DEGREE PROJECT

Phosphorus reduction in wastewater using microalgae with different phosphorus starvation periods

Fredrika Murby

Natural Resources Engineering, master's
2021

Luleå University of Technology
Department of Civil, Environmental and Natural Resources Engineering

LULEÅ UNIVERSITY OF TECHNOLOGY

Foreword

This report is the written documentation of my Master's thesis project at Luleå University of Technology (LTU). It is the final degree project in this education, adjourning the 5 year's master program Natural Resources Engineering with the specialisation Environment and Water. It was carried out in cooperation with the Water Research and Environmental Biotechnology Laboratory (WREBL) at Riga Technical University (RTU). The work responded to 30 hp, (equal to 30 ECTS-credits), which equates to 20 weeks of full-time studies.

The laboratory work, which was the main part of this thesis, was carried out in cooperation with- and supervised by PhD student Aigars Lavrinovics, to whom I cannot begin to express my thanks. Thank you for taking the time to teach me the laboratory work, for discussing the research topic and for letting me partake in your experiments. I would also like to express my deepest appreciation to Associate Senior Lecturer, Inga Herrmann, at LTU, my internal supervisor in this project. Thank you for your continual, spot-on feedback and support throughout this thesis.

I also wish to thank Professor Talis Juhna, for presenting me with this interesting research topic, welcoming me to RTU and encouraging me to do my project here. I am also grateful to the Latvian Council of Science, for funding the research project "Post-treatment of municipal wastewater using sequenced-batch photobioreactor technology", without which this thesis would not have materialized.

Finally, many thanks to friends, family, and colleagues (especially in the laboratory) for sharing your knowledge and support.

Riga, November 2020,

Fredrika Murby

Abstract

Anthropogenic induced nutrients in the Baltic Sea have led to 97% of it being eutrophic. Phosphorus is regarded the main regulating nutrient, and nearly 25% of the nutrients coming to the Baltic Sea originate from wastewater treatment plants. To reduce the nutrient concentrations in the effluents from treatment plants, tertiary treatment methods based on chemical dosing have been the principal answer. The chemicals create a sludge in addition to remediating the water, which needs disposal. Methods for remediating secondary wastewater with microalgae exist but are not common in conventional wastewater treatment. However, using microalgae could be beneficial, since they use inorganic carbon (from the atmosphere and wastewater) and inorganic nutrients, while producing biomass and oxygen. The biomass in turn has a potential to be used in production of bioenergy, food, and fertilizers.

This thesis investigated whether pre-phosphorus starvation of five different microalgae strains enhanced the removal rate of phosphorus from secondary wastewater. The aim was to determine the optimal starvation period of different algae strains and to achieve wastewater effluent concentrations below 0.1 mg/L at the shortest possible time. Algae were transferred to a phosphorus-free media for five, three, one and zero days before entering the wastewater in a batch reactor at a temperature of 27°C and a 16:8 hours light and dark regime. Phosphate and nitrate concentrations as well as biomass production were monitored during a period of ten days. The experiment was repeated three times using *Chlorella Vulgaris* and two times using *Tetradesmus Obliquus, Ankistrodesmus Falcatus, Botryococcus Braunii* and one time using *Desmodesmus Communis.* The secondary wastewater was obtained from a small wastewater treatment plant from the village Roja in Latvia. Prior to the experiments, it was filtered three times through filters with different pore sizes (the smallest pore size was 0.2 μm), and the average nitrate and phosphate concentrations were 21.3 ± 1.1 mg/L and 17.8 ± 0.56 mg/L, respectively. The nitrate to phosphate ratio was 1.8:1.

It was possible to remove the inorganic phosphorus to concentrations below 0.1 mg/L within ten days, although it did not happen in all the reactors. It was found that in most cases pre-phosphorus-starvation increased the removal rate of phosphorus. For two of the strains, *Chlorella Vulgaris* and *Ankistrodesmus Falcatus*, the three-day of pre-starvation period was optimal, while two to three days was optimal for *Tetradesmus Obliquus*, compared to other pre-starvation periods. For *Botryococcus Braunii* the one-day and the zero-days starved batches removed the phosphorus most efficiently. For *Chlorella Vulgaris* and *Ankistrodesmus falcatus* nearly a 100% of the phosphorus was removed within seven days after three days of pre-starvation. Without pre-starvation, these strains achieved the same result after ten days.

It was also found that the nitrogen was the limiting nutrient in the wastewater and that the different strains responded differently to the changes in environment brought on by the experiment. When using microalgae in wastewater treatment, the choice of strain greatly impacts the removal rate, as the likeliness for them to survive in a specific environment varies among strains. It was concluded that using microalgae as a wastewater treatment method could pose great benefits. However, more experiments with colder climate, non-pre-filtered wastewater, a less nutrient rich media, greater initial biomass concentrations and pilot tests are recommended. Another insight from this thesis was that the method for transferring algae between different media needs to be refined to reach the target concentration in a reactor (or other setup).

Sammanfattning

Människans utsläpp av näringsämnen till Östersjön har lett till att 97 % av den är övergödd. Fosfor anses vara ett av de viktigaste näringsämnena som reglerar övergödningen, och nästan 25 % av näringsämnena kommer från avloppsreningsverk. För att minska halterna av näringsämnena i Östersjön så har tertiär rening med kemisk dosering varit den vanliga lösningen. Kemikalierna skapar ett slam som behövs tas hand om på ett lämpligt sätt. Reningsmetoder för att behandla sekundärt avloppsvatten med hjälp av mikroalger existerar, men är inte vanliga i konventionella avloppsreningsverk. Att använda mikroalger som reningsmetod kan vara fördelaktigt då de slukar inorganiskt kol från både atmosfären och avloppsvattnet, samt äter inorganiska näringsämnen samtidigt som de producerar biomassa och syre. Biomassan kan sedan användas för att producera energi, mat och gödselmedel.

Detta examensarbete undersökte ifall fosforreduktionen i sekundärt avloppsvatten blev förstärkt då fem algarter som svultits på fosfor tillsattes för att rena det. Målet var att finna den optimala svältperioden hos de olika arterna, för att reducera den inorganiska fosforn i avloppsvattnet till koncentrationer lägre än 0.1 mg/L. Algerna planterades i ett fosforfritt medium i fem, tre, en och noll dagar innan de omplanterades i avloppsvattnet i en reaktor med temperaturen 27°C och ett 16 till 8 timmars ljus- och mörkerschema. Under de tio följande dagarna mättes regelbundet biomassaproduktionen, fosfat-, och nitrathalterna i vattnet. Experimentet upprepades tre gånger med arten *Chlorella Vulgaris* och två gånger med arterna *Tetradesmus Obliquus, Ankistrodesmus Falcatus, Botryococcus Braunii* och en gång med arten *Desmodesmus Communis*. Avloppsvattnet kom från ett litet avloppsreningsverk i byn Roja i Lettland. Det filtrerades tre gånger (med minsta porstorleken 0.2 μm) och medelvärde av nitrat- och fosfatkoncentrationen uppmättes till 21.3 ± 1.1 mg/L och 17.8 ± 0.56 mg/L. Förhållandet mellan nitratet och fosfatet i avloppsvattnet var 1.8:1.

Det var möjligt att nå en oorganisk fosforhalt lägre än 0.1 mg/L inom de tio dagarna som algerna renade vattnet, men det skedde inte i alla reaktorerna. I de flesta fallen ökade fosforsvälten algernas reduktionstakt av fosfor. För två av arterna, *Chlorella Vulgaris* och *Ankistrodesmus Falcatus*, var tre dagars försvältperiod optimal. För *Tetradesmus Obliquus* var en två till tre dagars försvältperiod optimal gentemot de andra försvältsperioderna. För *Botryococcus Braunii* hade en dag- och noll dagar samma effekt på reduktionstakten, dessa kulturer reducerade fosforn effektivast. *Chlorella Vulgaris* och *Ankistrodesmus Falcatus* som svultit i tre dagar reducerade närmare 100 % efter sju dagar i reaktorerna, gentemot de som svultit noll dagar, som nådde samma resultat efter tio dagar.

Det upptäcktes att kvävet var det begränsade näringsämnet i avloppsvattnet och att de olika arterna reagerade olika på de förändringarna i miljön som kom av experimentet. När mikroalger ska användas i reningsverk är det viktigt att tänka på vilken art som används, då deras trolighet att överleva i vissa miljöer varierar med arterna. Att använda mikroalger för tertiär rening är ett bra alternativ, men fler experiment med kallare klimat, filtrerat avloppsvatten, näringsfattigare tillväxtmedium, större initiala biomassakoncentrationer och pilotförsök rekommenderas. En annan insikt från detta arbete var att metoden för att beräkna och föra över alger mellan olika medium bör vidareutvecklas för att kunna nå den förbestämda biomassakoncentrationen i en reaktor eller annan uppsättning.

Table of contents

1 Introduction

1.1 Using microalgae in wastewater treatment to reduce eutrophication

Since the 1950s the eutrophication in the Baltic Sea has increased (Elmgren, 2001), and today about 97% of the Baltic Sea is eutrophic (Helcom, 2018a). There is strong evidence that anthropogenic induced nutrients are the dominant factor for this trend (Carstensen et al., 2014), especially phosphorus (P), which has long been regarded one of the main regulating nutrients of the eutrophication in the Baltic Sea (Helcom, 2018b). During the 1980s a peak in P load occurred, but has since decreased, mainly due to changes in the operation of wastewater treatment plants (WWTPs).

The main source of P coming into the Baltic Sea is the riverine load, which in the latest Helcom assessment (Baltic Sea environment proceedings No. 153, 2018), accounted for 94.8%. Riverine sources represent the P that enters the Baltic Sea through rivers and waterways, mainly transferring P from point-sources (principally WWTPs) which accounts for nearly a fourth of the total riverine P load.

Wastewater (WW) is usually treated physically, chemically, and biologically (Inc. Metcalf & Eddy, 2014). Nutrients are usually removed in the biological treatment process, as well as chemically precipitated in the tertiary treatment. Treating nutrient and organic rich WW with microalgae is already a proven method that offers an alternative and/or addition to the traditional biological and chemical treatments (Griffiths, 2013; Torres Bustillos, 2015). Microalgae capture inorganic and organic P and nitrogen (N) as well as gaseous CO_2, while producing biomass and oxygen (Torres Bustillos, 2015). These constituents occur naturally in tertiary wastewater and using microalgae for treatment, while simultaneously reducing atmospheric CO_2 and creating biomass, which can be used for other purposes, is both economically and sustainably adequate (Pires, 2017).

Reducing the P loads to the Baltic Sea could have positive impact on its eutrophication state, which concern all the surrounding countries. Increasing the P reduction in municipal wastewater treatment (WWT) is therefore of interest. This could not only improve the state of the Baltic Sea, but also help to increase recycling and reuse of P – a finite natural resource, which would in turn help to achieve a sustainable world. Therefore, this report focuses on tertiary treatment of WW by using microalgae, specifically on the removal of P from wastewater.

1.2 Aim

The aim of this thesis was to investigate microalgae's capacity to sequester P from secondary treated WW to reach ultra-low levels of P in the effluent (less than 0.1 mg/l), using laboratory experiments. More specifically, the aim was to determine the optimal P-starvation period and quantify its effect on the P uptake by five different algae strains. Further this thesis aimed at evaluating which of the strains were most suitable for further research, with the goal of implementing the algae in WWT.

1.3 Research questions

1. Is it possible to reach an inorganic P concentration of less than 0.1 mg/L of in wastewater by using microalgae?
2. How does pre-P-starvation affect the P uptake by the microalgae?
3. Is there an optimal starvation period for enhancing the P-reduction rate?
4. Which factors (e.g., light, temperature, and nutrient concentrations) are important to consider in a batch reactor, and analogously in a small-scale treatment plant, for productive P reduction?
5. How does the choice of microalgae strain impact the P reduction?
6. In what way do the experimental results indicate that implementing microalgae for tertiary treatment of WW in small-scale WWTPs could be meaningful?

1.4 Delimitations

This work is as stated focused on P removal and pre-P-starvations effect on microalgae. P appears in many different species, and the word P infers all of them. In this work, focus is on inorganic P in the form of phosphate (PO_4^{3-}) since P appears mainly in that species in secondary WW.

P removal by microalgae can be implemented in many different treatment steps of WW, however, this thesis only investigates using them in tertiary WW treatment. Further, using microalgae in small-scale treatment plants is evaluated, based on the findings in the literature and experiments.

The technology for separating the algae from the WW is not investigated in this thesis, however, it will affect the P removal unconditionally. The P will be removed from the WW and bound inside the algae. Thereby removing the algae from the WW is critical for removing the P from the WW.

2 Theoretical Background

The following chapters summarise a general background on the research topic, aiming to inform the reader on the present-day knowledge on P uptake in microalgae and microalgae's current role in wastewater treatment (WWT). It is necessary to have basic knowledge of the below described processes, to understand the experimental setup as well as the conclusions drawn from the data obtained in the experiments in this degree project. To facilitate connecting the research results to full-scale applications and to underline the necessity of this research subject, this chapter includes a short description of the state of the Baltic Sea regarding P and the role of WWT.

2.1 The sources and pathways of the phosphorus loads into the Baltic Sea

The Baltic Sea on the northern hemisphere is surrounded by nine countries and its drainage area is four times bigger than the surface area of the sea itself (Helcom, 2018a). It is one of the largest brackish waterbodies in the world, and it is home to both marine and freshwater species. The waterbody is shallow, nearly encircled by land and has low biodiversity, which makes it vulnerable to environmental pressures, such as eutrophication.

Although regional nutrients discharge from landbound anthropogenic sources to the Baltic sea have decreased between 2011–2016, the past and current inputs of nutrients still determine the state of the Baltic Sea (Helcom, 2018a). Eutrophication leads to consequences such as unclear waters, excess algal blooms, and low to non-existent oxygen levels at the sea bottoms due to degradation of excess primary producers (Helcom, 2018a).

The sources and pathways of the nutrients discharged into the Baltic Sea have been investigated and evaluated every fifth or sixth year since 1995, by the Helcom organisation (Helcom, 2018b). The latest evaluation regards year 2014, where the main sources of the P loading were riverine loads and direct-point sources, which accounted for 94.8 % and 5.2 % respectively. Riverine sources refer to P that enters the Baltic Sea through rivers and waterways, while direct sources entail that the loads enter the Baltic Sea directly, mainly from WWTPs. Before 2014 direct input has been a larger part of the influent loads, but has decreased since 1995, since it has been the subject of long-term focus. The evaluation period from 1995 to 2014 shows that the overall P load to the Baltic sea has decreased. The only subarea where a recessing trend was not found was in Latvia's total phosphorus (TP) release to the Baltic Sea, as well as the TP load to the Gulf of Riga (which both Latvia and Estonia discharge into). Both these loads have increased slightly since 1995. The TP load from Sweden has decreased since 1995, however, the reduction of the direct sources has not followed the same trend as for the other 8 countries surrounding the Baltic Sea but has increased and decreased every other evaluation period with 0-2 %-units.

Riverine loadings, being the main source, can be divided into four sub-sources, as seen in Figure 1 (Helcom, 2018b). The natural background accounts for about a third of the P load to the Baltic Sea, and the point-sources, mainly WWTPs, accounts for about 24 %. The diffuse sources consist of principally agriculture loads, while the transboundary sources refer to loads of P which are not connected to any specific source, as they originate in upstream countries.

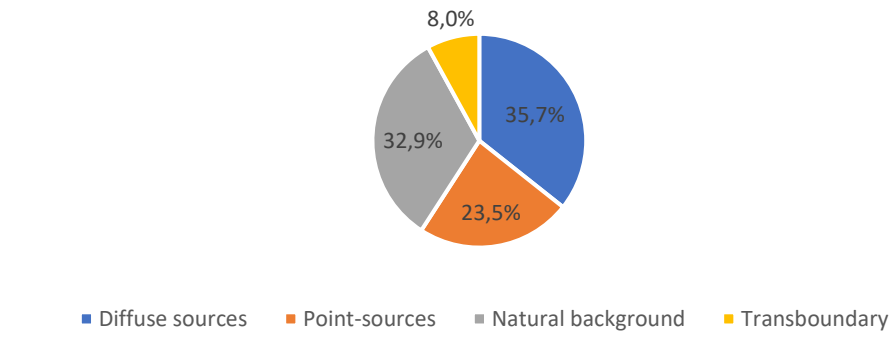


Figure 1: Riverine load of total phosphorus to the Baltic Sea in 2014. Source of data: Helcom, 2018b

2.2 General wastewater treatment process

The principal methods of wastewater treatment can be described by a unit of processes which are grouped together to provide primary, secondary, tertiary, and advanced treatment (Inc. Metcalf & Eddy, 2014). In general, the primary treatment consists of physical processes, removing components such as rags, toilet paper, sand, and other coarse particles. The secondary treatment refers to chemical and biological treatment, with the intention of removing readily biodegradable organics and suspended soils. Tertiary treatment refers to removing residual solids after secondary treatment. Also, disinfection and nutrient removal is often included in this step. The advanced treatment refers to the removal of dissolved solids after the biological treatment, where the processes depend on the purpose of the reuse of the water after the treatment. In Figure 2 below the treatment train of these processes are schematically described.

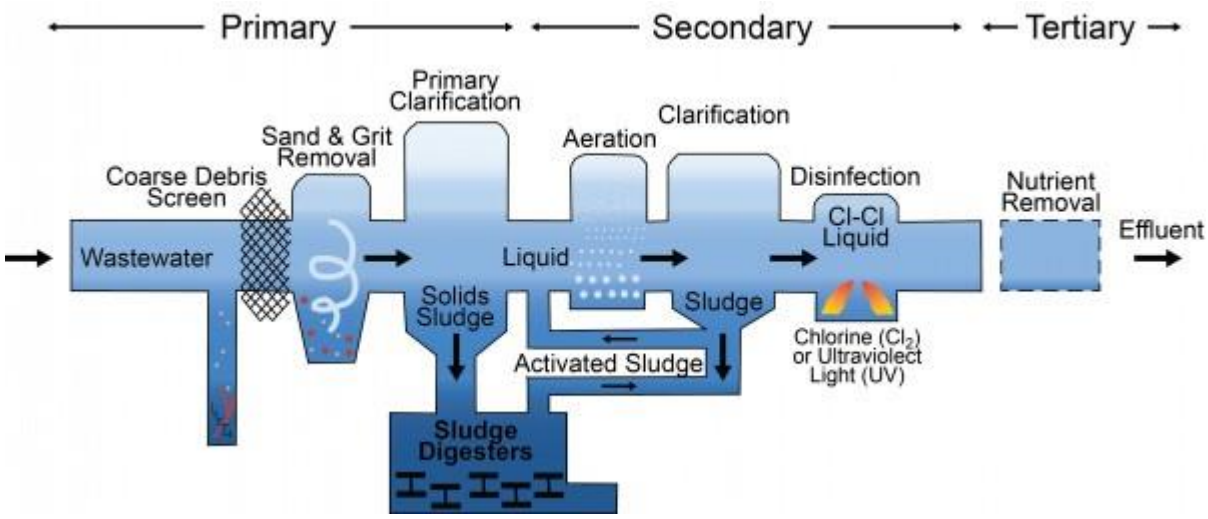


Figure 2: Wastewater treatment process [Factsheet]. From Center for Sustainable Systems, University of Michigan. (2020).

2.3 Microalgae

Microalgae are microscopic, mostly unicellular, photosynthetic organisms that reside naturally in the worlds' aquatic systems (Griffiths, 2013), and are also found on the surface of all type of soils (Tomaselli, 2004). They are primary producers of oxygen and they use organic and inorganic carbon, as well as inorganic N and P (nutrients) for their growth, and they have high growth rates (Pires, 2017; Torres Bustillos, 2015). This results in a reduction of the concentration of these substances, in the media they are residing in (Mohsenpour et al., 2020). They could be regarded the real lungs of the earth, as opposed to the forests, since they produce about half of the world's O_2 through photosynthesis (Williams, 2013). However, too many microalgae in a waterbody, which can be the product of an excess nutrient rich environment and warmer temperatures, can cause eutrophication. That is a problem with many negative environmental consequences, like oxygen free sea and lake beds as well as toxic algae blooms (Helcom, 2018a).

Phytoplankton, a type of microalgae, is the main primary producer in most marine and freshwater bodies (Griffiths, 2013). They are the foundation for numerous food chains, upon which most of the worlds' fishery industries rely and are widely used in other industries, such as production of pigments, lipids, foods, and renewable energy (Griffits, 2013; Torres Bustillos, 2015). The fact that microalgae are photosynthetic organisms means that light drives their metabolism. (Whitton et al., 2015). For microalgae to grow, not only the nutrients P and N are essential, but also other trace elements (Li et al., 2011). External critical factors, in addition to the nutrients, influencing the photosynthesis process and analogously the biomass production, are the light abundance and temperature in the growth environment. In secondary treated wastewater, inorganic carbon in not considered a limiting constituent.

