon via the Calvin cycle by utilising the reductant NADPH (nicotinamide adenine dinucleotide phosphate oxidised) and energy from ATP hydrolysis produced in the photosynthetic electron transport chain [67]. Inorganic carbon, as CO_2, is fixed to ribulose-1, 5-bisphosphate (RuBP), the acceptor molecule, yielding two molecules of 3-phosphoglycerate (3-PGA) in a reaction catalysed by the enzyme ribulose-1, 5-bisphosphate carboxylase oxygenase (RuBisCo) (Figure 2.4). The carboxyl carbon on each 3-PGA molecule is subsequently phosphorylated to form 1, 3-bisphosphoglycerate (3-bisPGA), and is successively reduced to glyceraldehyde-3-phosphate (G3P). In this reaction, for every three molecules of CO_2 fixed, four molecules of RuBP are produced with only three remain in the cycle. The additional G3P is transferred into storage or metabolised further to pyruvate through the glycolytic pathway and subsequently into the tricarboxylic acid cycle (TCA). The Calvin cycle provides the carbon skeletons necessary for other metabolic reactions to produce amino acids and lipids in microalgae [67].

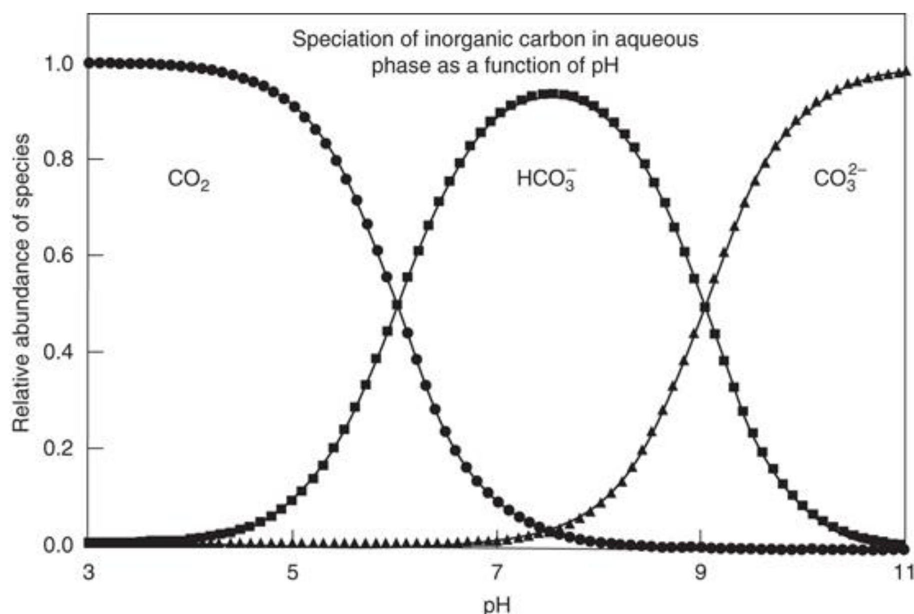


Figure 2.3 –Distribution of inorganic carbon speices as a function of pH [67]. The pH range between 7 and 8 favours HCO_3^- as the dominant dissolved inorganic carbon species, with CO_3^{2-} dominant at pH >10.3 and H_2CO_3 at pH <6.35 in freshwater environements at 25°C.

Previous studies have reported that, other than light, the quantity of carbon in wastewater to be one of the principal rate-limiting factors for microalgal growth [188–190]. Low availability in carbon, in particular inorganic carbon, can limit microalgal growth and directly the quantity of N and P assimilated by microalgae (described in more detail below). To increase the availability of carbon in the wastewater medium, and exogenous supply, in the form of CO_2 or bicarbonate salts, is commonly used [84, 191, 192]. The effect is a significant improvement in microalgal growth and remediation of N and P from wastewater, with the efficiency dependent on the CO_2 concentration and injection period. Shen et al., (2015) [193] reported on the remediation of TN from artificial wastewater by *S. obliquus* at CO_2-to-air ratios of 1%, 5%, 10% and 15%. In this treatment, a 99.6% removal efficiency of TN occurred within 2 days at 5% CO_2, with the concentration decreasing from an initial value of 25.0 to 0.08 mg L^{-1}. In comparison 1%, 10%, 15% CO_2 or ambient air were only capable of reducing the TN concentration to, respectively, 3.55, 3.0, 5.5 and 6.15 mg L^{-1} within 3 days.

A similar effect has been reported by other studies, with the supply of CO_2 in the range of 1 to 6% described as optimum to promote microalgal growth and nutrient removal [194–197]. Concentrations above this range have been found to reduce the beneficial effect of CO_2, with reported inhibitory effects on microalgal respiration [198]. It must be noted that the tolerance to CO_2 is strain dependent, with certain species capable of acclimating to elevated CO_2 concentrations up to 100% [199, 200]. Maeda et al., (1995) [201] observed that *Chlorella* sp. strain T-1 could grow in an atmosphere containing 100% CO_2 following a period of acclimation through sequential culturing at increasing CO_2 concentrations. Sakai et al., (1995) [202] demonstrated that a CO_2 concentration of 40% had no negative effect on the growth rate of the microalga *Chlorella* sp. strain H-84 when compared to its cultivation in 5%, 10% or 20% CO_2.

While the strategy of CO_2 injection is a viable option to augment carbon availability for microalgae in wastewater, its provision is energetically expensive, as detailed in Chapter 1. Furthermore, the supply of CO_2 may reduce the potential of the microalgae to use and therefore reduce the carbonaceous material in wastewater. Amblard et al., (1990) [203] surmised that because autotrophic carbon fixing is the primary pathway to increase net carbon in microalgae, CO_2 sparging may reduce the depuration of COD from the water as the metabolism of the microalgae is shifted to autotrophic metabolism only. Hu et al., (2012) [197] reported the occurrence of this effect, with the rate in COD reduction inversely related to the CO_2 concentration supplied. A potential strategy that may mitigate this is through intermittently supplying microalgae with CO_2, promoting autotrophic growth followed by heterotrophic consumption of the carbonaceous material. Indeed, it was observed that intermittent sparging for a specific period of time minimised carbon losses to microalgae when cultured in a raceway reactor, with higher CO_2 concentrations in the gas necessitating a lower gas flow [204].

Alternatively, certain microalgae can be cultivated on organic carbon substrates, in theory utilising the carbonaceous material in wastewater as a source. Some photosynthetic microalgae are facultative heterotrophs, able to metabolise organic carbon compounds, either in a mixotrophic mode with CO_2 and light or in a strict heterotrophic mode (without light) [205]. However, the complexity of the carbonaceous material in wastewater may limit its availability as a viable carbon source. Carbonaceous material in municipal wastewater is extremely heterogeneous with compounds ranging from simple low-molecular-weight compounds, like butyric acid, to more complex compounds such as polycyclic aromatic hydrocarbons and synthetic polymers [206, 207]. For example, Devi et al., (2012) [208] reported a COD reduction of only 18.3% with a final concentration of 328 mg L^{-1} O_2 in sterile municipal wastewater only when treated by a microalgae consortium under strict heterotrophic conditions.

Analysis of municipal wastewater has identified the majority of biological carbonaceous material to be composed of fibres and proteins while sugars account for only a total 10% or less [207, 209]. It has been suggested that in wastewater treatment the decomposition of complex organic carbon compounds by heterotrophic microorganisms (i.e. bacteria and fungi) is necessary to facilitate the conversion of the carbonaceous material to a suitable substrate in order to be a viable carbon source for microalgae [210, 211]. A premise corroborated by He et al., (2013) [212], the authors showed no substantial reduction in BOD_5 or DOC concentration from sterile secondary wastewater treated by *C. vulgaris* under mixotrophic conditions, whereas an average 90% BOD_5 removal efficiency was recorded in unsterilized secondary wastewater under the same conditions. A faster reduction with a lower final NH_4^+-N and PO_4-P concentration was recorded in the unsterilized wastewater microalgae treatment.

However, the capacity of microalgae to assimilate and metabolise carbonaceous material from wastewater may also be highly dependent on the composition of the wastewater itself, and not only the culture conditions. For example, Sacristán de Alva et al., (2013) [213] recorded 77.3% COD removal efficiency from sterile PSW treated by *S. obliquus*, decreasing from 782 to 177 mg L^{-1} O_2 under mixotrophic mode. In two independent studies treating sterile centrate wastewater, Li et al., (2011)

[214] reported a consistent COD removal efficiency (>80%) when treated by *C. vulgaris* strain UTEX 25 in either autotrophic, heterotrophic and mixotrophic mode, while Hu et al., (2010) [197] reported a similar COD removal efficiency (78.9%) when treated by *Auxenochlorella protothecoides* under mixotrophic conditions (5% CO_2). It is clear from the reported experimental evidence that the ability of microalgae to grow on and simultaneously reduce the carbonaceous material from wastewater is dependent on its composition, in addition to species and culture conditions. However, a paucity of information exists on the precise nature and mechanisms by which microalgae are capable of digesting and assimilating more complex carbon compounds from their aquatic environment [215, 216].

To improve the treatment efficiency of wastewater by microalgae, which may be limited by a labile source of carbon and without adopting CO_2 injection, supplementation with a source of readily-biodegradable carbon has been examined. Addition of organic carbon to microalgae cultures has predominantly focused on substrates such as glucose, glycerol, acetate or ethanol known to directly enter into the glyoxylate or glycolytic pathways [205, 217–220] (Figure 2.4). Other carbon sources include mono- and di-saccharides such as fructose, sucrose and lactose [215]. Chandra et al., (2014) [221] reported an improved efficiency in NO_3-N and PO_4-P removal from synthetic wastewater enriched with glucose using a natural microalgal consortium. In the treatment without amendment with glucose, the concentration of NO_3-N and PO_4-P decreased by 33% and 9.9%, respectively, whereas the glucose supplemented treatments (at concentrations of 0.5 to 3 g L^{-1}) effected a removal efficiency between 36% and 55% for NO_3-N, and 54% to 55% for PO_4-P. Interestingly, the authors observed a decrease in COD removal efficiency with an increase in glucose concentration. Perez-Garcia et al., (2011) [86] reported a higher rate of NH_4^+-N removal from both synthetic and real municipal wastewater when treated with *C. vulgaris* supplemented with either glucose or acetate. Although enrichment with organic carbon could be a strategy to improve the treatment efficiency of a microalgal wastewater treatment process, supplementing organic compounds increases production costs. Low-cost or waste organic carbon substrates have been researched mainly to improve biomass yield of microalgae, including food waste (e.g. dairy waste and cane molasses), polysaccharide hydrolysate (produced from starch or straw) and high strength domestic or livestock wastewater (centrate) [222–225].

2.2.2.2 Nitrogen

Microalgae are able to utilise N from a variety of inorganic (e.g. NH_4^+, NO_3, and NO_2) and organic sources (e.g. amino acids, urea, purines and nucleosides) [129, 226]. In regards to inorganic N, microalgae express a clear preference for NH_4^+ if available because its assimilation and incorporation is energetically more efficient [205, 227]. Ruiz-Marin et al., (2010) [228] demonstrated preference for NH_3 as an N source from wastewater to any other N source by the microalgae *S. obliquus* and *C. vulgaris*. Ammonium is assimilated by a group of membrane transporter proteins belonging to the ammonium transporter family, an evolutionarily common protein expressed in bacteria, yeast, algae and higher plants [229]. Once translocated across the membrane, NH_4^+ can directly be incorporated into amino acids necessary for growth and other metabolic functions (described below). In contrast, NO_3 and NO_2

must be reduced to NH_4^+; a reaction catalysed by the enzymes nitrate reductase and nitrite reductase, which require respectively the reductants NADH and ferredoxin [67]. Moreover, the transport of NO_3 into the cell is an energy-dependent process directly consuming ATP. Furthermore, NH_4^+ has quantitatively been linked to supresses the consumption of NO_3 and NO_2 until it is almost completely consumed [227, 230]. In contrast, organic N is considered a poor source because of the greater energy cost in acquiring the N [216, 231]. Urea and amino acids require an enzymatic deamination step, which predominantly occurs extracellularly [216, 232]. In some cases, the acquisition of organic N is found to be inhibited by the presence of NH_4^+ [233].

Although a decrease of NH_4^+ mediated by nitrification can be viewed as a benefit, from an operational viewpoint of a microalgae wastewater treatment process, the generation of NO_X is undesired as it is not eliminated by microalgae in the presence of NH_4^+. Therefore, in a microalgae-bacteria process in which nitrification occurs, either a denitrification step in the treatment train needs to be included or a sufficient long hydraulic retention period is necessary for the microalgae to effectively reduce the NH_4^+ and then NO_3 in order to meet the required total N discharge limits. Both approaches have the disadvantage of increasing operational cost and complexity. Furthermore, nitrification may induce N-limited conditions, with microalga growth rates reduced because of their competition for nutrients [234]. In a steady state microalgae-bacteria process, various authors have reported that an approximate 60 to 85% of NH_3 in the medium is oxidised to NO_3 with only 13 to 40% assimilated by the microalgae [235, 236].

Inorganic N assimilation in microalgae is inter-connected with the microalgae's carbon metabolism, requiring carbon skeletons in the form of keto-acids to incorporate N into organic compounds [67] (Figure 2.4). Anabolism of amino acids in microalgae requires inorganic N in the form of NH_4^+ as the primary N donor molecule. The integration of N is catalysed by the sequential action of the evolutionary conserved enzymes glutamine synthetase (GS) and glutamine 2-oxoglutarate amino transferase (GOGAT) [237–239] (Figure 2.4). GS fixes NH_4^+ on a glutamate molecule to yield glutamine, and the added amino group then can act as the N donor to 2-oxoglutarate in the NADPH dependent conversion to yield two glutamate compounds catalysed by GOGAT [237]. The assimilated N can then be further distributed to form other amino acids via transamination reactions. For example, aspartate aminotransferase transfers (AspAT) the amino group of glutamate to oxaloacetate yielding aspartate and 2-oxoglutarate, whereas asparagine synthetase (AS) transfers the amino group of glutamine to aspartate to form asparagine, both reactions are reversible [237]. Consequently, glutamine, glutamate, aspartate and asparagine are precursor substrates for the synthesis of organic N compounds, such as amino acids, nucleotides, chlorophylls, polyamines and alkaloids [237, 240]. Overall, this process is dependent on the supply of carbon skeletons in the form of keto acids, specifically 2-oxoglutarate and oxaloacetate which are metabolites in the tricarboxylic acid (TCA) cycle, along with ATP and reductants generated in the TCA cycle and mitochondrial electron transport chain [238].

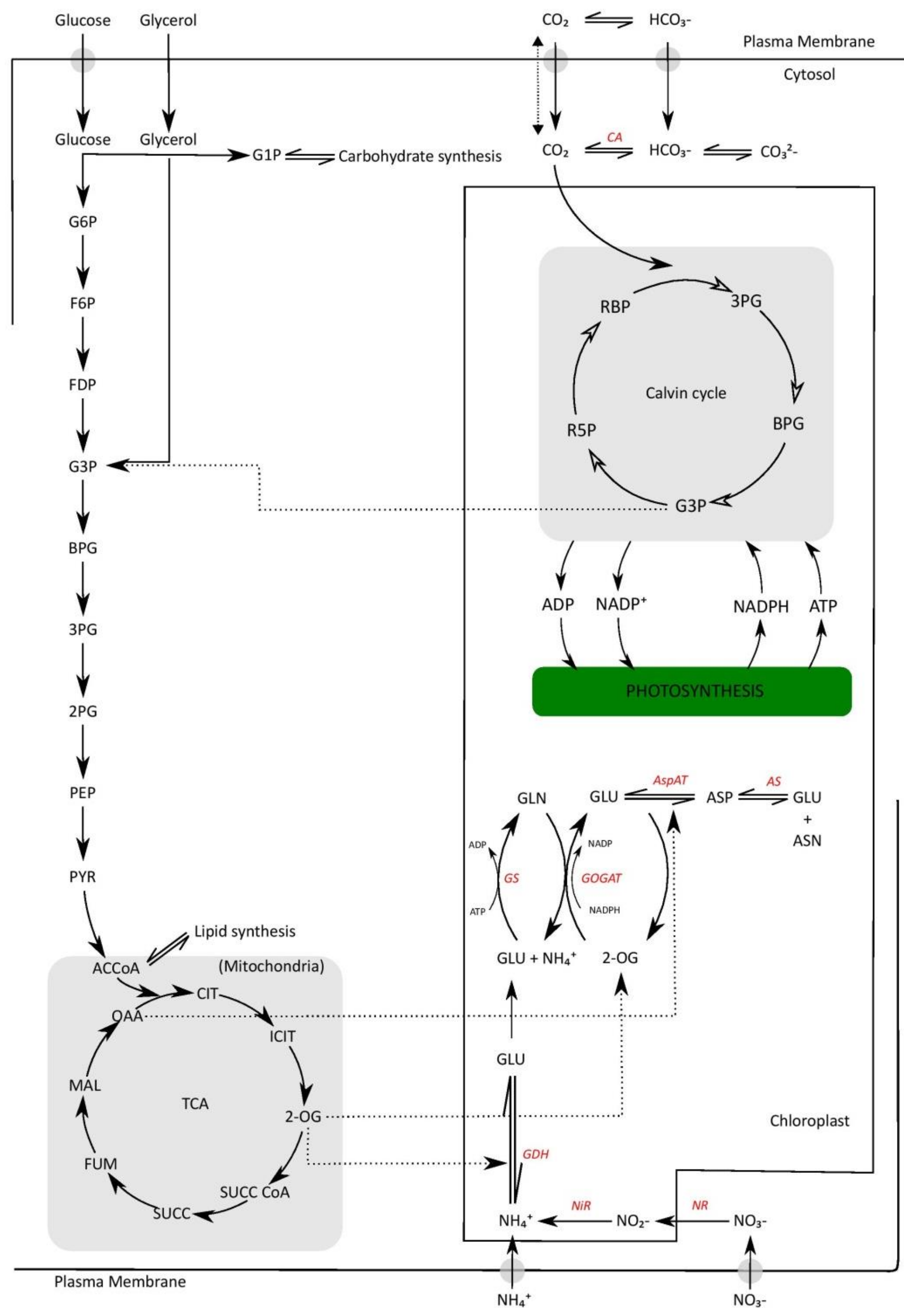


Figure 2.4 – Scheme of metabolic pathways for assimilation of carbon and nitrogen in the production of energy and amino acids in photoautotrophic and heterotrophic cultivation mode of microalgae (adapted from [205, 237]). Compound abbreviations are as follows: CIT, citrate; ICIT, isocitrate; 2-OG, 2-oxoglutarate; SUCC CoA, succinyl Coenzyme A; SUCC, succinate; FUM, fumarate; MAL, malate; OAA, oxaloacetate; GLU, glutamate; GLN, glutamine; ASP, aspartate; ASN, asparagine; ATP, adenosine triphosphate; ADP, adenosine diphosphate; NADP+ (NADPH), nicotinamide adenine dinucleotide phosphate oxidised (reduced); G3P, glyceraldehyde-3 phosphate; R5P, ribulose-5 phosphate; RBP, ribulose-1, 5 bisphosphate; 3PG, 3-phospho glycerate; BPG, 1, 3-bisphosphoglycerate; G6P, glucose-6 phosphate; F6P, fructose-6 phosphate; FDP, fructose 1,6-bisphosphate; 2PG, 2-phospho glycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; ACCoA, acetyl-Coenzyme A; NH_4^+, ammonium; NO_2^-, nitrite; NO_3^-, nitrate; CO_2, carbon dioxide; HCO_3^-, bicarbonate; and CO_3^{2-}, carbonic acid. Enzymes in red: NR, nitrate reductase; NiR, nitrite reductase; GDH, glutamate dehydrogenase; GS, glutamine synthetase; GOGAT, glutamine 2-oxoglutarate amino transferase; AspAT, aspartate aminotransferase; AS, asparagine synthetase; and CA, carbonic anhydrase. Grey circles on plasma membrane denote active transport and arrows diffusion only.

An auxiliary pathway in the regulation of NH_4^+ assimilation into amino acids was identified as the reversible reductive amination of 2-oxoglutarate regulated by the enzyme glutamate dehydrogenase (GDH) [241]. Although the pathway is highly conserved between microalgae species it is not thought to have a significant part in the formation of amino acids [237]. In fact, evidence suggests its main role is to catabolise glutamate which returns the carbon from the amino acid [242]. The activity of GDH is believed to be active under conditions of stress, particularly carbon shortage, and thus provides a feedback of necessary carbon skeletons to the TCA cycle in the mitochondria ensuring that energy production is not impaired [239, 242].

The carbon removed from the TCA cycle is replenished through anaplerotic reactions either involving the respiration of fixed CO_2 (autotrophic) or through assimilation of organic carbon (heterotrophic) [243]. In photoautotrophic mode, the inorganic carbon fixed in the Calvin cycle can enter the glycolytic pathway (also known as the Embden-Meyerhof pathway) as G3P, in which it is becomes metabolised into pyruvate [205, 244] (Figure 2.4). The generated pyruvate is then transported to the mitochondria upon which it enters the TCA cycle following its conversion to Acetyl-CoA. Through the TCA cycle, Acetyl-CoA is further metabolised to yield CO_2, reducing equivalents, ATP and carbon skeletons, including 2-oxoglutarate oxaloacetate for biosynthesis and further respiration as recycled substrates in the cycle [245] (Figure 2.4).

In heterotrophic mode, organic carbon substrates, as in the example for glucose, would be actively transported into the cytosol by the hexose/H^+ symporter system together with H^+ ions at a stoichiometry of 1:1 with the energy provided for this by the hydrolysis of one ATP molecule [246, 247]. In the cytosol, glucose becomes metabolically active through the glycolytic pathway, which transforms one glucose molecule into pyruvate [205]. Glucose may also be metabolised in the pentose phosphate pathway (PPP) producing ribose-5-phosphate and erythrose-4-phosphate, which are precursor substrates in nucleic acid and amino acid synthesis respectively [248]. The function of both pathways are considered anabolic and anaerobic because no O_2 is consumed and because ATP and reducing equivalents are required which are generated in alternative aerobic pathways, mainly from the mitochondria electron transport chain and oxidative phosphorylation. The main difference between the two pathways is the condition under which they are activated; PPP generally has a high rate of activity under dark conditions while glycolysis mainly takes place in light conditions [249]. Glycerol, as an alternative carbon substrate, can translocate across the membrane by passive diffusion into the cytosol of microalgae upon which it becomes sequentially phosphorylated and reduced to G3P and glycerate [205, 216]. The G3P is metabolised to Acetyl-CoA, amongst other metabolic intermediates, and directed into the TCA for energy [216]. It is impossible to precisely determine which substrate is preferred by any given microalgae. Overall, the carbon and N cycles in microalgae are integrally connected, with as much as 35% of carbon coupled to the incorporation of N in microalgae [67, 205, 250].

2.2.2.3 Phosphorus

In microalgae, P is an important element involved in innumerable metabolic pathways as well as a structural component of phospholipids, nucleotides and integral to the biological energy currency, ATP [68]. Inorganic P in wastewater exists in several ionic states and like inorganic carbon the specific species is dependent on the pH (H_3PO_4, <2.15; $H_2PO_4^-$, 2.15 to 7.20; HPO_4^{2-}, 7.20 to 12.33; and PO_4^{3-}, >12.33) [251]. Inorganic P is generally regarded as the most bioavailable form of P, with microalgae reported to preferentially assimilate HPO_4^{2-} and $H_2PO_4^-$ [67, 252]. In eukaryotic algae, PO_4^{3-} enters the cell by means of active transport through a symporter channel with H^+ or Na^+ ions providing the driving force, established by a plasma membrane H^+-ATPase pump [67]. It is increasingly recognised that soluble organic P compounds are a critical source of bioavailable P [68, 253]. These are made accessible to the microalgae by the expression of extracellular membrane-bound as well as free phosphatases, which non-specifically hydrolyse bound PO_4^{3-} groups [68, 254, 255]. Phosphorus is incorporated into organic compounds following phosphorylation of adenosine diphosphate (ADP). This is an endergonic reaction with the energy input obtained from either the oxidation of respiratory substrates or the photosynthetic electron transport chain [68, 140]. The produced ATP permits the transfer of the PO_4^{3-} group to organic compounds at the substrate level, as for example in the conversion of glucose to glucose-6-phosphate in the glycolytic pathway [67, 245]. Furthermore, in P-rich environments microalgae can accumulate P in excess of their metabolic needs and store it as acid-insoluble polyphosphate granules – a mechanism termed 'luxury uptake' which only occurs without a prior starvation period [256].

2.2.3 Abiotic and biotic factors influencing microalgae wastewater treatment

2.2.3.1 Bacteria

Extensive research in wastewater treatment has been carried out with single microalgal species or a consortium of different species. In reality, the presence of other microorganisms (e.g. bacteria and fungi) is unavoidable in a microalgal wastewater treatment system, as it is not feasible to previously sterilise the water because of the enormous volumes to be processed. In these conditions, the dynamics in community structure are generally a function of operational and environmental conditions, as well as the composition of wastewater being processed [257, 258]. With regards to bacteria, only a few studies report on community dynamics in microalgal-bacterial co-culture treatment processes. Su et al., (2011) [166] treating PSW with a microalgal consortium, reported the enrichment of certain bacterial species and which stabilised over the course of a semi-continuous treatment system. Notably, the bacterial community became dominated by members of the classes *Bacteroidia* (50%), *Flavobacteria* (25%), *Betaproteobacteria* (12.5%) and *Gammaproteobacteria*. In a subsequent study where different inoculation ratios of microalgae to sludge were investigated for their removal efficiency of contaminants from PSW, variations in the bacterial community composition occurred between the treatments of different inoculation ratios [259]. Bacterial species that were not detected in the original inoculum

became enriched to varying degrees during operation, which may have contributed to the difference in removal efficiency between the treatments. When cultured in digestate, the microbial community was dominated by *Gammaproteobacteria*, mainly *Pseudomonas stuzeri* followed by members of the class *Alphaproteobacteria* [260]; whereas in pig manure, 54% of the community was represented by members belonging to the phylum *Verrucomicrobium*, with also high representation by *Gammaproteobacteria* and members of the phylum *Firmicutes* [258]. Overall, microalgae have a significant effect on the microbial community and were found to reduce the diversity of bacteria present [261].

With regard to the treatment of wastewater, bacteria are necessary and indeed can be beneficial to microalgae. The bacteria may support the photoautotrophic growth of microalgae by providing CO_2 through their heterotrophic metabolism of organic matter, mineralising it to inorganic compounds that can be consumed directly by the microalgae, including NH_4^+ and PO_4^{3-} [262–264]. In return, microalgae provide O_2 generated via photosynthesis, required by the heterotrophic bacteria to degrade the organic matter, and microalgae during dark respiration [67]. In fact, photosynthetic oxygenation has the potential to meet dissolved O_2 needs to a treatment system without the use of mechanical aeration or mixing, thereby reducing the energy demands for the treatment process. To exemplify, Karya et al., (2013) [235] employed a sequence batch design with *Scenedesmus* sp. and nitrifying bacteria isolated from activated sludge to evaluate whether this co-culture system can support nitrification. Without mechanical aeration, the process was shown successful in reducing 81 to 85% of NH_4^+-N through its conversion to NO_3-N by nitrification, for which the O_2 for this process had been generated by the microalga. Similarly, Wang et al., (2015) [265] reported that photosynthesis by a microalgal consortium (predominantly *Chlorella* sp.) generated a sufficient quantity of dissolved O_2 to support nitrification in a photo-sequence batch reactor. In this process, centrate from anaerobically digested swine manure was cycled in the reactor between light and dark conditions, with the microalgae under illumination providing enough O_2 for complete nitritation, while in the dark condition denitrification occurred with the addition of acetate as a carbon source. Overall, 80% of the N was removed through nitritation and denitrification from an influent which was not aerated and had a mean NH_4^+-N concentration of 297 mg L^{-1}. González et al., (2008) [210] reported that the microalga *C. sorokiniana* was capable of providing a sufficient dissolved O_2 concentration for heterotrophic degradation of swine slurry medium when diluted 4 and 8 times, with O_2 concentrations reaching 2.5 mg L^{-1}.

The interaction between bacteria and microalgae is more complex than the exchange of just nutrients. Certain bacteria can promote microalgal growth by excreting growth-promoting compounds or vitamins (e.g. thiamine, biotin, etc.) [266–268]. De-Bashan et al., (2004) [269] found that the bacterium *Azospirillum brasilense* (strain Cd) promoted the growth and nutrient uptake rate of a microalga consortium (*C. vulgaris* and *C. sorokiniana*) when co-immobilised in alginate beads. The microalgal-bacterial co-culture was capable of removing 100% NH_4^+-N, 15% NO_3-N and 36% PO_4-P from municipal wastewater, while a corresponding culture with only microalgae achieved 75% NH_4^+-N, 6% NO_3-N and

19% PO_4-P removal within 6 days. Microalgae can promote bacterial growth through microalgal exudates that either stimulate their growth directly or can be assimilated as a source of carbon [270–272]. Hulatt and Thomas (2010) [273] quantified the amount of DOC excreted by microalgae in polythene photobioreactors, demonstrating a significant increase in bacterial population in microalgal-bacterial co-cultures as a result when compared to control cultures with only the bacteria. The authors of this study also showed that the DOC released by *C. vulgaris* and *Dunaliella tertiolectra* accounted for a maximum 6.4% and 17.3% of the total organic carbon in the culture, respectively. Conversely, metabolites presenting either bactericidal or fungicidal activity excreted by microalgae have been reported, including activity against the bacterium *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, as well as the fungus *Candida albicans* [274, 275]. Similarly, certain species of bacteria were found to be able to excrete algicidal compounds [276, 277].

2.2.3.2 pH

Several studies have reported on abiotic mechanisms by which bacteria and microalgae adversely affect each other. For example, an increased pH and dissolved O_2 concentration observed in microalgae cultures can have a detrimental effect on bacterial activity [278–280]. Assimilation of inorganic carbon by microalgae, if not replenished at an equivalent rate of consumption, can cause the pH to increase in the medium leading to an alkaline environment (pH >9) [67, 146]. In these conditions the benefit provided by aerobic and facultative bacteria in wastewater may be reduced as their growth and function becomes impaired. A strong correlation between heterotrophic bacteria abundance and pH is reported by other studies, which demonstrate an increased "inactivation" of bacteria with increasing pH [279, 281–283]. Reduction in coliform bacteria and other pathogenic microorganisms is reported to occur at pH 8.5 with pH 9.5 resulting in the highest elimination of the wastewater bacterial community [89, 284]. The effects are mediated through several different, potentially co-occurring mechanisms, such as conformational changes in bacterial membrane structure, respiratory chain damage and increased susceptibility to exogenous factors such as light [285–287]. Consequently, the reduced abundance of the microbial community in wastewater treated by microalgae will lead to a lower rate of CO_2 release via respiration that would otherwise serve the microalgae with an alternative source for photosynthesis [288, 289]. Notably, pH is also a determining factor of microalgal growth, with alkaline conditions having a negative effect on microalgae cycle completion because of changes in membrane lipid composition and increased metabolic activity [290, 291].

The optimal pH range for the majority of freshwater microalgae species is reported to be between 7 and 9 [292–294]. Environments outside of the optimum range for a particular species or consortium may adversely affect their growth rate and limit their capacity to remediate nutrients from the medium. From two independent experimental runs, Sutherland et al., (2015) [295] reported a decrease in removal efficiency of dissolved inorganic nitrogen (DIN), with increasing pH from PSW treated with a natural consortium of microalgae. In this study, pH 6.5 and 7 resulted in an approximate 62% DIN, whereas at pH 7.5 to 8 approximately 50% DIN removal was achieved. Martinez et al., (2000) [296]

observed that cell rupture of *S. obliquus* was associated with the point at which the pH of the medium reached its highest value (>11) when treating municipal STE. Overall, establishing an optimal environment for microalgal cultivation in wastewater for the purpose of nutrient remediation can be a critical step in preserving species dominance. These conditions are, however, highly dependent on the microalgal species and the cultivation method employed. Therefore, a suitable strategy might be to allow a natural species to acclimate to the subsequent processing conditions that naturally develop or are expected.

2.2.3.3 Temperature and light

As nutrients (e.g. N and P) become limiting, the autochthonous microbial community in wastewater may compete with exogenous microalgae (supplemented into the wastewater) for resources [261, 297]. It is therefore essential to establish an environment which promotes the growth of the microalgae growth above that of bacteria and fungi. In this context, temperature and light have a significant influence. In regards to light, its availability is fundamental for normal microalgal functioning. Energy captured from light drives the process of O_2 evolution and generates ATP and reducing agents necessary for fixing CO_2 into organic carbon [67, 298] (Figure 2.4). Below the light saturation point, the rate of photosynthetic activity is proportional to the irradiance intensity, with intensities above this point causing photo-inhibition as receptor systems become damaged [67, 298]. The illumination intensity at which saturation occurs may differ depending on the microalgal species and temperature. Dauta et al., (1990) [299] investigated the optimal light intensity of four microalgal species (*C. vulgaris*, *Fragilaria crotonensis*, *Staurastrum pingue*, *Synechocystis minima*) under a range of light intensities (5 to 800 µE m^{-2} s^{-1}) and temperature (10 to 35°C). The optimal light intensity increased with temperature until the optimal temperature was reached; thereafter the optimal light intensity remained constant or decreased with increasing temperature. In general, the saturation point for freshwater microalgae is reported to lie between 200 and 400 µE m^{-2} s^{-1} [289, 300, 301]. Maintaining an algal culture at or below the saturation point has a practical component because excess light is not utilised by the algae and thus becomes a waste of energy expenditure in the form of excess electricity.

The illumination period and intensity to which a microalgal-bacterial consortium is exposed to is demonstrated to significantly affect the ratio of bacteria to algae, and consequently the efficiency of carbon, N and P removal in wastewater. Under prolonged dark conditions, Lee et al., (2015) [302] reported a reduced capacity in N and P removal from municipal wastewater when treated with a microalgal-bacterial consortium. After 12 days of operation, the total dissolved nitrogen (TDN) concentration was reduced to 4.8, 14.0 and 25.6 mg L^{-1}, and the total dissolved phosphorus (TDP) concentration reduced to 0.6, 1.7 and 3.0 mg L^{-1} in photobioreactors under 12:12h, 36:12h and 60:12h dark-light cycles, respectively. Conversely, the soluble COD concentrations were reduced to 72, 56 and 35 mg L^{-1} O_2, respectively. A significant shift in the bacteria to microalgae ratio was observed following quantification by qPCR assay. Under prolonged dark conditions a higher ratio of bacteria to microalgae was recorded, with the lowest microbial biomass in terms of dry weight and chlorophyll *a* in the 60:12h

dark-light cycle treatment. González-Camejo et al., (2017) [303] examined the treatment response of a microalgal-bacterial consortium cultured in effluent from an anaerobic membrane system under varying light intensity. At the lowest set light intensity (40 µE m^{-2} s^{-1}) a higher activity of nitrifying bacteria was observed causing increased concentrations of NO_3 and NO_2 in the effluent with only 73.9% of NH_3 reduction credited to microalgae assimilation, and consequently the TN concentrations exceeded the permissible discharge standard (i.e. 10 mg L^{-1} TN). In comparison, light intensities of 85 and 125 µE m^{-2} s^{-1} favoured microalgae growth over nitrifying bacteria, with recorded NH_3 removal efficiencies by the microalgae of 98.3% and 99.3% respectively. Conversely, between the different light intensities examined, no difference in P removal efficiency was recorded (98.6, 99.2 and 99.5% at 40, 85 and 125 µE m^{-2} s^{-1} respectively). These observations indicated that the illumination period and intensity have a strong influence on the population dynamics in a microalgal-bacterial wastewater treatment system. Thus, in order to promote the growth of the microalgae above bacterial growth and to ensure an adequate response in treatment, these parameters must be adjusted accordingly.

The environmental temperature also has a significant influence on microalgal productivity and treatment efficiency in wastewater. Ruiz-Martínez et al., (2015) [304] assessed the NH_4^+-N removal rate by *Scenedesmus* sp. at various temperatures from effluent of a pilot scale submerged anaerobic membrane bioreactor; at a higher temperature the removal rate of NH_4^+-N increased, with 15°C, 18°C, 26°C and 34°C demonstrating a rate of 4.3, 6.7, 15.7 and 17 mg N L^{-1} d^{-1}, respectively. However, the optimal temperature has been shown to vary depending on the microalgal species and their acclimation to a particular environment. For instance, Filippino et al., (2015) [305] reported a high efficiency in nutrient removal within a shorter cultivation period by *C. vulgaris* at a lower temperature. A 90% reduction in TDN and PO_4-P was achieved within 4 days of cultivation at 15°C versus 12 days at 25°C. Similarly, Sforza et al., (2014) [156] reported a lower NH_4^+-N concentration in the effluent of treated PSW by *C. protothecoides* at lower temperatures (15°C) compared to temperate conditions (23°C to 30°C). Interestingly, the authors reported that specific growth rate, based on the parameter of cell number, was positively correlated with temperature, while total biomass (measured as total suspended solids (TSS)) tended to increase with decreasing temperature.

In general, most microalgae are capable of surviving at temperatures between 10°C to 30°C, with the optimal temperature within a more narrow range, often between 15°C and 25°C [301]. Although higher temperatures are generally associated with higher growth rates and increased nutrient uptake rates because of higher metabolic activity, these conditions are not always compatible with the conditions for wastewater treatment. Maintaining an optimum temperature in a microalgal wastewater treatment process through artificial heating is not feasible given the extreme volumes. Therefore, the microalgal species or consortium employed to treat the wastewater should be selected on their ability to thrive under the environmental conditions that are frequented at the treatment plant. The temperature of wastewater for mid-latitude climates has been reported to range between 3°C to 27°C [89]. In Scotland it is reported to range from 20°C in summer and 8°C in winter, with an average yearly temperature of

approximately 13.5°C [306]. Acclimation of a microalga to the treatment conditions can be performed to improve efficiency and tolerance to an environment that is otherwise unfavourable. In fact, algae acclimated to lower temperatures (5°C to 10°C) may be beneficial for use in wastewater treatment. Maxwell et al., (1994) [307] reported that *C. vulgaris* grown at 5°C exhibited physiological characteristics of cells acclimated to high irradiance conditions.

As photosynthetic carbon assimilation (i.e. Calvin cycle) is enzymatically mediated, the rate of the reaction is temperature-dependent with a lower reaction rate recorded at lower temperatures [67]. To compensate for the imbalance of more light being adsorbed than can be used for carbon fixation, microalgae respond by reducing their chlorophyll concentration at lower temperatures compared to cells at temperate conditions under the same illumination intensity. The reduction in chlorophyll was accompanied with an increase in the carotenoid xanthophyll [307, 308]. Xanthophyll forms part of the light harvesting antenna complex of photosystem II (PS II) and is proposed to modulate the transition of the antenna complex to a dissipative photo-protective state, protecting the complex against damage from light saturation [309]. Therefore, cultivation at low temperature may require lower light intensities to minimise light saturation and photo-inhibition and, hence, may reduce power consumption associated with the provision of illumination. A further benefit of a low operating temperature is the improved solubility of O_2 and reduced growth rates of indigenous microorganisms [1, 89, 308].

2.2.4 Microalgae bioreactor configuration for wastewater treatment

Wastewater treatment by microalgae faces several challenges that range from varying wastewater composition to the large volumes that need to be treated. Different microalgae cultivation techniques have been proposed and studied to ensure optimal microalgae productivity, high effectiveness in the removal of nutrients or contaminants, and to accommodate the large volumes of wastewater. The design and configuration of the reactor has a large effect on the treatment performance, with control over light and temperature influencing growth and in turn the assimilation and removal of contaminants from the wastewater [310]. The different microalgal cultivation techniques can be broadly categorised as either suspended or immobilised systems [311, 312]. These systems are further sub-categorised as being either open to the environment or enclosed. The main performance consideration of the bioreactor is its economic cost, with examples for a microalgae wastewater treatment system including (but not limited to) PBR, HRAP, matrix-immobilised microalgae and attached microalgal biofilms systems [146].

2.2.4.1 Immobilised

The concept of immobilised cell culturing is defined by the state in which living cells are prevented from moving independently to all parts of the aqueous phase of a system, either by natural or artificial means [313]. The immobilisation of microalgae can be achieved through the self-attachment (passive) to a bedding material, which is either completely or partially submerged to support biofilm development (i.e.

flat panel or rotating algal biofilm reactor), or through entrapment (active) in gel matrices that can be induced or mediated by flocculent or chemical agents [314–317].

Biofilm formation initially occurs because cations, inorganic and organic compounds adhere to the surface of the bedding material, in effect increasing the concentration relative to the aqueous phase and creating a favourable environment for microbial growth [318, 319]. Once colonised onto the surface, microalgae and bacteria secrete extracellular substances composed of nucleic acids, proteins, polysaccharides and phospholipids which serve to improve adherence to the bedding material but also to entrap and concentrate nutrients necessary for cell growth [319]. In general, microalgal biofilms are restricted to a single plain because of the need for light and gas exchange, with biofilm thickness between 0.052 to 2 mm for optimal performance [320, 321]. In the case of active immobilisation, the most widely used technique is the encapsulation of microalgae into polymer matrices made of artificial (e.g. acrylamide) or natural materials (e.g. carrageenans or alginates) [64, 317]. Manufactured to form beads, the microalgae are entrapped in a suspended form within the pores of the polymer matrix that are smaller than the cells, retaining them while allowing the diffusion of water and substances for their metabolisms and growth [322].

The principal advantage of immobilised microalgae systems is that they eliminate or reduce the processing cost associated with separating the algal biomass from the treated water before discharge [311, 314, 323]. Furthermore, by immobilising microalgae a higher concentration of cells relative to free suspended systems can be maintained in the water. Up to 3.3 g L^{-1} dry weight (DW) [324] compared to 1.5 to 1.7 g L^{-1} DW and 0.25 to 1 g L^{-1} DW in suspended tubular and raceway ponds respectively, has been reported [312]. It is thought that the high concentration of active biomass within biofilms or other matrices allows for an increased rate of biodegradation activity and therefore improved removal efficiency [322]. This effect could also be attributed to the fact that particulate, organic and inorganic compounds attach to the surface of the immobilising polymers or biofilms, increasing and sustaining a high concentration of these substances to the proximity of the microalgae and other microorganisms, in effect facilitating their biodegradation. However, no study has directly examined this occurrence to any great extent. Similarly, the close proximity of co-immobilised microalgae and bacteria, which generate O_2 and CO_2 respectively, can avoid gas diffusion problems inside the medium or immobilising matrix [289, 318, 325]. Conversely, Jiménez-Pérez et al., (2004) [326] found the N and P uptake rates of the microalga *S. intermedius* and *Nannochloris* sp. to be slightly higher when cultured in suspension compared to when immobilised. The authors argued this to be because of the additional resistance of nutrient diffusion across the polymer and impeded light penetration caused by the dense growth of cells within the inner surface of the beads, thereby reducing the photosynthetic activity.

The performance of immobilised microalgae systems to treat wastewater has been well documented (Table 3 and references therein). However, despite being effective at removing contaminants from wastewater, aspects of this technology still limit its commercial application. In active immobilisation, the polymers used to form the matrices are vulnerable to degradation over time, which can result in

cells leaching [64, 327]. Furthermore, the technical knowledge necessary for the manufacturing and high cost associated with the materials can prohibit their application, especially when the aim is to treat large volumes of wastewater [140, 311, 322]. On the other hand, microalgal biofilms require a large surface area. A theoretical analysis estimated 0.32 to 2.1 m^2 PE^{-1} is required to accommodate a microalgae biofilm treatment process in addition to the 0.2 to 0.3 m^2 PE^{-1} of the conventional wastewater treatment system when employed as a post-treatment process (i.e. tertiary) [87, 328]. Functioning as the primary biological treatment process for municipal wastewater, an estimated 0.76 m^2 PE^{-1} is required [328]. The aerial requirement compromises the environmental sustainability of this technology. Also, when exposed to the natural elements, fluctuations in both irradiance and temperature affect the performance, with low irradiance leading to low microalgal growth and O_2 generation in the biofilm and, hence, a reduced efficiency in nutrient removal [329, 330].

Attempts to optimize light utilization in algal biofilm-based systems have been directed to bioreactor design modification, typically designed with high surface area to volume ratio [331]. A rotating algal biofilm was designed and operated by Christenson and Sims (2012) [323] to allow periodic exposure of the biofilm between the medium and light. Posadas et al., (2014) [257] compared the treatment of domestic wastewater by two microalgae biofilm systems, one grown on an open surface and the other enclosed in clear tubes. Overall, the open surface algal-bacteria biofilm had higher efficiency in inorganic carbon, N and P removal compared to the enclosed biofilm reactor. The main hypothesis put forward to explain the difference in efficiency between the two biofilm systems was the location of the active microalgal population in respect to the light source. In the enclosed system, photosynthetic O_2 originated at the tubular surface and needed to diffuse to the centre of the tube in order for it to be utilised via heterotrophic metabolism; on the other hand, in the open biofilm O_2 originated in close contact to the contaminants at the biofilm-wastewater interface. Microalgal biofilms are also prone to sloughing, defined by the detachment of microalgae and other particulate matter from the matrix surface in the course of treatment. For example, Boelee et al., (2011) [329] noted an average suspended solids concentration of 3.2 mg L^{-1} in the final effluent, containing a high proportion of microalgae biomass. This corresponded to an average concentration of 0.13 mg L^{-1} N and 0.07 mg L^{-1} P. Taking this into account, under continuous operation the biomass requires separation from the water prior to discharge to minimise the input of these captured nutrients into receiving systems, effectively negating the main advantage of microalgae immobilisation [64, 319]. When managed incorrectly, microalgae biomass can account for a considerable proportion of the suspended solids content, contributing substantially to the effluent BOD [2].

2.2.4.2 Suspended cultures

Suspended cultivation of microalgae allows the cells to move freely in the aqueous phase and is amongst the most commonly applied algal cultivation technique for treating wastewater [146, 332–334]. Open suspended systems can be categorised into natural ponds, such as facultative ponds and lagoons, or artificial containers such as raceway ponds. In facultative ponds, different environments

naturally form as a result of the large depths (over 1 meter) and with minimal mixing as provided solely by wind, natural convection currents and water flow [335]. Consequently, stratification occurs as aerobic conditions form at the surface of the water because of microalgae photosynthesis, while anaerobic conditions form towards the bottom [335]. However, improved treatment efficiency as a result of the stratification has been reported, as it allowed different microbial communities with opposing roles in the treatment to become established [336]. In practice, high BOD, NH_3 and PO_4 removal rates and microalgal growth have been reported in facultative ponds with minimal operation cost and maintenance required [335, 337, 338]. HRAP can be considered as an improvement to the design of facultative ponds, with added operational control over mixing and culture conditions [334]. Generally designed with depths of 0.2 to 0.5 meters, HRAP are configured as a closed single canal, or meandering canal divided by central walls [335, 339]. To prevent sedimentation of the microalgae and to ensure periodic exposure to light, mixing is provide by means of a paddlewheel that is normally operated at velocities between 10 and 30 cm s^{-1}, while a CO_2 inlet can provide control over pH [80, 295, 340].

Photobioreactors are enclosed suspended cultivation systems, designed as an enclosed system composed of transparent plastic or glass materials which hold the algal biomass and growth medium within a confined system boundary [80, 334]. As a cultivation method, PBR have the benefit of better control over the culture environment. Temperature is easily controlled by heating or cooling the tubing, fluctuations in the pH are minimised through direct CO_2 injection or acid addition, and evaporation or contamination is greatly reduced because of the sealed system limiting the exposure of the culture environment [310]. The main advantage of PBRs is the improved light utilisation rate with a higher surface area to volume ratio compared to open pond systems [73, 341]. The increased irradiance to which the microalgae are exposed to promotes higher photosynthetic rates and cell densities. However, the use of PBRs for large-scale application is likely to be limited because of the high economic cost for materials, construction, and operation [73, 342, 343].

Both facultative and HRAPs are open cultivation systems and thus dependent on sunlight as the primary source of irradiance. As such, variations in effluent quality will occur between seasonal cycles with the most effective period being the summer months [330, 339]. Other factors that affect the performance of open reactors are temperature, evaporation and potentially inorganic carbon deficiencies. Evaporation helps maintain a stable temperature (during the day), however, the loss of water from the system can result in significant change in the ionic composition which can directly affect microalgal growth [344]. Likewise, CO_2 diffusion to the atmosphere can reduce the biodegradation activity and growth of the microalgae, leading to a less efficient treatment performance [81]. Open culturing systems (i.e. HRAP) are also susceptible to contamination by protozoa and zooplankton, which can reduce the algal concentration within a few days [345]. For example, Oswald (1980) [346] reported a 90% reduction in algal concentration within 2 days as a result of rotifers and cladocerans that can graze on microalgal cells.

A further disadvantage to microalgal pond systems is the large surface area required because of the shallow depths that are necessary to facilitate light penetration through the water [80]. Craggs et al., (2003) [347] reported that the surface area of HRAP at a depth of 0.45 and 0.3 meters operating at a volume of 37.5 m^3 would occupying an area of 85 and 128.1 m^2 respectively. Under the proposed loading rate in the study, the surface area required to treat 1 m^3 d^{-1} of wastewater was 17 and 25.6 m^2 based on the depth of the pond. In a study by Wang et al., (2015) [265], the authors estimated the surface area occupied by a HRAP using data from a laboratory pilot experiment. Depending on the N load the system required between 12 and 60 m^2 to treat 1 m^3 d^{-1} of centrate wastewater from anaerobically digested swine manure. In comparison, PBRs have inherent limitations associated to their design, such as high dissolved O_2 accumulation that can reduce photosynthetic activity, and biofouling with microbial films forming on the internal surfaces of the reactors which can adversely affect light penetration. PBRs placed outdoors are also susceptible to the seasonal variation in illumination intensity. Molina et al., (2001) [348] designed an outdoor tubular PBR with a working volume of 200 L, with vertical tubes made of plexi-glass connected to a 4 m tall airlift and degasser section to examine the pilot-scale production of the microalga *Phaeodactylum tricornutum*. In this reactor, a maximum biomass productivity of 1.9 g L^{-1} d^{-1} was obtained with a decline to 1.2 g L^{-1} d^{-1} in the spring cultivation period.

2.2.4.3 Treatment performance and duration

The COD, N and P concentration in the effluent and duration of the treatment are key criteria in assessing the performance of a bioreactor system of a microalgal wastewater treatment process. The performance of a microalgal treatment process must be able to meet current mandatory effluent concentrations, as set in Europe by the UWTD, with the prospect of achieving lower set standards (Chapter 1; [14, 26, 27]. Furthermore, the addition or integration of a microalgal biological treatment process within a conventional wastewater treatment train must complement the upstream and downstream processes by achieving a constant output and flow. In regards to hydraulic retention time, the shorter the time the smaller the reactor system necessary, which has benefits to capital costs and also surface area requirements [89, 349]. Table 3 lists the remediation data by microalgae reported from independent studies treating municipal wastewater cultured either by matrix-immobilised (active), biofilm-immobilised (passive), PBR suspended or HRAP suspended systems.

When comparing the N and P removal efficiency for the microalgal cultivation systems a vast difference is noted, not only between but also within the different cultivation systems (Table 4; Figure 2.5). Between immobilised and suspended cultivation systems, a consistently high N and P removal efficiency over the shortest treatment duration is noted for PBR suspended systems, despite the vast differences in operating parameters (i.e. biomass inoculation concentration, temperature and irradiance). In PBR suspended systems, an average 87.3% N and 82.9% P removal efficiency was achieved, within an average of 3.1 days (days or HRT). Of all the collated studies in this category, with the exception of that by Choi (2015), a final N concentration below 10 mg L^{-1} and P concentration below 1 mg L^{-1} was

reported, with the majority of studies reporting P concentrations below 0.5 mg L^{-1} (Table 3). The dominant species of microalgae used were of the family *Chlorophyceae*, which is well known for their N and P remediation abilities, including *Chlorella* sp. and *Scenedesmus* sp. A similar consistent rate of N and P removal but at a lower efficiency is noted in the biofilm-immobilised systems, at a respective 77.4% and 79.3% efficiency, taking an average treatment time of 4.3 days (days or HRT). Matrix-immobilised cultivation systems were operated for treatment duration of 4.1 days (days or HRT), during which the highest N removal efficiency was achieved compared to all other systems. The HRAP suspended systems did not performed as well, achieving the lowest P removal efficiency and requiring a longer treatment time with an average 8.6 days (days or HRT).

Table 3 – Carbon, nitrogen and phosphorus removal capacities from municipal wastewater by microalgae in different bioreactor types as reported in independent studies. Concentration values are in mg L^{-1} (C_i and C_f; % removal percentage).

