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NUTRIENT RECOVERY FROM HUMAN URINE BY MICROALGAE

Effects of trace elements and glucose

Faculty of Engineering and Natural Sciences
Master of Science Thesis
June 2020

ABSTRACT

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Master of Science Thesis
Tampere University
Degree programme of environmental, energy and bioengineering
June 2020

Human urine is a source of phosphorus and nitrogen, which are the major nutrients increasing both crop yields in agriculture and eutrophication in natural waters. Recovery and reuse of these nutrients promotes sustainability by reducing the need for industrially produced chemical fertilizers in agriculture and by decreasing nutrient discharges to natural waters. One potential method to recover nutrients from human urine is to use it for cultivation of microalgae, which assimilate nutrients and use them to produce biomass, which in turn can be used as a feedstock for various bioproducts, such as biofuels, fertilizers, or bioplastics.

In this study, nitrogen and phosphorus were recovered from 1:15 diluted source-separated urine by a co-culture of freshwater microalgae *Scenedesmus acuminatus* and indigenous bacteria. The study consisted of two complementary experiments, the first of which was conducted outdoors in a continuously operated raceway pond (RwP) for 91 days. The purpose of the experiment was to optimize the cultivation conditions to achieve maximum nutrient removal in northern climatic conditions. The specific objective was to investigate the impact of mixotrophic growth mode on nutrient removal in Nordic autumn conditions where autotrophic growth is limited by the availability of sunlight. The second experiment was carried out in batch flasks under laboratory conditions, and its purpose was to investigate the effects of individual trace elements on nutrient removal.

The highest removal efficiencies achieved in the RwP experiment were 100 % for PO_4^{3-} and 85 % for NH_4^+. The PO_4^{3-} removal efficiency was substantially increased under mixotrophic cultivation conditions, while the NH_4^+ removal efficiency was not affected by the trophic mode. The results showed that PO_4^{3-} removal can be enhanced in light-limited conditions prevailing in the Nordic autumn by supplementing the cultivation with an organic carbon source, such as glucose. The laboratory-scale batch experiment revealed that the trace elements whose supplementation promoted biomass growth and nutrient recovery from diluted human urine were magnesium, iron, and manganese. The addition of zinc, copper, or boron, on the other hand, had no growth-enhancing effect.

Keywords: microalgae, *Scenedesmus acuminatus*, nutrient recovery, source-separated human urine, raceway pond, trace elements, mixotrophy

TIIVISTELMÄ

Kirsi Järvi : Ravinteiden talteenotto ihmisvirtsasta mikrolevien avulla: Hivenaineiden ja glukoosin vaikutus
Diplomityö
Tampereen yliopisto
Ympäristö- ja energiatekniikan tutkinto-ohjelma
Kesäkuu 2020

Ihmisvirtsa sisältää fosforia ja typpeä, jotka ovat tärkeimmät maatalouden tuottoa sekä luonnonvesien rehevöitymistä lisäävät ravinteet. Näiden ravinteiden talteenotto ja uudelleenkäyttö edistää kestävää kehitystä pienentämällä teollisesti tuotettujen lannoitteiden tarvetta maataloudessa sekä vähentämällä ravinnepäästöjä luonnonvesiin. Yksi mahdollinen menetelmä ravinteiden talteenottoon ihmisvirtsasta on sen käyttäminen mikrolevien viljelyyn, sillä mikrolevät käyttävät ravinteita tuottaakseen biomassaa, jota puolestaan voidaan käyttää raaka-aineena erilaisiin biotuotteisiin, kuten biopolttoaineisiin, lannoitteisiin tai biomuoveihin.

Tässä tutkimuksessa typpi ja fosfori otettiin talteen suhteessa 1:15 laimennetusta erilliskerätystä virtsasta *Scenedesmus acuminatus* -lajin makeanveden mikrolevän sekä paikallisten bakteerien muodostaman sekaviljelmän avulla. Tutkimus koostui kahdesta toisiaan täydentävästä kokeesta, joista ensimmäinen suoritettiin ulkona jatkuvasyöttöisessä rengaskanava-altaassa 91 päivän aikana. Kokeen tarkoitus oli optimoida viljelyolosuhteet ravinteiden maksimaalisen poistumisen saavuttamiseksi pohjoisissa ilmasto-olosuhteissa. Erityisenä tavoitteena oli tutkia miksotrofisen viljelyn vaikutusta ravinteiden poistumiseen pohjoismaisissa syysolosuhteissa, joissa autotrofista kasvua rajoittaa auringonvalon saatavuus. Toinen koe suoritettiin panospulloissa laboratorio-olosuhteissa, ja sen tarkoitus oli tutkia yksittäisten hivenaineiden vaikutusta ravinteiden poistoon.

Allaskokeessa saavutetut suurimmat poistotehokkuudet olivat 100 % fosfaatille ja 85 % ammoniumille. Fosfaatin poistotehokkuus kasvoi merkittävästi miksotrofisen viljelyn aikana, mutta ammoniumin poistotehokkuuteen trofiatila ei vaikuttanut. Tulokset osoittivat, että fosfaatin poistumista voidaan tehostaa pohjoismaisissa syysolosuhteissa, joissa valoa on saatavilla rajallisesti, lisäämällä viljelmään orgaanista hiiltä esimerkiksi glukoosin muodossa. Laboratoriomittakaavan panoskokeet osoittivat, että hivenaineet, joiden lisääminen viljelmään edisti biomassan kasvua ja ravinteiden talteenottoa laimennetusta ihmisvirtsasta olivat magnesium, rauta ja mangaani. Sinkin, kuparin tai boorin lisäämisellä ei sen sijaan ollut kasvua parantavaa vaikutusta.

Avainsanat: mikrolevät, *Scenedesmus acuminatus*, ravinteiden talteenotto, erilliskerätty ihmisvirtsa, rengaskanava-allas, hivenaineet, miksotrofia

PREFACE

I would like to thank professor Jukka Rintala and assistant professor Aino-Maija Lakaniemi for their guidance and for the opportunity to be a part of this project and team. In addition, I wish to thank Ilmari Laaksonen for his assistance and company during this project. I also wish to thank for all the help and precious tips I got in laboratory and on the study field from my co-workers and laboratory staff.

Tampere 29.6.2020

Kirsi Järvi

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

3PG	3-phosphoglycerate
ATP	adenosine triphosphate
AVP	ammonia volatilization percentage
CO_2	carbon dioxide
COD	chemical oxygen demand
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
EDTA	ethylenediaminetetraacetic acid
G3P	glyceraldehyde-3-phosphate
H^+	proton
H_2O_2	hydrogen perokside
HCO_3^-	bicarbonate
HRT	hydraulic retention time
NADPH	nicotinamide adenine dinucleotide phosphate
NE	nitrification efficiency
NH_3	ammonia
NH_4^+	ammonium
$NH_4^+ - N$	Ammonium nitrogen
NO_2^-	nitrite
NO_3^-	nitrate
$NO_3^- - N$	nitrate nitrogen
$O_2^{\bullet -}$	superoxide radical
PBR	photobioreactor
PO_4^{3-}	phosphate
$PO_4^{3-} - P$	phosphate phosphorus
PUFA	polyunsaturated fatty acid
PSI	photosystem I
PSII	photosystem II
RBP	ribulose-1,5-bisphosphate
RE	removal efficiency
Redox	reduction-oxidation
RG-II	rhamnogalacturonan-II
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rotations per minute
RuBisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
RwP	raceway pond
SOD	superoxide dismutase
T	temperature
TAG	triacylglycerol
TDN	total dissolved nitrogen
TDP	total dissolved phosphorus
TE	trace element
TSS	total suspended solids
VSS	volatile suspended solids

1. INTRODUCTION

One of the biggest environmental challenges facing humanity is population growth, which, along with rising living standards, has led to the depletion of many natural resources (Galloway et al. 2008, Roy 2017). Deterioration of farmlands is a global phenomenon, which undermines food security and causes inequality (Galloway et al. 2008, Roy 2017, Smil 2000). Contemporary agriculture relies on chemical fertilizers whose key components are nitrogen and phosphorus. Currently, these indispensable nutrients are obtained by mining phosphorite (phosphate rock) and by industrial nitrogen fixation using a Haber-Bosch process (Galloway et al. 2004, Nagarajan et al. 2020, Roy 2017, Solovchenko et al. 2016). Both processes are expensive in many aspects – they consume money, chemicals, energy, and non-renewable resources, produce CO_2 emissions and other pollutants, and cause irreversible changes in ecosystems (Nagarajan et al. 2020, Solovchenko et al. 2016).

Another problem arising from the use of chemical fertilizers is that they potentially end up in the environment, and these nutrient emissions cause eutrophication and pollution in many ecosystems (Nagarajan et al. 2020, Roy 2017, Smil 2000, Solovchenko et al. 2016, Vitousek et al. 1997). Anthropogenic emissions include run-off from crop fields, wastewaters, and nitrous oxides released into the atmosphere as a result of e.g. combustion of biomass or biofuels (Galloway et al. 2004, Nagarajan et al. 2020, Smil 2000, Solovchenko et al. 2016, Vitousek et al. 1997). For decades, efforts have been made to prevent eutrophication and pollution caused by nutrient emissions, for example, by building wastewater treatment plants to remove nutrients from industrial and domestic wastewaters before discharging them into natural waters (Smill 2000). Recently, the idea of using wastewaters as a source of valuable resources has received more and more support, and general interest has shifted from nutrient removal to nutrient recovery (Nagarajan et al. 2020, Roy 2017).

In domestic wastewater, majority of the nutrient load originates from urine (Maurer et al. 2003), and therefore it makes sense to treat urine separately from other wastewaters. Nitrogen, phosphorus, and other nutrients can be recovered from source-separated human urine, for example, by using it for cultivation of microalgae (Chatterjee et al. 2019, Tuantet et al. 2014b). The biomass produced by microalgae can be used as a feedstock for various bioproducts, such as

biofuels, fertilizers, or bioplastics, and thus the method allows nutrients assimilated by microalgae to be recovered and re-used (Chatterjee et al. 2019, Hernándes et al. 2013, Lopez Rocha et al. 2020, Zhu et al. 2013). In addition, cultivation of microalgae helps in the combat against global warming by capturing CO_2 from the atmosphere or flue gas (Posadas et al. 2015, Praveenkumar et al. 2014, Razzak et al. 2013). The method involves certain challenges, of course, as it still needs to be optimized to achieve maximal biomass growth and nutrient removal as cost-effectively as possible (Chatterjee et al. 2019). In addition, the energy efficiency of some process steps, such as biomass harvesting, should be improved for the whole process to be viable (Razzak et al. 2013).

The purpose of this study was to remove and recover nitrogen and phosphorus from source-separated urine by a co-culture of fresh-water microalgae *Scenedesmus acuminatus* and indigenous bacteria, and to optimize the growth conditions for outdoor cultivation in the Nordic climate. The effects of trace element supplementation and mixotrophic cultivation conditions on nutrient removal efficiency were examined.

2. BACKGROUND

2.1 Natural nutrient cycles

In natural environments, circulation of nitrogen (N) and phosphorus (P) is crucial. Nitrogen and phosphorus are vital for all living organisms, as they are indispensable parts of many biomolecules (Alberts et al. 2002). In nature, elaborate recycling systems allow effective utilization of these valuable elements (Smil 2000).

In nitrogen cycle, nitrogen exists in multiple organic and inorganic forms and is circulated among atmosphere, terrestrial, and aquatic ecosystems (Galloway et al. 2004, Vitousek et al. 1997). Nitrogen cycle involves distinct processes for nitrogen fixation, nitrification, decomposition, anaerobic digestion, and denitrification (Figure 1). Nitrogen fixation is a process where prokaryotes such as soil microbes (bacteria and archaea) or aquatic cyanobacteria (blue-green algae) convert atmospheric dinitrogen (N_2) to ammonium (NH_4^+). which is available to other organisms (Galloway et al. 2004). NH_4^+ released by nitrogen-fixing prokaryotes can be further converted to nitrite (NO_2^-) and nitrate (NO_3^-) by ammonia-oxidizing prokaryotes (bacteria and archaea) in an aerobic process called nitrification (Davis 2010, Shen et al. 2008). In the form of NO_3^- or NH_4^+, nitrogen is available to photoautotrophic producers such as plants and algae (Glass et al. 2002). Nitrogen enters the food webs when it is converted to organic compounds such as proteins and polynucleotides by photoautotrophs, which in turn are exploited by (heterotrophic) consumers (Vanni 2002). Decomposers are microorganisms (bacteria and fungi) that remove nitrogen from the food web by converting the organic material from excrements or dead organisms back to inorganic NH_4^+ in aerobic conditions (Davis 2010, Vanni 2002). Anaerobic digestion is an analogous decomposition process, but the breakdown of organic material is performed by bacteria and archaea in anaerobic conditions (Davis 2010). Denitrification completes the nitrogen cycle through the conversion of NH_4^+ to N_2 by denitrifying bacteria in anaerobic conditions (Davis 2010, Galloway et al. 2004).

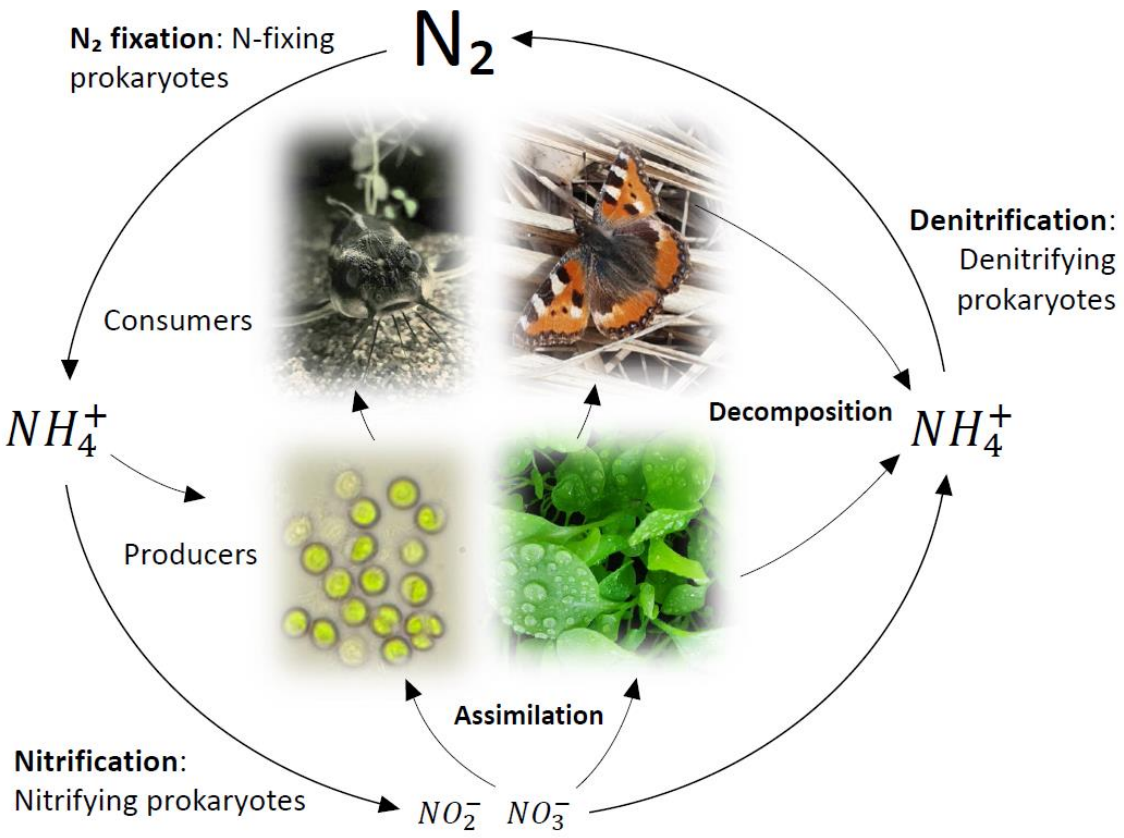

Figure 1. A schematic diagram of biotic processes in natural nitrogen cycle. Nitrogen is circulated among atmosphere, terrestrial, and aquatic ecosystems. The diagram is drawn according to the articles by Galloway et al. (2004), Glass et al. (2002), Shen et al. (2008), and Vanni (2002).

In phosphorus cycle, phosphorus is circulated among lithosphere, hydrosphere, and biosphere (Figure 2). Natural mobilization of phosphorus in lithosphere is caused by uplift and denudation of phosphorus rich minerals (Roy 2017, Smil 2000). Phosphorus is released as phosphate (PO_4^{3-}), and it is relocated and possibly transferred to the hydrosphere through runoff before it enters the biosphere through the nutrient uptake by producers (Smil 2000). In biosphere, phosphorus is transferred from producers to consumers until it is remineralized and released to lithosphere or hydrosphere through decaying processes (Smil 2000, Vanni 2002). In hydrosphere, PO_4^{3-} may end up in the pelagic zone, where it eventually sinks into sediments (Smil 2000, Vanni 2000). From sediments, PO_4^{3-} is restored to the phosphorus cycle by uplifting (Roy 2017, Smill 2000). The time scale of the geotectonic uplift-denudation cycle is millions of years, which along with the relatively low solubility of PO_4^{3-} and its fast conversion into insoluble forms leads to phosphorus commonly being the growth-limiting nutrient in many natural ecosystems (Roy 2017, Smil 2000).

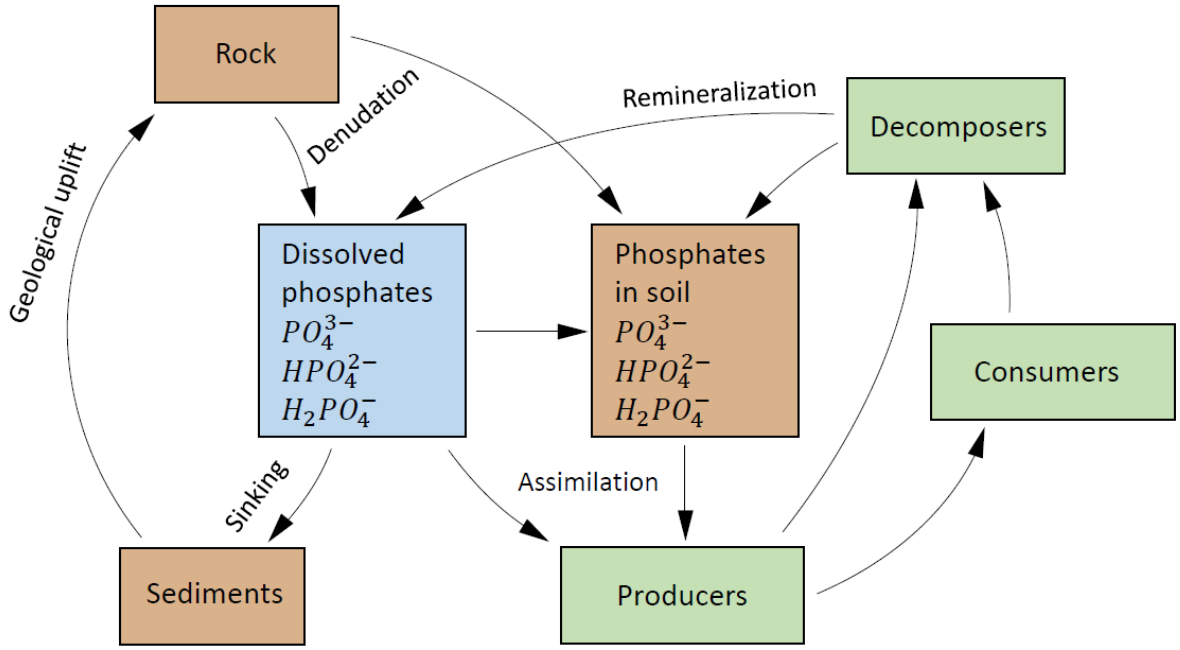

Figure 2. A schematic diagram of natural phosphorus cycle. Phosphorus is circulated among lithosphere (brown), hydrosphere (blue), and biosphere (green). The diagram is drawn according to the articles by Roy (2017), Smil (2000), and Vanni (2002).