2.4 Microalgae in wastewater treatment

Already in the 1950s it was shown that algae could be used in the secondary wastewater treatment, in the biological step (Oswald & Gotaas, 1957). The experiments with microalgae and bacteria at that time were carried out in oxidation ponds. Oxidation ponds are ponds open to the air, filled with WW which has been primarily treated. In these ponds, algae used nutrients from the WW, carbon from the atmosphere and the WW, and natural solar light for their growth. The growth process generated oxygen and oxidised ammonium (Griffiths, 2013). The oxygen generated by the algae was used by bacteria for degrading organic matter to CO_2, inorganic nutrients, and water (Oswald & Gotaas, 1957). These constituents were in turn used by the algae for growing. The circle was complete.

Since then, systems containing only algae, as well as algae and bacteria have evolved (Griffiths, 2013), like raceways and large-scale pond systems. The process of separating the algae from the water through flocculation and centrifugation are costly methods, which has led to alternative methods emerging. These methods mainly include immobilizing the algae in the water by fixing them to artificial substrates, like for example periphyton biofilms. The success of immobilization techniques has stimulated the development of numerous different treatment systems. However, another problem arises when growing large cultures of algae in reactors. When an algae culture becomes dense, less light gets through the water to the cells, leading to lower production of biomass. When the biomass stops growing, the nutrients stay in the media. Many algae are both phototrophic and heterotrophic, meaning that they can use both light and/or complex carbon

molecules (without light) to grow. This property is what many researchers try to use nowadays, to solve the interruption of nutrient reduction due to cultures becoming too dense (Murwanashyaka et al., 2020; Nzayisenga et al., 2018; Guldhe et al., 2017). Using mixotrophic algal cultures could also be handy when using microalgae in cold climate regions with seasonally little sun in case the plan is to use natural light.

Even though microalgae have a proven role as a bioremediation treatment method, they are not used frequently in conventional WWTP (Griffiths, 2013), although using them more widely could provide real benefits (Whitton et al., 2015), especially at small, rural and/or onsite WWTPs. The produced biomass from microalgal treatment of WW could be used for nutrient recovery and/or energy production. Microalgae could be used in WWTPs to induce a more circular economy regarding resource utilization in the future. As demands on lower effluents of P and N has increased, chemical-dosing-based technology has been the response. In small rural plants, implementing chemical dosing may also require better infrastructure of - and around the plant (like e.g., roads), potable water safety showers and chemical storage facilities. Treatment with microalgae could offer an alternative method to the chemical dosing technology and thereby decrease the surrounding necessities associated with the usual response to meet the demand of lower nutrients in the effluents. Microalgae could remediate the effluent from nutrients and simultaneously create a valuable product, biomass.

Microalgae are generally regarded most suited to work in the tertiary treatments step of WWTPs, where the residues in the water, desired to remove, mainly include inorganic nutrients such as ammonium (NH_4), nitrate (NO_3^-) and phosphate (PO_4^{3-}) (Griffiths, 2013). One reason for this is that many microalgae are sensitive to the most common pollutants, such as heavy metals, pesticides, oils, solvents, and other organic and inorganic chemicals, which are often encountered in industrial wastewater. Even though some strains can sequester these pollutants to varying extents, another problem withstands, the disposal of the contaminated biomass. Therefore, it is more common to use microalgae as remediation method of secondary and tertiary WW as well as nutrient rich effluents from farming systems. The biomass produced in these media is a valuable resource and could be further used as a product, rather than being disposed as a waste.

Microalgae for WWT is a topic currently being reinvestigated (Whitton et al., 2015). Many reports, from different fields of science, claim that now is the time to make a shift from a fossil fuel dominated world to green energy and living (Rockström, 2020), all technologies which decrease greenhouse-gases emissions are of interest. In an article by Lorenza Ferro et al. (2018) native Nordic microalgae were tested for their ability to grow in municipal WW in moderate winter conditions (6 h of light and 15°C) and at cold climate (5°C and constant light). It was found that the native Nordic strains could efficiently remove nutrients at both cold and dark stress. The degree of purification of nutrients reached 100 %. The article suggests that native species should be used when growing algae in Nordic climate, as this can be a solution to the common limits when growing algae in cold climate with seasonal light and temperature fluctuations. Among the algae tested it was found that the response to the stress differed greatly with the different strains. *Chlorella Vulgaris* and *Scenedesmus sp* were two of the four strains tested in these conditions, which performed very well. The WW used in these experiments had average total N content of 61.6 mg/L and average total phosphate content of 2.23 mg/L and pH 7.61. This WW came from the local WWTP, and it was filtered through a 2.5 μm cellulose filter and autoclaved for 5 minutes in 121°C. The experiments were continued until the algae had reached stationary phase, which was on average 40-45 days in the moderate winter conditions and 20-30 days at the cold stress conditions (Ferro et al., 2018).

2.5 The phosphorus removal process

2.5.1 Indirect and direct uptake

According to a review on nutrient removal in municipal wastewater by microalgae, the nutrients in the wastewater can be removed by either indirect or direct uptake (Whitton et al. 2015). The direct uptake of nutrients by microalgae refers to the processes of nutrients uptake through interconnected biochemical pathways into the biomass. This process depends on the microalgae and is analogous to biomass production. In the algae cells the nutrients are either stored in polyphosphate granules (for P) or assimilated into nucleic acids and proteins. The proteins and nucleic acids are used in the biomass growth. When algal biomass is growing in wastewater, the pH naturally rises due to inorganic carbon (HCO_3^- and CO_2) (Grobbelaar, 2004) and hydrogen (Whitton et al., 2015) being assimilated in the biomass. Inside the cell the hydrogen facilitates the chemical transformation of P and N into the necessary specification for biomass growth. The forms of P and N that can translocate across the cell membrane into the algae are NH_4^+, inorganic N, and organic N (for N), and HPO_4^{3-}/$H_2HPO_4^{3-}$ and organic P (for P). They are assimilated in the preferred order as written since this order costs the algae the least energy. Inside the cell, the algae use NH_4^+ and HPO_4^{3-} to create new biomass, or in the case for P, sometimes for storing it in the polyphosphate granules. This means that if there is only inorganic N ($NO_3^{2-}$and NO_2^-), organic N or organic P available, they will be transformed inside the cells to facilitate biomass growth or storage. Tertiary wastewater usually contains mainly PO_4^{3-}, $NO_3^{2-}$and NH_4^+ (Griffiths, 2013), therefore several hydrogen ions will be consumed when the biomass grows, resulting in reduction of H^+-ions in the localized environment, which contributes to an elevation of the pH therein (Whitton et al., 2015).

The indirect uptake refers to precipitation of P, which happens naturally and mainly due to the elevation of pH in the water, caused by the direct uptake processes. The foremost reason for the pH elevation is the carbon assimilation into the biomass (Larsdotter et al., 2007). The bicarbonate-carbonate system, which determines the pH in most aquatic systems, provides the CO_2 for the photosynthetic fixation, which results in an accumulation of OH^- in the growth solution (Grobbelaar, 2004). The elevation of the pH facilitates that P precipitates with cations that are present in the wastewater (Whitton et al., 2015). However, for the co-precipitation of P to happen, there must be a high content of dissolved oxygen present in the media as well. The cations which the P precipitates with can for example be iron, magnesium and calcium (Larsdotter, et al., 2007). Calcium is regarded the most important one, as it is usually present in sufficient amounts in most hard waters. P can also precipitate with calcium in WW at pH ranges common for WW. However, for this to happen, the WW must contain concentrations of at least 50 mg/L of P, and a 100 mg/L of calcium, which are not likely conditions in secondary treated WW.

2.5.2 The nitrogen to phosphorus ratio

It is found that algal biomass consists of mainly carbon, N and P, with these constituents responding to about 50 %, 1–10 % and less than 1 % of the biomass respectively (Grobbelaar, 2004). The variations of the ratios depend on the species and the nutrients available, but variations can also occur within an axenic culture. P is often the limiting nutrient in algal biotechnology, since it easily binds to other ions and precipitates, making it unavailable for the

algae. In 1958, the optimal N to P ratio (NPR) in marine phytoplankton was established to be 16:1, 16 mol of N to 1 mol of P (Redfield, 1958). This is a well-known and acknowledged ratio when discussing nutrients in aquatic systems (Hecky et al., 1993; Arbib, 2013). However, since then, many studies have found that the NPR is rather specific to the strain of microalgae (Rhee et al., 1980, Whitton et al., 2016) and may change depending on the conditions of the environment, advocating for microalgae's ability to adapt to the surrounding conditions (Arbib, 2013). It has been found that the NPR in biomass from freshwater microalgae ranges between 8:1 and 45:1 (Hecky et al., 1993). Although generally, the NPR is substantially higher in freshwater biomass than the established Redfield ratio, 16:1.

In a study on the growth kinetics of the algae *Scenedesmus obliquus* with varying NPR, it was found that for total nutrient removal and maximum biomass production, NPR ranging between 9–13 (9:1 and 13:1) was optimal (Arbib et al., 2013). This study also showed that varying NPRs had great effect on the total nutrient removal. For example, NPR of 1:1 generated 89 % and 16 % of N and P removal, respectively, while a NPR of 35:1, generated 42 % and 100 % removal of the N and P, respectively. The authors concluded that for *Scenedesmus obliquus*, N could be considered the limiting nutrient in wastewater, when the NPR is below 13. Another study on the NPR's effect on nutrient removal in municipal WW concluded that the optimum NPR for removing P varied greatly between 5:1 and 30:1, depending on the ecological conditions in the wastewater (Choi & Lee, 2015). The TP removal depends on the NPR, but factors such as the light intensity, the P concentration, the pH and the temperature had big impact as well. It was also found that the P uptake was inversely related to the internal P concentration of the cell. Algae with less P inside were inclined to take up more P. Therefore, the internal P concentration can also be considered a factor controlling the P uptake kinetics. It has also been concluded that an oversupply of N, P or carbon can cause stress within an algae culture, resulting in reduced growth rate (Grobbelaar, 2004).

2.5.3 Biomass growth conditions and limitations

Optimal biomass growth is analogous to optimal nutrient removal, since the nutrients are incorporated into the biomass (Grobbelaar, 2004). In an algal cell the inputs are nutrients (where P and N are the most important nutrients, but S, K, Na, Fe, Mg and Ca are necessary as well), trace elements (B, Cu, Mn, Zn, Mo, Co, V and Se) and carbon (in the form of CO_2 and HCO_3^-). The outputs are O_2 and algal biomass. The parameters in the environment determining the rate of the photosynthesis are the solar (or artificial) light (measured as irradiance per cell), the pH, the salinity (for marine algal species) and the temperature. These parameters and inputs will promote and/or limit the growth process. Some parameters have indirect influence on the light irradiance per cell, such mixing and vessel compartment size, and they are usually found in unnatural systems (Masojidek et al., 2004). They are therefore important to consider when growing algae in artificial systems like bioremediation facilities and laboratories. The design of the growth vessel will limit how much a culture can grow, as after a culture has become highly concentrated, the light per organism will decrease, as the cells shade each other. Similarly, for the mixing, if the algae grow in a reactor without mixing, the cells might settle and shade parts of the culture. Depending on which systems the biomass growth occurs in, different parameters might become the limiting factor.

Microalgae can grow in a broad temperature range, but they generally perform best in 20–25 °C according to Lorenza Ferro (2016). Other factors such as properties of the species, and the local

environment will also affect the temperature range the algae can reside in. The key parameters required for algae growth are summarized in Table 1.

Table 1: A generalized set of conditions for culturing micro-algae (modified from Anonymous, 1991). Modified from Food and Agriculture Organization of the United Nations (FAO) (1996)

Parameters	Range	Optimum
Temperature (°C)	16–27	18–24
Salinity (g/l)	12–40	20–24
Light intensity (lux)	1.000–10.000 (depends on volume and density)	2.500–5.000
Photoperiod (light: dark, hours)	16:8 (minimum) 24:0 (maximum)	
pH	7–9	8.2–8.7

An algae culture's growth curve usually follows the same pattern as displayed in Figure 3 (FAO, 1996). When introducing a group of algae cells into a new environment, it takes some time for the organisms to adjust. That time and stage is called the lag phase during which the culture does not grow. Once the algae have settled, the cells start to divide, and the culture grows exponentially. Eventually, one or more of the required growth factors (such as nutrients, carbon, light, etc.) limit the exponential increase in cells and the growth rate becomes stationary. At this point the growth rate is in balance with the limiting factor in the local environment (Figure 3).

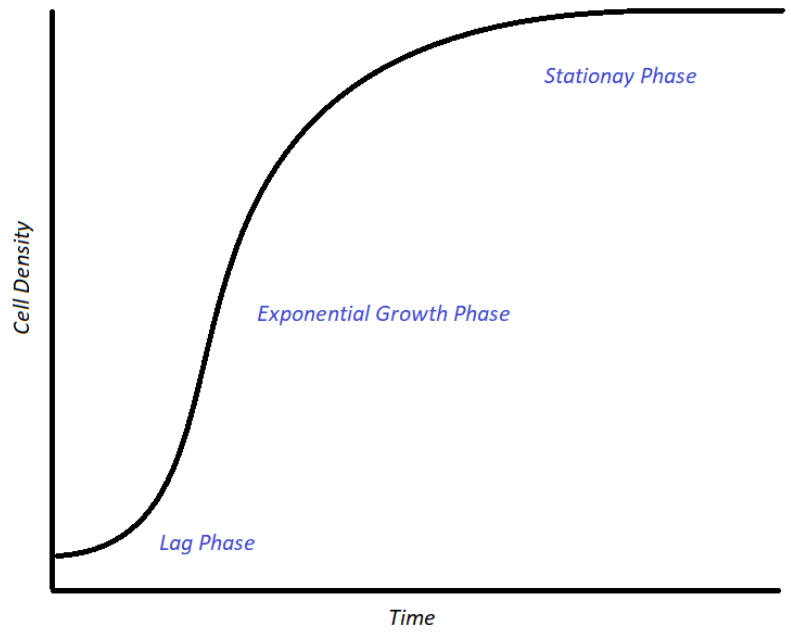


Figure 3: Typical algae growth curve Source of data: FAO, 1996.

2.5.4 Excess uptake of phosphorus

It has been found that microalgae can store P in polyphosphate granules for future use when the P might be limiting for growth in the local environment (Whitton et al., 2015). This happens naturally in lakes, where P often occurs in low concentrations (Brown & Shilton, 2014). If the P is limiting or non-existent, the microalgae can feed from its internal reserves to stay alive and reproduce. This means that the algae take up more P than necessary for survival. This excess uptake is divided into two processes, where the initiation mechanisms for the excess uptake differ. The first process is called over-compensation and it occurs when algae have been starved of P for a period of time and then is re-exposed to P, which results in an excess uptake of P (Brown & Shilton; 2015, Eixler, et al., 2006). However, it has been concluded that the influence of P-starvation on subsequent P-uptake is not consistent, it varies with different conditions set in the environment (such as the PO_4^{3-} concentration in the media and the length of pre-starvation period). The second excess uptake process is called luxury P uptake and is not a consequence of pre-P-starvation of the algae, but of algae being exposed to an excess P rich environment. Again, the algae store more P than it needs for mere survival, as polyphosphate granules (Crimp, et al., 2018; Grobbelaar, 2004).

3 Methods

In this chapter the laboratory work is outlined, including experiments performed, methods of analyses and the general laboratory work leading up to performing experiments with microalgae. Standards and recipes which have been used are referred to and can be found in the appendices.

A literature study was conducted in parallel to the laboratory work to learn about the research subject. When searching for literature the Luleå university library's databases were used. Most of the literary findings can be found in chapter 2 Theoretical Background.

3.1 Experimental work overview

In the laboratory, focus was on P removal in WW by using microalgae in batch reactors. A main experiment took place in the end of a three-month period of laboratory work. Before that, endeavours leading up to the main experiment occurred, which included growing algae, testing different analysis methods and equipment. Two pre-experiments were conducted before the main experiment, to determine initial algal biomass concentration to use in the experiment, as well as to determine pre-starvation periods for the algae. These two pre-experiments were not performed in repetitions, so no solid conclusions could be drawn for them. However, their purpose was to indicate how to set some parameters in the main experiment before commencing it.

Below in Figure 4 the general laboratory work and experiments, in their moment in time, are displayed. The media which the algae were residing in are also listed in the figure as this may have effect on the outcome of the experiments. In the following chapters all experiments will be described in detail as well the different growth media used (EG, BG11, BG0 and WW).

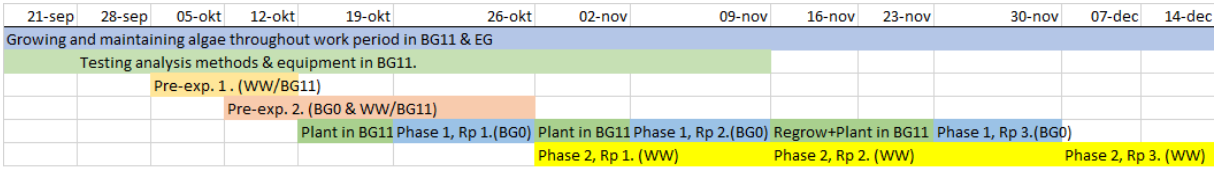


Figure 4: Laboratory work overview. Rp is short for replicate and pre-exp. for pree-experiment.

The main experiment, positioned on the bottom right corner of the figure, was performed in 3 replicates which could be divided into 2 phases. The starvation phase (phase 1) and the measurement phase (phase 2) when the algae had been replanted in wastewater. Leading up phase 1, the algae were replanted in BG11 about one week before phase 1 (and by that, two weeks before phase 2). As can be seen in the top-left part of the figure, growing and maintaining algae, as well as testing analyses, methods, and equipment, took place in parallel with the experiments.

3.2 Growing algae prior to wastewater experiments

3.2.1 Preparing growth media Euglena gracilis, the Blue-Green medium and BG0

Two different growth media were prepared and used while growing algae and testing the analysis methods. The media *Eugena gracilis* medium (EG) and the Blue-Green Medium (BG11) were used and their full recipes can be found in appendix 1, 2 and 3. The EG medium has more nutrients and less trace elements. The BG11 medium has the opposite characteristics, with a lower concentration of P and N, but more different constituents. The EG is prepared for use directly, while the BG11 is prepared by creating a stock solution of concentrate, which can be stored and diluted to the original concentration when it is needed.

The EG was used when planting a low mass of algae in growth a media, intending it to multiply in cells quickly. The EG was used in 250 ml bottles, which were kept throughout the experiments as a safety measure and used as constant source of algal biomass. The EG was prepared by weighing all constituents separately and adding them to a 1 litre flask. Then 990 ml of deionized water was added, as well as 10 ml of Calcium chloride solution. Then this medium was portioned out (usually 125 ml of EG per bottle) in smaller flasks and bacteria in the flasks and growth media were eliminated by placing all flasks inside an autoclave for 15 minutes at 121°C.

The BG11 was used in the 1 litre batch reactors prior to the real experiment, with the purpose to grow algae and testing the batch reactors construction. The BG11 media has more constituents which the algae need to grow. However, to start an algae culture in BG11, a lot of initial biomass needs be planted (minimum 0.05 g of dry weight per litre). If planting too few algae in the BG11 growth media, it is possible that bacteria will take over and out compete the algae.

The original stock solution (1–8) in the BG11 recipe in annex 2 was modified to have five times higher concentration than the original recipe, to reduce space requirements. Before using it however, it was diluted until the same concentration as the original recipes. The full measurements and calculations for each constituent can be found in annex 3.

A BG11 without any P was also used in the experiments. This growth media was given the name BG0 and was prepared exactly like the BG11, only without adding the K_2HPO_4 from the original BG11 recipe.

Before using the BG11 or the BG0, bacteria in the flasks were eliminated by placing all flasks inside a small an autoclave. For 15 minutes they were exposed to 121°C, which killed all living organisms.

3.2.2 Growing algae

Before starting to experiment with algae in secondary treated WW, enough algal biomass had to be produced from the indigenous samples, which was supplied by The Culture Collection of Algae and Protozoa (CCAP), an algae bank located in Scotland. The strains used in this thesis were: *Desmodesmus communis (D. communis), Tetradesmus obliquus (T. obliquus) Chlorella Vulgaris* (*C. vulgaris*) *Ankistrodesmus falcatus* (*A. falcatus*) and *Botryococcus braunii* (*B.*

braunii), see annex 5 for strain designation and origins. The indigenous algae had been started up and cultivated by PhD student Aigars Lavrinovics before this degree project was undertaken. When this degree project started, each algae strain was cultivated in (at minimum two) 250 ml PYREX® flasks and in a 1-litre PYREX® flask.