Algae	Waste water type	Treatment conditions and reactor	HRT	Treatment time (days)	Nitrogen			Phosphorus			Carbon			Reference
					C_i	C_f	%	C_i	C_f	%	C_i	C_f	%	
Immobilised - passive														
Centrate wastewater native algal-bacterial consortium	PSW	Algal biofilm reactor, fixed, V = 31 L, 0.5 m^2, artificial illumination, 21.9°C; 7.7 pH	10	40	91 TN	27.3	70	7 PO_4^{3-}-P	0.05	85	181 TOC	18.1	90	[350]
Consortia of *Chlorella* and *Phormidium* sp.	Grey water	Algal biofilm reactor, fixed, V = 3 L, 630 cm^2, natural sunlight, 7.3 pH	6	-	29 TAN	1.7	94	24.5 TDP	2.4	90	235 COD	71	69	[351]
Consortium of *Woronichinia* sp., *Actuodesmus* sp., *Aulacoseira* sp., *Desmodesmus quadricaudatus, Nitzschia* sp., *Limnothrix redekei* and *Gomphonema parvulum*	PSW	Algal biofilm reactor, fixed, V = 31 L, 0.5 m^2, artificial illumination, 21.7°C; 8.3 pH	10	40	86 TN	6.8	92	12 PO_4^{3-}-P	0.96	96	167 TOC	18.3	89	[257]
	PSW	Algal tubular biofilm reactor, fixed, V = 31 L, 0.5 m^2, artificial illumination	10	40	86 TN	17	80	12 PO_4^{3-}-P	3.84	68	167 TOC	25.0	85	[257]

Consortium of *Scenedesmus, Chlorella,* Cyanobacteria, *Oocystis, Ankistrodesmus* and *Synura*	STE	Rotating algal biofilm disk, fixed, V = 8 L, artificial illumination, 21 to 25°C, 8.5 to 9 pH	6	21	46.5 TN	8.7	81	15.15 TP	0.07	99	63.1 COD	-	-	[170]
Predominant strain was *Halochlorella rubescens*	STE	Twin-Layer PBR biofilm, fixed, V = 55 L, 3 x 2m^2 modules, artificial illumination, 18 to 32°C, 8.4 pH	1	8	7.5 NO_3-N	1.3	83	0.61	0.17	73	-	-	-	[352]
Scenedesmus sp. and natural bacteria population	STE	Algal biofilm reactor, fixed, V = 96 L, artificial illumination, 20 to 22°C, 7.76 pH	2	91	18.5 TN	11	36	1.32 TP	<0.5	62	60 COD	39	35	[353]
Chlorella vulgaris	Treated municipal waste water	Suspended carrier, suspended V = 20 L, aerated, artificial illumination, 25 to 30°C; 8.2 to 9 pH	0.1	37	17.4 DIN	6.7	61	3.07 TP	0.8	71	21 COD	-	-	[354]
Chlorella sp., *Scenedesmus, Pediastrum, Nitzschia, Navicula, Crucigenia, Synedra* and bacteria	Waste water Lagoon effluent	Rotating algal biofilm disk, fixed, V = 535 L, natural sunlight, 9.6 to 19.2°C; 8 to 10 pH	0.2	20	4.5 TN	1.1	75	2.1 TP	1.6	23	-	-	-	[323]
Nitzschia sp. and other green filamentous microorganisms	Municipal waste water	Algal biofilm, fixed, 1.8 m^2, artificial illumination, 22°C; 7 pH	0.7	10	5.5 NO_3-N	2.2	60	0.9 PO_4^{3-}-P	0.2*	88	-	-	-	[329]

Scenedesmus obliquus and bacteria	STE	Biofilm in a twin wall polycarbonate sheet, fixed, V = 5 L, 0.5 m^2, natural illumination, 21.7°C;7.6 pH	1	130	32 TAN	1.6	95	1.7 TP	0.1	94	61 COD	37	39	[355]
Immobilised - active														
Scenedesmus obliquus	STE	Sodium alginate beads, suspended, V = 2.5 L, aerated, batch mode, artificial illumination, 25°C; 9 to 9.5 pH	-	2	34 NH_4^+-N	1.2	96	2.5 PO_4^{3-}-P	1.12	55	-	-	-	[228]
Chlorella vulgaris	PSW	Carrageenan beads, suspended, V = 0.4 L, flask shaking, artificial illumination, 24°C; 7.1 pH	-	5	37NH_4^+-N	<2	95	3.1 PO_4^{3-}-P	0.04	99	-	-	-	[356]
Chlorella vulgaris	BNR treatment effluent	Alginate beads, V = 5 L, agitated, artificial illumination, 30°C; 6.7 pH	-	6	8.73 TN	0.1	99	0.8 TP	0.32	60	-	-	-	[305]
Chrlorella vulgaris	PSW	Soldium alginate - medium concentration, V = 1.6 L, aerated, artificial illumination, 25°C; 6.5 to 7.2 pH	-	2	42 NH_3-N	0.4	99	12 PO_4^{3-}-P	0.62	94	-	-	-	[357]

Chlorella vulgaris	PSW	Sodium alginate - low density, V = 1 L, aerated, artificial illumination, 20°C; 7.95 to 8.29 pH	-	7	36.2 NH_4^+-N	0.1	99	3.4 PO_4^{3-}-P	1.08	68	-	-	-	[358]
Phormidium sp.	STE	Chitosan, V = 0.5 L, aerated, artificial illumination, 20°C; .8.5 to 10 pH	-	0.25	9.5 NH_4^+-N	0.4	95	2.2 PO_4^{3-}-P	0.64	71	30 COD	-	-	[359]
Chlorella vulgaris	STE	Sodium alginate, V = 1 L, aerated, natural illumination, 30°C; 7.8 to 9.1 pH	0.7	20	24 NH_3-N	4.4	81	9.2 TP	2.7	70	257 COD	94	63	[360]
Consortium of algae and bacteria; main algae were *Scenedesmus* and *Chlorella*	STE	Alginate beads, V = 2.5 L, no mixing, artificial illumination, 23°C; 8.05 to 9 pH	-	10	36 NH_4^+-N	3.6	90	0.86 TP	0.03	97	49 COD	-	-	[361]
Suspended - PBR														
Consortium with the predominate strains *Actinastrum, Scenedesmus, Chlorella, Spirogyra.*	PSW	Semi-continuous mode, V = 1 L, aerated, artificial illumination, 23 to 25°C; 7 to 8 pH	3	10	39 NH_4^+-N	6.1	84	2.1PO_4^{3-}-P	<0.1	99	-	-	-	[362]

Prevalent microalgae species was *Scenedesmus*	STE	Batch, V = 15 L, pump mixed, artificial illumination, 20°C; 7.2 to 8.5 pH	-	1	36 NH_4^+-N	0.1	99	2.56 PO_4^{3-}-P	0.03	98	-	-	-	[363]
Prevalent microalgae species was *Scenedesmus*	STE	Batch, closed, V = 15 L, pump mixed, natural illumination, 4 to 28°C	-	7	21 NH_4^+-N	4.6	79	1.49 PO_4^{3-}-P	0.44	70	-	-	-	[363]
Scenedesmus obliquus and wastewater microbial community	STE	Batch, flat panel PBR, V = 4.5 L, aerated, artificial illumination, 20°C; 7 pH	1.1	-	19.7 TN	2	89	1.75 TP	0.09	84	-	-	-	[349]
Consortium of chlorococcales and cyanobacteria as well as natural wastewater microbial community	Anaerbic wastewater effleunt	Batch, flat panel PBR, V = 8 L, aerated CO_2, artificial illumination, 28 to 32°C;7.2 pH	2	-	59 NH_4^+-N	-	67	-	-	97	51 COD	-	-	[364]
Scenedesmus obliquus and wastewater microbial community	STE	Semi-continuous, tubular air lift reactor, V = 330 L, natural illumination, 13°C; 8.72 pH	5	110	26.16 TN	3.4	86	1.77 TP	0.21	88	76.6 COD		24	[365]
Scenedesmus obliquus	STE	Batch, V = 2.5 L, aerated, artificial illumination, 25°C; 9 to 9.5 pH	-	2	34 NH_4^+-N	0.1	99	2.5PO_4^{3-}-P	0.42	83	-	-	-	[228]

Chlamydomonas reinhardtii	STE	Batch, V = 5 L, mixed, artificial illumination, 7 to 10 pH	-	4	25 NH_4^+-N	0.1	99	1.7PO_4^{3-}-P	<0.1	98	30.2 COD	-	-	[366]
Chlorella vulgaris	Tertiary waste-water	Batch, V = 0.2 L, mixed, artificial illumination, 27°C; 7.3 to 5.7 pH	-	4	8.7 TN	0.1	99	1.71 TP	<0.1	99	22.6 TC	-	-	[367]
Chlorella vulgaris and natural wastewater microbial community	Municipal waste water	Membrane photobioreactor, closed, V = 10 L, aerated, artificial illumination, 25°C; <9 pH	2.5	-	8.3 TN	3.6	56	1.24 TP	<0.3	82	55.6 COD	-	-	[368]
Chlorella vulgaris and natural wastewater microbial community	Pre-PSW	Optical panel membrane PBR, closed, V = 40 L, aerated, artificial illumination, 25°C; 7.2 pH	3.4	150	40.2 TN	11	70	9.24 TP	4.37	52	209 COD	86	58	[181]
Chlorella sp. ADE4 and natural wastewater microbial community	Treated sewage effluent	Membrane PBR, closed, V = 7 L, aerated, artificial illumination, 25°C; 7.5 to 8.5 pH	2	18	18.8 TN	6.3	66	1.01 TP	<0.1	94	10.5 COD	-	-	[369]
Suspended - HRAP														

Scenedesmus obliquus and wastewater microbial community	STE	HRAP, V = 533 L, mixed, natural illumination, 13°C; 9.32 pH	10	110	26.16 TN	11	55	1.77 TP	0.64	64	76.6 COD		-12	[365]
Chlorella pyrenoidosa and wastewater microbial community	STE	HRAP, V = 165 L, mixed, natural illumination, 31 to 6°C; 7.8 to 9.3 pH	-	18	46 NH_4^+-N	2.1	95	3.22 TP	0.59	84	426 COD	90	78	[370]
Consortium of *Chlorella, Nitzschia* sp., *Navicula* sp., *Stigeoclonium* sp., ciliate, protozoa and bacteria	PSW	HRAP, V = 470 L, mixed, natural illumination, 23.7°C	6	-	36 NH_4^+-N	0.3	99	-	-	-	318 COD	64	80	[371]
Unspecified - algae and microorganisms	PSW	HRAP, mixed, natural illumination, 13 to 19°C;7.4 to 8.9 pH	8	-	51.2 TN	14	72	8.5 TP	4.8	43	260 COD	170	34	[372]
Micractinium pusillum, Desmodesmus communis, D. opliensis, Pediastrum boryanum, Actinastrum hantzshii, closterium and natural bacteria	PSW	HRAP (Spring), V = 4375 m^3, mixed, natural illumination, 13°C; 9.7 pH	7	-	22 NH_4^+-N	4	79	1.8 DRP	1.6	22	-	-	-	[373]
Prevalent organisms was *Coelastrum* amongst others	USAB effluent	HRAP, (Spring L-CO_2) V = 9600 m^3, mixed, natural illumination, 7.9 to 8.1 pH	7	-	48 NH_4^+-N	2.9	94	7.8PO_4^{3-}-P	3.2	58	167 COD	63	62	[374]

Chlorophyta	PSW	Experiment 1, V = 15 L, mixed, natural illumination, 23°C; 7.5 pH	4	16	29.1 DIN	15	48	4.1 DRP	3.56	13	-	-	-	[295]

COD, chemical oxygen demand; DIN, dissolved inorganic nitrogen; DRP, dissolved reactive phosphorous; NH_4^+-N, ammonium-nitrogen, NO_3-N, nitrate-nitrogen; PO_4^{3-}-P, phosphate-phosphorous; TAN, total ammonia nitrogen; TC, total carbon; TDP, total dissolved phosphorous; TN, total nitrogen; TOC, total organic carbon; TP, total phosphorous.

In regards to N removal, the high efficiency recorded in the matrix-immobilised systems cannot be completely attributed to the function of the microalgae. In part, the ionic interactions of the N cations and anions (i.e. NH_4^+ and NO_3^-) with the polymer used as the matrix would contribute to their reduction. For example, Fierro et al., (2008) [375] reported a higher inorganic N and P removal efficiency from synthetic wastewater by *Scenedesmus* sp. immobilised in chitosan (PO_4-P: 94% and NO_3-N: 70%) compared to that in a free-living suspended state (PO_4-P: 20% and NO_3-N: 30%). Despite the vast difference in removal efficiency no statistical difference between the treatments was computed following the removal efficiencies adjustment of the immobilised treatment with the control treatment, chitosan beads only (without microalgae). In the control experiment a 60% PO_4-P and 20% NO_3-N reduction was recorded, suggesting that the net removal efficiency contributed by microalgal assimilation was only 34% PO_4-P and 50% NO_3-N. The higher PO_4-P removal in the immobilised treatments was attributed to the release of calcium ions from the polymer that contributed to its precipitation rate [376]. The same effect was reported by Ruiz-Marin et al., (2010) [228] when comparing NH_4^+-N and PO_4-P removal from urban wastewater by both *C. vulgaris* and *S. obliquus*, with each microalga cultured either immobilised in sodium alginate or in free-living suspended state. A higher NH_4^+-N uptake rate and growth rate was recorded in the immobilised microalgae treatments, with *S. obliquus* more effective in removing the inorganic nutrients within the 2-day cultivation period.

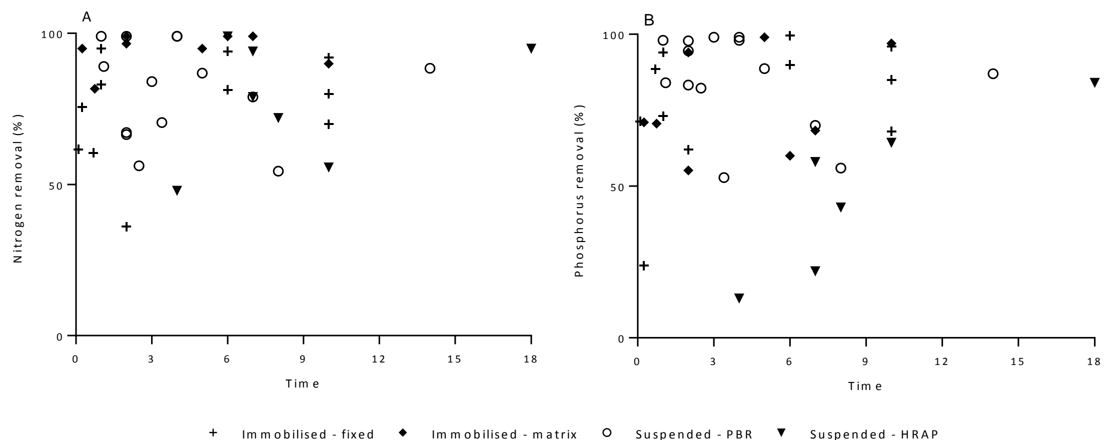


Figure 2.5 – Treatment period versus remediation efficiency for A) Nitrogen and B) Phosphorus from data reported in the literature (Table 3) for independent microalgal treatment studies cultured either matrix immobilised (active), biofilm immobilised (passive), PBR suspended or HRAP suspended.

The discrepancy in N and P removal efficiency between open and enclosed microalgal systems is mainly a result of the different environments that may form (i.e. nitrification and/or denitrification) and surface to volume ratios. In a comparative study, Molinuevo-Salces et al., (2010) [377] assessed the performance of a microalgal consortium treatment in an anaerobically-digested swine slurry in an open HRAP and enclosed PBR. Depuration of NH_4^+-N was recorded in both bioreactor types; however, in the

open HRAP NH_3 volatilisation was the dominant mechanism of removal, whereas in the enclosed PBR nitrification and denitrification became dominate. A higher N concentration was recorded in the biomass of the enclosed PBR, but interestingly a higher P concentration was recorded in biomass of the open HRAP. In a similar study, Arbib et al., (2013) [365] compared the treatment performance of a mesocosm HRAP (530 L) and airlift tubular-PBR (380 L) run in parallel under continuous operation fed with secondary treatment effluent. A statistically significant average TN and TP removal efficiency was recorded in both systems with a respective 65% and 58% in the HRAP, and 89% and 86% in the tubular-PBR over the course of the duration (157 days) of the treatment. The majority of inorganic N and P removal was attributed to assimilation by the microalgae and other microorganisms in the wastewater, with only a small fraction through chemical volatilisation or precipitation. The main reason for the better efficiency in the tubular-PBR was the higher surface to volume ratio of the system, which facilitated a greater photosynthetic rate, and which in turn promoted higher growth of the microalgae. Comparing both systems, a maximum suspended solids concentration of 733 mg L^{-1} was recorded in the tubular-PBR, whereas an average 188 mg L^{-1} was recorded in the HRAP. Furthermore, the input of atmospheric air to the tubular-PBR helped maintain a stable pH of the wastewater, with the elevated pH in the HRAP affecting the performance of the microalgae and other microorganism.

Table 4 – Average N and P removal efficiency for the culturing conditions (data from studies in Table 3)

Factor	No. of studies	P % removal	N % removal
Biofilm immobilised (passive)	11	77.4	79.3
Matrix immobilised (active)	8	76.9	94.4
HRAP Suspended	7	47.4	81.2
PBR Suspended	13	87.3	82.9

Following the evaluation of microalgae cultivation types, a suspended PBR-type system was selected as the cultivation system in the present work because of certain attributes which are desirable for a microalgae wastewater treatment system. These attributes include: 1) effective in treating municipal wastewater in compliance to current or lower effluent standards; 2) low technology footprint requirement; 3) on average able to accomplish contaminant removal within a shorter treatment time compared to other systems; and 4) improved control over the culture condition. Microalgae harvesting and downstream biomass application are beyond the scope of this thesis, but are discussed for further development.

Chapter 3 – Materials and Methods

The materials and methods described in this chapter are standard analytical techniques common for the analysis of wastewater and microalgae. Experimental designs are described in their respective chapters. Unless otherwise stated, all chemicals were of analytical grade and prepared in deionised water (18.2 MΩ cm^{-1}). Samples and chemicals were prepared, diluted and stored in type A borosilicate glass, unless otherwise specified. Weights measurements were recorded in grams (g) and volumes in millilitres (mL).

3.1 Wastewater source

PSW was obtained from Seafield Wastewater Treatment Plant located in Edinburgh, UK. The facility treats predominantly domestic wastewater received via a combined sewage system from Edinburgh City and the surrounding areas. The site treats an average flow of 283 million L a day, i.e. a PE of approximately 800,000, to comply with the standards set by the UWTD for BOD and COD of, respectively, 25 mg L^{-1} O_2 and 125 mg L^{-1} O_2 [14, 378]. The treatment process comprises 10 preliminary screens, 4 grit removal tanks, 4 primary settlement clarifiers and 4 plug flow secondary activated sludge lanes followed by 9 final settlement tanks before being discharged to the Firth of Forth via a long sea outflow (Mr. Skroblin of Veolia Ltd., personal communication).

The samples were collected from the same primary settling tank effluent channel for all the experimental work. The wastewater samples were grab samples taken on the same day that an experiment was to be commenced (around 8:00 am). Once collected, the samples were taken directly to Heriot-Watt University where they were processed within two hours. It should be noted that because sampling was conducted on different dates in order to accommodate the logistics of running experiments to test different parameters, the composition of the wastewater varied; this was addressed in the respective results chapters. Unless stated otherwise in the respective results chapters, each wastewater batch was filtered through a Whatman 113 filter (Ø 90mm, pore size 30 µm, Whatman International Ltd., UK) as a pre-treatment step to provide consistency in turbidity between samples.

3.2 Microalgae strains, medium and maintenance

Chlorella vulgaris (CCAP 211/79), *Heynigia riapria* (CCAP 222/47) and *Acutodesmus obliquus* (SAG 276-1; formerly known as *Scenedesmus obliquus*) were used in the experiments for this thesis work. All strains were non-axenic freshwater microalgae. Manipulations of the stock cultures and seed cultures were carried out under sterile conditions in a biological laminar flow hood to limit the contamination of the cultures with other microorganisms.

A modified version of Bold basal medium (BBM), adjusted to pH 7.2 was used as the maintenance medium. To 800 mL deionised water, 1 mL of each micro nutrient solution (Table 5) and 10 mL of each macro nutrient solution (Table 6) were added and the volume made up to 1 L with deionised water after pH adjustment. All glassware and medium was heat sterilised at 121°C for 15 minutes. Stock cultures of 100 mL were maintained in 250 mL Erlenmeyer flasks and grown statically with intermittent manual

shaking. Routine serial sub-culturing was performed every 14 days by transferring 10% v/v of each strain to fresh BBM.

For all experiments, seed cultures of the strains were grown for 7 days prior to use as inocula. These were cultured in 350 mL BBM in 500 mL round borosilicate bottles, which were aerated continuously with atmospheric air through a sterile In-Line HEPA filter (Ø 53 mm, pore size ≥0.3 µm, Whatman International Ltd., UK) at a volumetric flow rate of 0.15 of air volume per volume of liquid per minute (V/Vm). Environmental conditions were the same for both the stock cultures and the experimental runs – i.e. incubated at 15 ±1°C and at a 12:12 light-dark cycle (Fluora, Osram, Germany) with a photon flux of 100 µE m^{-2} s^{-1} (US-SQS/L probe, Walz, Germany).

Table 5 – Stock micro-nutrients for the preparation of BBM

Solution	Chemical	Formula	Concentration[2] (g L^{-1})
1	Iron (II) sulphate	$FeSO_4.7H_2O$	4.98
	Sulphuric acid	>98% H_2SO_4	1 mL
2	Manganese (II) chloride	$MnCl_2.4H_2O$	1.44
	Zinc sulphate	$ZnSO_4.7H_2O$	8.82
	Cobalt (II) chloride	$CoCl_2.6H_2O$	0.4
	Copper (II) sulphate	$CuSO_4.5H_2O$	1.57
	Sodium molybdate	$NaMoO_4.2H_2O$	1.18
3	EDTA salt	EDTA	50
	Potassium hydroxide	KOH	31
4	Boric acid	H_3BO_3	11.42
5	Vitamin B1	Thiamine-HCL	1.2
	Vitamin B7	Biotin	0.012
	Vitamin B12	Cyanocobalamin	0.01

[2]Reagents were made in various volumes to the stated concentration; 1 mL of each stock solution was added to 1 L of BBM medium.

Table 6 – Stock macro-nutrients for the preparation of BBM

Solution	Chemical	Formula	Concentration[1] (g L^{-1})
1	Sodium Nitrate	$NaNO_3$	75
2	Calcium chloride	$CaCl_2.2H_2O$	2.5
3	Magnesium sulphate	$MgSO_4.7H_2O$	7.5
4	Potassium hydrogenphosphate	K_2HPO_4	7.5
5	Potassium dihyrdogen phosphate	KH_2PO_4	17.5
6	Sodium chloride	NaCl	2.5
7	Sodium carbonate	Na_2CO_3	2

[1]Reagents were made in various volumes to the stated concentration; 10 mL of each stock solution was added to 1 L of BBM medium.

3.3 Analysis of Inorganics

Ammonia, nitrite, nitrate and phosphate analyses were performed following the methods described in Standard Methods for the Examination of Water and Wastewater [379]. These methods were modified to accommodate the analysis of smaller sample volumes – 5 mL instead of 25 mL – without affecting the chemistry of the reactions. Prior to analysis, all samples were centrifuged at 3500x*g* for 10 minutes (Heraeus Multifuge 3S) to minimise optical interference from either the microalgae or particular matter. Absorbance was measured on a Genesys 20 spectrophotometer with a 1 cm light path (Thermo Scientific, UK).

For each inorganic compound, a calibration graph of known concentrations versus their respective absorbances was plotted. The resultant calibration graphs followed a linear regression model (Equation 1) with the intensity of the reaction colour formed correlating to the concentration of the analyte. Thus, once the absorbance intensity (y) of the sample was known, the unknown analyte concentration (x) was calculated by inverting the linear equation 1 (i.e. Equation 2). Within the calibration range, the amount of analyte was the limiting factor in the reaction. Any experimental sample out of the range of absorbance (>1 Abs units) was diluted with deionised water prior to reagent addition and the dilution factor included in the final calculation.

$$y = mx + c \tag{1}$$

$$x = \frac{y-c}{m} \tag{2}$$

where y is the measured absorbance intensity, x the concentration of analyte, m the slope and c the y-intercept of the line of regression.

3.3.1 Validation

The trueness of the calibration graphs and standard deviations of the regression equation were calculated following the guidelines described in Miller and Miller (2010). The correlation coefficient, *R*, was calculated to assess the degree of correlation between x and y, and the manner of their dependency (Equation 3).

$$R = \frac{\sum[(xi-\bar{x})(yi-\bar{y})]}{\sqrt{[\sum(xi-\bar{x})^2][\sum(yi-\bar{y})^2]}} \quad (3)$$

where $\sum$ is the sum of the measured values, xi and yi the individual values of analyte concentration and absorbance respectively, $\bar{x}$ the mean of all the x-values, and $\overline{y}$ the mean of all the y-values.

A t-test was used to validate the relationship between the dependent y variable (absorbance) and the independent x variable (concentration) as linear (Equation 4). The calculated t value (t_{CAL}) was compared to the critical t value (t_{CRI}) with a significance level of 99% at n-2 degrees of freedom – t table from Emden (2008). The null hypothesis, which states there is no significant linear relationship between the independent and dependent variable as the slope equals 0, is rejected if t_{CAL} is greater than t_{CRI}, thus confirming linearity.

$$t = \frac{R\sqrt{n-2}}{\sqrt{1-R^2}} \quad (4)$$

The standard deviation of the regression ($SD_{y/x}$), which estimates the error in the y-direction as residuals, was calculated by equation 5, and the standard error of the slope (SD_m) and intercept (SD_c) by equation 6 and 7 respectively. The error of the calculated concentration (S_{xo}) was determined by equation 8, where v is number of replicate readings.

$$SD_{y/x} = \sqrt{\frac{\sum(yi-\hat{y})^2}{n-2}} \quad (5)$$

$$SD_m = \frac{SD_{y/x}}{\sqrt{\sum(xi-\bar{x})^2}} \quad (6)$$

$$SD_c = SD_{y/x}\sqrt{\frac{\sum xi^2}{n\sum(xi-\bar{x})^2}} \quad (7)$$

$$S_{xo} = \frac{S_{y/x}}{m}\sqrt{\frac{1}{v}+\frac{1}{n}+\frac{(yi-\bar{y})^2}{c^2\sum(xi-\bar{x})^2}} \quad (8)$$

The confidence interval (CI) at 99% at n-2 degrees of freedom for the slope and intercept were calculated by equation 9.

$$CI = t_{CRI}\, SD_m \text{ or } SD_c \quad (9)$$

The limit of detection (LOD) is classed as the analyte concentration giving a signal equal to the blank signal (y_B) plus three standard deviations of the blank (S_B) (Equation 10). In this equation S_B is substituted with $S_{y/x}$ as it is assumed each point, including the blank, has an equal variation in

distribution represented by the standard deviation of the regression (residuals) calculated as $S_{y/x}$. Additionally, the blank signal, which corresponds to the absorbance of the reagents without analyte is estimated as the calculated intercept, c.

$$\mathrm{LOD} = c + 3S_{y/x} \tag{10}$$

Each day of analysis for each inorganic compound, three standards and a sample of deionised water as the blank were treated in parallel to experimental samples to check reagent performance and precision.

3.3.2 Reporting of inorganic compound concentration

The concentration of each inorganic compound in the calibration and sample was recorded as the concentration of the element of interest in the compound. For example, NO_3-N refers to the concentration of N within the NO_3 compound. The method for the analysis of each inorganic compound is described below

3.3.3 Ammonia - Nitrogen

Ammonia was determined by the Phenate reaction with no preliminary distillation step, which was in compliance with the standard method 4500-NH_3.F. The concentration of NH_3-N is measured based on the intensity of indophenol, a blue compound formed by the reaction of NH_3 with phenol and hypochlorite as catalysed by sodium nitroprusside (Berthelot reaction) (Searle, 1984; Park *et al.*, 2009). In the sample, an alkaline environment is formed which allows NH_3 to react with sodium hypochlorite to form monochloramine. This reacts with phenol to form the intermediate *p*-benzoquinone chloramine before reacting with a further phenol molecule to form an indophenol dye. The reagents for this reaction were prepared as listed in Table 7 and were stable for three months at 4°C.

Calibration standards were prepared from a commercial 100 mg L^{-1} NH_3-N stock standard (Hach, UK). A working 1 mg L^{-1} NH_3-N standard was made by diluting 1 mL of the stock standard in a final volume of 100 mL deionised water. Calibration standards in the range of 1 to 0.025 mg L^{-1} NH_3-N were diluted from this solution.

Table 7 – Ammonia reagents

Reagent	Chemical	Formula	In 500 mL
NH_3.R1	Phenol	C_6H_6O	15 g
	Sodium nitroprusside	$Na_2[Fe(CN)_5NO]$	0.015 g
NH_3.R2	Sodium Hydroxide	NaOH	10 g
	Sodium hypochlorite solution (12%)	NaOCl	4 mL

For analysis, 0.5 mL NH_3.R1 and 0.5 mL NH_3.R2 reagent were added to a 5 mL sample and the mixture vortexed. Each reaction was carried out in a 12-mL test tube, which was sealed after mixing and left in

the dark between 22 to 27 °C for at least one hour for colour development. Absorbance intensity of the indophenol was measured at 635 nm. The final concentration of the sample was calculated using equation 11, which was derived from the calibration data (Figure 3.1, Table 8).

$$x = \frac{Abs_{635} - 0.0331}{1.1433} \quad (11)$$

where x is the concentration (mg L^{-1}), Abs_{635} the absorbance of the sample at 635 nm, 1.1433 the slope of the line of best fit, and 0.0331 the intercept of the line.

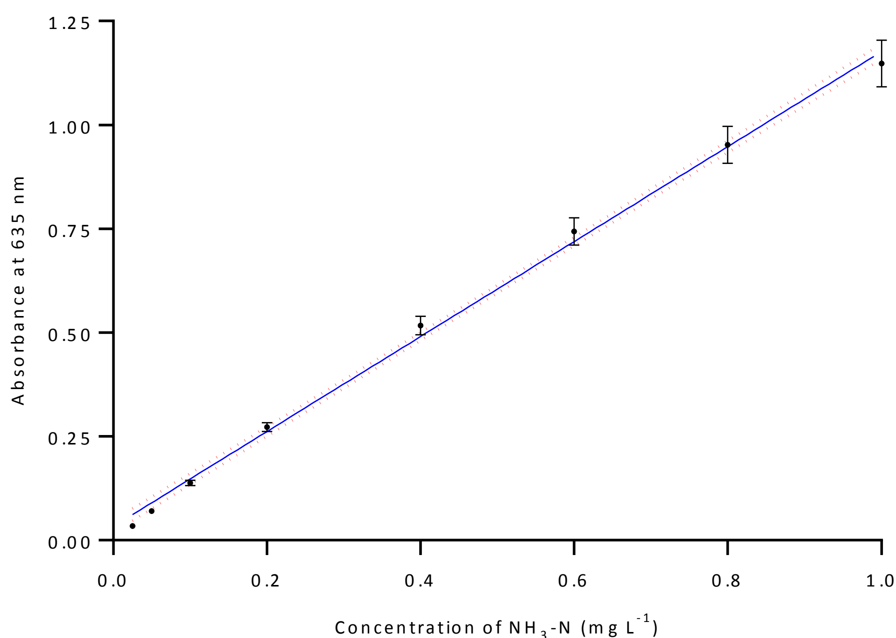


Figure 3.1 – Ammonia-Nitrogen calibration graph. Absorbance readings are mean ±SD, n = 14 (4 for concentration 0.025 mg L^{-1} NH_3-N) independent samples for each concentration. The dotted line represents the ±99% confidence interval for the regression line.

Table 8 – Ammonia calibration data

Definition	Value
Number of samples, *n*	102
Regression coefficient, *R*	0.9957
Slope (*m*) ± standard error (SD_m)	1.1433 ± 0.0106
Intercept (*c*) ± standard error (SD_c)	0.0331 ± 0.0058
Residual standard deviation, $SD_{y/x}$	0.0364
t_{CRI}	107.87
t_{CAL}	2.6259
Linearity significant	Yes
Measurement LOD (Abs at 635nm)	0.142
Concentration LOD (mg L^{-1})	0.096

3.3.4 Nitrite - Nitrogen

Nitrite was determined by the Diazotization reaction described in method SM 4500-NO_2^-.B in which an azo dye is formed in proportion to the amount of NO_2 present. Nitrite reacts with sulphanilamide at pH 2 to 2.5 to form a diazonium cation which couples with N-(1-napthyl)-ethylenediamine dihydrochloride (NED dihydrochloride) to produce a red-purple azo dye (modified Griess reaction) [383]. The reagents for this reaction were prepared as listed in Table 9 and were stable for one month when stored in the dark at 4°C.

Calibration standards were prepared from a 100 mg L^{-1} NO_2-N stock standard, which was made using sodium nitrite ($NaNO_2$). In a 1 L volumetric flask, 0.49243 g $NaNO_2$ was added to approximately 200 mL deionised water and, once dissolved, the remaining volume made up to 1 L. The stock standard was stored at 4°C and was stable for one week. A working 1 mg L^{-1} NO_2-N standard was made by diluting 1 mL of the stock standard in a final volume of 100 mL deionised water. Calibration standards in the range of 0.6 to 0.0125 mg L^{-1} NO_2-N were diluted from this solution.

Table 9 – Nitrite reagents

Reagent	Chemical	Formula	In 100 mL
NO_2.R1	Sulphanilamide	$H_2NC_6H_4SO_2NH_2$	1 g
	Hydrochloric acid	HCl (37%)	10 mL
NO_2.R2	NED dihydrochloride	$C_{10}H_7NHCH_2CH_2NH_2.2HCl$	1 g

For analysis, 0.1 mL NO_2.R1 and 0.1 mL NO_2.R2 reagent were added to a 5 mL sample and the mixture vortexed. Colour development was left to proceed for a minimum of 20 minutes at room temperature. Absorbance intensity of the azo dye was measured at 543 nm. The final concentration of the sample was calculated using equation 12, which was derived from the calibration data (Figure 3.2 and Table 10).

$$x = \frac{Abs_{543} - 0.0191}{3.3378} \qquad (12)$$

where x is the concentration (mg L^{-1}), Abs_{543} the absorbance of the sample at 543 nm, 3.3378 the slope of the line of best fit, and 0.0191 the intercept of the line.

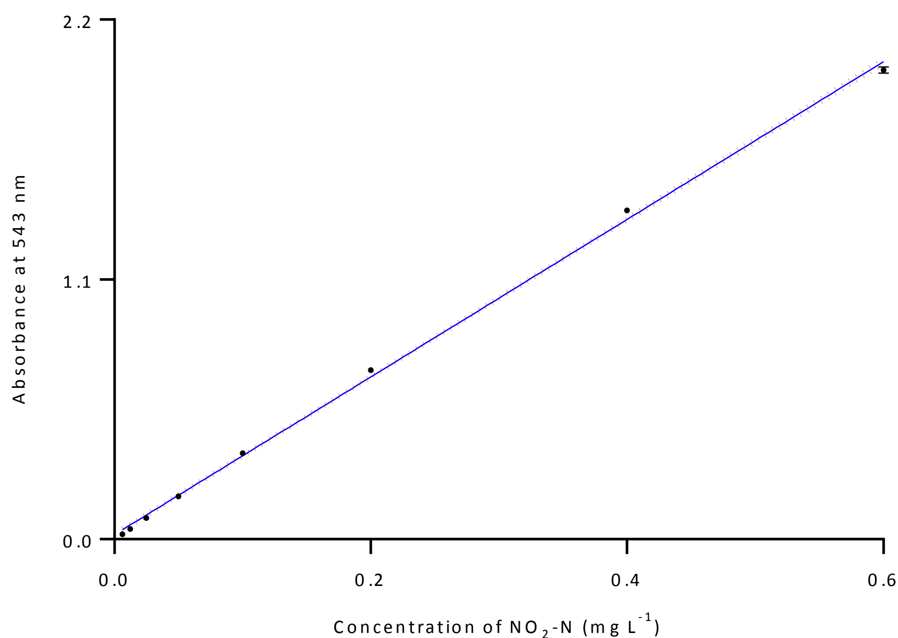


Figure 3.2 – Nitrite-Nitrogen calibration graph. Absorbance readings are mean ±SD, n = 14 for all concentration with the exception of 0.0125 mg L^{-1} NO_2-N where n = 9, independent samples for each concentration. The dotted line represents the ±99% confidence interval for the regression line.

Table 10 – Nitrite calibration data

Definition	Value
Number of samples, *n*	93
Regression coefficient, *R*	0.9993
Slope (*m*) ± standard error (SD_m)	3.3378 ± 0.0128
Intercept (*c*) ± standard error (SD_c)	0.0191 ± 0.0038
Residual standard deviation, $SD_{y/x}$	0.0266
t_{CRI}	2.631
t_{CAL}	260.88
Linearity significant	Yes
Measurement LOD (Abs at 543 nm)	0.099
Concentration LOD (mg L^{-1})	0.024

3.3.5 Nitrate - Nitrogen

Nitrate was determined by the formation of an azo dye using the reserved Hydrazine reduction reaction method SM 4500-NO_3^-.G. Nitrate is reduced to NO_2 by hydrazine sulphate catalysed by copper ions [383, 384]. Zinc ions are present to limit the formation and precipitation of copper oxide or hydroxide in the alkaline condition of the reaction, and to minimise the copper complexing with organic matter. The formed nitrite is then determined following the diazotization reaction through the addition of a colour reagent. The determined concentration of nitrate in the sample includes the fraction of nitrite which was present before reduction. To discriminate between the two compounds, the nitrate concentration in the sample is calculated as follows (Equation 13):

$$[NO_3\text{-}N]_A = [NO_3\text{-}N]_I - [NO_2\text{-}N] \qquad (13)$$

where $[NO_3\text{-}N]_A$ is the actual nitrate concentration, $[NO_3\text{-}N]_I$ is the measured concentration, and $[NO_2\text{-}N]$ the nitrite concentration measured directly from the Diazotization method in section 3.3.5. The reagents for the reducing reaction were prepared as listed in table 11, which were stable at room temperature, and the colour reagent listed in table 12, stable for one month when stored in the dark at 4°C.

Calibration standards were prepared from a commercial 100 mg L^{-1} NO_3-N stock standard (Hach, UK). A working 1 mg L^{-1} NO_3-N standard was made by diluting 1 mL of the stock standard in a final volume of 100 mL deionised water. Calibration standards in the range of 1 to 0.05 mg L^{-1} NO_3-N were diluted from this solution.

Table 11 –Reducing reagents

Reagents	Chemical	Formula	In 100 mL
NO_3.R1	Hydrazine sulphate	$NH_2NH_2.H_2SO_4$	2.7 g
NO_3.R2	Copper sulphate	$CuSO_4.5H_2O$	0.25 g
NO_3.R3	Zinc sulphate	$ZnSO_4.7H_2O$	5.3 g
NO_3.R4	Sodium hydroxide	$NaOH$	10 g

Table 12 –Colour reagent

Reagent	Chemical	Formula	In 100 mL
Colour reagent	Orthophosphoric acid	H_3PO_4 (>85%)	20 mL
	Sulphanilamide	$H_2NC_6H_4SO_2NH_2$	1 g
	NED dihydrochloride	$C_{10}H_7NHCH_2CH_2NH_2.2HCl$	0.08 g

The reducing reagent was prepared fresh when required. In a 20-mL test tube, 13.7 mL deionised water, 5 mL NO_3.R1, 0.750 mL NO_3.R2 and 0.550 mL NO_3.R3 were thoroughly mixed. The solution was kept sealed until subsequent use in order to minimise hydrazine loss by oxidation. For analysis, 0.730 mL

NO_3.R4 reagent was added to a 5 mL sample and vortexed, followed by the addition of 0.420 mL reducing reagent. The mixture was then vortexed and the reaction left standing to proceed for 10 minutes. A 0.730 mL volume of the combined colour reagent was then added and the sample vortexed again. Colour development was left to proceed for a minimum of 30 minutes. Absorbance intensity of the azo dye was measured at 535 nm. The final concentration of the sample was calculated using equation 14, which was derived from the calibration data (Figure 3.3 and Table 13).

$$x = \frac{Abs_{535}+0.0297}{0.9579} \qquad (14)$$

where x is the concentration (mg L^{-1}), Abs_{535} the absorbance of the sample at 535 nm, 0.9579 the slope of the line of best fit, and 0.0297 the intercept of the line.

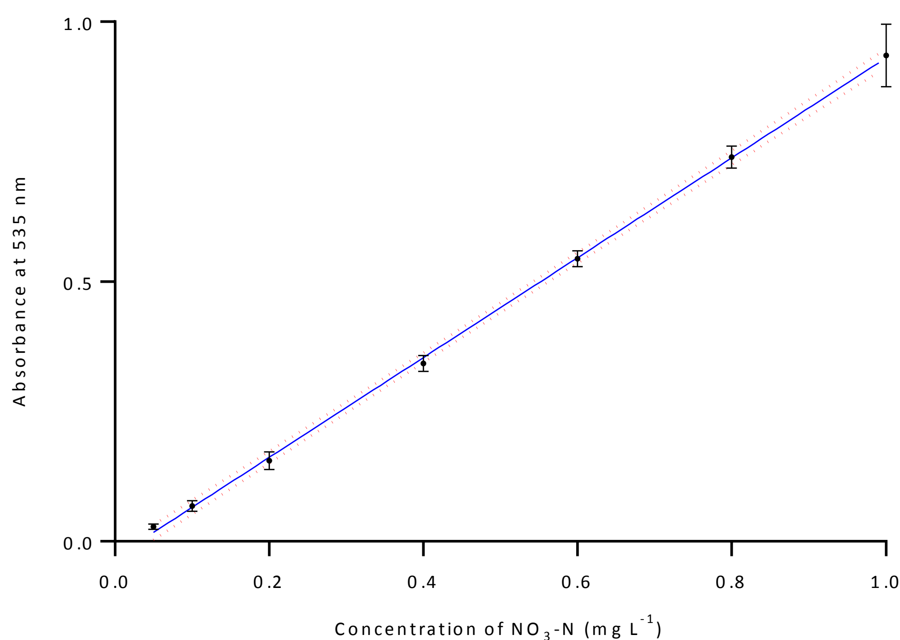


Figure 3.3 – Nitrate-Nitrogen calibration graph. Absorbance readings are mean ±SD, n = 10 for all concentration with the exception of 0.05 mg L^{-1} NO_3-N where n = 9, independent samples for each concentration. The dotted line represents the ±99% confidence interval for the regression line.

Table 13 – Nitrate calibration data

Definition	Value
Number of samples, *n*	69
Regression coefficient, *R*	0.9972
Slope (*m*) ± standard error (SD_m)	0.9579 ± 0.0088
Intercept (*c*) ± standard error (SD_c)	-0.0297 ± 0.0029
Residual standard deviation, $SD_{y/x}$	0.0147
t_{CRI}	2.6512
t_{CAL}	109.15
Linearity significant	Yes
Measurement LOD (Abs at 535 nm)	0.014
Concentration LOD (mg L^{-1})	0.046

3.3.6 Total Nitrogen

Total nitrogen includes the organic and inorganic bound N. Analysis was performed using Hach test kit *LCK238*, following the manufacture's guidelines with readings recorded on a DR1900 spectrophotometer (Hach, Loveland, CO, USA).

3.3.7 Phosphate - Phosphorus

Phosphate was determined by the Ascorbic acid reaction method SM 4500-P.E, which without any preliminary steps determines SRP. The concentration of SRP is measured based on the intensity of the formed phosphomolybdenum blue complex. The reaction proceeds with ammonium molybdate reacting with orthophosphate in acid conditions (pH <2) as catalysed by potassium antimony tartrate to form 12-phosphomolybdic acid (McKelvie *et al.*, 1995). The complex is reduced by ascorbic acid to an intensely blue coloured phosphomolybdenum complex. The reagents for this reaction were prepared as listed in table 14 and were stable for one month at room temperature.

Calibration standards were prepared from a 150 mg L^{-1} PO_4-P stock standard, which was made using potassium dihydrogen orthophosphate (KH_2PO_4). KH_2PO_4 was first oven dried at 105°C overnight and cooled in a desiccator. In a 1 L volumetric flask, 0.65913 g KH_2PO_4 was added to approximately 200 mL deionised water and, once dissolved, the remaining volume made up to 1 L. The stock standard was stable for 1 week when stored at 4°C. A working 1.5 mg L^{-1} PO_4-P standard was made by diluting 1 mL of the stock standard in a final volume of 100 mL deionised water. Calibration standards in the range of 1.2 to 0.0375 mg L^{-1} PO_4-P were diluted from this solution.

Table 14 – Phosphate reagents

Reagents	Chemical	Formula	In 100 mL
PO_4.R1	Ammonium molybdate	$(NH_4)_6Mo_7O_{24}.4H_2O$	4 g
PO_4.R2	Potassium antimony tartrate	$C_8H_4K_2O_{12}Sb_2.3H_2O$	0.274 g
PO_4.R3	Sulphuric acid	H_2SO_4 (>95%)	14 mL

The colour reagent was only stable for a maximum of 4 hours and therefore prepared fresh when required. In a 50-mL Falcon tube, 9 mL deionised water, 4.5 mL PO_4.R1, 1.5 mL PO_4.R2, 15 mL PO_4.R3 and 0.180 g ascorbic acid ($C_6H_8O_6$) were mixed. For analysis, 1 mL colour reagent was added to a 5 mL sample and vortexed. Colour development was left to proceed for 30 minutes. Absorbance intensity was measured at 882 nm. The final concentration of the sample was calculated using equation 15, which was derived from the calibration data (Figure 3.4 and Table 15).

$$x = \frac{Abs_{882}+0.0105}{0.5523} \quad (15)$$

where x is the concentration (mg L^{-1}), Abs_{882} the absorbance of the sample at 882 nm, 0.5523 the slope of the line of best fit, and 0.0105 the intercept of the line.

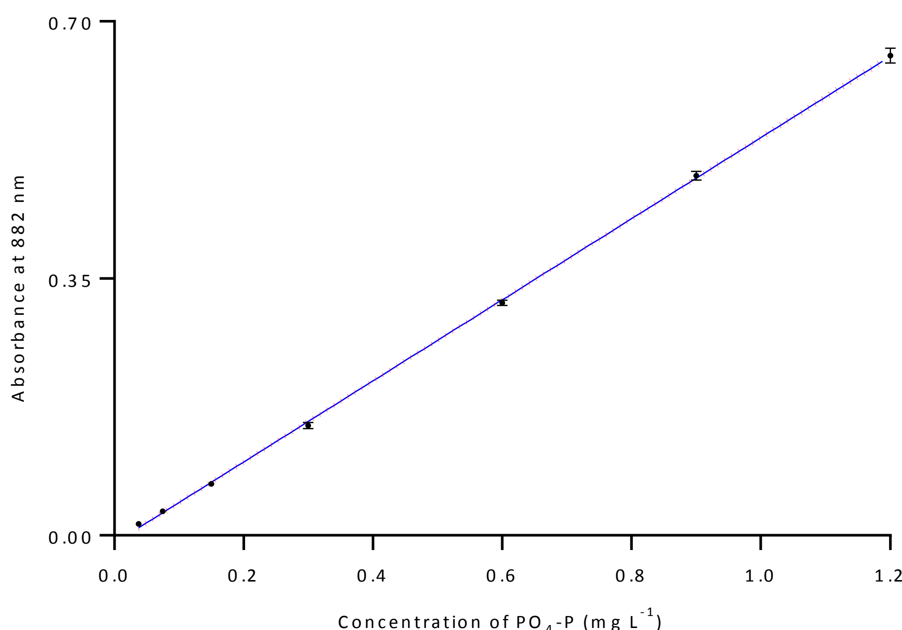


Figure 3.4 – Phosphate-Phosphorous calibration graph. Absorbance readings are mean ±SD, n = 17 independent samples for each concentration. The dotted line represents the ±99% confidence interval for the regression line.

Table 15 – Phosphate calibration data

Definition	Value
Number of samples, *n*	119
Regression coefficient, *R*	0.9996
Slope (*m*) ± standard error (SD_m)	0.5523 ± 0.0014
Intercept (*c*) ± standard error (SD_c)	-0.0105 ± 0.0001
Residual standard deviation, $SD_{y/x}$	0.0063
t_{CRI}	2.6185
t_{CAL}	398.66
Linearity significant	Yes
Measurement LOD (Abs at 882 nm)	0.008
Concentration LOD (mg L^{-1})	0.034

3.3.8 Total Phosphorus

Total phosphorus includes the organic and inorganic bound P in the sample which is measured as SRP once digested with potassium persulphate ($K_2S_2O_8$). To a 16 mm Ø borosilicate glass tube, 0.1 g $K_2S_2O_8$ was added followed by 5 mL sample and 0.1 mL 30% v/v H_2SO_4 (made from >98% H_2SO_4). The tube was sealed with Teflon lined caps and the sample digested at 148°C for 30 minutes. Once cooled, 0.4 mL 10% w/v NaOH solution was added and the sample vortexed. Thereafter, SRP was analysed in the sample by the Ascorbic acid method detailed in section 3.3.8. Calibration standards for TP were prepared in the same way as described for SRP in section 3.3.8. The final concentration of the sample was calculated using equation 16, which was derived from the calibration data (Figure 3.5 and Table 16).

$$x = \frac{Abs_{882} + 0.0059}{0.4895} \qquad (16)$$

where x the concentration (mg L^{-1}), Abs_{882} the absorbance of the sample at 882nm, 0.4895 the slope of the line of best fit, and 0.0059 the intercept of the line.

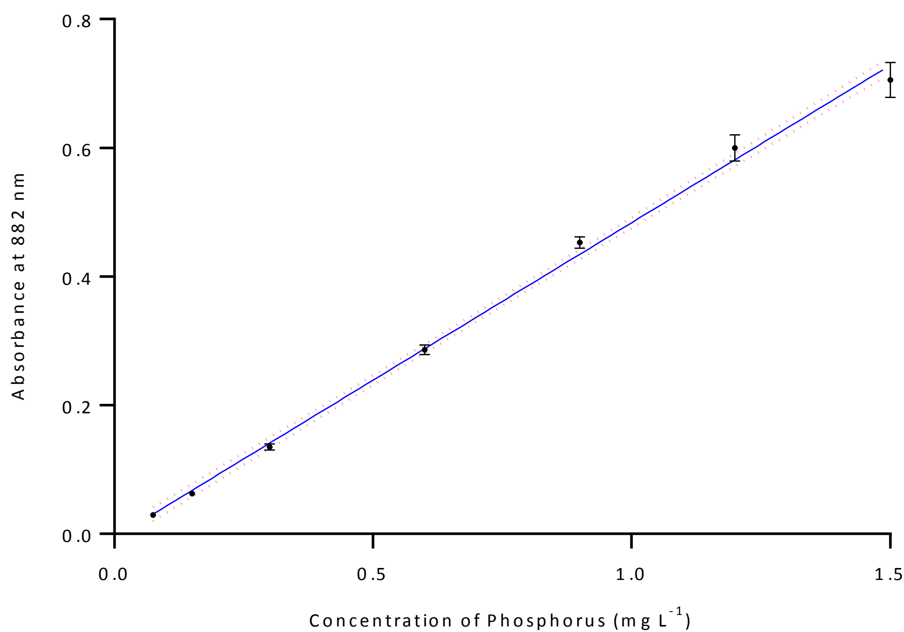


Figure 3.5 – Total phosphorus calibration graph. Absorbance readings are mean ±SD, n = 8 independent samples for each concentration. The dotted line represents the ±99% confidence interval for the regression line.

Table 16 – Total phosphorus calibration data

Definition	Value
Number of samples, *n*	55
Regression coefficient, *R*	0.9971
Slope (*m*) ± standard error (SD_m)	0.4895 ± 0.0051
Intercept (*c*) ± standard error (SD_c)	-0.0059 ± 0.0043
Residual standard deviation, $SD_{y/x}$	0.0192
t_{CRI}	2.6718
t_{CAL}	96.04
Linearity significant	Yes
Measurement LOD (Abs at 543 nm)	0.052
Concentration LOD (mg L^{-1})	0.118

3.4 Total carbohydrate analysis

The amount of carbohydrate in the experimental sample was quantified using the phenol-sulphuric acid method of DuBois *et al.*, (1956). Samples were centrifuged (15,000x*g*; 5 min) prior to analysis. Briefly, 0.5 mL samples were each mixed with 0.25 mL of 5% w/v phenol solution in a test tube, then 1.5 mL of >98% sulphuric acid was added. The mixture was vortexed vigorously and then allowed to stand for 10 minutes prior to spectrophotometric measurement at 490 nm. Each day the analysis was performed, a standards using D-glucose between the ranges of 10 to 100 mg L^{-1} were included. The final concentration of the sample was calculated using equation 17, which was derived from the calibration data (Figure 3.6 and Table 17).

$$x = \frac{Abs_{490} - 0.0098}{0.0124} \quad (17)$$

where x the concentration (mg L^{-1}), Abs_{490} the absorbance of the sample at 490 nm, 0.0124 the slope of the line of best fit, and 0.0098 the intercept of the line.

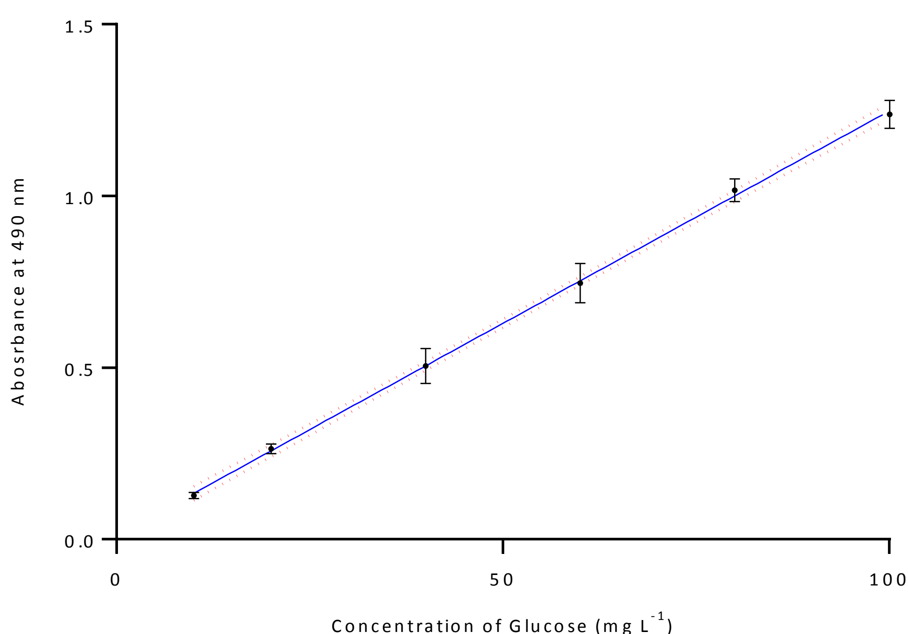


Figure 3.6 – Total carbohydrate calibration graph. Absorbance readings are mean ±SD, n = 10 independent samples for each concentration. The dotted line represents the ±99% confidence interval for the regression line.

Table 17 – Total carbohydrate calibration data

Definition	Value
Number of samples, *n*	60
Regression coefficient, *R*	0.9953
Slope (*m*) ± standard error (SD_m)	0.0124 ± 0.0002
Intercept (*c*) ± standard error (SD_c)	0.0098 ± 0.0096
Residual standard deviation, $SD_{y/x}$	0.0392
t_{CRI}	2.6633
t_{CAL}	77.91
Linearity significant	Yes
Measurement LOD (Abs at 543 nm)	0.127
Concentration LOD (mg L^{-1})	9.467

3.5 Chemical Oxygen Demand

Chemical oxygen demand measures the oxidation potential of a sample expressed in terms of oxygen equivalence as mg L^{-1} O_2, which is the mass of O_2 consumed per L of solution. The COD test is an empirical test used as an index of municipal waste pollution, thus making it an important parameter in wastewater treatment. It is an indication of how stable a particular waste solution is in terms of whether it will exert a harmful O_2-demand on the environment it is released into. Additionally, since it is a chemical reaction it is not subject to inhibition by toxic compounds that are known to affect tests that are biochemically based, such as for example the biological oxygen demand test.