Human activities have caused great disturbances in natural nitrogen and phosphorus cycles. Intensive agriculture has led to soil degradation and erosion, resulting in an increased need for chemical fertilizers (Galloway 2008, Smil 2000). At every stage of the process where nitrogen and phosphorus are used in the manufacture of fertilizers and transported through farms and dining tables to the effluent of the wastewater treatment plant, leaks causing nutrients to end up in natural waters are possible (Galloway et al. 2004, 2008, Solovchenko et al. 2016, Smil 2000, Vitousek et al. 1997). In natural waters, the anthropogenic nutrient emissions cause eutrophication, which has detrimental effects on aquatic ecosystems (Goncalves et al. 2017, Smil 2000, Solovchenko et al. 2016, Vitousek et al. 1997). The major consequences are algal blooms, which in turn cause a variety of secondary problems such as oxygen depletion, turbidity, and decrease in light transmission (Goncalves et al. 2017, Smil 2000, Vitousek et al. 1997). Eventually, these disruptions in normal functioning of the ecosystem lead to mass die-offs of delicate fish, molluscs, echinoderms, and cnidarians (Goncalves et al. 2017, Smil 2000, Vitousek et al. 1997).

The main sources of anthropogenic nutrient discharges to natural waters are various NH_4^+-, NO_3^--, and PO_4^{3-}-rich wastewaters produced by industry and municipalities. These emissions have

decreased over the years due to advances in wastewater treatment technology and tightened regulations (Goncalves et al. 2017).

2.2 Removal and recovery of nitrogen and phosphorus from wastewaters

In many countries, including Finland, municipal and industrial wastewaters are commonly treated in centralized wastewater treatment units, where a sequence of physical, chemical, and biological processes are employed to remove organic material and nutrients. Typically, the sequence begins with preliminary treatments, where trash and grit are removed (Carballa et al. 2004, Davis 2010). These are followed by a primary sedimentation, where suspended solids settle to the bottom of the tank by gravity (Carballa et al. 2004, Davis 2010). The resulting sludge is scraped from the bottom of the tank and directed to sludge treatment processes (Carballa et al. 2004, Davis 2010, Verlicchi and Zambello 2015). The primary sedimentation is followed by a biological treatment, where organic matter is decomposed by microorganisms, and NH_4^+ is removed in nitrification and denitrification processes (Carballa et al. 2004, Davis 2010). Biological processes are usually carried out in suspension cultures, and the oxygen required for decomposition and nitrification is produced by aeration (Carballa et al. 2004, Davis 2010). The biological treatment is followed by a secondary sedimentation, where the sludge containing microbial biomass settles to the bottom of the tank, from where it is directed to sludge treatment processes (Carballa et al. 2004, Davis 2010, Verlicchi and Zambello 2015). PO_4^{3-} is chemically precipitated in either primary or secondary sedimentation (Davis 2010, Solovchenko et al. 2016, Verlicchi and Zambello 2015).

Conventional technologies for wastewater treatment are effective in removing nutrients, but they have some downsides such as high energy and chemical consumption and sludge production (Nagarajan et al. 2020). Due to the wide range of contaminants, including heavy metals, pharmaceutical residues and microplastics, the disposal of sewage sludge is difficult to implement (Carballa et al. 2004, Kirchmann et al. 2017, Mahon et al. 2017, Tarpani et al. 2020, Verlicchi and Zambello 2015). Its use after treatment as a fertilizer for cereal crops is possible under current legislation, but currently for image reasons its use is decreasing (Kirchmann et al. 2017). In the wastewater treatment processes, nitrogen and phosphorus are largely lost, as nitrogen is converted to gaseous N_2 through nitrification-denitrification process and phosphorus is precipitated to forms (such as hydroxyapatite), which are generally difficult to recover and utilize (Davis 2010, Maurer et al. 2003, Solovchenko et al. 2016).

For a centralized wastewater treatment plant, a collection system consisting of pipelines and pumps must be constructed and maintained, which increases energy consumption and costs especially in scattered settlements (Maurer et al. 2005). The high costs related to the collection system increases the attractiveness of decentralized wastewater treatment systems where the raw wastewater is collected, treated, disposed, and reused near to its source (Roefs et al. 2017). These systems are especially well suited for arising residential areas that do not yet have an existing collection system. Typically, these systems involve separate collection and treatment for grey water from sinks, showers, washing machines, and dishwashers, and for black water containing the excrements (Maurer et al. 2005, Roefs et al. 2017).

An interesting premise for wastewater treatment could be based on the closure of the nutrient cycles broken down by human activity. This could be achieved through source-separation of urine, as it is the origin of most nutrients in domestic wastewater (Maurer et al. 2003). Moreover, as production volume of urine is just 0.6 – 2.6 L per person per day (Rose et al. 2015), significant savings could be achieved if the majority of expenses allocated for wastewater treatment could be concentrated on treatment of urine.

Due to its high nutrient content, urine can be used as a growth medium for cultivation of microalgae (e.g. Chatterjee et al. 2019, Jaatinen et al. 2016). The nutrients are assimilated by microalgae and used in biomass production. To close the nutrient cycle, the proper end use of microalgal biomass must be envisaged. This includes harvesting of the produced biomass and its utilization as a feedstock for various bioproducts such as fuels and/or plastics (Chatterjee et al. 2019, Hernándes et al. 2013, Lopez Rocha et al. 2020, Zhu et al. 2013).

2.3 Microalgae

Microalgae, or microphytes, are microscopic algae living mostly in aquatic environments. They are predominantly photoautotrophic i.e. they capture light energy and use it to convert inorganic carbon (carbon dioxide, CO_2, and bicarbonate, HCO_3^-) into carbohydrates and other organic biomolecules. They form the basis of many food webs, as heterotrophic organisms (grazers) utilize the chemical energy bound to biomolecules they have produced. As a byproduct, they release molecular oxygen (O_2) into the atmosphere.

Microalgae are found in all marine and freshwater ecosystems, including seas, lakes, rivers, and brooks. Globally, they are responsible for a significant portion of the primary production and CO_2

fixation (Arrigo et al. 1998, Falkowski et al. 1998, Field et al. 1998). Owing to the variety of morphological and physiological adaptations, they can thrive in a wide range of habitats from hot springs to cold and low-light environments under the sea ice (Samsonoff and MacColl 2001). Many species live in symbiotic relationships with fungi and cnidarians, forming composite organisms known as lichens and corals (Honegger 1991, Yellowlees et al. 2008).

Microalgae are not a monophyletic group but a diverse set of organisms that share two characteristics: microscopic size and an ability for oxygenic photosynthesis (Falkowski et al. 2004). According to the loosest definition, the group consists of organisms from two domains, Bacteria and Eukarya. Microalgae belonging to Bacteria domain are cyanobacteria, the simplest photosynthesizing organisms currently in existence (Xiong et al. 2000). This study focuses on eukaryotic microalgae, which are commonly used in wastewater remediation and production of various bioproducts.

2.3.1 Photosynthesis – evolution and principles

Photoautotrophy is an ancient trait that originated not long after the origin of life, but the first photoautotrophs were anoxygenic (Blankenship 2010, Xiong et al. 2010). Oxygenic photosynthesis evolved in late Archean or early Proterozoic eon. The first organisms that gained the ability to produce O_2 were the ancestors of the extant oxygenic cyanobacteria (Blankenship 2010, Soo et al. 2017, Xiong et al. 2010). O_2 was a mere byproduct of the photosynthesis, but its accumulation into the atmosphere triggered the evolution of aerobic respiration (Soo et al. 2017). Aerobic respiration, in turn, is one of the most significant evolutionary innovations because it boosted the cellular metabolism to a level that eventually allowed the complex multicellular life to evolve (Hedges et al. 2004).

Eukaryotic photosynthesis is thought to have arisen from the engulfment of a photosynthesizing cyanobacterium-like organism by a eukaryotic host cell that already contained a mitochondrion (Falkowski et al. 2004). This event led to what is known as endosymbiosis, in which the new energy-supplying cellular resident gained substantial benefits for their host cell. Over time, majority of the endosymbiont's own metabolic functions were lost, and it was assimilated into the host cell. This resulted in the evolution of the chloroplast, a cell organelle where the photosynthesis takes place in present-day eukaryotes (Falkowski et al. 2004, Leliaert et al. 2011).

Three extant chloroplast clades have evolved from this primary endosymbiosis event: green clade (Viridiplantae), red clade (Rhodophyta), and glaucophytes (Glaucocystophyta). Glaucophytes are a relict group of freshwater microalgae with very few extant species, whose chloroplasts have retained many characteristics, such as outer layer made of peptidoglycan, derived from their cyanobacterial ancestors (Sánches-Baracaldo et al. 2017). Rhodophyta (red algae) are a large and diverse group consisting of both planktonic and macroscopic species mainly found in oceanic ecosystems. Majority of the marine phytoplankton, including diatoms, coccolithophores, and red dinoflagellates, originate from secondary endosymbiosis, an event where an ancient red algae cell was engulfed by another eukaryotic cell (Falkowski et al. 2004). Viridiplantae (green algae and land plants) includes microscopic and macroscopic algae inhabiting freshwater and marine ecosystems, many of which originate from secondary endosymbiosis (Falkowski et al. 2004, Leliaert et al. 2011). One branch of the green clade (charophytes) gave rise to Embryophyta (land plants), which dominate the present-day terrestrial ecosystems (Falkowski et al. 2004, Leliaert et al. 2011, Sánches-Baracaldo et al. 2017).

Although there are slight differences among cyanobacteria and eukaryotic chloroplast clades, the basic function of photosynthesis is similar in all organisms capable of oxygenic photosynthesis. In eukaryotes, photosynthesis takes place in chloroplasts, which contain thylakoids surrounded by a stroma. Photosynthesis can be divided into two distinctive stages. In the first stage, light-dependent reactions convert the light energy into biochemical reductant nicotinamide adenine dinucleotide phosphate (NADPH) and high-energy carrier adenosine triphosphate (ATP), and split a water molecule into protons, electrons, and oxygen. These reactions take place in thylakoid membranes. In the second stage, light-independent reactions occurring in the stroma utilize the products formed in light-dependent reactions in assimilation of CO_2 and its reduction to carbohydrates. (McEvoy and Brudvig 2006, Razzak et al. 2013)

The light energy is captured by light harvesting complexes, where absorbed photons induce excitation of photosynthetic pigments. Chlorophyll a, a tetrapyrrole molecule with a Mg^{2+} bound to its center, is an essential pigment for all photosynthesizing organisms, but the presence and distribution of accessory pigments (e.g. chlorophyll b and carotenoids) show a relatively high interspecies diversity, which is thought to be an adaptation to different light conditions (Blankenship 2010, Leliaert et al. 2011). From light harvesting complexes, excitation energy is transferred to photosynthetic reaction centers Photosystem I (PSI) and Photosystem II (PSII). PSII

utilizes excitation energy passed on by light harvesting complex in water splitting reaction (McEvoy and Brudvig 2006, Razzak et al. 2013). It delivers the electrons through a cytochrome b_6f complex to PSI, simultaneously creating a cross-membrane proton (H^+) gradient that is used to generate ATP in photophosphorylation reaction (Allen 2003, Falkowski et al. 2004, Razzak et al. 2013). PSI, operating in a concert with PSII, generates NADPH, which is utilized in Calvin-Benson-Bassham cycle to drive enzymatic reduction of CO_2 (Allen 2003, Falkowski et al. 2004, Razzak et al. 2013).

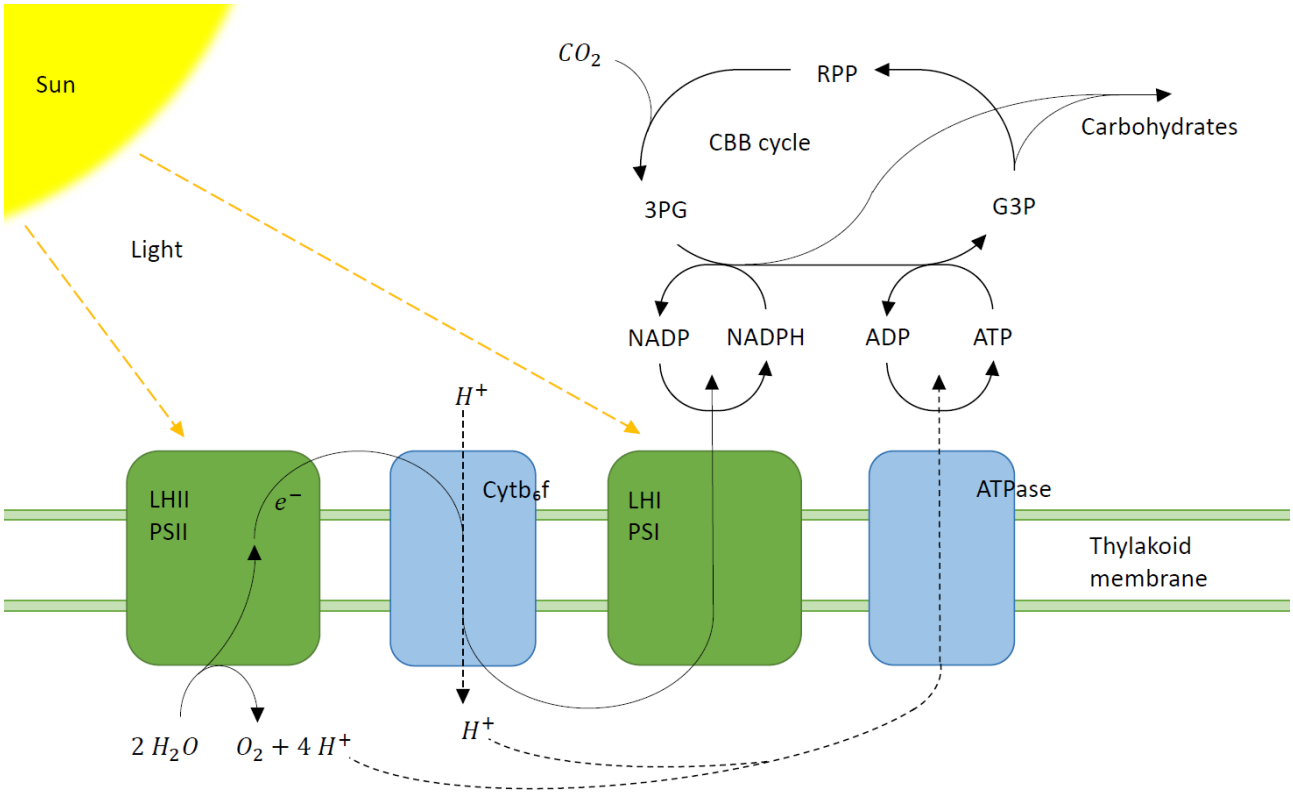

Figure 3. A schematic diagram of photosynthesis. The photosynthetic machinery consists of light harvesting complexes (LH) I and II, photosystems (PS) I and II, cytochrome b_6f complex (Cytb$_6$f), ATP synthase (ATPase), and Calvin-Benson-Basham (CBB) cycle. The diagram is drawn according to the articles by Allen (2003), Falkowski et al. (2004), McEvoy and Brudvig (2006), and Razzak et al. (2013).

In Calvin-Benson-Bassham cycle, CO_2 reacts with a five-carbon sugar ribulose-1,5-bisphosphate (RBP), forming two molecules of 3-phosphoglycerate (3PG), one of which is used in metabolic pathways and the other is converted to glyceraldehyde-3-phosphate (G3P; Razzak et al. 2013). Five out of six G3Ps are used in metabolic pathways and the rest are recycled back to RBP through a complex set of reactions (Razzak et al. 2013). The RBP carboxylation reaction is catalyzed by an enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), which may act as either carboxylase or oxygenase depending on whether the active site reacts with CO_2 or O_2 (Hemme et al. 2014, Kliphuis et al. 2011). Oxygenation results in a process called photorespiration where part

of the energy captured in light-dependent reactions is lost. Photorespiration increases in conditions where O_2/CO_2 ratio is high, such as closed photobioreactors where gas exchange is deficient (Kliphuis et al. 2011). In addition, elevated temperatures decrease the specificity of RuBisCO for CO_2 over O_2, thus promoting photorespiration (Hemme et al. 2014, Rowan and Kubien 2007).

Reduction-oxidation (redox) reactions, where electrons are passed from a donor to an acceptor, are pivotal for photosynthesis. Reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet -}$), are by-products generated in redox reactions of PSI and PSII (Cakmak 2000, Foyer 2018). Although they play a major role as signal transductors in regulation of photosynthesis, they also have detrimental effects on e.g. membrane structures (Cakmak 2000, Foyer 2018). Superoxide dismutases (SODs) are enzymes that catalyze the dismutation of O_2^- into O_2 and hydrogen peroxide (H_2O_2; Cakmak 2000, Cirulis et al. 2013, Foyer 2018). Plants and algae contain three types of SODs, two of which, CuZnSOD and FeSOD, are scavenging ROS generated in photosynthesis (Foyer 2018, Merchant et al. 2006, Millaleo et al. 2010). In addition to photosynthesis, ROS are produced in many other metabolic processes such as cellular respiration, and the third type of SODs, MnSOD, are located in mitochondria (Merchant et al. 2006, Millaleo et al. 2010).

2.3.2 Cultivation of microalgae

Microalgae have been cultivated for decades, because they have been considered as promising feedstocks for biofuels and high-value products such as cosmetics, nutritional supplements, and pharmaceuticals (Barkia et al. 2019, Borowitzka 2013, Cuevas-Castillo et al. 2020). The first commercial products were nutritional supplements made from microalgal species *Chlorella sp*., whose large-scale cultivation started in Japan in the 1960s (Richmond 2008), but the potential of microalgae as feedstocks for various bioproducts has been identified as early as the 1940s (Borowitzka 2013).

Microalgal cells are rich in natural oils, which include triacylglycerols (TAGs) and polyunsaturated fatty acids (PUFAs; Barkia et al. 2019, Cuevas-Castillo et al. 2020). TAGs are low-value precursors that can be used in biodiesel production but PUFAs are valuable as such because of their beneficial effects against inflammation and cardiovascular diseases (Barkia et al. 2019, Cuevas-Castillo et al. 2020). In addition, microalgae contain high amounts of polyphenols and various pigments, including β-carotene, astaxanthin, and lutein, which have antioxidant and anti-inflammatory

properties (Barkia et al. 2019, Cirulis et al. 2013, Cuevas-Castillo et al. 2020). Moreover, microalgal biomass contains up to 71 % protein, making it an excellent source of nourishment for both humans and animals (Cuevas-Castillo et al. 2020, Dineshbabu et al. 2019). However, only a fraction of the bioproducts derived from microalgae have been commercialized to date. Successfully commercialized microalgal products include some nutritional supplements (such as antioxidants and PUFAs), cosmetics, natural dyes, and algae-based foods for humans and animals (Dineshbabu et al. 2019), but commercialization of algal biofuels has faced problems caused by high production costs (mainly associated with cultivation, harvesting, and dewatering of the microalgal biomass) relative to low value of the final product (Nagarajan et al. 2020).

Because microalgae need nitrogen and phosphorus for their growth, they can be used to recover these nutrients from wastewaters. Many studies have investigated the potential use of microalgae for the treatment of various types of wastewaters, such as municipal, agricultural, and industrial wastewaters as well as source-separated human urine (Li et al. 2019, Chatterjee et al. 2019). Wastewater treatment can be coupled with feedstock production as the biomass yielded alongside nutrient removal can be used to produce e.g. biodiesel (Zhu et al. 2013), biomethane (Chatterjee et al. 2019, Hernándes et al. 2013) or bioplastics (Lopez Rocha et al. 2020). There are, however, some limitations on the utilization of microalgae if wastewater has been used in their cultivation. As a rule, they cannot be used in production of nutritional supplements or other high-value products intended for human consumption, but production of low-value feedstocks can be profitable when combined with wastewater treatment (Nagarajan et al. 2020).

Cultivation in suspension is the most commonly used way to produce microalgal biomass (Goncalves et al. 2017). Suspension cultures can be conducted in open or closed systems (Figure 4). Open systems are usually shallow (less than 0.3 m deep) raceway ponds (RwPs) typically equipped with a paddlewheel to mix the cultivation broth and enhance gas exchange at the liquid-gas interface (Cuevas-Castillo et al. 2020, Goncalves et al. 2017, Mendoza et al. 2013, Nagarajan et al. 2020, Razzak et al. 2013). RwPs are economical solutions to large scale cultivations as their construction and operation is easy and cost-effective (Barkia et al. 2019, Cuevas-Castillo et al. 2020, Nagarajan et al. 2020). They can be used in wastewater treatment where contamination with other microorganisms is not a primary concern (Nagarajan et al. 2020). Their downsides include inadequate mixing, low efficiency of sunlight utilization, and poor control over evaporation (Cuevas-Castillo et al. 2020, Goncalves et al. 2017). In addition, they require large areas of land (Cai

et al. 2013). Closed systems are different types of photobioreactors (PBRs), including tubular, airlift, bubble column, flat panel, and plastic bag PBRs, where various methods, such as mechanical stirrers and gas spargers are employed to achieve proper mixing and gas exchange (Goncalves et al. 2017, Razzak et al. 2013). PBRs are preferred in situations where the cultivation should be kept axenic, high biomass yields are required or the land prices are high (Barkia et al. 2019, Cai et al. 2013, Goncalves et al. 2017, Razzak et al. 2013). The disadvantages of PBRs are that their operational costs are relatively high, and they are hard to scale up (Cai et al. 2013, Cuevas-Castillo et al. 2020, Goncalves et al. 2017).