The 250 ml flasks were filled with 125 ml of EG solution and the 1 litre flasks were filled with BG11. The flasks were placed under an UV lamp in the laboratory with constant temperature of 25–28 degrees Celsius and a light regime of 16 h light and 8 h dark. These cultivations were kept throughout the experiment as a safety measure, as well as constant source of algal biomass. The flasks, just like the batch reactors, were regularly cleaned and refilled with new growth media to feed the algae. The flasks and batch reactors were cleaned by being disassembled, rinsed in the dishwasher, and autoclaved. Later the batch reactors were assembled in a laminar cabinet.

The algae in both growth media were replanted every 7–14 days, for different reasons; sometimes the algae consumed all nutrients, and other times bacteria overtook the environment. This could be observed by the colour of the solution and confirmed by different analyses. Other reasons for replanting of algae could be that the flasks got contaminated by other algae species. If a batch was contaminated it was thrown out and a new batch started. If the bacteria were outcompeting the algae in a batch, these algae were cleaned and replanted in new growth media. The replanting and cleaning processes are described below.

3.2.3 Replanting algae and cleaning off bacteria

When replanting algae, bacteria could be cleaned out at the same time. For replanting algae, the culture had to be separated from the growth media they were residing in. This was done by centrifuging the sample (with a Frontier 5000 Multi Pro Centrifuges, model FC5718R 230V manufactured by Ohaus), in 50 ml vials (Tube: 62.547.254 by Starstedt AG & Co.) which placed the heavier algae in the bottom of the vial, the bacteria layered above and the growth media on top. The centrifuging occurred during two minutes with 4000 rcf at 24 degrees Celsius. The algae were then cleaned with demineralized water and re-centrifuged until the bacteria had been washed off, and the water observed above the algae layer after centrifuging was clear. The bacteria could be washed off by carefully shaking the centrifuged sample until the bacteria dissolved with the water above. Some algae also mixed in and got discarded with the media/bacteria mix in this process. A vortex machine (Vortex-Genie, model G-560E, Manufactured by Scientific Industries, Inc.) was used in between the centrifuging to mix algae with added demineralised water. After centrifuging the biomass and discarding the above-lying supernatant, the residual biomass was mixed with a few ml of demineralised water, usually 10-15ml, to be able to move the biomass from the centrifuge vial to the new flask for replantation. Not only demineralised water could be used for the transfer but the media which was in the replantation bottle could be used as well, to eliminate dilution of the replantation media. This cleaning process did not eliminate all bacteria in the culture, but it reduced them enough so that the algae most of the time could outcompete them after being replanted.

The replanting did not only originate from existing strains in the 250 ml bottles but were also taken directly from the indigenous samples from Scotland, as well as scraped of agar plates which Aigars Lavrinovics had prepared before this master thesis project was started.

From the indigenous samples from the CCAP, 1 ml was pipetted into the 250 ml flasks filled with 125 ml of EG solution. When taking algae from the plates, the plates initially was examined

to avoid bacteria entering the EG solutions. The algae were scraped up with a sterile cart loop, from an area inside the plate which appeared bacteria free. About two full scrapes with the cart loops was enough to plant in the EG solution.

3.3 Wastewater sources

In the two pre-experiments, WW from the city Kuldiga in Latvia, was used. The WWTP in Kuldiga treats water from about 10 000 inhabitants, and the WW was collected from the secondary clarifier, at two different points in time. The PO_4^{3-} and NO_3^- concentrations were circa 17–18 and close to 0 mg/L respectively first time of collection. The second time of collection, the WW from the plant contained 0 mg/L of PO_4^{3-} and it was assumed that the NO_3^- concentration was still close to zero. To balance up the lack of PO_4^{3-} and NO_3^-, BG11 was added to the WW. Dimensions used are described in detail in the two pre-experiments below.

In the main experiment WW from Roja WWTP was used. The WW came from two small villages, Roja and Rude, and from a local fish processing factory. The villages had a total population of about 3 000 people. The fish processing factory operated seasonally and was responsible for greater variation of the nutrient content in the effluent. The water for the main experiment was taken from the secondary clarifier, after primary settling, activated sludge process and secondary settling. The wastewater used in the first replicate set the concentration of P and N which were used in the following replicates. In the second replicate the P and N occurred in more potent concentrations, therefore the WW used in the second replicate was diluted with demineralised water to obtain the same characteristics as the WW in replicate one. See Table 2 below for the average characteristics of the WW used in all three replicates in the main experiment.

Table 2: Average characteristics of the WW from Roja (± SD, n = 3)

PO_4^{3-}, mg/L	TOT-P mg/L	NO_3^- mg/L	TOT-N mg/L	pH	NPR
17.8 ± 0.6	20.2 ± 0.9	21.3 ± 1.1	24.9 ± 2.9	7.9 ± 0.5	1.8:1

The NPR was calculated by using the established relationship between the mass, the molar mass and the amount of substance (m=M/n), (see annex 10). The NPR displayed in this table is based on the PO_4^{3-} and the NO_3^-, as these had speciation and were the major contributors for their respective nutrient.

The WW in both the pre-experiments and in the main experiment was filtered 3 times using the filtration system in Figure 9 below, with pore sizes 1,2 µm, 0,45 µm and 0,2 µm, in listed order. The filtration was carried out to remove coarser particles and bacteria, however some bacteria was expected to remain in the WW. The reason why the filtration was performed was to avoid the bacteria culture being too large in the reactors, as it then risks them outcompeting the algae.

3.4 Determination of the initial biomass concentration and pre-phosphorus-starvation periods

To determine the initial biomass and pre-(P)-starvation periods, two pre-experiments were carried out in the batch reactors. Figure 5 displays six of the batch reactors, which in this picture were used for algae cultivation, and kept throughout the experiments. These reactors were called cultivation bottles. The batch reactors used for cultivation and experiments were assembled and functioning in the same way, as describe in the text below Figure 5. The algae used in all subsequent experiments were taken from the batch reactors (shown in Figure 5) and from the 250 ml bottles with EG described in *3.2.2 Growing algae*.

Figure 5: Batch reactors. Photograph by Murby, F. (2020).

The batch reactors consisted of 1-litre PYREX® flaks with cords attached to them. From the blue cords air was pumped in at a constant rate for mixing. A burst of CO_2 was added to the reactors from the same cord with regular time intervals, to supply the algae with carbon and to keep the pH at neutral. Out of the lid on top of the flasks came a cord which was attached to a filter, to let out gas. The third cord with the clip on, was used for sampling. A syringe or pipette could be attached to this cord and solution could be extracted. It was important to always shake the bottles before sampling, as in many of the reactors, algae settled despite the air mixing. This can be seen in the two leftmost flasks in Figure 5. Behind the flaks is a lamp providing fluorescent light on a regular schedule.

3.4.1 Pre-experiment 1: Determination of the initial biomass concentration

Before starting with the main experiment an initial algal biomass concentration to add to the WW was determined by performing an experiment with secondary treated WW from Kuldiga WWTP and BG11(see dimensions in Table 3). The aim was to find a concentration of initial biomass which was:

- *High enough to outcompete bacteria,*
- *Low enough to not consume all P within a day,*
- *Gradually reducing the PO_4^{3-} concentration,*
- *And high enough to perform analyses on.*

An initial biomass which did not consume all P within a day was sought after, since the effect of starvation would not be easily investigated if all the P was consumed within day.

To perform this pre-experiment, the algae strain *C. vulgaris* was used, since it had thrived in the growing process. The *C. vulgaris'* biomass concentration in the cultivation bottle was determined in the spectrophotometer and by using *C. vulgaris'* calibration curve (see annex 6). Volumes to extract from this bottle for replantation in the BG11/WW mix were calculated by using equation 1 below:

$$C1 * V1 = C2 * V2 \qquad (1)$$

Where C1 was the biomass concentration in the algae cultivation bottle (Figure 5) which the initial biomass was extracted from, in grams of dry weight per litre (g DW/L), V1 was the volume, in ml, that needed to be extracted and replanted in the BG11/WW bottle, C2 was the concentration which was decided to have in the BG11/WW bottle and V2 was the total volume in the BG11/WW bottle. The target initial biomass concentrations (C2) to be tested in 5 batch reactors were: 0.05, 0.10, 0.15, 0.20 and 0.25 g DW/L.

The PO_4^{3-} concentration and the biomass production, was measured once a day, in the following seven days, after starting the experiment. It was expected that the PO_4^{3-} concentration would be below 0.1 mg/L after this time. The pH was monitored irregularly to get a hint of how it changed under the biomass production.

Table 3: Conditions in pre-experiment 1.

Parameters		Setting	Comments
Temperature		26.5 ± 1 C°	Average, from pH meter.
Light	Light schedule	16h light 8h dark	
	Lamp	F54W T5 l 8 Gro-Lux Retail	
	Photosynthetically active radiation	120 µmol m^2 s^{-1}	Mean value, measured with a quantum flux meter (MQ-500, from Apogee)
Air mixing & CO_2	CO_2 volume	2.2 l	Pumped into each flask per 24 h
	Air Inflow	0–60 l/minute	O_2 Constantly pumped in, flow adjusted with handgrip
	Gas outlet	0.45 µm filter	
Growth media	Total volume	915 ml	
	Wastewater	500 ml	From Kuldiga WWTP
	BG11	400 ml	
	Transfer volume	15 ml	Demineralised water
	NPR	60:1	Approximate value, see annex 10

The airflow inflow into the reactors was manually set with a handgrip, making it possible to make the flow stronger and weaker. Its sole purpose was to mix the solution. An appropriate flow was determined and set by looking at the reactors.

To be able to calculate the relative P removal in each batch, equation 2 below was used:

$$R_P = (C_0 - C_i)/C_0 * 100 \qquad (2)$$

Where R_p was the removal rate of PO_4^{3-} in %, C_0 was the initial PO_4^{3-} concentration in mg/L and C_i was the PO_4^{3-} concentration at the end of the experiment or at a specific day i in mg/L.

3.4.2 Pre-experiment 2: Determination of pre-phosphorus-starvation periods

In this pre-experiment the starvation periods for the main experiment were determined. This happened by planting and detaining the algae *T. obliquus*, in the BG0 medium (the BG11 without any P), for different set of days. After the different starvation periods the algae were replanted in a P rich media - a mix of BG11 and WW (the BG11/WW-mix), and the biomass production and the PO_4^{3-} concentration were measured daily, for the following 8–11 days. For all set parameters, see Table 4.

Table 4: Conditions in pre-experiment 2

Parameters		Setting	Comments
Temperature		26.5 ± 1 C°	Average, from pH meter.
Light	Light schedule	16h light 8h dark	
	Lamp	F54W T5 l 8 Gro-Lux Retail	
	Photosynthetically active radiation	120 µmol m^2 s^{-1}	Mean value, measured with a quantum flux meter (MQ-500, from Apogee)
Air mixing & CO_2	CO_2 volume	1.7 l	Pumped into each flask per 24 h
	Air Inflow	0–60 l/minute	O_2 Constantly pumped in, flow adjusted with handgrip
	Gas outlet	0.45 µm filter	
Starvation Media	BG0	900 ml	
Growth media	Total volume	900 ml	
	Wastewater	500 ml	From Kuldiga WWTP
	BG11	400 ml	
	Transfer volume	15 ml	Demineralised water
	NPR	60:1	Approximate value, see annex 10

Before this experiment, the algae had been residing in P rich growth media. This pre-experiment was started in one reactor, and the experiment included a starvation phase and a measurement phase. Below in Figure 6, a schematic time schedule for the starvation periods (indicated by yellow colour) and the replanting in a P-rich media (indicated by green colour), can be observed. A reactor (denoted the starvation bottle) was filled with 900 ml of BG0 on day zero, and algae were planted in it. The starvation bottle had an initial biomass concentration of about 1.6 g DW/L on day zero. From that reactor, indicated by the big flask in Figure 6, the biomass concentration was measured every day. From that measurement, by using equation 1 (see pre-experiment 1 in chapter 3.4.1) and *T. obliquus'* calibration curve (annex 6), a volume was calculated, extracted, and replanted in a new 900 ml flask filled with the BG11/WW-mix, indicated by the small flasks in Figure 6.

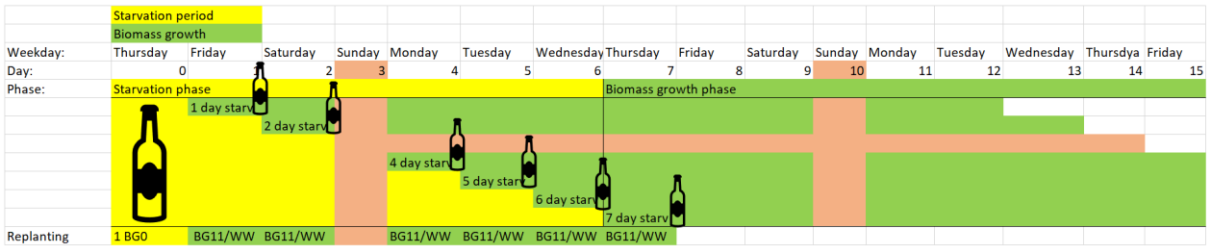


Figure 6: Schedule of starvation periods and replanting for pre-experiment 2. The big flask symbolises the starvation bottle - the reactor filled with algae and 900 ml of BG0. The smaller flasks symbolise the reactors filled with 900 ml of BG11/WW-mix and algae extracted from the starvation bottle. All reactors had the same size.

The aim was that the replanted biomass in the BG11/WW reactors should have the initial biomass concentration 0.25 g DW/L. The measurement of biomass in the starvation bottle, and replantation into a new WW/BG11 bottle was performed daily for six days.

To see how the reduction of PO_4^{3-} and the biomass growth was affected by the different initial starvation periods, measurements were taken once a day on the biomass and PO_4^{3-} concentration during the following days. The PO_4^{3-} measurements were stopped when the PO_4^{3-} concentration in the reactors had reached below 0.1 mg/L. The biomass concentration in the BG11/WW reactors were measured until eight, nine, ten or eleven days after plantation, depending on when the initial plantation of a batch took place. Observation of the data during the experiment lead to the decision that eight days as minimum of biomass measurements was enough to draw conclusions.

The WW came from Kuldiga WWTP. The first three flasks in pre-experiment 2, had WW from the first collection from Kuldiga WWTP. The fourth to sixth reactor, with five, six and seven days of pre-starvation (see Figure 6) had WW from the second collection, where the PO_4^{3-} concentration was close to zero. Therefore, the initial PO_4^{3-} came solely from the BG11 in the reactor with five days of pre-starvation. In reactor number 5 and 6 (which entails six and seven days of pre-starvation) $K_2HPO_4^{3-}$ was added, until the initial PO_4^{3-} concentration was about 20 mg/L. This was done to get a clearer picture of the PO_4^{3-} reduction over time. To be able to calculate the relative P removal, equation 2 in pre-experiment 1 (chapter 3.4.1) was used.

3.5 Main Experiment: Phosphorus reduction in wastewater by five algae strains with varying pre-phosphorus-starvation periods

3.5.1 Aim

The aim of the main experiment was to investigate the P reduction performance of five different algae strains, in a controlled environment under different pre-P-starvation periods. In other words, to access their ability to reduce the amount of P as greatly as possible, in as short time as possible. The hope was that these experiments could contribute valuable information on how pre-P-starvation of the algae affects the P reduction.

3.5.2 Overview of experiment

The experiment was carried out in the batch reactors described in chapter 3.4 and displayed in Figure 7.

Figure 7: Main experiment, replicate one. Photograph by Murby, F. (2020).

On the bottom right shelf are 4 reactors containing algae for cultivation in BG11. The rest of the reactors are the 20 batch reactors used in the main experiment, containing algae and WW.

On the bottom left corner is the blank reactor, containing only WW. This picture was taken on day zero of replicate one, when the algae had just been planted.

Five algae strains were used, and each strain had four batch reactors, which responded to different pre-starvation periods executed prior to replantation in a P-rich media. The algae strains used were; A. falcatus, C. vulgaris, T. obliquus, D. communis and B. braunii. The batch reactors were set up with all parameters controlling the environment at fixed values as in Table 5 The only varying parameter within a strain was the length of the pre-starvation period.

Table 5: Conditions in main experiment

Parameters		Setting	Comments
Temperature		27.3 ± 1 C°	Average, from pH meter
Light	Light schedule	16h light 8h dark	
	Lamp	F54W T5 l 8 Gro-Lux Retail	
	Photosynthetically active radiation	120 μmol m^2 s^{-1}	Average, measured with a quantum flux meter (MQ-500, from Apogee)
Air mixing & CO_2	CO_2 volume	4.2 l	Pumped into each reactor/24 h (average from 3 it.)
	Air Mixing	0–60 l/minute	O_2 Constantly pumped in, flow adjusted with handgrip
	Gas outlet	0.45 μm filter	
Starvation periods	Periods used	5, 3, 1and 0 Days	
Starvation media	Total volume	150 ml	Per Erlenmeyer flask
	BG0	140 ml	
	Demineralised water	10 ml	Used for transferring centrifuged algae to BG0
Growth media	Total volume	820–830 ml	Per reactor
	Wastewater	800 ml	From Roja WWTP
	Transfer volume	30 ml	Demineralised water
	NPR	1.8:1	Approximate value, see annex 10

The experiment could be divided into two phases. In the first phase, the algae were planted in the P free media BG0, for different set of days. No measurements were performed here. After that, the algae were replanted in filtered WW from Roja WWTP, which initiated the second phase of the experiment, where all measurements took place. The main parameters examined were the P concentration, the biomass production, the temperature, and the pH. Other parameters which, examined more rarely like the total-P, the total-N, the NO_3^- concentrations, were measured to get a more nuanced picture of what was going on within in the algae culture and the WW. All analyses performed, in their moment in time, can be seen in Figure 8 in the section below (3.5.3).

3.5.3 Time and sampling plan

The experiment took place in three replicates. Each replicate was 15 days long, where phase one responded to five days, due to the longest starvation period being five days. The other starvation periods started regularly after this, with a two-days interval, according to the determined periods in Pre-experiment 2. The zero-days starvation-period is used as a reference, displaying what happens in a batch reactor with no prior P starvation. The names "the zero-days starved batch" and "the reference batch" are used interchangeably in this thesis.

The second phase responded to ten days. A blank sample was run alongside phase two in each replicate, to distinguish background data of from the results, like P reduction from bacteria and precipitation. Figure 8 below displays the schedule of the planting and analyses performed in each replicate.

Main experiment Replicate schedule																
Days	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	10
Planting	Pre starv 5		Pre starv 3		Pre starv 1	Pre starv 0 (Planting all batches into WW)										
Biomass						Biomass	Biomass	Biomass	Biomass	Biomass	Biomass		Biomass			Biomass
PO_4^{3-}						PO_4^{3-}	PO_4^{3-}	PO_4^{3-}	PO_4^{3-}		PO_4^{3-}		PO_4^{3-}			PO_4^{3-}
pH						pH	pH	pH	pH	pH	pH		pH			pH
Temp						Temp	Temp	Temp	Temp	Temp	Temp		Temp			Temp
NO_3^-						NO_3^-	NO_3^-	NO_3^-	NO_3^-		NO_3^-		NO_3^-			NO_3^-
TOTAL-P						TOTAL-P					TOTAL-P					TOTAL-P
TOTAL-N						TOTAL-N					TOTAL-N					TOTAL-N

Figure 8: Time and sampling/analyse schedule of the replicates.

As can be seen in Figure 8, the PO_4^{3-} measurements occur daily the first three days, after plantation, and then the measurements faded out. That was because it was expected that the majority of the PO_4^{3-} would be consumed within the first three days. Once the PO_4^{3-} concentration in a batch was below 0.1 mg/L, it was no longer measured.

In the first replicate the NO_3^- was sampled on day zero, two, five, seven and ten, displayed by the green cells on these days and the light green gridded cell on day two. After the first replicate it was decided that NO_3^- should be sampled on day zero, one, three, five, seven, and ten, and not on day two. The two additional days are displayed by the dotted darker green NO_3^- cells.

3.5.4 Experiment setup

Around one week before the first phase with pre-starvation was initiated, the algae were replanted in BG11 in the cultivation reactors so that all algae came from the same conditions before entering the starvation media. The cultivation bottles were regularly refilled with BG11 and cleaned, throughout all replicates, to facilitate biomass growth.

Initiating the starvation period, a mass which responded to 0.16 g of dry weight (which corresponded to 0.20 g DW/L in 800 ml of solution) of each algae strain were centrifuged and planted in an Erlenmeyer flask, filled with 140 ml of sterilised BG0. When moving the centrifuged algae into the Erlenmeyer flasks, 10 ml of demineralised water was used. The Erlenmeyer flasks were placed under the lamps described in Table 5. for their starvation period. They were covered with aluminium foil, loosely fastened to allow gas exchange. The flasks were shaken every day manually to expose the algae to more light by hindering them being settled for a long time.