Under high temperature and acidic conditions, a known excess quantity of the oxidant dichromate ($Cr_2O_7^{2-}$) reacts with the sample matter, thereby becoming reduced to chromic acid (Cr^{3+}). The stoichiometry of the reaction allows for the quantity of unreduced dichromate to be determined by titration with a known concentration of iron (II) ammonium sulphate solution, thus the amount of oxidant consumed to be quantified providing the O_2-demand of the material in the sample. Both organic and inorganic compounds in a sample are susceptible to oxidation, although appreciably organic material is the predominant substrate of the reaction.

The analysis was performed using the mercury-free, small-scale reflux digestion procedure described and approved by the [387]. The choosing of this procedure was to avoid exposure to mercury (II) sulphate, which is used in the Standard Methods for the Examination of Water and Wastewater because of its toxic nature [379]. In the mercury-free method, chromium (III) potassium sulphate and an excess of silver nitrate are added as substitutes to suppress chlorine and ammonium ions, which interfere with the reaction. The reagents for the procedure are listed in table 18.

Table 18 – Chemical oxygen demand reagents

Reagent	Chemical	Formula	Concentration[3] (g L^{-1})
COD.R1	Silver nitrate	$AgNO_3$	1200
COD.R2	Chromium (III) potassium sulphate	$KCr(SO_4)_2.12H_2O$	250
COD.R3	Iron (II) ammonium sulphate	$(NH_4)_2Fe(SO_4)_2.6H_2O$	9.8
COD.R4	Potassium dichromate	$K_2Cr_2O_7$	6.129
COD.R5	Silver sulphate in sulphuric acid (commercial)	Ag_2SO_4, >98% acid	10 (in acid)

[3]Reagents were made in various volumes to the stated concentration.

Four blank samples of deionised water and standards (in triplicate) were prepared and digested in parallel when analysing any experimental sample to verify the reagents and procedure of the method. Glucose was used as the reference material and its theoretical COD (COD_t) calculated by equation 18:

$$COD_t,\ C_xH_yO_z = 8(4a + b - 2c)/(12a + b + 16c) \tag{18}$$

where a is the number of carbon atoms, b the number of hydrogen atoms, and c the number of oxygen atoms in the organic compound. Thus, 1 g L^{-1} of glucose ($C_6H_{12}O_6$) solution has a COD_t of 1.067 g L^{-1} O_2 when completely oxidised. Prior to use with samples, a series of standards (50, 100, 200 and 300 mg L^{-1} O_2) diluted from 1000 mg L^{-1} O_2 stock standard (0.93720 g L^{-1} glucose, previously dried at 105°C) were run to establish the method (Figure 3.7). For each digestion, a fresh COD stock standard was made. The range of detection for the method was between 9 to 400 mg L^{-1} O_2, with 1 mL 0.025 M iron (II) ammonium sulphate solution corresponding to 100 mg L^{-1} O_2.

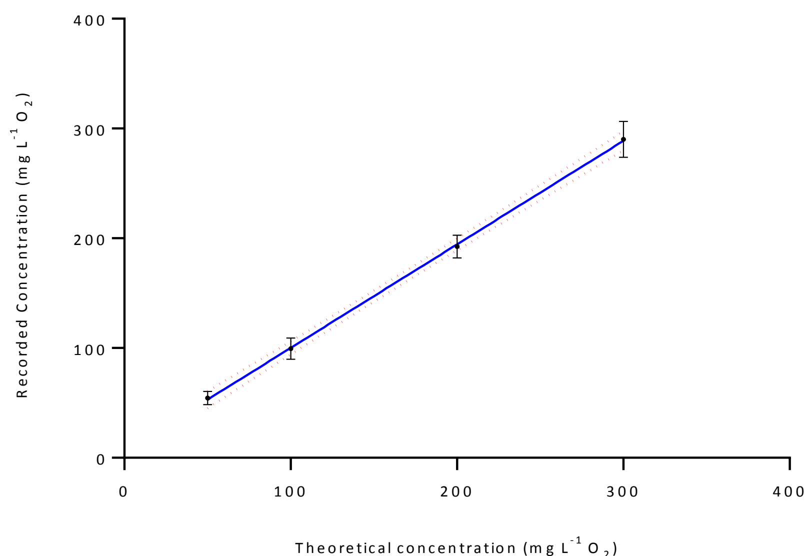


Figure 3.7 – Theoretical Chemical Oxygen Demand (x-axis) plotted against the experimental Chemical Oxygen Demand concentration (y-axis) from the method. Values are mean ±SD; n = 9 for each concentration; *R* = 0.9999. The dotted line represents the ±99% confidence interval for the regression line.

All experimental samples were filtered through a non-sterile 0.45 µm cellulose acetate filter (Whatman International Ltd., UK) prior to digestion in order to analyse for the soluble oxidising fractions only (COD_S). For analysis, a 2 mL sample was mixed with 0.1 mL COD.R1, placed into a digestion tube (16 mm Ø, borosilicate glass with Teflon caps), and left to stand for two minutes. The following was then carried out in a fume hood: to the digestion tube, 0.1 mL COD.R2 was added followed with 1 mL COD.R4 and 3 mL COD.R5. The digestion tube was gently vortexed for 30 seconds allowing any evolved gas to escape through the loose cap. With caps sealed, samples and blanks were placed in a COD heating block at 148°C for 2 hours.

Once cold, a sample (or blank) was poured into a 50-mL conical flask. In order to recover all of the sample volume in the tube, 10 mL deionised water was added to the digestion tube, capped, shaken and the solution added to the conical flask. One drop (using a glass Pasteur pipette) of Ferroin indicator was added and the sample titrated with 0.025 M iron (II) ammonium sulphate solution (i.e. COD.R3). The colour of Ferroin indicator changes sharply from blue-green (Fe^{2+}) to copper-red (Fe^{3+}) at the end point of the titration. The residual dichromate in the analyte was dependent on the volume of titrant necessary for the colour change, allowing the COD to be calculated using equation 19.

$$\text{COD as mg L}^{-1}\text{ O}_2 = (4000M(V_b - V_s))DF \tag{19}$$

where V_b is the volume (mL) of titrant reacted with the blank samples, V_s the volume of titrant reacted with the samples, 4000 a constant, DF the dilution factor, and M the molarity of the standardised iron (II) ammonium sulphate solution.

For the standardisation of the iron (II) ammonium sulphate solution, 10 mL of >98% sulphuric acid was added to 50 mL of deionised water in a 200-mL conical flask. The mixture was left to cool prior to addition of 5 mL of 0.02083 M potassium dichromate (COD.R4 reagent) and two drops of Ferroin indicator. The solution was titrated with the iron (II) ammonium sulphate solution (COD.R3) and the volume necessary to reach the end point recorded. The molarity of the iron (II) ammonium sulphate solution was calculated by equation 20, where V is the volume used.

$$M = 5/(8V) \tag{20}$$

3.6 Cleaning procedure

All glass necessary for the analysis of inorganics, COD and total carbohydrate was acid washed prior to use. For the analysis of inorganics and total carbohydrate, all glassware was washed in a 50% v/v HCl solution (made from 37% HCl), and borosilicate tubes for COD were washed in a 50% v/v HNO_3 solution (made from 70% HNO_3). For this, all glassware was submerged in the respective acid solution, allowed to stand for 30 minutes and then rinsed 3 times with deionised water. Sampling bottles for wastewater were washed with 1% v/v HCl for 30 minutes and rinsed with deionised water.

3.7 Total suspended solids

Whatman GF/C filters (Ø 25 mm, pore size = 1.2 µm, Whatman International, Ltd, UK) were used to determine the biomass dry weight as total suspended solids (TSS). Prior to use, each filter was washed and dried at 105°C for a minimum of 6 hours, and then placed in a desiccator to cool before being weighed. For sample analysis, a filter was placed onto the filtration unit and pre-wetted with deionised water. A recorded volume of sample was added under a constant vacuum. The filter was rinsed with deionised water, dried (105°C) and allowed to cool before being weighed. Each sample was measured in triplicate. The biomass dry weight was calculated using equation 21, which determined the difference between the final weight (W_F) and initial weight (W_I) of the filter and concentration recorded as g L^{-1}.

$$\text{TSS g L}^{-1} = \left((W_F - W_I) \times 1000\right) / \textit{Sample volume}\ (\text{mL}) \qquad (21)$$

3.8 Microalgae biomass dry-weight measurement

Each experiment was inoculated with a microalgae biomass dry weight of 0.1 g L^{-1}. A calibration graph of known weight concentrations versus their respective absorbance measurements was plotted for each micro-algal species. The biomass dry weight was determined following the procedure outlined in section 2.7. The calibration graphs are shown in Figure 3.8, 3.9 and 3.10, and calibration data in Table 19, 20 and 21, for *C. vulgaris*, *H. riparia* and *A. obliquus*, respectively.

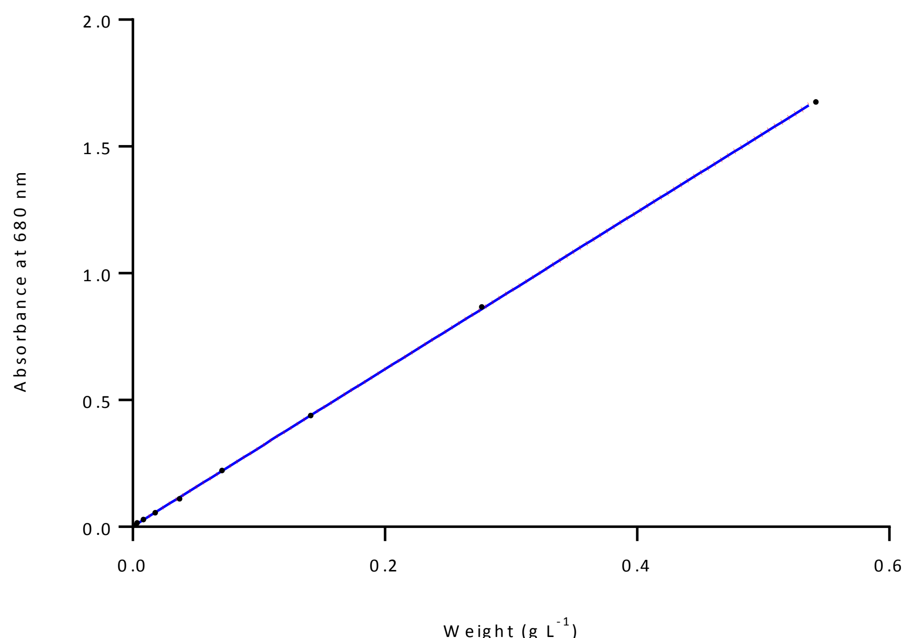


Figure 3.8 – *Chlorella vulgaris* calibration graph. Absorbance readings are mean ±SD, n = 18. The dotted line represents the ±99% confidence interval for the regression line.

Table 19 – *C. vulgaris* calibration data

Definition	Value
Number of samples, *n*	18
Regression coefficient, *R*	0.9997
Slope (*m*) ± standard error (SD_m)	3.0961 ± 0.0203
Intercept (*c*) ± standard error (SD_c)	0.0021 ± 0.0043
Residual standard deviation, $SD_{y/x}$	0.0147
t_{CRI}	2.9208
t_{CAL}	152.38
Linearity significant	Yes
Measurement LOD (Abs at 543 nm)	0.046
Concentration LOD (mg L^{-1})	0.014

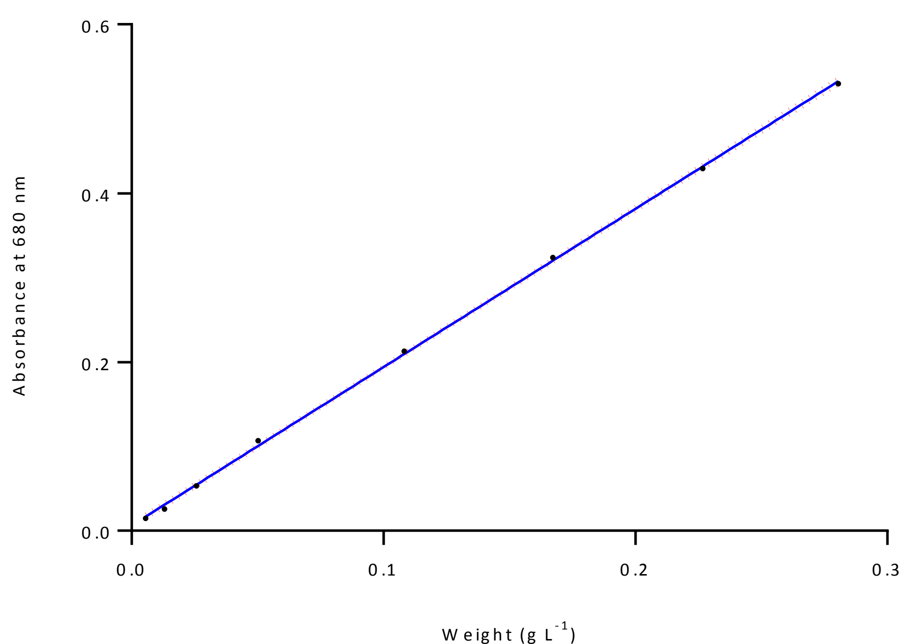


Figure 3.9 – *Heynigia riparia* calibration graph. Absorbance readings are mean ±SD, n = 24. The dotted line represents the ±99% confidence interval for the regression line.

Table 20 – *H. riparia* calibration data

Definition	Value
Number of samples, *n*	24
Regression coefficient, *R*	0.9989
Slope (*m*) ± standard error (SD_m)	1.8729 ± 0.0185
Intercept (*c*) ± standard error (SD_c)	0.0066 ± 0.0027
Residual standard deviation, $SD_{y/x}$	0.0089
t_{CRI}	2.8188
t_{CAL}	100.98
Linearity significant	Yes
Measurement LOD (Abs at 543 nm)	0.033
Concentration LOD (mg L^{-1})	0.014

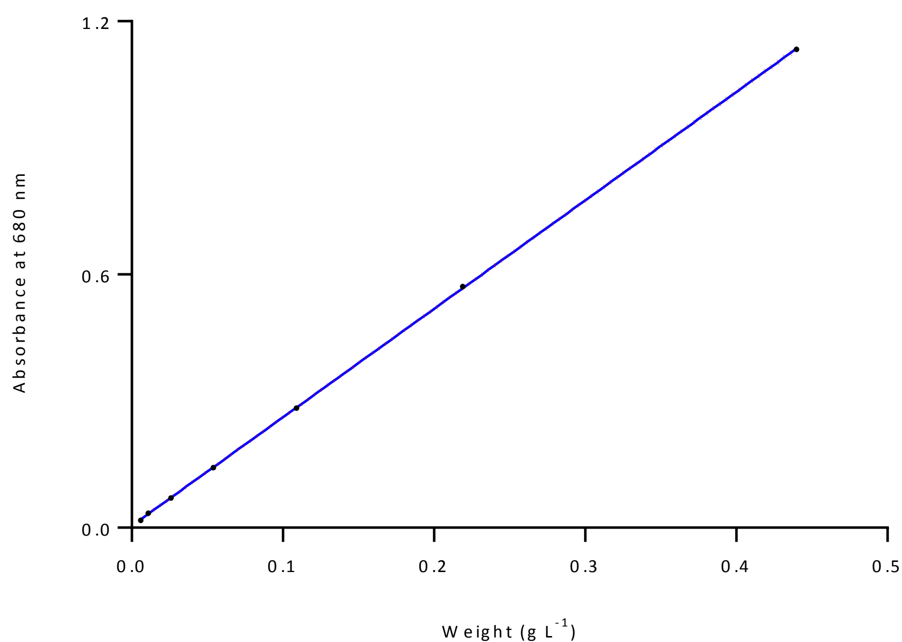


Figure 3.10 – *Acutodesmus obliquus* calibration graph. Absorbance readings are mean ±SD, n = 24. The dotted line represents the ±99% confidence interval for the regression line.

Table 21 – *A. obliquus* calibration data

Definition	Value
Number of samples, *n*	21
Regression coefficient, *R*	0.9998
Slope (*m*) ± standard error (SD_m)	2.5682 ± 0.0127
Intercept (*c*) ± standard error (SD_c)	0.0048 ± 0.0024
Residual standard deviation, $SD_{y/x}$	0.0085
t_{CRI}	2.8609
t_{CAL}	202.53
Linearity significant	Yes
Measurement LOD (Abs at 543 nm)	0.03
Concentration LOD (mg L^{-1})	0.01

3.9 Cell counts

The concentration of microalgal cells in liquid was determined by direct counting using a Neubauer improved haemocytometer with a depth of 0.1 mm. Samples were agitated to ensure the microalgae were homogenous prior to taking an aliquot and transferring to a Micro tube (1.5 mL). When necessary, the samples were diluted with deionised water to obtain a cell concentration range that could be counted. To each cell suspension used for counting, Lugols solution (to 0.1% v/v final concentration) was added and the mixture allowed to stand for approximately one hour. The treated suspensions were then thoroughly mixed and the cells counted and concentrations recorded as cells mL^{-1}.

$$\text{Cell mL}^{-1} = (x\ 10000)DF \quad (22)$$

where x the number of cells counted per square (1 mm x 1 mm), 10,000 the conversion factor from mm^3 to mL, and DF the dilution factor.

3.10 pH

The pH was measured with a HI1230 pH probe and HI8424 meter (Hanna Instruments Inc., UK). Prior to use, the apparatus was calibrated with pH 7.02 (HI7007) and 10.02 (HI7010) commercial buffers. Sample measurements were performed in the same tube that the sample was collected; the electrode probe was rinsed with distilled water before and between measurements. Under constant gentle mixing, readings were taken after they were observed to have stabilised.

3.11 Dissolved Oxygen

Dissolved oxygen (DO) was measured with the luminescent DO probe, LDO101 and HD40q meter (Hach, UK) with results reported as mg L^{-1} O_2. Measurements were performed in the same tube that the sample was collected; with the probe rinsed with deionised water before and between measurements. Under constant gentle mixing, readings were taken after they were observed to have stabilised.

The probe was calibrated in 100% water-saturated air. This was performed by filling a BOD bottle with approximately 1/3 distilled water, shaking it for 30 seconds to saturate the entrapped air and allowing the contents to equilibrate for 30 minutes. The bottle stopper was removed and the probe inserted to the centre of the bottle and allowed to calibrate following the meter's displayed guide. The meter automatically adjusts for barometric pressure and temperature, while conductivity was entered manually.

Chapter 4 – Preliminary evaluation of microalga to treat settled municipal wastewater effluent

4.1 Introduction

Microalgae are ubiquitous to wastewater environments indicating that the nutrient concentration and composition as a suitable medium for growth [129, 388]. Despite their abundance, however, experimental evidence from microalgae wastewater treatment studies reported in the literature highlight an extreme variation in COD, N and P remediation between studies (see references in Table 4). The main factors affecting treatment performance are reported to be the choice of microalgal species, wastewater composition and cultivation conditions. In this context, selection of a microalgal species for wastewater treatment from the literature for another wastewater source cannot be made conclusively on the reported values alone because of the variable nature of the wastewater between studies and cultivation conditions. Even wastewater from the same treatment plant will vary between seasons, let alone from one day to the next.

Several studies to date have screened a vast number of microalgae for their wastewater remediation potential and growth in different municipal wastewater streams [214, 389–393]. Microalgae belonging to the genera *Chlorella* and *Scenedesmus* are described as the best performing species because of their consistent high N and P remediation efficiencies and growth rate. The reasons for the dominance of these genera in wastewater treatment has been accredited to their robustness, tolerance and quick acclimation under varying environmental factors (i.e. temperature, pH, microbial community etc.) compared to other species, but also the ease in which they are cultured [390, 394, 395]. However, both *Chlorella* and *Scenedesmus* genera are extremely diverse with certain species exhibiting a better wastewater treatment performance than others. For example, Bohutskyi et al., (2015) [393] reported on the TN and TP removal efficiency and growth of multiple microalgae species in municipal wastewater from different streams (i.e. primary, secondary and centrate), including 14 *Chlorella* spp. and 4 *Scenedesmus* spp. The results of this study demonstrated the suitability of the strains *C. sorokiniana* CCTCC M209220, *C. sorokiniana* BRWWTP001 and *Scenedesmus alternans* UTEX B72 only in treating municipal wastewater. These algal strains exhibited a higher biomass productivity and TN and TP removal efficiency compared to the other strains assessed, with some *Chlorella* and *Scenedesmus* strains exhibiting no growth or change in TN and TP concentration. The enhanced performance of these types of wastewater autochthonous microalgae has been suggested to be related to the existence of certain genetic traits in regards to acclimation response, growth, cell wall composition as well as tolerance to, and production of, xenobiotic substances (i.e. chloreline) [393, 394, 396]. Some studies have even demonstrated certain *Chlorella* sp. to have a higher tolerance to anionic detergents compared to species such as *Dunaliella* sp. [397, 398].

Wastewater composition naturally varies as a result of the location and natural environment [1]. In this context, it is critical to select a microalgae species which is tolerant (or acclimated) to the expected composition and environment of the wastewater, in particular to toxic and/or synthetic chemicals that

may potentially be present (e.g. heavy metals, herbicides, antibiotics etc.) [396, 399]. Furthermore, the cultivation conditions can significantly impact on the treatment performance, with a single microalgae species response varying according to the conditions under which it is grown. Environmental factors, including temperature, light-dark cycle and light intensity all have a significant influence on microalgal productivity, as well as affect cellular metabolism [68, 400, 401]. It is therefore necessary to ensure an appropriate environment for a given microalgae strain, especially considering each species (or strain) will have a unique optimum requirement. Additionally, the autochthonous microbial community of wastewater can negatively impact microalgae productivity and compete for organic and inorganic resources [402]. It is therefore essential for the cultivation conditions to promote microalgae growth to minimise any negative interference from other microorganisms present naturally in the wastewater (e.g. bacteria and fungi). In light of the reported factors, it is essential to systematically evaluate a microalgal species tolerance and treatment performance to a distinct wastewater source under the chosen cultivation conditions, to verify its ability to achieve an adequate level of treatment.

In the present study, the main objective was to assess the COD, NH_3-N and PO_4-P remediation efficiency and growth of the microalgal species *Chlorella vulgaris*, *Heynigia riparia* and *Acutodesmus obliquus* in experiments with municipal PSW, in view of selecting the most effective species for subsequent studies. The unicellular species *C. vulgaris* and *A. obliquus* were selected for this study because of their natural abundance in wastewater systems and the extensive research available pertaining to their use in wastewater treatment. The application of *H. riparia* for the purpose of bioremediation of wastewater has not been extensively assessed since its identification as a new genus in 2009 [403]. The main interest in this species lies in its formation of large colonies, which were qualitatively found to grow at a lower maximum density than *C. vulgaris* and *A. obliquus*, offering a potential solution to the issue of self-shading and reduced light penetration in bioreactors. Colonies of *H. riparia* are symmetrical, comprised of small spherical cells connected together (up to 64 cells) via mucilaginous stalks grow to total colony diameters of 56 to 68 µm [403]. All species chosen are facultative heterotrophs, which is an essential attribute in their metabolic utilisation and reduction of the organic carbon (i.e. carbonaceous material) in the wastewater [205, 404].

In order to identify a robust microalga strain for wastewater treatment, the three selected strains where inoculated in unsterilized PSW to evaluate their treatment efficiency and any effect the wastewater had on growth and treatment performance. Furthermore, this study evaluated whether the culture conditions were appropriate for promoting microalgal growth in view of being co-cultured in an environment containing bacteria and fungi; the microalgae were cultured as free-living suspended cultures at 15°C with a 12:12 light:dark cycle at 100 µE m^{-2} s^{-1}. In a laboratory setting, the COD, NH_3-N and PO_4-P removal efficiency for each species was investigated under aerated and static (non-aerated or mixed) cultivation conditions. The latter condition was performed in order to evaluate and identify any limitations this strategy may have on microalgal productivity and treatment performance in order to establish an energy-efficient and cost-effective microalgal treatment process compared to conventional

wastewater systems. These aerated and static cultivation experiments were run in parallel and the results on their performance compared for each of the three microalgal strains in order to determine the strain and optimum culturing environment for treating wastewater.

4.2 Materials and Methods

4.2.1 Experimental Set-up

The experimental set-up consisted of each microalga species cultivated in a separate batch of PSW. Whilst this was not ideal, this experimental design was used because of a limited available space in the incubation chamber. As such, only one microalgal species could be accommodated in the chamber and tested under static (non-aerated) and aerated conditions, including the corresponding controls. Each treatment and corresponding controls were performed in triplicate. The PSW physiochemical composition of each batch used in this study are summarised in Table 22, with PSW batch 1, 2 and 3 used to investigate the growth and treatment efficiency of *C. vulgaris*, *H. riparia* and *A. obliquus* respectively.

Table 22 – Composition of PSW of the individual batches

Parameter (mg L^{-1})	PSW composition		
	Batch 1	Batch 2	Batch 3
NH_3-N	30.5 ±0.4	28.9 ±0.3	32.3 ±0.05
PO_4-P	3.2 ±0.0	3.0 ±0.03	3.8 ±0.03
NO_3-N	0.7 ±0.0	<DL	<D.L
NO_2-N	0.2 ±0.0	<DL	<D.L
COD_S	106.7 ±4.2	145.0 ±0.1	156.6 ±2.7
TSS	22.8 ±1.4	63.3 ±0.3	57.3 ±2.1
pH	7.13	8.07	7.2
COD/NH_3-N/PO_4-P (g/g/g)	100/42/4	100/20/2	100/21/2

DL = detection limit

The experiments were conducted using 800 mL of PSW that was placed in 1 L borosilicate bottle. All experiments were inoculated with washed microalgae at a biomass dry weight concentration of 0.1 g L^{-1}. For this, a culture of microalgae grown on BBM (Section 3.2) was concentrated by centrifugation (3500g; 10 min) in 50 mL Falcon tubes and washed twice with 10 mL of the collected wastewater to remove residual nutrients. To ensure consistency in the inoculation density across the microalgae treatments within the wastewater sample, 5 litres of filtered PSW was transferred to a 5 L glass bottle and inoculated with the washed microalgae. This was mixed and divided between six 1 L borosilicate bottles. Although the different wastewater samples used in the experiments were filtered (30 µm) to control the turbidity, the natural microbial community of the wastewater was not eliminated, which would potentially contribute an influence upon the COD, N and P removal of the wastewater sample. To evaluate this, control samples of 800 mL PSW, without addition of microalgae, were set-up. In total, four treatments, each in triplicate were prepared and labelled as follows: Wastewater Control (WWC),

Wastewater with aeration (WW+Air), Wastewater with microalgae (WW+*C.v* or *A.o* or *H.r*) and Wastewater with aeration and microalgae (WW+Air+*C.v* or *A.o* or *H.r*).

4.2.2 Glassware, sampling and analysis

All glass bottles for the four treatments were capped with foam plugs and incubated for a period of 7 days. Before use, all glassware with the relevant syphoning and aeration tubes was autoclaved (121°C; 15 minutes). Atmospheric air was continuously supplied directly into the aerated samples through a sterile In-Line HEPA filter at a rate of 0.2 V/Vm. Liquid samples were withdrawn daily to measure the concentration of NH_3-N, PO_4-P, NO_3-N and NO_2-N, microalgal cell growth (cell mL^{-1}) and pH (described in Chapter 3, sections 3.3.4, 3.3.5, 3.3.6, 3.3.8, 3.9 and 3.10 respectively). Dry weight (as a proxy for biomass) and COD were measured on the initial and final day of the experiment only (Chapter 3, sections 3.7 and 3.5 respectively). The analysis of these two parameters was limited because the experimental design had to take into account a maximum final volume withdrawal of 10% of the initial volume from each bottle. The reason for this conservative maximum sampling volume was to prevent experimental interference to microalgal growth, particular in static treatments caused by a large variation in the illuminated surface to volume ratio. All treatments were briefly mixed (by swirling) prior to taking an aliquot to ensure a homogenous sample.

The average NH_3-N and PO_4-P removal rates were determined using equation 23:

$$R_i = (S_0 - S_i)/(t_i - t_0) \quad (23)$$

where R_i represents the substrate removal rate (NH_3-N or PO_4-P), S_0 the initial concentration, S_i the corresponding concentration at t_i which is the time at which the concentration of the inorganic compound was reduced to its lowest.

The percentage removal efficiency (*RE*) was calculated using equation 24:

$$RE\ (\%) = (S_0 - S_i)/S_0 * 100 \quad (24)$$

Specific growth rate of the microalgae was calculated using equation 25

$$\mu\ (d^{-1}) = \frac{ln N_T - ln N_O}{\Delta t} \quad (25)$$

where N_O is the cell concentration at the beginning of a time interval, N_T the cell concentration at the end of the time interval and Δt the length of the time interval in days.

4.2.3 Statistical analysis

Figures were generated using Prism version 6.02 (GraphPad Software, USA) and statistical analysis was performed using SPSS version 22 (IBM Corporation, Armonk, NY). A two-way mixed ANOVA was applied to determine whether there were differences between the treatments within each PSW batch in the concentration of the inorganic compounds over time. For the test, the treatment type (between-subject

factor) and the time in days (within-subject factor) were the independent variables, while the concentration of the inorganic compounds the dependent variable. The data was initially analysed for outliers by Studentised residual plots, the Shapiro-Wilk test to determine whether the assumption of normality is met ($p > 0.05$), the Levene's test to determine whether the assumption of homogeneity is met ($p > 0.05$), and Mauchly's test to determine whether the assumption of sphericity is met (i.e. interaction, $p > 0.05$). The final significance is calculated by the two-way mixed ANOVA with the test satisfied when $p < 0.05$. To determine the point (day) at which the nutrient concentration became significantly different between the treatments, a univariate general linear analysis was applied at each day with a Tukey's HSD post-hoc test for multiple comparisons ($p < 0.01$). Unless stated otherwise, the p-value reported refers to the comparison of a treatment to the control treatment (WWC) in the experiment with the day stated at which the effect became significant.

4.3 Results and Discussion

4.3.1 Influence of aerated and non-aerated cultivation conditions on microalgae growth

The strains *C. vulgaris*, *H. riparia* and *A. obliquus* were evaluated with respect to their productivity and removal of COD, NH_3-N and PO_4-P from PSW under aerated and non-aerated cultivation conditions. This was performed in order to identify a suitable strain for use in subsequent experiments, with a view to developing a static microalgae wastewater treatment process. Figure 4.1 shows the time-course cell concentration for each species for the different cultivation conditions, and specific growth and final biomass concentrations are summarised in Table 23. In general, microalgal growth typically consists of a lag phase, an exponential or arithmetic growth phase and then stationary phase which, depending on the duration of cultivation, is followed by a phase of cell decline (death phase). From the recorded cell concentrations, slight variations in this behaviour were observed for each of the species, variations observed in this respect not only between the different microalgae species in the same condition but also for the same microalgae species in the different cultivation conditions (i.e. aerated vs non-aerated).

In the aerated cultivation condition, both *C. vulgaris* and *A. obliquus* exhibited a higher specific growth rate (0.22 d^{-1}) compared to *H. riparia* (0.04 d^{-1}). The growth of *C. vulgaris* in the WW+Air+*C.v* treatment was characterised by an initial increase at day 1 and slight decline at day 2, before arithmetic growth with a maximum cell density of 2.6 $x10^7$ ($\pm 3.9x10^6$) cells mL^{-1} achieved by day 3 (Figure 4.1A). Thereafter the cell concentration of *C. vulgaris* declined as of day 4 until becoming undetectable at day 7. The growth curve indicates a short adaptation phase for *C. vulgaris* under the aerated conditions, occurring within 24 hours (first sampling point). A similar observation was reported by Ruiz-Marin et al., (2010) [228] with *C. vulgaris* expressing a lag phase of no more than 20 hours when grown suspended in secondary effluent. Growth of *A. obliquus* in the WW+Air+*A.o* treatment was characterised by a continuous arithmetic growth phase following a 1-day lag (Figure 4.1A). A lower maximum *A. obliquus* concentration of 1.3 $x10^7$ ($\pm 3.8x10^5$) cell mL^{-1} was achieved at day 7 compared to *C. vulgaris*. From these results, it can be suggested that *C. vulgaris* had a better adaptive response to PSW compared to *A. obliquus* under aerated cultivation conditions. The lowest growth achieved was with *H. riparia* with an approximate 3-day lag indicating a long adaptation period that preceded a small increase over one day, with a maximum cell concentration of 3.4 $x10^6$ ($\pm 8.5x10^5$) cell mL^{-1} at day 7 (Figure 4.1A).

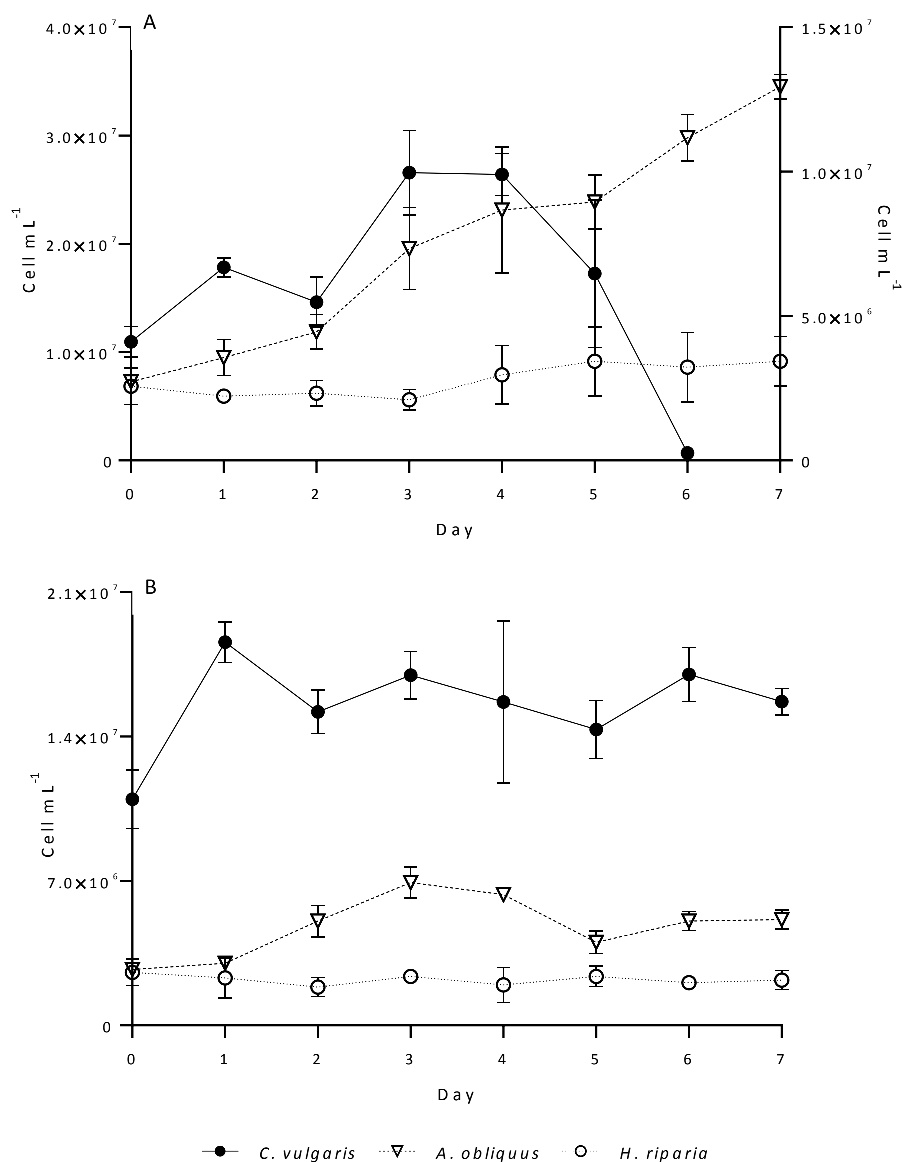


Figure 4.1 – Temporal changes in microalgal cell concentration for *C. vulgaris*, *H. riparia* and *A. obliquus* cultured in PSW under aerated (**A**) and non-aerated (**B**) cultivation conditions. In plot A, the left y-axis denotes cell concentration of *C. vulgaris* only and right y-axis denotes cell concentration of *H. riparia* and *A. obliquus*.

Table 23 – Specific growth rate and final biomass concentration (mg L^{-1}) of microalgae cultures when incubated under aerated and non-aerated conditions

Cultivation Conditions	*C. vulgaris*		*H. riparia*		*A. obliquus*	
	μ (d^{-1})	Biomass conc.	μ (d^{-1})	Biomass conc.	μ (d^{-1})	Biomass conc.
Aerated	0.22*	132.8 ±1.6	0.04	196.7 ±14.7	0.22	429.6 ±13.8
Non-aerated	0.05	217.3 ±6.4	-0.02	186.7 ±2.3	0.09	349.4 ±9.2

*Specific growth rate calculated between the initial day and day 5

The observed difference in growth between the three species in the aerated cultivation conditions can in part be explained by their individual morphological, phenological and genotypic features. The physiological properties of microalgae have been demonstrated to govern ecological performance, such as nutrient assimilation and adaptive mechanisms, including growth [405]. Microalgae, like many other organisms, express a relationship between growth rate and organisms size [406, 407]. Nielsen (2006) [408] quantitatively noted a significant allometric relationship in microalgae with smaller celled species exhibiting a higher maximum growth rate. A possible reason for the inadequate growth and performance of *H. riparia* may be explained by its colony formation, characterised by aggregates of well-differentiated, morphologically identical cells connected together via mucilaginous stalks, resulting in an overall large organism size (56 to 68 µm) [403]. As cell size increases, diffusion rates and intracellular transport rates are reduced becoming increasingly inadequate at maintaining required intracellular conditions and consequently limiting the maximum growth rate achievable [409]. In comparison; *C. vulgaris* size ranges between 2 to 10 µm, with daughter cells between 2 to 4 µm during exponential growth [410, 411]; while the size of *A. obliquus* ranges between 3 to 20 µm, with the potential of 4 to 8 celled colony's forming in which the individual cells are linearly arranged along their axes, a physiological adaptation that depends on various abiotic and biotic factors [412]. It must be noted that throughout these experiments *A. obliquus* was predominantly observed to be in a unicellular state.

An additional aspect governing growth and subsequently treatment performance (discussed below) could likely stem from the biochemical composition of the microalgae, dictating their robustness and vulnerability to the environment in PSW. For example, cell wall composition in *Chlorella* spp. and *Scendesmus* spp. has been described to contain the bio-polyester algaenan [413, 414]. Although the precise function of algaenan is still under review, the long chain fatty acid is hypothesised to confer or in part improve resistance to infection, based on the observations of being non-hydrolysable by common lytic enzymes and relatively impermeable to various organisms (i.e. fungi and bacteria) [415, 416]. Furthermore, algaenan-free microalgae are found to be more susceptible to chemical toxins [415]. The production of algaenan is noted to be strain-specific rather than species-specific [413]. In view of this fact, the particular species of *Chlorella* used in this study (*Chlorella vulgaris*) has been proven to not produce algaenan based on analysis with the stain calcofluor [417]. Despite not containing algaenan, *C. vulgaris* was found to be resistant to the actions of various concentrated mixtures of commercial enzymes, including β-glucanase, cellulase, and pectinase, suggesting a complex cell wall structure with tolerance to degradation [417]. Approximately 20% of the cell population was affected by enzyme action, with those cells affected likely undergoing auto-spore release or in the death phase of the cell cycle, which will have increased their susceptibility. Although the presence of algaenan in *H. riparia* was not investigated in the present study, a possibility exists that *H. riparia* lacks algaenan in its cell wall structure and therefore reduces the microalgae's robustness in the unsterile PSW environment. This assumption is based on the observation that colony forming microalgae identified to express algaenan in their cell wall (e.g. *Botryococcus braunii* and *Coelastrum sphaericum*) demonstrate good robustness and growth when cultured in unsterile wastewater, similar to unicellular microalga [339, 413, 418].

In the non-aerated cultures (static) the microalgae demonstrated a substantial lack of growth compared to that in the aerated cultures (Figure 4.1B). Cell concentrations of *C. vulgaris* and *A. obliquus* in the non-aerated conditions showed a similar response in growth for the initial couple of days of treatment with respect to their corresponding aerated cultures. In the WW+*C.v* treatment, *C. vulgaris* cell concentration increased at day 1 followed by a decline at day 2; however, the cell concentration remained relatively constant for the remaining 5-days, reaching a final concentration of 1.6 $x10^7$ (±6.6$x10^5$) cell mL^{-1}. *A. obliquus* in the WW+*A.o* treatment exhibited a 1-day lag followed by a small arithmetic increase over two days before declining to a cell concentration averaging 4.7 $x10^6$ (±6.1$x10^5$) cell mL^{-1} over the last 3-days of treatment. In comparison, no distinctive growth phase was discernible by *H. riparia* in the WW+*H.r* treatment; in fact, cell concentration in this treatment declined slightly over the course of the treatment period from an initial concentration of 2.5 $x10^6$ (±6.4$x10^5$) cell mL^{-1} to 2.2 $x10^6$ (±4.5$x10^5$) cell mL^{-1} at day 7. As a result of the low growth of all the microalgal cultures during the treatment period, the specific growth rates were small compared to under aerated conditions (Table 23).

Independent from any physiological aspects that govern microalgae growth, the substantial rise in pH following the elimination of carbon in the form of CO_2 from the PSW in the non-aerated cultures of *C. vulgaris* and *A. obliquus* may explain the low microalgae growth in these treatments. Temporal changes in pH for the treatments with or without microalgae, cultured under aerated and non-aerated conditions, are presented in Figure 4.2. The initial value of pH varied slightly between PSW batches in each of the experiments, with approximately pH 7 in PSW batch 1 and 3, and pH 8 for PSW batch 2 (Table 22). In the WW+*C.v* treatment (PSW batch 1), the pH increased in the first day of treatment to 9.5 ±0.1 and further thereafter, albeit at a slower rate, until day 4 at which point the pH stabilised to approximately 10.9 ±0.1 until the end of the treatment (Figure 4.2C). In the WW+*A.o* treatment (PSW batch 3) the pH increased at a more gradual and steady rate over the whole treatment period, reaching 10.6 ±0.1 at day 7 (Figure 4.2C). In autotrophic growth, microalgae uptake dissolved inorganic carbon (DIC) predominantly in the form of HCO_3^-, which is converted to CO_2 and fixed in the Calvin cycle [67]. Consequently, OH^- ions are produced that are either expelled to the immediate environment or are neutralised intracellularly following H^+ uptake, shifting the equilibrium to an alkaline environment [419–421]. In an aquatic environment, the concentration of DIC is strongly correlated to pH, with pH increasing as DIC decreases. Sutherland et al., (2015) [295] quantitatively noted this inverse correlation in which DIC decreased from 441 to 23 mg L^{-1} as the pH increased from <8 to 10.7 in a high rate algal mesocosm system without pH control. In carbon limited conditions, cell division and consequently growth are reduced owing to an arrest in photosynthetic activity and synthesis of carbon skeletons necessary for cell maintenance [67, 188, 189]. The magnitude in pH change in the WW+*C.v* and WW+*A.o* treatments suggests the concentration of DIC became limited in the PSW affecting microalgae growth potential. Furthermore, the high pH will have exacerbated the existing carbon limitations owing to a shift in DIC equilibrium, with pH >10 leading to CO_3^{2-} becoming the dominant inorganic carbon species [419, 422]. Most microalgae preferentially take up CO_2 over HCO_3^-, while CO_3^{2-} is not known to

be readily utilised by most microalgae [67, 423]. Therefore, as the microalgae actively fix inorganic carbon, not only did *C. vulgaris* and *A. obliquus* reduce the quantity of inorganic carbon but also the buffering capacity of the medium, which led to a shift in pH that further reduced the pool of bioavailable inorganic carbon species. As the treatments were cultured statically, the contribution of atmospheric CO_2 was considered to be negligible.

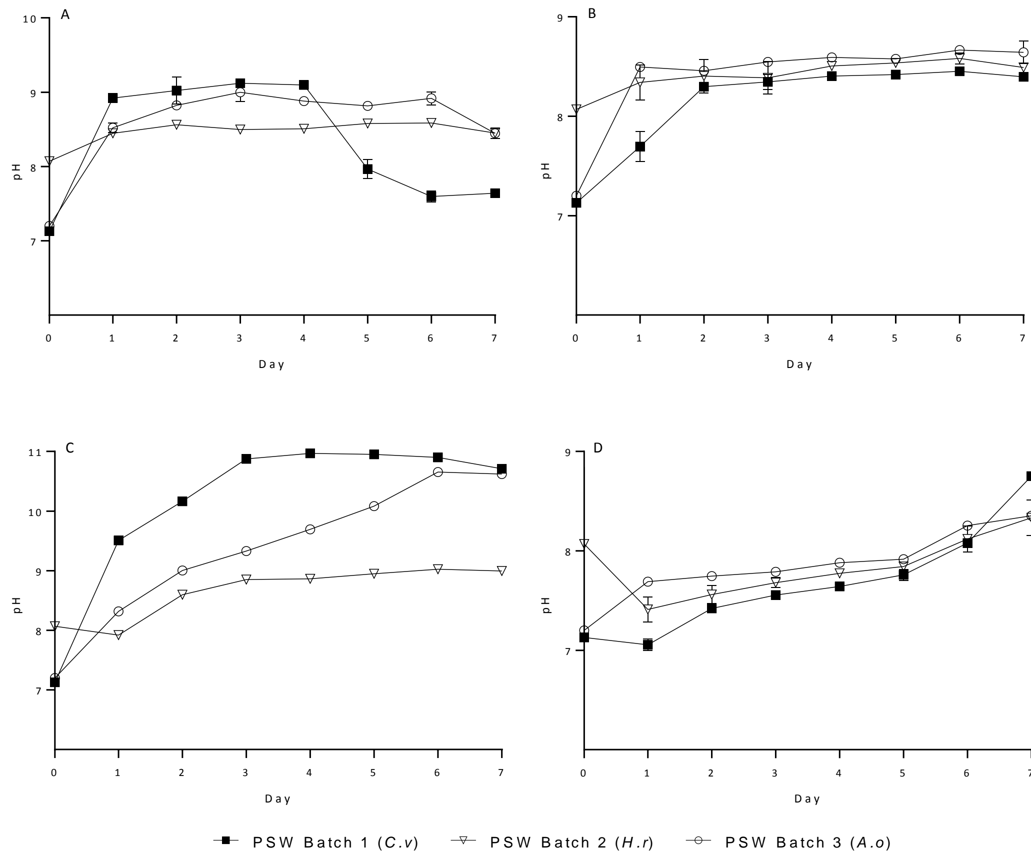


Figure 4.2 – Temporal changes in pH for the treatments with (**A, C**) or without (**B, D**) microalgae, cultured under aerated (**A, B**) and non-aerated conditions (**C, D**) for PSW batch 1 (squares; *C. vulgaris* treatment experiment), PSW batch 2 (open circles; *H. riparia* treatment experiment) and PSW batch 3 (open triangle; *A. obliquus* treatment experiment). Each point is a mean ±SD, of n= 3 independent replicates.

The change in pH was not as pronounced in the WW+*H.r* treatment compared to the WW+*C.v* and WW+*A.o* treatments (Figure 4.2C). In the WW+*H.r* treatment (PSW batch 2) a slight decline in pH was observed at day 1 before increasing again to a maximum value of 8.9 ±0.1 at day 7 (Figure 4.2C). The small effect in pH change and decline in *H. riparia* concentration in the WW+*H.r* treatment suggests a low photosynthetic efficiency by the microalgae. In autotrophic microalgae, the amount of light energy received and captured has a direct relationship to the carbon fixation capacity, which affects the productivity in microalgae growth [424, 425]. Both Jacob-Lopes et al., (2009) [426] and Goncalves et al.,

(2014) [427] respectively describe an increase in the CO_2 fixation quantity and rate in various algae species (green algae and cyanobacteria) when exposed to an increased light period and intensity (below the saturation point). Conversely, under low photo period conditions (>16:8 dark:light), CO_2 fixation rates depreciate significantly as a result of carbon-fixation reactions ceasing because of limited photosynthetic activity.

The pH dynamics for the aerated microalgae treatments are shown in Figure 4.2A. The WW+Air+*C.v* and WW+Air+*A.o* treatments exhibited an increase in pH from their initial value to 8.9 ±0.1 and 8.5 ±0.1 at day 1 respectively. The pH in the WW+Air+*A.o* treatment after day 1 stabilised for the exact period of *A. obliquus* exponential growth. Similarly, in the WW+Air+*C.v* treatment the pH remained constant at approximately 9.1 ±0.1 over days 1 to 4 which corresponded to the growth phase of *C. vulgaris*. These results indicate that the input of CO_2 into the aqueous phase was in equilibrium with the rate of consumption, sufficiently to avoid significant depletion of CO_2 in the medium and pH increase, as observed in the non-aerated microalgae treatments. Furthermore, the reduction in NH_3-N in the WW+Air+*C.v* and WW+Air+*A.o* treatments will have aided in minimising the effects of excess OH^- ion formed in the medium during inorganic carbon fixing (Figure 4.3A, C). This is because the translocation and assimilation of NH_3 into amino acids is accompanied by the translocation of H^+ ion out of the cell to maintain cytosolic pH following the reaction of the NH_3 with the carboxylic acid group [428–430]. The decline in *C. vulgaris* concentration corresponded to a pH drop between days 5 to 7, reaching a final value of 7.6 ±0.1. The resultant decrease in pH can be accredited to the continuous input of CO_2 present in the atmospheric air injected; the CO_2 concentration in the culturing system will have increased until CO_2 solubility in the medium reached atmospheric pressure saturation levels (i.e. equilibrated) as inorganic carbon input exceeded the rate of consumption resulting in a decrease in pH [393, 422]. In contrast to the microalgae treatments, the change in pH of the control treatments (without microalgae) was minor, with only small variations occurring throughout the 7-day treatment period because of the activity of the wastewaters microbial community (Figure 4.2B, D). For example, in the WWC treatment of PSW batch 1 the pH increased to 8.7 ±0.1 at day 7. In this treatment a decrease in the concentrations of NO_3-N and COD were noted, which is indicative of denitrification, a reaction in which OH^- ions are released, which explains the pH increase [89] (Figure 4.4; Table 25). This effect, however, cannot explain the increase in pH to 8.3 ±0.1 at day 7 in the WWC treatments of PSW batch 3, as indirectly no denitrification was recorded, i.e. increase and decrease in NO_3-N concentration.

In comparison to algal growth and yield for *Chlorella* sp. and *Acutodesmus* sp. reported in other studies, the specific growth rates and final biomass concentrations were lower in this study for both aerated and non-aerated conditions. For instance, specific growth rates between 0.25 and 2.4 d^{-1} for *C. vulgaris*, and between 0.28 and 1.19 d^{-1} for *A. obliquus*, have been reported during their growth in wastewater [148, 158, 228, 431–433]. However, the cultivation conditions, such as light intensity, inoculation density, light duration, temperature, CO_2 injection etc., and the types of wastewater used in the present study were quite different to those applied in other studies. Although the conditions under which the

microalgae were evaluated in the present study appeared to support growth in PSW, further studies to determine the optimal cultivation conditions for an industrial-scale process are recommended to improve microalgae productivity. This observation holds true for the *H. riparia* strain tested here. In order to fully elucidate whether *H. riparia* was in a major way limited by the cultivation conditions (specifically light) or by its inherent physiology (and biochemistry) for treating the wastewater medium, further experiments under varying conditions with either synthetic or sterile wastewater are required. These experiments should include the evaluation of the Fv/Fm ratio, a measure of the alga's maximum quantum yield of charge separation in PS II, a proxy for quantifying the environmental effects on algal health.

4.3.2 Influence of aerated and non-aerated cultivation conditions on microalgae Nitrogen and Phosphorus removal

Inorganic or organic forms of N and P are essential for growth and cellular function for all organisms. In microalgae, N is required as a substrate in the synthesis of proteins, chlorophylls and other biological molecules (i.e. ribonucleic acid (RNA), deoxyribonucleic acid (DNA) and ATP) [67]. Similarly, P is a key element in energy metabolism (i.e. ATP), a substrate of phospholipids and an essential component in various metabolic pathways functioning as a signalling or activating component (i.e. kinase and phosphorylation reactions) [68, 245]. The N:P ratio available to microalgae in the medium is considered one of the most influential parameters affecting N and P removal performance [141]. Limitations in one of these elements may reduce the removal of the other element [180]. Although each microalga was investigated for their growth and removal of N, P and COD in separate PSW samples, the initial inorganic N and P concentrations between the batches were found to be very similar (NH_3-N: 28.9 to 32.3 mg L^{-1}; PO_4-P: 3.0 to 3.8 mg L^{-1}) with an approximate 10:1 ratio (Table 22). As a result of NH_3-N and PO_4-P being the primary N and P compounds in microalgae metabolism, and present as the major fraction of inorganic N and P in all PSW batches at an equal ratio, this allowed comparisons to be made in the treatment performance of *C. vulgaris*, *H. riparia* and *A. obliquus* irrespective of the PSW batch [67].