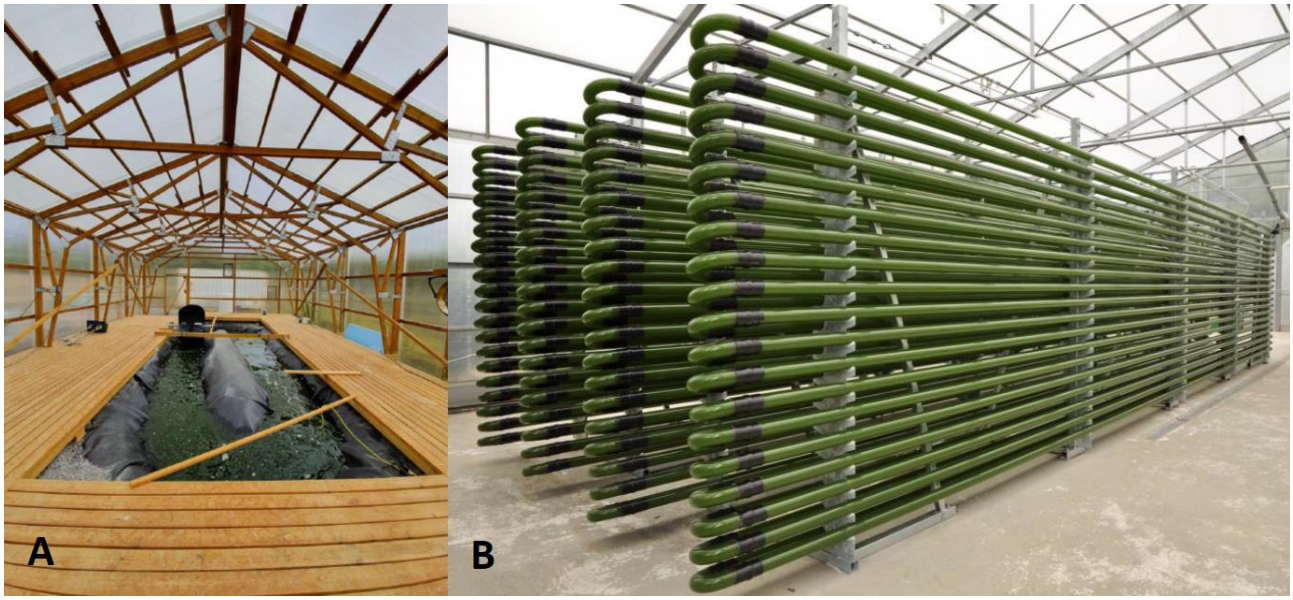

Figure 4. Examples of open and closed systems for cultivation of microalgae. A) A raceway pond (RwP) with an operational volume of 2000 L. The RwP was used in this study. B) A tubular glass photobioreactor (PBR) with an operational volume of 4000 L. The picture of the PBR is by IVG Biotech (2013).

In addition to traditional suspension cultures, various immobilization and attachment technologies where microalgae are trapped in gelatinous material or attached on solid surfaces are currently being developed. The immobilization and attachment methods have certain advantages over conventional suspension cultures, such as increased biomass density, cell-free effluent, and easier biomass harvesting, but applications of these methods are still mostly in the proof-of-concept phase. (Cai et al. 2013, Zhuang et al. 2020)

Cultivations can be operated in batch, fed-batch, semi-continuous or continuous mode (Doran 2012). In batch cultivations, the algae and the nutrients required for growth are added to the culture medium at the beginning of the cultivation. Growth ceases when the limiting nutrient is depleted, at which point the cultivation is ended and the biomass harvested (Doran 2012). In fed-

batch cultivations, fresh culture medium is added either continuously or discontinuously, and the biomass is harvested at the end of the cultivation (Doran 2012). Semi-continuously operated cultivations are similar to fed-batch cultivations, but the effluent is removed during the cultivation period (Doran 2012). In continuously operated cultivations, the influent containing fresh nutrients is continuously fed to the cultivation, and the effluent containing the algal biomass is continuously withdrawn from the reactor (Doran 2012). A central measure in continuously and semi-continuously operated systems is hydraulic retention time (HRT), which refers to an average length of time that a soluble compound remains inside the reactor (Davis 2010, Doran 2012). In microalgal cultivations, HRTs typically vary from one to fourteen days (Chatterjee et al. 2019, Luo et al. 2019).

Microalgae can be cultivated both indoors and outdoors. Although the operational parameters such as temperature and light intensity are easier to control indoors, outdoor cultivation is preferred due to its cost-effectiveness. Outdoor cultivations utilize sunlight as energy source, which reduces costs significantly compared to indoor cultivations that use artificial light (Nagarajan et al. 2020). In outdoor cultivations, diurnal and seasonal variations in temperature and light intensity cause fluctuations in biomass growth and nutrient consumption, but they are usually compensated for by savings in construction and operational costs (Nagarajan et al. 2020).

2.3.3 Growth requirements of microalgae

Microalgae are considered as easy to cultivate because their basic needs include just water, light, CO_2, and nutrients. However, in practice, effective growth requires optimization of several factors such as temperature (Li et al. 2011, Ras et al. 2013, Ruiz-Martinez et al. 2015), culture pH (Adamsson 2000), mixing (Barbosa et al. 2003), and light intensity and quality (Wang et al. 2014).

Light

Light intensity is regarded as one of the main controllers of algal growth in outdoor cultivations, which typically do not use artificial light sources and are therefore subjected to fluctuations in sunlight intensity due to diurnal, seasonal, and weather variations (Sutherland et al. 2014). Overall, the availability of light is known to be a major limiting resource in all algal cultivations because increasing biomass concentration reduces light transmission through the culture broth (Barbosa et

al. 2003, Sutherland et al. 2015, Tuantet et al. 2014b). The resulting light gradient across the reactor is a considerable problem in industrial scale cultivations where reactor volumes are typically large. Along with biomass density, the design of the reactor defines the depth of the euphotic zone (Sutherland et al. 2015). Careful design is particularly important for RwPs, as they are lit from one direction only. Because PBRs are typically made of transparent material, they receive scattered light from the direction from which they are not directly illuminated, and thus the aphotic zone in proportion to the reactor volume is generally smaller in PBRs than in RwPs. Light attenuation can be counteracted by agitation, as the turbulence allows periodic migration of cells from aphotic to euphotic zone (Barbosa et al. 2003). In continuously operated cultivations, transmittance of the culture broth can be improved by optimizing HRT in order to achieve an optimal biomass density that allows adequate light transmission without excessive washout of algal cells (Sutherland et al. 2015).

Inorganic carbon

Gas exchange is another important factor controlling algal growth. During photosynthesis, algae use CO_2 and release O_2. This results in CO_2 depletion in cultivations where CO_2 is not added and can be observed in increasing culture pH (Jaatinen et al. 2016, Hulatt et al. 2012). In closed systems, O_2 begins to accumulate in the culture medium, which in turn reduces biomass production due to increased photorespiration (Kliphuis et al. 2011). CO_2 depletion can be remedied by sparging air or flue gas into the algal cultivation. The use of air is technically simple but not very effective, as the CO_2 concentration in the atmosphere is just 0.04 % by volume (Praveenkumar et al. 2014). A sustainable and cost-effective solution is to use flue gas, as its CO_2 is captured and used to produce algal biomass instead of being released into the atmosphere (Posadas et al. 2015, Praveenkumar et al. 2014, Razzak et al. 2013). CO_2 sparging is relatively easy to implement in most PBRs, but in open and shallow RwPs, majority CO_2 is lost due to poor mass transfer caused by limited contact time between gas bubbles and culture medium (Mendoza et al. 2013). The mass transfer of CO_2 can be improved by equipping the RwP with a deep sump, which prolongs the gas/liquid contact time (Mendoza et al. 2013, Posadas et al. 2015). Another way to improve CO_2 dissolution is to use a closed mixing column, through which the algal cultivation is fed counter-current relative to the CO_2 influent (Putt et al. 2011).

Macronutrients

In addition to light and CO_2, algae need certain nutrients for their growth. Nitrogen, phosphorus, and potassium are macronutrients needed in relatively high amounts. Nitrogen and phosphorus are pivotal in biosynthesis and energy metabolism, as for example, polynucleotides, the high-energy carrier ATP as well as redox agents NADPH and nicotinamide adenine dinucleotide (NADH) contain both nitrogen and phosphorus (Alberts et al. 2002). In addition, nitrogen is an indispensable part of amino acids and chlorophylls (Alberts et al. 2002). Potassium is the most prevalent ion in all cells, and it has a key role in maintenance of electrolyte balance and providing the optimal ionic environment for metabolic processes such as glycolysis and protein synthesis (Leigh and Jones 1984).

Microalgae can utilize nitrogen in various forms, such as ammonium (NH_4^+), nitrate (NO_3^-), or urea ($NH_2(CO)NH_2$). Urea is an attractive nitrogen source as it is inexpensive and promotes algal growth relatively well (Arumugam et al. 2013, Li et al. 2008) but in non-sterile solution it hydrolyzes readily to form NH_4^+, leading to an elevated pH of the solution (Udert et al. 2006). Typically, NO_3^- is used as a nitrogen source in synthetic growth media such as modified N8 (Praveenkumar et al. 2014, Procházková et al. 2014), probably because of its easy availability, long shelf life, and stability in solution. It is generally thought that algae prefer NH_4^+ over NO_3^- as a nitrogen source, because NH_4^+ can be directly assimilated and used in amino acid synthesis but NO_3^- must be reduced to NH_4^+ before it can be incorporated into algal cells (Goncalves et al. 2017, Lachmann et al. 2019). Very often, however, the biomass production is higher in cultivations supplemented with NO_3^- compared to cultivations supplemented with NH_4^+ (Arumugam et al. 2013, Lachmann et al. 2019, Li et al. 2008). The slower growth in cultivations grown on NH_4^+ might be associated with toxicity of the growth medium caused by deprotonation of NH_4^+ to NH_3 at high culture pH (Adamsson 2000, Azov and Goldman 1982, Zheng et al. 2019). In addition, assimilation of NH_4^+ leads to H^+ production, which may provide an additional stress to microalgae (Lachmann et al. 2019) and may even lead to an excessive drop in culture pH and consequent collapse of the culture if the buffering capacity of the growth medium is insufficient (Hulatt et al. 2012).

Phosphorus is incorporated into algal cells through active transport in the plasma membrane in the forms of $H_2PO_4^-$ and HPO_4^{2-} (Solovchenko et al. 2016). $PO_4^{3-} - P$ is incorporated into biomolecules through phosphorylation, where ATP is produced from adenosine diphosphate (ADP)

and an energy input, which is obtained from cellular respiration or light-dependent reactions of photosynthesis (Goncalves et al. 2017, Solovchenko et al. 2016).

Nitrogen and phosphorus should also be provided in proportion to each other. It is generally thought that the average intracellular ratio of nitrogen and phosphorus in microalgae follows approximately the molar stoichiometry of 16:1 proposed by Redfield (1958), but significant species-specific variation has been observed in marine and freshwater microalgae (Klausmeier et al. 2004). In addition, many environmental variables such as nutrient availability and light intensity affect the microalgal uptake of nitrogen and phosphorus (Beuckels et al. 2015, Klausmeier et al. 2004, Solovchenko et al. 2016). For example, microalgae are capable of excess uptake (i.e. luxury uptake) of phosphorus in conditions where PO_4^{3-} concentration and light intensity are high (Solovchenko et al. 2016). In addition, Beuckels et al. (2015) observed that the ability of microalgae to accumulate phosphorus was improved in high nitrogen concentration compared to low nitrogen concentration. The N:P ratio of the synthetic growth medium should be optimized for species and growth conditions, and in wastewater treatment, growth conditions should be optimized to facilitate maximal uptake of both nutrients (Solovchenko et al. 2016).

Trace elements

Some of the nutrients essential for algal growth are needed in very small quantities. These nutrients are called micronutrients or trace elements (TEs). They are pivotal in many biochemical and physiological processes, which are well conserved in Viridiplantae. Among the most important TEs are magnesium, iron, manganese, zinc, copper, and boron. A research by Tuantet et al. (2014a) showed that supplementation with a mixture of these six trace elements improved biomass production and nutrient removal in microalgae grown on diluted human urine.

Magnesium (Mg) is the most abundant TE in algal cells (Merchant et al. 2006), and its biological functions include key roles in photosynthesis, protein synthesis, and nucleotide metabolism (Verbruggen and Hermans 2013). The most vital physiological role for Mg is in light absorption by occupying the central position in the molecule structure of chlorophyll pigments (Verbruggen and Hermans 2013). In addition, Mg acts as a cofactor and allosteric modulator for more than 300 enzymes including carboxylases, phosphatases, kinases, RNA polymerases, and ATPases (Verbruggen and Hermans 2013).

Iron (Fe) is another abundant TE in algal cells, and it is the most versatile and important TE for biochemical processes. Within cells, iron exists in two oxidation states, Fe(II) and Fe(III), which allows it to function as a reducing or oxidizing agent in biochemical reactions. As a redox-active metal, it has central roles in photosynthesis, cellular respiration, nitrogen assimilation, and scavenging of ROS (Hänsch and Mendel 2009, Procházková et al. 2014). There are many iron-containing protein families i.e. groups of evolutionarily-related proteins, which have similar functions in diverse biochemical processes (Hänsch and Mendel 2009). For example, ferredoxins are iron-sulphur proteins, which are used as electron carriers in many metabolic reactions such as photophosphorylation reactions of photosynthesis and reduction of NO_3^- to NH_4^+ in nitrogen assimilation (Goncalves et al. 2017, Hänsch and Mendel 2009). Redox activity also makes iron toxic, as Fe(II) can react with H_2O_2 to produce hydroxyl radicals, thus initiating oxidative stress and lipid peroxidation in biomembranes (Procházková et al. 2014). To reduce toxicity and precipitation, iron is usually added to the growth medium as ferric chelates, such as ferric ethylenediaminetetraacetic acid (Fe(III)EDTA). When iron is complexed with organic ligands such as EDTA, an equilibrium establishes between the complexed and dissolved forms. As Fe ions are assimilated by algae, they are replaced by dissociation of an equivalent concentration from the ferric chelate (Procházková et al. 2014).

Manganese (Mn) is essential for algal metabolism, serving as a catalytically active metal in e.g. MnSOD, which protects mitochondria from damaging effects of free radicals (Hänsch and Mendel 2009, Millaleo et al. 2010), and the Mn-containing water splitting system of PSII (Hänsch and Mendel 2009, McEvoy and Brudvig 2006, Millaleo et al. 2010). In addition, manganese exerts an activating role on many enzymes, including RNA polymerases (Hänsch and Mendel 2009). Manganese exist in seven oxidation states, three of which, Mn(II), Mn(III), and Mn(IV), occur in biological systems (Hänsch and Mendel 2009, Millaleo et al. 2010). Enzyme functions involve extensive redox cycles between these states. Like other redox-active metals, manganese is toxic in excess concentrations (Millaleo et al. 2010).

Zinc (Zn) is an important component of enzymes participating in e.g. protein synthesis and energy conversion. In addition, it protects biomembranes from damages caused by oxidative stress (Cakmak 2000, Hänsch and Mendel 2009). More than 1200 proteins are predicted to contain, bind, or transport zinc, including oxidoreductases, hydrolytic enzymes such as metalloproteases, and all proteins containing zinc-finger motifs (Hänsch and Mendel 2009). Zinc is particularly important for

DNA and RNA synthesis, RNA processing, and translation, because DNA and RNA polymerases, transcription factors, histone deacetylases, splicing factors, and enzymes involved in RNA editing are zinc-dependent (Berg and Shi 1996, Hänsch and Mendel 2009). Because zinc is not redox-active, it is well suited to its role as a structural element in nucleic acid binding proteins without entailing the risk of unwanted radical reactions (Berg and Shi 1996). In chloroplasts, zinc-dependent proteins fulfill many essential functions in e.g. repair processes of photodamaged PSII and ROS scavenging (Cakmak 2000, Hänsch and Mendel 2009).

Copper (Cu) is essential for photosynthesis and cellular respiration, carbon and nitrogen metabolism, and oxidative stress responses (Hänsch and Mendel 2009). Under physiological conditions, copper exists in two oxidation states, Cu(I) and Cu(II), and can easily interchange between these states (Burkhead et al. 2009, Hänsch and Mendel 2009). This allows copper to function in a wide variety of redox reactions, but as with other redox-active metals, the redox-activity of copper is associated with toxicity (Burkhead et al. 2009, Hänsch and Mendel 2009, Moenne et al. 2016). Because of copper's toxicity, a variety of chaperoning and scavenging systems exist in cells, that enable copper to be safely transported to where it is needed (Burkhead et al. 2009, Hänsch and Mendel 2009, Moenne et al. 2016). Interestingly, copper and iron metabolisms are intimately linked to each other, as many biochemical reactions can be catalyzed with either copper or iron binding enzyme, and the use of one catalyst over the other is governed by the bioavailability of copper and iron (Burkhead et al. 2009, Hänsch and Mendel 2009, Merchant et al. 2006). One example of such an enzyme pair is CuZnSOD and FeSOD, both of which have identical roles in ROS scavenging in chloroplasts (Burkhead et al. 2009, Hänsch and Mendel 2009, Merchant et al. 2006).

The importance of boron (B) for algal growth is still unclear. The necessity of boron for the normal growth of land plants is unquestionable, as it has been shown to have a key role in e.g. maintaining cell wall structures (Bolanos et al. 2004, Hänsch and Mendel 2009). In vascular plants, boron is complexed with rhamnogalacturonan-II (RG-II), which is a polysaccharide found in plant cell walls (Bolanos et al. 2004). Thus far, RG-II has not been detected in green algae, suggesting that although algal cell wall closely resembles plant cell wall, it is not completely similar. (Mikkelsen et al. 2014). In plants, boron has also some other functions in e.g. supporting metabolic activities (Bolanos et al. 2004, Hänsch and Mendel 2009), but it is not clear whether these functions exist in algae (Kropat et al. 2011).

It has been shown that supplementing TEs to microalgal cultivation enhances biomass production (Adamsson 2000, Kropat et al. 2011, Merchant et al. 2006, Procházková et al. 2014, Tuantet et al. 2014a). The exact TE requirements of microalgae are still largely unresolved, although it is known that the need for a particular TE depends on the species, growth conditions, concentrations of other TEs, and the level of abiotic stress (Cakmak 2000, Merchant et al. 2006, Procházková et al. 2014). In addition, supplementation of iron in particular has proved tricky because iron readily precipitates as oxyhydroxides, which are largely unavailable to algae (Procházková et al. 2014).

Organic carbon

In photoautotrophic growth, microalgae use inorganic carbon as a sole carbon source. Since light tends to be growth-limiting factor in most ecosystems, many algal species are able to utilize organic compounds in order to maintain growth in low-light conditions (Nirmalakhandan et al. 2019). A trophic strategy, in which an organism utilizes a mix of different sources of energy and carbon, is called mixotrophy.

In microalgal cultivations, mixotrophic metabolism has been shown to increase biomass production, which in turn promotes nutrient removal (Gao et al. 2019, Kim et al. 2013, Ma et al. 2016, Zheng et al. 2018, 2019). In projects aiming for biodiesel production, mixotrophic cultivation has been used to increase intracellular lipid content of algae. Enhanced microalgal lipid production was observed by Ma et al. (2016) in their experiment using waste glycerol as an organic carbon source in cultivation of microalgal species *Chlorella vulgaris*, and by Praveenkumar et al. (2014), who cultivated *Chlorella sp*. strain KR-1 using glucose as an organic carbon source. Similar enhance in lipid production in mixotrophic growth conditions was observed by Gao et al. (2019) in their experiment where *Chlorella sp.* strain G-9 was cultivated on synthetic wastewater and various concentrations of glucose.