To calculate how big of a volume to transfer between different reactors and flasks to obtain a certain biomass concentration, the spectrophotometer was used as well as the equations in Table 6, to determine the biomass concentration (see annex 6). After the biomass concentration had been established, equation 1 (see chapter 3.4.1) was used to calculate the volume needed for the replanting, to reach a certain concentration in the bottle where the algae were planted.

Table 6: Calibration curves 2020 [Excel sheet]. Modified from Lavrinovics, A. (2020).

Algae	Calibration curve	R^2	Notation	Explanation
C. vulgaris	y = 0,4076x - 0,0052	0,999	y	Grams of dry weight per litre (g DW/L)
D. communis	y = 0,8953x + 0,0013	0,9981	x	Absorbed light at 680 nm
T. obliquus	y = 0,5817x - 0,0129	0,9976	R^2	Coefficient of determination
A. falcatus	y = 0,5421x - 0,003	0,9926		
B. braunii	y = 0,5183x - 0,0054	0,9892		

After the pre-starvation periods had finished, the BG0-algae-solution was centrifuged, and the algae were separated from the supernatant and then diluted with 30 ml of demineralised water. The biomass concentration was measured, and transferral volumes calculated by using the spectrophotometer, the calibration curves in Table 6 and equation 1. The calculated volumes responded to a volume inside the 20–30 ml interval, and these volumes were planted in the WW reactors in Figure 7. Even though 0.20 g DW/L (in 800 ml of solution) was planted into the BG0 solution when the starvation periods were initiated, it was important to measure again, as the biomass concentration could have changed during the starvation period.

At the same day, volumes responding to 0.20 g DW/L in 800 ml of solution, from the cultivation bottles were planted in the WW reactors, to have a batch with zero days of pre-starvation. This meant that each strain had four bottles with the starvation periods: five, three, one and zero days. In total 20 batches reactors with algae were used. Alongside this was a WW batch reactor without algae, which was analysed for nutrients, pH, and temperature. It was called the blank reactor. Now 21 reactors were connected to the gas installation. After all plantations were done, all parameters described in Figure 8 were measured and analysed, according to the timetable. The CO_2 flow which is displayed in Table 5 was enough to keep the pH on a mainly neutral level, with fluctuations in the 7–8 pH interval occurring over the ten days period, due to biomass production and the CO_2 gas being injected into the reactors.

3.5.5 Data presentation, calculations, and demarcations

3.5.5.1 The phosphate reduction

The calculations, assumptions and demarcations made to display the PO_4^{3-} concentration reduction, relative to the initial PO_4^{3-} concentration were performed as explained below. It was assumed that the reduction in the blank reactor originated from bacteria and P precipitation, with bacteria being the main remover as the pH was kept close to neutral in all replicates (see annex 11).

The total PO_4^{3-} reduction in an algae-reactor relative to its initial PO_4^{3-} concentration was calculated by equation 2:

$$R_t = (C_0 - C_i)/C_0 * 100 \qquad (2)$$

Where R_t was the total removal of PO_4^{3-}, in %, by both algae and other processes (bacteria and precipitation) inside an algae-reactor, C_0 was the initial PO_4^{3-} concentration in mg/L and C_i was the PO_4^{3-} concentration at a specific day i.

The relative PO_4^{3-} removal, in %, in the blank reactor, denoted $R_{b,}$ was calculated the same way by using equation 2. R_b was the total relative removal of PO_4^{3-}, in %, in the blank reactor and C_0 was the initial PO_4^{3-} concentration in mg/L in the blank reactor and C_i was the PO_4^{3-} concentration at a specific day i, in the blank reactor.

R_t and R_b was calculated for each day i, in the sampling schedule (Figure 8), for each reactor in each replicate. Then an average value for each day i of all three replicates was calculated with equation 4, for all algae reactors and the blank reactor.

$$R_{t.mean.i.ps.s} = \frac{R_{t..i.n.ps.s} + R_{t.i.n.ps.s} + R_{t.i.n.ps.s}}{n_t} \qquad (4)$$

Where $R_{t.mean.i.ps.s}$ was the average relative total removal (t) on day a specific day (i), by a specific strain (s) with a specific pre-starvation period (ps) participating in replicate (n) and n_t was the sum of the replicates the strain participated in. $R_{b.mean.i}$ was the average relative total removal on day a specific day i, by the processes in the blank reactor and it was calculated in the same way.

The standard deviation (SD) was calculated for the whole population, for each $R_{t.imen.i.n.ps.s}$ and for each $R_{b.mean.i}$ by equation 6 below:

$$SD = \sqrt{\frac{\Sigma(R_{t.i.n.ps.s} - R_{t.mean.i.ps.s.})^2}{n_t}} \qquad (6)$$

And was demonstrated in the diagrams as well. By this the variations between the replicates could easily be exhibited.

3.5.5.2 The nitrate reduction

The average day when the NO_3^- concentration reached below or equal to (≤) 1 mg/L for each strain with a specific pre-starvation period, was calculated according to equation 7, as a basis for deeper discussion on the NPR.

$$i_{m.N\leq 1.s.ps} = \frac{(i_{N\leq 1.s.ps.n} + i_{N\leq 1.s.ps.n} + i_{N\leq 1.s.ps.n})}{n_t} \qquad (7)$$

Where $i_{m.N\leq 1.s.ps}$ indicated the average day when the batch with a specific strain (s) and pre-starvation period (ps) reached a NO_3^- concentration ≤ 1 mg/L, and n_t indicated the number of replicates a strain participated in. Each $iN\leq 1.s.ps.n$ indicated the day the NO_3^- concentration reached ≤ 1 mg/L for the specific strain (s) and specific pre-starvation period (ps) from a specific replicate (n).

The standard deviation was calculated for each specific strain with a specific pre-starvation period, according to equation 6 above, but now with the values for the NO_3^- instead of PO_4^{3-}, as seen below in equation 8.

$$SD = \sqrt{\frac{\Sigma(i_{N\leq 1.s.ps.n} - i_{m.N\leq 0.1.s.ps})^2}{n}} \qquad (8)$$

Where SD was the standard deviation for the whole population of a specific strain with a specific pre-starvation period, and the other components ($i_{m.N\leq 1.s.ps}$ and $iN\leq 1.s.ps.n$) are explained above.

3.6 Analytical methods

In this section the analyses performed in the laboratory to obtain the data are described according to how they were performed. The standards/manuals which have been followed are referred to and can be found in the annexes.

3.6.1 Observation of colour

The first and quickest way to get an indication of whether the algae were thriving or not was to check the colour of the growth. Before looking, the batch had to be mixed readily by shaking the reactor. If the water was green, this could imply that the algae were alive and well. If the water had a more yellow-green tone, bacteria could have been outcompeting the algae. If the water had a dark green tone, it could mean that the biomass concentration was high. If the water was white, the algae were most likely dead. These observations in combination with other analysis methods gave a clearer picture of what was going on inside the colony.

3.6.2 Nutrients analyses

To measure the removal of nutrients, the initial nutrient concentration was measured, and after a certain period of time it was measured again, and the new concentration was subtracted from the initial one, to obtain a removal rate. See equation 2 in chapter 3.4.1 on page *16*. To measure the nutrients a DR/890 Portable Colorimeter was used. The nutrients analysed in this thesis was the total P (Tot-P), the total N (Tot-N), the nitrates (NO_3^-) and the phosphates (PO_4^{3-}). Since PO_4^{3-} was the main parameter examined in all experiments, the procedure to measure it is generally summarised below, and it was performed according to the "*PhosVer 3 Method, Test 'N Tube Procedure*", in the HACH DR/890 Portable Colorimeter Procedures Manual (see annex 4). The Tot-P, Tot-N and NO_3^- measurements are performed similarly to the PO_4^{3-}, measurement, and their detailed procedures from the same manual can be found in annex 7, 8 and 9, respectively.

To measure the PO_3^{4-} concentration, a sample from a batch reactor was taken in a syringe, and filtered through a 0.45 μm filter, to remove organics such as algae and bacteria. Then a colourless liquid reagent was added to the sample and the sample-reagent solution was inverted slowly 10 times to mix the liquids. Then the colorimeter was calibrated to the sample, meaning that the wavelength read at the current sample was set to equal zero concentration of PO_3^{4-}. After that, a powder reagent was added to the sample, which coloured the PO_3^{4-} blue, and the sample was shaken readily for 15 seconds. After the mixing, the colouring reaction occurred for 2 minutes, after which a new reading of the concentration was taken. There was a limit of how much mg/L of PO_3^{4-} that could be detected by using the colorimeter. If the PO_3^{4-} concentration exceed the maximum limit (5 mg/L), the sample was diluted before adding the reagents and doing the reading.

In the growth media, BG11 and EG, there was only PO_3^{4-} present. In the WW other forms of P could occur, however, since secondary treated WW was used, where mainly PO_3^{4-} was left, this was expected to be the main source of the P. In the main experiments, total P was measured in the WW to ensure that this was the case.

In Table 7 below, the precision of the different nutrient analyses performed, and the estimated lowest detection limit can be observed. Note that the estimated detection limit for NO_3^- concentration is slightly higher than for the PO_3^{4-} concentration.

Table 7: Precision and detection limits for nutrients analysed with the HACH DR/890 Portable Colorimeter. Values obtained from HACH DR/890 Portable Colorimeter Procedures Manual.

Nutrient	PO_4^{3-}	P-tot	NO_3^-	Tot-N
Precision	± 0.08 mg/L	± 0.06 mg/L	± 0.5 mg/L	< 3 mg/L
Estimated detection limit	0.07 mg/L	0.07 mg/L	0.3 mg/L	7 mg/L

3.6.3 Biomass measurements

To measure the amount of biomass in a sample, a spectrophotometer, *Thermo Scientific GENESYS™ 150 UV-Visible Light Spectrophotometer,* was used. The light absorbance from waves with the wavelength 680 nm was measured, and it responded to a certain dry weight, in grams, of algal biomass per litre of water (g DW/L). The correlation between the light absorbance and g DW/L was calculated by Aigars Lavrinovics before this degree project was undertaken. The method for calibration as well as the equation to convert light absorbance to biomass can be found in annex 6.

The procedure to measure the absorbed light started with calibrating the zero absorbance in the spectrophotometer. A sample with demineralised water was used, and its light absorbance was set to indicate the absorbed wavelength of zero colours. When working with WW in the main experiment, filtered WW from the blank bottle (the WW without algae) was used as the zero-absorbance calibration. After the zero-absorbance had been established, samples from the reactors were poured into the small cups which was placed in the spectrophotometer, and their absorbed wavelengths were noted. The samples were mixed with a vortex machine before being poured into the cups, to avoid settling of the biomass. The biomass measurement worked within a range of 0.1–1.2 nm. If the biomass was more concentrated than that, it was diluted before measuring. If there was too little biomass, it could be concentrated before measuring. However, this rarely happened. Later the absorbed wavelengths were recalculated to biomass concentrations, using the equations in Table 6, described in annexe 6.

3.6.4 PH and temperature measurement

The pH and temperature were measured by using the pH meter function on the *WTW™ inoLab™ Multi 9420 IDS™ Digital Benchtop Multiparameter,* which also measured the temperature.

The pH measurement occurred by taking a sample from the batch reactor with a syringe and placing it in a 12 ml vial. Then the pH sensor was placed in the vail for measuring. In between the measurements the pH meter was cleaned with tap water and dried with paper. The temperature was measured by moving around a 1-litre bottle filled with 800 ml of water on the three shelves and measuring the temperature on each shelf, 24 h after moving it there. In the main experiment the temperature was slightly higher than in the two pre-experiments, likely due to more bottles crowding the shelves, maintaining the heat from the fluorescent lamps.

3.6.5 Microscopy

Microscope examination of the algae was done to ensure that the cultures were axenic. If a culture had more strains than the intended one in the sample, the batch was discarded, and a new batch was started with new algae.

First 50 µl of sample was diluted in an Eppendorf tube with 1 ml of water. Then the samples were poured into the cups of a filtration system. The filtration systems consisted cardinally of a pump, wastewater collection and removable cups where removable membranes were placed in the in the bottom for filtration. See Figure 9 below:

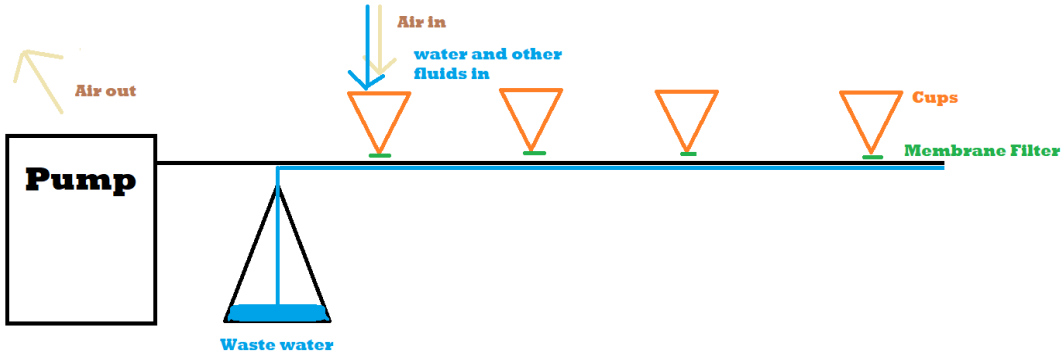


Figure 9: Sketch of the filtration system. The green membrane is what was examined in the microscope. Murby, F. (2020).

The filtration system had been prepared with 0.45 nm membrane filters, which would be examined in the microscope after having been prepared in the filtration system. The diluted sample was cleared of fluid and rinsed with 50 ml of demineralised water. After that 3–4 %-Formaldehyde solution was added, just covering the surface of the membrane, to break open the cell walls of the algae. The cups with the formaldehyde were let to rest for 10 minutes. Then the formaldehyde was sucked out and the membranes were rinsed 2 times with 50 ml of demineralised water. After that Tritons solution was added to the samples. The volume of Tritons was just enough to cover the surface of the membranes. Then 200 µg ml−1 4′, 6-diamidino-2-phenylindole (DAPI) colour solution was poured on top of the Tritons layer. In total these two solutions reached about a few mm above the membranes. The Tritons solution made the microscope picture sharper by breaking up the pores where the colour could enter. The DAPI solution coloured the DNA/RNA to vibrant colours, which made it possible to extinguish lipids and phosphates in the algae in the epifluorescence microscope (DM6000B, Leica, Germany, with digital camera DFC400 C, Leica, Germany). The cups with these solutions were set to rest for 20 minutes, and they were covered with aluminium foil to eliminate light entering the solution. The colouring deteriorated under light. After that, the solution was sucked out, and again, the samples were rinsed with 50 ml of demineralised water. After this the membranes were put on marked glass plates and set to dry under heated fan.

After the samples were dried, a small glass plate was put on top of the membrane and a drop of oil and was put in between the sample and the glass, so to keep the glass fixed. Before using the epifluorescence microscope, a drop of oil was added on top of the uppermost glass plate, now

lying between the microscope lens and the topmost glass. Finally, the samples were put under the epifluorescence microscope and they were examined under a fluorescent filter with excitation wavelength at 370 nm and emission at 526 nm.

4 Results

4.1 Pre-experiment 1: Determination of the initial biomass concentration

As can be seen in Table 8 below, the initial biomass measured in each bottle on day 0, same day as plantation, was not the same as the target initial biomasses (0.05, 0.10, 0.15, 0.20 and 0.25 g DW/L). Especially batch number 4 and 5 had almost the same initial biomass (0.168 and 0.160 g DW/L). Therefore, only conclusions on the initial biomasses shown in Table 8 could be drawn.

Table 8: Initial measured biomass and PO_4^{3-} concentration in the reactors in Pre-experiment 1, and final PO_4^{3-} concentrations measured seven days after plantation.

No.	Initial biomass (g DW/L)	PO_4^{3-}, mg/L, day 0	PO_4^{3-}, mg/L, day 7
1	0.038	16.0	0.21
2	0.078	17.5	0.11
3	0.130	16.7	0.12
4	0.168	16.0	0.18
5	0.160	14.8	0.10

The daily PO_4^{3-} reduction relative to the initial PO_4^{3-} concentration was calculated with equation 2 and is presented in Figure 10. The reduction is similar for each initial biomass (Figure 10) however, the lowest initial biomass (0,038 g DW/L) generates the slowest reduction of PO_4^{3-} in the observed timespan. Note that on day one, no reduction has occurred for this batch. The batch where the PO_4^{3-} was reduced most efficiently was the one with an initial biomass of 0.078 g DW/L, which also had the highest initial PO_4^{3-} concentration. The reduction of PO_4^{3-} to concentrations near 0.1 mg/L in this batch, was achieved within four days, while for the batch with initial biomass concentration 0.038 g DW/L, it took six days. For the batches with initial biomasses concentrations 0.130, 0.160 and 0.168 it took about five days to reduce the PO_4^{3-} to levels near 0.1 mg/L. However, within the span of seven days since planting the biomass, no batch reached below 0.1 mg/L (Table 8).

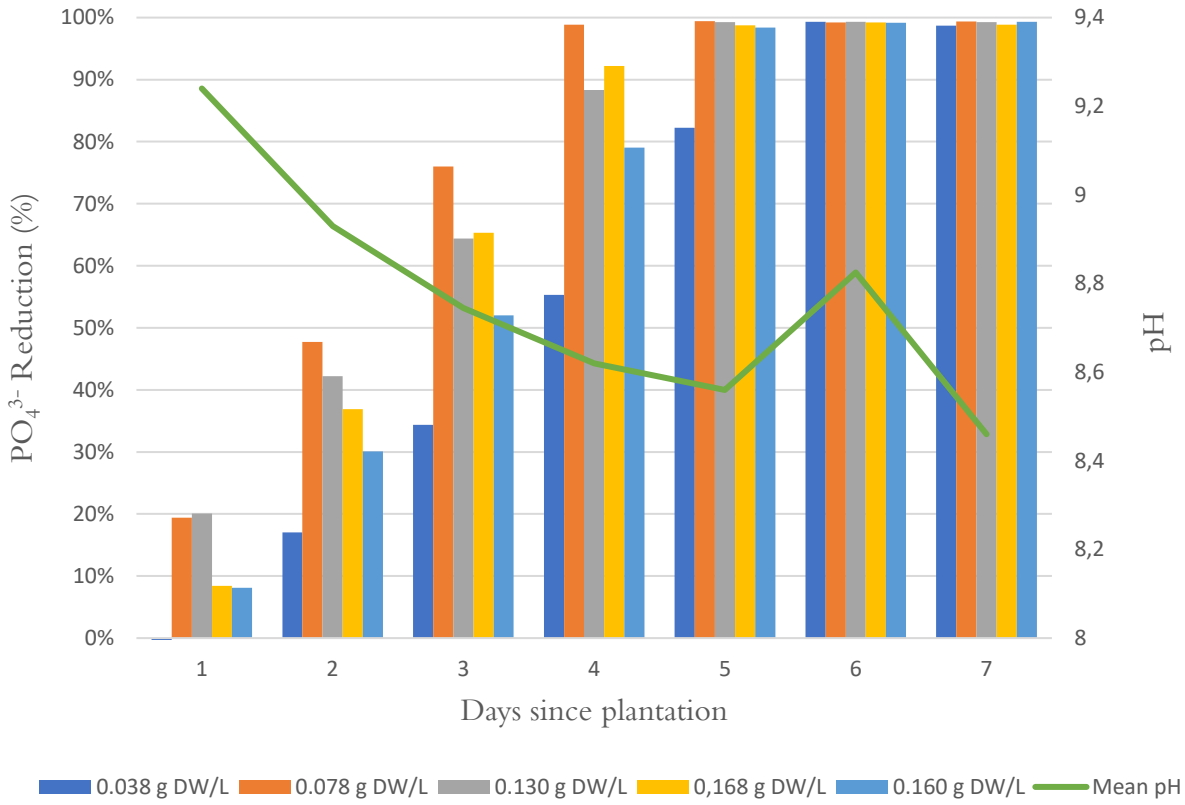


Figure 10: PO_4^{3-} removal in percent, relative to the initial PO_4^{3-} concentration of five reactors with different initial biomass concentration.

No samples were taken on day two and an average has been calculated from day one and three, for each batch, on this day. The average pH was calculated with measurement from each of the reactors. On day two and three no pH measurements were taken, and an average has been calculated on day two and three to get an approximate pH perception.