4.3.2.1 Inorganic Nitrogen removal

Concerning inorganic N removal, the graphs in Figure 4.3 plots A, C and E show the concentration of NH_3-N over the 7-day treatment period of all the treatments for PSW batch 1, 2 and 3 respectively. Table 24 shows the removal kinetic parameters and efficiencies of NH_3-N and PO_4-P for all microalgae treatments. Regardless of the treatment process, aerated or non-aerated, the addition of any of the three microalgal strains to PSW resulted in the enhanced removal performance of NH_3-N, and indeed markedly more so compared to the un-inoculated controls. In general, the highest rate of removal in the microalgae-inoculated treatments occurred within 24 hours, between 32.6 and 8.7% for aerated, and between 17.7 and 20.1% for non-aerated cultivation conditions of total NH_3-N reduction. The highest removal efficiency over the shortest retention period occurred in the WW+Air+*C.v* treatment. In this treatment, NH_3-N was removed below the detection limit within 5 days of cultivation, following a

steady but rapid decline at a rate of 6.08 mg L^{-1} d^{-1} from an initial concentration of 30.5 ±0.3 to 0.09 ±0.01 mg L^{-1} ($p < 0.01$ at day 1) (Figure 4.3A). However, the concentration of NH_3-N increased over the course of the remaining 2 days of cultivation, reaching a final concentration of 7.9 ±0.7 mg L^{-1}. Conversely, in the corresponding treatment with *A. obliquus*, the NH_3-N concentration declined at a continuous, albeit slower rate, over the whole 7-day treatment period ($p < 0.01$ at day 1) (Figure 4.2C; Table 24). The concentration of NH_3-N in the WW+Air+*A.o* treatment, however, did not achieve greater reduction as recorded in the WW+Air+*C.v* treatment, with a final NH_3-N concentration of 4.3 ±2.2 mg L^{-1}. In the WW+Air+*H.r* treatment the concentration of NH_3-N decreased only slight in comparison, from an initial value of 28.9 ±0.3 to 13.2 ±1.7 mg L^{-1} at day 7, with the greatest effect occurring within the first 2 days. This was the lowest performing strain, with a removal rate of 2.25 mg L^{-1} d^{-1}, which equates to nearly 3 times lower than observed in the WW+Air+*C.v* treatment. Moreover, no statistically significant interaction effect between the four treatments of PSW batch 2 was determined (Mauchly's test $p < 0.05$). The reported NH_3-N removal efficiencies in the *C. vulgaris* and *A. obliquus* aerated treatments (PSW batches 1 and 3) were comparable to the values reported in previous studies treating municipal wastewater under similar cultivation conditions (i.e. aerated and/or mixed) (Table 24). For example, in a study performed by Tam and Wong (1989) [136], the microalgae *C. pyrenoidosa* and *Scenedesmus* sp. were able to remove 93.9% and 98.1% of NH_4^+-N, respectively, from primary settled effluent after 13 days of cultivation with an initial NH_4^+-N concentration of 22.5 mg L^{-1}. Ruiz-Marin et al., (2010) [228] obtained a NH_4^+-N removal efficiency of 99% by *A. obliquus* from urban wastewater within 2 days, while Su et al., (2012) [366] reported NH_4^+-N removal efficiency of 90% by *C. vulgaris* within 6 days.

The recorded increase in NH_3-N concentration in the WW+Air+*C.v* treatment after day 5 corresponded with the decline in *C. vulgaris* concentration. From this observation it can be suggested that the decline in *C. vulgaris* and consequently cell death may have resulted in the release of intracellular N fractions, including NH_3-N, from the algal cells into the PSW. The decline in *C. vulgaris* concentration may in part be attributed to the concentration of inorganic N becoming limited in the PSW after arithmetic growth and consequently the nutrient requirements to sustain growth and microalgae function were not available. At day 4 the concentration of NH_3-N in the WW+Air+*C.v* treatment was below the detection limit (0.08 ±0.01 mg L^{-1}). Similarly, at day 4 both NO_2-N and NO_3-N concentrations in this treatment were on the border or below the detection limit at 0.01 ±0.0 mg L^{-1} and 0.09 ±0.0 mg L^{-1}, respectively. The low concentration of PO_4-P in the WW+Air+*C.v* treatment at day 4 (0.17 ±0.05 mg L^{-1}) may have contributed to these effects, which led to the observed decline in *C. vulgaris* concentration (Figure 4.3B). The resultant N:P ratio (based on NH_3-N:PO_4-P) at day 4 was 1:1, which denotes a severe limitation in N [180]. Conversely, the WW+Air+*A.o* treatment exhibited a constant N:P ratio within the optimal range of 8:1 to 11:1 for *A. obliquus* throughout the 7-day treatment period [178].

In the non-aerated microalgae treatments, the trend in NH_3-N removal was similar for all treatments, with removal rates between 1.25 and 1.87 d^{-1}, and efficiencies between 30 and 40% (Table 24). In these treatments the concentration of NH_3-N was characterised by an initial rapid decline by day 1, and

thereafter a slower but continuous rate of decline that occurred for the remaining treatment period (Figure 4.3A, C, E). In the WW+*C.v*, WW+*A.o* and WW+*H.r* treatments the NH_3-N concentration declined to 22.3 ±0.6, 26.6 ±0.2 and 23.1 ±0.1 mg L^{-1} at day 1, with final concentrations of only 18.2 ±0.2, 19.2 ±0.1 and 20.2 ±0.1 mg L^{-1}, respectively. No statistical interaction in NH_3-N concentration between any of the non-aerated microalgae treatments and their respective WWC treatments occurred (Mauchly's test, $p < 0.05$). These results were expected given that the amount of nutrients utilised by algae directly relates to their productivity [136]. This is because the demand for nutrients to sustain cellular function is lower than required for cell growth and division, and hence a reduced rate in nutrient assimilation is observed. Although this observation is not extensively reported on in microalgae wastewater studies, several studies have consistently found that a higher nutrient removal rate and capacity is coupled to higher specific growth rates in microalgae cultures [142, 252, 310, 326, 434, 435].

Table 24 – Removal of NH_3-N and PO_4-P values of microalgae treatments under aerated and non-aerated cultivation conditions

Parameter	*C. vulgaris*		*H. riparia*		*A. obliquus*	
	Static	Air[1]	Static	Air	Static	Air
$R_{NH3\text{-}N}$ (mg L^{-1} d^{-1})	1.76	6.08	1.87	4.00	1.25	2.25
$R_{PO4\text{-}P}$ (mg L^{-1} d^{-1})	0.22	0.63	0.17	0.18	0.52	0.47
RE NH_3-N (%)	40.3	99.7	30.1	54.4	40.5	86.6
RE PO_4-P (%)	47.8	98.7	38.6	42.3	95.7	86.6

[1]All calculations for the treatment WW+Air+*C.v* are based for the first 5 days

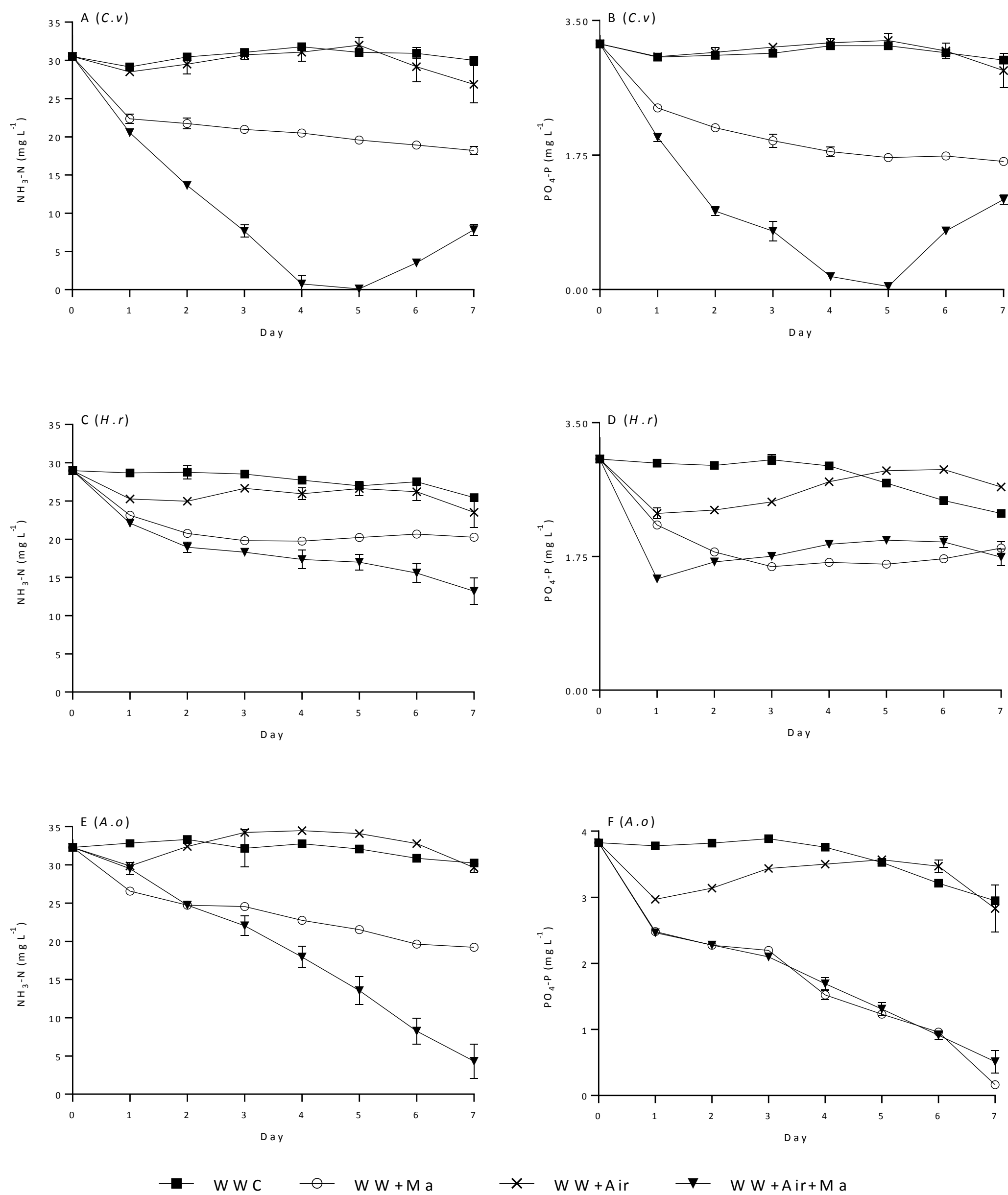


Figure 4.3 – Changes in PSW concentration for NH_3-N (**A, C, E**) and PO_4-P (**B, D, F**) in mg L^{-1} for PSW batch 1 (**A, B**), batch 2 (**C, D**) and batch 3 (**E, F**) treated under conditions with and without microalgae, supplied with or without air. Each point is a mean ±SD, of n= 3 independent replicates. Treatment WWC (Wastewater only); Treatment WW+Ma (Wastewater with microalgae: either with *C.v*, *A.o* or *H.r*); Treatment WW+Air (Wastewater with air); and Treatment WW+Air+Ma (Wastewater with air and microalgae: either with *C.v*, *A.o* or *H.r*).

The low growth and NH_3-N removal achieved in the WW+*C.v* and WW+*A.o* treatments was a result of the combined effects of inorganic carbon becoming limited initially and then likely from subsequent NH_3 toxicity because of the increase in the pH above 9. Ammonia assimilation and incorporation into amino acids in microalgae is inextricably dependent on carbon skeletons from the TCA cycle [67, 189]. Microalgae require CO_2 fixation to replenish the carbon in the TCA cycle via anaplerotic reactions, such as phosphoenolpyruvate carboxylase and/or RuBisCo [189, 436]. Thus, a deficiency in inorganic carbon reduces the capacity of microalgae to assimilate NH_3 because the carbon is preferentially fed into anaplerotic reactions necessary to sustain TCA cycle function [189, 437]. Comparison of Figure 4.2C and Figure 4.3A and E show that the decrease in NH_3-N uptake after day 1 in both the treatments correlated with the largest increase in the pH of the PSW. Although pH is not a direct quantification of inorganic carbon concentration, an alkaline pH in non-buffered or low alkalinity microalgae culture are indicative of a low DIC concentration available [295]. In studies by Pereira et al., (2016) [434] and Huertas et al., (2010) [399], both authors observed dependence in the inorganic carbon concentration on the capacity by the microalgae to assimilate inorganic N; higher inorganic carbon levels were associated with a higher and faster rates of inorganic N uptake. Similar to the results obtained in the WW+*C.v* and WW+*A.o* treatments in the present study, Huertas et al., (2010) [399] reported an increase in pH accompanied by a reduced capacity of the marine microalga *Nannochloropsis gaditana* to assimilate NO_3, the main inorganic N fraction, when cultured on air stripped of CO_2 (0.0001% v/v) compared to lower or neutral pH value when aerated on ambient and elevated CO_2-air.

The alkaline environment in the WW+*C.v* and WW+*A.o* treatment will have subsequently reduced the NH_3-N removal efficiency. This result is in agreement with work by Azov and Goldman (1982) [438], who observed a substantial decline in NH_4Cl removal efficiency, based on V/V_{max} ratio, at higher pH (i.e. >8.4 compared to 8) for *S. obliquus* grown in flask cultures. Ammonium is the preferred inorganic N species in medium for microalgae, as NH_3 at a high concentration becomes toxic to microalgae, as well as to other aquatic organisms [429]. The toxic effect of NH_3 on microalgae is because the compound can readily diffuse through the membrane unhindered as a result of its uncharged nature [439, 440]. A high intracellular concentration of NH_3 is reported to disrupt the photosynthetic apparatus in a light-dependent manner, either through the direct binding of NH_3 with the Mn complex of PS II, which is involved in the H_2O oxidation reaction, or by disrupting the intracellular pH stasis, or both [438, 439, 441]. Although, free NH_3 in solution can dissipate freely from liquid, this effect was considered to have a negligible influence on NH_3-N removal because the treatments were kept static [429].

Abeliovich and Azov (1976) [440] examined the effect of ammonia concentration at varying pH values and reported that photosynthesis and growth of *S. obliquus* was inhibited at an NH_3 concentration over 2 mM (approximately 34.06 mg L^{-1}) when pH values exceeded 8. Based on the conditions these authors used (i.e. pH 8 and temperature of 30°C), it can be deduced that approximated 7% (c.a. 2.5 mg L^{-1}) of the total NH_3-N was present as free NH_3, with the dissociation constant (pK_a) between NH_4^+ and NH_3 approximately 9.25 (25°C) [442]. Tolerance to NH_3 is however species specific, and has been described

as an important selection criterion for a suitable microalga in the wastewater treatment process because of the potential for high concentrations of free NH_3 to form [264]. In this context, it can be inferred that both *C. vulgaris* and *A. obliquus* are relatively resilient to the combined effects of high pH and free NH_3 formation, given the high concentrations that remained in the wastewater of both the microalgae treatments, highlighting these strains as suitable species for wastewater treatment. In the WW+*C.v* and WW+*A.o* treatments at day 7, the concentration of free NH_3 is approximated to be 16.9 and 17.6 mg L^{-1} respectively. Although no noteworthy microalgae growth in these cultures was recorded, the cell concentration did not decline below the inoculation concentration indicating a high tolerance.

In conventional wastewater treatment, NH_3-N reduction is achieved through its conversion to NO_2, then into NO_3 and N_2 by nitrification and denitrification, respectively. In this investigation, both the NO_2-N and NO_3-N concentrations were found to be different, not only between the four treatments in each PSW batch, but also between the same treatments in the different PSW batches (Figure 4.4). The environment of each treatment formed differently and independently, resulting in a different inorganic N profile because of the various mechanisms underlying NH_3-N removal. For instance, in the WWC treatment of PSW batch 1, by day 7 the concentration of NO_2-N declined from an initial 0.20 ±0.01 to 0.13 ±0.01 mg L^{-1}, and that of NO_3-N from an initial 0.69 ±0.01 to 0.11 ±0.02 mg L^{-1} (Figure 4.4A, B). This reduction can be ascribed to the action of denitrifying bacteria in the PSW, which utilise N-bound O_2 for aerobic respiration in an anoxic environment [89]. The decline in both NO_2-N and NO_3-N concentrations corroborates the understanding that the environment in this particular WWC treatment became anoxic, likely because denitrification ceased to function in the presence of even a low O_2 concentration (i.e. <0.3 mg L^{-1} O_2) [443–445]. The anoxic environment was a result of the treatment being incubated statically. The lack of O_2 will also have limited the ability of the nitrifying bacteria, if present, from converting the NH_3-N, and thus limiting its removal from PSW. This was evident by the fact that the NH_3-N concentration remaining constant over the 7-day treatment period (Figure 4.3A). This inference also explains the minimal removal efficiency of NH_3-N in the WWC treatment of PSW batches 2 and 3. Both NO_2-N and NO_3-N concentrations in these treatments were consistently below the detection limit from the commencement and duration of this investigation (Figure 4.4C - F), while the concentration of NH_3-N remained constant at approximately 25.4 ±0.4 and 30.2 ±0.4mg L^{-1} respectively (Figure 4.3A, C and E).

The increase in either, or both, NO_2-N and NO_3-N concentrations in the WW+Air treatments in this investigation substantiates the inference that the WWC treatments were O_2-limited and not just deficient in nitrifiers (Figure 4.4). In the WW+Air treatment of PSW batch 1 and 3, a gradual increase in NO_2-N concentration is noted at day 4, with each treatment reaching a respective concentration of 0.49 ±0.16 and 0.58 ±0.06 mg L^{-1} at day 7 (Figure 4.4A, E). In the WW+Air treatment of PSW batch 1, the increase in NO_2-N concentration was not accompanied by an increase in NO_3-N, which remained constant at approximately 0.76 mg L^{-1} (Figure 4.4B). While in the WW+Air treatment PSW batch 3, NO_3-N increased at day 5 from 0.07 ±0.01 to 0.50 ±0.1 mg L^{-1} at day 7 (Figure 4.4F). A similar result is

recorded in the WW+Air treatment of PSW batch 2 in which the concentration of NO_2-N increased to 0.63 ±0.03 mg L^{-1}, and of NO_3-N to 0.33 ±0.07 mg L^{-1}, at day 7. The lag in nitrification may be attributed to the long generation times exhibited by the nitrifying organisms [89]. The energy yield from NH_3 and NO_2 oxidation is low and consequently, AOBs exhibit a doubling time that can range between 16 and 189 hours, and NOBs between 18 and 69 hours, under optimal conditions [446].

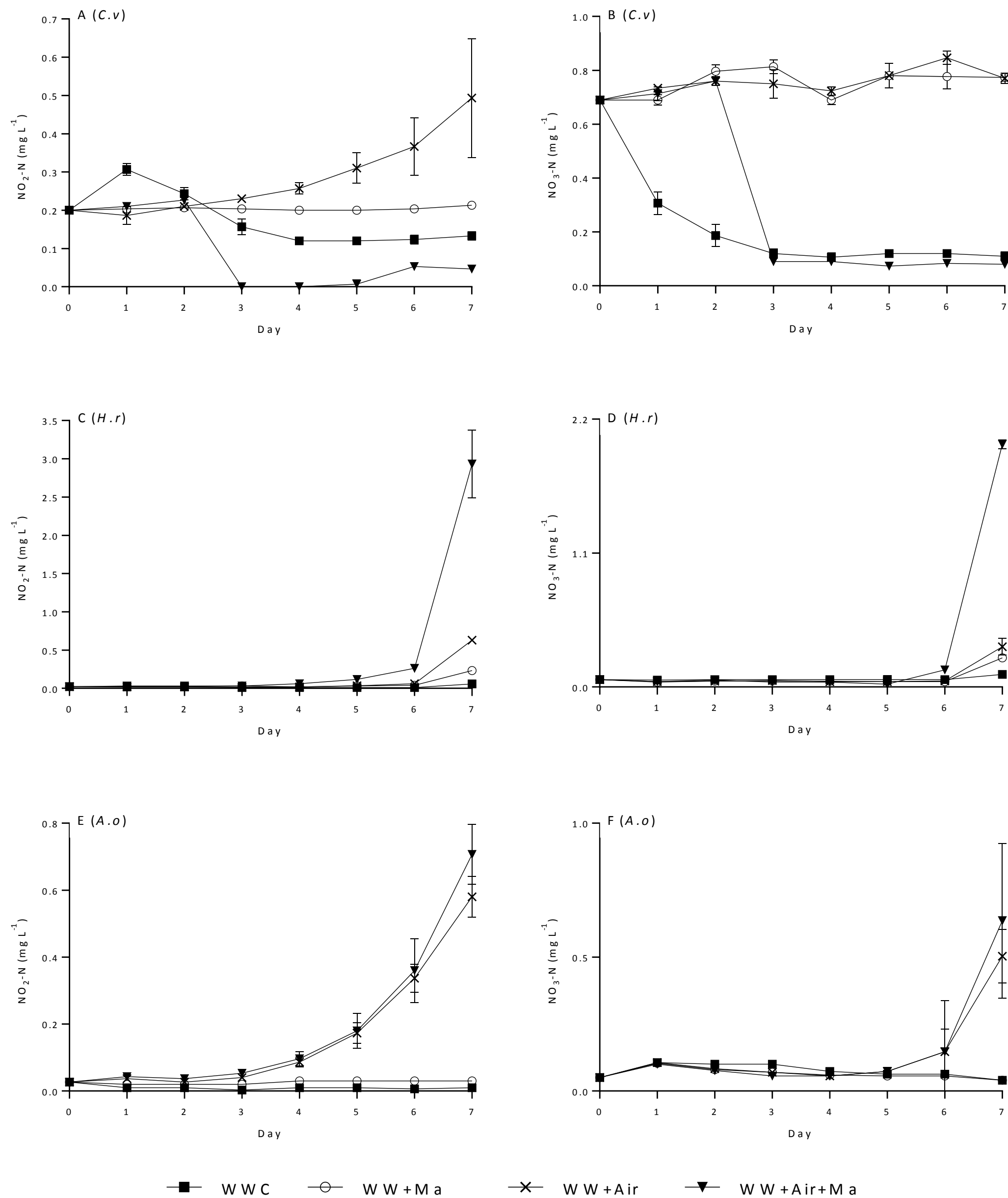


Figure 4.4 – Changes in PSW concentration for NO_2-N (**A, C, E**) and NO_3-N (**B, D, F**) in mg L^{-1} for PSW batch 1 (**A, B**), batch 2 (**C, D**) and batch 3 (**E, F**) treated under conditions with and without microalgae, supplied with or without air. Each point is a mean ±SD, of n= 3 independent replicates. Treatment WWC (Wastewater only); Treatment WW+Ma (Wastewater with microalgae: either with *C.v*, *A.o* or *H.r*); Treatment WW+Air (Wastewater with air); and Treatment WW+Air+Ma (Wastewater with air and microalgae: either with *C.v*, *A.o* or *H.r*).

Nitrifying bacteria are sensitive to pH, exhibiting an optimal range between pH 7 and 8.5, and inhibition occurring below pH 6.5 and above pH 9 [89, 447, 448]. Furthermore, without an adequate supply of inorganic carbon the nitrification reaction and growth of nitrifying bacteria are inhibited [449]. The high rate of inorganic carbon utilisation by phototrophs in microalgae-bacteria co-cultures can cause a lack of resource availability to nitrifiers and is linked to the cessation of nitrification [450]. In the WW+*C.v* and WW+*A.o* treatments, the pH increase and low inorganic carbon availability will have limited the formation of NO_2-N and subsequently NO_3-N. While the pH in the WW+*H.r* treatment remained below 9, the small increase in NO_2-N and NO_3-N detected (i.e. from 0.02 to 0.24 mg L^{-1} and 0.06 to 0.24 mg L^{-1} respectively) did not corresponded to the equivalent amount of NH_3-N removed, approximately 8.72 mg over the 7-day experiment, indicating that nitrification was not the dominant pathway in reducing the inorganic N from the PSW (Figure 4.2C; Figure 4.3E; Figure 4.4E, F). A low dissolved O_2 availability in the medium may explain the limited occurrence of nitrification in this treatment given the assumed low photosynthetic efficiency of *H. riparia* as suggested under the present conditions. However, further experiments are needed which directly address this hypothesis.

The highest concentration of NO_2-N and NO_3-N throughout this investigation was recorded in the WW+Air+*H.r* treatment. In this treatment the concentrations of NO_2-N and NO_3-N increased substantially as of day 6. The concentration of NO_2-N increased from 0.26 ±0.04 to 2.93 ±0.44 mg L^{-1} and the concentration of NO_3-N from 0.14 ±0.01 to 1.99 ±0.03 mg L^{-1}, at day 7 (Figure 4.4C, D). A similar increase was observed in the WW+Air+*A.o* treatment, although the increase in NO_2-N occurred after a shorter lag phase, with NH_3 conversion to NO_2 and NO_3 as of day 4 (Figure 4.4E, F). The occurrence of these compounds is a direct result of the O_2 provided by the aeration, together with the CO_2 availability and stable pH of the culture, directly promoting a higher activity of the nitrifying bacteria in the PSW [89]. The lack of nitrification in the WW+Air+*C.v* treatment is a result of NH_3-N becoming a limited resource, decreasing to below the detection limit at day 4 and likely before nitrification had a chance to begin. Interestingly, the decline in NO_2-N and NO_3-N in the WW+Air+*C.v* treatment occurred after the complete reduction in NH_3-N. The delay in NO_3-N and NO_2-N removal and uptake by *C. vulgaris* shows that this species prefers NH_3-N. This observation was expected since NH_3 is known to be preferentially assimilated by microalgae because NO_2 and NO_3 need to be reduced to NH_3 in an endogenic reaction [67, 451]. Both Silva et al., (2015) [252] and Ruiz-Marin et al., (2010) [228] demonstrated that *C. vulgaris* preferred NH_3-N in wastewater to other potentially available inorganic nitrogen sources.

4.3.2.2 Inorganic Phosphorus removal

Figure 4.3B, D and F show the temporal variation in PO_4-P concentration for each treatment in PSW batch 1, 2 and 3 respectively. The trend in PO_4-P depuration in each treatment across all PSW batches exhibited a similar trend to their respective NH_3-N profile, with the exception of the WW+*A.o* treatment. The aerated microalgae treatments presented the highest removal efficiencies, achieving maximum removal rates in the range of 0.18 to 0.63 mg L^{-1} d^{-1} (Table 24). The PO_4-P removal efficiencies obtained in this study were in the same order of magnitude than the ones reported by Wang et al., (2014) [154]

(0.17 to 0.32 mg L^{-1} d^{-1}), but substantially lower than reported by Ruiz et al., (2013) [452] (2.0 to 8.7 mg L^{-1} d^{-1}). The higher removal rates by Ruiz et al., (2013) [452] are justified by the continuous supply of 5% CO_2 compared to ambient air in this study, which ensured an adequate supply of dissolved inorganic carbon and, thus, promoted a greater growth response and nutrient removal by the microalgae. However, a similar TP removal rate to this study is also reported by Ji et al., (2013) [133] for *S. obliquus* and *C. vulgaris* cultured on municipal wastewater at 15% CO_2 (approximately 0.425 mg L^{-1} d^{-1}), while Silva et al., (2015) [252] reported a large variation in removal rate depending on the N:P ratio in the culture medium (0.48 to 2.61 mg L^{-1} d^{-1}). The discrepancy in P removal rate in microalgae cultures reported in the literature highlight the different propensity in microalgae performance and behaviour depending on the culture conditions and/or strain, and which further highlights those comparisons to select a favourable microalgal strain(s) for treatment must be done on a case-by-case basis.

In the WW+Air+*C.v* treatment, the PO_4-P concentration rapidly declined to below the detection limit by day 5, from an initial concentration of 3.2 ±0.1 to 0.04 ±0.05 mg L^{-1} (p <0.01 at day 1), and thereafter increased to a final concentration of 1.2 ±0.1 mg L^{-1}. A continuous steady rate of decline in PO_4-P was recorded in the WW+Air+*A.o* treatment from an initial concentration of 3.8 ±0.1 mg L^{-1} to 0.2 ±0.1 mg L^{-1} at day 7 (p <0.01 at day 1). In the WW+Air+*H.r* treatment PO_4-P decreased by day 1 from an initial concentration of 3.0 ±0.1 to 1.5 ±0.1 mg L^{-1}, followed by a gradual increase to a final concentration of 1.7 ±0.1 mg L^{-1}. In microalgae cultures it has been demonstrated that the removal of P from the medium is influenced by the available N concentration. Beuckels et al., (2015) [453] described the assimilation of PO_4^{3-} into microalgal biomass as dependent on the supply of bioavailable N. Their study identified that biomass P concentrations were low when the N concentration in the biomass was low because the algae were grown on N-limited medium, irrespective of the amount of P in the medium. This mechanism is in agreement with the results in the present study as the PO_4-P and NH_3-N concentrations in the aerated microalga treatments displayed an equivalent trend in removal.

The dependence of inorganic N uptake and incorporation into the microalgal biomass influencing inorganic P uptake is more pronounced in the non-aerated microalgae treatments. In these treatments the highest NH_3-N removal efficiency occurred at day 1, in conjunction with the highest PO_4-P removal efficiency. Thereafter, the removal rate of NH_3-N declined drastically and concomitantly with a decline in PO_4-P removal. For example, in the WW+*C.v* treatment PO_4-P decreased from an initial concentration of 3.2 ±0.0 to 2.4 ±0.1 mg L^{-1} by day 1, with only a further 0.7 mg PO_4-P removed over the remaining six days. This trend in PO_4-P concentration was also observed in the WW+*H.r* treatment (Figure 4.3D). The PO_4-P concentration in the WW+*A.o* treatment, on the other hand, was characterised by a steady and continuous rate of decline, identical to that recorded in the WW+Air+*A.o* treatment (Figure 4.3F). While PO_4-P removal was higher in the WW+*A.o* treatment compared to the WW+*C.v*, the mechanism of removal after day 1 will likely have been a result of P precipitation in both treatments.

In an alkaline environment, PO_4^{3-} ions precipitates from solution following their reaction with metal ions. Phosphate reacts with calcium ions at a pH of approximately 8 to form hydroxyapatite ($Ca_5(PO_4)_3$),

among other species, or it can also react with magnesium ions when the pH is above 7.5 to form struvite ($NH_4MgPO_4{\cdot}6H_2O$); both are insoluble mineral salts [126, 454]. The degree of precipitation is influenced by the concentrations of PO_4^{3-} and cations in the medium, as well as temperature and pH [376]. In the WW+*C.v* and WW+*A.o* treatments of this study, the decline in PO_4-P concentration can be attributed to its assimilation initially by the microalgae and subsequently by precipitation as the pH of the treatments had been observed to increase to approximately 9 at day 1. However, it could not be determined why the reduction in PO_4-P in the WW+*A.o* treatment, compared to the WW+*C.v* treatment, was more efficient despite both treatments resulting in a similar pH increase over the 7-day treatment period. One possible explanation may be the difference in the PSW composition of batches 1 and 3. A higher concentration of cations allows for a greater amount of PO_4-P to react with and precipitate out, while a higher ionic strength of the water decreases the potential for precipitation to occur [376]. Additional experimentation is needed to address this hypothesis.

4.3.2.3 Influence of the indigenous microbial community in PSW on Nitrogen and Phosphorus removal

Comparing the capacity to remove inorganic N and P between the treatments, the results indicate that regardless of the PSW batch and treatment condition, with or without aeration, the removal can be directly attributed to the microalgae because the control treatments (without microalgae) showed no significant decrease in NH_3-N and PO_4-P (Mauchly's test $p < 0.05$) (Figure 4.3). In the WWC treatments of PSW batch 2 and 3, the concentration of NH_3-N gradually decreased to 25.4 ±0.4 and 30.2 ±0.4 mg L^{-1} by day 7, respectively. In the same treatments the PO_4-P concentrations decreased marginally to a final concentration of 2.3 ±0.03 and 2.9 ±0.03 mg L^{-1} respectively. In comparison a slight initial decline, either at day 1 or 2, in both NH_3-N and PO_4-P concentrations is noted in the WW+Air treatments of PSW batch 2 and 3, with a gradual increase thereafter (Figure 4.3). For PSW batch 1, only a small deviation from the initial NH_3-N and PO_4-P concentration was recorded in both the WWC and WW+Air treatments by the end of the experiment. The gradual decrease in NH_3-N and PO_4-P in these treatments will have been because of the presence of the natural microbial community of the PSW, which either converted or assimilated the inorganic compounds. Although a small degree of nitrification and denitrification occurred in the control treatments, the results suggest that the natural microbial community was not able to effectively remove or convert the inorganic compounds to any great extent under the culture conditions presented.

Although the influence of the microbial community cannot be completely disregarded with respect to eliminating the inorganic N and P in the microalgae cultures, based on the results of the control treatments their ability to significantly do so is limited. This finding is consistent with previous studies employing microalgae-bacteria co-cultures. For example, Su et al. (2012b) investigated the potential of a co-culture composed of wastewater-born algae consortium (majority filamentous blue-green algae) and activated sludge, inoculated with different ratios (w/w: weight per weight) of nutrients removed from pre-treated wastewater. The removal efficiencies of total Kjeldahl N and PO_4-P removal at day 10 were respectively 95.5% and 93.5% in the 5:1 algae-bacteria co-culture, whereas in the reactor with only

sludge the concentrations declined by 31.4% and <10% respectively. Ma et al., (2014) [455] directly examined the influence of bacteria removing nutrients from centrate, a waste stream following sludge dewatering, with *C. vulgaris* by varying the initial concentration of bacteria in the co-culture. Their results revealed no significant difference in nutrient removal from the wastewater with increasing bacteria concentrations, implying that the presence of bacteria had little effect on the removal of the inorganic N and P compounds, at least within the investigated range. In the present study, the contribution of the bacteria in the microalgae treatments to remove the inorganic N and P may have been limited by the composition of the microbial community and environment of the treatment. Biological nutrient removal from wastewater is dependent on specific microorganisms (i.e. nitrifying, denitrifying and PAOs), which are encouraged to grow and function by cycling the wastewater through anaerobic, aerobic and anoxic environments [1, 89]. The presence of these microorganisms is naturally low in influent and settled wastewater, and without these specific microorganisms the removal of inorganic N and P in wastewater treatment tends to be minimal [456–458]. It can be suggested that the microbial population in the PSW, and therefore in the microalgae treatments, did not contain an adequate abundance of these microorganisms to effectively facilitate inorganic N and P removal.

Another aspect that may have limited the microbial population in reducing the inorganic N and P in the microalgae treatments may have been the environmental and cultivation conditions of the experiment. As previously discussed, nitrification and denitrification in the microalgae treatments may have been inhibited by the elevated pH or limited supply of inorganic carbon and oxygenation of the medium via photosynthesis [1]. In general, the elevated pH in the microalgae treatments may have potentially reduced the abundance of the microbial community of the PSW by means of photo-oxidative destruction of coliform bacteria [281, 285, 287]. As a result, this will have led to a lower rate of CO_2 release via heterotrophic carbon-oxidation that would have otherwise served the microalgae with an alternative source of this essential compound for photosynthesis [288, 289]. In regards to the decline in PO_4-P concentration after 24 hours in the WW+Air treatments of PSW batches 2 and 3, this may have been a result of PAOs in the wastewater sample. In anaerobic environments these bacteria hydrolyse their stored polyphosphate to drive carbon assimilation and storage [2]. When conditions change to aerobic, PAOs actively consume inorganic P beyond their need for balanced growth. It is possible that this particular group of bacteria were present in the PSW batches 2 and 3 at a sufficient abundance to accomplish the observed decline in PO_4-P, but not in the PSW batch 1. Between the collection and set-up of the treatments, the PSW may have become anaerobic and consequently induced the phase of polyphosphate release and carbon assimilation, and upon aeration of the PSW, the bacteria will have actively assimilated the inorganic P. This observation is supported by the fact that the concentration in PO_4-P did not increase in the WWC treatments as O_2 was not supplied, which maintained an anaerobic environment. As a result of the continuous aeration, no subsequent cycle in PO_4-P release and uptake could occur. Additional experimentation is needed to confirm this conjecture primarily by community analysis of the PSW at the various time points to confirm the presence and abundance of PAOs [459].

4.3.2.4 Influence of operating conditions on COD reduction

The influence of the treatment condition on the reduction of COD_S was also analysed. The final COD_S concentrations for each treatment in PSW batch 1, 2 and 3 are presented in Table 25. It was expected that the reduction in COD_S concentration would be generally higher in the treatments with aeration and/or inoculated with microalgae. This is because an aerobic condition is necessary for oxidative biodegradation and incorporation of the carbonaceous material by heterotrophic bacteria, with the provision of O_2 supplied in the atmospheric air injected and/or via photosynthetic O_2 evolution in the microalgae treatments [1, 67]. Interestingly, COD reduction in the WWC treatments of each PSW batch was equal to the reduction in the WW+Air treatments, and in some cases greater than in the microalgae treatments. For instance, in the WW+*C.v* treatment the COD declined by 44%, whereas in the respective WWC and WW+Air treatments of the PSW batch a 57 and 56% reduction was recorded, respectively. Similarly, in the WW+*H.r* treatment only a 9% reduction occurred (from 145 to 131 mg L^{-1} O_2), while in the WW+Air treatment of this PSW batch 26% of the soluble carbonaceous material was removed. In PSW batch 3, a removal efficiency of approximately 49% was recorded in all the treatments. It must be noted that the low COD_S removal efficiency of 20.5% recorded in the WW+Air+*C.v* treatment was most likely a consequence of a net increase in oxidisable organic matter in the PSW following algal death as a result of NH_3-N and PO_4-P shortage after day 5 – an effect also observed in a microalgae wastewater study by Sforza et al., (2014) [156].

Table 25 – Final COD_S concentration (mg L^{-1}) of treatments with or without microalgae, under aerated and non-aerated conditions

PSW batch	Initial	WWC	WW+Algae	WW+Air	WW+Air+Algae
PSW 1 (*C. vulgaris*)	106.7 ±4.2	45.3 ±4.6	59.6 ±6.4	46.31 ±7.2	84.80 ±5.0
PSW 2 (*H. riparia*)	145.0 ±0.1	120.0 ±2.7	131.3 ±5.9	106.33 ±4.5	89.0 ±7.0
PSW 3 (*A. olbiquus*)	156.6 ±2.7	85.6 ±2.5	78.6 ±2.3	81.3 ±10.1	70.31 ±6.0

These results suggest that the naturally occurring heterotrophic organisms are chiefly responsible for consuming and reducing the carbonaceous material in the PSW across all batches and treatments. Although it has been reported that microalgae can utilise organic compounds (i.e. glucose, acetone, etc.), either under heterotrophic or mixotrophic culture conditions, various studies have highlighted a limited capacity of microalgae that are able to utilise carbonaceous material from wastewater sources. For example, in a study by Lau et al., (1995) [150], evaluating the treatment of PSW by *C. vulgaris* cultured under mixotrophic mode, the authors reported that the trend in COD concentration over the 10-day retention period was similar between the control (without microalgae) and microalgal treatments employing different inoculation densities - ranging between 1 $x10^7$ and 5 $x10^5$ cells mL^{-1}. From this observation the authors inferred that under the experimental conditions and wastewater sample used, the microalgae were unable to utilise the carbonaceous material as a source or carbon

because of the high and variable complexity in the composition of the compounds; with the COD from the wastewater reduced primarily by the bacterial population. A similar finding was reported in a study by He et al., (2013) [212], in which an average 90% BOD_5 removal from unsterilized secondary wastewater was recorded, while in their sterile treatment no change in BOD_5 or dissolved organic carbon was recorded when both treatments were inoculated with *C. vulgaris*. However, the capacity of microalgae to assimilate and utilise the organic carbon material in wastewater is dependent on the wastewater type and source. Posadas et al., (2014) [460] observed a high variation in total organic carbon (TOC) removed by a microalgal consortium in different wastewater types, ranging from an 18% TOC removal in lyophilized coffee manufacturing wastewater to 56% TOC removal in fish processing wastewater. Overall, in the cultivation conditions of the present study, it can be inferred that the microalgae could not effectively remove and utilise the COD_S as a carbon source even under inorganic carbon limited conditions. To assess the capacity of each of the microalgal strains to utilise the carbonaceous material in wastewater sourced from Seafield, experiments using axenic cultures inoculated into sterile wastewater would ideally need to be conducted.

4.4 Conclusion

In this work three microalgal strains were assessed for their suitability to treat PSW based on i) their ability to remediate carbonaceous, nitrogenous and phosphorus material from the water, and ii) their growth. Each microalgal species was cultured in real and unsterilized PSW under aerated and non-aerated (static) conditions. The response in growth and treatment performance in aerated conditions, which represented the optimal condition in this study, varied substantially between the different microalgae species. *C. vulgaris* exhibited the ability to acclimatise better to the PSW medium and its environment based on the observed growth and higher inorganic N and P removal efficiencies as compared to that of *A. obliquus* and *H. riparia*, highlighting it as the preferred species in treating PSW. A possible reason for the better response exhibited by *C. vulgaris* was attributed to its physiology, as for a smaller unicellular microalga a higher rate of productivity and inorganic N and P uptake from PSW could be achieved. Concerning the performance in the non-aerated conditions, demise in growth and inorganic N and P removal was observed following the initial days after inoculation into the wastewater. In the non-aerated *C. vulgaris* and *A. obliquus* treatments, the demise in NH_3-N and PO_4-P reduction coincided with the formation of an alkaline environment, indicative of inorganic carbon being depleted in microalgae cultures. Given that the main difference between aerated and non-aerated microalgae treatments was the provision of CO_2 in the atmospheric air supplied, it can be inferred that the PSW was deficient in inorganic carbon necessary to support microalgae growth and inorganic nutrients for assimilation. Although organic carbon was present in the wastewater (measured as oxidisable-carbon), the consumption of it by the microalgae was considered to be minimal. This observation is based on the equal levels of deprivation achieved in COD concentration between the microalgae treatments and the control treatments (without microalgae). This data suggests that the carbonaceous material was primarily degraded by the autochthonous microbial community of the wastewater. Taking this into

account, it can be suggested that the majority of organic carbon fractions in the wastewater could either not be assimilated and/or metabolised by the microalgae potentially owing to the complexity of the compounds. In comparison the microalgae were chiefly responsible for removing the inorganic P and N. Overall, no negative effects on the microalgae could be assigned to the composition of the PSW itself (i.e. toxic compounds), while the possible competition with native microflora was considered, it is not seen to affect algal growth and function. Based on its performance, *C. vulgaris* was chosen as the test species for subsequent experiments in this thesis.

Chapter 5 – Effect of organic carbon enrichment on the treatment efficiency of primary settled wastewater by *Chlorella vulgaris*

5.1 Introduction

In Chapter 4, the characterisation of the PSW in the non-aerated microalgae treatments indicated that the concentration and fractions of carbon present in the wastewater were either of an insufficient quantity or in a non-bioavailable form, thus resulting in a low microalgal growth and inorganic N and P depuration from the water. The potential of establishing an energy-efficient and cost-effective microalgae treatment process by means of eliminating aeration is thereby restricted by the quantity of carbon naturally present in a given source of PSW. Microalgae acquire the majority of their carbon via photosynthetic carbon fixation in which inorganic carbon is incorporated into organic carbon substrates [67]. However, a number of microalgae have demonstrated to have facultative heterotroph capabilities, consuming organic carbon substrates over CO_2 fixation [461]. Alternatively, certain microalgal species are mixotrophic in which photoautotrophic and heterotrophic carbon assimilation and metabolism occur simultaneously [205, 300, 462, 463]. In the presence of a suitable organic carbon source, the synergistic effect of the two processes has been shown to enhance microalgal productivity. For example, when *C. vulgaris* was cultured in BG-11 medium under mixotrophic conditions the biomass yield was 2.08 g L^{-1} (at a glucose concentration of 7.22 mM) compared to just 1.64 g L^{-1} under photoautotrophic conditions [464]. Similarly, Mondal et al., (2017) [465] observed a 4-fold increase in *Chlorella* species BTA9031 productivity under conditions promoting mixotrophic growth compared to photoautotrophic conditions, with dry biomass yields of 1.45 and 0.7 g L^{-1} respectively. The effects of other organic carbon sources, including glycerol, fructose or sodium acetate have also been studied in mixotrophic cultivation of freshwater microalgae. The influence of the organic carbon source on microalgae productivity varies not only between organic carbon sources, but also the concentrations present at in the medium and even among different microalgae species cultured with the same carbon source [198, 205, 219, 466–469].

Some studies have reported the cultivation of microalgae in wastewater with an exogenous organic carbon source, herein representing a strategy to concurrently improve the treatment efficiency and microalgal productivity. For example, Gupta et al., (2016) [85] observed maximum 58.1% TN and 74.8% TP removal efficiency by *C. vulgaris* from municipal wastewater supplemented with 5 g L^{-1} glucose, compared to a respective 15.8% and 22.8% reduction when the organism was cultured in wastewater only. Higher removal efficiency in COD were also observed in the glucose-supplemented wastewater treatments compared to without. Interestingly, an enhanced reduction in COD was noted when glucose was present at a low concentration compared to high concentration (glucose concentration ranged from 2 to 30 g L^{-1} in the study). Perez-Garcia et al., (2011) [86] found that organic carbon supplementation of secondary municipal wastewater was necessary as the present fractions were not in a bio-available form for microalgae to assimilate when cultured under heterotrophic conditions. In the study, NH_4^+ removal by *C. vulgaris* from the wastewater was statistically higher when supplemented with 0.12 M acetate or

glucose compared to without any exogenous carbon. It is worth noting that the addition of organic carbon in microalgae wastewater treatment can also become a strategy to overcome light limitations that are caused by either the opaque nature of wastewater or high cell densities. Particulate and colloidal matter attenuate the light intensity to microalgae as well as increase the scattering of photons, reducing photosynthetic efficiency directly and detrimentally impacting on growth and remediation of pollutants from the wastewater [16, 146, 470]. Moreover, reduced light capturing is caused by self-shading in which the microalgae in suspension inadvertently shield each other from receiving light, which becomes accentuated as cultures become denser over time or if mixing is limited [150, 471–474].

It is critical to extrapolate carefully the results reported in previous studies. Many studies, including the above mentioned, assessed the effect that exogenous organic carbon has on a microalgae wastewater treatment process in either real or synthetic wastewater that had been sterilised by filtration or autoclaving (similar studies: [86, 218, 475, 476]). This is problematic given the presence of the natural microflora of the wastewater (i.e. bacteria, fungi, viruses, grazers etc.) that may negatively affect algal growth and nutrient removal efficiencies more acutely in the presence of a simple organic carbon source (e.g. glucose) that is known to stimulate non-photosynthetic organism growth. Analysis on the microbial dynamics in algal-bacteria co-cultures in wastewater containing a readily available source of exogenous organic carbon for either microorganism is limited. Mayo and Noike (1994) [477] reported on the effect that various glucose loading concentrations had on the growth and the culture dynamics between *C. vulgaris* and heterotrophic bacteria collected from settled activated sludge. Under the specific operating conditions, Mayo and Noike (1994) [477] observed *C. vulgaris* growth rate to increase with an increase in glucose loading rate, with the highest growth achieved at 150 mg L^{-1} d^{-1} glucose loading. Above this glucose loading rate the dissolved O_2 became the growth-limiting factor and VFA began to accumulate in the reactors, resulting in a decline in both bacterial and algal density. Above this glucose-loading rate (higher rates were 300 and 700 mg L^{-1} d^{-1}) the conditions formed indicated a prevalence of heterotrophic carbon-oxidation exceeding photosynthetic O_2 evolution. Yun et al., (2017) [478] reported a higher biomass yield in axenic algae culture in BG-11 medium amended with glucose under mixotrophic conditions compared to microalgal-bacterial co-cultures cultured with the same concentration of glucose. This result is in agreement with the findings reported by Zhang et al., (2012) [479], in which a higher biomass yield was achieved in a pure *C. pyrenoidosa* culture with 10 g L^{-1} glucose amended soybean wastewater under heterotrophic conditions compared to its corresponding microalgal-bacteria co-culture. However, the difference in algal biomass between the axenic and bacteria contaminated culture was reported as not significant. Interestingly, a lower final TN and TP concentration was recorded in the microalgal-bacterial co-culture (TN: 22.89 mg L^{-1}; TP: 0.69 mg L^{-1}) compared to the pure *C. pyrenoidosa* culture (TN: 64.71 mg L^{-1}; TP: 2.76 mg L^{-1}). Zhang et al., (2012) [479] attributes the success of the algae, which were in competition with the bacteria, in part because of the cultivation conditions and medium composition, which permitted to maintaining a higher active algal growth rate capable of competing with bacterial growth. A similar result was reported by Perez-Garcia et al., (2010) [480] using a co-culture of *C. vulgaris* and *Azospirillum brasilense* cultivated under

mixotrophic conditions with glucose (10 g L^{-1}), where a statistically significant removal of 44% NH_3-N compared to <30% under autotropic cultivation was observed without any negative effects on microalgal growth linked to the presence of the bacteria. In fact, a previous study found that the interaction between microalgae and bacteria can be dependent on the respective cell densities [481]; the study noted that a low bacterial cell density (5 x10^6 cell mL^{-1}) improved the growth of microalgae, whereas high bacterial cell density (between 10 and 20 x 10^6 cell mL^{-1}) inhibited microalgae growth and, conversely a high microalgae cell density inhibited the growth of bacteria. With the objective of developing a static microalgae wastewater treatment process, supplementation of a suitable organic carbon source may improve the remediation efficiency. Empirical studies aiming to investigate this effect should initially use a universally, readily available organic carbon source that can be metabolised by both microalgae and bacteria.

In this regard, various organic carbon substrates present themselves as a viable source in the evaluation of a static microalgae treatment process, including glucose or glycerol. However, in view of development a static microalgae wastewater treatment process for industrialisation and large-scale application, the significant cost of the proposed carbon sources is a major drawback. Both glucose and glycerol are commercial commodities and as such would increase the material cost of the overall process, with the quantity required dependent on the volumes of wastewater [482]. Moreover, glucose is primarily produced for human consumption and any deviation from this purpose is generally considered inappropriate with regards to the fuel versus food debate [483]. Glycerol is an organic carbon source often used in the mixotrophic production of microalgae and is an industrial by-product. However, the use of crude or pre-treated glycerol as a substrate in the manufacturing of value-added products has expanded in recent years with emerging processes resulting in resource competition [484–486]. Furthermore, glycerol as a by-product from microalgae biofuel production is often recycled as a carbon source in the medium as it positively affects net lipid productivity [464, 487, 488]. Therefore, the economic need to use a non-commercial organic carbon source(s) is imperative to ensure the sustainable development of a low-cost, static microalgae wastewater treatment process.

Various organic carbon by-products generated from manufacturing processes have been successfully proven to support microalgal growth, both in heterotrophic or mixotrophic conditions. For instance, sugar cane juice [223], cassava starch hydrolysate [224], corn powder hydrolysate [222, 225], cheese whey effluents [220, 489, 490], dairy waste (Woertz et al, 2009), and brewery waste [218, 491, 492] amongst others have proven their worth in this respect. The main focus of these studies was to improve microalgae biomass and lipid yield. An alternative opportunity could be to supplement PSW with a carbon-rich by-product as a relatively inexpensive source to enhance the treatment efficiency by *C. vulgaris* under static cultivation.

A potential carbon-rich by-product to enhance microalgae wastewater treatment efficiency is pot ale, a residue remaining in the pot sill after the first distillation step in whisky production [493, 494]. Characterised as an acidic (<4 pH) brown-red turbid liquid, pot ale is mainly composed of yeast and

barley fractions that are present in both the solid and soluble phase. Carbohydrates account for approximately 2.5% (w/v) of the soluble content of pot ale, while proteins account for approximately 1% (w/v) [493]. Consequently, high COD concentrations between 31 and 62 g L^{-1} O_2, and high BOD concentrations between 24 and 35 g L^{-1} O_2 have been reported for different pot ale sources [494–496]. The main fractions of carbohydrates are the non-fermentable sugar dextrin (polysaccharide of four or more glucose units linked by glycosidic bonds, and which can be branched) as we all residual fractions of glucose, fructose, maltose and formed organic acids [495, 497, 498]. Moreover, pot ale contains a high concentration of TN (>2 g L^{-1}) and TP (<1 g L^{-1}) that is released into solution from the milled malt and yeast fractions throughout the manufacturing process [496]. Presently, the disposal of pot ale is a concern as its high COD, N and P content are associated with expensive treatment processes [499, 500]. Conventional treatment of pot ale is expensive, achieved through anaerobic digestion with the co-generation of methane, followed by phosphate precipitation and biological nitrification and denitrification [495, 499]. Despite the high depurative efficiency achieved (<90%), effluents of pot ale still retain high organic loads with COD concentrations around 10 g L^{-1} O_2 [496, 501]. This is the result of the inherent variation in its composition following the distillation process that makes stable anaerobic digestion difficult to maintain [494, 499]. In some instances, the methane produced is contaminated with hydrogen sulphite, with concentrations reaching as high as 2 g L^{-1} and, hence, making it an unsuitable product without further processing. Being produced at an estimated 8 L per L of alcohol, approximately 2 to 3 million tonnes of pot ale are generated in Scotland annually, thus presenting a substantial source of a carbon-rich by-product [493, 502]. With the need for a more cost effective and sustainable disposal process, the coupling of pot ale with PSW treatment by microalgae is a potential solution.

The objective of this study is to evaluate the effect that enrichment of PSW with organic carbon substrates has on a static microalgal treatment process, to better understand this process, and to give a contribution towards its potential development for industrialisation and large-scale application. It was hypothesised that organic carbon enrichment of PSW improved the treatment efficacy by the mixotrophic microalga, *C. vulgaris*, under static culture conditions. To this end, three consecutive experiments in a laboratory setting were carried out to systematically assess this organism's performance in response to different organic carbon substrates and to compositional changes of the wastewater. In the first experiment, PSW was enriched with a small quantity of glucose as a representative organic carbon substrate to facilitate the bioremediation by *C. vulgaris*. This experiment was carried out on a single wastewater grab sample in order to verify the potential of the treatments and to provide a baseline response in removal efficiency, with a primary focus on NH_3, PO_4 and COD concentration. Moreover, in view of developing an integrated system where bacteria and microalgae coexist, the condition tested was to also qualitatively monitor the response that organic carbon enrichment of PSW had on the natural microflora of the wastewater in view of potential bacterial contamination and their effect on the treatment process. The second experiment aimed to validate the efficiency and reproducibility of this process, taking into account natural fluctuations in the composition

(biological/chemical) of wastewater, by additionally conducting three independent batch studies with PSW obtained on different days of the year (grab samples). In addition to enriching with glucose, when evaluating the reproducibility of the static microalgae treatment process, independent treatments enriched with either glycerol or CO_2 were also included to compare between different organic and inorganic carbon source. The third experiment aimed to evaluate the effect PSW enrichment with pot ale has on the treatment efficiency by *C. vulgaris*. This was done to verify pot ale as a potentially alternative and relatively cheap organic carbon source compared to glucose or glycerol, in order to improving the cost-effectiveness of a static microalgae treatment process. The pot ale experiment was repeated on a total of three PSW batches (grab samples), collected and treated separately and sequentially to ensure the reliability and reproducibility to include natural abiotic and biotic variability of the wastewater in the assessment of the pot ale enriched treatment process.