The optimal molar ratio of carbon and nitrogen for biomass production and/or nutrient removal in mixotrophic cultivation has been investigated in several studies. In the study by Zheng et al. (2019), the highest biomass production and nutrient removal was achieved with C:N molar ratios of 4:1 and 21:1, while Gao et al. (2019) found molar ratios of 21:1 and 26:1 to be the most suitable. Zheng et al. (2018) obtained very interesting results by cultivating microalgae on a mix of biggery and brewery wastewaters. After thorough tests with various mixing ratios, they reached a

conclusion that the optimal C:N molar ratio was 7:1, which was achieved by mixing biggery wastewater and brewery wastewater by 1:5. They also demonstrated that microalgae are able to utilize the complex organic compounds often present in wastewaters, which opens up intriguing opportunities for the utilization of diverse sidestreams produced in industry.

Co-cultivation with other microorganisms

Microalgae can be cultivated as axenic or (either intentionally or non-intentionally) as co-cultivations with other microalgal species or even with other microorganism, such as cyanobacteria or heterotrophic bacteria (Goncalves et al. 2016, Ramanan et al. 2016, Thomas et al. 2019). Co-cultivation of algae with bacteria has certain advantages in processes designed to recover nutrients from different wastewaters. To begin with, in most applications, it is not economically viable to expend resources to maintain the cultivation axenic. Furthermore, oxygenation of the culture broth by algal photosynthesis supports the decomposition of organic and inorganic matter by heterotrophic bacteria (Goncalves et al. 2016, Karya et al. 2013, Su et al. 2012), which in turn enhances removal of organic matter with minimal aeration costs. When cultured alone in photoautotrophic growth conditions, algae release dissolved organic carbon (DOC; Higgins et al. 2018, Hulatt et al. 2010, Marjakangas et al. 2015), which is not a desirable phenomenon in wastewater treatment. Moreover, co-cultivation of algae with heterotrophic bacteria gives rise to a mutualistic interactions where bacteria grow on organic matter released by algae, while algae benefit from the B-vitamin cofactors, such as biotin, cobalamin, and thiamine, secreted by bacteria (Goncalves et al. 2016, Higgins et al. 2018, Ramanan et al. 2016). The higher nutrient removal efficiencies in algal-bacterial co-cultures compared to algal monocultures (Higgins et al. 2018, Su et al. 2012) can be explained by bacterial uptake of the nutrients, but also by enhanced algal growth due to the bacterial secretions. Finally, co-cultivation of algae with bacteria derived from activated sludge may enhance flocculation and subsequent settling of the produced biomass (Goncalves et al. 2016, Lee et al. 2013, Su et al. 2012), which is a useful feature in biomass harvesting. However, growing microorganisms in a consortium may also result in competitive interactions (Goncalves et al. 2016, Ramanan et al. 2016), which must be taken into account when planning the co-cultivation.

2.3.4 Hydrolyzed urine as a nutrient and carbon source

Due to high concentrations of nitrogen and phosphorus, urine is seen as an attractive growth medium for cultivation of microalgae (Table 1). In addition, fresh urine is rich in many other essential macronutrients and TEs. The exact composition of fresh urine varies depending on many factors such as person's age, health, physical activity, and dietary intake (Heitland and Köster 2006, Moore et al. 2018, Rose et al. 2015).

Typically, urine is stored for some time before it is used as a growth medium for the cultivation of microalgae. Fresh urine may contain pathogens, but they are scarce in stored urine because they are generally adapted to life within humans and other homeothermic animals and do not survive indefinitely under the storage conditions. Storage for a period of approximately six months should be enough to ensure the destruction of most pathogens (Udert et al. 2006). However, urine stored in an open container is never completely sterile, as the nutrients and organic matter it contains provide an excellent growth substrate for a wide variety of heterotrophic microbes. Due to the microbial metabolism, the urine composition changes during storage (Udert et al. 2006, Wohlsager et al. 2010).

In fresh urine, approximately 85% of the nitrogen is fixed as urea and 5% as total ammonia (NH_4^+ + NH_3), while the remainder of the nitrogen compounds include creatinine, uric acid, and amino acids (Udert et al. 2006). During storage, urea is hydrolysed to NH_4^+, NH_3, and HCO_3^- by microbial activity. After hydrolysis, NH_4^+ and NH_3 account for 90% of the total nitrogen. Hydrolysis leads to a drastic increase in pH, which in turn triggers spontaneous precipitation of struvite ($MgNH_4PO_4 * 6H_2O$) and hydroxyapatite ($Ca_5(PO_4)_3OH$), causes deprotonation of NH_4^+ to NH_3, and contributes to the destruction of pathogens (Hansen et al. 1998, Ronteltap et al. 2007b, Udert et al. 2006). The loss NH_3 due to volatilization occurs in high temperatures (Hansen et al. 1998, Udert et al. 2006).

In fresh urine, 95 to 100% of phosphorus is fixed as dissolved phosphate (Udert et al. 2006). During urine hydrolysis, the phosphate concentration decreases in various precipitation processes (including but not limited to struvite precipitation), and up to 40% of the soluble phosphate is incorporated in the solid phase when the hydrolysis is complete (Ronteltap et al. 2007a, 2007b, Udert et al. 2006, Wohlsager et al. 2010). The effect is even more pronounced for calcium and magnesium, which are almost completely precipitated (Ronteltap et al. 2007a, 2007b, Udert et al. 2006, Wohlsager et al. 2010).

Table 1. The range of macronutrient and trace element concentrations (mg L^{-1}) in human urine.

Element		conc. (mg L^{-1})	References
Nitrogen	N	2200 - 9000	Akpan-Idiok et al. 2012, Chatterjee et al. 2019, Jaatinen et al. 2016, Maurer et al. 2003, Pandorf et al. 2019, Tuantet et al. 2014a, Udert et al. 2006, Viskari et al. 2018, Wohlsager et al. 2010
Phosphorus	P	160 - 800	Akpan-Idiok et al. 2012, Chatterjee et al. 2019, Jaatinen et al. 2016, Maurer et al. 2003, Pandorf et al. 2019, Viskari et al. 2018, Wohlsager et al. 2010
Potassium	K	500 – 2800	Akpan-Idiok et al. 2012, Chatterjee et al. 2019, Moore et al. 2018, Pandorf et al. 2019, Tuantet et al. 2014a, Udert et al. 2006, Viskari et al. 2018, Wohlsager et al. 2010
Sodium	Na	500 – 3500	Akpan-Idiok et al. 2012, Chatterjee et al. 2019, Moore et al. 2018, Tuantet et al. 2014a, Udert et al. 2006, Viskari et al. 2018, Wohlsager et al. 2010
Calcium	Ca	12 – 190	Akpan-Idiok et al. 2012, Chatterjee et al. 2019, Moore et al. 2018, Pandorf et al. 2019, Tuantet et al. 2014a, Udert et al. 2006, Viskari et al. 2018, Wohlsager et al. 2010
Iron	Fe	0.3	Viskari et al. 2018
Magnesium	Mg	1.4 – 100	Akpan-Idiok et al. 2012, Chatterjee et al. 2019, Moore et al. 2018, Pandorf et al. 2019, Tuantet et al. 2014a, Udert et al. 2006, Viskari et al. 2018, Wohlsager et al. 2010
Manganese	Mn	0.0001 – 0.01	Heitland and Köster 2006, Viskari et al. 2018
Zinc	Zn	0.01 – 0.3	Heitland and Köster 2006, Moore et al. 2018, Viskari et al. 2018, Wohlsager et al. 2010
Copper	Cu	0.01 – 0.02	Heitland and Köster 2006, Moore et al. 2018, Wohlsager et al. 2010
Boron	B	2 – 14	Tuantet et al. 2014a

In addition to calcium and magnesium, many other TEs have been found to precipitate during storage. For example, Wohlsager et al. (2010) observed that 44% of the zinc in the human urine was precipitated during eight weeks of storage. The precipitation of TEs may occur during or after hydrolysis. In their experiment, Ronteltap et al. (2007a) observed that more than half of the copper was precipitated in hydrolysed urine spiked with copper at 500 µg L^{-1} over 15 days. Precipitation of TEs involves a large number of complexation reactions, whose kinetics are affected by, for example, temperature, pH, and the presence of other elements (Ronteltap et al. 2007a, 2007b). Thus, it is difficult to predict how individual TEs will behave in urine, which may vary in composition and be stored under changing conditions.

The concentration of dissolved organic carbon (DOC) in urine is relatively high, approximately 2400 – 5600 mg L^{-1} (Akpan-Idiok et al. 2012, Jaatinen et al. 2016). The chemical oxygen demand (COD) of urine varies between 2500 and 10000 mgO_2 L^{-1} (Chatterjee et al. 2019, Tuantet et al. 2014a, 2014b, Udert et al. 2006). Organic acids, creatinine, amino acids, and carbon hydrates are the major components increasing COD (Udert et al. 2006). The most abundant organic substance in fresh urine is urea, but since it is already fully oxidised, it does not increase COD of stored urine. Organic substances provide a carbon source for various microbes incl. mixotrophic microalgae, while HCO_3^- produced in urea hydrolysis is utilized as an inorganic carbon source in photoautotrophic growth.

The molar ratio of nitrogen and phosphorus in fresh urine varies and changes with NH_3 volatilization during storage. Molar ratios from 18:1 to 48:1 have been observed (Akpan-Idiok et al. 2012, Chatterjee et al. 2019, Jaatinen et al. 2016, Maurer et al. 2003, Pandorf et al. 1019, Tuantet et al. 2014b, Wohlsager et al. 2010). In general, the concentration of total ammonia in hydrolysed urine is high, which is toxic to microalgae, causing biomass growth to slow down (Collos et al. 2014). To prevent toxicity, urine is diluted in ratios ranging from 1:300 to 1:2 (Jaatinen et al. 2016, Tuantet et al. 2014b).

3. MATERIALS AND METHODS

This study consists of two complementary experiments that were started simultaneously. The first experiment was conducted in a continuously operated outdoor raceway pond (RwP). The aim was to optimize the cultivation conditions for microalgae in Nordic climate conditions to maximize nutrient removal from source-separated urine. The second experiment was carried out in batch flasks under laboratory conditions, and its purpose was to investigate the effects of individual trace elements on nutrient removal.

3.1 Raceway pond experiment

3.1.1 Overview of the experimental setup

The study was conducted in Hiedanranta, an upcoming suburb of Tampere that also serves as an experimental urban living lab dedicated to projects promoting sustainability and circular economy. The RwP was located in an unheated greenhouse made of preserved wood and 10 mm transparent polycarbonate sheet (Figure 5). The greenhouse was equipped with two 107 W electric fans (Master, USA), which started automatically when the indoor air temperature rose above 40 °C. Out of the two RwPs in the greenhouse, the larger one with a liquid volume of approximately 2000 L was used in this study. The smaller RwP with a capacity of approximately 400 L was used to produce the inoculum for the experiment. Both RwPs were constructed on gravel and lined with a 1.5 mm high-density polyethylene sheet. Both RwPs were also equipped with a paddlewheel operated with a 0.5 horsepower gear wheel motor (Regal Beloit, Marathon motors, USA).

Masterflex L/S pump drives and Masterflex L/S Easy-Load II pump heads (Cole-Parmer, USA) were employed to feed and withdraw the influent and the effluent. The RwP was connected to the influent and effluent tanks with 5 mm polyvinyl chloride tubes (Oy Toppi Ab, Finland) except at the pump heads where Masterflex Tygon L/S 25 tubes (Cole-Parmer, USA) were used. The tubes were linked with polypropylene tubing connectors (Kartell, Italy).

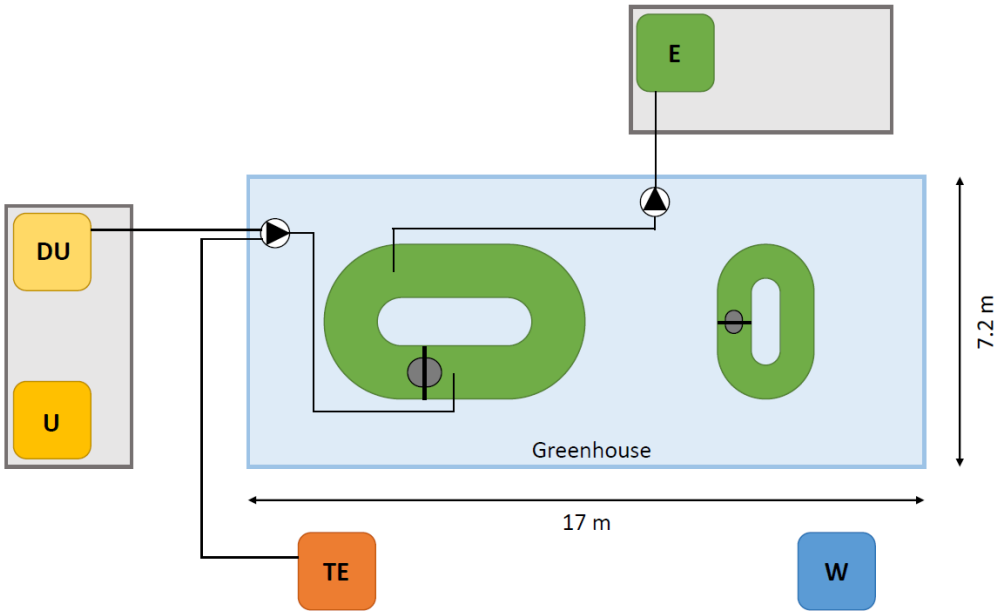

Figure 5. The schematic of the greenhouse and its surroundings. The greenhouse contained two raceway ponds, of which the larger one was used in this study. Urine, diluted urine, and effluent tanks were stored inside two shipping containers. Trace element mixture tank and clean water tank were placed outside. U: urine, DU: diluted urine, E: effluent, TE: trace element mixture, W: clean water.

3.1.1 Urine and water supply

Urine was collected with separating dry toilets. It was received from a nearby collection facility in Hiedanranta, where it was initially stored for at least six months at ambient temperature (5 – 20 °C) to kill pathogens and hydrolyze the urea. During the RwP operation, urine was stored in a 1000 L plastic tank placed in a shipping container beside the greenhouse (Figure 5). The urine stock was changed on days 22 and 77 from the start of the continuous operation (Table 3).

Tap water was stored in 1000 L plastic tanks placed outdoors beside the greenhouse (Figure 5). Urine was diluted with water at a ratio of approximately 1:7.5 (v:v). The dilution was prepared in a 1000 L plastic tank, which was stored in the same shipping container as undiluted urine. The tank was filled when the volume decreased below 200 L (Table 3).

3.1.2 Trace element mixture

The trace element (TE) mixture contained six elements (Fe, Mg, Mn, Cu, Zn, and B) with concentrations equal to the lower concentrations used by Tuantet et al. (2014a). Separate 1000x stock solutions of each TE were prepared by dissolving a weighed amount of the chemical in MQ

water (Table 2). The TE mixture was prepared by diluting the stocks with tap water at a ratio of 1:500 (v:v). The TE mixture was mixed with diluted urine at a ratio of 1:1 (v:v).

The TE mixture was stored outside in a 1000 L tank (Figure 5), which was covered with a tarpaulin to protect the mixture from sunlight. A fresh TE mixture was prepared in a washed tank when the volume of the previous mixture decreased below 200 L (Table 3).

Table 2. Trace element stocks: the chemicals, their concentrations in 1000x stock solutions, and the final concentrations in RwP influent / batch flasks.

Element	Chemical	Conc. in RwP influent / batch flask (µM)
Fe	$FeNaEDTA$	32
	$Na_2EDTA * 2H_2O$	10
Mg	$MgCl_2 * 6H_2O$	20
Mn	$MnCl_2 * 4H_2O$	6.6
Cu	$CuSO_4 * 5H_2O$	0.7
Zn	$ZnSO_4 * 7H_2O$	1.1
B	H_3BO_3	0.1

3.1.3 Inoculation of the RwP

The RwP was inoculated with a mixed culture from previous year's field experiment conducted at Hiedanranta. The culture had been maintained in the laboratory over the winter by transferring it to fresh 1:20 diluted urine approximately once a month. It was kept in shake flasks at constant light intensity (54 µmol m^{-2} s^{-1}) and agitation speed (150 rpm). The culture was found to be dominated by a green alga *Scenedesmus acuminatus* but contained also some procaryotic and eukaryotic microorganisms of local origin. The use of the mixed culture was justified by the difficulty of avoiding contamination of a monoculture in an open pond. In addition, a diverse microbial

consortium was expected to enhance nutrient recovery through cooperative interactions and higher acclimatization potential (Higgins et al. 2018, Thomas et al. 2019, Xu et al. 2020).

The inoculum was pre-grown in the laboratory in five 1-L photobioreactors for 7 days. Air at a flow rate of 0.2 L min^{-1} was sparged from the bottom of the photobioreactors through aquarium air stones. The cultures were mixed with magnetic stirrers and the photobioreactors were illuminated from two sides at constant light intensity of 150 µmol m^{-2} s^{-1}. A total of 5 L of suspended culture was used to inoculate the small RwP (Figure 5). The culture was grown in the small RwP on 1:20 diluted urine supplemented with 400 mL of each TE stock for two weeks before it was used to inoculate the large RwP. The large RwP was inoculated on June 3rd (day -24) by transferring 100 L of suspended culture from the small RwP. The large RwP was filled with urine and tap water at a ratio of 1:15, and 2 L of each TE stock were added to reach the concentrations shown in Table 2.

3.1.4 Operation and sampling

The RwP was first operated as a batch system for 17 days, after which it was fed twice on day -7 (June 20th) and day -3 (June 24th) by adding 67.5 L of undiluted urine to replace the evaporated water. Continuous operation was started on day 0 (June 27th).

Initially, diluted urine and TE mixture were mixed by connecting the inlet tubes with a Y connector downstream of the pump heads. This was expected to reduce the concentration gradients in the influent stream. To achieve a 14-day hydraulic retention time, diluted urine and TE mixture flow rates were set to 72 L/d and the effluent flow rate to 144 L/d. The flow rates were kept constant throughout the experiment except for on day 11, when a water shortage forced to stop pumps for five hours, and at the end of September (day 91), when the flow rates dropped approximately 20 % due to increase in liquid viscosity at near-zero outdoor air temperature. The paddlewheel velocity was initially set to 10 rpm.

Starting day 0, the RwP was sampled twice a week (on Monday and Thursday). The RwP was filled (to replace the evaporated water) and the biomass adhering to its sides was brushed off before sampling. Samples were taken from the center of the water column. On Mondays, samples were analyzed for pH, total suspended solids (TSS), volatile suspended solids (VSS), ammonium nitrogen ($NH_4^+ - N$), and anions. On Thursdays, total dissolved nitrogen (TDN) and total soluble phosphorus (TDP) were also determined. Dissolved organic carbon (DOC) was measured once every two weeks

in July and August, and twice a week in September. Temperature was measured twice a week starting on day 18.

The influent was sampled once a week (on Thursday). Samples were taken from the end of the influent tube. Sampling was performed prior to filling the diluted urine tank or changing the TE mixture except on day 77, when the diluted urine tank was mistakenly filled before the sample was taken. Samples were analyzed for anions, ammonium nitrogen, TDN, and TDP. DOC was measured once every two weeks in July and August, and twice a week in September.

All samples were taken in the morning (8 – 11 a.m.). Ammonium nitrogen and pH were measured on site. Other measurements were made in the laboratory during the same day, except for anions and DOC, for which the filtered samples were stored at -20 °C. All measurements were performed in duplicate.

On day 49, diluted urine and TE mixture influents were separated into two individual tubes. The purpose was to avoid the precipitation of TEs and the accumulation of microbial biomass in the influent tube. In addition, the paddlewheel velocity was increased to 13 rpm, and an aquarium aerator was added to the RwP. On day 53, a second aerator was added to the RwP.

In September, carbon:nitrogen mass ratio of the influent was increased to 5:1 to enhance nutrient recovery in decreasing daylight. This was achieved by adding 2 kg of D-glucose to the TE mixture tank on day 70. The tank was estimated to contain approximately 850 L of TE mixture, resulting in a glucose concentration of 2.4 g L^{-1} in the TE mixture and 1.2 g L^{-1} in the influent containing the diluted urine. A fresh TE mixture with added glucose (2.5 g L^{-1}) was prepared on day 81.

The experiment was terminated on September 26th. The RwP had been in operation for 115 days, of which 91 were in continuous mode. Summary of the operation of the RwP is presented in Table 3.

Table 3. Summary of the operation of the RwP. DU: diluted urine, TE: trace elements, PW: paddlewheel.