From the result of pre-experiment 1 it could be concluded that 0.78 g DW/L should be the initial biomass concentration used in the main experiment. However, after discussing the setup and analyses of the main experiment, it was decided that an initial biomass concentration of 0.25 g DW/L would be used. In this pre-experiment, the concentration 0.25 g DW/L was not achieved in flask number 5 as planned, therefore no data from pre-experiment 1 was used when deciding on the initial biomass concentration in the main experiment. This decision was based on Aigars Lavrinovics', the PhD student leading this experiment, previous experience, and this biomass concentration was chosen mainly for having enough biomass to perform all analyses which were to take place in the main experiment.

4.2 Pre-experiment 2: Determination of pre-phosphorus-starvation periods

Figure 11 below displays the daily change in the PO_4^{3-} concentration relative to the initial PO_4^{3-} concentration. This was calculated using equation 2 (see methods, Pre-experiment 1).

As can be seen in Figure 11, the batches which had been starved for one, four and five days, reached 99–100 % of removal after four days (where a 99–100 % removal inferred that the PO_4^{3-} concentration was less than 0.1 mg/L), while the batch which had been starved for two days, reached a 100 % on day three, since replantation. It can also be seen that the batches which were starved for six and seven days, had similar removal patterns, slowly decreasing the PO_4^{3-} concentration compared to the other batches, and reached a 100 % on day six. The batch which had been pre-starved for four days had a marginally faster removal rate than the batch that had been pre-starved five days (Figure 11).

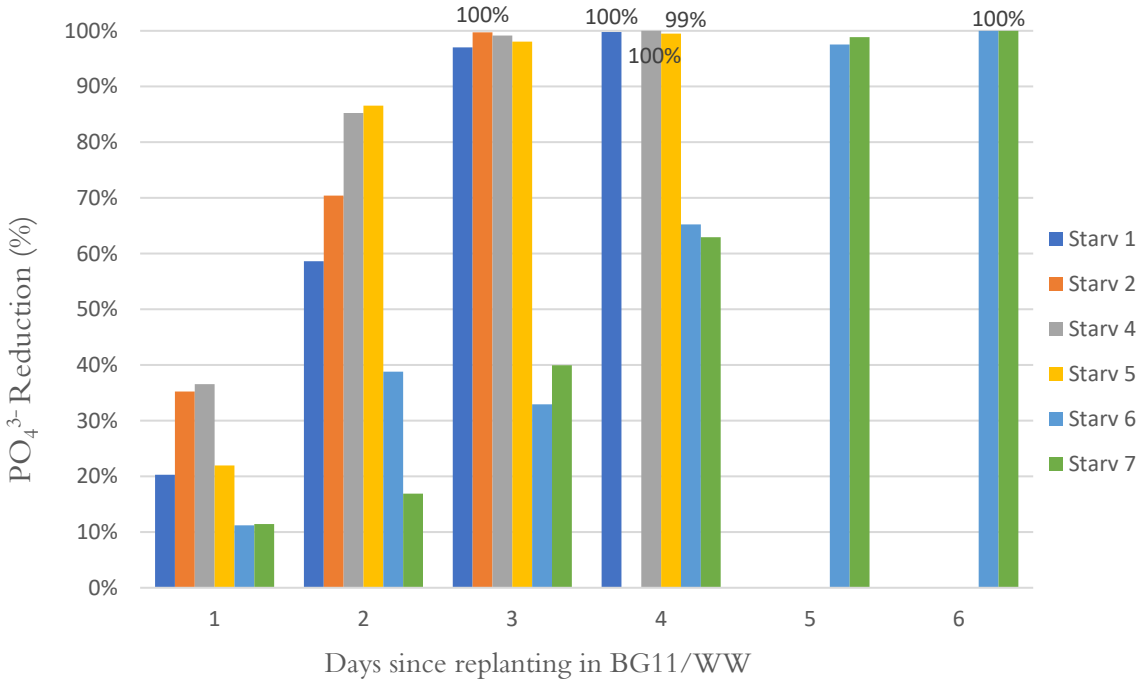


Figure 11: PO_4^{3-} removal in percent, in each batch, relative to their initial PO_4^{3-} concentration.

Once the concentration had reached below 0.1 mg/L of PO_4^{3-}, there was no more data, which can be seen on day five and six, where only the batches which had been starved for a longer time are displayed. For some days (Sundays) data was missing and an average was used, from the measurements on the previous and following day. Day zero, which was the day of the replantation in the WW/BG11 mix, is not included in this diagram since no PO_4^{3-} reduction had occurred yet. The first PO_4^{3-} concentration was measured 24 h after replanting.

In Figure 12 below, a clear relationship between the biomass produced, in g DW/L and the decrease in the PO_4^{3-} concentration from Figure 11 above can be observed. The batches which had been starved for one, two and four days have the highest produced biomass. It is important

to note that the bottles which were starved for five, six and seven days do not have measurements for 11 days like the rest, but for ten, nine and eight days, respectively. However, it can be seen, looking at day eight, that the batches starved for less days, had higher biomass production all along, until day eight.

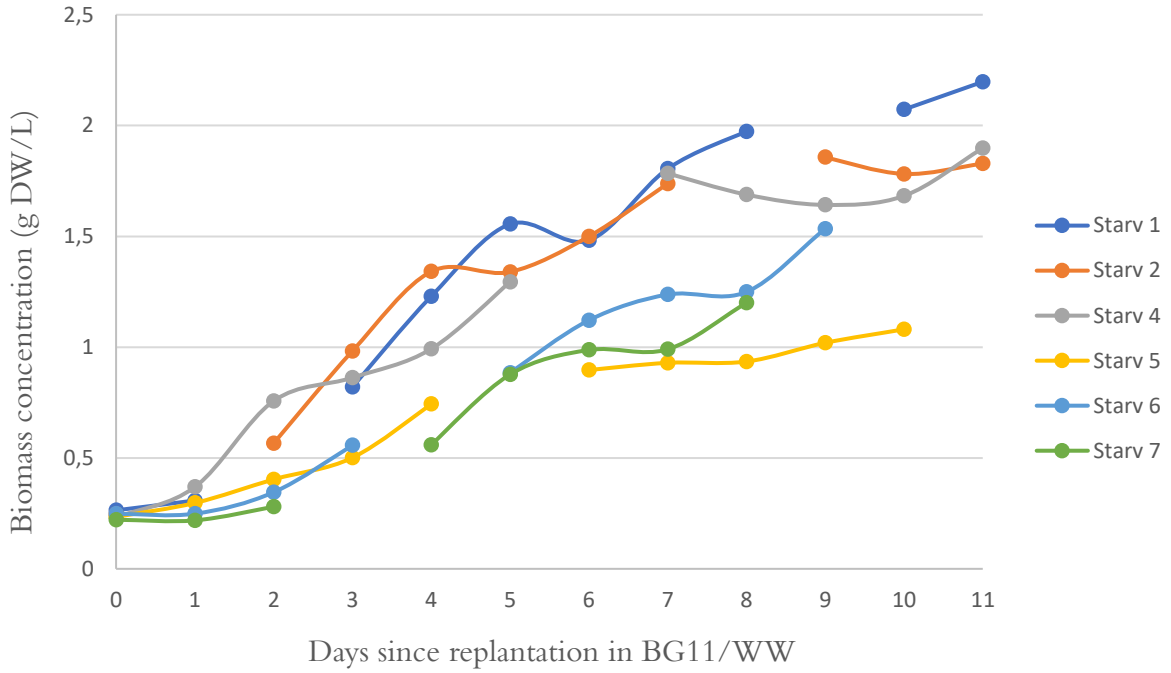


Figure 12: Biomass produced daily by T. obliquus replanted (initial biomass concentration of on average 0.24 g DW/L) from BG0 into a BG11/WW mix.

From these observations it can be concluded that pre-starvation periods ranging between one to five days (especially two days), for removing PO_4^{3-} efficiently, was optimal for *T. obliquus.* With these results it was decided that in the main experiment, the starvation periods as following were to be used; one, three and five days. These starvation periods were chosen as the main experiment aimed at investigating the optimal starvation period, and at quantifying the effects of starvation time on the efficiency of the algae to remove the PO_4^{3-}. It was expected that three days was the optimal starvation period, since two and four days of pre-starvation removed PO_4^{3-} the fastest (Figure 11) in this experiment. For the other algae strains used in the main experiment it was impossible to know if these pre-starvation periods would prove this statement.

4.3 Main experiment: Phosphorus reduction in wastewater by five algae strains with varying pre-phosphorus-starvation periods

When starting with the main experiment, it was found that there was not enough biomass in the cultivation bottles to start with 0.25 g DW/L for each strain, therefore an initial biomass concentration of 0.20 g DW/L was decided to be used in the main experiments, as it was after pre-experiment 1 and 2, thought to be enough to reduce the P gradually within the 10 days of experimental period. However, after measuring the initial biomass concentrations in the batch reactors after plantation, the concentrations in Table 9 were obtained. Therefore, the target concentrations in the following two replicates were set to 0.15 g DW/L, which was the average initial biomass concentration, measured in replicate one, excluding D. communis. D. communis was not used in the following replicates since there was not enough biomass of that strain. The new target initial biomass concentration of 0.15 g DW/L was set to mimic the empiric setup in replicate one. In replicate two, the initial biomasses concentration was on average 0.13 g DW/L and in replicate three it was on average 0.12 g DW/L (Table 9). *T. obliquus* did not grow in replicate two and was therefore discarded halfway into the experiment. For replicate three, only cultivated *T. obliquus* and *C. vulgaris* could be used, since there was not enough *B. braunii* and *A. falcatus* left. This entailed that most strains produced data in only two replicates, but *C. vulgaris* produced data in three replicates and *D. communis* only in one replicate.

Table 9: Average initial biomass concentrations (g DW/L) of each strain in each replicate on day zero. Each strain had four reactors and their initial biomass concentration was measured shortly after plantation.

Replicate	*D. communis*	*T. obliquus*	*C. vulgaris*	*A. falcatus*	*B. braunii*	All strains
1	0.10	0.16	0.15	0.14	0.15	0.14
2	N/A	0.13	0.12	0.14	0.13	0.13
3	N/A	0.12	0.12	N/A	N/A	0.12

In table 10 the number of reactors where the PO_4^{3-} concentration had reached below 0.1 mg/L and below 1 mg/L are displayed in both numbers and percent. As can be seen, a minority of the reactors reached the goal concentration, however, a majority reached below 1 mg/L, which shows that the removal was still quite comprehensive, as the average initial PO_4^{3-} concentration in the WW was about 17.8 mg/L (Table 2).

Table 10: Number of reactors in which the PO_4^{3-} concentration reached below 0.1 mg/L and below 1 mg/L out of the 40 reactors tested.

PO_4^{3-} < 0.1 mg/L	Percent	PO_4^{3-} < 1 mg/L	Percent
14/40	35%	28/40	70%

In the diagrams below (Figure 13–17) the relative reduction in concentration of PO_4^{3-} to the initial concentration is displayed for each strain and pre-starvation period. The reduction which occurred in the blank reactor is also displayed, always portrayed as the light green curve named "Blank". The reduction curves (the curves named with two letters and a number, where the two letters notate the strain and the number entails the length of the pre-starvation period) respond to the average reduction for a strain and specific pre-starvation period, based on data

from the replicates the strain participated in. The blank curve responds to the average reduction in the blank bottle, from the same replicates as the species participated in. For example, *T. obliquus* participated in replicate one and three (Figure 14), therefore the reduction displayed by the blank curve is the average reduction in the blank bottle from replicate one and three.

As can be seen in all diagrams below (Figure 13–17) the algae that had been pre-starved either three days or one day usually removed the P the quickest. The zero-days starved batch (the reference) never reduced faster than the pre-starved batches, except for in *B. brauniis'* case, where the one-day pre-starved and reference removed P at an equal rate. Most algae reduced nearly a 100 % of the P within the ten days interval, except for *D. communis* and *T. obliquus*. *D. communis* only participated in one replicate and judging from the colour of the reactors and the biomass growth (annex 11), it was concluded that the culture did not thrive. The same pattern was observed for most of *T. obliquus* reactors in replicate one and three.

It can be observed in Figure 15 and Figure 16 that for *C. vulgaris* and *A. falcatus* the three-day pre-starved batch reduced nearly a 100 % of the P on day seven, while the reference reduced nearly a 100 % on day 10. In *B. brauniis'* case all batches reached nearly a 100 % removal on day 10, with the reference and one-day pre-starved batch having the fastest removal rate.

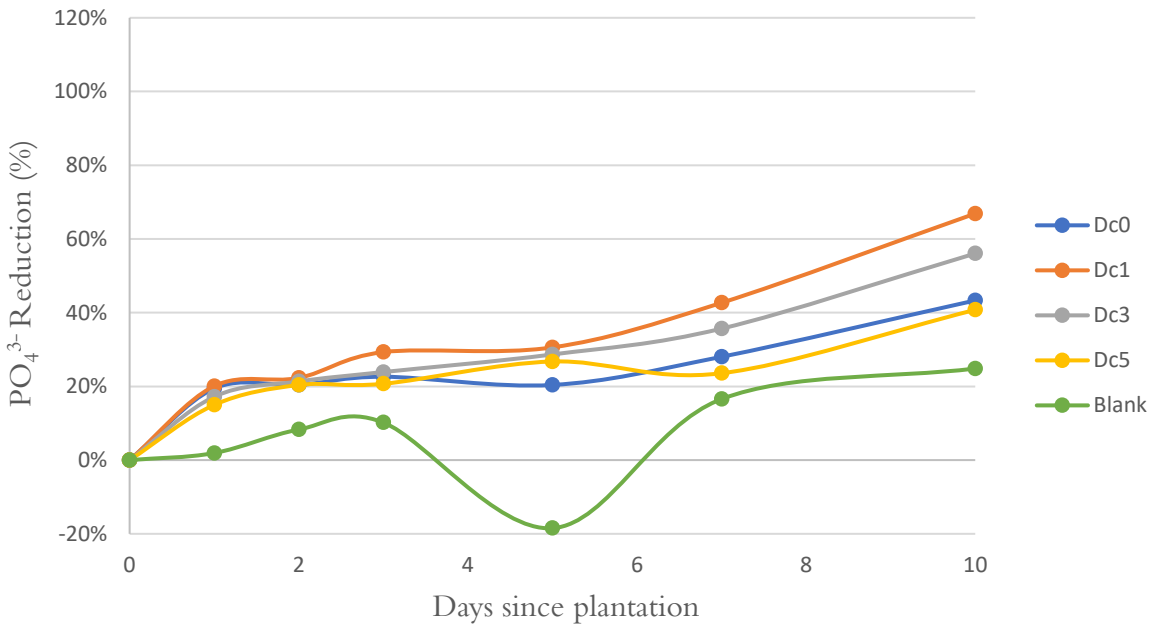


Figure 13: Reduction of PO_4^{3-} by D. communis. Data from replicate one. No SD is displayed since D. communis only participated in one replicate. Dc0 to Dc5 indicate the number of pre-starvation days.

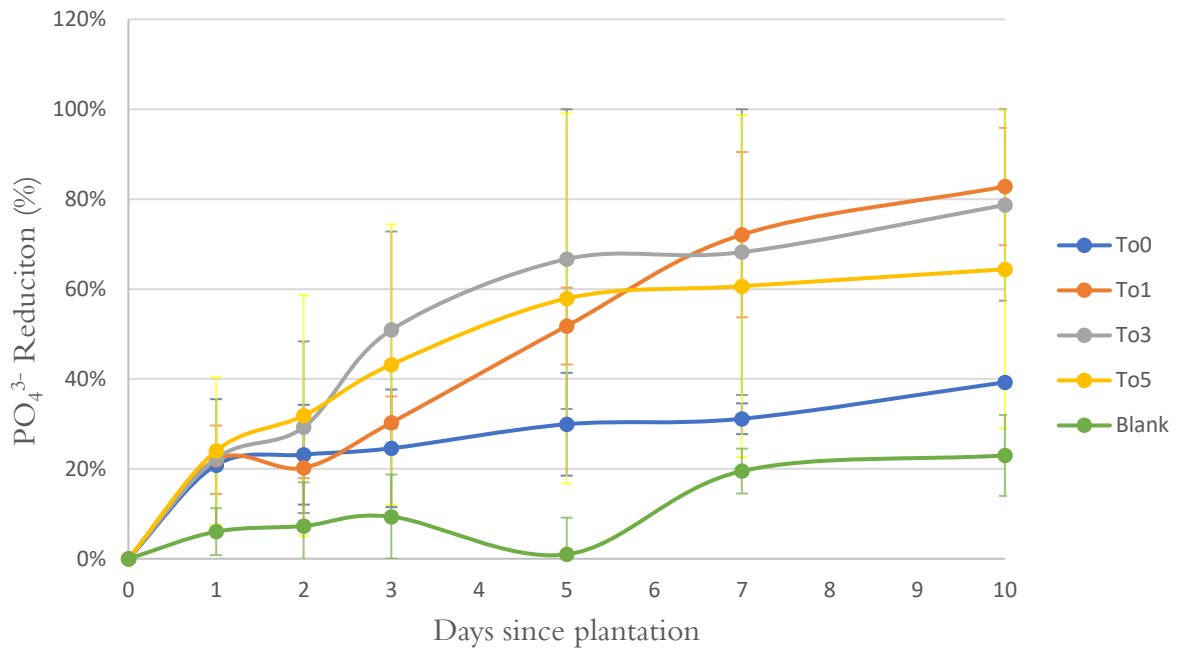


Figure 14: T. obliquus, data from replicate one and three. ± SD is given for each point and marked in the same colour as the data series. To0 to To5 indicate the number of pre-starvation days.

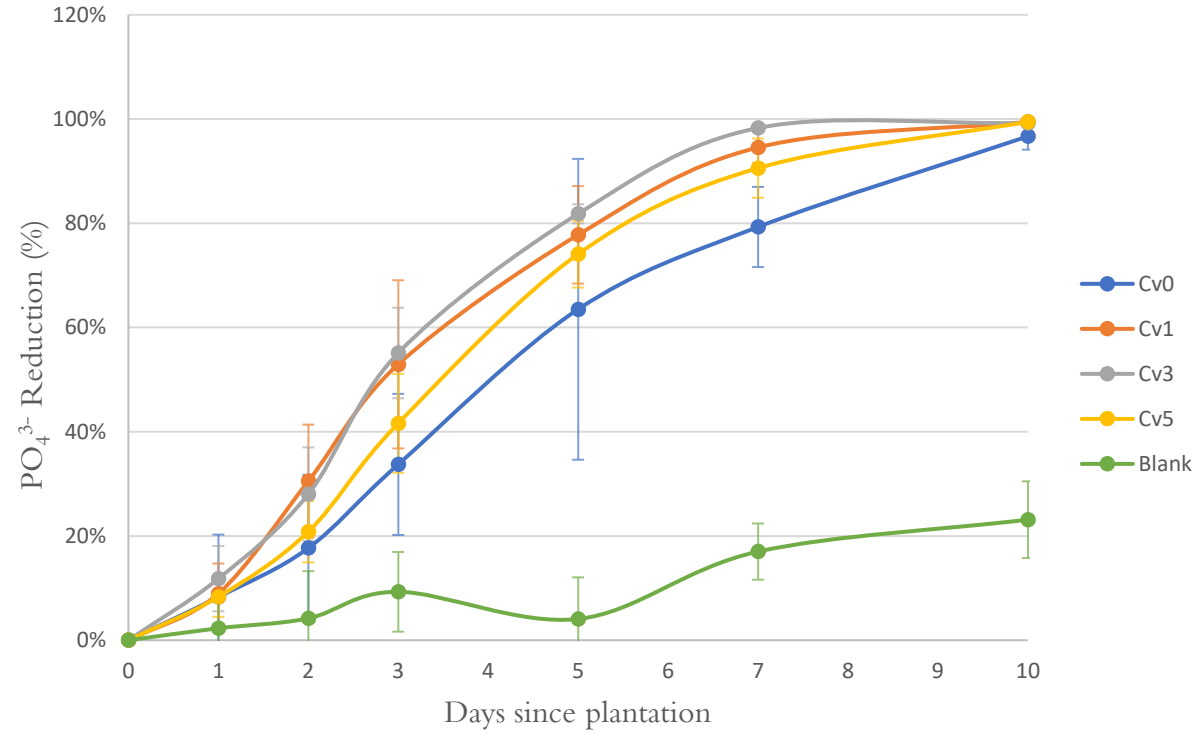


Figure 15: C. vulgaris, data from replicate one, two and three. ± SD is given for each point, marked in the same colour as the data series. Cv0 to Cv5 indicate the number of pre-starvation days.