5.2 Material and Methods

5.2.1 Experimental conditions and set-up

5.2.1.1 Quantities of organic and inorganic carbon used for each experiment

In the first and second experiment the amount of organic carbon added to the PSW samples throughout this study was set to generate an equivalent COD of 300 mg L^{-1} O_2. For glucose, this equated to 281.1 mg L^{-1}, whereas for glycerol this was 245.9 mg L^{-1} (quantity calculated using equation 18). Prior to use, D-glucose (as powder: ACROS Organics, UK) was oven-dried overnight at 105°C. For glycerol, several millilitres were autoclaved (121°C; 15 minutes), then allowed to cool to room temperature and the quantity required accurately weighed in a pre-weighed Falcon tube. A small amount of PSW sample was added to the glycerol in the tube to reduce its viscosity and facilitate its transfer. In order to recover all of the glycerol in the tube, aliquots of wastewater from the sample were used to wash the tube three times. For inorganic carbon enrichment, CO_2 (75%) was bubbled directly into the wastewater sample through a sterile In-Line HEPA filter at a rate of 0.2 V/Vm for 1 minute every 8 hours. The gas flow was measured by a rotameter (FL-2010, Omega Engineering Ltd., UK) with injection time regulated by a solenoid valve (CO2Art Ltd., UK) connected to a programmable 24-hour time switch.

The pot ale used in this study was previously subjected to a protein extraction process [493]. The de-proteination process is reported to consistently achieve removal of >60% soluble protein fraction, with a maximum 90% removal [503]. To avoid the introduction of other microorganisms other than the autochthonous microbial community of the PSW and bacteria associated with the microalga, upon receipt the pot ale was filter sterilised (0.22 µm) and stored at 4°C until use. No pH adjustment or amendments with nutrient salts was performed. The quantity of pot ale added to the PSW for each batch run was set at a ratio of 1:150 (v/v); this resulted in an equivalent COD increase between 250 and 260 mg L^{-1} O_2.

5.2.1.2 Initial glucose enrichment experiment

Glucose enrichment in PSW with microalgae was performed in 450 mL of wastewater contained in 500 mL round borosilicate bottles. For this, a cell suspension of *C. vulgaris* grown on BBM was concentrated by centrifugation (3500x*g*; 10 min) in 50 mL Falcon tubes and washed twice with 10 mL of the collected wastewater. Three litres of filtered PSW was transferred to a 5 L borosilicate bottle and inoculated with the washed microalgae at a biomass dry weight concentration of 0.1 g L^{-1}. For enrichment, 1.5 L of the wastewater with *C. vulgaris* was transferred to a clean 2 L borosilicate bottle and amended with glucose (section 5.2.1.1), and then the sample was divided between three 500 mL borosilicate bottles. This step was repeated separately for the enrichment of the wastewater only treatment without the addition of the microalga. In total, four conditions (each in triplicate) were set up and labelled as follows: Wastewater control (WWC), Wastewater with glucose (WWG), Wastewater with *C. vulgaris* (WW+*C.v*) and Wastewater with glucose and *C. vulgaris* (WWG+*C.v*).

5.2.1.3 Evaluating the reproducibility of the treatment efficiency by C. vulgaris with either glucose, glycerol or CO_2 enrichment across different PSW samples

In addition to glucose, the effect of glycerol and CO_2 enrichment was also investigated as additional independent treatments. The volume treated was increased to 950 mL, and for each batch of PSW one bottle for each treatment was set up. For each PSW batch treated, 4 L of filtered PSW was transferred to a 5 L borosilicate bottle and inoculated with washed microalgae (as prepared in section 5.2.1.2) at a biomass dry weight concentration of 0.1 g L^{-1}. A 950-mL volume of the wastewater with *C. vulgaris* was then transferred to each bottle. Glucose and glycerol were added directly to the PSW to the concentrations stated in section 5.2.1.1. The treatment conditions were labelled as follows: Wastewater control (WWC), Wastewater with *C. vulgaris* (WW+*C.v*), Wastewater with glucose and *C. vulgaris* (WWG+*C.v*), Wastewater with glycerol and *C. vulgaris* (WWGY+*C.v*), and Wastewater with CO_2 and *C. vulgaris* ($WWCO_2$+*C.v*). This experiment was repeated a total of three times with each run treating a different sample of PSW. From hereafter, R1, R2 and R3 refer to the experimental runs performed with PSW batches 1, 2 and 3 respectively.

5.2.1.4 Pot ale enrichment experiment

Pot ale enrichment in PSW with *C. vulgaris* was performed in 450 mL of wastewater contained in 500 mL borosilicate bottles. The experimental set up was the same as described for the initial glucose enrichment experiment in section 5.2.1.2, with the exception of pot ale as the exogenous organic carbon source, added directly to the PSW to the ratio stated in section 5.2.1.1. In total, four conditions, each in triplicate were set up and labelled as follows: Wastewater control (WWC), Wastewater with pot ale (WWPA), Wastewater with *C. vulgaris* (WW+*C.v*), and Wastewater with pot ale and *C. vulgaris* (WWPA+*C.v*). This experiment was repeated a total of three times with each run treating a different sample of PSW enriched with a different sample of pot ale. Hereafter, R4, R5 and R6 refer to the experiments performed with PSW batch 4, 5 and 6, and pot ale batch 1, 2 and 3, respectively.

5.2.1.5 Glassware, sampling and analysis

For the experiments all glassware was capped with a foam bung and incubated for a period of 5 days. Before use, all glassware with the relevant syphoning and aeration tubes was autoclaved (121°C; 15 minutes). Liquid samples were withdrawn daily to measure microalgal cell growth (cell mL^{-1}), pH and concentration of NH_3-N, PO_4-P, NO_3-N and NO_2-N (described in Chapter 3, sections 3.3.4, 3.3.5, 3.3.6, 3.3.8, 3.9 and 3.10 respectively). In the initial glucose experiment only the concentration of glucose was measured by total carbohydrate analysis on a daily basis. Dry cell weight (as a proxy for biomass) and COD were measured on the initial and final day of each experiment only (Chapter 3, sections 3.7 and 3.5 respectively). All treatments were briefly mixed (by swirling) prior to taking an aliquot to ensure a homogenous sample.

5.2.2 Statistics

Figures were generated using Prism version 6.02 (GraphPad Software, USA) and statistical analysis was performed using SPSS version 22 (IBM Corporation, Armonk, NY). Normality and homogeneity of variances for the data was tested with a Shapiro-Wilk test and Levene's test respectively. Since the data were found not to comply with a normal distribution, a nonparametric Kruskal-Wallis test by rank was run to determine if a difference in the median concentration values of an inorganic compound occurred between the treatments at a selected time point. The null hypothesis states that the distribution of concentration in each treatment is the same, and the alternative hypothesis states that the concentration differs in its distribution between at least two treatments. A statistically significant difference is noted when $p < 0.05$, rejecting the null and accepting the alternative hypothesis. If a statistically significant difference is calculated ($p < 0.05$), a pairwise comparison using Dunn's procedure with a Bonferroni correction for multiple comparisons was followed. Unless otherwise stated, the reported significance refers to a comparison of a treatment to the control treatment (WWC) at the time point (day) stated.

5.3 Results and Discussion

5.3.1 Effect of enrichment with glucose

5.3.1.1 Inorganic Nitrogen and Phosphorus removal

Bioavailable organic carbon, in the form of glucose, had a strong influence on the ability of *C. vulgaris* to remove inorganic N and P from the PSW. In the case of NH_3-N, this was the most abundant form of inorganic N available to the microalga in the PSW (Figure 5.1A), and its removal was more effective in wastewater that was enriched with glucose compared to the untreated (no glucose) control. In the WWG+*C.v* treatment, NH_3-N concentration significantly declined from an initial concentration of 28.6 ±0.1 to 4.1 ±0.3 mg L^{-1} at day 1, and reached 0.1 ±0.05 mg L^{-1} at day 2 ($H(3) = 10.421$, $p = 0.002$ at day 1). Conversely, in the WW+*C.v* treatment without enrichment with glucose, concentrations of NH_3-N decreased at a slower rate, reaching 19.7 ±0.7 mg L^{-1} at day 1, after which only a total of 2.1 mg NH_3-N was further removed over the remaining four days. In the treatments without the microalgae, NH_3-N decreased to no more than 19.1 ±0.2 mg L^{-1} in the WWG treatment, and no reduction was recorded in the WWC treatment. No statistically significant difference was calculated between the control and the treatments WW+*C.v* and WWG. Concerning TN, a final concentration of 5.29 ±0.22 mg L^{-1} was achieved in the WWG+*C.v* treatment, which is below the maximum 10 mg L^{-1} imposed by the UWTD [14]. No other treatment in this experiment achieved the required minimum TN discharge concentration (Table 26). However, the limited removal in TN for these treatments is not surprising given that the majority of N present in the PSW was NH_3-N; 28.6 ±0.1 mg L^{-1} NH_3-N with the remaining N fractions (organic N + NO_2-N + NO_3-N) equating to approximately 6.43 mg L^{-1}. Therefore, a low NH_3-N reduction was expected to result in a relatively high TN concentration.

Table 26 – Initial and final concentrations of TN and COD for treatments enriched or not enriched with glucose, with or without *C. vulgaris*; concentration in mg L^{-1} and mg L^{-1} O_2 respectively.

Treatment	TN		COD	
	Initial	Final	Initial	Final
WW	35.03 ±0.48	32.63 ±0.18	141.9 ±4.2	101.6 ±5.6
WWG		25.34 ±0.21	416.3 ±15	138.3 ±3.1
WW+*C.v*		19.12 ±0.83	141.9 ±4.2	106.6 ±8.4
WWG+*C.v*		5.29 ±0.22	422.4 ±5.8	133.6 ±9.1

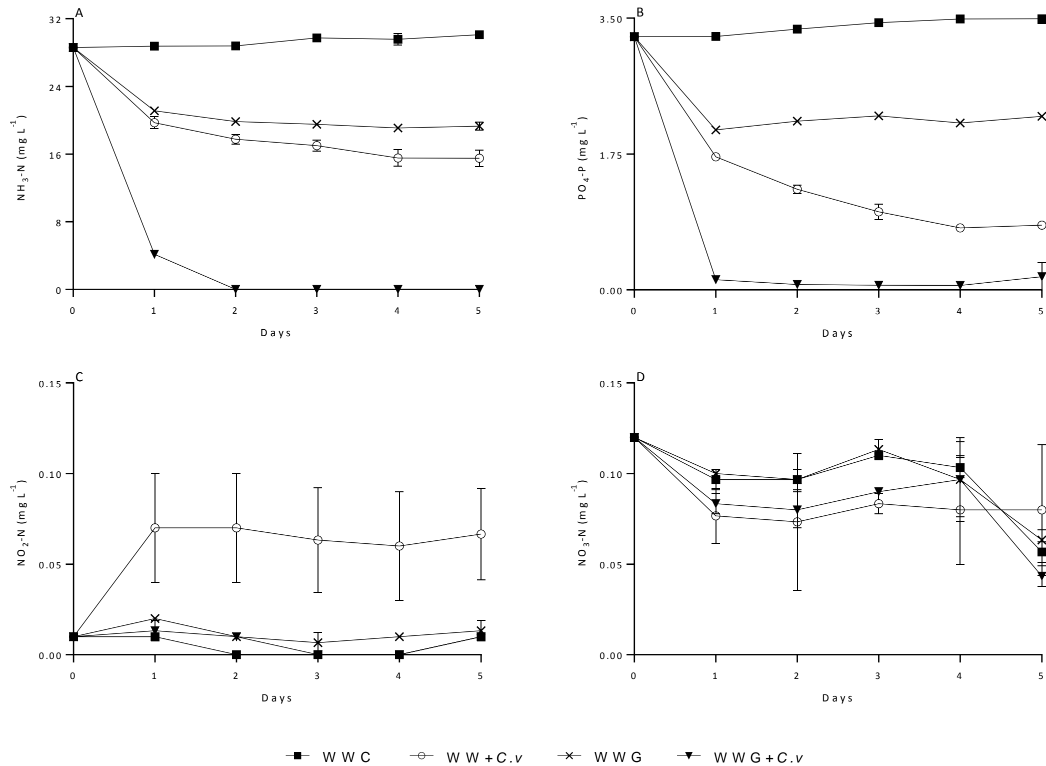


Figure 5.1 – Changes in the PSW concentrations for NH_3-N (**A**), PO_4-P (**B**), NO_2-N (**C**) and NO_3-N (**D**) in mg L^{-1} treated with and without C. *vulgaris*, enriched with or without glucose. Each data point is the mean ±SD, of n = 3 independent replicates. Some error bars are smaller than the symbols. Treatment WWC (wastewater only); treatment WW+*C.v* (wastewater with *C. vulgaris*); treatment WWG (wastewater with glucose); and treatment WWG+*C.v* (wastewater with glucose and *C. vulgaris*).

In view of the marked reduction in NH_3-N concentration recorded in the WWG+*C.v* treatment, it can be argued that this effect was a direct result of the additional organic carbon (as glucose) to the PSW. As detailed in section 2.2.2.2 and Figure 2.4, inorganic N assimilation and synthesis into amino acids in microalgae is inextricable dependent on carbon skeletons and energy in the form of ATP and NADPH/NADH [67, 238]. In microalgae, the incorporation of NH_3 has been shown to increase the demand for TCA cycle intermediates, with 2-oxogluterate and oxaloacetate being the main metabolites [238]. Carbon substrates, which have been appropriated for N assimilation from the TCA cycle, are replenished by anaplerotic reactions involving carbon fixation or assimilation in autotrophic or heterotrophic pathways, respectively, of mixotrophic algae like *C. vulgaris*. In the WWG+*C.v* treatment the glucose will have been assimilated and metabolised by the microalgae, initially to glucose-6-phosphate and other intermediates to pyruvate through the glycolytic pathway and subsequently into the TCA providing a direct input of necessary carbon substrates [205, 245]. In comparison, microalgae growth was minimal in the WW+*C.v* treatment for the duration of the 5-day treatment period and alkalisation of the PSW above pH 10 occurred, symptomatic of inorganic carbon limitation as observed also for the non-aerated *C. vulgaris* treatment in Chapter 4 (Figure 4.3; Figure 5.2B, D). Moreover, the

reduction of COD in the WW+*C.v* treatment was minimal with a removal efficiency of 24%, which was slightly lower than that in the WWC treatment (28%) (Table 26). The similar final COD concentrations recorded in the WW+*C.v* and WWC treatments suggest: i) the readily available source of organic carbon in the wastewater was limited and depleted fast; ii) the naturally occurring heterotrophic organisms were chiefly responsible at consuming the carbonaceous material since either treatment resulted in a similar final COD concentration; and iii) the residual carbonaceous material in the wastewater could not be degraded further by either microalgal or microflora community under the conditions of the treatments. Based on these observations, it can be argued that bioavailable carbon (organic or inorganic) to the microalgae in the wastewater of the WW+*C.v* treatment was limited, thus explaining the minimal reduction in NH_3-N. Consequently, when compared to the WW+*C.v* treatment, the significantly higher NH_3-N removal efficiency in the WWG+*C.v* treatment can be attributed to higher availability of bioavailable carbon, mainly to *C. vulgaris*, herein in the form of glucose. A similar finding was reported by Eisele and Ullrich (1997) [504], where inorganic N assimilation by *Ankistrodesmus braunii* was enhanced by glucose (10 mM) cultivated in CO_2-free air. By comparison, in the carbon-deplete culture condition (with glucose or CO_2) the majority of the NO_3 assimilated by the algae was released as NH_3 following NO_3 reduction, as the microalgae were not able to incorporate the inorganic N into organic compounds because of a lack of bioavailable carbon.

With respect to other studies treating wastewater with microalgae under aerated conditions, a similar or higher N removal efficiency was recorded in the present glucose-enriched PSW treated with *C. vulgaris* under static conditions. For example, in the study performed by Sforza et al., (2014) [156], the microalga *C. protothecoides* was able to remove 71% TN from unsterile settled municipal wastewater with an initial TN concentration of 38.71 mg L^{-1} when cultivated under continuous aeration with 5% v/v CO_2 at 10°C. Choi (2015) [181] obtained a 99.8% NH_4^+-N removal efficiency from unsterile preliminary sedimentation effluent with an average 25.38 mg L^{-1} NH_4^+-N by *C. vulgaris* cultured in a microalgae membrane bioreactor supplied with continuous atmospheric air at a hydraulic retention time of 3.4 days. In fact, in this experiment the WWG+*C.v* treatment achieved the same NH_3-N removal efficiency within a shorter retention period than the WW+Air+*C.v* treatment in the preliminary evaluation study described in Chapter 4. When Figure 5.1A is juxtaposed with Figure 4.3A, it can be clearly seen that the WWG+*C.v* treatment achieved a 99% NH_3-N removal by day 2 compared to by day 4 in the WW+Air+*C.v* treatment. This observation suggests the presence of glucose facilitates NH_3-N removal from wastewater by microalgae without aeration, and as, or more effective than in aerated cultures.

The variation in N removal efficiency and time taken between the non-aerated WWG+*C.v* treatment and the aerated microalgal wastewater treatment studies mentioned above (amongst others) may be attributed to the different carbon sources present as a result of the cultivation strategy. In addition to being metabolised directly in anaplerotic reactions, the metabolism of glucose through the glycolytic pathway yields a net gain of two ATP and two NADH molecules, providing energy and reducing power for cellular metabolism, including for amino acid synthesis [205, 244]. Moreover, products formed in

glucose metabolism can be further metabolised for ATP production in the mitochondrial oxidative phosphorylation pathway, which is functional also in the absence of light [505]. On the other hand, inorganic carbon fixation is achieved at the expense of ATP and NADPH. In brief, energy captured in the form of photons by chlorophylls, phycobilins and carotenoids enables electrons donated by water to transfer to PS I and II. Through the electron transfer chain, the reaction yields sufficient energy to regenerate ADP and $NADP^+$ to ATP and NADPH, respectively [67, 506]. The products produced in the light reaction are then utilised for inorganic carbon fixation in the Calvin cycle and other metabolic reactions, which are also functional in the dark [67, 506]. Therefore, in autotrophic condition inorganic N incorporation into amino acids is dependent on photosynthesis in comparison, under mixotrophic condition the presence of an organic carbon source means that microalgae are not dependent on photosynthesis and light stops being a growth limiting factor. It can be argued that under mainly the autotrophic condition, the incorporation of N into amino acids requires a higher photosynthetic efficiency to meet the additional demand in ATP and NADPH for both N and carbon fixation. Consequently, not only does a limited resource of inorganic carbon affect N incorporation in microalgae, but also a low photosynthetic efficiency. Therefore, the aforementioned phenomenon may explain the slower and/or reduced N removal efficiency in microalgal cultured with inorganic carbon as their primary source compared to organic carbon, as observed in the present experiment.

In all the treatments, both the NO_2-N and NO_3-N concentrations were consistently on the border of the detection limit from the commencement and duration of these experiments (Figure 5.1C, D). Although N_2 was not analysed for, the likelihood of inorganic N being removed through its conversion to N_2 (i.e. nitrification and denitrification) will have been limited by various chemical and physical factors associated with the treatments, albeit independent from each other. For all treatments, the main limitation to nitrification will have been the relatively short duration of the experiment (day 5), terminating likely before a sufficient abundance of nitrifying bacteria could become established to elicit a detectable difference in NO_2-N concentration given their long generation time [1]. Additionally, the observed pH changes, O_2 availability and inorganic or organic carbon concentrations occurring in each treatment to varying degrees may also have limited these pathways [89]. For instance, nitrification rates are reduced by a high concentration of carbonaceous-BOD in wastewater, a situation that would have been exacerbated by the deliberate organic carbon enrichment with glucose carried out in the WWG and WWG+*C.v* treatments in the experiment reported here [507, 508]. Furthermore, the removal of NH_3-N to almost below detection limits in the WWG+*C.v* treatment occurred within only 2 days, resulting in resource limitation for nitrifying bacteria and probably well before nitrification had a chance to begin. The pH increase in the WW+*C.v* treatment, and inferred low inorganic carbon availability will have limited the activity of nitrifying bacteria and formation of NO_2-N or NO_3-N [449]. Although a small increase in NO_2-N was detected in this treatment (i.e. from 0.02 to 0.07 mg L^{-1} at day 1), this did not coincide with an equivalent amount of NH_3-N removed over the 5-day duration, indicating that nitrification was not the dominant pathway in reducing NH_3-N from the PSW. Inorganic N concentrations in the control treatments (WWC and WWG) remained fairly constant over the 5-day

duration of these experiments, with the exception of NH_3-N declining slightly within the first day in the WWG treatment, but which was not significant (*H* (3) = 10.421, *p* = 0.307 at day 1). This reduction can be ascribed to a high metabolic activity of the microbial community present in the PSW as a result of the exogenous glucose, which coincided with a decrease in total carbohydrate concentration (Figure 5.2A). A major limitation to these control treatments was the low concentration of dissolved O_2, which can be attributed to the cultures having been incubated statically (Figure 5.2C). In the WWC and WWG treatments, O_2 concentrations were 1.36 ±0.18 and 0.54 ±0.11 mg L^{-1} respectively. This will have not only impacted on the metabolic activity of the endogenous microorganisms in digesting and assimilating inorganic N, but also nitrifying bacteria dependent on O_2 for converting them by nitrification and thus limiting their removal [1, 89].

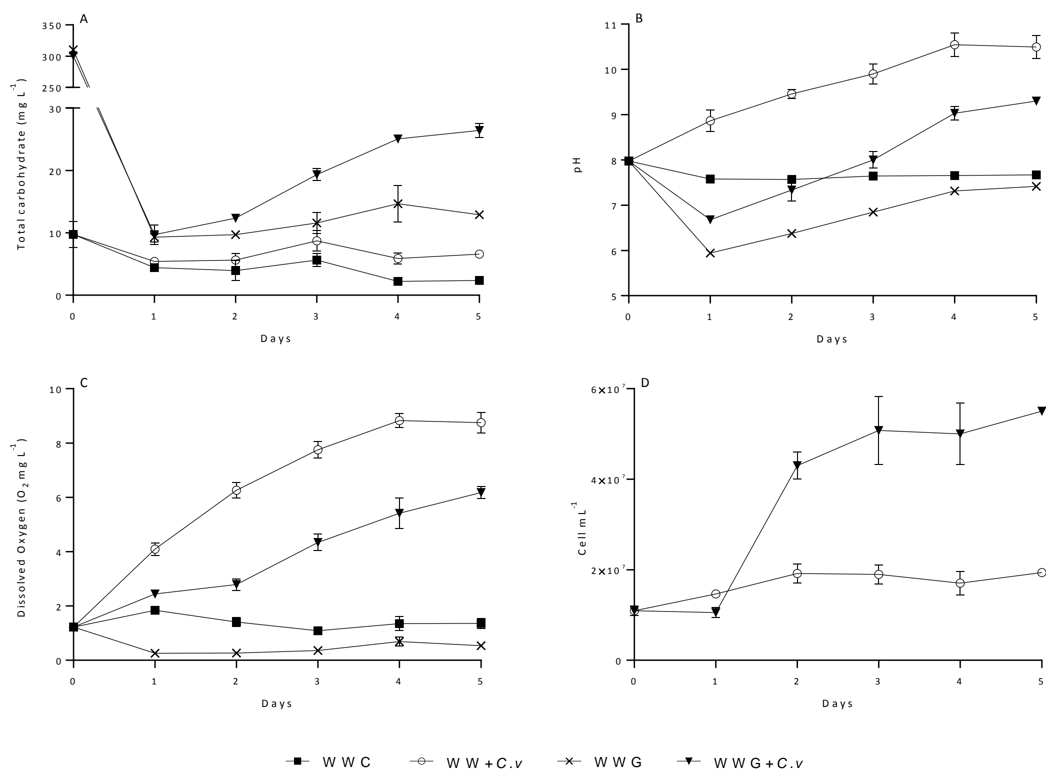


Figure 5.2 – Changes in PSW total carbohydrate concentration (**A**) in mg L^{-1}, pH (**B**), dissolved O_2 concentration (**C**) in mg L^{-1} O_2 and *C. vulgaris* concentration (**D**) in cell $mL^{-1}$for each treatment for the duration of the experiment. Data points are mean ±SD, of n = 3 independent replicates. Some error bars are smaller than the symbols. Treatment WWC (wastewater only); treatment WW+*C.v* (wastewater with *C. vulgaris*); treatment WWG (wastewater with glucose); and treatment WWG+*C.v* (wastewater with glucose and *C. vulgaris*).

The addition of glucose also had a significant effect on inorganic P removal. In the WWG+*C.v* treatment, PO_4-P was drastically reduced from 3.2 ±0.02 to 0.1 ±0.01 mg L^{-1} at day 1 and remained at this concentration until the end of the treatment period (Figure 5.1B) (*H* (3) = 10.385, *p* = 0.002 at day 1).

This was a maximal removal efficiency of 96% within a period of 1 day. Notably, this is a far higher recorded rate than reported in previous studies using PSW which had reported removal efficiencies of less than 50% for the same retention time [136, 150, 509]. Similar to the observed difference in NH_3-N removal between the WWG+*C.v* treatment and the WW+Air+*C.v* treatment discussed in Chapter 4, the reduction of PO_4-P below the detection limit in the WWG+*C.v* treatment was achieved within a shorter retention period at day 2 compared to day 4 (Figure 4.3B). As previously detailed (Section 4.3.2.2), the efficiency of P removal is affected by both abiotic and biotic factors. In pH environments of approximately 9 or above, for example, soluble PO_4^{3-} precipitates as a result of chemically reacting with cations in solution, mostly magnesium and calcium ions [376, 454]. In regards to biotic influences, research has indicated that P assimilation by algae is dependent on the overall bioavailable N concentration in the water, whereas N uptake is independent of P [453]. The reason for this is unclear, but a working theory suggests the dependence of P assimilation on N is because of the nutrients respective functions in cellular metabolism. Nitrogen is mainly integrated into proteins necessary for biological activities in a cell, so a low supply of N will thus limit the synthesis of proteins [510, 511]. A reduction in protein synthesis is accompanied by a reduction in ribosome abundance as well as the quantity of RNA transcribed. Given that the majority of intracellular P is present in ribosomal RNA, low protein expression levels as a result of N limitation will most likely result in lower cellular demand for P because of a reduction in ribosomes required [512]. Indeed, this physiological explanation has been observed in studies in which the supply of N to microalgae was limited, resulting not only in a lower concentration of N but also of P in the biomass [513].

Given the high removal efficiency of NH_3-N under neutral pH in the WWG+*C.v* treatment and exponential growth of *C. vulgaris*, it can be inferred that the main mechanism for PO_4-P removal was through assimilation by *C. vulgaris* and other microorganisms, such as bacteria, present in the wastewater and/or associated with the microalga mainly for direct metabolic use (Figure 5.1A; Figure 5.2B, D). In comparison, PO_4-P removal in the WW+*C.v* treatment was a result of its assimilation initially and subsequent precipitation after day 1 because of a gradual increase in the pH above 9 (Figure 5.1B; Figure 5.2B). Here, PO_4-P concentrations decrease from 3.2 ±0.02 to 1.7 ±0.02 mg L^{-1} at day 1, and then continued to decrease reaching a minimal concentration of 0.8 ±0.02 mg L^{-1} at day 4. The low removal and consequent assimilation rate of NH_3-N by *C. vulgaris* will have likely influenced the internal N concentration of the microalgae, thus also affecting the assimilation of P in this treatment [453]. However, the continuous removal of P by the microalgae through luxury uptake after day 1 in the WW+*C.v* treatment cannot be ruled out [514]. This same trend of a slow decrease in PO_4-P after day 1 was not observed in the WWG treatment despite displaying a similar reduction in NH_3-N and PO_4-P for the initial day of treatment as in the WW+*C.v* treatment. The reduction in PO_4-P concentration in the WWG treatment by day 1 was likely through its assimilation and incorporation by the indigenous microbial community present in the PSW, concurrent with the reduction of NH_3-N. As anoxic conditions developed in the control treatments, assimilation and degradation of the inorganic compounds will have

slowed (Figure 5.2C). However, as the pH did not increase above 8 in these treatments, no substantial decrease in PO_4-P could be attributed to PO_4^{3-} precipitation.

In general, the characterisation of the wastewater revealed significant reductions in NH_3-N and PO_4-P in the WWG+*C.v* treatment within just 2 days. In the control treatments (without microalgae) the most effective decline in NH_3-N and PO_4-P was in the WWG treatment by day 1, while WWC exhibited no noteworthy change from the initial concentrations of the PSW. Although the degree of NH_3-N and PO_4-P removed in the WWG treatment by the naturally occurring heterotrophic organisms will have also contributed to the removal of these inorganics in the WWG+*C.v* treatment, their overall contribution can be considered minimal. Based on the values obtained, in the WWG treatment at total of 7.4 mg NH_3-N and 1.2 mg PO_4-P were removed by day 1, whereas in the WWG+*C.v* treatment 24.4 mg NH_3-N and 3.1 mg PO_4-P were removed by day 1. In view of the higher reduction in NH_3-N and PO_4-P achieved when inoculated with *C. vulgaris*, from the net difference between the WWG and WWG+*C.v* treatment it can be argued that the microalgae were chiefly responsible at remediating the inorganic N and P from the PSW. As previously discussed in Section 4.3.2.3 of Chapter 4, a potential factor limiting the influence of the microbial community in removing inorganic N and P from the wastewater was the microbial-community not being composed of microorganisms able to consume substantial quantities of these compounds. To fully elucidate the precise quantities and removal efficiencies of NH_3-N and PO_4-P from the wastewater by the microalgae alone, sterile glucose enriched PSW should be treated with an axenic strain of *Chlorella* under the present culture conditions.

Furthermore, the high pH environment and dissolved O_2 concentrations formed in the microalgae treatments may have limited the microbial population in removing inorganic N and P from the wastewater, in particularly in the WW+*C.v* treatment (Figure 5.2B, C). An alkaline environment (discussed below) in conjunction with a high dissolved O_2 concentration in a light environment mediates photo-oxidative destruction of coliform bacteria [515, 516]. Ansa et al., (2012) [283] observed that the increase in faecal coliform rate of decay in raw domestic wastewater correlated with an increase in chlorophyll-*a* concentration, with the microalgae responsible for a rise in pH and dissolved O_2. Marchello et al., (2015) [394] reported no difference in coliform and *Escherichia coli* concentrations between aerated and non-aerated microalgae-bacteria co-cultures treating secondary treatment effluent. In both these treatments the concentration of bacteria declined abruptly by day 2, resulting in a 99% reduction in colony forming units concurrent with a high pH environment. Overall, the dissolved O_2 concentration in the WW+*C.v* and WWG+*C.v* treatments had a tendency to increase during the 5-day treatment period to 8.7 ±0.4 and 6.2 ±0.2 mg L^{-1}, respectively, indicating a prevalence of photosynthetic activity over heterotrophic carbon-oxidation.

However, the high dissolved O_2 concentration in the microalgae treatments may have had a negative effect on *C. vulgaris*. Dissolved O2 generated *in situ* via photosynthesis can negatively affect microalgal productivity. For example, Molina et al., (2001) [348] observed a 17 and 25% reduction in photosynthetic activity by the microalga *Phaeodactylum tricornutum* in medium with dissolved O_2

saturation levels of 200 and 300%, respectively. The negative effect is mainly a result of the competitive nature of O_2 with CO_2 for the active site of RuBisCO [67]. In the Calvin cycle, RuBisCO actively fixes CO_2 to form two compounds of 3-phosphoglycerate in the carboxylation reaction of ribulose 1, 5-bisphosphate. These compounds are recycled in the Calvin cycle and the formed products are used in carbon metabolism of respiration or storage components. However, in the presence of O_2, RuBisCO fixes O_2 to form one molecule of 2-phosphoglycolate and one of 3-phosphoglycerate. Although 2-phosphoglycolate can be converted back to 3-phosphoglycerate following its conversion into glyoxylate, additional expense of ATP is necessary. Furthermore, more CO_2 needs to be fixed in the Calvin cycle to compensate for the loss of the carbon substrates. This requires additional ATP and NADPH, which are generated in the light reaction of photosynthesis thereby increasing the indirect cost associated with O_2 fixing. Overall, O_2 fixing diverts energy and carbon compounds that would otherwise be used to support microalgal growth and thus reduce microalgae productivity [517]. Although the minimal growth observed in the WW+*C.v* treatment can mainly be assigned to the lack of readily available carbon substrates, the high dissolved O_2 concentration will also have impacted on *C. vulgaris* ability to fix inorganic carbon under the conditions which formed. Another aspect caused by a high O_2 concentration is photo-oxidative damage to PS II through the formation of reactive oxygen species, thus a dissolved O_2 concentration of 1.9 mg L^{-1} or less in microalgae cultures is recommended [518, 519].

5.3.1.2 Organic nutrient removal

Under aerobic conditions, organic substrates in wastewater are removed through oxidative biodegradation and incorporation for biosynthesis predominantly by heterotrophic bacteria [1]. Owing to the mixotrophic nature of *C. vulgaris*, it will have participated together with the indigenous bacterial community in the PSW and that associated with the micro-alga for the collective removal of bioavailable organics from wastewater [205]. Figure 5.2A shows the total carbohydrate (TC) concentrations for each of the treatments throughout the culture period. Without enrichment with glucose, the initial TC concentration was 9.7 ±2.1 mg L^{-1}, which was lower than the theoretical range of 50 to 120 mg L^{-1} for municipal wastewater, as suggested by Gray (2004) [1]. The TC concentration in the WW+*C.v* and WWC treatments declined only slightly to an average 4.6 ±6.5 mg L^{-1} after 1 day for both treatments, with no substantial change thereafter. In the enriched treatments (WWG and WWG+*C.v*) the TC concentration declined rapidly from an initial concentration of 305.1 ±6.5 to 9.2 ±1.1mg L^{-1} after 1 day. It can be inferred that glucose was completely removed within this time since its concentration reached initial concentrations recorded in the non-enriched (WWC) treatment. The COD results further confirm the removal of the glucose from the enriched treatments (Table 26), as shown by a removal of approximately 67% in the WWG and WWG+*C.v* treatments, with final COD readings of 138.3 ±3.1 and 133.6 ±9.1 mg L^{-1} O_2, respectively. Based on the TC profiles of both glucose-enriched treatments, it cannot be conclusively stated whether the microbial community present in the PSW or microalgae were chiefly responsible for the removal of the exogenous glucose. The necessary active hexose/H^+ symporter system, by which glucose from the medium is assimilated in microalgae, including in *Chlorella*

spp., is known to be induced (by protein expression) within 15 to 18 minutes upon glucose detection [520, 521]. Despite this fast acclimation response, previous research identified heterotrophic bacteria to always exhibit higher glucose-specific uptake rates compared to *Chlorophyta* in either batch, chemostat or discontinuous feed cultivation conditions [522]. However, a lower residual glucose concentration was achieved in experiments with only alga than that in cultures with only heterotrophic bacteria. A potential study to clarify the degree of glucose consumption by the algae and bacteria would be to use ^{13}C isotopic labelled glucose [523].

Interestingly, the beginning of the *C. vulgaris* stationary growth phase at day 2 in the WWG+*C.v* treatment coincided with an increase in TC concentrations (Fig. 5.2A, B). Henderson et al., (2008) [524] reported an increased production of dissolved organic carbon during the stationary growth phase for various microalgal species, and this was attributed to the excretion of extracellular polysaccharide substances (EPS) by the microalgae. Hence, the observed increase in TC concentrations after day 2 in the WWG+*C.v* treatment could be attributed to EPS production during the stationary phase [525]. Various EPS compounds are known to be produced by different microalgae, including *Chlorella* spp., with glucose as a common carbon source in the culture media [526, 527].

5.3.1.3 Growth and pH

It was initially proposed that indigenous microorganisms, particularly bacteria, in the PSW samples would out-compete *C. vulgaris* for organic and inorganic resources, and result in limiting the alga's growth and ability to the remove N, P and the exogenous glucose that was added. The results, however, indicate that the removal of these components in PSW is enhanced by the inoculation of *C. vulgaris* together with the supplementation of glucose. Indeed, the addition of glucose had a distinctly positive effect on the growth of *C. vulgaris* (treatment WWG+*C.v*) compared to no substantial growth observed in the absence of glucose (treatment WW+*C.v*) (Figure 5.2B). Although cell counts in the WW+*C.v* treatment did not indicate any growth of the microalgae by cell numbers, the biomass measurements were seven times higher compared to that in the WWC treatment which did not contain glucose and was not inoculated with the alga, with dry weights of 280.8 ±16.6 and 42.8 ±2.2 mg L^{-1} for the treatments respectively. The WWG+*C.v* treatment had the highest biomass yield with 419.1 ±4.5 compared to 111.7 ±13.0 mg L^{-1} for the WWG treatment.

Variations in pH occurred in all four treatments, with the highest degree of change observed in the WW+*C.v* treatment (Figure 5.2D). As previously detailed in sections 2.2.2.1 and 4.3.1, the alkalisation of the PSW in this treatment is a consequence of the fixation of CO_2 by RuBisCO, which is converted from HCO_3^-. In brief, this photosynthetic-driven process leaves OH^- ions in the cell which have to be neutralised with H^+ ions that are taken up from the extracellular environment, resulting in an increased extracellular pH [419]. The knock-on effect is a decrease in the CO_2 to bicarbonate ratio, and eventually a reduced absolute CO_2 concentration. The alkalisation also suggests a reduction and consequent limitation in inorganic carbon because of its ability to buffer pH changes in the medium environment.

Moreover, the unfavourable (high pH) environment present may also have limited the growth of other members of the microbial community in the PSW and thus reduced their production of CO_2 via respiration that would have otherwise served *C. vulgaris* with an alternative source of this essential compound for photosynthesis. Furthermore, the pH increase in the WW+*C.v* treatment will have had a strong influence on its NH_3-N removal efficiency (Figure 5.1A). While NH_4^+ is the preferred inorganic N source for microalgae, a rise in pH above 8 leads to its dissociation to form free NH_3, which is toxic to microalgae [429]. The pH in this treatment increased from 7.97 to 10.49 at a relatively constant rate over the 5-day duration of these experiments (Figure 5.2D). This will have contributed to the formation of free NH_3, creating an unfavourable environment for nutrient assimilation and microalgae growth. The resultant drop in NH_3-N removal after day 1 in the treatment supports the lack of available carbon before the onset of ammonia toxicity, most likely because of the low inorganic carbon to the microalgae will have limited the assimilation of NH_3-N.

Conversely, the pH in the glucose-enriched treatments decreased rapidly within the first day to below 6.6 for the WWG+*C.v* and to below 5.9 for the WWG treatments (Figure 5.2D). This drop in pH coincided with the removal of the added glucose in both treatments, suggesting that acidification of the PSW did not negatively affect the consumption of this substrate (Figure 5.2A). The anoxic environment in the WWG treatment will have driven the degradation of organic compounds, including glucose, to produce organic acids through the process of acidogenesis and acetogenesis, and thus also the observed pH reduction in this treatment (Figure 5.2C) [89]. It should also be noted that the pronounced removal of NH_3-N and PO_4-P will have also influenced the overall extracellular H^+ concentration and thus influencing the observed shifts in pH values. An abrupt decline in the concentrations of these nutrient pollutants can lower the pH, as observed in the WWG+*C.v* treatment with NH_3-N the main N source in the PSW [143, 429].

5.3.2 Treatment reproducibility assessed across PSW samples and alternative carbon sources

The small-scale treatment of PSW with the addition of exogenous glucose was used to evaluate the growth of *C. vulgaris*, its removal of inorganic compounds, and to analyse for other biochemical and physical changes under the different treatment regimens evaluated. This provided a useful understanding of the treatment performance under static culturing conditions revealing that it was limited, either because of the limited bioavailability of carbon to the microalga or detrimental effects from pH changes. In order to upscale this into a commercially-viable system, we would need to demonstrate that this process can be consistently replicated with PSW collected at any time to take into consideration biotic and abiotic variability of the wastewater throughout the year. To investigate this, a further three batches of PSW were collected and treated separately and sequentially with *C. vulgaris* employing the same static culturing approach as described and evaluated above. In addition to enriching with glucose, treatments with glycerol and CO_2 were also included to compare between the use of a different organic and inorganic carbon source.

The NH_3-N and PO_4-P concentration profiles for each treatment in the different PSW batches are shown in Figure 5.3. In general, the efficiency in NH_3-N and PO_4-P removal across the batches of PSW was effective and reliable in the treatments with exogenous organic carbon. The treatments enriched with glucose or glycerol exhibited a similar trend with respect to their removal of NH_3-N and PO_4-P, with a respective 91% and 98% final average efficiency in both treatments (both $p < 0.01$ at day 2). Notably, when comparing between the individual experimental runs, the minimum NH_3-N concentration achieved increased with an increase in initial concentration. For instance, in PSW batch R1 the concentration of NH_3-N declined from an initial concentration of 23.4 ±0.2 mg L^{-1} to below the detection limit (LOD 0.09 mg L^{-1}) in both the WWG+*C.v* and WWGY+*C.v* treatments at day 2 (Table 27; Figure 5.3A). Conversely, a higher minimum NH_3-N concentration was achieved for the same treatments of PSW batch R2 and R3 at day 3; these PSW batches had a higher initial NH_3-N concentration of 34.9 ±0.5 and 34.7 ±0.2 mg L^{-1}, respectively (Table 27). The concentration of NH_3-N in the WWG+*C.v* and WWGY+*C.v* treatments at day 3 were, respectively, 1.03 ±0.01 and 0.41 ±0.01 mg L^{-1} in PSW batch R2, and 2.31 ±0.02 and 3.04 ±0.01 mg L^{-1} in PSW batch R3 respectively (Figure 5.3C, E). A similar observation can be drawn based on the TN concentrations of the organic carbon enriched treatments in each of the different PSW batches, as a higher initial concentration led to a higher final concentration (Table 28). For example, the highest initial TN concentration was recorded in PSW batch R3 and, consequently, this PSW batch exhibited the highest final TN concentration which was above the permissible maximum 10 mg L^{-1} by the UWTD in the organic carbon enriched treatments. No such effect was observed for the PO_4-P concentration in the organic carbon enriched treatments (Figure 5.3B, D, and F).

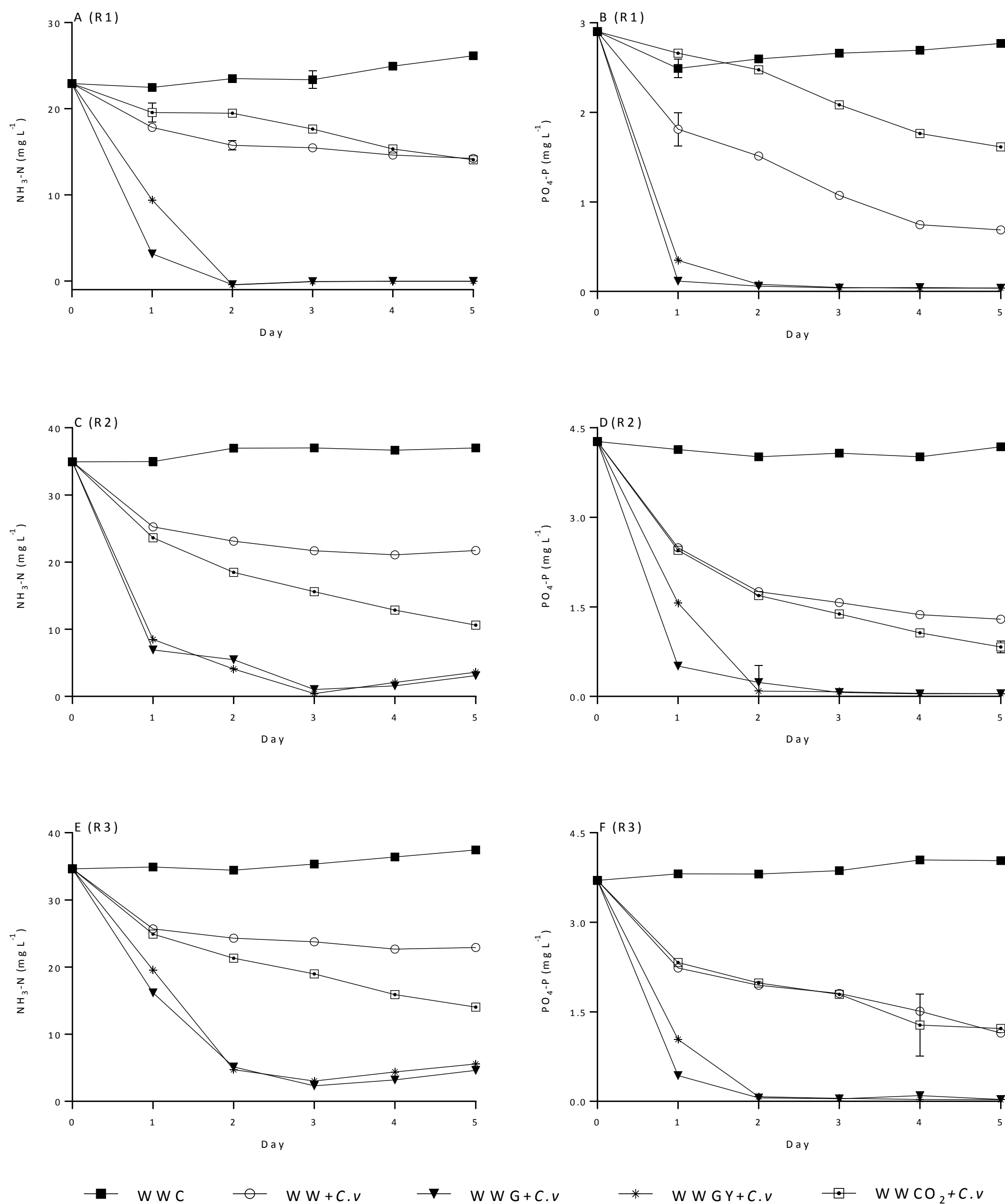


Figure 5.3 – Changes in the PSW concentrations for NH_3-N (**A**, **C**, **E**) and PO_4-P (**B**, **D**, **F**) in mg L^{-1} for PSW batch R1 (**A**, **B**), R2 (**C**, **D**) and R3 (**E**, **F**) treated under the conditions with or without *C. vulgaris*, and enriched with or without glucose, glycerol or CO_2. Each point is a mean ±SD, of n = 3 (pseudo replicates for each treatment per batch). Treatment WWC (Wastewater only); treatment WW+*C.v* (Wastewater with *C. vulgaris*); treatment WWG+*C.v* (Wastewater with glucose and *C. vulgaris*); treatment WWGY+*C.v* (Wastewater with glycerol and *C. vulgaris*); and treatment $WWCO_2$+*C.v* (Wastewater with CO_2 and *C. vulgaris*).

Table 27 – Initial inorganic N and P concentrations and pH of each PSW batch used in the experiment to validate the reproducibility of the static treatment process; values are a mean ±SD, of n = 3 (pseudo replicates) with concentrations reported in mg L^{-1}.

PSW batch	NH_3-N	PO_4-P	NO_2-N	NO_3-N	pH
R1	23.4 ±0.2	2.9 ±0.1	0.30 ±0.0	0.41 ±0.0	7.42
R2	34.9 ±0.5	4.3 ±0.3	0.03 ±0.0	0.06 ±0.0	7.36
R3	34.7 ±0.2	3.7 ±0.1	0.03 ±0.0	0.06 ±0.0	7.42

Table 28 – Initial and final concentrations of COD (mg L^{-1} O_2) and TN (mg L^{-1}) for each PSW batch (R1, R2 and R3) when inoculated with or without *C. vulgaris*, and enriched with or without glucose, glycerol or CO_2; values are a mean ±SD, of n = 3 (pseudo replicates).

PSW batch	Treatment	COD		TN	
		Initial	Final	Initial	Final
R1	WWC	113 ±5.3	65 ±0.7	29.8 ±0.2	24.4 ±0.8
	WW+*C.v*	113 ±5.3	85 ±3.5		17.5 ±0.2
	WWG+*C.v*	379 ±4.1	109 ±0.7		5.3 ±0.0
	WWGY+*C.v*	392 ±7.0	95 ±3.5		5.1 ±0.1
	$WWCO_2$+*C.v*	113 ±5.3	75 ±2.1		15.5 ±0.5
R2	WWC	219 ±10.0	92 ±3.5	38.7 ±1.8	36.3 ±0.5
	WW+*C.v*	219 ±10.0	82 ±2.1		23.8 ±0.9
	WWG+*C.v*	513 ±9.1	103 ±2.1		9.4 ±0.8
	WWGY+*C.v*	520 ±16.3	119 ±7.7		9.6 ±0.3
	$WWCO_2$+*C.v*	219 ±10.0	94 ±6.3		16.0 ±0.0
R3	WWC	182 ±6.1	104 ±1.4	44.5 ±0.7	36.8 ±0.4
	WW+*C.v*	182 ±6.1	83 ±0.7		26.0 ±0.4
	WWG+*C.v*	482 ±3.1	153 ±0.7		12.0 ±0.1
	WWGY+*C.v*	477 ±4.2	116 ±0.7		11.9 ±0.1
	$WWCO_2$+*C.v*	182 ±6.1	89 ±0.7		20.5 ±0.3

These observations suggest that there is a limitation between the maximum N concentrations that could be treated in the presence of the enriched carbon quantities added to the PSW batches in this study. Given the necessity for keto-skeletons required for the incorporation of inorganic N into microalgal biomass, this observation can be explained by the fact that a higher N load in the PSW would inferably require a greater quantity of carbon by the microalgae. In the present study, a similar COD removal efficiency in all the organic carbon-enriched treatments was recorded (Table 28). Across all these treatments an average 74% COD was removed with a maximum 79% and a minimum 68% COD removal achieved in the WWG+*C.v* treatment of PSW batch R2 and R3 respectively. In all these treatments the decline in COD was equivalent to the quantity of organic carbon added (Table 28). In the WWG+*C.v* and WWGY+*C.v* treatments of PSW R2 the quantity of carbonaceous material removed was higher than just the quantity of exogenous organic carbon, at a respective 410 and 401 mg. The higher quantity of carbonaceous removal in the PSW batch R2 may be a result of higher levels of readily available carbonaceous material. However, given the higher maximum NH_3-N concentration achieved, despite

the higher concentration of carbonaceous material consumed, it can be suggested that additional reduction may have been by the naturally occurring microbial community and not exclusively by *C. vulgaris*. Moreover, the higher COD removal did not appear to reflect itself in microalgae growth. The maximum microalgal cell concentrations reached were lower in PSW batches R2 and R3 (Figure 5.4A, C and E). *C. vulgaris* increased to above 4.5 $x10^7$ cells mL^{-1} in PSW batch R1, with a maximum cell concentration of 6.08 $x10^7$ ($\pm 3.2x10^6$) cells mL^{-1} at day 3, and 4.65 $x10^7$ ($\pm 2.8x10^6$) cells mL^{-1} at day 4 for the treatments enriched with glucose and glycerol, respectively. In comparison, in PSW batches R2 and R3 the maximum cell concentration reached in either of the organic carbon enriched treatments was below 4.5 $x10^7$ cells mL^{-1}.

Previous research has demonstrated that adjustments in carbon availability affect depuration of N from wastewater or other medium, and vice versa, by microalgae. For instance, Petrovic and Simonic (2015) [528] noted that at lower NO_3 concentration, the N content of the medium was more efficiently removed than under higher NO_3 concentration when each condition contained an equal quantity of 0.5 mg L^{-1} sucrose. In this study, NO_3-N removal efficiency was 92% at an initial concentration of 50 mg L^{-1} by *C. sorokiniana*, whereas 88% removal efficiency was achieved at an initial concentration of 100 mg L^{-1}. Similarly, Gupta et al., (2016) [85] recorded a significantly higher quantity of TN and TP removed by *C. vulgaris* in wastewater supplemented with 5 g L^{-1} glucose compared to with 2 g L^{-1} glucose. Interestingly, however, an increase in glucose concentration above 5 g L^{-1} did not result in a significant difference in final N and P removed, but rather in a slight variation in the rate of removal. A higher glucose concentration promoted a higher removal rate with the fastest rate achieved at 30 g L^{-1}, which was the maximum glucose concentration evaluated in this study. Overall, the findings from this study and those of the above-mentioned studies indicate that a certain quantity of bioavailable carbon is necessary in order to ensure specific quantity of NH_3-N removal.

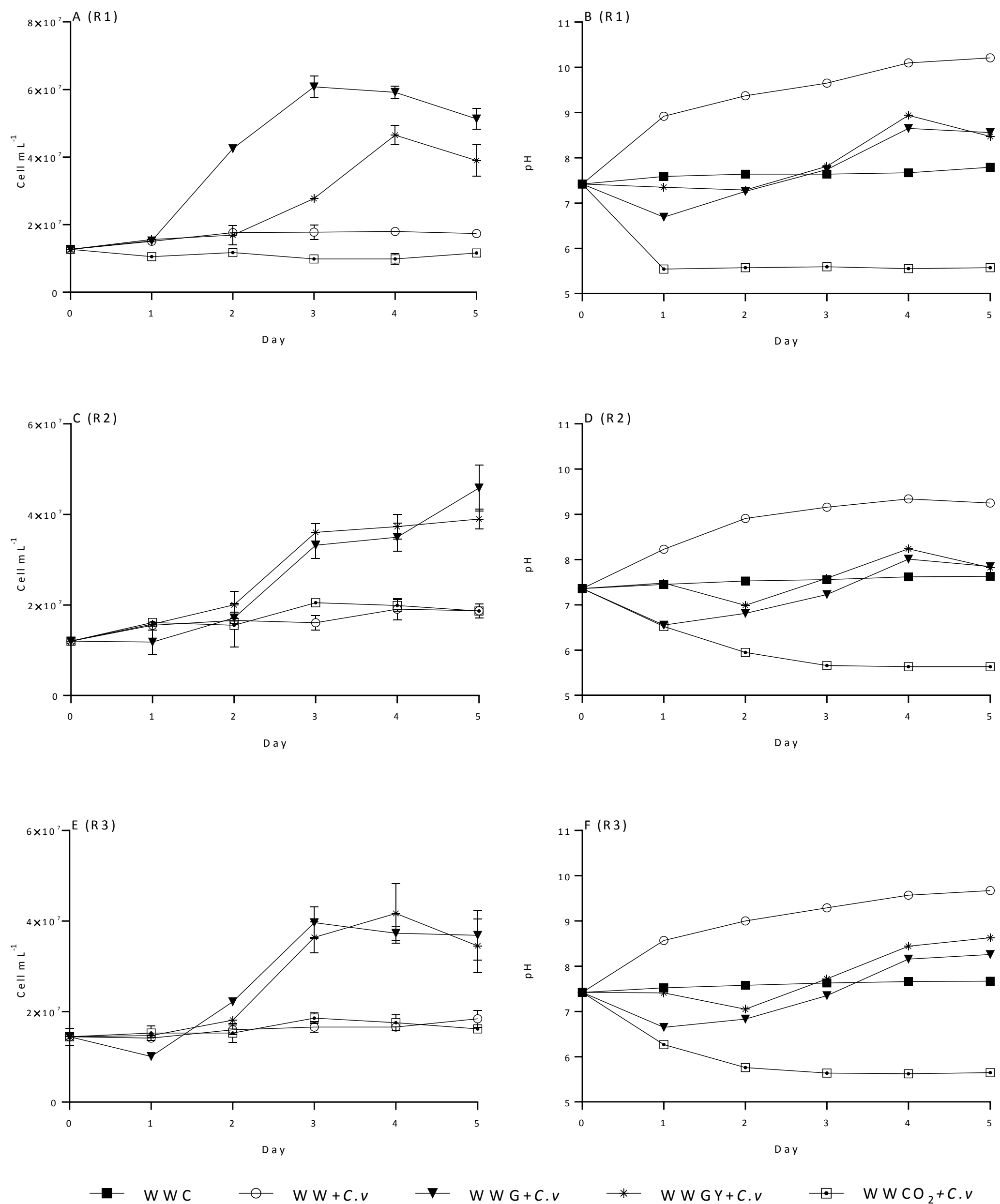


Figure 5.4 – Change in *C. vulgaris* concentration (**A, C, E**) in cell mL^{-1}, and pH (**B**, **D**, **F**) for PSW batch R1 (**A**, **B**), R2 (**C**, **D**) and R3 (**E**, **F**) treated under the conditions with or without *C. vulgaris*, and enriched with or without glucose, glycerol or CO_2. Cell concentration is expressed a mean ±SD, of n = 3 (pseudo replicates for each treatment per batch) and for pH one measurement was recorded per sample for each treatment. Treatment WWC (Wastewater only); treatment WW+*C.v* (Wastewater with *C. vulgaris*); treatment WWG+*C.v* (Wastewater with glucose and *C. vulgaris*); treatment WWGY+*C.v* (Wastewater with glycerol and *C. vulgaris*); and treatment WWCO$_2$+*C.v* (Wastewater with CO_2 and *C. vulgaris*).