Time (d)	Date	Action
-24	3. Jun.	Inoculation, feeding (urine + TE)
-7	20. Jun.	Feeding (urine)
-3	24. Jun.	Feeding (urine)
0	27. Jun.	Start of continuous operation
11	8. Jul.	DU tank fill, fresh TE mixture
22	19. Jul.	New urine stock, DU tank fill, fresh TE mixture
32	29. Jul.	DU tank fill, fresh TE mixture
42	8. Aug.	DU tank fill, fresh TE mixture
49	15. Aug.	Aerator #1 added, PW velocity increased, DU and TE influents separated
53	19. Aug.	Aerator #2 added
54	20. Aug.	DU tank fill, fresh TE mixture
67	2. Sept.	DU tank fill, fresh TE mixture
70	5. Sept.	Glucose added to TE mixture
77	12. Sept.	New urine stock, DU tank fill
81	16. Sept.	Fresh TE + glucose mixture
88	23. Sept.	DU tank fill
91	26. Sept.	The end of the experiment

3.2 Optimization of trace element mixture composition

3.2.1 Experimental design

The purpose of this experiment was to optimize the composition of the trace element (TE) mixture so that the final mixture would contain only the necessary TEs that are not available in diluted urine in sufficient quantities. The experiment was based on the TE mixture used by Tuantet et al. (2014a) in their second experiment, and it was composed of treatments where TEs were omitted one at a time.

The experiment was divided into three set-ups, each with three treatments (one of which served as a control). All treatments were conducted as duplicates so that each set-up was made up of a total of six flasks. All experiments were performed in 1 L Erlenmeyer flasks for 14 days at constant light intensity (54 µmol m^{-2} s^{-1}) and agitation speed (150 rpm). The flasks were maintained at 22 – 28 °C.

The experimental cultures were inoculated with the same mixed culture dominated by *Scenedesmus acuminatus* that was used in the RwP experiment. Each experiment was inoculated with a pre-culture grown for 2 weeks under conditions similar to those of the experimental cultures. The purpose was to ensure that the inoculate was at the same stage of growth in all experiments.

To inoculate the experimental cultures, a 70 mL aliquot was harvested from the pre-cultures. The growth medium contained 33 mL of urine diluted in MQ water to a volume of 500 mL (approximately 1:15 dilution). Each treatment contained a specific mixture of TEs prepared from the same stock solutions used in RwP experiment (Table 2). The urine used in growth experiments was from the first urine batch used in RwP experiment.

The TE mixtures used in each set-up are shown in Table 9. In set-up I, the control treatment was made up of a mixture of six elements (Fe, Mg, Mn, Cu, Zn, and B) used by Tuantet et al. (2014a) in their second experiment. The second treatment contained only Fe and Mg, and the third treatment contained all elements except B. In set-ups II and III, the control treatments contained Fe, Mg, Mn, Cu, and Zn. In set-up II, Cu was omitted in the second treatment and Zn in the third treatment. In set-up III, Mn was omitted in the second treatment and both Cu and Zn were omitted in the third treatment.

3.2.2 Sampling

Flasks were sampled on days 0, 2, 5, 7, 9, 12, and 14. At the start of the experiment (day 0), a 7 mL sample was withdrawn from each inoculated flask, of which 4 mL was used for pH measurement and the remainder was filtered and assayed for anions, ammonium nitrogen, TDN, and TDP. TSS and VSS were determined from the inoculum and their concentrations in the experimental cultures were estimated by calculation.

On sampling days 2 to 12, a 34 mL sample was withdrawn from each flask, of which 4 mL was used for pH measurement and the remainder was filtered for TSS determination. The filtrate was collected and analyzed for anions and $NH_4^+ - N$. At the end of the experiment (day 14), sampling for set-up I was performed as on days 2 to 12 but the TSS filtrate was also analyzed for TDN and TDP. In order to save time, sampling protocol was simplified for set-ups II and III and soluble nutrients (anions, $NH_4^+ - N$, TDN, and TDP) were determined from a separately collected aliquot instead of the TSS filtrate.

Sampling was performed in the afternoon (12 – 14 p.m.). All measurements were made during the same day except for anions, for which the filtered samples were stored at -20 °C. All measurements were performed in duplicate.

3.3 Analytical methods and calculations

3.3.1 Analytical methods

Culture pH was measured with WTW pH 315i pH meter and SenTix® 41 electrode. The RwP temperature was measured with a digital thermometer. The greenhouse temperature was monitored with a digital thermometer, which, in addition to indicating the current temperature, also recorded the highest and the lowest temperatures.

TSS was determined by filtering a measured volume of the sample through a glass fiber filter (Whatman® GF/A, 47 mm), followed by drying the filter at 105 °C for at least 20 h. VSS was determined by igniting the dried filter at 550 °C for 2 hours. The filter was weighed before sample insertion and after drying and ignition. VSS was calculated by subtracting the weight of the ignited filter from the weight of the dried filter and dividing the result by the volume of the sample. It was assumed that VSS measures the amount of living algal and bacterial biomass as well as dead cells present in the RwP and batch flasks. Culture composition was also monitored using a bright-field

microscope (Axio-Imager, Carl Zeiss, Germany) and some selected samples were photographed using an AxioCam HRc CCD camera accompanied with ZEN 2 (blue edition) software (Carl Zeiss, Germany).

Prior to the analyses of dissolved nutrients, the samples were filtered with 0.20 µm polyester syringe filters (CHROMAFIL® Xtra PET-20/25). Ammonium nitrogen ($NH_4^+ - N$) was determined with HACH® Lange ammonium cuvette test LCK303 and HACH® DR1900 (RwP experiment) and HACH® DR3900 (optimization experiment) spectrophotometers. For RwP experiment, the $NH_4^+ - N$ analysis was performed on site to avoid systematic error towards lower values caused by deprotonation of ammonium to ammonia and volatilization of the ammonia. TDN and TDP were determined with HACH® Lange LCK238 total nitrogen cuvette test and LCK348 total phosphorus cuvette test, respectively. Digestions were performed in HACH® DRB200 heating system and concentrations were determined with HACH® DR3900 spectrophotometer. Concentrations of phosphate (PO_4^{3-}), nitrate (NO_3^-), and nitrite (NO_2^-) ions were measured with ion chromatography (IC, Dionex ICS-1600). Dionex IonPack AS22 4 mm anion exchange column with ASRS-300 suppressor was used as a separating column. Eluent buffer contained 4.5 mM Na_2CO_3 and 1.4 mM $NaHCO_3$ and eluent flow rate was set to 1.2 mL/minute. Analysis was performed according to the ion chromatography standard SFS-EN ISO 10304-1:en. Dissolved organic carbon (DOC) was determined using a Shimadzu TOC-5000 analyzer according to the standard SFS-EN 1484:en. Organic acids were analyzed with Agilent series 1100 high-performance liquid chromatography (HPLC) system equipped with diode-array detector G1315A (Agilent Technologies, California) were used to analyze the samples. Eluent buffer contained 20 mm H_2SO_4 and eluent flow rate was set to 600 mL/minute. RHM-monosaccharide H+ (8%) 300 mm x 7.8 mm column (Phenomenex, California) was used as a separating column. The column was heated to 50 °C.

Outdoor temperature data was obtained from Finnish Meteorological Institute. The temperature was measured at Siilinkari observation station, which is 3.6 km west of the Hiedanranta experimental site.

3.3.2 Calculations

Removal efficiencies (RE, %) for $NH_4^+ - N$ and $PO_4^{3-} - P$ were defined as:

$$RE(\%) = \frac{C_{influent} - C_{RwP}}{C_{influent}} * 100$$

where $C_{influent}$ is the concentration of $NH_4^+ - N$ or $PO_4^{3-} - P$ (mg L^{-1}) measured from the influent at time t and C_{RwP} is the concentration of $NH_4^+ - N$ or $PO_4^{3-} - P$ (mg L^{-1}) measured from the RwP at time t.

Nitrification efficiency (NE, %) of $NH_4^+ - N$ was was defined as:

$$NE\ (\%) = \frac{C_{NO_3-N}}{C_{influent\ NH_4^+-N}} * 100$$

where C_{NO_3-N} is the concentration of $NO_3^- - N$ (mg L^{-1}) measured from the RwP at time t and $C_{influent\ NH_4^+-N}$ is the concentration of $NH_4^+ - N$ measured from the influent at time t.

Ammonia volatilization percentage (AVP, %) was calculated according to Hansen et al. (1998) as follows:

$$AVP\ (\%) = (1 + \frac{10^{-pH}}{10^{-\left(0.09018+\frac{2729.2}{T(K)}\right)}} * 100$$

where pH is the culture pH and $T(K)$ is the temperature of the culture broth in Kelvins.

For optimization experiments, statistical hypothesis test was based on nested ANOVA model:

$$y_{ijk} = m + a_i + b_{j(i)} + \varepsilon_{ijk}$$

where y_{ijk} represents the response variable (the final concentrations of $NH_4^+ - N$, $PO_4^{3-} - P$ or VSS), m represents the intercept, a_i represents the groups formed by individual TE treatments, $b_{j(i)}$ represents the subgroups formed by two biological replicates nested within a_i, and ε_{ijk} represents the residual. The final concentrations of $NH_4^+ - N$, $PO_4^{3-} - P$, and VSS in each treatment where TEs were omitted were individually tested against the corresponding concentrations in control treatment of the same set-up. In addition, the control treatments were tested against each other.

All analyses were conducted with R 3.6.1 (R Core Team 2019). Packages ‘ggplot2’ (Wickham 2016) and ‘gridExtra’ (Auguié 2017) were used to draw the graphs. Functions ‘lme’ and ‘anova.lme’ from a package ‘nlme’ (Pinheiro et al. 2019) were used to build the statistical model and perform the hypothesis test.

4. RESULTS

4.1 Raceway pond experiment

4.1.1 Temperatures and culture pH

The RwP was monitored for a total of 91 days from June 27th to September 26th. During this period, the RwP was operated in continuous mode with influent and effluent flow rates of 144 L d^{-1}.

Outdoor air temperature recorded at a nearby Siilinkari observation station varied between 28 °C (on day 30) and 3 °C (on day 88). The mean temperatures were 16.8 °C in July, 16.6 °C in August, and 11.1 °C in September (Figure 6A). Temperature in the greenhouse followed the changes in the outdoor air temperature and varied between 47.4 °C and 2.0 °C while the temperature measured from the RwP (Figure 6A) in the morning varied between 23.8 °C (on day 28) and 9.7 °C (on day 91).

The pH was at its highest (8.1 – 8.5) at the start of the continuous mode operation but decreased quite drastically to 7.4 on day 32 (Figure 6B), while it remained quite steady at approximately 7 (except for occasional fluctuations) on days 35 – 70. The lowest pH values were observed on days 53 and 74 (6.7 and 6.5, respectively). After day 74, the pH started to rise again, eventually reaching values above 7.5.

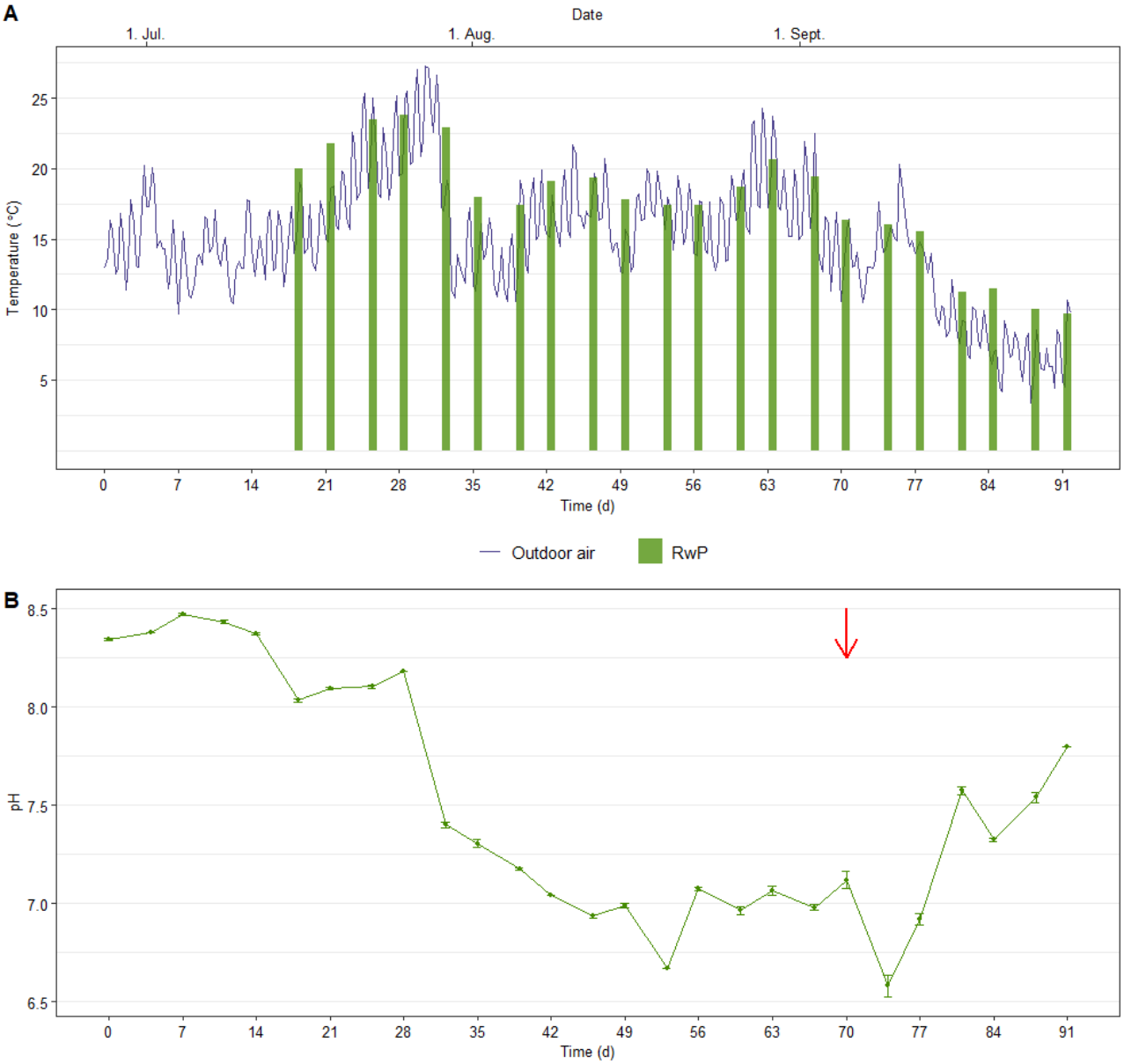

Figure 6. A) Outdoor air temperature and RwP temperature. Outdoor air temperature was recorded at Siilinkari observation station 3.6 km west of the experimental field B) Culture pH. Red arrow indicates the onset of mixotrophic cultivation by adding glucose to the TE mixture tank.

4.1.2 Biomass growth and dissolved organic carbon

The biomass concentration measured as VSS was at its highest (0.80 g L^{-1}) at the start of the continuous mode operation (Figure 8A). Thereafter, the biomass concentration started to decrease and remained relatively constant (0.33 – 0.44 g L^{-1}) from mid-July until the end of August. In September, the biomass concentration decreased below 0.3 g L^{-1}. On day 74 (four days after the growth conditions were changed to mixotrophic), the biomass concentration increased to 0.51 g

L^{-1}. After that, it began to decrease again, reaching the lowest value (0.24 g L^{-1}) at the end of the experiment.

Changes in species composition can be seen in microscopic images from August 22nd (day 56; Figure 7A) and September 19th (day 84; Figure 7B). Both images show abundant algal cells, which are green in August but pale yellowish in September. Some of the cells are larger compared to others, and these have retained their green color in September as well. The biomass also included some grayish circular structures that became remarkably more abundant during mixotrophic cultivation (Figure 7A,B).

Appearance of the cultivation broth changed after the growth conditions were changed to mixotrophic. On day 70, the amount of biomass was low, and cells formed dense clumps (Figure 7C). On day 74, the culture had turned solid green and the broth was quite uniform (Figure 7D). On day 77, the color of the culture had turned yellowish, but the broth remained uniform (Figure 7E).

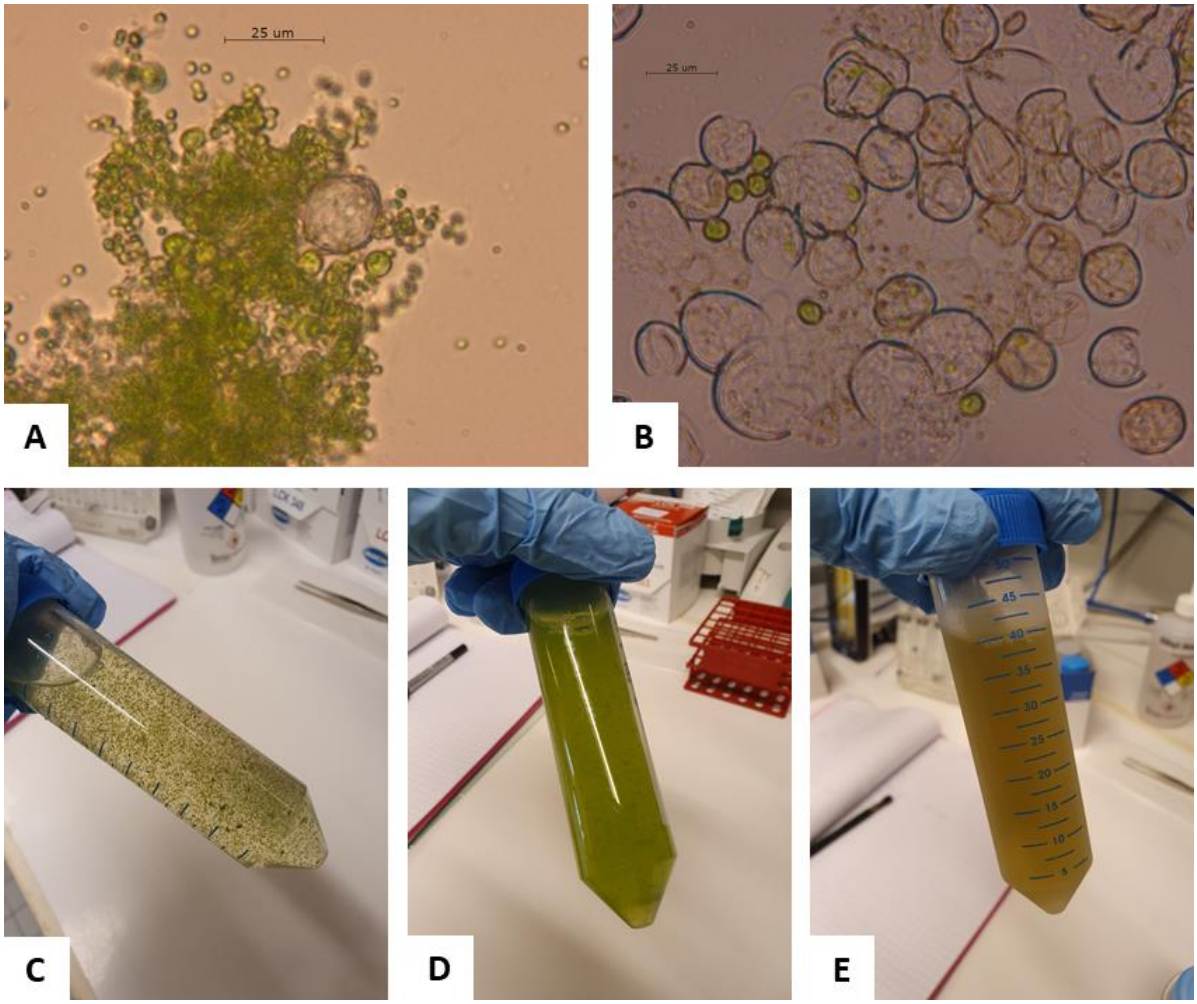

Figure 7. Microscopic images of the biomass growing in the RwP on a) August 22nd (day 56) and b) September 19th (day 84) and appearances of the cultivation broths on c) September 5th (day 70), d) September 9th (day 74), and e) September 12th (day 77).

Dissolved organic carbon (DOC) concentrations were measured from the RwP and the influent (Figure 8B). In the influent, DOC remained relatively stable (93 – 121 mg L^{-1}) on days 7 – 70. On days 70 and 81, D-glucose was added to the influent, which is reflected in the DOC concentrations in the following samples. Interestingly, the concentrations measured on days 74 and 84 (828 mg L^{-1} and 1077 mg L^{-1}, respectively) were 180 and 220 % of the concentrations expected based on the added amount of glucose. In both batches, concentrations decreased over time to 272 mg L^{-1} (on day 81) and 284 mg L^{-1} (on day 91), which were 60 % of the concentrations expected based on the added amount of glucose.