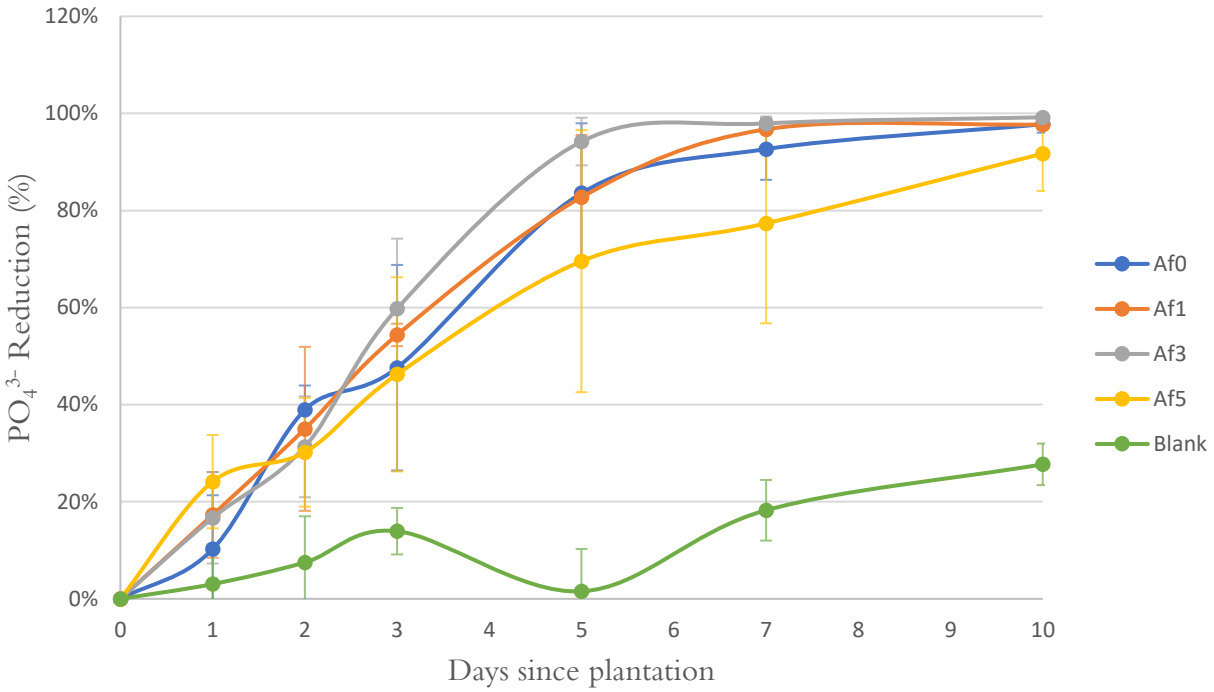


Figure 16: A. falcatus, data from replicate one and two. ± SD is given for each point, marked in the same colour as the data series. Af0 to Af5 indicate the number of pre-starvation days.

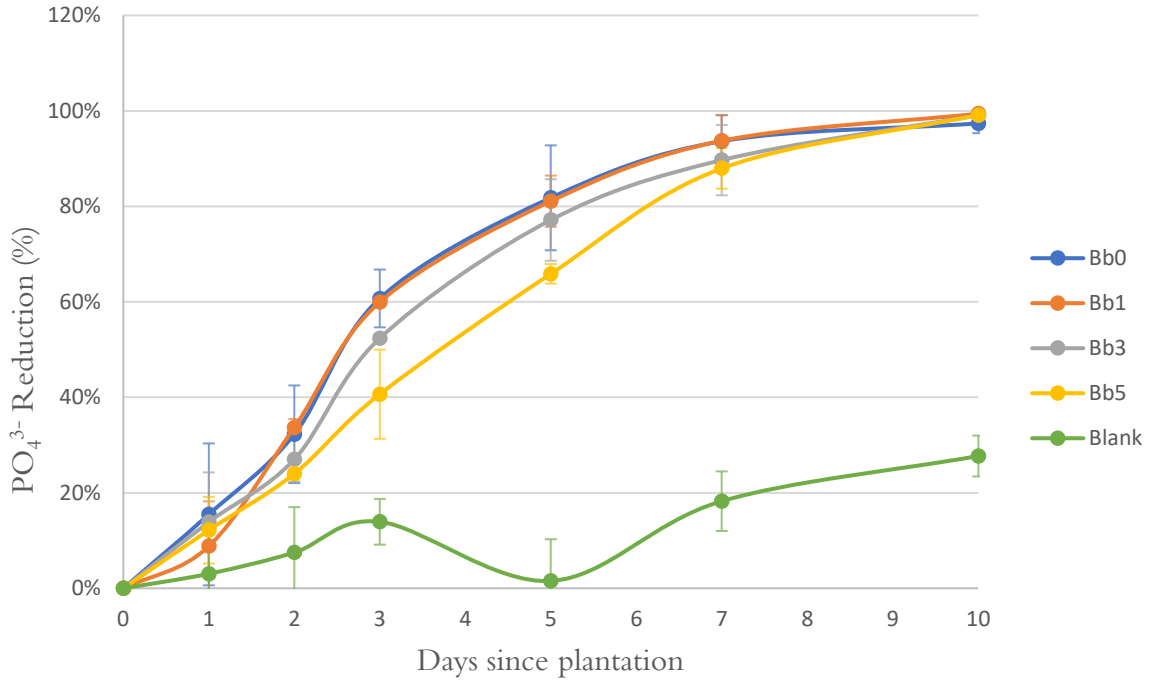


Figure 17: B. braunii, data from replicate one and two. ± SD is given for each point, marked in the same colour as the data series. Bb0 to Bb5 indicate the number of pre-starvation days.

The average day for a strain with a specific pre-starvation period, when the NO_3^- concentration was ≤ 1.0 mg/L ranges from four to seven and a half days as can be seen in Table 11 below. *D. communis* and *T. obliquus* were excluded since the NO_3^- concentrations in the reactors with these strains never reached ≤ 1.0 mg/L, except for *T. obliquus* pre-starved three days in replicate three, and *T. obliquus* pre-staved five days in replicate one.

Table 11: Days from planting until the concentration of NO_3^- was ≤ 1.0 mg/L. in a reactor for a strain with a specific pre-starvation period as well as its belonging SD.

Strain	pre-starvation days	Average day, [NO3-] ≤ 1.0 mg/L	σ
C. vulgaris	0	6,3	0,9
	1	5,0	0,0
	3	5,0	0,0
	5	5,0	1,6
A. falcatus	0	7,5	2,5
	1	6,0	1,0
	3	5,0	0,0
	5	5,0	0,0
B. braunii	0	4,0	1,0
	1	4,0	1,0
	3	5,0	0,0
	5	6,0	1,0

5 Discussion

5.1 Pre-experiment 1: Determination of the initial biomass concentration

As could be seen in Table 8 the initial biomasses in the batches did not respond to the calculated and planned ones. Because of this greater care was taken when calculating the transfer volumes between a cultivation bottle to an experiment bottle for pre-experiment 2, which was successful. One mistake which was understood after the experiment had been terminated was that when using equation 2 for calculating the volume to extract from the cultivation bottle, the transferal volume (the 15 ml of demineralised water) was not considered. However, the V2 in the plantation bottle should include the total volume, which in pre-experiment 1 corresponded to 915 ml and not the 900 ml which was used in the calculations.

In Figure 10 the initial biomass concentration of 0.078 g DW/L was the fastest batch to reduce the PO_4^{3-} to below 0.1 mg/l, which was unexpected. It was thought that a higher initial biomass concentration would decrease the PO_4^{3-} faster. It should be noted that the batch with initial biomass concentration of 0.078 g DW/L also had initial PO_4^{3-} concentration of 17.8 mg/L, while the other batches had lower initial PO_4^{3-} concentrations, mainly around 16 mg/L (Table 8). Which leads us to the question: what effect does the initial PO_4^{3-} concentration have on the removal rate of PO_4^{3-}? This was not investigated experimentally in this thesis, however, further down a discussion on the NPR will be debated.

Pre-experiment 1 did not serve its purpose to help determine the initial biomass concentrations in the main experiment. However, it contributed with practise for the people working with the experiments as well as insight on P removal and equation 1. There can also be other faults which contributed to the initial biomass concentrations not responding to the planned ones. For example, faults within the preparation works, within the measurement technology, within the sampling procedure, as well as some other, yet undetected fault could have contributed.

5.2 Pre-experiment 2: Determination of pre-phosphorus-starvation periods

From the second pre-experiment the pre-starvation periods to use in the main experiment could be successfully determined. It was also found that the initial biomass concentrations calculations and transferral process worked quite well, which was not the result in pre-experiment 1 (nor in the main experiment, which is discussed below). In pre-experiment 2 the initial biomasses concentration of the six starvation reactors was 0.24 g DW/L on average, and the target initial biomass concentration was 0.25 g DW/L. Some biomass loss in the transferral process during replanting, was expected. To refine the transferral method is a suggestion for future experiments. For example, when using equation 1, (V1 = (V2*C2)/C1), a constant could be added to C2, to account for the biomass loss in the transferral process. The equation could look something like this: V1 = (V2*(C2+A))/C1, where A is a constant, increasing the target biomass a little, so that the real biomass measured after plantation is closer to the target concentration. The number of A would have to be determined experimentally but judging from the pre-experiment 1 and 2 it could have been within the range 0.01 to 0.09.

5.3 Main experiment: Phosphorus reduction in wastewater by five algae strains with varying pre-starvation periods

5.3.1 Initial biomass concentration

5.3.1.1 Initial biomass concentration anomaly

Even though careful calculations were performed to ensure that the initial biomass concentration in the batch reactors should lie around 0.20 g DW/L for each strain, by using the spectrophotometer, the calibration curves in annex 6 and the knowledge obtained in pre-experiment 1 and 2, the initial biomass concentrations were a lot lower than expected, as can be seen in Table 9, where all strains together had average initial biomass concentration 0.14 g DW/L in replicate one and even lower in the following two replications (however the two following replicates were closer to their new target biomass concentration which was set to 0.15 g DW/L). This likely affected the result of the main experiment, since it is assumed that too low initial biomass concentrations made it difficult for the algae to start up a culture quickly, leading to the PO_4^{3-} reduction occurring slower than expected. The sampling schedule (Figure 8 in chapter 3.5.3), is designed with the thought that most of the PO_4^{3-} would be removed after three to four days, which did not happen. It is believed that low initial biomass concentration originated from two steps in the transferral process: when centrifuging and when replanting the algae. In the main experiment, the transferral process included moving algae first from the cultivation reactor to the BG0 filled Erlenmeyer flasks, and secondly from the Erlenmeyer flasks to the batch experiment reactors.

In replicate one, when planting the biomass in the BG0 flasks, biomass was taken from cultivation reactors with quite low biomass concentrations. This meant that usually two up to six vials were used for centrifuging. When the biomass had been centrifuged the separated BG0 was discarded. This is the first step where some biomass likely got discarded, together with the supernatant.

Then, the second step where it was likely that biomass got lost, was when pouring the biomass between the centrifuge vials to collect all biomass in one vial, before pouring the biomass into a plantation reactor. Every time when transferring biomasses between vials like this, it is likely that some biomass stays in the vial and is not transferred.

There are two simple ways of avoiding this problem, one which was used in replicate two and three. First, one could wait with transferring biomass from the cultivation reactor to other locations, until the biomass concentration in the cultivation reactor is high enough to make the transferral volumes low enough to use only one centrifuge vial. The second way to solve the problem is to use only one centrifuge vial and refill it with biomass solution after centrifuging it until the total transferral volume have been centrifuged in one vial. This is what was done in replicate two and three.

Even though the way the replantation was performed was changed, it was not enough to reach the target initial biomass concentration which was set 0.15 g DW/L in replicate two and three. Another change which could have been made, was to use a modified equation 1, as described in discussion 5.2.

5.3.1.2 The initial biomass concentration's effect on the phosphate removal

If a great number of algae cells are planted in a reactor, it can be assumed that they will consume the nutrients faster than if a low initial biomass concentration had been planted. This discussion is not on that - but if there is a lower limit of initial biomass concentration, where the biomass cannot grow.

In the first replicate, *D. communis* did not grow much at all (Figure 13). The total and greatest reduction of the PO_4^{3-} was performed by the algae that had been pre-starved one day and it reached to reduce 67 % of the PO_4^{3-} on day 10 (orange curve). Given that the reduction in the blank reactor reached 25 % on day 10 (Figure 13, grey curve) the *D. communis* did not remove much at all compared with the other strains (e.g., Figure 15). As it had the lowest initial biomass concentration of 0.10 g DW/L, (Table 9), setting it apart from the other strains, this could be a reason for why it did not thrive. *D. communis* did not regrow in time to be used in the other two replicates and this could not be further investigated. However, some data speaking against the theory that "a too low initial biomass concentration makes it difficult for the algae to grow" is the results of *T. obliquus*. In the second replicate *T. obliquus,* with an average initial biomass concentration of 0.13 g DW/L (Table 9), was discarded half-way into the experiment, as it did not grow at all. In replicate three, the average initial biomass concentration was 0.12 g DW/L, and it grew well (Figure 14). The *T. obliquus* algae which had been pre-starved for 3 days reduced the PO_4^{3-} the fastest and greatest (annex 11). Looking at the raw data (annex 11) it can be observed that the 3 days pre-starved *T. obliquus* in replicate three had the initial biomass concentration of 0.116 g DW/L, the lowest of all the *T. obliquus* examined in that replicate. It was also observed in pre-experiment 1 that one of the *C. vulgaris* batches with low initial biomass concentration (0.078 g DW/L) most efficiently removed the PO_4^{3-} (Figure 10).

It is likely that if there exists a limit of "the lowest initial biomass concentration which can yield biomass growth and nutrient reduction", it is specific to the strain. Therefore, this aspect of the nutrient removal remains a mystery. Albeit, in theory, if all other parameters are set to optimal conditions for PO_4^{3-} reduction (aka biomass production), the initial biomass concentration might not matter, however the timespan of the nutrient removal will be affected by the initial biomass concentration. The thing that could have made it difficult for the algae to grow in our experiment, could have been the bacteria in combination with the low initial biomass concentration. Perhaps what happened with *D. communis* in replicate one and *T. obliquus* in replicate two was that the bacteria ate the nutrients ahead of the algae.

5.3.2 Comparing the two pre-experiments with the main experiment on the nitrogen to phosphorus ratio and the pH

One reason for why the P was reduced slower than expected, could have been the NPR. Even with a low initial biomass concentration in pre-experiment 1, nearly a 100 % of the PO_4^{3-} was removed after six days (Figure 10). Meanwhile, in the main experiment, the PO_4^{3-} reduction took longer time (around 10 days) to reach nearly a 100 % removal (Figure 15–17). This could be because in pre-experiment 1, a mix of BG11 and WW was used, where the BG11 had an NPR of 77:1 (annex 10). It is estimated that the NPR in the two pre-experiments was around 60:1 (annex 10), and it is very likely that in these two pre-experiments P was the limiting nutrient. It was calculated that the WW from Roja WWTP had a NPR around 1.8:1 (Table 2 & annex 10 for calculations), based on the PO_4^{3-} and NO_3^-. Comparing this ratio with the

Redfield ratio 16:1 (Redfield, 1958) and numerous other studies (Arbib, et al., 2013; Choi & Lee, 2015; Hecky, et al., 1993), it can be concluded that the N was the limiting nutrient in the wastewater, affecting the P removal rate. The N was diminished quite fast (Table 11), by the strains that thrived in the main experiment (*C. vulgaris, A. falcatus* and *B. braunii*). Some of the *T. obliquus* batches thrived as well (pre-starved five days in replicate one and pre-starved three days in replicate three) and they consumed all NO_3^- as well (annex 11). It seems that the Redfield ratio, (16:1), can be used as a guide rather than a rule, and that the optimal NPR ratio depends on the algal strain and the parameters controlling the growth process.

Comparing the two pre-experiments to the main experiment, another difference is that in pre-experiment 1, the pH was not neutral, but around 8–9 (Figure 10). According to theory (Larsdotter, 2007) this would have led to substantial amounts of P precipitating, due to the presence of cations (see annex 3) and possible high concentrations of dissolved oxygen (provided by the mixing equipment). In the second pre-experiment the pH was not monitored, and whether P precepted or not is impossible to know.

5.3.3 The pre-phosphorus-starvation's influence on phosphorus reduction

It appears by looking at Figure 14–17 that the three-day pre-starvation period promoted the P reduction (especially for *C. vulgaris* in Figure 15, and for *A. falcatus* Figure 16). It also appears that one-day pre-starvation is the second-most efficient reducer in some cases (again Figure 15 and Figure 16) and the most efficient reducer in other cases (Figure 13, for *D. communis* and equal to the reference in Figure 17, *B. braunii*). For *T. obliquus*, both three-day and one-day of pre-starvation seemed to enhance the reduction (Figure 14), but one can argue that a three-day pre-starvation was the optimal one of the two, by looking at the raw data in annex 11 and pre-experiment 2.

In pre-experiment 2 it was observed that *T. obliquus* could remove P to ultralow levels within three days, when the algae had been pre-starved of P for two days ahead (Figure 11). This efficient removal can also be credited to bacteria and P precipitation as these two factors were not investigated in pre-experiment 2. However, relative to the other pre-starved batches which would have been affected by that as well, the two-days pre-starved batch was the most efficient one. In the main experiment the measurements inferred vastly different results in the two replicates *T. obliquus* participated in, which is reflected in the great spans of the standard deviations (Figure 14). *T. obliquus* only removed approximately 80 % of P within 10 days, for all pre-starvation periods. However, the raw data (annex 11) shows that in the first replicate, all *T. obliquus* batches except for the five days pre-starved batch, performed badly. The five days pre-starved batch reached a 100 % removal on day 10. In the third replicate the three-day pre-starved batch consumed all PO_4^{3-} withing five days, as opposed to *T. obliquus* pre-starved one day, consuming up to 96 % on day 10. In Figure 14 it can be observed that the three-day pre-starved and one day, compete on being the fastest reducer. Based on pre-experiment 2 and the raw data in replicate three (annex 11), it indicates that two to three days is the optimal pre-starvation period for *T. obliquus,* given that the algae are thriving (which was not always the case). The reference batch was never thriving in the main experiment and was not investigated in pre-experiment 2, and therefore the pre-starved batches could not be compared to it. The two to three-day pre-starved batch was the most efficient relative to the other pre-starved batches, not to the reference.

For *D. communis* the one day and three-day pre-starved batches worked the most efficiently (in that order) (Figure 13) of the batches, however none of the reactors reduced more than 67 % in the 10 days. As the algae only sufficed for one replicate, not much can be said, but this data grouped together with three of the other strains data, implies that pre-starvation enhances the P reduction rate.

For *C. vulgaris* all pre-starvation periods reduced the P at a quite similar rate (Figure 15), but the three-day pre-starved batch removed P the fastest. The one-day pre-starved batch was the second fastest and the reference batch was the slowest. Comparing the three-day pre-starved batch to the reference, it reached nearly a 100 % removal on day seven, whereas the reference achieved almost the same result on day 10. The *C. vulgaris* produced the most accountable data since it was used in all three replicates and only the reference batch had varied results in the replicates, as can be seen by the blue SD, spanning up to 30 % on day five. One can argue that a three-day pre-starvation period is optimal for *C. vulgaris*, once used in the same conditions as in this experiment. However, since a two-day starvation period was not investigated, and both three and one-day of pre-starvation seemed to enhance the P removal, perhaps a two-days pre-starvation period could work even better than a three-day pre-starvation period.

B. braunii had the opposite result of the others (Figure 17). The reference and one-day starved batches removed P most efficiently, in that order. It seems that the longer *B. braunii* is starved the slower it removed the P. *B. braunii* displayed the least SD, both regarding the PO_4^{3-} reduction and the NO_3^- reduction, attributing it a predictable reduction curve.

For *A. falcatus* the three-day pre-starvation period appeared to be the optimal one, removing nearly a 100 % after seven days (Figure 16). The one-day pre-starved batch ran up to a close second most efficient P remover, both implying that pre-starvation within the span of one to three days enhanced the P removal rate in the batch reactor. For *A. falcatus* and *C. vulgaris* the three-day pre-starved batch removed nearly a 100 % of the P 1.42 times faster than the reference.

5.4 Conditions to consider when using microalgae for nutrient removal

As can be seen in the literature study and the experiments, the conditions in which the algae grow, significantly affects the nutrient removal rate (Ferro et al, 2018; Arbib et al, 2013). The ranges in which the algae can operate vary among the species, and it was not thoroughly investigated in this thesis (only pre-P-starvation). However, from the findings in the experiments and the literature, it seems that the temperature, light, pH, and the NPR greatly impact the nutrient removal rate. For marine algae, the salinity of the growth media also affects the growth rate (FAO, 1996), and inversely, the absence of salt likely has importance when cultivating freshwater algae.