Unexpectedly, the addition of CO_2 in the WWCO_2+*C.v* treatments seemed to provide little benefit in promoting microalgal growth and the removal of inorganic N and P (Figure 5.3). In fact, the WW+*C.v* treatments, which had no additional carbon or were mixed, demonstrated similar rates in growth and inorganic N and P removal. Although no direct negative impact was observed on the performance of *C. vulgaris* cultured with CO_2 at the concentration and frequency supplied in these experiments, the results suggest that the CO_2 had an inhibitory effect on the capacity of the microalgae to remediate N and P. It is known that the provision of inorganic carbon to microalgae improves photosynthetic efficiency and further improvement of productivity and growth rate [156, 200, 529, 530]. Acidification of the wastewater to a pH of approximately 5.6 in all WWCO_2+*C.v* treatments occurred, presumably because of an increase in carbonic acid from the CO_2 suggesting an accumulation of inorganic carbon in the medium. Conversely, the pH in organic carbon enriched treatments decreased slightly in the initial few days of treatment and increased once *C. vulgaris* stationary phase was reached, with none of the treatments increasing above pH 9 (Figure 5.4B, D and F). In comparison to the organic carbon enriched treatments, intermittent enrichment with CO_2 did not yield an improvement in microalgal growth or nutrient removal from the wastewater (Figure 5.3, Figure 5.4).

According to previous research, the supply of concentrated CO_2 to non-acclimated microalgae can lower or inhibit respiration because of its strong influence on photosynthetic efficiency [198], an effect described by [531]. This effect can be explained by the activity of carbonic anhydrase. In brief, the abundance of intracellular carbonic anhydrase is known to increase in microalgae cells when grown under atmospheric concentrations of CO_2, and to decrease when grown on air enriched with CO_2 [532] (study used 5% CO_2). When transferred to a high CO_2 environment, the upregulation of carbonic anhydrases in non-acclimatised microalgae can have a negative impact [531]. In these conditions, the high catalytic activity of intracellular carbonic anhydrase can cause acidification of the stroma, an environment which is physiologically alkaline [533, 534]. The resultant loss in pH control leads to an inhibition of the Calvin cycle (enzymes are pH dependent) and a loss of the proton gradient established across the thylakoid membranes necessary for ATP production, hence leading to a reduction in photosynthetic efficiency and the ability to fix CO_2. Moreover, CO_2 uptake is not easily controlled in microalgae unlike HCO_3^-, as CO_2 can easily diffuse through the membrane resulting in uncontrolled uptake [535]. Based on this physiological explanation, it can be inferred that *C. vulgaris* was not acclimated to the conditions of CO_2 enrichment that this strain was subjected to in the WWCO_2+C.v treatment. Prior to its inoculation in the wastewater, *C. vulgaris* was grown under atmospheric air conditions and consequently may have contained a high number of carbonic anhydrase, which increased its susceptibility to the intracellular inhibitory effects of CO_2. Moreover, this assumption supports the decline in removal efficiency of NH_3-N despite the presence of excess inorganic carbon (low pH) in the WWCO_2+*C.v* treatments. The rate of photosynthesis will have been impaired and directly impacting on the generation of carbon skeletons and ATP necessary for microalgal N assimilation and growth [244]. It is clear this point needs further investigation to drive firmer conclusions. Since the acidification of the stroma itself is hard to assess, the intensity of chlorophyll fluorescence could be measured as a proxy of

cell health to monitor photosynthetic efficiency [531]. Although the high concentration of CO_2 was probably the main cause of the effect, intermittent aeration is considered a suitable strategy for microalgae treatment of wastewater at a lower energetic cost, but control optimisation is needed in its application [536].

Results presented in the previous initial glucose enrichment experiment (Section 5.3.1) showed that nitrification and denitrification were not dominant pathways in reducing the NH_3-N in the organic carbon enriched static microalgae wastewater treatment process. Similar results were obtained in the reproducibility experiment, which substantiates this observation. Slight variations in the initial NO_2-N and NO_3-N concentrations were recorded between the PSW batches, however, this seemed to have no noteworthy effect on the treatment performance of the process (Table 27). The highest initial NO_2-N and NO_3-N concentrations were in PSW batch R1. In this PSW batch the NO_2-N concentration was reduced to below the limit of detection in all treatments, except in the WW+*C.v* treatment, and the NO_3-N concentration declined below the detection limit, except in the WW+*C.v* and WWCO_2+*C.v* treatments (Figure 5.5A, B). Slight fluctuations in both the NO_2-N and NO_3-N concentrations were recorded in all treatments in PSW batch R2 and R3 over the 5-day treatment period, nonetheless no substantial change occurred (Figure 5.5C – F).

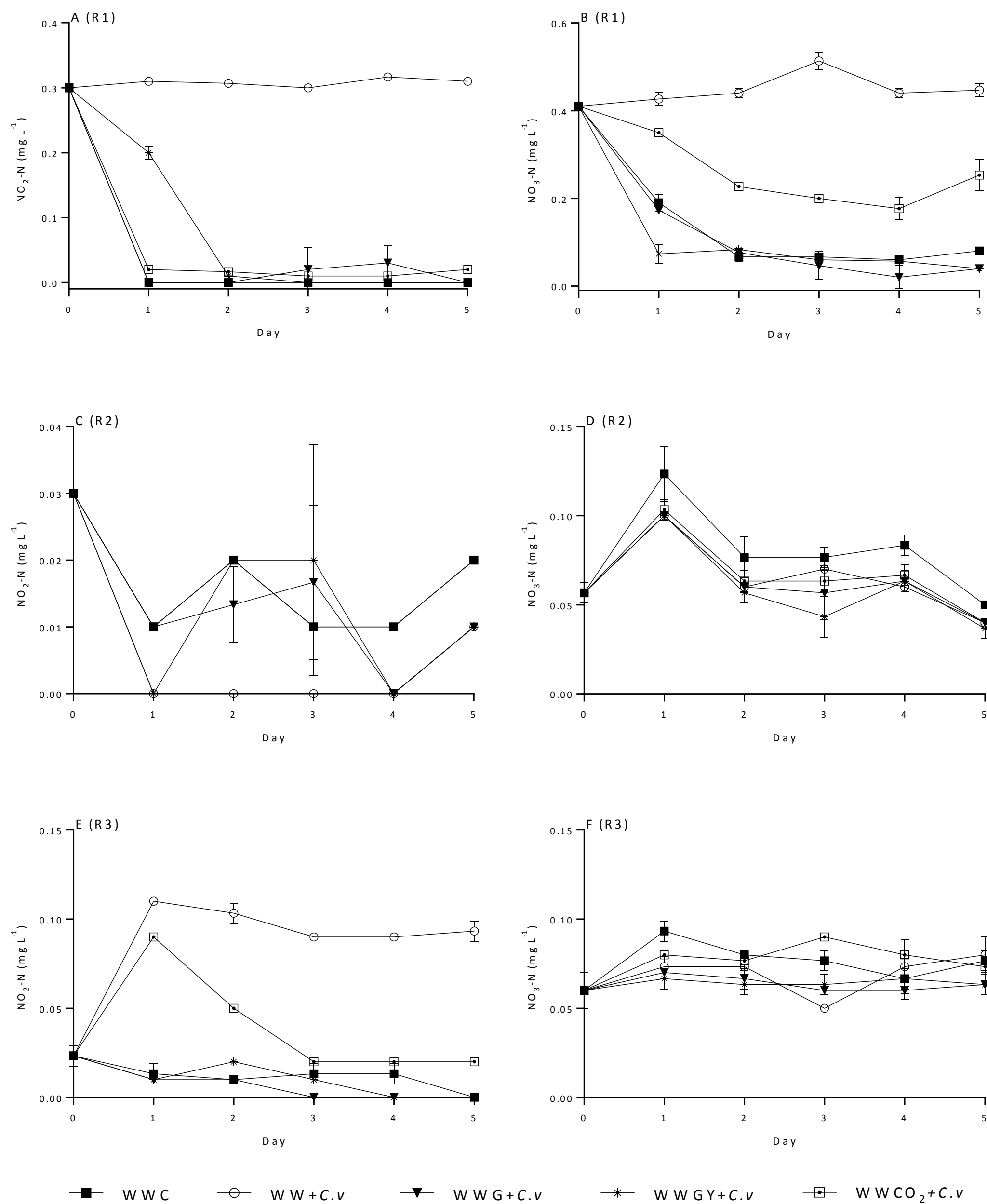


Figure 5.5 – Changes in the PSW concentrations for NO_2-N (**A**, **C**, **E**) and NO_3-N (**B**, **D**, **F**) in mg L^{-1} for PSW batch R1 (**A**, **B**), R2 (**C**, **D**) and R3 (**E**, **F**) treated under the conditions with or without *C. vulgaris*, and enriched with or without glucose, glycerol or CO_2. Each point is a mean ±SD, of n = 3 (pseudo replicates for each treatment per batch). Treatment WWC (Wastewater only); treatment WW+*C.v* (Wastewater with *C. vulgaris*); treatment WWG+*C.v* (Wastewater with glucose and *C. vulgaris*); treatment WWGY+*C.v* (Wastewater with glycerol and *C. vulgaris*); and treatment WWCO$_2$+*C.v* (Wastewater with CO_2 and *C. vulgaris*).

5.3.3 Pot ale enrichment of PSW

Results in the above experiments demonstrate that organic carbon enrichment had a positive effect on the performance of a microalgal process to treat unsterilized PSW without aeration. Although using glucose or glycerol as organic substrates is pertinent for research in a laboratory setting, from a commercial perspective these substrates entail a high cost at an industrial and large-scale application. Consequently, it is imperative that alternative, low-cost organic carbon substrates are identified and assessed for their applicability, ideally from a waste source. The following experiments investigated the effect of pot ale enrichment, a whiskey by-product, on the treatment efficiency of PSW by *C. vulgaris*. To verify its suitability, the experiment was replicated on three separate batches of PSW each with independently deproteinated pot ale samples, collected and treated independently and sequentially to understand any abiotic and biotic variability the wastewater or pot ale may have on the performance of treating the PSW.

5.3.3.1 Characterisation of pot ale and PSW batches

Pot ale composition, as well as the initial composition of each PSW batch (with and without pot ale amendment) was analysed immediately before commencing the experiments with the inoculation of the microalga see Table 29 and Table 30, respectively. Pot ale COD concentration was consistent across all samples, with a mean concentration of 42.8 ±1.9 g L^{-1} O_2, indicating a high oxidisable-carbon content. Inorganic analysis revealed NO_3-N to be the main inorganic N species, however, the average concentration across all the pot ale samples was low, at 0.36 ±0.1 mg L^{-1}. Both NO_2-N and NH_3-N concentrations were found to be negligible or below the limit of quantification for the assays used. To accurately determine NH_3-N and NO_3-N concentrations, it is worth to mention that an additional analysis was conducted on pot ale adjusted to pH 7. This was done to enable an alkaline environment to form, which is necessary for the chemical reaction of the assay following the addition of the reagents, but also to eliminate any potential interference that cations may have on the reaction (i.e. copper, magnesium or calcium) [379]. In brief, after pH adjustment the pot ale was left to stand for 1 hour under continuous shaking (100 rpm) and then re-filtered to 0.2 µm to remove any precipitation. No difference in NH_3-N and NO_3-N concentration was recorded between the non-adjusted (~3.3) and pH adjusted (7.0) pot ale sample (data not shown). In regards to TN, analysis revealed pot ale contained a high concentration, which will have come from organic fractions and varied in concentration between the samples. A similar result was reported by Barrena et al., (2017) [503] in which TN concentration varied between 440 and 1100 mg L^{-1} for deproteinated pot ale processed from four independent malt whiskey distilleries. The pot ale used in this study also contained a high amount of PO_4-P and TP, with average concentrations of 420.49 ±50.26 and 599.05 ±66.01 mg L^{-1}, respectively.

Table 29 – Physicochemical characterisation of three deproteinated pot ale sample from undisclosed malt whiskey distilleries; values are a mean ±SD, of n = 3 (pseudo replicates) with organic and inorganic N or P concentrations reported in mg L^{-1}, and COD concentration in mg L_{-1} O_2.

PA batch	NH_3-N	NO_2-N	NO_3-N	TN	PO_4-P	TP	COD	pH	mS/cm
P1	<0.1	<0.02	0.26	886	436	622	43100	3.32	4.33
P2	<0.1	0.14	0.57	696	442	634	41400	3.30	3.74
P3	<0.1	0.03	0.31	327	334	482	40700	3.28	4.18

NH_3-N and NO_3-N concentrations from pot ale with pH 7.

The initial inorganic N and P concentrations varied not only between the batches of PSW, but also within the same batch of PSW when enriched with pot ale (Table 30). Since pot ale is rich in inorganic P and carbonaceous material, whist containing trace quantities of inorganic N, the enrichment resulted in a substantial shift with respect to the canonical C/N/P ratio (herein based on COD/NH_3-N/PO_4-P concentrations) for freshwater algal growth (i.e. 100/15/1) [145]. In the pot ale enriched PSW, the C/N/P ratios ranged between 100/11/2 and 100/5/2.5, denoting a limitation in inorganic N, while without pot ale C/N/P ratios ranged between 100/25/3 and 100/16/4.

Table 30 – Physicochemical characterisation of each PSW batch; values are a mean ±SD, of n = 3 (pseudo replicates) with concentrations reported in mg L^{-1}.

PSW Batch	NH_3-N		PO_4-P		NO_2-N		NO_3-N		COD (mg L^{-1} O_2)	
	-	+	-	+	-	+	-	+	-	+
R4	20.9	19.6	5.7	9.8	0.02	0.02	0.05	0.05	130	393
R5	47.8	46.8	5.9	9.1	0.03	0.02	0.06	0.11	191	440
R6	35.2	34.4	4.4	7.2	0.03	0.02	0.05	0.06	168	415

"-", PSW without pot ale; "+", PSW with pot ale

5.3.3.2 Effect of enrichment with pot ale

Pot ale had a significant effect on the removal of both the NH_3-N and PO_4-P concentrations in PSW inoculated with *C. vulgaris* under static culture conditions. As shown in Figure 5.6, a clear depuration of these compounds in the WWPA+*C.v* treatment occurred in all three wastewater batches examined. In the case of NH_3-N, its concentration declined to below the detection limit only in the WWPA+*C.v* treatment of PSW batch R4, from an initial 20.9 ±0.09 to 0.09 ±0.0 mg L^{-1} at day 2 ($H(3) = 10.385$, $p = 0.016$ at day 1) (Figure 5.6A). In comparison, a higher final NH_3-N concentration was recorded in the WWPA+*C.v* treatments of PSW batch R5 and R6 after the 5-day treatment period; initial and final concentrations were respectively 47.8 ±0.09 and 17.7 ±0.9 mg L^{-1} in PSW batch R5 ($H(3) = 10.385$, $p = 0.013$ at day 1)(Figure 5.6C), and 35.2 ±0.03 and 4.7 ±0.2 mg L^{-1} in PSW batch R6 ($H(3) = 10.421$, $p = 0.013$ at day 1) (Figure 5.6E). This was a direct result of the wastewater containing a higher initial NH_3-N

concentration. Tam & Wong (1996) observed a similar response in *C. vulgaris* cultures with varying NH_3-N concentrations, in which a higher initial N concentration resulted in a lower removal efficiency and consequently higher residual N concentration. The effect described in the present experiment is in agreement with the observed response described in the reproducibility experiments when using glucose or glycerol as the organic carbon source (section 5.3.2).

As postulated in the reproducibility experiments, the resultant demise in NH_3-N removal in the WWPA+*C.v* treatments of PSW batch R5 and R6 may be a result of the wastewater having become limited in bioavailable carbon for the microalgae to utilise. This inference is based on the trend in COD concentration recorded daily in this experiment. In the WWPA+*C.v* treatment of PSW batches R5 and R6, the concentration of COD gradually declined after an initial rapid drop at day 1, with final COD concentrations at 154 ±2.9 and 122 ±6.6 mg L^{-1} O_2, respectively (Figure 5.7C, E). For PSW batch R5 and R6, a correlation is noted when the concentration of NH_3-N and COD of the WWPA+*C.v* treatments are juxtaposed in respect to their wastewater batch. In the WWPA+*C.v* treatments of PSW batch R5 and R6, the trend in COD concentration coincided with the decline in NH_3-N concentration, with the lowest recorded concentration of both parameters occurring at day 3. Thereafter, no substantial change in both the NH_3-N and COD concentrations were recorded for the remaining treatment period, indicating that further carbonaceous material and NH_3-N was not taken up by the microalgal-bacterial co-culture. At this time point the concentration of COD corresponded in part to the COD concentration recorded in the WW+*C.v* and WWC treatments of the respective PSW batch, from which it can be inferred that the exogenous carbonaceous material in the form of pot ale was completely removed. Furthermore, the high concentration of dissolved O_2 in these treatments compared to the controls (WWC and WWPA) can be considered as evidence of the near complete depletion of the bioavailable fractions of carbonaceous material in the PSW as no further aerobic degradation occurred (Figure 5.7B, D, F). In WWPA+*C.v* treatment in PSW batch R4, the trend in COD concentration was characterised by a slower initial decline untill day 3, at which point the concentration slightly increased before decreasing again to 172 ±14.2 mg L^{-1} O_2 at day 5 (Figure 5.7A). The slight increase in COD concentration at day 4 was probably a result of the accumulation of soluble degradable matter in suspension related to cell death. The increase in COD concentration coincided with a decline in *C. vulgaris* concentration (Figure 5.7A; Figure 5.8B).

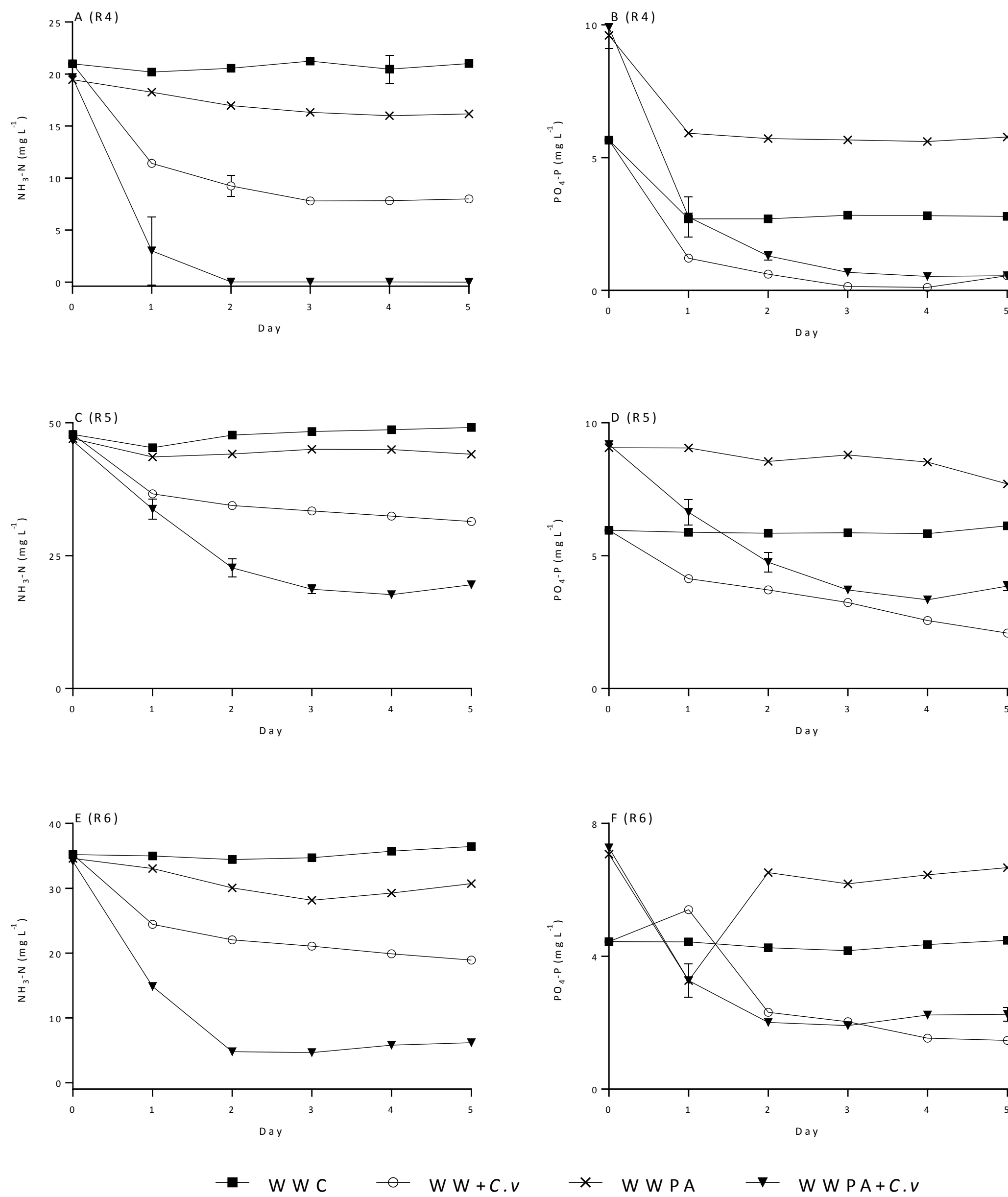


Figure 5.6 – Changes in PSW concentrations for NH_3-N (**A, C, E**) and PO_4-P (**B, D, F**) in mg L^{-1} for PSW batch R4 (**A, B**), R5 (**C, D**) and R6 (**E, F**) treated under the conditions with and without *C. vulgaris*, enriched with or without pot ale. Each data point is the mean ±SD, of n = 3 independent replicates. Some error bars are smaller than the symbols. Treatment WWC (Wastewater only); Treatment WW+*C.v* (Wastewater with *C. vulgaris*); Treatment WWPA (Wastewater with pot ale); and Treatment WWPA+*C.v* (Wastewater with pot ale and *C. vulgaris*).

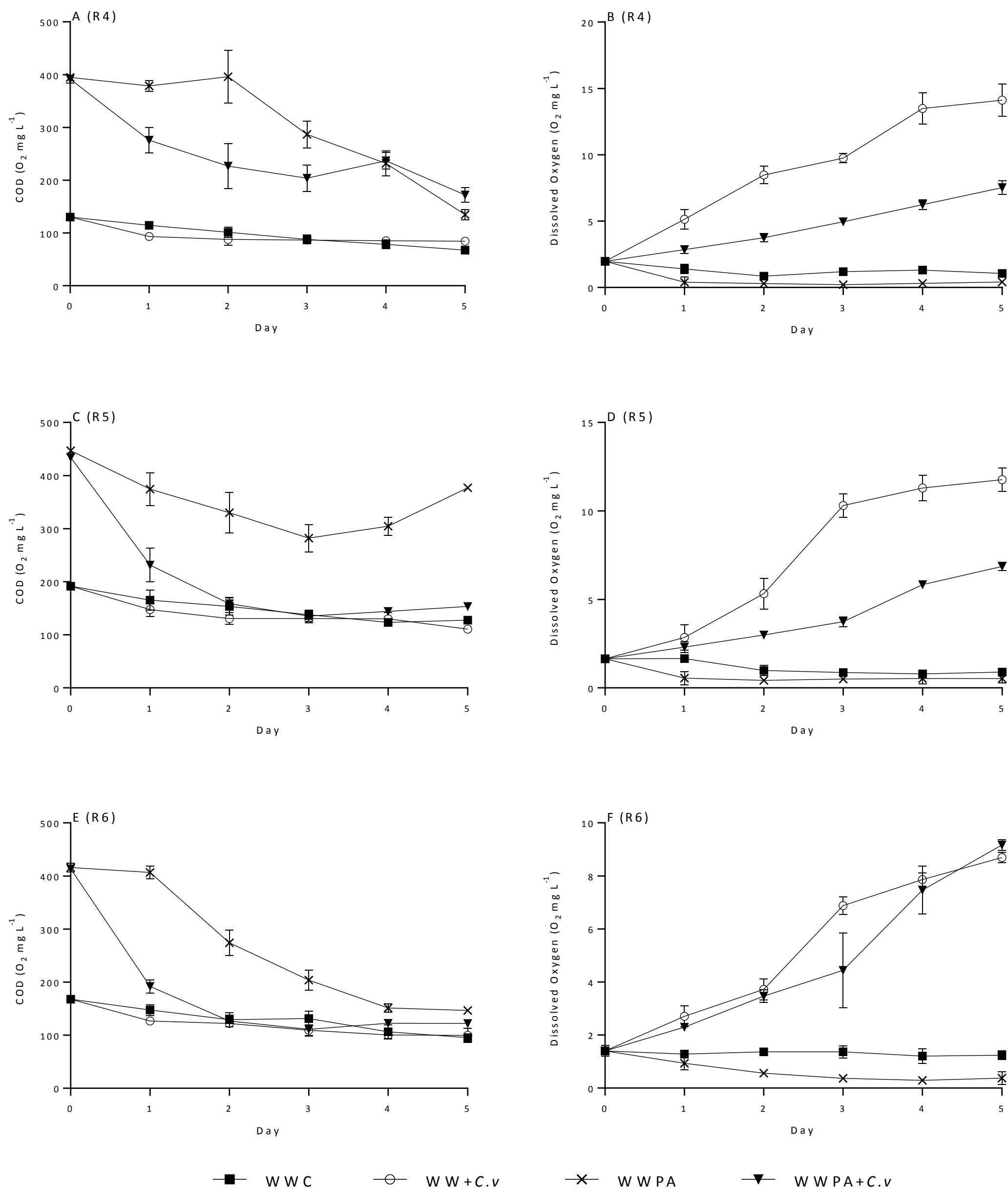


Figure 5.7 – Changes in COD concentration (**A, C, E**) and dissolved O_2 concentration (**B, D, F**) in mg L^{-1} O_2 for PSW batch R4 (**A, B**), R5 (**C, D**) and R6 (**E, F**) treated under the conditions with and without *C. vulgaris*, enriched with or without pot ale. Each data point is the mean ±SD, of n = 3 independent replicates. Some error bars are smaller than the symbols. Treatment WWC (Wastewater only); Treatment WW+*C.v* (Wastewater with *C. vulgaris*); Treatment WWPA (Wastewater with pot ale); and Treatment WWPA+*C.v* (Wastewater with pot ale and *C. vulgaris*).

Similar to the glucose and glycerol enriched treatments in the reproducibility experiment, the results from the WWPA+*C.v* treatments suggest a maximum removal capacity of NH_3-N that can be achieved by the microalgal-bacterial co-culture in relation to the quantity of pot ale added. In the WWPA+*C.v* treatments, the total quantity of NH_3-N removed at day 3 was 27.9 mg in PSW batch R5 and 29.5 mg in PSW batch R6. Given that pot ale in both these treatments amounted to an approximate 250 mg L^{-1} O_2 COD, it can be inferred that this quantity of carbonaceous material supported the removal of the above

amount of NH_3-N from the PSW in the experimental conditions. In line with this observation, the initial NH_3-N concentration of PSW batch R4 was below the suggested maximum and as such a complete reduction below detection limit in the WWPA+*C.v* treatment was achievable. However, based on data reported in the literature, it cannot be posited that increasing the concentration of bioavailable carbon will necessarily facilitate a greater quantity in N removal from the wastewater to permissible discharge concentrations (i.e. 10 mg L^{-1} TN: [14]). Growth and nutrient removal characteristics of *C. vulgaris* using artificial wastewater showed a complete removal up to a 21.2 mg L^{-1} NH_4-N concentration, while the removal efficiency dropped 50% at initial concentrations between 41.8 and 92.8 mg L^{-1} NH_4-N [538]. A similar observation was reported by Choi and Lee (2013) [539], with *C. vulgaris* cultured in sterile municipal wastewater amended with ammonium salt. A complete reduction in NH_4-N was achieved up to an initial concentration of 25.2 mg L^{-1}, a 50% decline in NH_4-N removal efficiency was recorded in the wastewater at concentrations exceeding 85.5 mg L^{-1}, and further decreased to less than 30% at concentrations above 105.4 mg L^{-1}. In both these studies, inorganic carbon (as CO_2) was supplied to the medium, either by direct aeration or shaking of the cultures, and as such the cultures were not carbon limited. These data suggest that the microalga *C. vulgaris* cannot remediate NH_3-N when its initial concentration is higher than a specific threshold value. However, this effect may be a result of the culture conditions and not because of the microalgae's ability. Both of the above studies were conducted in batch cultures with the microalgae reaching stationary growth. As a result, N uptake may have been limited because uptake is closely related to growth. As such it would be of value and interest to examine if a limitation occurred under continuous cultivation in which the microalgae are maintained in a perpetual state of exponential growth.

In regards to the use of brewery or distillery wastewater to grow microalgae, only a few studies have reported on the subject. Solovchenko et al., (2014) [540] carried out research on a semi-batch operated 50 L microalgae-bacteria treatment process of alcohol distillery wastewater using the mixotrophic strain *C. sorokiniana*. In this study, the treatment process was operated for 3 cycles, each run for a period of 4 days, with each cycle achieving significant COD removals, from approximately 20 to 1.5 g L^{-1} O_2. In a preliminary test, alcohol distillery wastewater treated by the endogenous microflora under aerated conditions with atmospheric air achieved no noteworthy COD reduction, demonstrating that the microalgae were vital to the reduce COD. In this study, it must be noted that the alcohol distillery wastewater was adjusted to pH 7 to ensure an optimum environment for the microalgae. In the present study, adjustment of the pot ale was not necessary mainly because of the dilution factor – 10 mL in 1.5 L. Although the addition of pot ale was accompanied by a small decrease to the pH of the wastewater, this did not negatively affect the treatment performance in the WWPA+*C.v* treatment. In other studies, Yang et al., (2008) [541] reported a 76% reduction in COD from cassava ethanol fermented wastewater treated by the microalgae *C. pyrenoidosa* in both batch and continuous operated mode. O'Rourke et al., (2016) [542] demonstrated successful mixotrophic cultivation of the microalga *Parachlorella kessleri* on waste residue from fermented wort, with the carbonaceous material composed of residual glucose, maltose and maltodextrin.

The assimilation and metabolism of the non-fermented sugars in pot ale by *C. vulgaris* in the PSW may have occurred through a less indirect pathway compared to that for glucose or glycerol (discussed in Chapter 4). Compared to simple organic carbon compounds such as glucose, glycerol or acetate, there is a paucity of information detailing the precise nature and mechanism by which microalgae assimilate and digest more complex carbon substrates in their aquatic environment [215, 216, 543]. Carbohydrate metabolism in microalgae has been extensively studied, primarily in an attempt to attain a higher starch or lipid content for the manufacturing of biofuels from algal biomass [544, 545]. This research demonstrated carbohydrate synthesis and catabolism to predominantly occur in the chloroplast of microalgae belonging to the phyla *Chlorophyta*, *Dinophyta*, *Glaucophyta*, and *Rhodophyta* [546–550]. No studies have robustly proven extracellular expression of the enzymes necessary for starch hydrolysis (i.e. isoamylase, β-amylase or α-amylase) in microalgae, either membrane bound or released into the medium. Therefore, it cannot be stated with absolute certainty as to whether *C. vulgaris* was able to hydrolysis and degrade the non-fermented sugars of the pot ale extracellularly.

However, as the COD concentration declined in the WWPA+*C.v* treatments for each PSW batch, it is possible that *C. vulgaris* was able to assimilate the organic carbon provided in the form of pot ale by other means. In the WWPA treatments of each PSW batch a reduction in COD was observed, indicating the ability of the endogenous microflora in the PSW to digest the carbonaceous matter, including the pot ale (Figure 5.7A, C, E). Bacteria are known to release as well as display membrane bound enzymes, including glucosidases such as β- and α-amylase [551–554]. In the WWPA+*C.v* treatments the concentration of dissolved O_2 increased above 2 mg L^{-1}, achieving required levels for heterotrophic microorganisms to oxidise the organic material as well as autotrophic bacteria to carry out nitrification (Figure 5.7B, D, F) [89]. Therefore, it is feasible that the endogenous microflora aided in the digestion of the non-fermented sugars to a form that was more readily available to the microalgae; by hydrolysing the polysaccharides to monomers or disaccharides, such as glucose or maltose [2, 543]. Additionally, heterotrophic respiration would have increased the availability of inorganic carbon. Analysis of the carbohydrate fractions in the wastewater of this treatment is necessary to drive a firmer conclusion. An alternative mechanism may have been via endocytosis. However, substrate uptake by this mechanism in *Chlorophyta* has not been clearly demonstrated. Wang et al., (2011) [555] reported on the internalisation of copper nano particles via endocytosis in the prokaryotic alga *Microcytis aeruginosa*. Domozych (1991) [556] reviewed vesicle trafficking in *Chlorophyta*, highlighting the complexity of endomembrane system in relation to other eukaryotic cell processes, although no information was presented in regards to substrate uptake by endocytosis. To fully elucidate the mechanism by which the organic carbon of pot ale is utilised by the microalgae, heterotrophic culturing under axenic conditions would need to be performed. To further investigate the mechanism of cellular uptake and verify if the endocytic pathway confers a mechanism for the internalisation of soluble organic carbon, microalgae could be pre-treated with an endocytic inhibitor such as Wortmanin or sodium azide [557, 558].

Based on the time-course of *C. vulgaris* concentration, the high initial NH_3-N concentration present in PSW batch R5 and R6 combined with the elevated pH (discussed below) are likely the reasons underlying the slower growth rate and extended lag period exhibited by the microalgae. Based on morphological observations of *C. vulgaris* grown under alkaline conditions, pH-induced effects resulted in a greater flexibility of *Chlorella* cell walls and is suggested to prevent its rupture and, therefore, inhibiting autospore release [290]. *C. vulgaris* concentration in the WWPA+*C.v* treatment of PSW batch R6 exhibited a 1-day lag followed by a gradual rate of growth, increasing from 1.2 $x10^7$ ($\pm 1.6x10^6$) cells mL^{-1} at day 1 to a maximum 3.2 $x10^7$ ($\pm 1.9x10^6$) cells mL^{-1} at day 4 (Figure 5.8F). In PSW batch R5, the cell concentration in the WWPA+*C.v* treatment displayed a lower growth rate over the course of the initial 4 days of treatment, increasing only marginally before substantially increasing at day 5 (Figure 5.8D). *C. vulgaris* concentration in this treatment was respectively 1.3 $x10^7$ ($\pm 5x10^5$) and 3.1 $x10^7$ ($\pm 2x10^6$) cells mL^{-1} at day 1 and day 4, and 5.8 $x10^7$ ($\pm 3x10^6$) cells mL^{-1} at day 5. In comparison, *C. vulgaris* concentration in the WWPA+*C.v* treatment of PSW batch R4 (lowest recorded NH_3-N concentration in this experiment) exhibited a 1 day lag period with a clear exponential phase, with maximum cells concentration reached by day 3 from an initial 1.1 $x10^7$ ($\pm 1x10^6$) to 3.5 $x10^7$ ($\pm 2x10^6$) cells mL^{-1} respectively, followed by a small decline at day 4 before increasing again to an equivalent concentration as recorded on day 3 (Figure 5.8B). At present no explanation can account for the sudden increase in *C. vulgaris* concentration recorded in the WWPA+*C.v* treatment of PSW batch R5 after day 4, yielding the highest cell concentration of all three experimental runs. A similar response was observed in the WW+*C.v* treatment of the same PSW batch, but not for PSW batch R4 or R6, which suggests that the cause may be a result of the wastewater itself rather than be treatment specific. However, a lack of complete analysis of the wastewater (i.e. metal content, microbial abundance, characterisation of organic compounds etc.) imposes limitations on this interpretation and its precise cause. It is interesting to note that despite the discrepancy in *C. vulgaris* concentration over the 5-day treatment period between the WWPA+*C.v* treatments, the concentration of final biomass in each treatment was similar; in the PSW batch R4, R5 and R6 final yields were 476 ±25, 410 ±26 and 426 ±11 mg L^{-1} respectively.

The trend in microalgae growth in the WWPA+*C.v* treatments of PSW batch R5 and R6 bear comparison to microalgae growth in conditions with similar or higher NH_3 concentrations, in which a prolonged acclimation phase or reduced productivity was noted relative to conditions of lower NH_3 concentration [440, 537, 559]. For instance, the specific growth rate of *C. vulgaris* cultured on wastewater dropped by a third when the NH_4^+-N concentration was doubled, from 0.92 d^{-1} at 17 mg L^{-1} to 0.33 d^{-1} at 39 mg L^{-1}, displaying a longer acclimation period on a time scale of hours [559]. It must be noted that tolerance to NH_3 is species dependent, which explains the discrepancy in microalgae growth observed in the present study when compared to other microalgae wastewater studies with higher NH_3-N concentration in which no effect on microalgae growth and metabolism is observed (Godos et al. 2010; Collos & Harrison 2014 and references therein). Tolerance of *C. vulgaris* to NH_3-N concentrations of 170 mg L^{-1} or higher have been reported [538, 559, 560].

The dissolved O_2 concentration increased in the treatments with microalgae, despite the prevailing high free NH_3 formation as a result of pH increase, indicating a prevalence of photosynthetic activity over heterotrophic carbon-oxidation. The WWPA+*C.v* treatments of PSW batch R4, R5 and R6 achieved maximum dissolved O_2 concentrations of 7.5 ±0.5, 6.8 ±0.2 and 9.1 ±0.2 mg L^{-1} O_2, respectively (Figure 5.8B, D and F), and maximum pH values of 10.8 ±0.09, 9.0 ±0.02 and 8.9 ±0.16, respectively (Figure 5.7B, D, F). Previous research has demonstrated that accumulation of free NH_3 in the extracellular environments, which can penetrate internally into algal cells cause an intracellular pH disturbance, damage PS II and reduce photosynthetic efficiency and O_2 evolution [439, 561]. For example, NH_3-N concentration of 2 mM (i.e. about 34 mg L^{-1}) at pH 8 was reported to inhibit photosynthesis and growth of *S. obliquus* in HRAP treating domestic sewage [440]. In a further investigation, Azov and Goldman (1982) [438] observed a 50 and 90% decline in *S. obliquus* photosynthesis when the pH increased to 9.5 at 25°C, at NH_3-N concentrations of 2 and 3 mM respectively. In comparison to the results reported in the studies above, it is clear that *C. vulgaris* used in these experiments is tolerant to NH_3 and elevated pH and, hence, its suitability in wastewater treatment. Despite the presence of O_2 and NH_3-N in the WWPA+*C.v* treatment of PSW batch R5 and R6, the occurrence of nitrification was ruled out based on the absence of no substantial increase in both NO_2-N and NO_3-N concentrations and the elevated pH values present in the wastewater (Figure 5.9C – F). The same observation holds true for the WWPA+*C.v* treatment in PSW batch R4 with the amendment that NH_3-N was limited in the wastewater following its decline at day 2 (Figure 5.9A, B).

In regards to P, the addition of pot ale resulted in a higher initial and consequently final PO_4-P concentration compared to that for the experiments using glucose or glycerol as the organic carbon source. In the WWPA+*C.v* treatment in PSW batch R4, the concentration of PO_4-P declined rapidly at day 1 to 2.8 ±0.8 mg L^{-1} after which the rate slowed before reaching a final concentration of 0.5 ±0.06 mg L^{-1} (Figure 5.6B), but which was not significant compared to the WWPA treatment ($H(3) = 10.495$, $p = 0.243\ 6$ at day 5). Similarly, for the same treatment in PSW batch R6 the highest removal effect was recorded at day 1, declining to 3.3 ±0.04 mg L^{-1}, with a final PO_4-P concentration of 2.3 ±0.2 mg L^{-1}, which was found to be significant compared to WWPA ($H(3) = 10.152$, $p = 0.018$ at day 3) (Figure 5.6F). Whereas in PSW batch R5 the PO_4-P concentration in this treatment declined at a steadier rate from an initial concentration of 9.1 ±0.06 to 3.3 ±0.12 mg L^{-1} at day 4, before slightly increasing to 3.9 ±0.17 mg L^{-1} at day 5 (Figure 5.6D). This decline in PO_4-P concentration was, however, found as insignificant compared to the WWPA treatment ($H(3) = 10.385$, $p = 0.249\ 6$ at day 5). In these treatments the decline in PO_4-P was in part a consequence of microalgal uptake, together with chemical precipitation following an increase in pH above 8 in the culture condition (Figure 5.8A, C, E). Although the precise partitioning of PO_4-P removed by the microalgal-bacterial co-culture and its precipitation was not conducted, the decline in PO_4-P removal rate after the initial days of treatment may have been a response to limited NH_3-N uptake by the microalgae as P assimilation is shown to be dependent on N uptake [453]. In general, final PO_4-P concentration in the WWPA+*C.v* treatments declined to a similar PO_4-P concentration recorded in the WW+*C.v* treatments (Figure 5.6B, D and F).

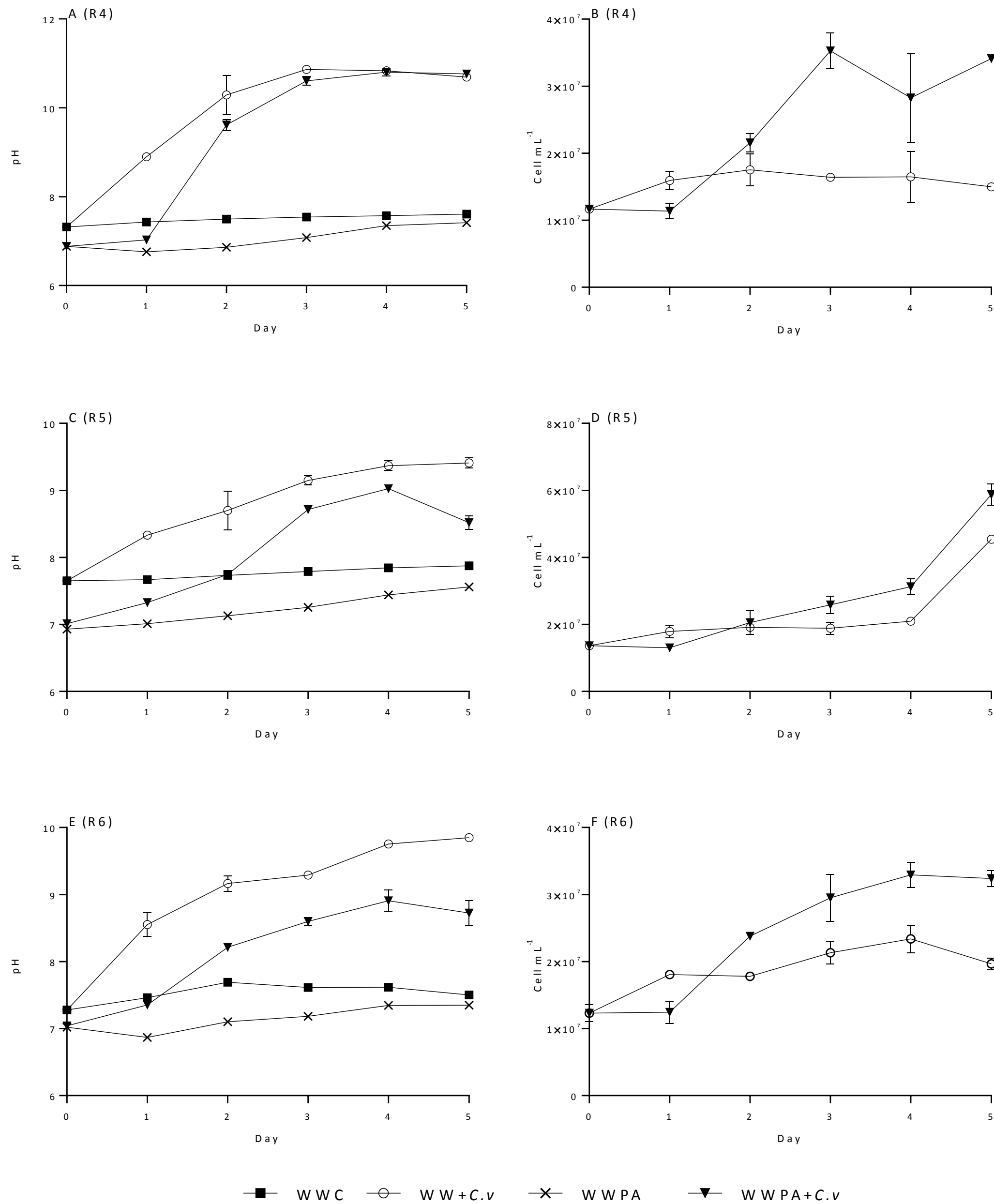


Figure 5.8 – Change in PSW pH (**A, C, E**) and *C. vulgaris* concentration (**B, D, F**) in cell mL^{-1} for PSW batch R4 (**A, B**), R5 (**C, D**) and R6 (**E, F**) treated under the conditions with and without *C. vulgaris*, enriched with or without PA. Each data point is the mean ±SD, of n = 3 independent replicates. Some error bars are smaller than the symbols. Treatment WWC (wastewater only); Treatment WW+*C.v* (wastewater with *C. vulgaris*); Treatment WWPA (wastewater with pot ale); and Treatment WWPA+*C.v* (wastewater with pot ale and *C. vulgaris*).

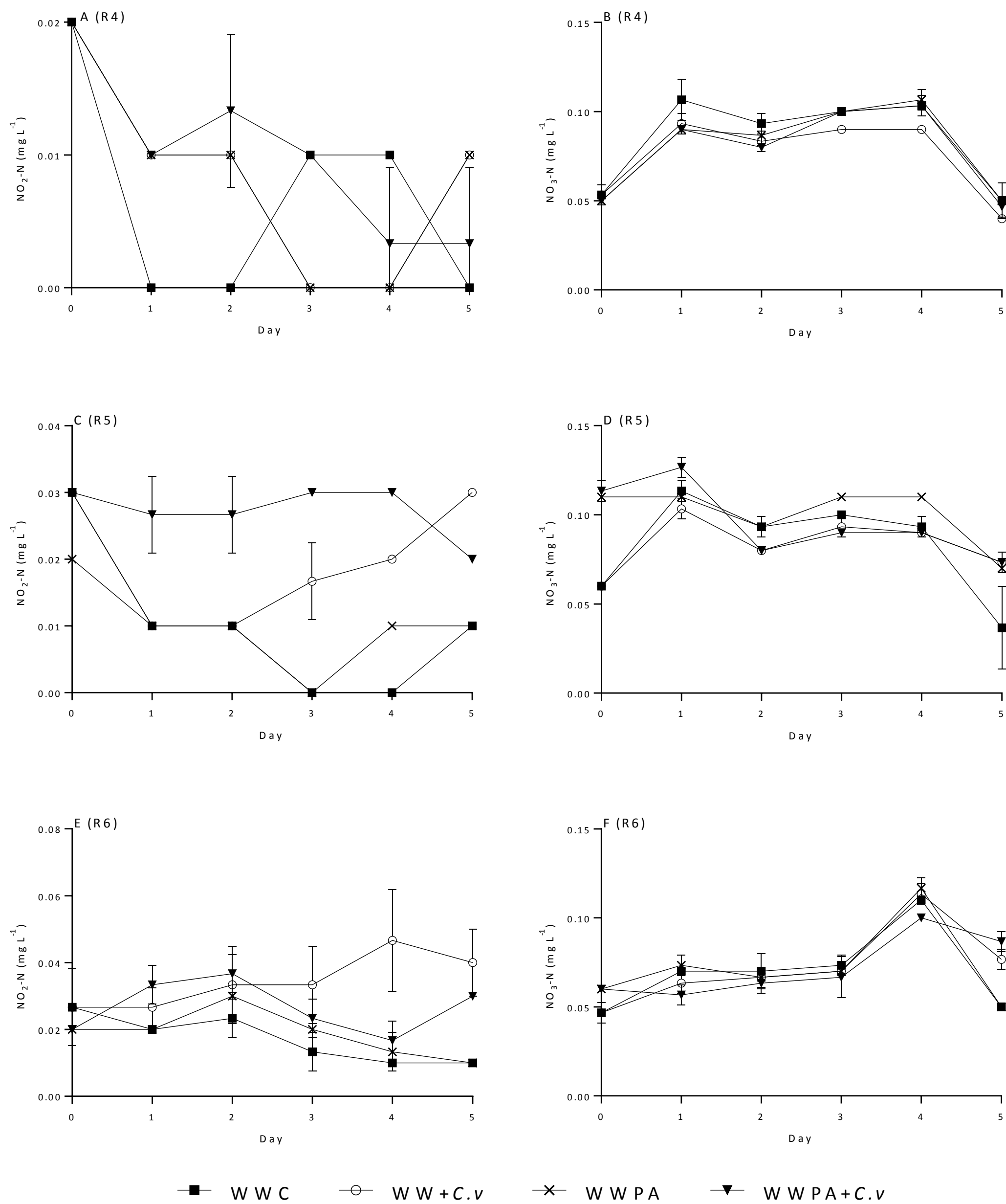


Figure 5.9 – Changes in PSW concentrations for NO_2-N (**A, C, E**) and NO_3-N (**B, D, F**) in mg L^{-1} for PSW batch R4 (**A, B**), R5 (**C, D**) and R6 (**E, F**) treated under the conditions with and without *C. vulgaris*, enriched with or without PA. Each data point is the mean ±SD, of n = 3 independent replicates. Some error bars are smaller than the symbols. Treatment WWC (wastewater only); Treatment WW+*C.v* (wastewater with *C. vulgaris*); Treatment WWPA (wastewater with pot ale); and Treatment WWPA+*C.v* (wastewater with pot ale and *C. vulgaris*).

Whilst it is pertinent to analyse for TP concentration in future studies to ensure the static microalgae treatment process complies with the UWTD, the high residual concentration of PO_4-P as a result of pot ale amendment highlights a potential limitation to the use of this carbon sources in a static microalgae wastewater treatment process. Future work on optimising the static microalgae treatment using pot ale should focus on lowering PO_4-P concentration, and directly TP concentration, to about 2 to 3 orders of magnitude before the wastewater can be safely discarded into the environment. While microalgae accumulate PO_4-P in the form of polyphosphates, this mechanism increases upon starvation of the cells [562]. The microalga in the inocula used in these experiments were not starved before being inoculated into the PSW. A potential strategy to improve PO_4-P removal may be to starve the cultures to induce the accumulation of more PO_4-P than the levels demonstrated in these experiments. However, this may have implications upstream of the process that could entail a financial cost because of a further cultivation step required. Alternatively, the P content of the pot ale could be extracted prior to addition in PSW, either through precipitation, adsorption or electrodialysis methods [563–566].

In the present experimental design, it is difficult to determine precisely the individual contribution the microalgae and the heterotrophic (i.e. bacteria, fungi etc.) constituents in the co-culture had in the removal of either the inorganic and organic fractions from the PSW. Nonetheless, characterisation of the wastewater in the microalgae treatments compared with the control treatments (WWC and WWPA) highlight that *C. vulgaris* was a key organism in the consortium responsible for achieving inorganic N and P removal. The decline in N and P were, however, lower in the WW+*C.v* treatments (without pot ale) relative to the WWPA+*C.v* treatments (Figure 5.6). This result is congruent with the observed response in the equivalent treatments in the initial glucose experiment. As previously inferred this is because of a limitation in inorganic and organic carbon based on the prevalence of high pH values and minimal reduction in COD, similar to results in the present (pot ale experiment) WW+*C.v* treatments. In regards to COD, the WWPA treatments indicated a varied capacity at removing the additional carbonaceous material provided in the form of pot ale between the treatments, albeit at a slower rate compared to the WWPA+*C.v* treatments (Figure 5.7A, C, E). Final COD concentrations in the WWPA treatment in PSW batch R4, R5 and R6 were 135 ±10.0, 377 ±4.0 and 147 ±5.2 mg L^{-1} O_2, respectively (Table 28). Despite the reduction in carbonaceous material in the WWPA treatments, no significant change in NH_3-N concentration was recorded, whereas slight variations in PO_4-P concentration were and more so in the PSW batch R4 than in PSW batch R5 and R6. In comparison, a noticeable decline in the NH_3-N concentrations was observed in the equivalent WWG treatment in the initial glucose experiment (Figure 5.1A). Additionally, in the WWG treatment the observed decline in pH at day 1 was attributed to the degradation of the carbonaceous material, including glucose, which resulted in organic acid formation through the process of acidogenesis and acetogenesis as a result of the anoxic environment that formed (Figure 5.2B, C) [89]. A similar response was not observed in the WWPA treatments despite the culture condition becoming anoxic, inferring that withou O_2 the soluble carbonaceous material, predominantly pot ale, was not as easily digestible compared with glucose. The final dissolved O_2 concentration in the WWPA treatments were 0.42 ±0.15, 0.53 ±0.24 and 0.38 ±0.24 mg L^{-1} O_2 in PSW batch R4, R5 and R6

respectively (Figure 5.7B, D, F). In general, these observations suggest that the microalgae were chiefly responsible for removing the inorganic N and P, and the naturally occurring heterotrophic organisms the carbonaceous material.