In the RwP, the DOC concentration started from 81 mg L^{-1} on day 7 and declined smoothly to 37 – 47 mg L^{-1} on days 35 – 63 (Figure 8B). The concentration was at its lowest (24 mg L^{-1}) on day 70. After the onset of mixotrophic cultivation, the RwP's DOC concentration followed the fluctuations in the influent's DOC concentration with a delay of three to four days. The highest concentrations were measured on days 77 and 91 (59 mg L^{-1} and 80.3 mg L^{-1}, respectively). Between the two peaks, the lowest concentration (48 mg L^{-1}) was observed on day 84.

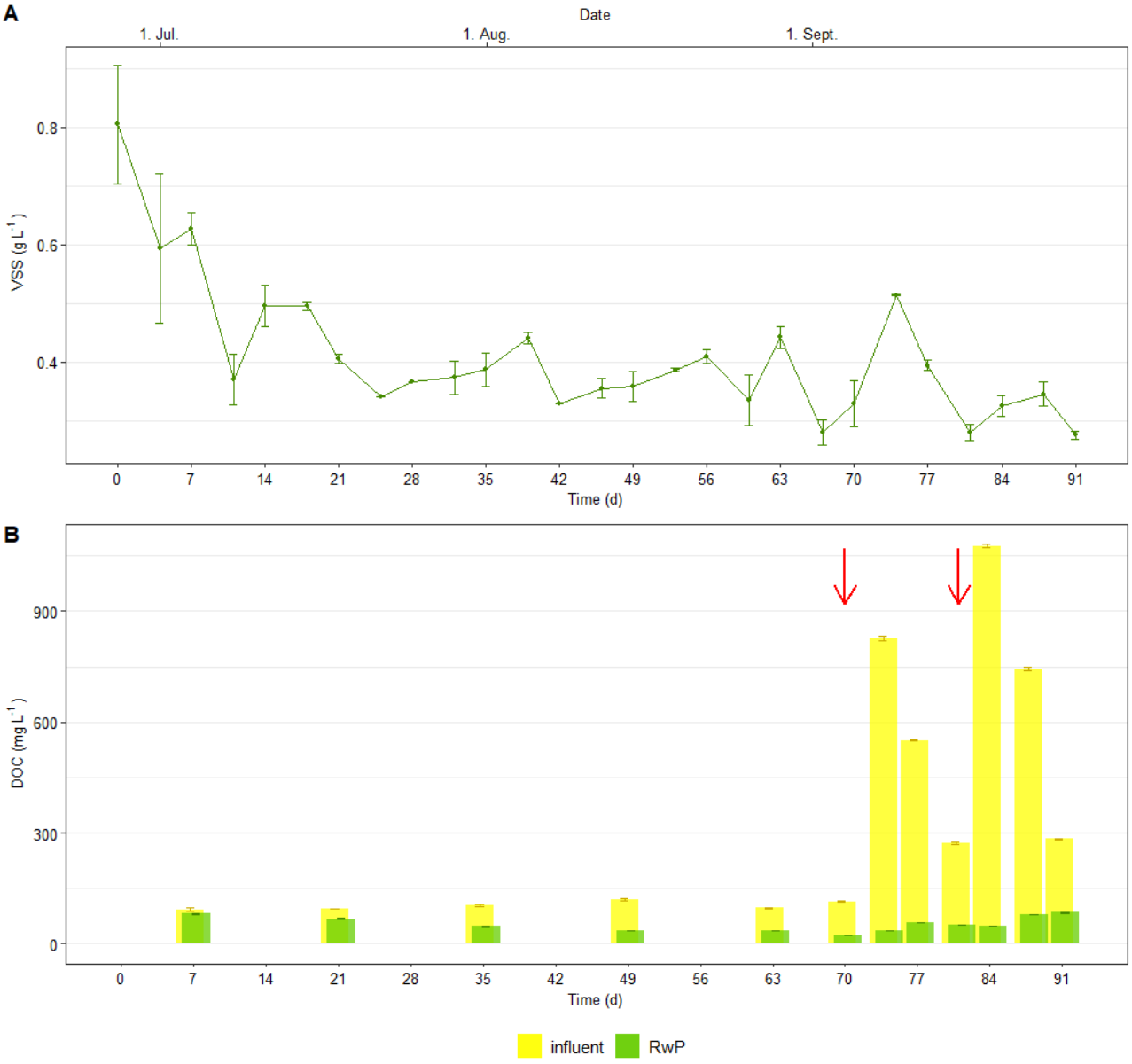

Figure 8. A) Changes in biomass concentration measured as volatile suspended solids (VSS) in the RwP. B) Changes in dissolved organic carbon (DOC) concentration in the RwP and the influent. The first red arrow indicates the onset of mixotrophic cultivation by adding glucose to the TE mixture tank and the second red arrow indicates the time point when fresh TE mixture and glucose stock were prepared.

An unidentified organic acid was found to accumulate in the RwP. One distinct peak with a retention time of 6.2 minutes was observed in the chromatogram. The areas of the chromatogram peaks increased steadily in subsequent samples from day 7 to day 63 but decreased to approximately one third on day 77 (Table 4). Despite many attempts, the organic acid was not identified, so its concentrations in the samples could also not be determined. The organic acid was not observed in the influent.

Table 4. The peak areas (Vmin.) of the unidentified organic acid observed in the RwP.

Time (d)	Peak area (Vmin)
7	454
21	538
35	7945
49	15102
63	21412
77	6741

4.1.3 Nutrient removal

In the influent, the $NH_4^+ - N$ concentration ranged from 137 to 256 mg L^{-1} (Figure 9A). $NH_4^+ - N$ accounted for 92 – 100 % of the TDN. Overall, the $NH_4^+ - N$ and TDN concentrations in the influent remained relatively stable throughout the experiment except for one peak that occurred two weeks after the start of the experiment. In addition, there was a slight but constant increase in both $NH_4^+ - N$ and TDN concentrations, which coincides with replacement of the urine batch on day 77. The nitrate (NO_3^-) and nitrite (NO_2^-) concentrations remained below detection limit throughout the experiment.

In the RwP, the $NH_4^+ - N$ was at its highest (225 mg L^{-1}) at the start of the experiment but dropped to 58 mg L^{-1} after 11 days (Figure 9A). The sudden peak observed in the influent's $NH_4^+ - N$ concentration at day 14 was reproduced in the RwP seven days later, when the $NH_4^+ - N$ concentration increased to 85 mg L^{-1}. The lowest $NH_4^+ - N$ concentration (17 mg L^{-1}) was observed on day 32 (July 29th). Thereafter, the $NH_4^+ - N$ concentration gradually increased to 45 – 52 mg L^{-1}, where it remained from day 46 until the onset of mixotrophic cultivation on day 70. On day 74, the $NH_4^+ - N$ concentration decreased to 43 mg L^{-1}, but then began to increase again, eventually reaching 115 mg L^{-1} on day 91. The TDN concentration followed the fluctuations of the $NH_4^+ - N$ concentration being 6 – 26 mg L^{-1} higher at the beginning of the experiment (Figure 9A). From day 35, the difference increased to 32 – 57 mg L^{-1}. The $NO_3^- - N$ concentration increased from an

initial 0 mg L^{-1} to 1 mg L^{-1} on day 32 and 5 – 10 mg L^{-1} on days 35 – 70, after which it decreased to 1 – 2 mg L^{-1} (Figure 9A). NO_2^- concentration remained below detection limit throughout the experiment.

In the influent, the $PO_4^{3-} - P$ concentration ranged from 6 to 13 mg L^{-1} (Figure 9B). $PO_4^{3-} - P$ accounted for 57 – 87 % of the TDP. $PO_4^{3-} - P$ concentrations remained relatively constant except for the peak observed on day 14. In addition, concentrations increased slightly after the urine batch was replaced on day 77.

In the RwP, the $PO_4^{3-} - P$ concentration was at its highest (28 mg L^{-1}) at the start of the experiment (Figure 9B). Initially, the concentration decreased slowly, and it fell below the $PO_4^{3-} - P$ concentration of the influent on day 60. The $PO_4^{3-} - P$ concentration decreased steadily during July and August, but a slightly steeper decrease from 14 mg L^{-1} to 10 mg L^{-1} was observed on day 28 (July 25th). On day 70, the concentration was 6 mg L^{-1}. After the onset of mixotrophic cultivation, the concentration dropped sharply below the detection limit from day 77 until the end of the experiment. The TDP concentration followed the fluctuations of the $PO_4^{3-} - P$ concentration being 0 – 6 mg L^{-1} higher (Figure 9B).

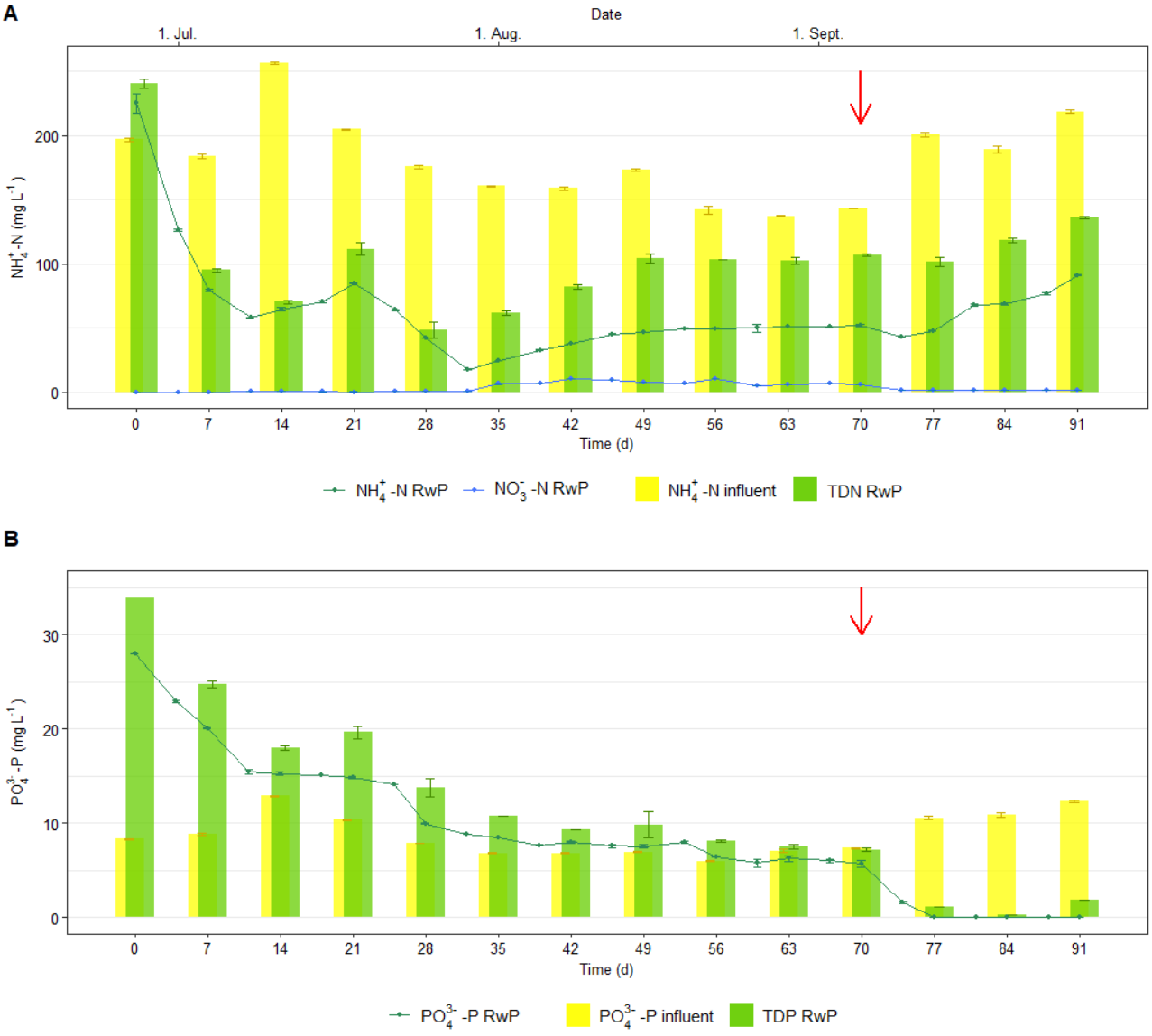

Figure 9. A) Changes in $NH_4^+ - N$, $NO_3^- - N$, and TDN concentrations in the RwP and the influent. B) Changes in $PO_4^{3-} - P$ and TDP concentrations in the RwP and the influent. Red arrow indicates the onset of mixotrophic cultivation by adding glucose to the TE mixture tank.

REs were calculated for $PO_4^{3-} - P$ and $NH_4^+ - N$ over the time period when the concentration in the RwP was lower than in the influent. REs ranged from 12 to 100 % for $PO_4^{3-} - P$ and from 57 to 85 % for $NH_4^+ - N$, whereas nitrification accounted for 0.1 – 7.3 % and ammonium volatilization for 0.2 – 7.3 % reduction in the $NH_4^+ - N$ concentration (Table 5).

Table 5. Removal efficiencies (RE, %) for $PO_4^{3-} - P$ and $NH_4^+ - N$, nitrification efficiency (NE, %), and ammonia volatilization percentage (AVP, %) in the RwP.

Time (d)	$PO_4^{3-} - P$ RE (%)	$NH_4^+ - N$ RE (%)	NE (%)	AVP (%)
7		57	0.1	
14		75	0.1	
21		58	0.1	5.3
28		76	0.2	7.3
35		85	4.0	0.7
42		76	6.4	0.4
49		73	4.4	0.3
56		65	7.3	0.4
63	12	63	4.6	0.5
70	22	64	4.0	0.4
77	100	76	0.8	0.2
84	100	64	0.9	0.4
91	100	58	0.6	1.1

4.2 Optimization of trace element mixture composition

4.2.1 Set-up I

Figure 10 shows changes in culture pH, VSS, $NH_4^+ - N$, and $PO_4^{3-} - P$ in set-up I during the 14-day experiment. Each culture started at a relatively uniform pH (8.8 – 8.9), $NH_4^+ - N$ concentration (214 – 226 mg L^{-1}), and $PO_4^{3-} - P$ concentration (14 – 15 mg L^{-1}). Initial biomass concentration measured as VSS (0.04 g L^{-1}) was calculated from the value determined from the inoculum.

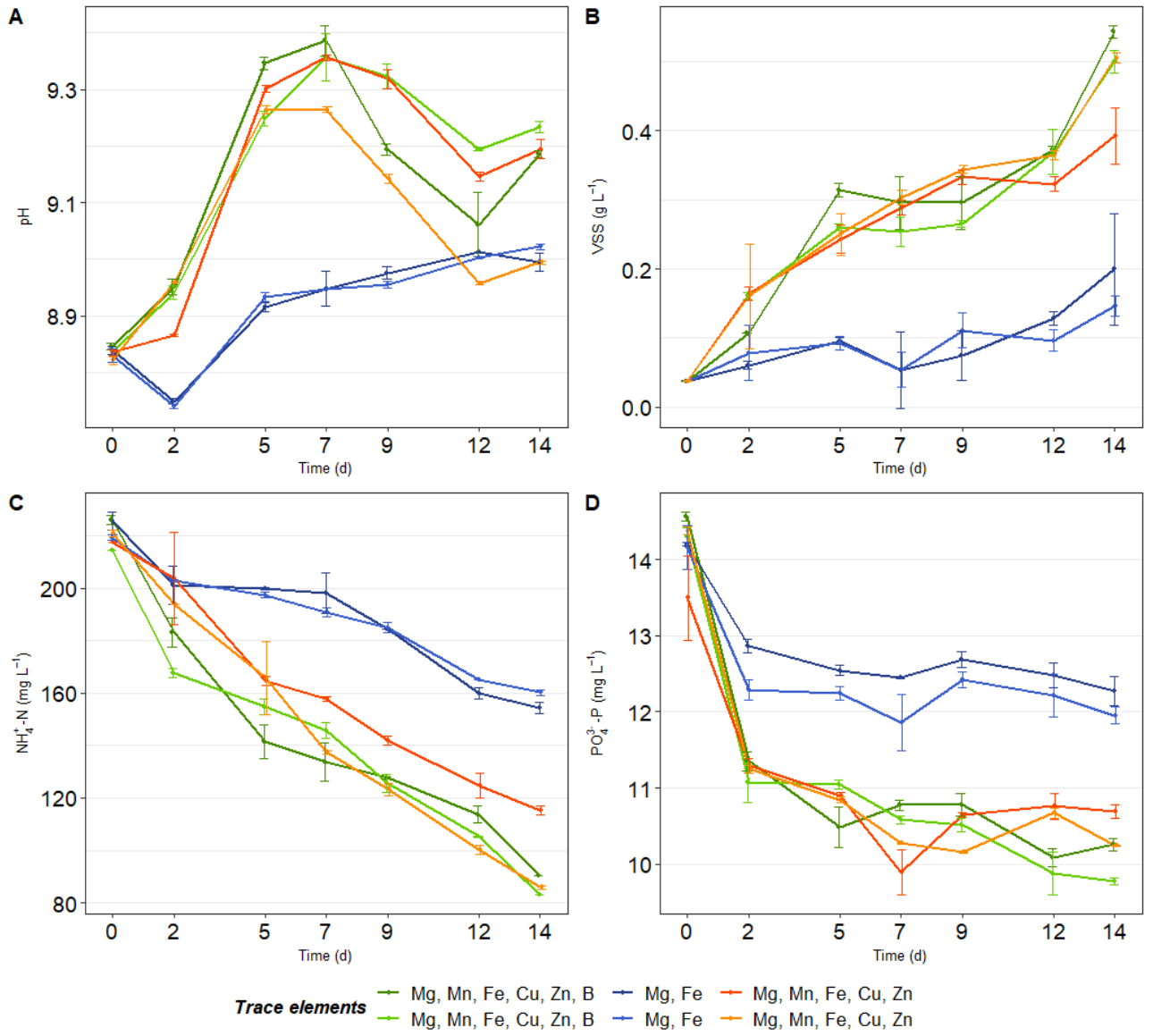

Figure 10. Set-up I: changes in A) culture pH, B) biomass concentration measured as volatile suspended solids (VSS), C) $NH_4^+ - N$, and D) $PO_4^{3-} - P$ in the batch flasks during the 14-day experiment. The error bars represent the standard error of technical duplicates.

During the experiment, changes in pH varied with the treatments. In cultures supplemented with five (Mg, Mn, Fe, Cu, and Zn) or six (Mg, Mn, Fe, Cu, Zn, and B) TEs, the pH increased rapidly, reaching a peak in the middle of the experiment, and then began to decrease. In cultures supplemented with only two TEs (Mg and Fe), the pH dropped slightly on day 2, after which it started to increase slowly. The highest pH values were 9.3 – 9.4 for five or six TE containing treatments (days 5 – 7) and 9.0 for two TE containing treatments (days 12 – 14).

As the experiment progressed, $NH_4^+ - N$ and $PO_4^{3-} - P$ concentrations decreased and VSS concentrations increased in all batch flasks, but the rate varied with the treatments. The final concentrations of $NH_4^+ - N$, $PO_4^{3-} - P$ and VSS as well as the nutrient removal efficiencies were as shown in Table 6. $NH_4^+ - N$ and $PO_4^{3-} - P$ removal efficiencies as well as the biomass production were significantly lower in cultures supplemented with Mg and Fe alone compared to cultures supplemented with complete set of TEs (Table 9). The highest biomass production and nutrient removal efficiencies were observed in treatments supplemented with five (Mg, Mn, Fe, Cu, and Zn) or six (Mg, Mn, Fe, Cu, Zn, and B) trace elements. The absence of boron did not appear to have a statistically significant effect on either biomass growth or nutrient removal efficiency, and therefore it was decided to exclude boron from control treatments in the subsequent set-ups. NO_3^- and NO_2^- concentrations remained below the detection limit throughout the experiment. Concentrations of TDN and TDP measured at the start and the end of the experiment were identical with those of $NH_4^+ - N$ and $PO_4^{3-} - P$, respectively, indicating that the cultures initially did not contain any other soluble N or P compounds and none was formed during the experiment.

Table 6. Set-up I: residual concentrations and removal efficiencies (in parenthesis) of $NH_4^+ - N$ and $PO_4^{3-} - P$, as well as biomass production measured as volatile suspended solids (VSS) at the end of the experiment. Values are averaged over technical duplicates.