Another observation originating from the literature study and the experiments is that the choice of strain also greatly impacts the P removal. Primarily, for the P-removal to occur the strain must thrive in the local conditions. Some strains cannot handle the Nordic climate (Ferro et al., 2108) and some strains appeared to be sensitive to the changes in the main experiment (which inferred moving between different environments and P-starvation). Especially *D. communis* and *T. obliquus* were difficult to regrow in the WW in the main experiment. It was observed that the NPR in the algal biomass is different for different species (Arbib et al., 2013; Hecky, et al., 1993), which means they will incorporate different amounts of P relative to N molecules depending on their genetic setup.

A parameter which was seldom mentioned in the literature, but was essential in the experiments, was the competition of nutrients between algae and bacteria. The reason for why all growth media was sterilised and the WW was filtered, was to minimise the chance of bacteria outcompeting the algae. This is also important to consider when growing algae, as the bacteria can be a reason for why an algae culture dies. Either sufficient algal biomass must be induced in a new environment, or the bacteria load must be decreased, when culturing algae.

When searching for a specific strain to use as remediation treatment, one should search for an algae strain which thrives in a climate similar to the media which needs remediation. If possible, local strains which already exist in the media are to be preferred, as it is already proven that they can grow there.

5.5 Connecting the results to further experiments and future use

The purpose of this experiment was to investigate the P removal rate of algae, depending on how long they have been starved of P before being entering the WW. However, connecting this experiment to commercial use, it is thought that microalgae could be used in smaller WWTPs and on-site systems in the future, as these likely have similar properties to the WW tested in the experiments. It is important to remember that in these experiments the average temperature was quite high (around 27°C) and that the secondary wastewater was nutrient rich, with tot-P around 20 mg/l and tot-N around 24 mg/L (Table 2).

Before any commercial use of microalgae will take place at full scale, a thorough mapping of different strains behaviour regarding P uptake and pre-starvation, can be of good use. Especially since P-starvation for one to three days could be induced in a WWTP, to totally reduce the retention time. As seen in Figure 15 and 16, the three-day pre-starvation of *C. vulgaris* and *A. falcatus* made the reduction of PO_4^{3-} (to nearly a 100 %) appear three days faster than the reference batches. Perhaps a future solution could be to work with two (or more) reactors, where one is in starvation mode and the other is in reduction mode. They could switch mode regularly, so that the algae are always either eating - or being starved of P.

When investigating how pre-starvation of P affects the rate of the P removal, all parameters controlling the biomass production should be set to promote P reduction. In this experiment it was found that N was limited in the wastewater and that a too low initial biomass concentration took a long time to grow - and analogously a long time to remove the P. If this had not been the case, if there had always been enough N and a higher initial algal biomass concentration, perhaps a clearer differentiation on how pre-starvation affected the P reduction could have emerged.

In this experiment the WW from the secondary clarifier was filtered three times to remove coarser particles and bacteria, to avoid bacteria outcompeting the algae in the reactors. If the algae were to be used in small scale treatment plants in the future, this filtration would not occur. If this becomes a treatment method, perhaps a larger initial biomass concentration could be implemented in the WW, equalising the competition of nutrients between the bacteria and algae. Perhaps a treatment method where bacteria and algae work in symbiosis can be refined, since this method already exists, as described by Oswald & Gotaas in 1957.

A benefit with using microalgae in WWT instead of chemical dosing technology in tertiary treatment has been highlighted in a review by Whitton et al (2018). The authors state that microalgal treatment is not expected to demand safety showers, chemical storage facilities and better infrastructure inside and around the plant, which might contribute to them being an easier solution to implement in small-scale (sometimes rural) treatment plants. However, this is not for certain since the technology of separating the algae from the water might be advanced and less easy to implement. However, this was not investigated in this thesis and is something which needs be considered when developing WWT methods with microalgae. In addition to that, even if the PO_4^{3-} concentration reaches below 0.1 mg/L by the microalgae, if the algae are not completely separated from the water, the effluent will still contain P - organically bound in algae. In a review by Branyikova et al. (2018), it is reported that harvesting techniques of microalgae from water were able obtain in general at most 90% of the cultivation from the water. However, in small scale treatment plants, when choosing between implementing chemical dosing

technology and microalgae for reducing P, a similar removal efficiency could potentially be reached with both treatment technologies (if 90 % of microalgae (obtaining about a 100% of the PO_4^3) can be removed), only microalgae doing it in a more sustainable way. That is something that could be interesting to investigate further – comparing chemical dosing technology and microalgae, in small-scale treatment plants, regarding parameters such as energy use, waste generation, costs and effluent P concentrations.

Microalgae are living things and need specific conditions to survive and reproduce as described in section 5.4 and therefore can be difficult to maintain, since the influent WW might have varying properties. Also, even if some algae perform well in moderate winter climate, like *C. vulgaris* in the study by Lorenza Ferro et al. (2018), the time it took for the algae to reach a 100 % removal, (40–45 days), is likely too long to be practical in a conventional treatment plant, where retention time usually needs to be shorter.

To be able to use microalgae in WWTPs, further experimentation with cold-climate setups, non-pre-filtered WW and a less nutrient rich media is recommended as well as inducing greater biomass concentrations. If N is often the limiting constituent in tertiary WW, perhaps an algae strain which uses less N in relation to P could be found in natural environment which mimics that setup.

5.6 The Baltic Sea and wastewater treatment

The obvious benefit of using microalgae to reduce P in the effluent of WWTPs, would be to reduce the P load to the Baltic Sea. Using microalgae instead of chemical dosing technology when conducting tertiary treatment of WW could also be preferred since they use atmospheric CO_2, while producing O_2, which is a great way to reduce greenhouse gas emissions. Especially since atmospheric CO_2 is constantly dissolved into the sea, affecting the carbonate system, and reducing the pH in the surface waters. The effects of this can be observed in the Great Barrier Reef, where the acidified state has led to chalk constellations, such as shells and corals, dissolving to balance out the increase of hydrogen ions appearing when the sea is absorbing CO_2 (Australian government, 2020).

However, small-scale treatment plants are just one of the many contributors of the P loads to the Baltic Sea, and how big their impact is in comparison to bigger municipal WWTP was not investigated in this thesis. As described in the theory (section 2.1) agricultural loads as well as transboundary sources also accounts for the P load to the Baltic Sea. To reduce the eutrophication, the nutrient loads (both N and P) need to be decreased in as many pathways as possible. Using microalgae to reach low concentrations of inorganic P in secondary WW is just one of many ways to achieve that. For example, to decrease the number of sewer overflows and implementing wastewater source-separating systems are two other ways to decrease the nutrient load to the Baltic Sea. Also, changing the way fertilizers are used in farming systems could likely have a great impact of the P load to the Baltic Sea as well.

5.7 Sources of errors

The list of faults can be made long, mainly due to human errors. Here, only the ones which most certainly occurred, are mentioned.

The data presentation of the main experiment (Figure 13–17) displays the total reduction in the reactors, which can mislead the reader to believe that the reduction in the algae reactors was made by solely algae. The total reduction infers the reduction of PO_4^{3-} by both algae and other processes. It can be seen in the diagrams that in the blank reactor, the other processes accounts for nearly 14–32 % of the PO_4^{3-} concentration reduction. The reason for why the blank reactors' reduction is not subtracted from the total reductions is because that could lead to an even greater error. Doing that would mean that it is assumed that the bacteria and precipitation in the algae-reactors would reduce the same percent of PO_4^{3-} as the bacteria and precipitation in the blank reactor, which might not be the case. It can be assumed that the algae bring some bacteria of their own to the reactors, and if these bacteria (or the algae itself) outcompete the bacteria from the wastewater, is impossible to know. The precipitation is directly connected to the pH and will therefore respond to the pH in the specific reactor. Therefore, the total reduction is displayed, albeit it is important to remember that the total reduction infers the reduction by algae, bacteria and possibly precipitation of P. For the future, to see the effect of the bacteria present in the algae strains, could perhaps be done by isolating the bacteria from the algae, and then run the experiments with that bacteria plus the bacteria present in the WW.

Another source of faults could have been the timespan between the sampling and the analyses performed. In the main experiment there were a lot of parameters examined, where some of them, like the polyphosphates and alkaline enzyme activity, took a long time. Information about these parameters were not included in this thesis, as there was not enough time and space in this degree project. However, these analyses contributed to the workload and time-delay between sampling and analysing. Especially when measuring the temperature in the main experiment, where often two or three hours passed between sampling and measurement. Since the samples were put in 12 ml vials, it can be assumed that they had acquired room temperature after a short time. For this reason, the average temperature stated in Table 5 comes from five samples from replicate one, measured directly after sampling. The temperature does not originate from the raw data and is therefore excluded from annex 11. To obtain a correct temperature in the two pre-experiments the average temperature (in Table 3 and Table 4) was obtained by filling a reactor with tap water, moving it around the shelves with 24 h interval, and measuring the temperature on each shelf. However, this temperature was not deemed to be the same as in the main experiment. In the main experiment the shelves were full of reactors (in the two pre-experiments less reactors were used) which absorbed and retained heat from the lamps, contributing to the slightly higher temperature in the main experiment.

The fact that the PO_4^{3-} concentration was measured with such long timespans in between the analyses at the end of the ten days period could contribute to faults. Perhaps the concentration less than 0.1 mg/L of PO_4^{3-} was reached on day, four, six, eight or nine, and not on day five, seven and ten. But it is impossible to know since it was not measured on these days.

Another misleading factor in this thesis is that a two-days pre-starvation period was not investigated in the main experiment and thus is not named as the optimal period. However, since three-day and one-day of pre-starvation seemed to enhance the P removal rate for most of the strains, it is very possible that a two-days pre-starvation period could have been the optimal starvation period for some strains.

6 Conclusions

The use of microalgae to reduce phosphorus concentrations in wastewater to less than 0.1 mg/L is possible if the conditions are set for optimal nutrient reduction. From the findings in the literature these conditions included: a strain which thrived in the local environment, a nitrogen to phosphorus ratio which made the nutrient removal break even or was not limited by nitrogen (in which case the nitrogen might be completely removed, but not the phosphorus), an adequate temperature, enough light, proper mixing, and enough CO_2 to substrate the biomass production and to keep the pH neutral. From the experiments it was confirmed that the nitrogen to phosphorus ratio as well as the choice of strain effected the result. In the experiments it was also found that an adequate initial algal biomass concentration was required to avoid bacteria outcompeting the algae, a parameter which was seldomly mentioned in the literature, but essential in the experiments. If these conditions are not met, the nutrient removal might not reach ultralow levels within the desired timespan, or ever - if one key condition is limiting the algae growth it analogously limits the phosphorus uptake in the algae.

In this study, pre-phosphorus-starvation enhanced the phosphorus uptake rate of most of the investigated microalgae strains, especially pre-starvation within a one to three days span. In this study a three-day pre-starvation period was optimal for *C. vulgaris* and *A. fâlcatus*. For *T. obliquus* two to three days of pre-starvation was optimal compared to the other pre-starvation periods. During the experimental time, the reference batch of *T. obliquus* did not thrive well, therefore no comparison between the pre-starved batches and the reference batch could be made. For *B. braunii*, zero to one days of pre-starvation was optimal, and longer pre-starvation retarded the phosphorus reduction rate. For *D. communis* no clear conclusions could be drawn. Two-days of pre-starvation was not thoroughly investigated in this thesis but could also be optimal for some strains. Concentrations less than 0.1 mg/L phosphate were reached in a minority of the reactors (in 35 %) within the 10 days of phosphorus reduction by algae. Concentrations less than 1 mg/L was more common on day 10, reached in 70 % of the reactors.

Alkaline pH can contribute to phosphorus precipitation, which can be mistakenly interpreted as removed by algae. Phosphorus precipitation and removal by bacteria most likely contributed to the phosphorus removal rate explored in these experiments.

If implementing microalgae as a treatment method, it is important to consider the choice of strain, as the strains endurance towards different environmental changes appears to differ greatly. For example, *T. obliquus* removed phosphate to below 0.1 mg/L within five days, when it was thriving. In many of the reactors *T. obliquus* did not thrive, for unknown reasons, as the conditions were the same in all reactors. Therefore, selecting a more durable strain like *C. vulgaris*, might be wiser since it thrived in all reactors and regrew fast enough to participate in all three experiments. *C. vulgaris* was also the strain that on average removed the phosphate most efficiently in the main experiment. Both *C. vulgaris* and *T. obliquus* achieved these results under three days of pre-phosphorus-starvation. The most suitable strains for further research with pre-starvation aiming for usage in WWT are thereby *C. vulgaris* and *T. obliquus*.

The use of microalgae for treatment of wastewater could be meaningful in small-scale treatment plants where nutrient concentrations might be higher, and the infrastructure around- and inside the plant is sometimes not very advanced. That is because microalgal treatment as opposed to chemical dosing treatment does not require safety equipment such as potable water safety showers, chemical storage facilities and better infrastructure inside and around the plant. There could be many more benefits gained by implementing microalgae as a treatment method, since they reduce atmospheric and dissolved carbon dioxide while producing oxygen and biomass. The biomass is a valuable product as opposed to chemical sludge, which is a waste. However, more experiments with colder climate, non-pre-filtered wastewater, a less nutrient rich media, greater initial biomass concentrations and pilot tests are recommended before implementing microalgae in already existing treatment plants. In Table 12 below the advantages and disadvantages of implementing microalgae as a treatment method investigated in this thesis are summarised.

Table 12: List of advantages and disadvantages of using microalgae as tertiary treatment.

Advantages	Disadvantages
Remove inorganic phosphorus below 0.1 mg/L.	Long retention time.
Consume carbon dioxide.	Nitrogen might be limited in wastewater which decreases efficiency of method.
Produce oxygen.	Sensitive to surrounding conditions.
Do not create a waste sludge, but a valuable product.	Method for separating algae from water might be advanced.
Facilitate nutrient recovery.	

Another insight found while performing the experiments was that the equation ($V1 \star C1 = V2 \star C2$) and methods used for transferring algal biomass between two medias could be refined, to facilitate reaching the target biomass in the media which the biomass is being transferred into.

7 References

Arbib, Z., Ruiz, J., Álvarez-Díaz, Pablo, Garrido-Pérez, C., Barragan, J. & Perales, J. A. (2013). Photobiotreatment: influence of nitrogen and phosphorus ratio in wastewater on growth kinetics of Scenedesmus Obliquus. (2013). *International Journal of Phytoremediation, 15*(8), 774–788.https://doi.org/10.1080/15226514.2012.735291

Australian government/Great Barrier Reef Marine Park Authority (2020). *Ocean acidification.* https://www.gbrmpa.gov.au/our-work/threats-to-the-reef/climate-change/ocean-acidification

Bránykiková, I., Prochazkova, G., Potočár, T., Jezkova, Z. & Brányik, T. (2018). Harvesting of Microalgae by Flocculation. *Fermentation 4*(93). DOI: 10.3390/fermentation4040093

Brown, N. & Shilton, A. (2014). Luxury uptake of phosphorus by microalgae in waste stabilisation ponds: current understanding and future direction. *Reviews in Environ Science and Bio/technology, 13*(3), 321–328. https://doi.org/10.1007/s11157-014-9337-3

Carstensen, J., Gustafsson, B., Andersen, J. H. & Conley, D. J. (2014). Deoxygenation of the Baltic Sea during the last century. *Proceedings of the National Academy of Science of the United States of America 111*(15), 5628–5633.

Center for Sustainable Systems, University of Michigan. (2020). *U.S. Wastewater Treatment Factsheet* [Factsheet]. Pub. No. CSS04-14.

Choi, H. J., & Lee, S. M. (2015). Effect of the N/P ratio on biomass productivity and nutrient removal from municipal wastewater. *Bioprocess and biosystems engineering, 38*(4), 761–766. https://doi.org/10.1007/s00449-014-1317-z

Crimp, A., Brown, N., & Shilton, A. (2018). Microalgal luxury uptake of phosphorus in waste stabilization ponds - frequency of occurrence and high performing genera. *Water Science and Technology: A Journal of the International Association on Water Pollution Research, 78*(1–2), 165–173. https://doi.org/10.2166/wst.2017.632

Eixler, S., Karsten, U. & Selig, U. (2006). Phosphorus storage in Chlorella vulgaris *(Trebouxiophyceae, Chlorophyta)* cells and its dependence on phosphate supply. *Phycologia, 45*(1), 53-60. https://doi.org/10.2216/04-79.1

Elmgren, R. (2001). Understanding Human Impact on the Baltic Ecosystem: Changing Views in Recent Decades. *AMBIO, 30*(4-5), 222-31. http://www.jstor.org/stable/4315140

Ferro, L., Gorzsás, A., Gentili, F., & Funk., C. (2018). Subarctic microalgal strains treat wastewater and produce biomass at low temperature and short photoperiod. *Algal Research 35*, 160–167. https://doi.org/10.1016/j.algal.2018.08.031

Food and Agriculture Organization of the United Nations. (1996). *Manual on the Production and Use of Live Food for Aquaculture* (FAO Fisheries Technical Paper. No. 361). Rome, FAO. *http://www.fao.org/3/w3732e/w3732e00.htm#Contents*

Griffiths, D. J. (2013). *Microalgae and Man* [Electronic recourse]. Nova Science Publishers, Inc. https://libris-kb-se.proxy.lib.ltu.se/bib/18696352?vw=full&tab3=full

Grobbelar, U, J. (2004). Algal nutrition: Mineral nutrition. Richmond (Editor). *Handbook of microalgal culture: biotechnology and applied phycology* (p.97-115). Blackwell Science Ltd.

Guldhe, A., Ansari, A., F., Singh, P., & Faizal Bux. (2017). Heterotrophic cultivation of microalgae using aquaculture wastewater: A biorefinery concept for biomass production and nutrient remediation. *Ecological Engineering,* 99, 47-53. https://doi.org/10.1016/j.ecoleng.2016.11.013

Hecky, R, E., Campbell, P. & Hendzel, L, L. (1993). The stoichiometry of carbon, nitrogen, and phosphorus in particulate matter of lakes and oceans. *Limnology and oceanography. 38(4), 709-724.* https://doi.org/10.4319/lo.1993.38.4.0709

HELCOM. (2018A). *State of the Baltic Sea - Second HELCOM holistic assessment 2011-2016* (Baltic Sea Environmental Proceedings 155). Helsinki: Helsinki Commission.

HELCOM. (2018B). *Sources and pathways of nutrients to the Baltic Sea* (Baltic Sea Environment Proceedings No. 153). Helsinki: Helsinki Commission.

Inc. Metcalf & Eddy., Bowden, G., Abu-Orf, M., Pfrang, W. (2014). Wastewater Engineering: Treatment and Resource Recovery (Fifth edition). McGraw-Hill Publication.

Larsdotter, K., Cour Jansen, J., & Dalhammar, G. (2007). Biologically mediated phosphorus precipitation in wastewater treatment with microalgae. *Environmental Technology, 28(9), 953-960.* DOI: 10.1080/09593332808618855

Lavens, P., & Sorgeloos, P (Eds.). (1996). *Manual on the Production and Use of Live Food for Aquaculture* (FAO fisheries technical paper 361). Food and Agriculture Organization of the United Nations. http://www.fao.org/3/w3732e/w3732e00.htm#Contents.

Li, Y., Chen, Y., Chen, P., Min, M., Zhou, W., Martinez, B., Zhu, J., & Ruan, R. (2011). Characterization of a microalga Chlorella sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. *Bioresource Technology, Volume 102*(8). 5138-5144, https://doi.org/10.1016/j.biortech.2011.01.091.