5.4 Conclusion

This study aimed to evaluate the influence of organic carbon enrichment on *C. vulgaris* performance in order to reduce both the carbonaceous and inorganic N and P load in PSW under static cultivation conditions. Exogenously supplied organic carbon to the wastewater proved to improve the depuration of these contaminants in the microalgae treatment. An initial experiment with PSW enriched with glucose revealed significant reductions in NH_3-N (28.6 ±0.1 to 0.1 ±0.05 mg L^{-1}) and PO_4-P (3.2 ±0.02 to 0.1 ±0.01 mg L^{-1}) in the WWG+*C.v* treatment within 2 days. The degree of removal compared to the WW+*C.v* treatment was attributed to the higher availability of carbon that is postulated to feed into anaplerotic reactions replacing key intermediates in the TCA cycle that would otherwise have been sequestered in the anabolic reaction of inorganic N incorporation into amino acids. Performance of the organic carbon enriched microalgae wastewater treatment on a further three individually PSW batches, either with glucose or glycerol enrichment, yielded consistent rates of inorganic N and P reduction. Characterisation of the wastewater revealed removal efficiencies of 90% and above (at day 2) for NH_3-N and PO_4-P, irrespective of the initial concentration of these inorganics in the wastewater. However, higher initial concentrations of these inorganics did not lead to their reduction to levels as low as those achieved when their initial concentrations were lower, hence suggesting that the capacity of the microalgae in this respect for treating PSW may be limited by the availability of organic carbon or the cultivation mode (i.e. batch).

Further investigation using the deproteinated pot ale as an organic carbon source to improve the economic feasibility of the treatment process demonstrated a comparative inorganic N and P removal response in the microalgae treatments to those achieved with either glucose or glycerol enrichment. With the treatment repeated on three PSW batches, collected and treated separately and sequentially, a similar observation was recorded to the reproducibility experiment in that the final achievable NH_3-N concentration was affected by its initial concentration. Under the culture conditions used, enrichment of PSW with pot ale at a ratio of 1:150 v/v, which accounted for an equivalent COD of approximately 250 mg L^{-1} O_2, prompted the removal of approximately 28.7 mg L^{-1} NH_3-N in the WWPA+*C.v* treatments. As a consequence, wastewater with a higher initial concentration (than the quantified theoretical maximum) exhibited higher final concentrations. However, further research on additional wastewater samples with controlled N loads, and adequate pH and dissolved O_2 control measures is needed to draw firmer conclusions with the aim of addressing how to overcome this limitation.

Interestingly, in all microalgae treatments of the experiments no significant formation of NO_2-N or NO_3-N was detected across all treatments, indicating that nitrification activity was limited for various reasons, albeit independently from each other. Using a readily available organic carbon source in

unsterilized PSW presented the possibility of the naturally occurring heterotrophic microorganisms from out-competing the microalgae in the enriched treatments. *C. vulgaris* cell concentration and final biomass dry weights indicate that *C. vulgaris* was a good competitor in a mixed population, since within 3 to 5 days this alga reached highest cell concentration and consistent final biomass yields across the different PSW batch enriched treatments. Inclusion of community analysis in any future experiments is recommended to better understand the interaction and influences between the microalgae and bacteria under the present experimental design. The findings presented here suggest that the microalgae were chiefly responsible for removing the inorganic N and P, while the endogenous microbial community in the wastewater had consumed the majority of the carbonaceous material.

Chapter 6 – Evaluation of the treatment efficiency of pot ale enriched primary settled wastewater by *Chlorella vulgaris* operated as a static semi-continuous process

6.1 Introduction

A practical step needed for the implementation of microalgae into wastewater treatment is to develop a process able to treat the wastewater in a timely manner that is up to speed with the rate at which it is produced and at scale. To this end, research has been conducted on developing semi-continuous and continuous operated microalgae treatment processes (Ho et al., (2014) [567] and references therein). In a continuous operated process, fresh wastewater is continuously fed into the PBR at the same rate at which spent wastewater (i.e. treated) is withdrawn. Under steady state conditions, a constant rate of nutrient removal is achieved as the active microalgal-bacterial culture is maintained in a constant state of growth to promote a high assimilation rate of inorganic N and P [543, 567]. In a semi-continuous operated process, a proportion of the wastewater in the PBR is withdrawn and replaced with fresh wastewater when the water is appropriately cleaned and the microalgae have reached late logarithmic growth [543, 567]. The culture is then maintained under batch operation to allow cell densities to increase and contaminant removal to occur before a next replacement. In both processes, the ratio between the total culture volume and the replaced volume for the specific period of treatment defines the hydraulic retention time (HRT), normally determined per day. The main advantages that either of these processes offer compared to a batch process are that they have shorter HRTs required for microalgae growth and contaminant removal as the initial time required for the microalgae to acclimate is limited to the start-up of the culture only.

In regards to the use of PBRs for wastewater treatment, a major limitation for scale up is the delivery of light. PBRs which are externally illuminated require a large surface area to volume ratio in order to ensure sufficient light with an equal distribution reaches the microalga to support photosynthesis [568]. Current commercial PBR designs follow this principle, but in order to accommodate large volumes, as would be necessary when treating wastewater, a large illuminated surface area is necessary. This aspect increases the complexity in PBR design and marks a serious contribution towards reactor cost [569]. Furthermore, light attenuation can result from biofilm formation on the internal surface of the reactors during the course of cultivation, resulting in photo limitation affecting algal productivity and subsequently treatment [365, 570, 571]. The foregoing problems can be overcome by internalising the light source, either through the use of plastic light guides, or fluorescent or light-emitting diode (LED) strips [571–574]. These approaches have successfully been applied in PBRs with microalgae, achieving similar or higher biomass yields to external illuminated PBRs [575–577].

As an extension of the batch-wise operated treatments investigated previously (Chapter 5), the specific aim of this chapter was to evaluate the treatment of a static microalgae process under semi-continuous operation. The objective was to evaluate the effects that wastewater, when being replaced with fresh sample at designated intervals, has on COD and inorganic N and P removal by the microalgae, in this

case using *Chlorella vulgaris*. Furthermore, an experiment was conducted to evaluate the performance of the microalgae semi-continuous static process at treating pot ale enriched PSW at a greater volume (7 L) in a reactor configured with internal LEDs as the source of light.

6.2 Material and Methods

6.2.1 Semi-continuous treatment experiment

The same four treatments described in the pot ale batch experiment in Section 5.2.1.4 were set up under identical conditions to investigate the treatment performance under semi-continuous operation, with the exception of the wastewater not being filtered. Semi-continuous treatment was started as a batch culture, thereafter the treatments were fed semi-continuously (i.e. every 4th day), for a total of three cycles by discarding half of the initial volume and replacing it with the corresponding wastewater samples: PSW in the WWC and WW+*C.v* treatments, and pot ale enriched PSW in the WWPA and WWPA+*C.v* treatments. The pot ale-enriched PSW was prepared as described in section 5.2.1.1. Throughout this experiment, fresh PSW was used for each cycle, collected on the day the experiments were commenced, while the same batch of pot ale was used for the enrichment. In this experiment the composition of the pot ale sample was comparable to the previous pot ale samples used in the batch experiments listed in Table 29, and was as follows (mg L^{-1}): COD, 43325; NH_3-N, 0.21; PO_4-P, 463; NO_3-N, 0.34; NO_2-N, 0.04; TN, 573; TP, 463. The pH was 3.26.

6.2.2 Laboratory large-volume semi-continuous treatment experiment

An 8 L capacity transparent polypropylene bottle, with a diameter of 19.3 cm and height of 40.9 cm, was used as the reactor bottle (Nalgene, USA, product number DS2205-0020). The operating volume of each reactor for the experiment was set at 7 L. Internalised at the centre of the bottles were waterproof RGB-LED strips fixed firmly to a central pole in a spiral arrangement (AquaWhite Flexi-LED strip, Tropical Marine Centre Ltd., Chorleywood, UK). The suitability of the light spectrum of the LEDs was checked prior to use. Internal light levels were determined in dry conditions and set at approximately 100 µE m^{-2} s^{-1} photon flux (US-SQS/L probe, Walz, Germany) measured internally at a distance of 5 cm from the wall of the bottle. In this experiment, only the WWPA+*C.v* treatment was assessed, and performed in triplicate. The culture was started as a batch culture with washed *C. vulgaris* inoculated into 21 L of unfiltered PSW (described in section 5.2.1.2), and then dispensed equally between three reactor bottles. The treatment was run for 23 consecutive days with half of the initial medium volume decanted and replaced with fresh PSW on designated days. Throughout this experiment, the same batch of pot ale was used for the enrichment, and had the following composition (mg L^{-1}): COD, 45600; NH_3-N, 0.17; PO_4-P, 425; NO_3-N, 0.32; NO_2-N, 0.04; TN, 221; TP, 613. The pH was 3.26.

6.2.3 Glassware, sampling and analysis

For all experiments, all glassware and reactor bottles were capped with a foam bung. Before use, all glassware and reactor bottles with the relevant syphoning tubes were autoclaved (121°C; 15 minutes). Liquid samples were withdrawn daily to measure microalgal cell growth (cell mL^{-1}), pH and concentration of NH_3-N, PO_4-P, NO_3-N and NO_2-N (described in Chapter 3, sections 3.3.4, 3.3.5, 3.3.6, 3.3.8, 3.9 and 3.10, respectively). Dry cell weight (as a proxy for biomass) and COD_S were measured on

the initial and final day of each experiment only (Chapter 3, section 3.7 and 3.5 respectively). All treatments were briefly mixed (by swirling) prior to taking an aliquot to ensure a homogenous sample. In the case of the large-volume experiments, samples were mixed for 30 seconds using an internal magnetic stirrer (flea) for 1 minute.

6.3 Results and Discussion

6.3.1 Small-volume treatment of semi-continuous pot ale enriched PSW

Previous experiments highlight that *C. vulgaris* is a key organisms in the consortium necessary for the effective removal of the inorganic N and P from the wastewater, and as such it is imperative to retain a sufficient active population when operating the treatment as a semi-continuous or continuous process. The shorter the HRT, the greater the limitation is on algal growth, which can consequently affect treatment efficiency as the cell population may be washed out from the reactor if the HRT is shorter than the microalgal growth rate [578]. Inorganic N and P concentrations recorded in the WWPA+*C.v* treatments operated as a batch process (Section 5.3.3) indicated a maximum removal effect within 3 days of cultivation, with the achievable levels dependent on the initial concentrations of these inorganics in the wastewater. The highest concentration of *C. vulgaris* achieved in these treatments was within 4 to 5 days. Although inorganic N and P removal occurred faster compared to *C. vulgaris* growth, in the present experiment emphasis was placed on retaining a high cell concentration. Therefore, PSW in the semi-continuous experiment was replaced every 4 days with a resultant HRT of approximately 8 days. As the active biomass was not recycled in this study, the solids retention time was equal to the HRT.

6.3.1.1 Evaluation of the treatment performance

The efficiency of a static microalga wastewater treatment process, operated under semi-continuous mode, was evaluated based on the removal of COD, NH_3-N and PO_4-P from PSW. Figure 6.1 represents the change in both NH_3-N and PO_4-P concentrations in the microalgae treatments with and without pot ale-enrichment during the 4 consecutive cycles of the experiment. In the first cycle of the semi-continuous process, which can be consider operating in a batch mode, the NH_3-N concentration declined below the limit of detection in the WWPA+*C.v* treatment (Figure 6.1A). Here, the concentration of NH_3-N decreased from 29.2 ±0.5 to 0.01 ±0.01 mg L^{-1}, corresponding to a removal rate of 7.3 mg L^{-1} d^{-1} and efficiency of 99% (Figure 6.1A; Table 31). Based on the NH_3-N data obtained in the pot ale batch experiments previously discussed in Chapter 5, the achieved level of NH_3-N removed in cycle 1 is in agreement with the proposed maximum quantity achievable in response to the enriched carbon quantity added to the PSW (i.e. <30 mg L^{-1} NH_3-N). Thereafter, the efficiency of the treatment decreased with recorded percentages of 89, 82 and 74% by the end of cycle 2, 3, and 4, respectively, corresponding to removal rates of 3.6, 4.6 and 4.7 mg L^{-1} d^{-1}. Although inorganic N and P removal was more proficient in the WWPA+*C.v* treatment compared to the WW+*C.v* treatment, because of a higher concentration of bioavailable carbon, a similar decline in NH_3-N removal efficiency was also observed in the WW+*C.v* treatment in the consecutive cycles. In the WW+*C.v* treatment, NH_3-N concentration decreased from an initial value of 29.5 ±0.2 to 12.2 ±0.5 mg L^{-1} at day 4, corresponding to a removal rate of 4.3 mg L^{-1} d^{-1} and efficiency of 59% (Figure 6.1B; Table 31). Thereafter, the concentration of NH_3-N decreased in a consistent manner over the 4-day duration in cycle 2, 3 and 4 at a rate of 1.7, 2.8 and 2.9

mg L^{-1} d^{-1}, respectively, which corresponded to removal efficiencies of 29, 39 and 37%. As a result of the decrease in NH_3-N removal per cycle, a higher final NH_3-N concentration was recorded in each subsequent cycle in both the microalgae treatments.

Table 31 – Removal efficiency of NH_3-N and PO_4-P from PSW in semi-continuous operated conditions either with or without *C. vulgaris*, and either enriched with or without pot ale. Treatment WWC, wastewater only; treatment WW+*C.v*, wastewater with *C. vulgaris*; treatment WWPA, wastewater with pot ale; and treatment WWPA+*C.v*, wastewater with pot ale and *C. vulgaris*. Each value is the mean ±SD, of n = 3. The days in bold represent the removal rates prior to PSW replenishment at the end of each cycle.

Treatment	NH_3-N (% removal)							
	Day 2	**Day 4**	Day 6	**Day 8**	Day 10	**Day 12**	Day 14	**Day 16**
WWC	0.9 ±1	0.2 ±2	-1.8 ±1	-3.1 ±0.5	1.4 ±0.1	3.5 ±0.2	4 ±1.6	-0.8 ±2
WW+*C.v*	58 ±0.9	59 ±1	17 ±0.6	29 ±2	31 ±3	39 ±3	31 ±0.2	37 ±2
WWPA	12 ±0.2	22 ±1	12 ±0.5	9.6 ±1	8.7 ±2	6.3 ±5	10 ±1	6.6 ±4
WWPA+*C.v*	78 ±6	99 ±0.4	75 ±6	89 ±2	78 ±5	82 ±3	69 ±2	74 ±0.8
	PO_4-P (% removal)							
WWC	3.7 ±3	-2.4 ±2	-0.3 ±0.2	-0.7 ±0.2	2.0 ±0.4	1.4 ±2	6.2 ±1	3.4 ±4
WW+*C.v*	78 ±0.9	77 ±5	23 ±4	56 ±2	46 ±4	59 ±4	41 ±1	59 ±4
WWPA	1.9 ±0.8	8.3 ±0.7	11 ±2	8.7 ±2	9.4 ±4	2.9 ±3	5.5 ±1	5.6 ±4
WWPA+*C.v*	49 ±4	71 ±2	43 ±4	50 ±4	46 ±5	63 ±4	41 ±3	62 ±2

Reports of a decline in removal efficiency in a semi-continuous microalgae treatment process are found in the literature. For example, Ruiz-Marin et al., (2010) [228] evaluated the treatment efficiency of encapsulated *S. obliquus* in unsterilized urban wastewater. A 90% NH_4^+-N removal efficiency was achieved within the first 2 days (cycle 1). In subsequent cycles, the removal efficiency was found to decline to 87%, a rate which was maintained for a further four cycles before a substantial decline to 10% was recorded in the last cycle. De-Bashan et al., (2002) [579] cultured *C. vulgaris* co-immobilised with the bacterium *Azospirillum brasilense* under semi-continuous operation in artificial sterile wastewater for six consecutive cycles. The wastewater was completely replaced every 48 hours, denoting the end of a cycle, while the biomass was recycled. A near complete removal of NH_4^+-N was maintained for the first four cycles, thereafter the efficiency dropped and only 67% of NH_4^+-N was removed at the end of the sixth cycle. Ruiz-Marin et al., (2010) [228] attributes the main reason for the decline in removal efficiency to a collapse in the culture after the fourth cycle, although no indication is given as to why (e.g. pH change, nutrient limitation etc.); and de-Bashan et al., (2002) [2002] ascribed the decline to the microalgae becoming saturated, however, growth data is only reported for the first 144 hours (i.e. for the first 3 cycles). It can be speculated that the algae entered a stationary phase of growth, which would have reduced nutrient assimilation capacity. In the present study, the concurrent demise in NH_3-N removal efficiency after cycle 1 in both the microalgae treatments suggests the occurrence of a

common effect. As the same fresh wastewater sample was used in each consecutive cycle for all treatments, it can be suggested that the same environmental factor occurring during the course of each cycle may be responsible. The dominant factor between the treatments was the alkaline conditions which formed in the microalgae treatments and likely the reason underlying the decline in NH_3-N removal efficiency. A substantial increase in pH is observed in the course of each cycle in the WW+*C.v* treatment and in the WWPA+*C.v* treatment after cycle 1, likely resulting from the use of inorganic carbon as a carbon source in photosynthesis leading to the release in OH^- ions into the wastewater that induce the formation of alkaline conditions ([67]; Figure 6.2). An increase in alkalinity has been shown to affect enzyme activity, nutrient assimilation, and viability and growth rate of microalgal [290, 423, 580].

In the WW+*C.v* treatment, the microalgae acclimated well in the PSW during the initial first days as indicated by an immediate growth response (Figure 6.3). *C. vulgaris* concentration increased from 1.5 $x10^7$ ($\pm 5.1x10^5$) to 2.2 $x10^7$ ($\pm 2.6x10^6$) at day 2, with no further growth in microalgae thereafter. Notably, the cessation in arithmetic growth of *C. vulgaris* at day 2 in the WW+*C.v* treatment coincided with the cessation in NH_3-N removal (Figure 6.1B; Figure 6.3). Concomitantly, the pH rapidly increased from 7.5 ±0.0 to 8.7 ±0.02 at day 2, and further to 8.9 ±0.1 at day 4 (Figure 6.2). Although the transient change in the pH was within the relatively wide pH range (8.6 – 9.1) that is reported to not affect microalgal growth, the observed change in pH of cycle 1 may have been too abrupt and leading to less optimal microalgae growth under the sudden stress induced by the change in pH conditions in this experiment [581]. Furthermore, the pH change will have increased the concentration of free NH_3 dissolved in the water [442]. Alkaline conditions have been noted to impact on microalgae NH_3 removal capabilities as well as negatively affect the health of microalgal cells (discussed below) [429, 440]. Kang et al., (2014) [582] obtained a significant reduction in NH_4^+-N removal efficiency at pH 8 compared to the culture conditions between pH 5 to 7, likely a result of a greater dissociation effect of NH_3 in the medium under alkali conditions. Additionally, the sharp pH increase denotes an appreciable reduction in alkalinity and buffering capacity of the wastewater, indicating a limitation in bioavailable inorganic carbon which will have impacted on the ability of the microalgae to effectively assimilate and incorporate inorganic N into the cell.

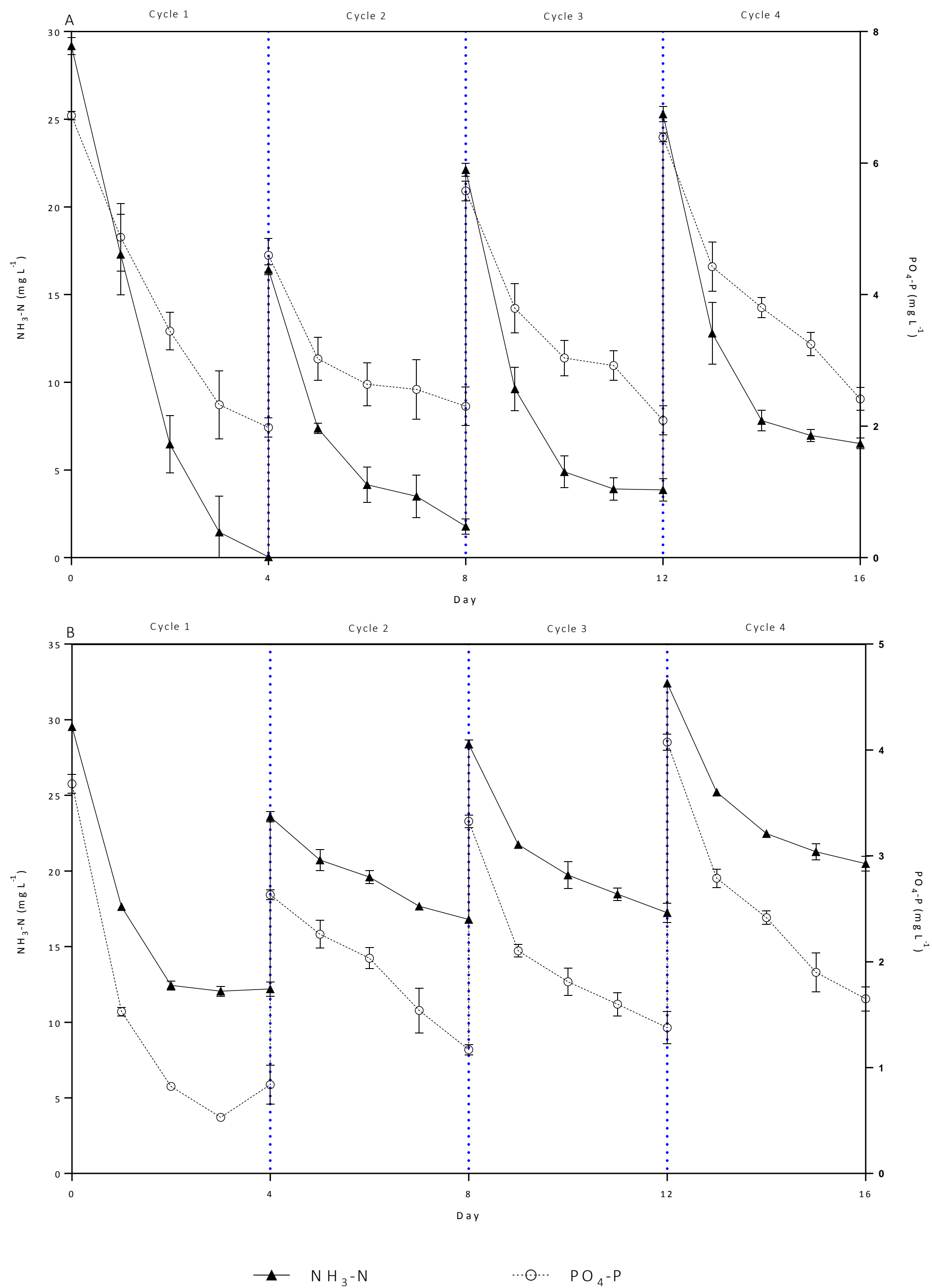


Figure 6.1 – Time-course of NH_3-N and PO_4-P concentrations in mg L^{-1} of PSW treated by semi-continuous operation in the WWPA+*C.v* treatment (**A**; wastewater with pot ale and *C. vulgaris*) and WW+*C.v* treatment (**B**; wastewater with *C. vulgaris*). Each point is the mean ±SD of n = 3 independent replicates. Some error bars are smaller than the symbols. The dotted line represents the duration of each cycle.

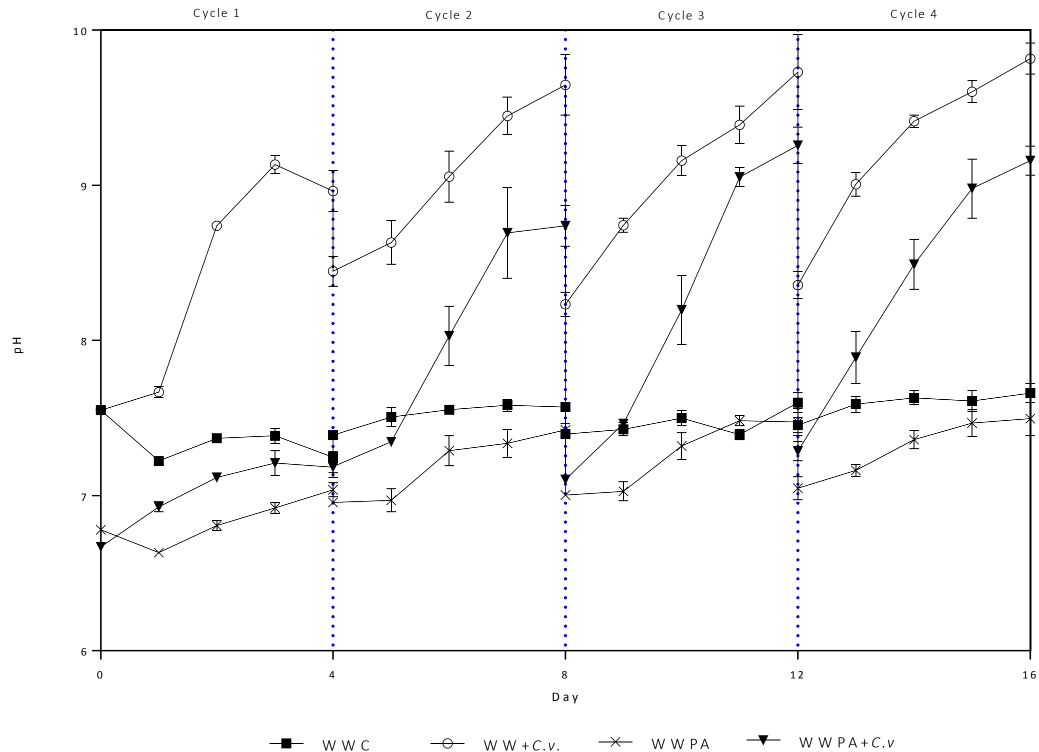


Figure 6.2 – Time course of pH of PSW treated by semi-continuous operation under the conditions with and without microalgae, and enriched with or without pot ale. Each point is the mean ±SD of n = 3 independent replicates. Some error bars are smaller than the symbols. The dotted line represents the duration of each cycle. Treatment WWC (wastewater only); Treatment WW+*C.v* (wastewater with *C. vulgaris*); Treatment WWPA (wastewater with pot ale); and Treatment WWPA+*C.v* (wastewater with pot ale and *C. vulgaris*).

The pH value dropped slightly with the change in PSW between cycles in the WW+*C.v* treatment as a result of dilution and replenishment of inorganic carbon present in the fresh wastewater. However, the pH increased rapidly again and reached values above 9.5 at the end cycles 2 to 4 (Figure 6.2). In addition to the maximum cell concentration achieved being lower in each proceeding cycle, the period of arithmetic growth was shorter, lasting only one day (Figure 6.3). The initial and final cell concentrations in the WW+*C.v* treatment were respectively 1.1 $x10^7$ (±1.6$x10^6$) and 2.1 $x10^7$ (±6.3$x10^5$) in cycle 2, 1.1 $x10^7$ (±1.1$x10^6$) and 1.8 $x10^7$ (±6.5$x10^5$) in cycle 3, and 8.7 $x10^6$ (±1.3$x10^6$) and 1.7 $x10^7$ (±1.9$x10^6$) in cycle 4. A decline in final biomass concentration was also recorded between cycles, with values of 340 ±11, 296 ±6, 251 ±13 and 218 ±7 mg L^{-1} obtained for cycles 1, 2, 3, and 4, respectively. A similar response to increasing pH levels on growth was observed by Ge and Champagne (2016) [583] when treating sterile synthetic centrate with *C. vulgaris* in a semi-continuous operated PBR at a HRT of 8 days. As a result of increasing pH levels (8.53 – 9.21), a decline in biomass yield was observed in the two treatments with the highest centrate loading rates during phase 2 of the experiment. When the pH of the treatments was adjusted (through the addition of 2 M HCl) to 7.5 in phase 4 of the experiment, a significant increase in biomass yield was obtained from an approximate 0.28 – 0.31 g L^{-1} (phase 2 values)

to 0.49 – 0.51 g L^{-1}. Simultaneously, pH adjustment improved the NH_4^+-N removal efficiency of the treatment from 86% to 92%.

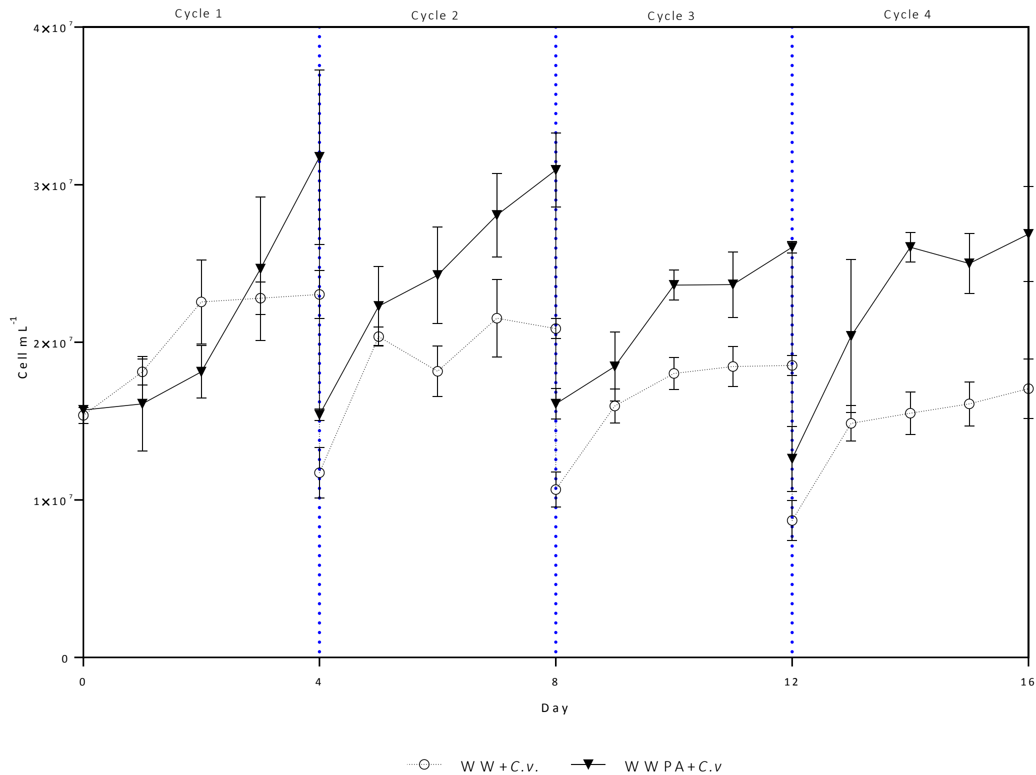


Figure 6.3 – Changes in *C. vulgaris* concentration in cell mL^{-1} of PSW treated by semi-continuous operation under the conditions with microalgae either enriched with or without pot ale. Each point is a mean ±SD, of n = 3 independent replicates. Some error bars are smaller than the symbols. The dotted line represents the duration of each cycle. Treatment WW+*C.v* (wastewater with *C. vulgaris*); and Treatment WWPA+*C.v* (wastewater with pot ale and *C. vulgaris*).

The assumption of pH having a negative effect on microalgae growth and consequently NH_3-N removal in the microalgae treatments of this experiment is supported by the observed trend in these parameters in the WWPA+*C.v* treatment. During cycle 1 of the WWPA+*C.v* treatment, the decline in NH_3-N concentration was accompanied with a continuous increase in *C. vulgaris* and biomass concentration over its 4-day duration, while the pH remained below 7.5, ranging between 6.6 and 7.2 (Figure 6.2). Thereafter, a substantial increase in pH (>8.5) was recorded during the subsequent cycles that was accompanied with a decline in cell and biomass concentrations. The final cell and biomass concentrations were 3.2 $x10^7$ (±5.5x106) cell mL^{-1} and 500 ±33 mg L^{-1} in cycle 1, 3.1 $x10^7$ (±2.3$x10^6$) and 429 ±45 in cycle 2, 2.6 $x10^7$ (±3.5$x10^5$) and 424 ±19 in cycle 3, and 2.6 $x10^7$ (±3.0$x10^6$) and 321 ±24 in cycle 4. Based on these results, it can be implied that the increase in pH impeded cell and biomass productivity in the WWPA+*C.v* treatment, and affected NH_3-N removal efficiency by reducing the abundance of microalgae and solubility of NH_3-N.

The minimal change in pH during cycle 1 in the WWPA+*C.v* treatment was not consistent with the pH profiles previously described in the same treatment operating in batch mode for the equivalent treatment period (Figure 5.8). In the batch operated WWPA+*C.v* treatments, the pH increased to above 8.9 by day 4 in all three trials. Based on the fact that the wastewater in the present experiment was not filtered prior to use, it can be suggested that the difference in pH between the batch and semi-continuous WWPA+*C.v* treatment during cycle 1 was a result of a higher abundance in heterotrophic microorganisms and concentration of suspended solids in the wastewater. It has been observed that the pH in microalgal cultures is more stable or decreases, depending on the O_2 availability, when co-cultured with a suitable bacterial concentration. Su et al., (2012) [259] found that the pH in the reactor with microalgae treating unsterilized municipal wastewater increased above 9.2, whereas in the microalgal treatments with added activated sludge (applied at various ratios) maintained a pH of approximately 7.5 for the duration of the experiment. The stability of pH in the microalgal-activated sludge treatments investigated can initially be attributed to an improved rate of inorganic carbon production by the heterotrophic bacteria via respiration, which counter-balanced the rate of depletion by microalgae inorganic carbon fixing initially, and subsequently the nitrification process as dissolved O_2 accumulated in the medium. Introduction of bacteria to axenic microalgae cultures during mid cultivation has been shown to have an ameliorating effect, with a reduction in pH from an alkali condition occurring simultaneously with bacterial growth [584]. Additionally, production of acidic substances during the degradation of organic compounds (e.g. acetic acid, poly-(γ-glutamic acid)) by bacteria can lower the pH in microalgal cultures [479, 585, 586].

It is therefore plausible that a higher abundance of heterotopic organisms in the wastewater of the present experiment aided in mitigating the formation of alkali conditions in the WWPA+*C.v* treatment during cycle 1. This is supported by the rate of inorganic carbon formation from heterotrophic respiration was equal, or close to, the rate of consumption by microalgae [587]. This scenario was limited in the WW+*C.v* treatment since sufficient organic material must be present for significant bacterial CO_2 production (Table 33). Analysis of the dissolved O_2 concentration achieved in cycle 1 indirectly substantiated this effect. Despite a higher cell concentration in the WWPA+*C.v* treatment compared to the WW+*C.v* treatment in cycle 1, the achieved dissolved O_2 concentration at the end of the cycle was lower, at 3.2 ±04 compared to 6.7 ±0.2 mg L^{-1}, respectively (Table 32). These values show that the O_2 levels were above the minimum required (>2 mg L^{-1} O_2) to sustain heterotrophic microorganisms to oxidise the carbonaceous material, as well as autotrophic bacteria to carry out nitrification [2, 89]. The lower dissolved O_2 concentration in the WWPA+*C.v* treatment relative to the WW+*C.v* treatment will have been in response to the added pot ale, as the heterotrophic population in the wastewater will have consumed a higher amount of O_2 during the degradation and metabolism of the added carbonaceous material in the wastewater. Additionally, the demand and rate of inorganic carbon fixing by the microalgae in the WWPA+*C.v* treatment may have been lower as the pot ale permitted the microalgae to compensate the demand of carbon with an organic source via mixotrophic metabolism.

Table 32 – Dissolved O_2 concentrations in mg L^{-1} of PSW at the beginning and end of each treatment cycle in the presence or absence of microalgae, and enriched with or without pot ale. Treatment WWC, wastewater only; treatment WW+*C.v*, wastewater with *C. vulgaris*; treatment WWPA, wastewater with pot ale; and treatment WWPA+*C.v*, wastewater with pot ale and *C. vulgaris*. Each value is the mean ±SD, of n = 3 independent replicates.

Cycle	Period	Treatment			
		WWC	WW+*C.v*	WWPA	WWPA+*C.v*
1	Initial	1.7 ±0.1	1.7 ±0.1	1.7 ±0.1	1.7 ±0.1
	Final	1.1 ±0.0	6.7 ±0.2	0.7 ±0.1	3.2 ±04
2	Initial	1.5 ±0.4	4.1 ±0.6	1.2 ±0.2	3.6 ±0.2
	Final	0.8 ±0.4	5.4 ±0.1	0.5 ±0.0	4.5 ±0.3
3	Initial	1.3 ±0.3	3.6 ±0.8	1.1 ±0.0	2.7 ±0.3
	Final	1.1 ±0.2	3.9 ±0.2	0.4 ±0.1	3.8 ±0.2
4	Initial	1.4 ±0.4	1.8 ±0.7	0.9 ±0.2	2.2 ±0.4
	Final	0.9 ±0.2	2.1 ±0.4	0.3 ±0.0	3.4 ±0.2

The increase in pH observed in the subsequent cycles after cycle 1 in the WWPA+*C.v* treatment could be because of the following reason. In comparison to cycle 1, the COD loadings of the wastewater were lower in cycles 2 to 4 (Table 33). Consequently, the respiration rate of bacteria will have declined as a result of the bioavailable carbon sources depleting sooner, directly affecting the rate of CO_2 formation. Thereafter, the quantity of inorganic carbon consumed by the microalgae may have been greater than the quantity produced during respiration of the heterotrophic bacteria, resulting in a pH increase. The alkali conditions will have also had a direct impact of the bacterial population, and should be included in additional studies. However, it should be noted that the main limitation to this interpretation is that the bacterial population was not quantified in the wastewater at any stage of this experiment. Concurrently, as the loading COD concentration was lower in cycle 2 to 4, it is possible that the microalgae increased their rate of inorganic carbon fixing to compensate for the demand in carbon required to sustain growth.

On the other hand, the reduction in NH_3-N removal efficiency in the WWPA+*C.v* treatment may have been in response to the low N:P ratio present in the wastewater used. The optimal ratio for maximum N and P removal by a microalgal-bacterial consortium is reported to lie between 6:1 and 10:1, with ranges extending to 5:1 and 30:1, depending on the algal species and culture conditions [148, 180, 588]. The initial N and P ratio, based on NH_3-N and PO_4-P concentrations, in the PSW of the WWPA+*C.v* treatment were in the range of 4.3:1 and 3.5:1 and declined to between 2.7:1 and 0.01:1 at the end of the cycles, overall indicating a limitation in NH_3-N throughout the experiment. However, as the N:P ratio in the WW+*C.v* treatment remained within the recommended range throughout the whole experiment (15:1 – 8:1), it can be argued that the increase in pH was the main effector reducing NH_3-N removal efficiency as

both microalgae treatments were affected. As such, further studies should place emphasis on controlling the pH when optimising the treatment process.

Table 33 – COD concentrations in mg L^{-1} O_2 of PSW at the beginning and end of each treatment cycle with or without microalgae, and enriched with or without pot ale. Each data point is a mean ±SD, of n = 3 independent replicates.

Cycle	Period	Treatment			
		WWC	WW+*C.v*	WWPA	WWPA+*C.v*
1	Initial	200 ±2.5	200 ±2.5	481 ±1.1	474 ±2.0
	Final	108 ±2.0	99 ±3.6	238 ±2.8	125 ±7.1
	RE (%)	46	50	51	74
2	Initial	120 ±3.0	113 ±6.8	360 ±1.5	245 ±8.5
	Final	88 ±1.5	101 ±2.3	75 ±2.1	63 ±1.5
	RE (%)	27	11	79	75
3	Initial	160 ±0.5	161 ±2.0	311 ±6.7	294 ±1.7
	Final	96 ±1.9	99 ±2.6	141 ±5.5	127 ±11
	RE (%)	40	39	55	57
4	Initial	157 ±2.0	147 ±4.3	309 ±5.3	300 ±27
	Final	105 ±5.9	115 ±20	144 ±6.6	131 ±3.2
	RE (%)	33	22	53	56

This point has further relevant implications in the continual performance of a static semi-continuous treatment process, not only in controlling inorganic N removal, but also in maintaining a healthy population of microalgae. An indirect effect in response to the elevated pH conditions in both the microalgae treatments is noted in regards to the dissolved O_2 concentrations. Initial and final dissolved O_2 concentrations for each cycle are summarised in Table 32. A decline in the maximum dissolved O_2 concentration achieved in each consecutive cycle is noted in both the microalgae treatments. For instance, in the WW+*C.v* treatment a concentration of 6.7 ±0.2 mg L^{-1} was achieved at the end of cycle 1 compared to 2.1 mg L^{-1} at the end of cycle 4. As previously mentioned, the accumulation of free NH_3 in microalgal cultures caused by alkali conditions can interfere with the O_2-evolution complex in PS II because of the unregulated passive inflow of free NH_3 across the cell membrane [439, 561]. Although a similar response in the microalgae treatments in the batch operated pot ale experiment was not noted, based on the observed accumulation of dissolved O_2, it should be pointed out that the period of cultivation (5 days) may have been too short a period to have noted any longer-term effects. As the microalgae in the semi-continuous treatment were not replaced with fresh inoculum, chronic exposure to NH_3 at high pH may have adversely affected their health and the photosynthetic reactions, and consequently growth and treatment performance on a long term basis. Consequences of this effect over longer treatment periods will likely result in the culture condition turning anaerobic, which may

further negatively affect treatment performance and microalgae growth, in particular under natural diurnal light dark periods [290, 440].

In regards to the WWPA+*C.v* treatments the high copper content in PA may have also contributed to the decline in O_2 evolution and the metabolic function of the microalgae. Copper is an essential trace element required by microalgae for cellular growth and enzyme activity, including in the synthesis and function of the copper containing electron carrier's plastocyanin and cytochrome oxidase (Baron et al, 1995; Borowitzka, 2016). The uptake of metal ions by microalgae, including copper, occurs in two stages. Metal ions firstly adsorb onto the external surface of the microalgae membrane followed by the internalisation across the membrane facilitated by ion pores, channels or protein transporters in the algal cell membrane (Levy et al, 2007; Tessier, 1995; Knauer et al, 1997; Kaplan, 2013). However, a variety of adverse effects have been reported in response to elevated copper concentration in the medium of microalgae impairing the biochemical functions governing growth, photosynthesis and respiration. The most notable effect is on the PSI and PSII in microalgae, with copper demonstrated to inhibit the electron flow in the reaction centres (Hadjoudja et al, 2009; Samson et al, 1988; Stauber et al 1987). In regards to cellular growth, high internal copper concentrations are reported to influence mitosis by inhibiting spindle formation by reacting with glutathione, an essential compound in the cellular division mechanism (Stauber et al 1987; Stoiber et al, 2007). Furthermore, copper may inhibit enzymes in the cytoplasm or disrupt the mechanism regulating intracellular pH (Cid et al, 1996; Franklin et al, 2001a). Unfortunately, the concentration of copper in the PSW of all these experiments in this study was not quantified. It is feasible that the addition of PA increased the exposure of the microalgae to ionic copper which would have impacted on their growth and biochemical functions. Furthermore, the effects of copper toxicity have been shown to increase with increasing pH. Wilde et al. (2006) reported a 20-fold increase in copper toxicity to Chlorella sp. as the pH increased, with IC_{50} values (concentration required to inhibit algal growth rate by 50%) decreasing from 19 to 1 µg Cu L^{-1} as the pH increased from 5.5 to 8. A similar 20-fold increase was reported by Franklin et al. (2000) as the pH increased from 5.7 to 6.5. Traub (2015) reported that approximately 70% of the copper present in PA was present in the solids fractions (yeast), which were removed prior to use in these experiments, leaving a residual concentration after deprotonation of the PA between approximately 0.5 and 1 mg L^{-1}. Although the dilution factor at which PA was added to the PSW would have reduced the copper content to a negligible concentration, copper accumulation from repeated dosing following PSW replenishment may have occurred. Therefore an effect of copper toxicity would be expected in the semi-continuous experiment compared to the batch experiments, as a fresh microalga inoculum was used to treat each PSW batch in the batch experiment. Analysis of the microalgae biomass copper concentration might be beneficial not only to elucidate whether an accumulation in the microalgae occurs, but also to assess the suitability of the produced biomass in downstream processes. On a large scale operation, excess microalgae-bacteria biomass produced during the treatment process could be used in the production of biogas through anaerobic digestion. In the process of anaerobic digestion copper toxicity could become an issue (Yenigun et al, 2010; Lin et al, 1993).

Autotrophic microbial nitrification is known to be highly sensitive to pH, with optimal conditions found to lie within a narrow pH range of 7 to 8.5 [89, 589]. The decrease in wastewater NH_3-N concentration in both the microalgae treatments in the present experiment was not accompanied by an increase in concentration of either NO_2-N or NO_3-N (Figure 6.4). The maximum concentrations of NO_2-N and NO_3-N recorded in either of the microalgae treatments throughout this experiment were 0.05 mg L^{-1} and 0.11 mg L^{-1}, respectively. The low concentration in each cycle suggests that the process of NH_3 conversion to NO_3 was limited, and the NO_3 present or formed was rapidly assimilated by the microalgae. The possibility of NO_3 being converted to N gas will have been unlikely as aerobic conditions were maintained in the reactors by the microalgae via photosynthesis throughout the 16 day experimental period (Table 32). Moreover, reduction of NH_3 via volatilisation was considered to be negligible as the treatments were cultured statically.

Similar to the reduction pattern of wastewater NH_3-N, the PO_4-P removal efficiency in the microalgae treatments was highest in cycle 1 compared to that in cycles 2, 3 and 4 (Table 31). By the end of cycle 1, the PO_4-P concentration was reduced in the WWPA+*C.v* treatment from an initial 6.7 ±0.07 to 1.9 ±0.15 mg L^{-1}, and in the WW+*C.v* treatment from an initial 3.7 ±0.09 to 0.8 ±0.19 mg L^{-1} (Figure 6.1). This corresponded to a removal efficiency of 77% and 71% in the WWPA+*C.v* treatment and the WW+*C.v* treatment, respectively, at a removal rate of 1.2 and 0.7 mg L^{-1} d^{-1} (Table 31). Thereafter, the PO_4-P removal efficiency in cycles 2 to 4 for both the microalgae treatments were comparable at approximately 60%, with removal rates between 1 and 0.6 mg L^{-1} d^{-1} in the WWPA+*C.v* treatment, and 0.6 and 0.4 mg L^{-1} d^{-1} in the WW+*C.v* treatment, in cycles 2 to 4. The residual PO_4-P concentrations at the end of cycles 2 to 4 were similar in the respective microalgae treatments despite a slight increase in the initial concentration occurring in each cycle. However, the final concentration of PO_4-P of cycles 2 to 4 in both the microalgae treatments was higher compared to the respective final concentration reached in cycle 1 (Figure 6.1).

In this experiment, the removal of PO_4 in the microalgae treatments is suggested to have been achieved by two mechanisms – directly by microalgal-bacterial assimilation, and indirectly by chemical precipitation as a result of alkaline conditions. The decline in PO_4-P removal efficiency after cycle 1 will likely have been a consequence of the microalgae becoming P-saturated as well as in response to a reduction in NH_3-N assimilation and incorporation. Previous studies have demonstrated that P-starved microalgae or cyanobacteria could attain a far higher rate of nutrient uptake than saturated cells [562, 590, 591]. As the microalgal-bacteria biomass was not recycled between cycles, it is plausible that the microalgae attained saturation levels during cycle 1, and consequently were unable to effectively reduce PO_4-P during cycles 2 to 4 with a limited quantity assimilated. According to Powell et al., (2008) [592], microalgae can accumulate P beyond their metabolic needs independent of N, although the maximum achieved percentage of P in the biomass is limited to approximately 3%. From data in other small-scale experiments, a similar effect can be inferred in which the microalgae apparently become saturated with high polyphosphate accumulation before all the PO_4 is removed, and consequently PO_4 remains present

in the wastewater [269, 514]. In regards to the influence of N on P uptake, the uptake of P is dependent on the availability and quantity of N [453]. As a result of NH_3-N removal efficiency declining in the microalgae treatments, a decline in PO_4-P removal can be expected. On the other hand, the presence of metal ions in the wastewater, together with elevated pH values, can favour the precipitation of PO_4-P. Inorganic P precipitation is dependent on pH, temperature and cation concentration in the culture condition, and can account for 29 to 77% of phosphate removal when pH >8.5 [593].

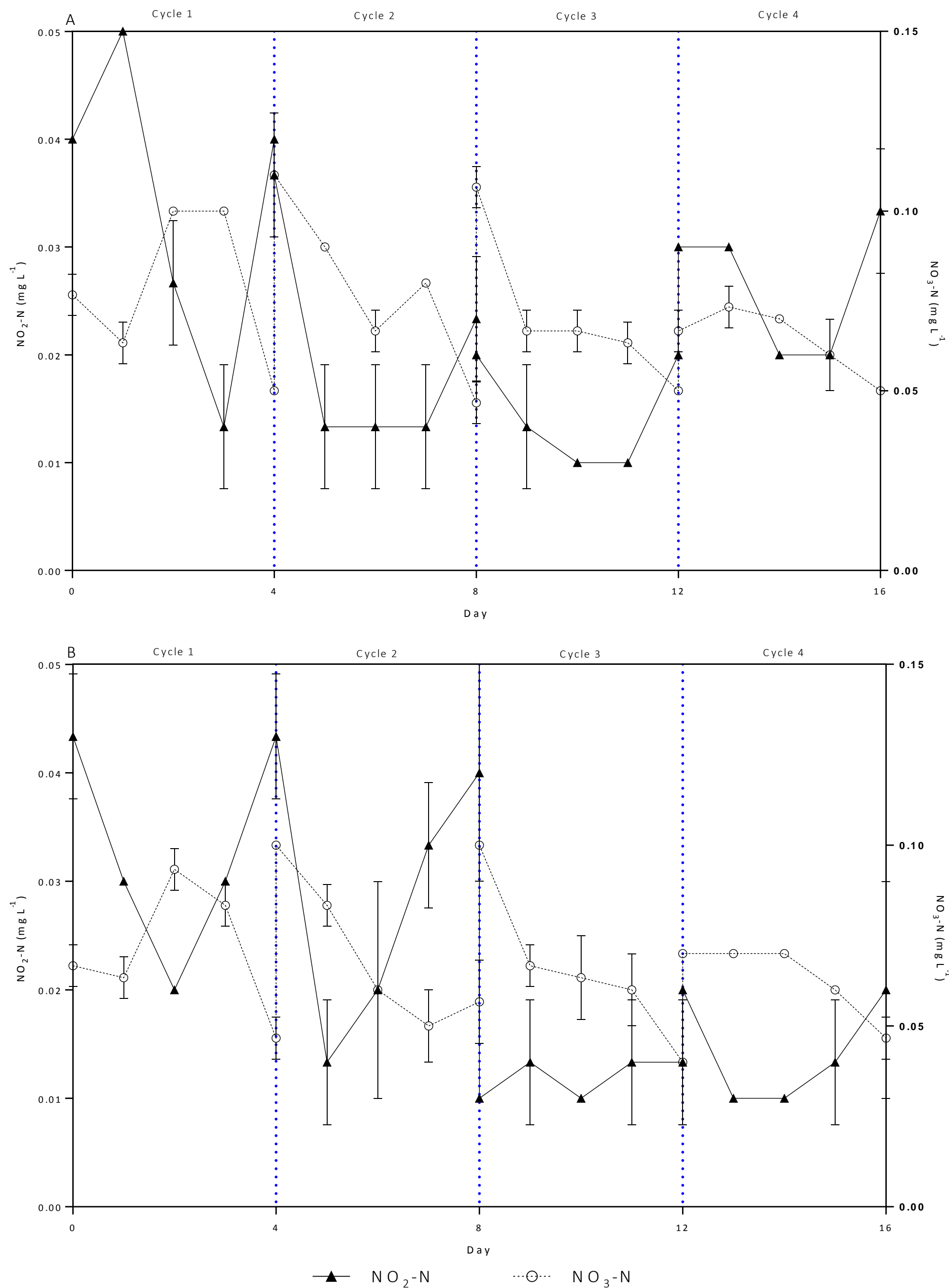


Figure 6.4 – Time-course of NO_2-N and NO_3-N concentrations in mg L^{-1} of PSW treated by semi-continuous operation in the WWPA+*C.v* treatment (**A**; wastewater with pot ale and *C. vulgaris*) and WW+*C.v* treatment (**B**; wastewater with *C. vulgaris*). Each point is a mean ±SD, of n = 3 independent replicates. Some error bars are smaller than the symbols. The dotted line represents the duration of each cycle.

Overall, the NH_3-N and PO_4-P removal efficiencies obtained in the WWPA+*C.v* treatment for all the cycles were in range with data reported in other microalgae wastewater treatment studies operating semi-continuously or continuously under more favourable conditions, such that employed optimised pH control or air/CO_2 aeration and mixing [156, 228, 467, 578, 583, 594]. For example, Arcila and Buitron (2016) [595] achieved 99% NH_4^+-N and 49% PO_4-P removal from settled municipal wastewater in a 50 L HRAP operated in continuous mode at 10 HRT inoculated with a naturally occurring microalgal-bacterial consortium. In a 22 L flat panel PBR, Anbalagan et al., (2016) [596] achieved an average 83% TN and 78% TP removal efficiency from municipal wastewater in a semi-continuous microalgae process over three consecutive cycles operating at a HRT of 6 days.

The NH_3-N and PO_4-P removal in the wastewater was mainly attributed to the microalgae as the control reactors without microalgae showed no appreciable decline. In fact, in the WWC treatment a slight increase in NH_3-N and PO_4-P was detected during cycle 2 based on the concentrations recorded, likely from the degradation of organic N and P fractions in the wastewater or release of cellular matter following cell death (Table 31). Between the cycles, the initial concentration of NH_3-N and PO_4-P was higher than the preceding cycle, presumably because of a higher concentration in the wastewater collected from the plant for each subsequent cycle (Figure 6.5B). However, it must be noted that the composition of the wastewater was not analysed prior to use in the treatments. In the WWPA treatment, a maximum NH_3-N removal efficiency of 22% was recorded by the end of cycle 1, and a maximum PO_4-P removal efficiency of 8% by the end of cycle 2 over the whole duration of these experiments (Figure 6.5A; Table 31). This decrement can be ascribed to a higher metabolic activity of the microbial community present in the PSW as a result of the exogenous pot ale, which coincided with a decrease in COD concentration (Table 33). However, no difference in biomass concentration was recorded at the end of each cycle, with an average final concentration of 167 ±12 mg L^{-1} for all cycles. Although a small change in NH_3-N concentration is noted in the WWPA treatment, no substantial difference in both the NO_2-N and NO_3-N concentrations were recorded, with the concentrations trending to be below the detection limit throughout the duration of the experiment (Figure 6.6). A major limitation to these control treatments was the low concentration of dissolved O_2, which can be attributed to the cultures having been incubated statically (Table 32). This will have impacted on the metabolic activity of the endogenous microorganisms in digesting and assimilating inorganic N compounds or converting them by nitrification and, thus, limiting their removal.