Trace elements	$NH_4^+ - N$ (mg L^{-1})	$PO_4^{3-} - P$ (mg L^{-1})	VSS (g L^{-1})
Mg, Mn, Fe, Cu, Zn, B	91 (60 %)	10 (30 %)	0.54
	83 (61 %)	10 (32 %)	0.50
Mg, Fe	155 (32 %)	12 (14 %)	0.20
	160 (27 %)	12 (16 %)	0.15
Mg, Mn, Fe, Cu, Zn	115 (47 %)	11 (21 %)	0.39
	86 (61 %)	10 (29 %)	0.51

4.2.2. Set-up II

Figure 11 shows changes in culture pH, VSS, $NH_4^+ - N$, and $PO_4^{3-} - P$ in set-up II during the 14-day experiment. Initial conditions were similar to set-up I and each culture started at relatively uniform pH (8.7 – 8.8), $NH_4^+ - N$ concentration (218 – 224 mg L^{-1}), and $PO_4^{3-} - P$ concentration (13 – 14 mg L^{-1}). Initial VSS concentration (0.04 g L^{-1}) was estimated from the value determined from the inoculum.

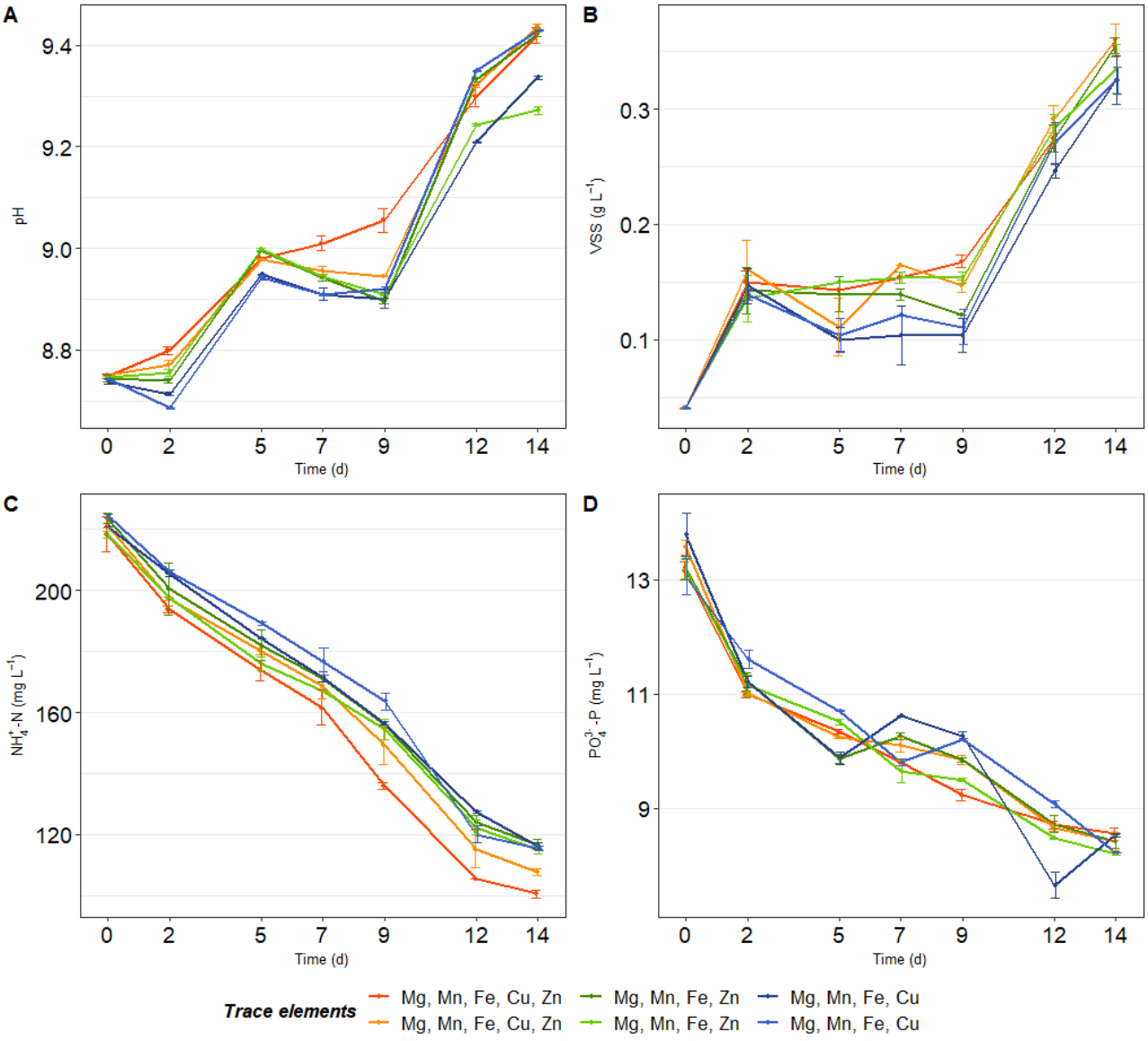

Figure 11. Set-up II: changes in A) culture pH, B) biomass concentration measured as volatile suspended solids (VSS), C) $NH_4^+ - N$, and D) $PO_4^{3-} - P$ in the batch flasks during the 14-day experiment. The error bars represent the standard error of technical duplicates.

Midway through the experiment, the temperature in the laboratory was up to 5 °C higher compared to other set-ups. As the experiment progressed, $NH_4^+ - N$ and $PO_4^{3-} - P$ concentrations decreased rather steadily in all treatments, although the changes on days 5 – 9 were smaller compared to set-ups I and III. The increase in VSS concentrations ceased on day 5 and continued after day 9, resulting in slightly lower biomass growth compared to set-ups I and III. Culture pH followed a similar pattern where the sharp rise at the start of the experiment ceased on day 5 and continued after day 9, resulting in pH values 9.3 – 9.4 at the end of the experiment.

The final concentrations of $NH_4^+ - N$, $PO_4^{3-} - P$, and VSS along with nutrient removal efficiencies were as shown in Table 7. Although the control treatments (supplemented with Mg, Mn, Fe, Cu, and Zn) seemed to perform slightly better in biomass growth and $NH_4^+ - N$ removal compared to the treatments where either copper or zinc was omitted, the difference was not statistically significant (Table 9). NO_3^- and NO_2^- concentrations remained below the detection limit throughout the experiment. Concentrations of TDN and TDP measured from the cultures at the start and the end of the experiment indicated that all soluble N and P were in the form of ammonium and phosphate, respectively.

Table 7. Set-up II: residual concentrations and removal efficiencies (in parenthesis) of $NH_4^+ - N$ and $PO_4^{3-} - P$, as well as biomass production measured as volatile suspended solids (VSS) at the end of the experiment. Values are averaged over technical duplicates.

Trace elements	$NH_4^+ - N$ (mg L^{-1})	$PO_4^{3-} - P$ (mg L^{-1})	VSS (g L^{-1})
Mg, Mn, Fe, Cu, Zn	101 (54 %)	9 (35 %)	0.36
	108 (51 %)	8 (38 %)	0.36
Mg, Mn, Fe, Zn	117 (48 %)	8 (36 %)	0.36
	115 (47 %)	8 (38 %)	0.34
Mg, Mn, Fe, Cu	116 (47 %)	9 (38 %)	0.33
	116 (49 %)	8 (37 %)	0.33

4.2.3. Set-up III

Figure 12 shows changes in culture pH, VSS, $NH_4^+ - N$, and $PO_4^{3-} - P$ in set-up III during the 14-day experiment. Initial conditions were similar to set-ups I and II, and each culture started at relatively uniform pH (8.8), $NH_4^+ - N$ concentration (220 – 224 mg L^{-1}), and $PO_4^{3-} - P$ concentration (13 – 14 mg L^{-1}). VSS concentration (0.04 g L^{-1}) was estimated from the value determined from the inoculum.

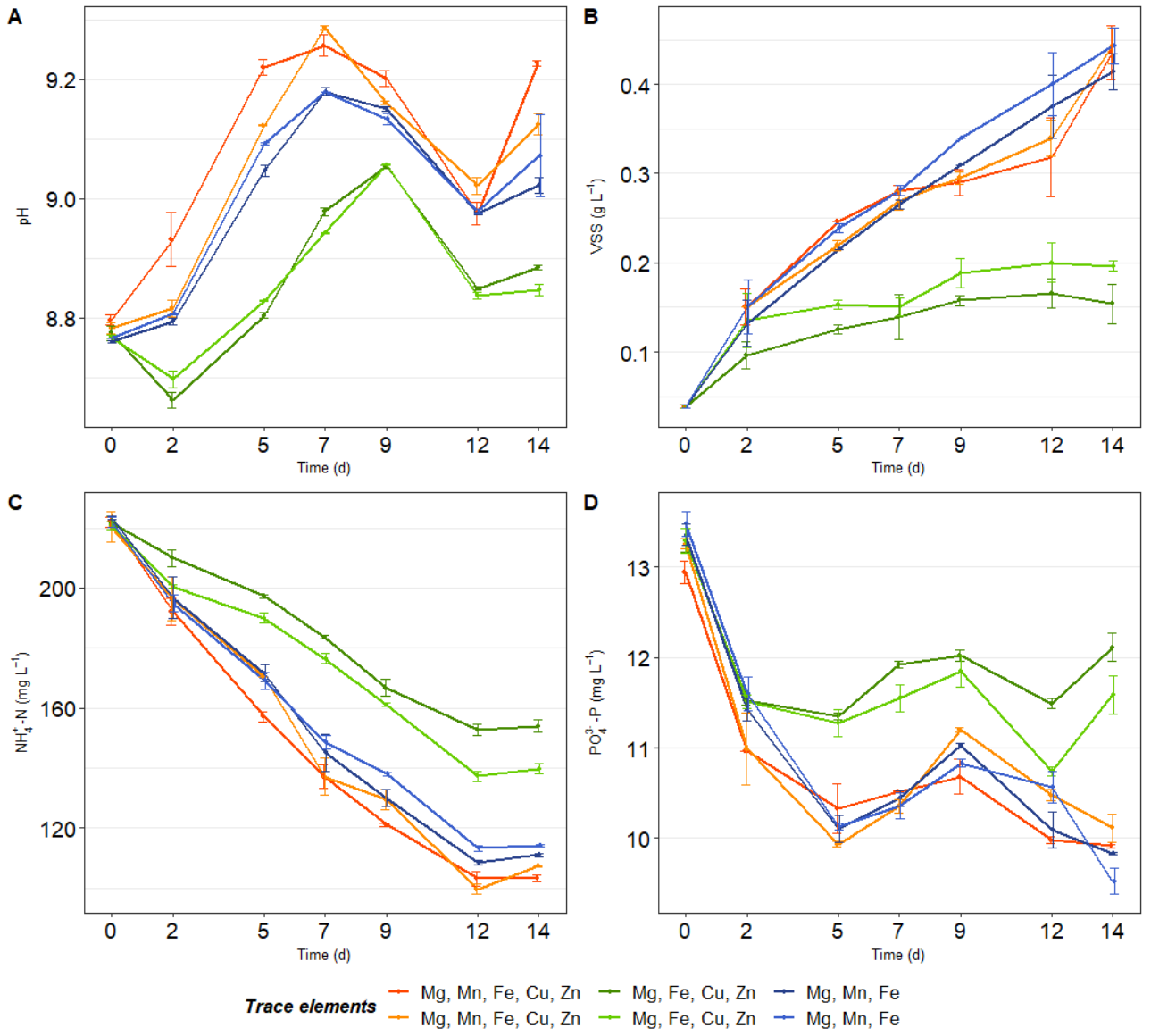

Figure 12. Set-up III: changes in A) culture pH, B) biomass concentration measured as volatile suspended solids (VSS), C) $NH_4^+ - N$, and D) $PO_4^{3-} - P$ in the batch flasks during the 14-day experiment. The error bars represent the standard error of technical duplicates.

During the experiment, changes in pH varied with the treatments. In control treatments (supplemented with Mg, Mn, Fe, Cu, and Zn) and treatments where both copper and zinc were omitted, the pH increased rapidly, reaching a peak on seventh day, and then began to decrease. In treatments where manganese was omitted, pH dropped slightly on the second day, after which it began to increase (reaching a peak on the ninth day) and then decrease again. The highest pH values were 9.2 – 9.3 for control treatments and treatments where Cu and Zn were omitted and 9.1 for treatments where Mn was omitted.

The final concentrations of $NH_4^+ - N$, $PO_4^{3-} - P$, and VSS along with nutrient removal efficiencies were as shown in Table 8. $NH_4^+ - N$ and $PO_4^{3-} - P$ removal efficiencies and biomass production were significantly lower in cultures where Mn was omitted compared to control treatments (Table 9). Instead, the absence of both copper and zinc did not appear to have a statistically significant effect on biomass growth or nutrient removal. NO_3^- and NO_2^- concentrations remained below the detection limit throughout the experiment. Concentrations of TDN and TDP measured from the cultures at the start and the end of the experiment indicated that all soluble N and P were in the form of ammonium and phosphate, respectively.

Table 8. Set-up III: residual concentrations and removal efficiencies (in parenthesis) of $NH_4^+ - N$ and $PO_4^{3-} - P$, as well as biomass production measured as volatile suspended solids (VSS) at the end of the experiment. Values are averaged over technical duplicates.

Trace elements	$NH_4^+ - N$ (mg L^{-1})	$PO_4^{3-} - P$ (mg L^{-1})	VSS (g L^{-1})
Mg, Mn, Fe, Cu, Zn	103 (53 %)	10 (23 %)	0.44
	107 (51 %)	10 (24 %)	0.44
Mg, Fe, Cu, Zn	154 (31 %)	12 (9 %)	0.15
	140 (37 %)	12 (13 %)	0.20
Mg, Mn, Fe	111 (50 %)	10 (26 %)	0.41
	114 (49 %)	10 (29 %)	0.44

4.2.4. Statistical analysis and comparison between set-ups

Table 9 shows the *p*-values for the statistical tests that were used to determine whether there was a significant difference in biomass growth and nutrient removal between the control treatments and the treatments with certain TEs omitted. Possible differences between set-ups were also statistically tested. It was found that the treatments supplemented with five TEs (Mg, Mn, Fe, Cu, and Zn) in all three set-ups were similar with respect to the final concentrations of VSS (*p*-value 0.816), $NH_4^+ - N$ (*p*-value 0.512), and $PO_4^{3-} - P$ (*p*-value 0.506), which indicates that the set-ups are comparable to each other.

*Table 9. Experimental designs with supplemented trace elements for the three set-ups and p-values of nested ANOVA models testing effects of trace element omissions on residual concentrations of $NH_4^+ - N$ and $PO_4^{3-} - P$, as well as on biomass production measured as volatile suspended solids (VSS). * : statistical significance.*

Experimental design			*p*-values		
Set-up	Control	Treatment	$NH_4^+ - N$	$PO_4^{3-} - P$	VSS
I	Mg, Mn, Fe, Cu, Zn, B	Mg, Fe	0.0044 *	0.0187 *	0.0097 *
		Mg, Mn, Fe, Cu, Zn	0.4551	0.3036	0.3523
II	Mg, Mn, Fe, Cu, Zn	Mg, Mn, Fe, Zn	0.0877	0.3032	0.3491
		Mg, Mn, Fe, Cu	0.0842	0.6063	0.0619
III	Mg, Mn, Fe, Cu, Zn	Mg, Fe, Cu, Zn	0.0302 *	0.0227 *	0.0065 *
		Mg, Mn, Fe	0.1035	0.2095	0.5685

Batch flasks of each set-up were photographed at the beginning of sampling on days 0, 7, and 14 (Figure 13). In flasks containing at least Mg, Mn, and Fe, the colour of the cultivation broth turned from pale green to dark green due to intense algae growth. This occurred by the middle of the experiment in all set-ups except for II, where growth was ceased on day 5 and continued after day 9.

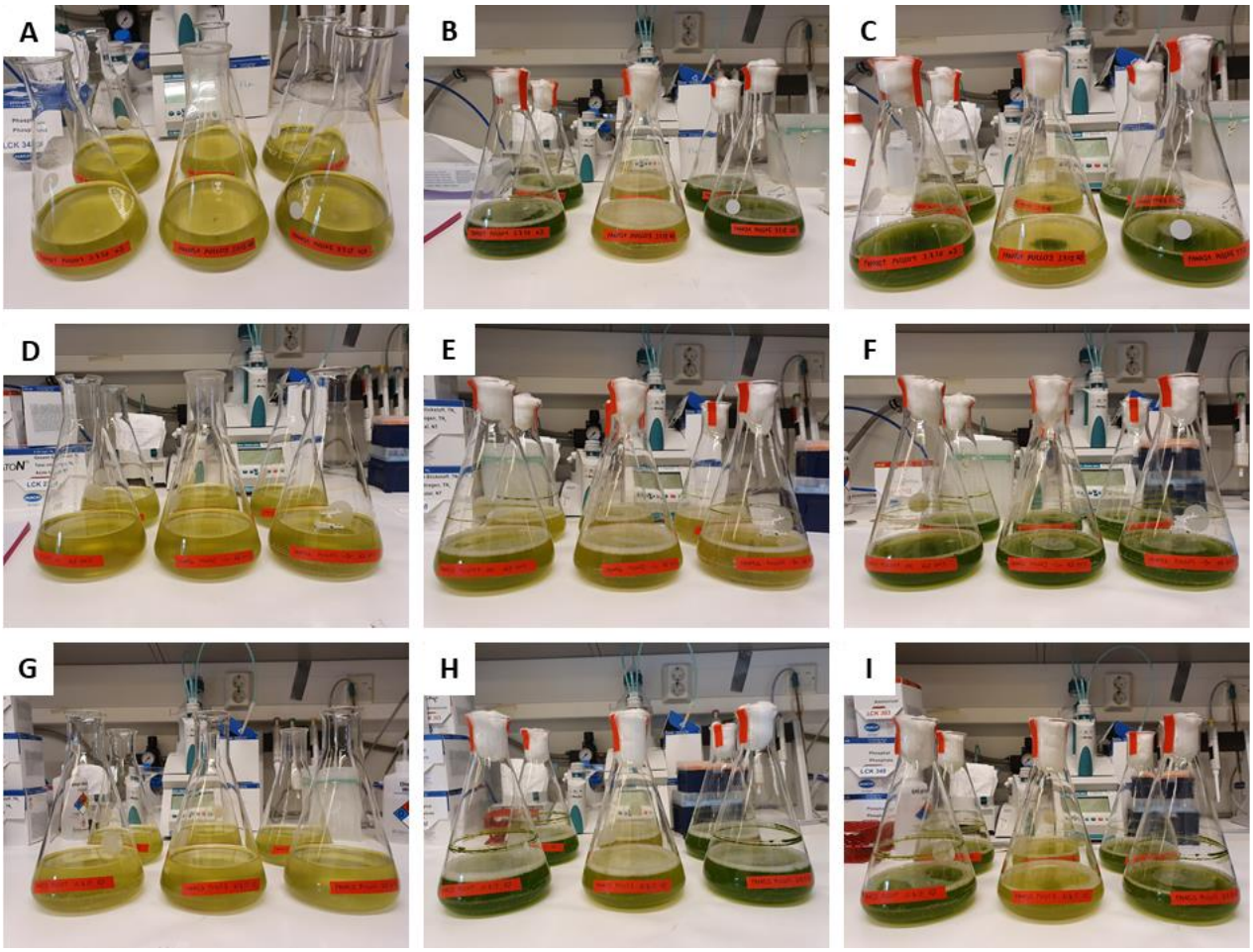

Figure 13. The batch flasks of each set-up photographed at the beginning of sampling. The flasks treated with the same trace element mixture (biological replicates) were arranged sequentially. A – C): Set-up I on days 0, 7, and 14. Treatments from left to right: six trace elements (Mg, Mn, Fe, Cu, Zn, and B), two trace elements (Mg and Fe), and five trace elements (Mg, Mn, Fe, Cu, and Zn). D – F): Set-up II on days 0, 7, and 14. Treatments from left to right: five trace elements (Mg, Mn, Fe, Cu, and Zn), Cu omitted, and Zn omitted. G – I): Set-up III on days 0, 7, and 14. Treatments from left to right: five trace elements (Mg, Mn, Fe, Cu, and Zn), Mn omitted, and Cu + Zn omitted.

5. DISCUSSION

5.1 Nutrient removal efficiency

The RwP experiment showed that NH_4^+ and PO_4^{3-} can be removed from source-separated human urine by using it as a growth medium for microalgal cultivation, and nutrient removal was enhanced by adding glucose to the culture. At best, an 85 % reduction in $NH_4^+ - N$ concentration and 100 % reduction in $PO_4^{3-} - P$ concentration was achieved during the experiment conducted in the RwP. The average REs were 68 % for $NH_4^+ - N$ and 67 % for $PO_4^{3-} - P$. The average $NH_4^+ - N$ RE was in line with $NH_4^+ - N$ REs obtained in the laboratory-scale batch experiment, while the average $PO_4^{3-} - P$ RE was increased by high $PO_4^{3-} - P$ REs achieved during the mixotrophic cultivation, resulting in substantially higher $PO_4^{3-} - P$ RE compared to those obtained in the batch experiment.

The effluent nitrogen concentration obtained in this study did not meet the requirements for discharge of total nitrogen (15 mg L^{-1}) from urban wastewater treatment plants established in Directive 98/15/EC. The minimum nitrogen RE (70 %) established in the Directive was momentarily met on days 14 and 28, but from day 35, an unidentified nitrogenous metabolite began to accumulate in the RwP, reducing the nitrogen RE below the required level. The average $NH_4^+ - N$ RE achieved in this study was in line with those observed by Chatterjee et al. (2019) and Saarnio (2019), whose experimental set-ups were similar to this study. Posadas et al. (2015) achieved higher $NH_4^+ - N$ REs in their experiment using a co-culture of *Scenedesmus sp.* and activated sludge from the wastewater treatment plant grown on domestic wastewater in semi-continuously operated open RwPs in three sizes ranging from 700 L to 850 L, but the $PO_4^{3-} - P$ REs were substantially lower than obtained in this study during the mixotrophic cultivation.

The maximum phosphorus RE of 100 % achieved during the mixotrophic cultivation resulted in an effluent phosphorus concentration of less than 1 mg L^{-1}, which complies the requirements for discharge of total phosphorus (2 mg L^{-1}) from urban wastewater treatment plants established in Directive 98/15/EC. This study is unique in a way that 100 % reduction in phosphorus concentration has not previously been achieved in continuously operated pilot-scale RwP. As high $PO_4^{3-} - P$ RE was observed by Tuantet et al. (2014b) in their experiment using algal species *Chlorella sorokiniana* grown on diluted urine in a 1 L continuously operated photobioreactor with a light path of 10 mm under high artificial illumination. With their exceptionally high REs (both

$PO_4^{3-} - P$ and $NH_4^+ - N$), the experiments of Tuantet et al. (2014b) represent an important milestone in research of nutrient removal from urine by microalgae. However, since the experiments were performed in laboratory scale, the results are not fully comparable to those obtained from studies conducted in larger scale. The ultra-thin reactor and bright artificial light applied by Tuantet et al. (2014b) are likely not feasible beyond laboratory scale.

Availability of light was likely limiting algal growth and nutrient removal in the RwP experiment. In this study, the biomass concentration was rather high at the beginning of the continuous mode, which likely reduced the light transmittance of the culture broth. However, rapidly with the onset the continuous operation, the biomass concentration began to decrease (due to washout of algal cells) and therefore lost its significance in determining the depth of the euphotic zone. On the other hand, some dense flocs consisting of cells and debris were forming in the culture broth, and within these flocs, light transmittance was likely poor. The major problem, in any case, was the structure of the RwP, which allows it to be lit from only one direction, leaving the bottom in the shade. The shading can be compensated by sufficient agitation, which allows the cells to migrate from aphotic to euphotic zone (Barbosa et al. 2003). In addition, growth conditions should be optimized so that no flocs are formed.

The high pH values observed in the RwP during the first four weeks after the onset of the continuous mode indicated that the culture was depleted of CO_2. In August, two aquarium aerators were placed at the bottom of the RwP to increase the CO_2 assimilation. However, aeration did not improve algal growth or nutrient removal, which was probably due to weak CO_2 dissolution caused by too short contact time between the gas bubbles and water. Dissolution of CO_2 can be enhanced by equipping the RwP with a mixing column, which can be installed on existing RwPs (Putt et al. 2011).

5.1.1 Effect of influent's N:P ratio on nutrient removal

The continuous mode of the RwP experiment began with $NH_4^+ - N$ and $PO_4^{3-} - P$ concentrations being higher in the RwP than in the influent. Starting from day 0, both concentrations decreased in the RwP, but at different rates. The $NH_4^+ - N$ concentration decreased rapidly and was lower in the RwP than in the influent by day 7. In contrast, for $PO_4^{3-} - P$, it took nine weeks before the concentration in the RwP decreased below the concentration in the influent, and before the onset

of mixotrophic cultivation, the $PO_4^{3-} - P$ RE was only 22 %. Similarly, the $PO_4^{3-} - P$ RE was substantially lower than the $NH_4^+ - N$ RE in the laboratory-scale batch experiment.

Interestingly, the disparity in nitrogen and phosphorus uptake was reversed in the RwP experiment after the onset of mixotrophic cultivation, when $PO_4^{3-} - P$ was removed completely but the $NH_4^+ - N$ RE remained at 58 – 76 %. During mixotrophic cultivation, biomass growth and NH_4^+ uptake were possibly limited by PO_4^{3-} availability. Sometimes, however, phosphorus removal can be 100 % without the phosphorus being a limiting resource (Tuantet et al. 2014b). This can be explained by luxury uptake of phosphorus, which will be discussed further in section 5.3.

The disparity in $NH_4^+ - N$ and $PO_4^{3-} - P$ REs indicates that the influent's N:P ratio was not stoichiometric for the mixed culture under the conditions prevailing in the summer. An optimal N:P ratio is crucial for high biomass productivity and nutrient removal (Choi and Lee 2015), but it is known to vary with species and environmental conditions (Klausmeier et al. 2004, Solovchenko et al. 2016). In addition, many algae are able to adapt their nutrient uptake according to the availability of N and P (Beuckels et al. 2015). In this study, the molar ratio of N and P in the influent varied between 30:1 and 37:1, which falls within the range of structural N:P ratios observed in marine and freshwater algae (Klausmeier et al. 2004), but the algal-bacterial use of nitrogen and phosphorus varied with environmental conditions. Comparing of these results to the literature is, however, complicated by the fact that the culture used in this study was a mix of *S. acuminatus* and unknown indigenous microorganisms.

5.1.2 Effect of the ambient temperature on nutrient removal

During the RwP experiment, the highest $NH_4^+ - N$ RE was observed on day 32 (July 29th) at the end of approximately a week-long period when daytime temperatures recorded at Sillinkari observation station were above 25 °C. This was reflected in the RwP where the highest temperature of the growth season was observed four days before the $NH_4^+ - N$ RE was at its highest. It is possible that warm temperature combined with abundant solar irradiance enhanced the ammonium uptake of the algae. Studies have shown that biomass growth and nutrient uptake of many temperate microalgal species are at their highest at temperatures above 20 °C (Ras et al. 2013, Li et al. 2011). However, favourable temperature did not seem to increase biomass production or phosphate removal in the RwP of this study. On the contrary, warm temperature combined with relatively high culture pH created favourable conditions for ammonia volatilization

(Hansen et al. 1998), and thus up to one tenth of $NH_4^+ - N$ removal was caused by deprotonation and subsequent volatilization of ammonium. After day 32, the $NH_4^+ - N$ RE decreased in parallel with the decline in the culture pH.

Temperature also affected the results of the laboratory-scale batch experiment aiming to optimize the TE mixture composition. Midway through the set-up II, the laboratory temperature rised above normal room temperature (22 °C) and the batch flasks placed under the fluorescent lamps were exposed to higher temperatures compared to other set-ups. The partial cessation of biomass production observed on days 5 – 9 was likely caused by photorespiration, a metabolic process where the photosynthetic enzyme RuBisCO misidentifies O_2 as CO_2 and therefore acts as an oxygenase instead of carboxylase (Hemme et al. 2014, Kliphuis et al. 2011). During days 5 – 9 of set-up II, the conditions were favouring photorespiration, because the O_2/CO_2 ratio was high due to preceding photosynthesis, and the specificity of RuBisCO for CO_2 was decreased due to elevated temperatures. Photorespiration reduces biomass productivity, but it is a benign phenomenon because it decreases instantly as soon as conditions become more favourable for photosynthesis. Another mechanism by which high temperatures may have inhibited growth is ammonia toxicity, but because elevated ammonia levels can directly damage PSII, the recovery from toxic levels of ammonia is slow (Collos and Harrison 2014). Interestingly, nutrient removal per produced biomass was higher in set-up II compared to other set-ups, which indicates that ammonia volatilization along with struvite precipitation were involved in nutrient removal during the period of low biomass production. In fact, these processes have most likely occurred in the background throughout the experimental series.

5.1.3 Effect of the culture pH on nutrient removal

During the RwP experiment, a drastic decrease in the culture pH was observed on the same day the $NH_4^+ - N$ concentration in the RwP was at its lowest. A similar phenomenon of a sudden fall in the culture pH was observed by Saarnio (2019) in an experiment using the similar mixed culture of *S. acuminatus* and indigenous microorganisms grown on 1:15 diluted urine in a continuously operated open RwP. At that time, the culture pH suddenly plummeted from 8 to 6 on day 60 of the experiment. Steep decreases in pH values have been observed in batch cultivations of algal species *Chlorella vulgaris* on NH_4^+ (Hulatt et al. 2012) and diluted urine (Jaatinen et al. 2016). In general,

sudden decreases (and increases) in the culture pH should be avoided as they may lead to the collapse of the cultivation (Hulatt et al. 2012).

The decrease in the culture pH can be caused by assimilation of NH_4^+ into algal cells (Brewer and Goldman 1976, Hulatt et al. 2012, Lachmann et al. 2019), but in this study and the study conducted by Saarnio (2019), the drastic decreases in the culture pHs were not associated with the level of NH_4^+ uptake. However, concurrently with the decrease in the culture pH, an unidentified organic acid began to accumulate in the RwP along with the unknown nitrogenous metabolite(s). Microalgae are known to produce and excrete extracellular metabolites, which commonly include various nitrogenous and/or anionic compounds such as Aminolevulinic and uronic acids (Liu et al. 2016). However, since the identification of the unknown compounds was not in the scope of this study, their source and possible role in the drastic fall in the culture pH remained unresolved.

5.1.4 Effects of other biological processes on nutrient removal

During the RwP experiment, the $NO_3^- - N$ concentration in the RwP started to increase on day 32. On days 35 – 70, up to approximately one tenth of $NH_4^+ - N$ removal was explained by nitrification. Nitrification is known to occur in co-cultures of algae and bacteria (González-Fernández et al. 2011, Luo et al. 2019, Posadas et al. 2013, Su et al. 2012) but it is sometimes considered undesirable in processes designed to recover nitrogen into algal biomass (Viruela et al. 2016). However, several studies have demonstrated that both processes can occur simultaneously (González-Fernández et al. 2011, Karya et al. 2013, Posadas et al. 2013, Su et al. 2012). Algae can utilize NO_3^- as a secondary nitrogen source when all NH_4^+ has been used (Karya et al. 2013), and partial conversion of NH_4^+ to NO_3^- could reduce toxic effect of ammonia as well as loss of nitrogen through ammonia volatilization in high culture pH. Moreover, it has been demonstrated that in certain conditions, denitrification may occur within the anoxic zones formed within the biomass flocks (González-Fernández et al. 2011). The ability to conduct nitrogen recovery and nitrification-denitrification processes in parallel could be useful in an environment where abiotic conditions are variable and can rapidly change in favor of either process. In this study, the partial shift in algal-bacterial consortium's metabolism from NH_4^+ uptake towards nitrification was likely triggered by some kind of change in the abiotic and/or biotic conditions in the RwP.

NO_3^- and NO_3^- formation were also monitored in the experimental series dedicated to optimizing the TE mixture composition, but concentrations remained below the detection limit in all

treatments. This indicates that the occurrence of nitrification in the cultures was negligible because environmental conditions favoured algal growth and nutrient uptake over nitrification.

5.2 Effect of trace element addition on biomass growth and nutrient removal

A research by Adamsson's (2000) showed that biomass production of *S. acuminatus* grown in diluted human urine was increased by supplementation with iron and magnesium, and thus the first goal of this study was to test whether iron and magnesium alone were sufficient for optimal growth and nutrient removal. Based on the results of this study, biomass growth and nutrient removal rates were significantly lower in cultures supplemented with iron and magnesium compared to cultures supplemented with the full set of TEs, which indicates that the optimal TE mixture should contain some other TE(s) in addition to iron and magnesium. As a result, the importance of boron, zinc, copper, and manganese supplementation was also investigated.

Supplementation of boron, zinc or copper had no statistically significant effect on algal growth and nutrient removal. In contrast, omission of manganese resulted in weakened growth and nutrient removal, and manganese was thus the third vital TE to achieve optimal growth and nutrient uptake on hydrolysed human urine.

There are three possible explanations why supplementation of boron, zinc, and copper was not essential for optimal growth. The first explanation is obviously that *S. acuminatus* does not require these three TEs for growth, but this is not very likely as the need for zinc and copper in particular is well established among Viridiplantae (Burkhead et al. 2009, Kropat et al. 2011, Hänsch and Mendel 2009). The need for boron, on the other hand, is not that undisputed. For example, according to study by Kropat et al. (2011), supplementation of boron had no effect on biomass production of green alga *Chlamydomonas reinhardtii* grown on synthetic growth medium.

The second explanation for the redundancy of boron, zinc, and copper in the TE mixture is that these TEs are already available at sufficient concentrations in the hydrolysed human urine. Fresh human urine is known to contain boron, zinc, and copper in relatively high quantities (Heitland and Köster 2005, Moore et al. 2018, Nielsen 1997), but at least zinc and copper are known to precipitate during hydrolysis (Ronteltap et al. 2007a, Wohlsager et al. 2010). Since the concentrations of zinc and copper vary in fresh urine (Heitland and Köster 2005, Moore et al. 2018) and formation of the precipitates during hydrolysis depends on the storage conditions (Ronteltap et al. 2007a), it is impossible to predict the residual concentrations in the hydrolysed urine as they

were not measured. It is possible that the concentrations were sufficient to support optimal algal growth.

The third explanation is a hybrid of the two above mentioned explanations. To some extent, the uptake of TEs is governed by various environmental factors such as the availability of other essential resources (Merchant et al. 2006, Procházková et al. 2014), metabolic mode (Procházková et al. 2014), and the level of abiotic stress (Cakmak 2000, Merchant et al. 2006), the need for supplementation of boron, zinc, and/or copper might be low in certain conditions. Furthermore, as copper and iron have similar catalytic potentials, the deficiency of copper may be compensated for by an excess of iron (Burkhead et al. 2009, Merchant et al. 2006). Thus, it is possible that the growth conditions in concert with the TE concentrations in the batch of the hydrolysed urine used in this study led to the redundancy of boron, zinc, and copper in the TE mixture. Whether this is applicable to all cultivations of *S. acuminatus* in diluted urine remains to be confirmed.

It should be noted, however, that the mixed culture used in this study did not only contain *S. acuminatus* but a diverse set of microorganisms with varying TE requirements. Moreover, because the species ratios in co-cultivations may change with time (Thomas et al. 2019), and these changes are likely associated with changes in the environmental conditions (Cole 1982, Interlander and Kilham 2001), the TE requirements for the mixed culture used in this study is fully applicable only under similar growth conditions.

In the RwP experiment, the culture was supplemented with all six TEs used in the study by Tuantet et al. (2014a). However, the results were not as promising as in the laboratory-scale batch experiment. $NH_4^+ - N$ and $PO_4^{3-} - P$ REs obtained before the onset of mixotrophic cultivation were similar to those obtained by Saarnio (2019) in an experiment performed in similar conditions without the continuous TE supplementation. It may be that other environmental factors were limiting algal growth, and thus they were not able to fully exploit the TE supplementation, but it is also possible that the TEs were used or precipitated before they were fed to the RwP. The colour of the first batch of the TE mixture had turned light brownish after three days of preparation, suggesting that some kind of colloidal precipitate formed in the mixture. It is known that ferric chelates are susceptible to photodegradation, and in certain circumstances they readily form insoluble hydrous ferric oxides, which can subsequently absorb other TEs (Procházková et al. 2014). Hereafter, the TE mixture tank was covered with a tarpaulin to protect the mixture from sunlight. Furthermore, a precipitate made of dark deposit and unidentified microorganisms formed

inside the influent tube feeding the mix of diluted urine and the TE mixture into the RwP. It is possible that the microbes in the urine already utilized the TE mixture within the shared influent tube. It is also possible that some organic substances in the urine were complexing with the TEs, thus hampering TE bioavailability. The two influents were separated into individual tubes to prevent precipitation and microbial exploitation of TEs before they reached the RwP. Although the colour of the TE mixture remained clear and the tubes were not gathering up any debris for the rest of the summer, it cannot be ruled out that TEs were still exploited and precipitating to some extent.

5.3 Effect of organic carbon addition on biomass growth and nutrient removal

In the autumn, the light availability starts to decrease, which results in reduced photosynthesis, biomass production, and nutrient uptake. Addition of organic carbon, such as glucose, acetate, and glycerol, has been shown to increase biomass production and nutrient uptake in algal cultures (Gao et al. 2019, Kim et al. 2013, Ma et al. 2016, Zheng et al. 2018, 2019) but mixotrophic growth mode has not been previously tested in pilot-scale cultivations.

In the RwP experiment, glucose was added to the influent during the last 20 days in order to support mixotrophic growth of the microalgae. As a result, $PO_4^{3-} - P$ RE increased to 100 %, a level not previously observed in pilot-scale experiments. A small increase in biomass concentration was also observable right after the start of mixotrophic growth mode, but it leveled of very soon, probably because night temperatures decreased below 10 °C. DOC was quickly removed and it was probably used by heterotrophic microorganisms in addition to algae. Interestingly, the DOC concentration in the RwP remained relatively constant throughout the summer and was not affected by the change in the influent DOC concentration.

On day 77, the color of the cultivation broth turned yellowish. Microscopic images revealed that the culture contained high numbers of algal cells but the color change in the cultivation broth was due to the loss of green color of the algae, which indicates that the chlorophyll was degraded and/or its production was ceased because it was unnecessary under conditions favoring mixotrophic growth (Kuai et al. 2018). A drastic decrease in chlorophyll concentration in cultures where heterotrophic or mixotrophic growth was promoted by adding glucose was observed by Caporgno et al. (2019) and Chavoshi et al. (2019), and Caporgno et al. (2019) also reported a reduced greenish coloration in heterotrophic and mixotrophic cultivations.

Cessation of photosynthesis may partly explain why NH_4^+ uptake was not increased with mixotrophic growth – the rate of protein synthesis had decreased as there was no longer need to produce the heavy machinery for photosynthesis. In addition, because mixotrophic cultivation did not significantly increase the biomass production, the nitrogen requirements of microalgae and other microorganisms remained at approximately the same level as before. On the other hand, the drastic increase in the $PO_4^{3-} - P$ RE is a bit harder to explain, because it is not in line with the biomass production or $NH_4^+ - N$ RE. Increased light intensities and temperatures are known to promote luxury uptake of PO_4^{3-} (Solovchenko et al. 2016, Tuantet et al. 2014b), but during the mixotrophic cultivation in late September, light intensities and temperatures were low. Luxury uptake of PO_4^{3-} is, however, a common trait in microalgae, and it has been thought to have evolved as an adaptation to uneven availability of phosphorus in many aquatic environments (Solovchenko et al. 2016). It is possible that the mixotrophic cultivation in the Nordic autumn provided another mechanism for luxury uptake of phosphorus.

In this study, glucose was used as the source of organic carbon. Glucose was chosen because of the feasibility of its use. The most sustainable solution would, however, involve the use of various industrial sidestreams as sources of organic carbon. This could be achieved by adding e.g. waste glycerol to the growth medium (Ma et al. 2016), or by combining two different wastewater streams, one with a high proportion of organic carbon and the other with high nutrient concentrations (Zheng et al. 2018).

6. CONCLUSIONS

The results showed that PO_4^{3-} removal can be enhanced in light-limited conditions prevailing in the Nordic autumn by supplementing the cultivation with an organic carbon source. In cultivation conditions promoting mixotrophic growth, the $PO_4^{3-} - P$ RE of 100 % was achieved, which is among the highest $PO_4^{3-} - P$ REs reported for pilot-scale cultivations to date. In laboratory-scale batch cultivations, the biomass growth and nutrient removal was enhanced by supplementing the cultivation with a trace element mixture containing Mg, Fe, and Mn. The addition of Zn, Cu, or B, on the other hand, had no growth-enhancing effect.

The future studies should focus on process optimization to achieve maximal growth and nutrient uptake of microalgae. The mass balance of nitrogen and phosphorus along with cultivation conditions should be optimized to achieve complete removal of both $NH_4^+ - N$ and $PO_4^{3-} - P$, and the possibility of using industrial side streams, such as waste glycerol, as a source of organic carbon in mixotrophic cultivation should be explored.

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