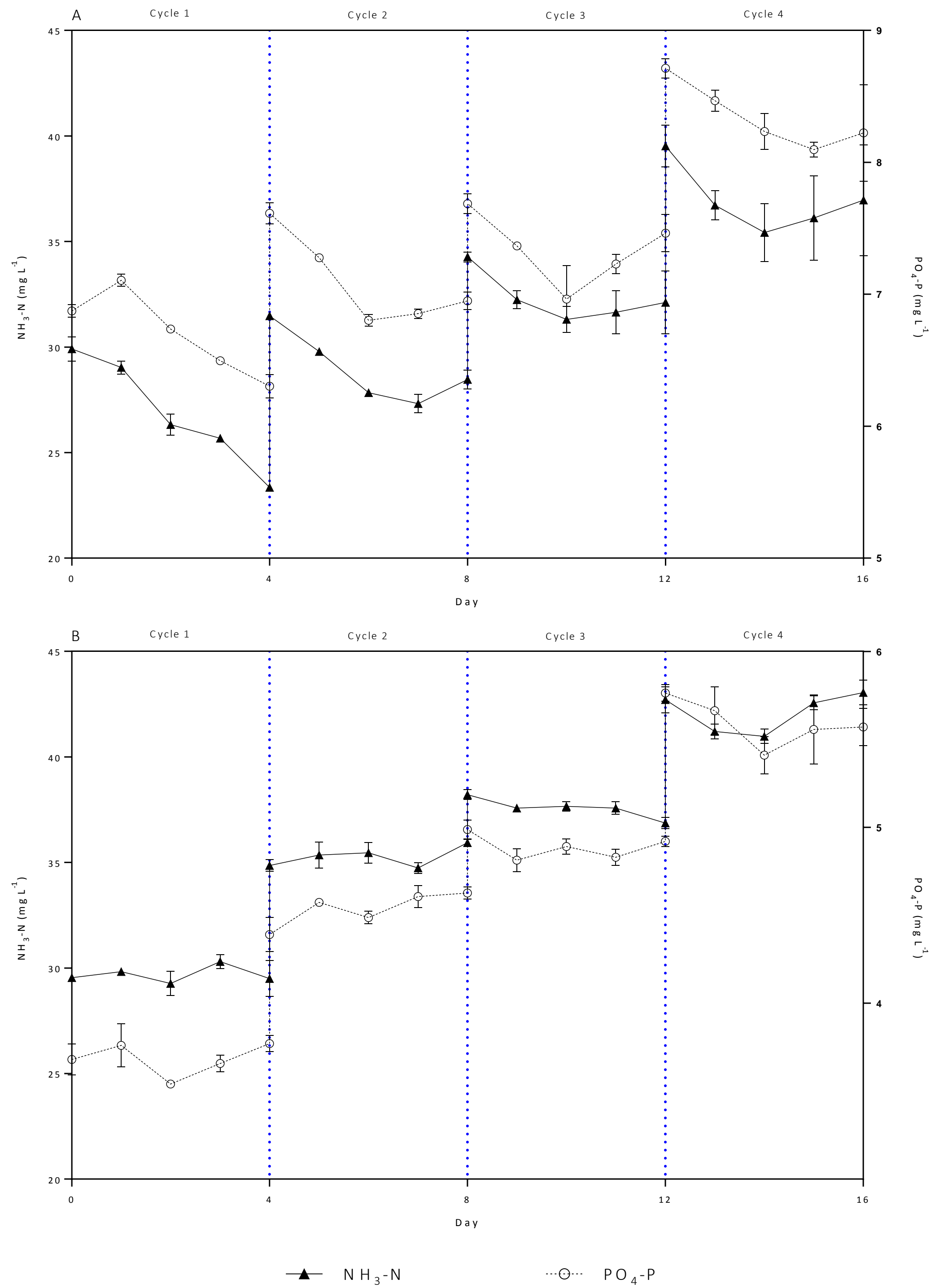


Figure 6.5 – Time-course of NH_3-N and PO_4-P concentrations in mg L^{-1} of PSW treated by semi-continuous operation in the WWPA treatment (**A**; wastewater with pot ale) and WWC treatment (**B**; wastewater only). Each point is a mean ±SD, of n = 3 independent replicates. Some error bars are smaller than the symbols. The dotted line represents the duration of each cycle.

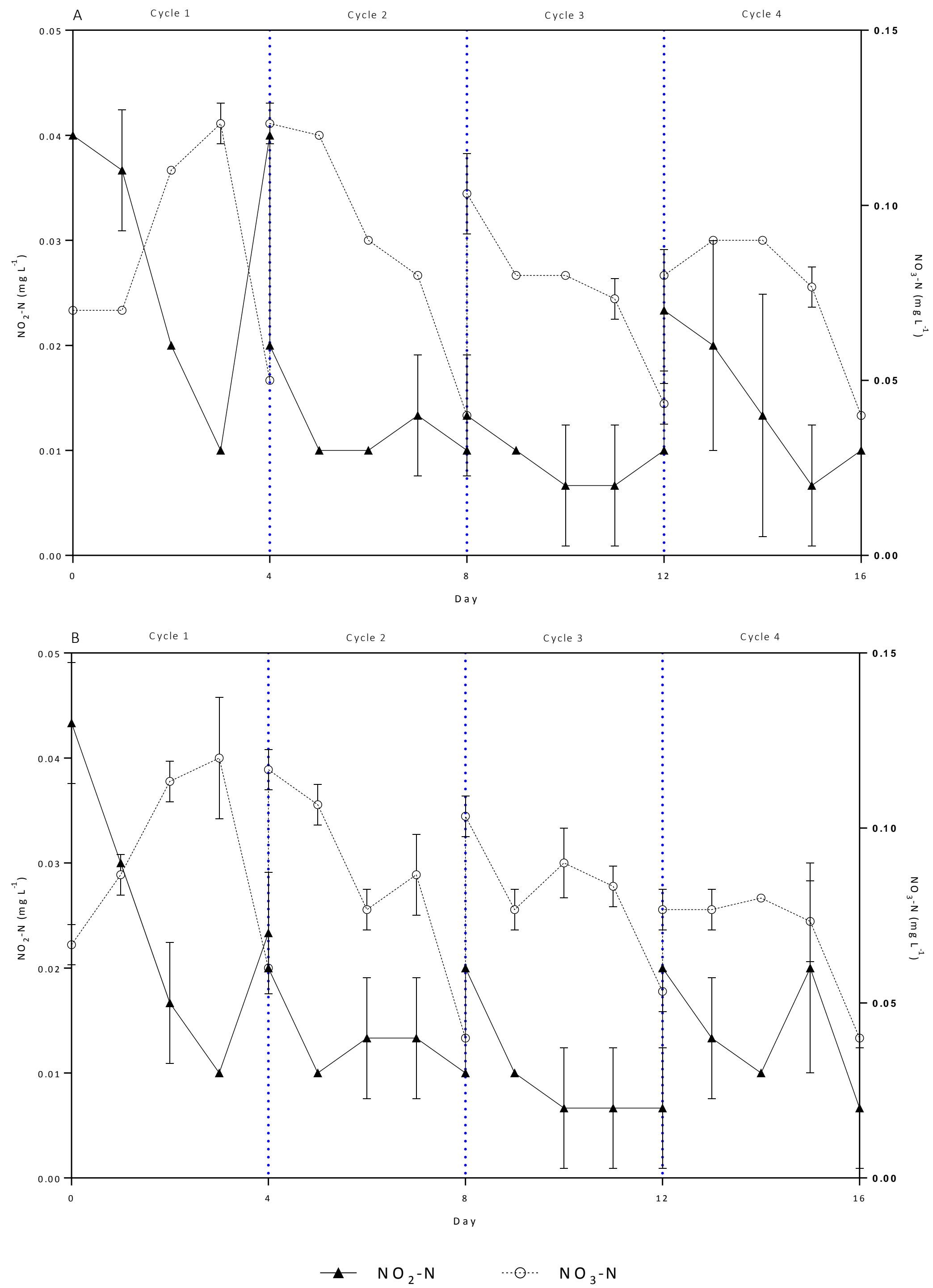


Figure 6.6 – Time-course of NO_2-N and NO_3-N concentrations in mg L^{-1} of PSW treated by semi-continuous operation in the WWPA treatment (**A**; wastewater with pot ale) and WWC treatment (**B**; wastewater only). Each point is a mean ±SD, of n = 3 independent replicates. Some error bars are smaller than the symbols. The dotted line represents the duration of each cycle.

6.3.2 Performance of a large-volume microalgae semi-continuous process treating pot ale enriched PSW

Figure 6.8 represents the changes in both the NH_3-N and PO_4-P concentrations in the 7 L semi-continuous operated WWPA+*C.v* treatment. The concentration of these inorganics in the initial two cycles was low relative to wastewater samples used in the previous experiments of this thesis (Chapters 4 and 5), because of the unfortunate circumstance of each sample being collected after a period of heavy rain. The initial concentrations of NH_3-N and PO_4-P were respectively 6.1 ±0.01 mg L^{-1} and 3.1 ±0.02 mg L^{-1} in cycle 1, and 6.1 ±0.5 mg L^{-1} and 2.3 ±0.06 mg L^{-1} in cycle 2. As a result of the low initial concentration, the demand exceeded the availability, and NH_3-N rapidly declined below the detection limit by the second day of treatment in both cycle 1 and 2 (Figure 6.8). In cycle 1, both the NO_2-N and NO_3-N concentrations declined from an initial 0.07 ±0.0 mg L^{-1} and 0.87 ±0.01 mg L^{-1}, respectively, to below the detection limit at day 2, with no change thereafter (Figure 6.9). Apart from a small increase in NO_3-N at the beginning of cycle 2, as a result of fresh PSW being supplied, both the NO_2-N and NO_3-N concentrations were below the detection limit for the whole duration of the cycle (Figure 6.9). Consequently, it can be stated that as of the second treatment day in cycle 1 and 2 there was an insufficient supply of inorganic N in the wastewater.

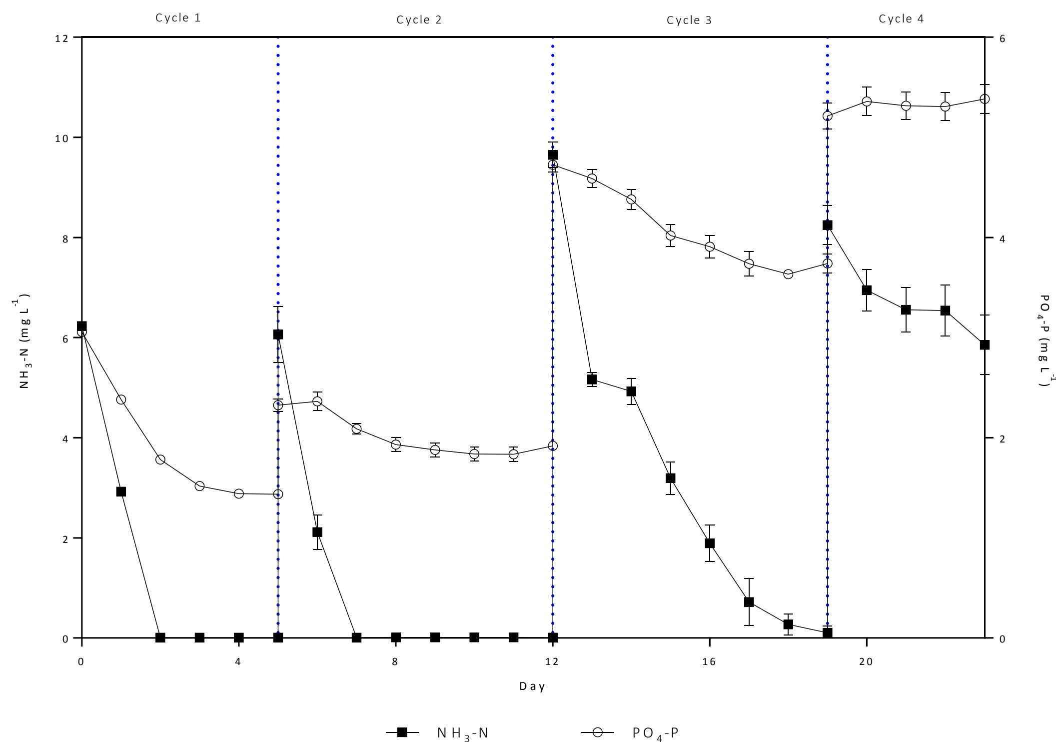


Figure 6.8 – Time course of NH_3-N and PO_4-P concentrations in mg L^{-1} of pot ale enriched PSW treated by semi-continuous operation under the conditions with microalgae (WWPA+*C.v* treatment) in 7 L internally illuminated reactor. Each data point is the mean ±SD, of n = 3 independent replicates. Some error bars are smaller than the symbols. The dotted line represents the duration of each cycle.

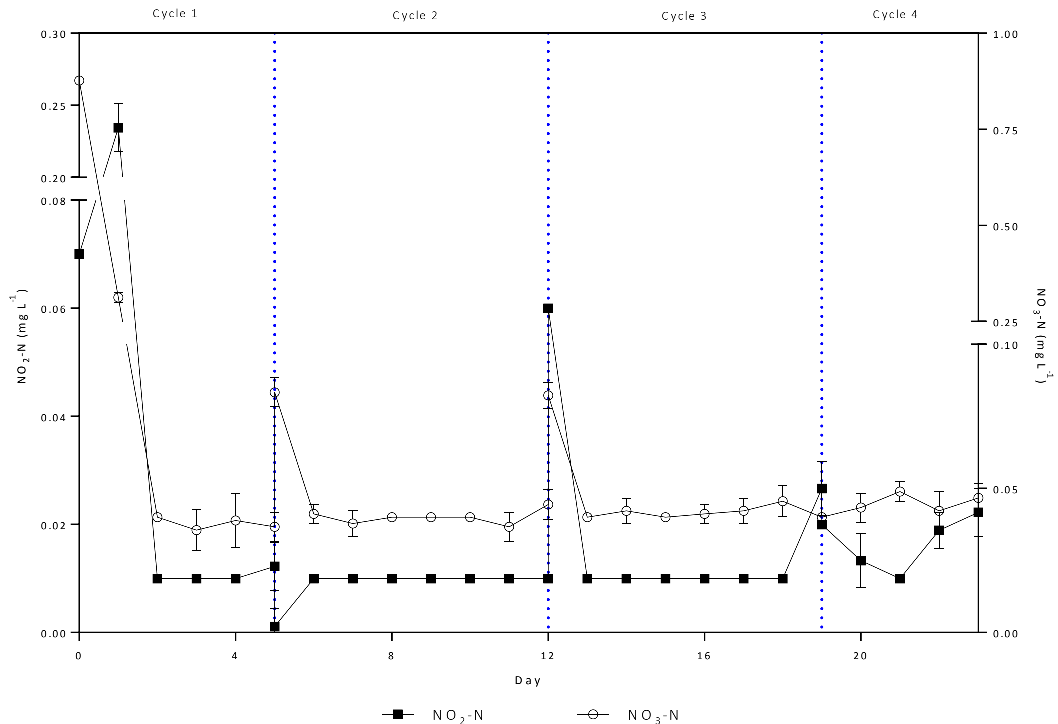


Figure 6.9 – Time course of NO_2-N and NO_3-N concentrations in mg L^{-1} of pot ale enriched PSW treated by semi-continuous operation under the conditions with microalgae (WWPA+*C.v* treatment) in 7 L internally illuminated reactor. Each data point is the mean ±SD, of n = 3 independent replicates. Some error bars are smaller than the symbols. The dotted line represents the duration of each cycle.

The supply of N is known to be a critical factor in regulating lipid synthesis and yield in microalgae [146, 597]. Microalgae have been reported to typically have a lipid content of less than 20% based on the final dry weight of cell biomass when cultured under normal (non-stressed) conditions [598]. When N becomes the growth limiting factor, the lipid content increases with final yields of 60 to 80% reported as a result of the microalgae transitioning to anabolism energy-rich storage compounds such as starch and lipids [334, 599, 600]. However, this effect is accompanied by cell-cycle cessation and reduction in productivity as a result of N not being available for protein synthesis, which is essential for cellular metabolism and growth [68, 339]. Previous studies have observed targeted nutrient recycling of endogenous N-rich compounds such as proteins, previously required in various anabolic pathways which become superfluous in an adaptive response to N limitation in their environment [601, 602]. Lavin and Lourenco (2005) [603] observed a drop in intracellular inorganic N pools in various marine microalgae between the mid-exponential and the late-exponential phases to sustain growth for a short period of time, following N-deplete conditions in mid-exponential phase. Park et al., (2015) [604] observed a continuation in *C. reinhardtii* (strain CC-400 cw15 mt$^+$) growth up to 12 hours after N deprivation occurred, based on optical density and cell abundance data. The interpretation of the above studies confirms the physiological accumulation of N reserves and *in vivo* N recycling in microalgae.

Figure 6.10 presents the growth of *C. vulgaris* in the WWPA+*C.v* treatment over a period of 23 days. Based on its growth, *C. vulgaris* can be suggested to have utilised the assimilated NH_3-N initially and subsequent intracellular N compounds to sustain growth following N limitation as of the second day of treatment in the first two cycles. A clear acclimation phase is noticed in cycle 1, indicated by a 3-day lag, after which the cell concentration increased from 1.0 $x10^7$ ($5.8x10^5$) at day 3 to 1.5 $x10^7$ ($1.7x10^6$) at day 5. In cycle 2, the microalgae exhibited a small increase in concentration after the addition of fresh PSW, followed by a sudden increase at day 9. Here, *C. vulgaris* concentration increased from 9.9 $x10^6$ ($\pm 1.1x10^6$) at day 9, to 1.9 $x10^7$ ($\pm 1.3x10^6$) at day 12 (the end of cycle 2). The reason for the extended treatment period from 5 days in cycle 1 to 7 days in cycle 2 was to allow the microalgae to grow to ensure a sufficient concentration in the subsequent cycles after being diluted with fresh PSW. Although the cell concentration increased in cycle 2, this was not accompanied by an increase in biomass yield. At the end of cycle 1 and 2, biomass concentrations were 385 ±5 and 255 ±1 mg L^{-1} respectively.

The data obtained for both cycles define a clear contradiction in the biomass concentration and the cell concentration, indicating that biomass content per cell might have changed. Sforza et al., (2014) [156] obtained a comparable result when cultivating *C. protothecoides* continuously in unsterilized settled municipal wastewater. The authors note a significant reduction in biomass concentration between the dark and light phases of the culture conditions (12:12 light:dark), despite the cell concentration remaining constant. The reduction of biomass was attributed to an intracellular biomass loss following the consumption of stored compounds during dark respiration. The biomass concentration increased during the light period, accompanied with the consumption of NH_3-N and PO_4-P. Given that the microalgae in this study were nutrient limited, it is possible that following dark respiration during the 12 hour dark period, the microalgae could not replenish intracellular resources consumed to sustain cellular metabolise and respiration, and in the long-term leading to a reduction in biomass between cycle 1 and 2. This inference is based on the assumption that further to inorganic N being limited, so may have been carbon. In addition to the dilute inorganic N and P concentrations in the PSW of cycles 1 and 2, the initial COD concentrations were low relative to the concentrations recorded in the previous batch and small-volume semi-continuous WWPA+*C.v* treatments (Table 34). Based on the trend in COD concentration for the WWPA+*C.v* treatments operated under batch mode (Section 5.3.3; Figure 5.7), it is reasonable to suggest that the bioavailable carbon in the wastewater of the WWPA+*C.v* treatment in this experiment was removed by the third day of treatment in each cycle. Consequently, the microalgae will have been carbon and N limited, directly resulting in biomass loss as the required metabolites could not be replaced following their consumption in catabolic reactions. However, analysis of the microalgae's composition in regards to carbon, N and P content is necessary to drive firmer conclusions.

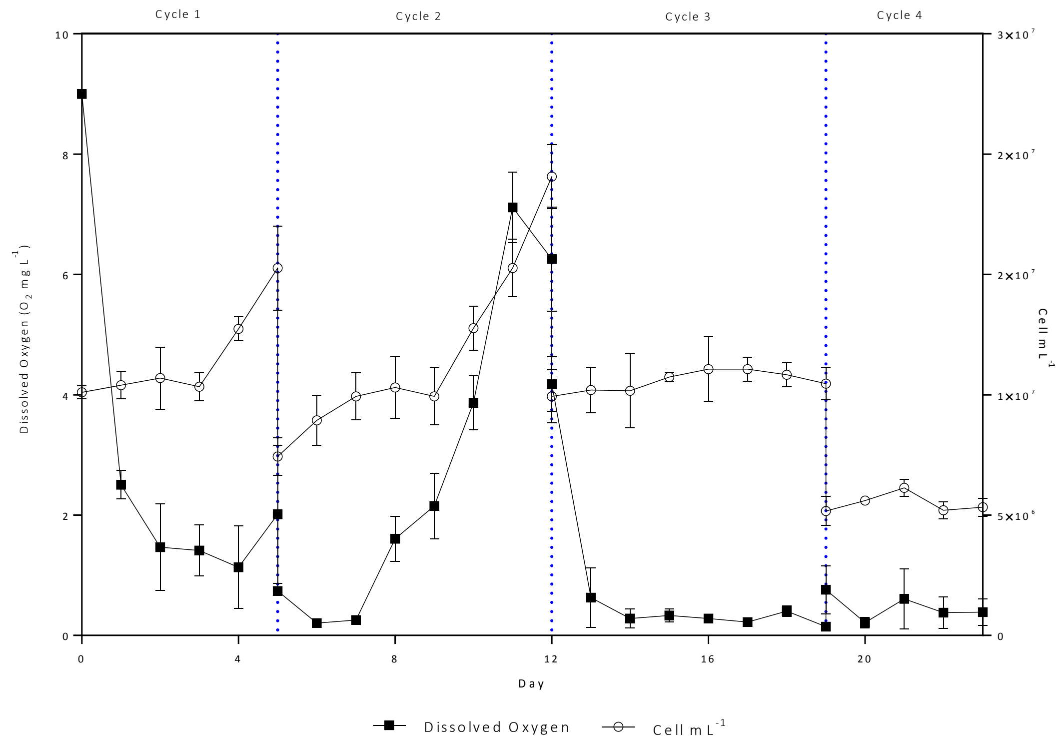


Figure 6.10 – Changes in *C. vulgaris* concentration in cell mL^{-1} and dissolved O_2 concentration in mg L^{-1}, of pot ale enriched PSW treated by semi-continuous operation under the conditions with microalgae (WWPA+*C.v* treatment) in 7 L internally illuminated reactor. Each data point is the mean ±SD, of n = 3 independent replicates. Some error bars are smaller than the symbols. The dotted line represents the duration of each cycle.

Table 34 – Initial and final COD concentrations in mg L^{-1} O_2 of PSW treated under the condition with microalgae enriched with pot ale. Each data point is a mean ±SD, of n = 3 independent replicates.

Phase	Cycle 1	Cycle 2	Cycle 3	Cycle 4
Initial	289 ±3.8	240 ±11.1	383 ±3.5	368 ±7.2
Final	113 ±1.5	118 ±2.9	205 ±3.5	284 ±10.1
Removal efficiency (%)	60	50	46	22

In regards to PO_4-P, a small decline in concentration is observed during cycle 1 until day 3, at which point the concentration remained constant at approximately 1.5 ±0.5 mg L^{-1} for the remaining 2 days of the cycle (Figure 6.8). In cycle 2, the concentration of PO_4-P declined by a total of 0.5 mg over the 7-day treatment period reaching a concentration of 1.8 ±0.08 mg L^{-1} at day 12. In all the reactors, the pH remained in the range of 6.6 to 7.3 over the 23-day duration of this experiment, suggesting that the small quantity of PO_4-P removed was mainly by means of assimilation by the microalgal-bacterial co-culture and not mediated by inorganic P precipitation [376].

A large variation in *C. vulgaris* growth and inorganic N and P removal is noticed for cycles 3 and 4 compared to cycles 1 and 2. In cycle 3, the concentration of NH_3-N and PO_4-P was high relative to the previous cycles, likely because the wastewater was collected during a period of dry weather. The

concentration of NH_3-N declined to below the detection limit during the 7-day duration of cycle 3, from 9.5 ±0.3 to 0.09 ±0.1 mg L^{-1} (Figure 6.8). The high NH_3-N removal efficiency was associated with its re-addition to the microalgae. Boonchai et al., (2015) [605] recorded an improved response in N and P by *Chlorella* sp. when treating synthetic wastewater and grown under N-deplete conditions compared to the control culture which was grown in medium with excess N and P, for 40 hours prior to inoculation. After 48 hours of treatment, a total of 82% TN and 92% TP were removed by the N-deplete *Chlorella* sp. culture and a total of 60% TN and 89% TP by the control *Chlorella* sp. culture. This effect has also been described by Wang et al., (2016) [606] and proposed as a strategy to enhance NH_3-N from wastewater by microalgae. In this study the decline in NH_3-N was not accompanied with an equivalent response in PO_4-P assimilation by the microalgal-bacterial co-culture. Only 1 mg PO_4-P was removed from the wastewater over the 7-day duration of cycle 3, reaching a concentration of 3.7 ±0.09 mg L^{-1} by day 19 (Figure 6.8). Similarly no discernible change in *C. vulgaris* concentration was recorded in the whole cycle, whereas the biomass concentration increased from 169 ±0.01 mg L^{-1} at the beginning to 253 ±0.01mg L^{-1} at the end of cycle 3. Re-addition of N in microalgae cultures has been reported to be a slow enhancer of protein content, with the activation and expression of enzyme necessary for N assimilating and storage [604, 607].

Although the increase in biomass concentration in cycle 3 may in part be an increase in microalgae density, it must be noted that the wastewater became more turbid, accompanied with a decolouration from green to yellow-green. A main factor in this development will have been the fact that the wastewater was not filtered prior to use. Consequently, as microalgal biomass was removed following each cycle and not recycled, an increase in total suspended solids may in part be attributed to the increase in biomass concentration. The addition of fresh PSW will have also been accompanied by the addition of naturally occurring heterotrophic organisms. In fact, small aggregates became visible with the naked eye as of day 2 of treatment in cycle 3, exhibiting a floc-like appearance. The occurrence of this was not observed in the WWPA+C.v small-volume semi-continuous treatment. A wastewater sample collected before the reactors were replenished with fresh PSW on day 12 (end of cycle 2) is shown in Figure 6.11A, and on day 19 (end of cycle 3) in Figure 6.11B. Overall, a greater quantity of insoluble matter was observed at the bottom of each reactor in cycle 3 compared to cycle 2, prior to mixing and sample collection. Moreover, compared to cycle 2 in which the increase in cell concentration was accompanied by an increase in dissolved O_2, reaching 6.2 ±0.9 mg L^{-1} at day 12, the concentration of dissolved O_2 declined to below 0.5 mg L^{-1} by the second day in the cycle 3 (Figure 6.10). The low dissolved O_2 concentration in cycle 3 indicates a prevalence of heterotrophic carbon-oxidation over photosynthetic O_2 evolution. Based on the formation of small flocs, the increase in insoluble matter in the reactor and a substantial decline in dissolved O_2 concentration, it can be suggested that the population of heterotrophic organisms had increased beyond a suitable ratio under which the microalgae could compete with. Unfortunately, once the experiment had been set up it was noticed that the arrangement of the internalised LED strips fixed around the central pole resulted in a non-uniformed distribution of light, with distinct dark zones occurring (Figure 6.12). While this set-up

indicated not to be an issue in supporting growth and photosynthetic activity of the microalgae, based on the dissolved O_2 and cell concentration increase in cycle 2, the attenuation of light to the microalgae due to the shading effects caused by an increase in suspended solids may have been conducive in reducing photosynthetic activity in cycle 3. During cycle 4, the colour of the wastewater turned grey and was accompanied by a rancid odour. As a result of microalgae showing no growth and effective removal of NH_3-N and PO_4-P the experiment was terminated.

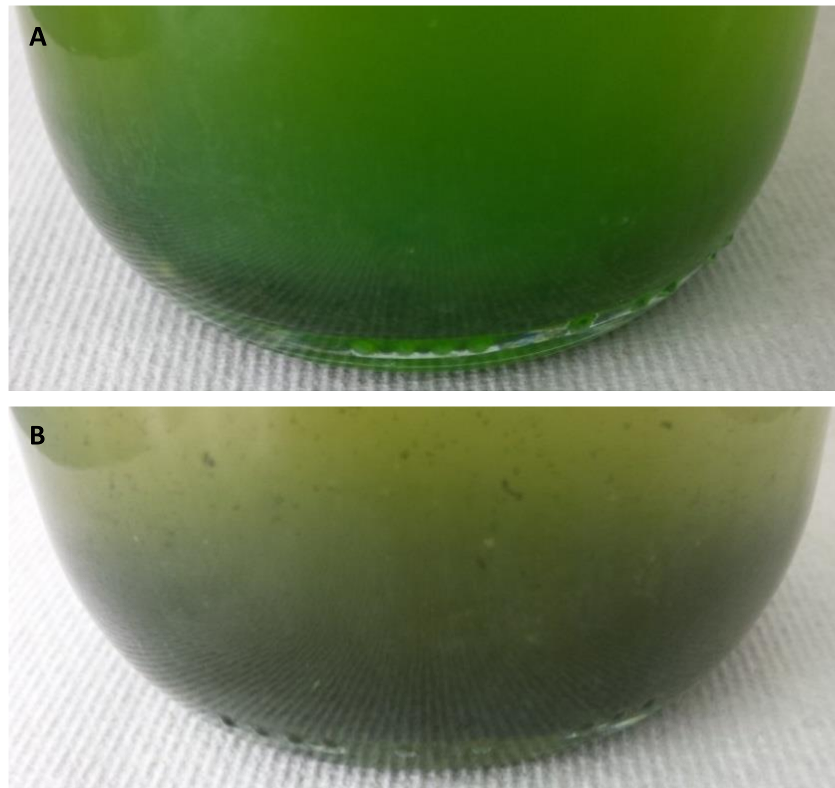


Figure 6.11 – Depiction of the progressive decline in colouration of PSW treated under the conditions with microalgae and enriched with pot ale. Image **A** taken at the end of cycle 2 (day 12), and image **B** at the end of cycle 3 (day 19).

Figure 6.12 – Reactor set-up for the large-volume semi-continuous WWPA+*C.v* treatment with internalised LED strips. Red arrows denote dark zones. Image taken on day 4.

6.4 Conclusion

The present study demonstrates that a static microalgae wastewater treatment process under semi-continuous operation at bench scale was effective at removing inorganic N and P from PSW enriched with pot ale compared to those treatments without *C. vulgaris*, either enriched with or without pot ale. A complete removal of NH_3-N (from 29.2 ±0.5 to 0.01 ±0.01 mg L^{-1}) and 71% removal of PO_4-P (from 6.7 ±.0.07 to 1.9 ±0.15 mg L^{-1}) were achieved within 4 days (cycle 1) in the WWPA+*C.v* treatment. Additionally, a substantial reduction in COD concentration (74%) and a stable dissolved O_2 concentration were reached, as well as an increase in *C. vulgaris* concentration. However, results in the subsequent three cycles in the WWPA+*C.v* treatment highlight that inorganic N and P removal was less effective. This was accompanied by a lower concentration of *C. vulgaris* at the end of each cycle. A similar observation was noted in the WW+*C.v* treatment. The constant alkali conditions, which formed during each cycle in both the microalgae treatments, is suspected to be the cause of the decrease in inorganic N and P removal efficiencies and microalgal growth.

A further experiment was conducted to evaluate the performance of the WWPA+*C.v* semi-continuous treatment process at 7 L in a reactor configured with internalised lights. However, as a result of the inorganic N and P concentrations being low in the wastewater samples of the initial two cycles, poor performance and microalgae growth were observed throughout the duration of the experiment. The initial imbalance in inorganic N availability was suggested to be the limiting factor affecting inorganic P removal and microalgae growth. Consequently, it is possible that the culture conditions facilitated the growth of the native microflora of the wastewater at the expense of microalgal population. This

inference was based on the observed decline in *C. vulgaris* and total biomass concentration between the cycles, and difference in colour (from green to grey) and odour.

Overall, based on the experimental results, the stability of a static semi-continuous microalgae wastewater treatment process for long-term operation still remains to be determined. Additional measures, such as biomass recycling, that would help increase the solids retention time and to control pH should be considered in order to sustain nutrient removal and abundance of the active microalgae population.

Chapter 7 – General Conclusion

The application of microalgae to treatment municipal or industrial wastewaters for remediation of carbonaceous, nitrogenous and phosphorus materials was first proposed almost 70 years ago, operated as sewage oxidation ponds [63, 608]. In the decades since, a large body of work on the development of microalgae wastewater treatment processes has substantiated their application and efficiency, not only in respect to remediating the eutrophic fractions (i.e. carbon, N and P) contained in the wastewater, but also in pathogen reduction, toxic metal adsorption and removal of other emerging contaminants (e.g. pharmaceuticals, personal care products and trace organic compounds (i.e. phenol, etc.). Despite their success, however, much of the applied research on using microalgae for wastewater treatment has focused on optimising the process to achieve high biomass yields and productivity for bioenergy production, either by selecting for naturally high energy-rich algal species or by evaluating their response to stress factors associated with the production of value-added products. The use of wastewater as a medium in these studies is mainly used to reduce production cost, with the treatment thereof considered as a concurrent benefit and a secondary objective. As a result, much of the work has been carried out (either in a laboratory setting or small-scale industrial trials) in optimal culture conditions in which the microalgae in photobioreactors are continuously mixed or aerated. As detailed in Chapter 1, the application of continuous mixing or aeration in microalgae cultivation consumes a significant proportion of the total energy used. This directly affects the economic viability and sustainability of a microalgae wastewater treatment process, as well as its competitiveness against established secondary processes widely used in the industry today. A further aspect which must be considered is the stage in the treatment at which the microalgae are introduced. The application of microalgae to treat secondary treatment effluent – i.e. after the energy intensive secondary treatment stage – would not result in the much-desired reduction in overall energy demands of wastewater treatment.

To improve the economic feasibility of microalgal to treat wastewater, the research described herein aimed to evaluate the performance of a static microalgae treatment process. In brief, initial experiments evaluated carbonaceous, nitrogenous and phosphorous removal of selected microalgae strains from PSW cultured in two modes: aerated and static. The latter condition was performed in order to evaluate and identify any limitations this strategy may have on microalgal productivity and treatment performance in order to establish an energy-efficient and cost-effective microalgal treatment process compared to conventional wastewater systems. Further experiments were carried out to assess the treatment efficiency of PSW by *C. vulgaris* in response to exogenous carbon loading under static culture conditions. The major findings for each of the chapters are described below, followed by description of the impact of the work and suggestions for further work.

7.1 Summary of main findings

7.1.1 Microalgae selection for contaminant removal from wastewater (Chapter 4)

The selection of a suitable microalgal strain is important in establishing a robust microalgae wastewater treatment process. Therefore, the aim of the work described in Chapter 4 was to carry out a selection between *C. vulgaris*, *A. obliquus* and *H. riparia* species under the same experimental conditions, evaluating the aptitude to grow in unsterilized PSW in terms of biomass productivity and wastewater treatment efficiency. The results of the laboratory experiments showed potential application for effective treatment by the strains *C. vulgaris* and *A. obliquus*. Characterisation of the PSW revealed significant NH_3-N and PO_4-P reductions at respectively 6.08 and 0.63 mg L^{-1} d^{-1} in the WW+Air+*C.v* treatment, and 2.25 and 0.47 mg L^{-1} d^{-1} in WW+Air+*A.o* treatment. The removal rates translated to an approximate efficiency of 98% in the WW+Air+*C.v* treatment and 86% in the WW+Air+*A.o* treatment. A clear arithmetic phase of growth in *C. vulgaris* and *A. obliquus* was recorded in these treatments, at an approximate rate of 0.22 d^{-1}. In comparison, inorganic N and P removal rates by *H. riparia* in the WW+Air+*H.r* treatment were notable lower at 4 mg L^{-1} d^{-1} NH_3-N and 0.18 mg L^{-1} d^{-1} PO_4-P, with efficiencies of 54% NH_3-N and 42% PO_4-P. The variation in removal efficiency and productivity between *C. vulgaris*, *A. obliquus* and *H. riparia* was in part explained by their individual morphological, phenological and genotypic features. In previous studies, it has been demonstrated that cell size has an influence on the achievable maximum growth rate of a microalga [406–408]. As high N and P assimilation by microalga is closely related to growth, it was postulated that the high inorganic N and P removal by *C. vulgaris* was in relation to an improved acclimation and growth response because of its smaller cell size, compared to *A. obliquus* and *H. riparia* which are naturally bigger species. Although it was not concluded that the species of *C. vulgaris* used produced algaenan, it was suggested that differences in its cell wall structure compared to A. obliquus or H. riparia improved the its resistance and negative interference to allelopathic interactions.

In the static cultured treatments the removal efficiency of inorganic N and P did not manage to satisfy European Commission Directive limits. Indeed, low removal rates ranging from 1.25 to 1.87 mg L^{-1} d^{-1} NH_3-N and 0.52 to 0.17 mg L^{-1} d^{-1} PO_4-P were recorded in the microalgae treatments for the 7 day duration of the experiment. As a consequence of the low removal rate final concentrations of NH_3-N and PO_4-P were respectively 18.2 ±0.2 and 1.67 ±0.04 mg L^{-1} in the WW+*C.v* treatment, and 19.2 ±0.1 and 0.16 ±0.04 mg L^{-1} in the WW+*A.o* treatment. Analysis of the PSW in these treatments highlighted the formation of an alkaline condition which occurred concurrently with the demise in inorganic N and P removal. The pH in the WW+*C.v* treatment increased in the first day of treatment to 9.5 ±0.1 and further thereafter reaching a maximum of 10.9 ±0.1 by day 4. Similarly, in the WW+*A.o* treatment pH increased at a more gradual rate over the whole treatment period, reaching 10.6 ±0.1 at day 7. Considering the main difference between the aerated and non-aerated culture modes was the provision of inorganic carbon supplied to the microalgae, in the form of CO_2, it was inferred that the availability of a carbon source was the limiting factor in the wastewater used in this particular study. Indeed, analysis

of the COD in the static microalgae treatments highlighted a limited capacity of the algae to reduce and therefore utilise the carbonaceous material in the PSW. This was most likely because of the high variability and complexity in the compositions of the compounds. Similar observations have been reported in the literature in which studies demonstrated a reduced efficiency and assimilation of inorganic N and P because of limited carbon resources, both as inorganic and organic carbon which are metabolised via photosynthesis and the TCA cycle respectively. It was concluded from the results that a major limitation to the wastewater treatment efficiency by the microalgae under static cultivation was the low availability of carbon. Among the selected strains, *C. vulgaris* was selected for further experiments in which the PSW was enriched with exogenous organic carbon with the aim of improving treatment performance.

7.1.2 - Effects of carbon enrichment on inorganic N and P removal from PSW by C. vulgaris (Chapter 5)

The addition of a bioavailable organic carbon source to PSW had a significant effect on *C. vulgaris* inorganic N and P removal efficiency under static culture conditions. In an initial experiment conducted on PSW enriched with glucose, to an equivalent COD concentration of 300 mg L^{-1} O_2, the concentration of NH_3-N and PO_4-P was reduced below the detection limit by day 2 in the microalgae treatment (WWG+*C.v*). Here, the NH_3-N declined from an initial concentration 28.6 ±0.1 to 0.1 ±0.05 mg L^{-1} and the PO_4-P from an initial concentration 3.2 ±0.02 to 0.1 ±0.01 mg L^{-1} by day 2. In addition, exogenous glucose promoted cell productivity and biomass yield, as well as a more stable pH profile for the 5 day duration of the experiment (ranging between 6.6 and 9.3). Conversely, in the WW+*C.v* treatment without enrichment with glucose the concentration of NH_3-N and PO_4-P decreased to a final value of 15.5 ±0.5 mg L^{-1} and 0.8 ±0.01 mg L^{-1} respectively. From these results it was clear that whilst *C. vulgaris* naturally aided in inorganic N and P removal from the PSW by means of assimilation, the addition of glucose significantly improved their capability. According to metabolic pathways, the absorbed glucose was utilised in the assimilation and anabolism of NH_3-N to amino acids via its metabolism in the glycolysis pathway and TCA cycle. Conversion of glucose in these biochemical pathways assisted in replenishing the intermediates 2-oxoglutarate and oxaloacetate which are sequester from the TCA cycle in the GS-GOGAT pathway for glutamate synthesis (i.e. anaplerotic reactions). Furthermore, the metabolism of glucose was a source of ATP and NADH generation critical to PO_4-P assimilation and integration into RNA for protein translation necessary for cell growth and maintenance.

Further experiments were carried out on different wastewater grab samples to evaluate the reproducibility of the static microalgae process treating organic carbon enriched PSW, to take into account the natural fluctuations in the composition of wastewater (i.e. biological/chemical). In addition to enriching with glucose, treatments with glycerol and intermittent CO_2 injection were also included to compare between the use of a different organic and inorganic carbon source. From the results, performance of the microalgae treatments enriched with organic carbon for all three PSW batches yielded consistent responses of inorganic N and P removal, at an efficiency of 90% and above. The observed effect was accompanied with promising microalgae growth and favourable pH conditions in

the wastewater for the duration of the experimental period. In comparison, no significant difference in contaminant depuration and microalgae growth was observed between the CO_2 enriched microalgae treatment (WWCO_2+*C.v*) and the microalgae only treatment (WW+*C.v*). The average inorganic removal efficiencies in the WWCO_2+*C.v* treatments and WW+*C.v* treatments were 55% and 33% for NH_3-N, and 63% and 70% for PO_4-P, respectively. The difference in treatment response between the inorganic carbon and organic carbon treatments can be explained by the effect of high CO_2 concentration on non-acclimated microalgae. Based on microalgae physiology a high CO_2 concentration supplied to non-acclimated algae, as is the case in these experiments, can negatively influence photosynthetic efficiency and respiration, directly affecting the ability of the microalgae to assimilate N, P and carbon. Therefore the tolerance of *C. vulgaris* to high CO_2 concentrations limited the ability of the alga to sequester the inorganic carbon in an appropriate manner for utilisation in inorganic N and P assimilation and growth.

The same experimental design was employed to investigate the suitability of deproteinated pot ale as an alternative carbon source to glucose or glycerol for the enrichment of PSW. Across the three pot ale enrichment PSW batches treated with *C. vulgaris* (WWPA+*C.v*) the pattern of inorganic N and P removal was similar to the response recorded in the microalgae treatments with glucose or glycerol. However, it must be noted that when comparing the experimental runs, higher initial concentrations of these inorganics did not lead to their reduction to levels as low as those achieved when their initial concentrations were lower, hence suggesting that the capacity of the microalgae in this respect for treating PSW may be limited by the availability of organic carbon. Furthermore, the efficiency of NH_3-N depuration from the PSW by the microalgae in these treatments (WWPA+*C.v*) was observed to have an influence on the PO_4-P removal efficiency. For instance, in the WWPA+*C.v* treatment of the initial experimental run (R4) the NH_3-N concentration declined from 20.9 ±0.09 to 0.09 ±0.0 mg L^{-1} and PO_4-P from 9.8 ±0.02 to 0.5 ±0.06 mg L^{-1}. The removal of inorganic N and P in the repeated WWPA+*C.v* treatments was respectively; from 47.8 ±0.09 to 17.7 ±0.9 NH_3-N mg L^{-1}, and from 9.1 ±0.06 to 3.9 ±0.17 PO_4-P mg L^{-1} in the experimental run R5; and from 35.2 ±0.03 to 4.7 ±0.2 NH_3-N mg L^{-1} and from 7.2 ±0.2 to 2.3 ±0.2 PO_4-P mg L^{-1} in the experimental run R6. When the profile of the inorganic N and P concentrations were juxtaposed with the profile of their respective COD concentration, the cessation in COD removal corresponded to the cessation in inorganic removal. It was concluded from the results that the removal capacity of NH_3-N and PO_4-P that can be achieved by the microalgae was dependent on the concentration of pot ale added under the present experimental design. Since the enrichment of pot ale in the PSW was fixed at a ratio of 1:150 v/v (approximate COD equivalent of 250 mg L_{-1} O^2) it was concluded that this quantity of carbonaceous material promoted the assimilation of approximately <30 mg NH_3-N and <6 mg PO_4-P by the algal cells.

7.1.3 Semi-continuous treatment (Chapter 6)

It is important for microalgae treatment process not only to be sustainable but also capable of continuous treatment of wastewater. To this end, the organic carbon enriched static microalgae treatment process was assessed in a semi-continuous manner for four consecutive cycles. In the small-

volume experiment, the initial cycle of the WWPA+*C.v* treatment demonstrated removal efficiency consistent with that observed in the previous organic carbon enriched microalgae treatments under batch operation. However, a decline in removal efficiency was noted in the subsequent cycles. The underlying reason for this was ascribed to the high pH levels that occurred within each cycle and the potential influence of repeated exposure to copper in the pot ale. As the microalgae were not recycled or replenished between cycles, the prolonged exposure will have negatively affected the cells health as well as facilitated the formation of free NH_3. A slight decline in biomass was noted in the subsequent cycles as a result which will have affected the overall capability of the microalgae treatments at remediating inorganic N and P from the wastewater. In the large-volume semi-continuous WWPA+*C.v* treatment, the dilute composition of the wastewater samples collected for the initial two cycles highlight that the effectiveness of the system is strongly affected by low concentrations of inorganic N and P.

7.2 Impact of research study

The findings obtained from this study have considerable implications towards improving the sustainability and treatment performance of municipal wastewater by microalgae. The application of microalgae as a treatment stage in present wastewater treatment trains is predominantly implemented as a tertiary process after the energy intensive secondary treatment stage, with the aim to reduce the concentrations of inorganic N and P. Consequently, their application is not considered a viable option by the wastewater industry as the additional cost in microalgae cultivation (i.e. mixing/aeration) and complexities in operation do not align to the concept of a circular economy model in respect to energy reduction and improving sustainability of wastewater treatment. The aspiration is to move towards solutions with wider environmental benefits which contribute to the delivery of a circular economy. The results obtained in this thesis demonstrated that the application of microalgae to treat PSW without aeration offers a key area to develop low energy biological wastewater treatment compared to conventional secondary processes. For instance, in the static microalgae treatments treating organic carbon enriched PSW, significant reductions in inorganic N and P were attained. In some of the wastewater samples used the level of reduction in these compounds was below the required limit set by the UWTD (European Commission, 1991). This observation is potentially relevant for countries in the European Union as the implementation of more stringent effluent standards is being proposed with the aim to improve water quality. However, optimisation of the proposed static microalgae treatment process to achieve consistent removal of inorganic N and P under fluctuations in wastewater composition is needed. If this can be achieved adoption of a low energy biological wastewater treatment process by microalgae would be viable. Further benefits of the assessed static microalgae treatment process include the high generation of O_2 by photosynthesis as well as the suppression of a nitrification/denitrification reaction in the process, with the latter aspect having the advantage of reducing operational cost and complexity of a wastewater treatment process.

7.3 Suggestions for future work

The use of algae of as the biological treatment stage in a wastewater treatment train, to reduce nitrogenous, phosphorous and carbonaceous material, has the potential to operate at a lower footprint in terms of energy consumption and greenhouse gas generation compared to conventional biological wastewater treatment processes. Irrespective of these requirements in the cultivation and operation of a microalgae wastewater treatment process, a major challenge that still limits the application of microalgae to treat wastewater is the non-sterile environment associated with the process. Although the work in this thesis demonstrated a robust and reproducible trend in inorganic N and P removal using monocultures of microalgae as the inocula, future work would benefit by improving the stability of the microalgae community used by adopting a native microalgae-bacterial consortium. To this end, a naturally forming microalgae-bacterial consortium should be grown and used from a sample of the wastewater that it is intended to treat. The main reason being that native algal-bacterium consortia are demonstrated to have higher treatment efficiencies in regards to contaminant depuration, as well as faster settling rates leading to improved effluent characteristics (i.e. lower turbidity) compared to non-native wastewater microalgal-bacterial consortia [259, 609]. In respect to the latter parameter, bacteria whose growth and abundance in microalgal-bacterial flocs were permitted to establish naturally improved the flocculation and settling characteristic of the biomass [609]. This has major implications on the operating cost of a microalgae treatment process. For instance in the commercial production of microalgae biomass, its separation from the aqueous phase is estimated to account between 20% to 30% of the total operating expenditure, usually achieved via centrifugation [609]. In a study by Su et al., (2012) [366] evaluating the settleability of microalgae, algae biomass settling rate was enhanced when co-cultured with an inoculum of natural occurring activated sludge bacteria, exhibiting a reduction in TSS concentration from 1.64 to 0.05 g L^{-1} within 30 minutes compared to a reduction from 1.68 to 0.41 g L^{-1} in the algae only culture. This level of reduction is within the maximum TSS concentration limit of 0.05 g L^{-1} set by the UWTD [14]. Although the assessment and discussion pertaining to the establishment and settling characteristics of microalgal-bacterial consortium was out of the scope of this thesis, researchers should be encouraged on the co-culturing of natural community of algae and other microorganisms such as yeasts, fungi and bacteria in order to create stable communities that perform in a predictable manner while filling all ecological niches to limit the potential for contamination and culture crashes. Therefore, future work should assess the organic carbon enriched wastewater treatment strategy on a naturally formed microalgal-bacterial consortium in regards to both its treatment performance and settling characteristics, in order to further reduce operating cost and improve treatment efficiency.

Furthermore, by using an established community of microorganisms, including the microalgae, for wastewater treatment, known transcription factor(s) expressed by certain microorganisms in the consortium could be used as indicators of community health in response to variations in wastewater composition. Various compounds which may be present in the wastewater have been identified to

induce oxidative stress responses within algae thereby affecting cellular metabolic pathways and other essential cellular functions [610]. To monitor the health of the microalgal-bacterial consortium, a quantitative PCR assay could routinely be used to monitor the expression of known genes involved in oxidative stress response, for example the antioxidant enzyme ascorbate perioxidase in algae cells. This would be beneficial to the treatment process as it would permit relatively quick changes in operating parameters such as light intensity, HRT, STR and aeration which may alleviate against the acute change in wastewater composition and aid in stabilising the treatment performance. Adoption of such an assay would require further understanding and identification of genes and their transcription factor(s) involved in the response mechanism to known compounds ubiquitous to wastewater, for example pharmaceuticals and personal care products.

Although it must be acknowledged that the use of a microalgal-bacterial consortium with good settling characteristics may not be practical under static culture conditions, ironically in practice the static microalgae treatment process evaluated in this work will require mixing to a certain degree if implemented at an industrial scale. In this regard intermittent aeration is advised as it reduces the energy consumption for aeration. For instance, a 33% to 50% reduction in electricity cost for aeration was reported by [611] in macroalgae cultures supplied with intermittent aeration (16 hours on: 8 hours off) compared to continuous aerated cultures. Thus, further development should be carried out in a reactor configuration that is aerated intermittently, potentially coupled with CO_2 injection. This would have a dual benefit of providing a means of maintaining microalgal-bacterial flocs in suspension during the treatment phase of the process and provide an economic and effective means of pH control. Although the work described in this thesis provided an insight into the treatment response of a static microalgae wastewater treatment process, a major limitation to the inference of its suitability as a process was the lack of pH control in the treatments, which is one of the important culture parameters. In general, it is highly recommended that subsequent experiments assess the effects of various parameters such as osmotic, pH, O_2 and temperature on the treatment efficiency and biomass productivity to obtain the optimal conditions for industrial scale cultivation. Furthermore, long term studies of the static microalgae treatment process in various reactor configurations designed with internalised lights could offer a better insight towards optimising culture performance and indirectly treatment efficiency, especially in regards to substantiating the effects of biofilm growth inside the reactors.

The treatment performance of a microalgal-bacteria consortium in PSW enriched with other organic carbon sources is necessary to establish this process in geographical locations restricted by the supply of pot ale. While deproteinated pot ale was a suitable choice for enrichment in the present work to theoretically control the treatment cost, it is not necessary an economical source for use outside of Scotland. According to previous research, various other food industry by-product streams, including industrial by-product streams from the fruit processing industry, dairy industry and brewing industry including molasses streams, contain high concentrations of saccharides. Besides the enrichment of

saccharides, certain food industry by-product streams may not contain toxic compounds such as copper or exhibit an unbalanced concentration of inorganic N or P as in the case of pot ale, which was described as an effect that negatively impact on algal growth and treatment efficiency in the semi-continuous experiments. However, analysis of alternative by-product streams containing high concentrations of organic carbon in regards to how they are utilised by algal cells would be beneficial in respect to optimising the process through establishing suitable microalgal-bacterial communities. For instance, extracellular hydrolases secreted by wastewater-borne bacteria may be required to convert polysaccharides, such as starch, sucrose, and cellulose, into glucose suitable for microalgae assimilation and metabolism. This strategy, however, would require establishing an appropriate community of relevant and suitable bacteria which can associate in a beneficial manner with the algae through exposer experiments. Therefore, in future, the use of alternative food industry by-product streams as a source of organic carbon for enrichment in PSW to be treated by a microalgal-bacterial consortium require studies analysing the bio-conversion of algae indigestible carbon.

A final route of further investigation would be to conduct a life cycle assessment (LCA) of the static microalgae wastewater treatment process, ideally using experimental data on contaminate depuration using a naturally formed microalgal-bacterial consortium. The use of LCA would provide insight on the overall sustainability of a static microalgal-bacterial treatment process (or intermittently aerated process) by considering the processing method, its energy investment and environmental impact compared to conventional secondary biological treatment process. Downstream biomass processing for methane gas generation or as a source of fertiliser following further processing, such as curing the biomass, would be invaluable towards improving the overall sustainability and environmental impact of the treatment process by contributing to a circular economy model.

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