Masojidek, J., Koblızek, M., & Torzillo, G. (2004). Photosynthesis in Microalgae. Richmond (Editor). *Handbook of microalgal culture: biotechnology and applied phycology,* 20-40. Blackwell Science Ltd

Mohsenpour, F. S., Hennige, S., Willoughby. N., Adeloye, A., & Gutierrez, T. (2021). Integrating micro-algae into wastewater treatment: A review. *Science of The Total Environment*, 752, article 142168 X-X. https://doi.org/10.1016/j.scitotenv.2020.142168

Murwanashyaka, T., Shen, L., Yang, Z., Chang, J.-S., Manirafasha, E., Ndikubwimana, T., Chen, C., & Lu, Y. (2020). Kinetic modelling of heterotrophic microalgae culture in wastewater: Storage molecule generation and pollutants mitigation. *Biochemical Engineering Journal, 157*, article 107523. https://doi-org.proxy.lib.ltu.se/10.1016/j.bej.2020.107523

Nationalencyklopedin. (n.d.). Fotosyntes [Photosynthesis]. Retrieved 2020-09-16 from http://www.ne.se.proxy.lib.ltu.se/uppslagsverk/encyklopedi/lång/

Nzayisenga, J. C., Eriksson, K., & Sellstedt, A. (2018). Mixotrophic and heterotrophic production of lipids and carbohydrates by a locally isolated microalga using wastewater as a

growth medium. *Bioresource Technology, 257*, 260–265. https://doi.org/10.1016/j.biortech.2018.02.085

Oswald, W., & Gotaas, H. (1957). Photosynthesis in Sewage Treatment. *Transactions of the American Society of Civil Engineers, 122*(1), 73-105. Retrieved November 25, 2020, from http://content-calpoly-edu.s3.amazonaws.com/ceenve/1/images/57_photosynthesis_in_sewage_treatment.pdf

Pires, J. (2017). *Microalgae as a Source of Bioenergy: Products, Processes and Economics* [Electronic recourse]. Bentham Ebooks. 978-1-68108-522-7

Redfield,C, A. (1958). The biological control of chemical factors in the environment. *American Scientist 46*(3), 205–221. Retrieved November 17, 2020, from https://ebme.marine.rutgers.edu/HistoryEarthSystems/HistEarthSystems_Fall2008/Week4b/Redfield_AmSci_1958.pdf

Rhee, G, Y. & Gotham, I, J. (1980) Optimum N:P ratios and coexistence of plankton Algae. (1980). *Journal of Phycology, 16(*4), 486–489. DOI:10.1111/j.1529-8817.1980.tb03065.x

Rockström, J (producent) (2020). Webbinarium: *Jordens framtid i Antropocen* [Akademiföreläsning] [The future of the earth in Anthropocene. Academy Lecture, only available in Swedish]. Kungliga Vetenskaps-akademien. https://www.kva.se/sv/kalendarium/webbinarum-jordens-framtid-i-antropocen

Tomaselli, L. (2004). The Microalgal Cell. Richmond (Editor). *Handbook of microalgal culture: biotechnology and applied phycology* (p.3). Blackwell Science Ltd

Torres Bustillos, L. G. (2015). Microalgae and Other Phototrophic Bacteria: Culture, Processing, Recovery and New Products [Electronic Recourse]. Nova Science Publishers Incorporated. https://libris-kb-se.proxy.lib.ltu.se/bib/21073112

Whitton, R., Le Mével, A., Pidou, M., Ometto, F., Villa, R. & Jefferson, B. Influence of microalgal N and P composition on wastewater nutrient remediation. (2016). *Water research 91,* 371-778. https://doi.org/10.1016/j.watres.2015.12.054

Whitton, R., Ometto, Pidou, M.,F., Jarvis, P., Jefferson, B., Raffaela, V., & Jefferson, B. (2015). Microalgae for municipal wastewater nutrient remediation: mechanisms, reactors and outlook for tertiary treatment. *Environmental Technology Reviews*, *4*(1), 133-148. https://doi.org/10.1080/21622515.2015.1105308

Williams, R. (26-Oct-2013). *Microscopic algae produce half the oxygen we breathe* [Audio]. The Science Show. URL. https://www.abc.net.au/radionational/programs/scienceshow/microscopic-algae-produce-half-the-oxygen-we-breathe/5041338

Annex 1 – The *Euglena gracilis* medium recipe

culturecollection of algae and protozoa

media recipes

EG (Euglena gracilis medium)

Freshwater algae and protozoa

Stock		**per litre**
(1)	$CaCl_2$ stock solution:	
	$CaCl_2$	1.0 g

Medium	**per litre**
Sodium acetate trihydrate	1.0 g
"Lab-Lemco" powder (Oxoid L29)*	1.0 g
Tryptone (Oxoid L42)*	2.0 g
Yeast extract (Oxoid L21)*	2.0 g
$CaCl_2$ stock solution (1)	10.0 ml

Add constituents above and make up to 1 litre with deionized water. For agar add 15 g per litre *Bacteriological Agar (Oxoid L11). Autoclave at 15 psi for 15 minutes.

Supply
* Unipath Ltd, Wade Road, Basingstoke, Hants RG24 0PW, UK

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

Annex 2 – The Blue-Green Medium recipe

culturecollection of algae and protozoa

media recipes

BG11 (Blue-Green Medium)

Freshwater algae and protozoa

Stocks		**per 500ml**
(1)	$NaNO_3$	75.0 g
		per 500 ml
(2)	K_2HPO_4	2.0 g
(3)	$MgSO_4.7H_2O$	3.75 g
(4)	$CaCl_2.2H_2O$	1.80 g
(5)	Citric acid	0.30 g
(6)	Ammonium ferric citrate green	0.30 g
(7)	$EDTANa_2$	0.05 g
(8)	Na_2CO_3	1.00 g
(9)	Trace metal solution:	**per litre**
	H_3BO_3	2.86 g
	$MnCl_2.4H_2O$	1.81 g
	$ZnSO_4.7H_2O$	0.22 g
	$Na_2MoO_4.2H_2O$	0.39 g
	$CuSO_4.5H_2O$	0.08 g
	$Co(NO_3)2.6H_2O$	0.05 g

Medium	**per litre**
Stock solutions 1 - 8	10.0 ml each
Stock solution 9	1.0 ml

Make up to 1 litre with deionized water. Adjust pH to 7.1 with 1M NaOH or HCl. For agar add 15.0 g per litre of Bacteriological Agar (Oxoid L11)*. Autoclave at 15 psi for 15 minutes.

To reduce precipitation autoclave stocks 5 + 6 separately in 100ml deionized water and then add to rest of medium (autoclaved) aseptically when cool.

To make up medium from stock solutions supplied by CCAP, please refer to the labels on each stock for volume per litre.

Make up to 1 litre with deionized water. Adjust pH to 7.1 with 1M NaOH or HCl. For agar add 15.0 g per litre of Bacteriological Agar (Oxoid L11)*. Autoclave at 15 psi for 15 minutes.

Supply

*Unipath Ltd, Wade Road, Basingstoke, Hants, RG24 0PW, UK

Reference

Stanier RY, Kunisawa R, Mandel M & Cohen-Bazire G (1971) Purification and properties of unicellular blue-green algae (Order Chroococcales). Bacteriol. Rev. **35**: 171-205.

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

Annex 3 - The 5 × concentrated Blue-Green Medium recipe

When preparing the Stock solutions 1-8 (SS(1-8)), instead of preparing 500 ml stock for each constituent, as in the original recipe, a 100 ml for each constituent of SS(1-8) was prepared, except for $NaNO_3$ (stock 1), where the weight of the $NaNO_3$ was divided by 5, but then 1000 ml of denaturalized water was added to it. Then instead of adding 10 ml from each 500 ml SS(1-8) stock + 1 ml of trace metal solution (9) to 919 ml of deionized water, to create the final BG11, 50 ml of each 100 ml of stock was added into a 1-litre flask, as well as 500 ml of the one litre of $NaNO_3$. Also 10 ml of trace metal solution (9) was added to this. The trace metal solution (9) had been prepared earlier by Aigars Lavrinovics and was also concentrated 5 times. After this, 0,5-2 ml of 1 molar Hydrochloric acid (HCl) was added to the 1 litre flask of concentrated BG11 until the pH reached 7.0-7.2. The pH in the SS(1-8) solution settles on alkalic values, where phosphorus starts to precipitate. This could be observed by the naked eye, as a white fog-looking appearance in the flask. When adding acid until the pH descend withing the range 7.0-7.2, the medium clarifies, and phosphorus stays dissolved.
Finally, demineralised water was added until the total volume of the flask was 1 litre. This produced a final BG11 which was 5 times concentrated. The growth media was kept in the fridge for storage, and when needing it; $1/5^{th}$ of the stock media (1-9) was mixed with $4/5^{th}$ of denaturalized water. The full measurements and calculations are shown below.

	Original Recipe				Our 5 × Concentrated Recipe		
Nr	Stocks	g/500 ml	ml/stock-bottle	Add to 1 litre flask (ml)	g /100 ml	ml/stock-bottle	Add to 1 litre flask (ml)
1	$NaNO_3$	75	500	10	15	1000	500
2	K_2HPO_4	2	500	10	0,4	100	50
3	$MgSO_4 \star 7H_2O$	3,75	500	10	0,75	100	50
4	$CaCl_2 \star 2H_2O$	1,8	500	10	0,36	100	50
5	Citric acid	0,3	500	10	0,06	100	50
6	Ammonium ferric citrate green	0,3	500	10	0,06	100	50
7	$EDTANa_2$	0,05	500	10	0,01	100	10
8	Na_2CO_3	1	500	10	0,2	100	50
9	Trace metal solution		1000	1			5
Total ml or g produced		84,2	5000		16,84	1700	
Total ml of stocks/1-litre bottle				81			815
Add H2O-De to 1 litre bottle				919			185

Original recipe $NaNO_3$: 75 g/ 500 ml = 150 g/1000 ml We do 15g/1000 ml instead of 15g/100 ml. Then we add 500 ml to each flask which results in 7,5 g per flask, which is NaNO3 concentrated 5 times like the rest of the stocks

The EDTA Na_2 stock is to light to weigh if divided by 5. A 5 times concentrated stock media of 100 ml was prepared, and then only 5 ml of that stock was added to the 5⋆concentrated BG11 stock.

Annex 4 – HACH procedure manual, orthophosphate measurement

Method 8048

PHOSPHORUS, REACTIVE (0.00 to 5.00 mg/L PO_4^{3-})

PhosVer 3 Method, Test 'N Tube Procedure **For water, wastewater, and seawater**
USEPA accepted for reporting wastewater analysis*

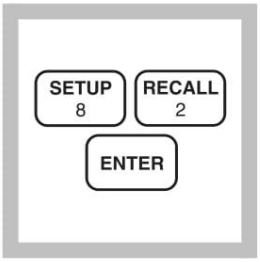
1. Enter the stored program number for reactive phosphorus (PO_4^{3-}), Test 'N Tube.

Press: **PRGM**

The display will show:

PRGM ?

Note: *For most accurate results, perform a Reagent Blank Correction using deionized water (see Section 1).*



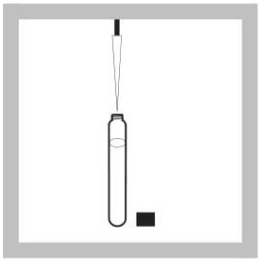
2. Press: **82 ENTER**

The display will show **mg/L, PO4** and the **ZERO** icon.

Note: *For alternate forms (P, P_2O_5), press the* **CONC** *key.*

3. Insert the COD/TNT Adapter into the cell holder by rotating the adapter until it drops into place. Then push down to fully insert it.

Note: *A diffuser band covers the light path holes on the adapter to give increased performance.The band should NOT be removed.*



4. Use a TenSette Pipet to add 5.0 mL of sample to a Reactive Phosphorus Test 'N Tube Dilution Vial. Cap and mix.

Note: *For samples with extreme pH, see the* Interference *section.*

* Procedure is equivalent to USEPA Method 365.2 and Standard Method 4500-P E for wastewater.

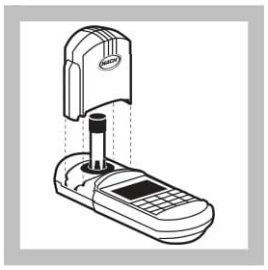
5. Clean the outside of the vial with a towel.

Note: *Wiping with a damp towel, followed by a dry one, will remove fingerprints or other marks.*

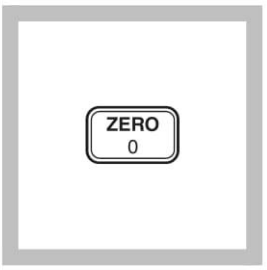
6. Place the sample vial into the adapter.

Push straight down on the top of the vial until it seats solidly into the adapter.

Note: *Do not move the vial from side to side as this can cause errors.*



7. Tightly cover the sample vial with the instrument cap.

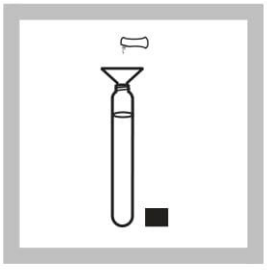


8. Press: **ZERO**

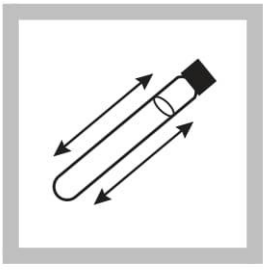
The cursor will move to the right, then the display will show:

0.00 mg/L PO4

Note: *For multiple samples, zero only on the first sample. Read the remaining samples after adding the PhosVer 3 Reagent.*

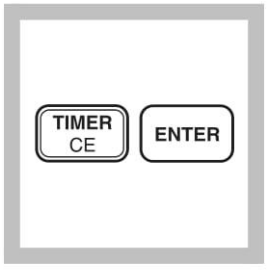


9. Using a funnel, add the contents of one PhosVer 3 Phosphate Powder Pillow to the vial.



10. Cap the vial tightly and shake for 10-15 seconds.

Note: *The powder will not completely dissolve.*



11. Press:

TIMER ENTER

A 2-minute reaction time will begin.

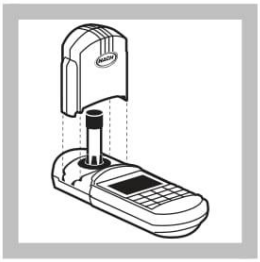
Note: *Read samples between 2 and 8 minutes after the addition of the PhosVer 3 reagent.*

Note: *A blue color will develop if phosphate is present.*

12. Immediately after the timer beeps, place the sample vial in the adapter.

Push straight down on the top of the vial until it seats solidly into the adapter.

Note: *Do not move the vial from side to side as this can cause errors.*



13. Tightly cover the vial with the instrument cap.

14. Press: **READ**

The cursor will move to the right, then the result in mg/L phosphate (PO_4^{3-}) will be displayed.

Note: *Standard Adjust may be performed using a prepared standard (see Section 1).*

Sampling and Storage

Collect samples in plastic or glass bottles that have been acid cleaned with 1:1 Hydrochloric Acid Solution and rinsed with deionized water. Do not use commercial detergents containing phosphate for cleaning glassware used in this test.

Analyze samples immediately after collection for best results. If prompt analysis is impossible, preserve samples for up to 48 hours by filtering immediately and storing at 4 °C. Warm to room temperature before analyzing the sample.

Accuracy Check

Note: *Clean glassware with 1:1 hydrochloric acid solution. Rinse again with deionized water. Do not use detergents containing phosphates to clean glassware.*

Standard Additions Method

a) Fill three 25-mL graduated mixing cylinders with 25 mL of sample.

b) Snap the neck off a Phosphate PourRite Ampule Standard, 50 mg/L as PO_4^{3-}.

c) Use the TenSette Pipet to add 0.1 mL, 0.2 mL and 0.3 mL, respectively, to the three 25-mL aliquots of sample prepared in *step a*. Mix well.

Annex 5 - Algae strains

Algae strain	Strain designation	Origin
Ankistrodesmus falcatus	CCAP 202/5C	Freshwater; pond, Botanical Garden, Lund, Sweden
Chlorella vulgaris	CCAP 211/11B	Freshwater; Madison, Wisconsin
Tetradesmus obliquus	CCAP 276/10	Freshwater; Lund, Sweden
Desmodesmus communis	CCAP 276/4B	Freshwater; garden basin, Cambridge, England, UK
Botryococcus braunii	CCAP 807/2	Freshwater; Grasmere, Cumbria, England, UK

Annex 6 – Calibration curves for algae

The calibration curves for each strain was determined experimentally by Aigars Lavrinovics at the RTU Water Research and Environmental Biotechnology Laboratory before this degree project was undertaken. The relationship between light absorbance at 680 nm of a sample of algae and dry weight of algal biomass per litre of water (g DW/L) was calculated as following: A collection of samples with cumulative increasing dilutions were measured in the spectrophotometer. After that, the samples were filtrated using membrane filters with 0.45 µm pore size and dried at 80 °C for 4 hours. Then the filters were weighed to get the dry weight of each sample, the filter weight itself was subtracted from the total weight. The dry mass weight and the light absorbance were plotted in excel. Through the linear relationship between the light absorbance and the biomass (g DW/L) a $Y = K\star X + M$ equation could be found for each strain. Y equals the biomass concentration in g DW/L, X equals the light absorbance, K and M are constants. The light absorbance worked within the range of 0.1 to 1.2 nm for all the algal strains. If the sample had a light absorbance outside of this range the sample either had to be diluted or concentrated.

See table 1 and figure 1 below for an example of how one of the calibration curves were obtained. This example is from the calibration curve for Botrycocus braunii.

Table 13: Lavrinovics, A, (2020). Botrycocus braunii calibration curve values [Table]. The filter weighted 0,801 g and has been considered when calculated the biomass g per DWL^{-1}

NR	Biomass. ml	Water. ml	Vol of filtrate. ml	OD. λ_{680}	Biomass. g DW 27 ml Inc. filter	Biomass. g DW/L
1	30	0	27	0.270	0.0836	0.130
2	27	3	27	0.236	0.0834	0.122
3	24	6	27	0.208	0.083	0.107
4	21	9	27	0.184	0.0825	0.089
5	18	12	27	0.158	0.082	0.070
6	15	15	27	0.132	0.0817	0.059
7	12	18	27	0.109	0.0816	0.056
8	9	21	27	0.082	0.0812	0.041
9	6	24	27	0.055	0.0807	0.022
10	3	27	27	0.028	0.0803	0.007

Figure 18: Lavrinovics, A. (2020). Linear relationship between DWL^{-1} and light absorbance [Figure].

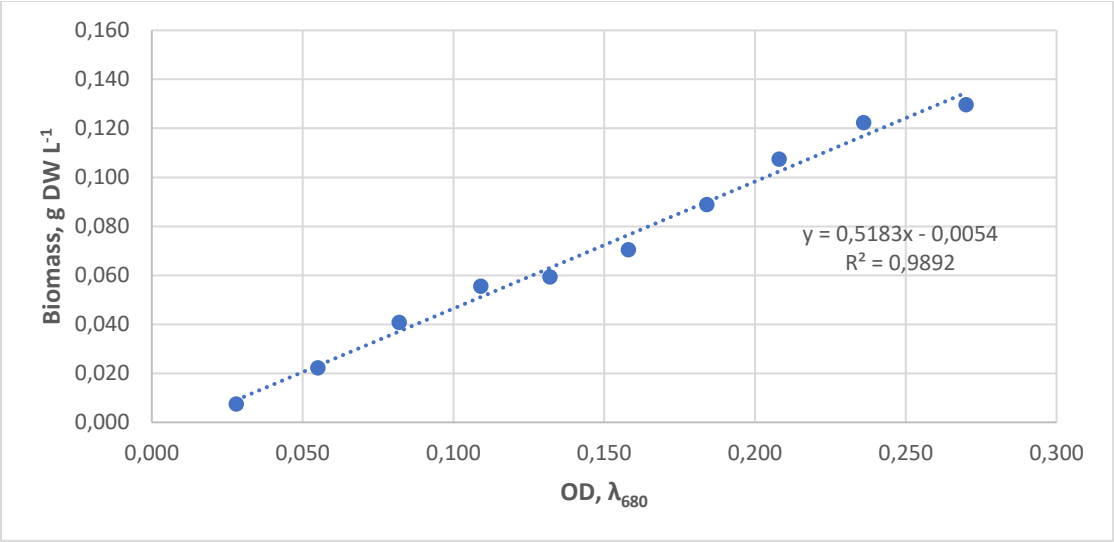


Table 14: Modified from Lavrinovics, A. (2020). Calibration curves 2020 [Excel sheet].

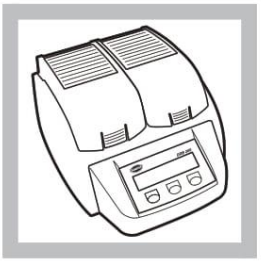
Algae	Calibration curve	R^2	Notation	Explanation
C. vulgaris	y = 0,4076x - 0,0052	R^2 = 0,999	y	g of Dry weight per litre (g DW/L)
D. communis	y = 0,8953x + 0,0013	R^2 = 0,9981	x	Absorbed light at wavelength 680 nm
T. obliquus	y = 0,5817x - 0,0129	R^2 = 0,9976	R^2	Coefficient of determination
A. falcatus	y = 0,5421x - 0,003	R^2 = 0,9926		
B. braunii	y = 0,5183x - 0,0054	R^2 = 0,9892		

Annex 7 - HACH procedure manual, Total-phosphorus measurement

Method 8190

PHOSPHORUS, TOTAL (0.00 to 3.50 mg/L PO_4^{3-}) For water, wastewater and seawater

PhosVer 3 with Acid Persulfate Digestion* USEPA Accepted for reporting wastewater analysis
Test 'N Tube Procedure



1. Turn on the DRB200 Reactor. Heat the reactor to 150 °C.

Note: *See DRB200 instrument manual for selecting preprogrammed temperature applications.*

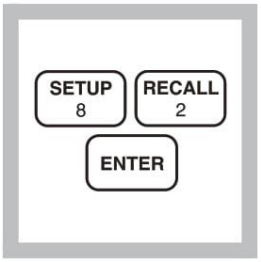
2. Enter the stored program number for total phosphorus, (PO_4^{3-}), Test 'N Tube.

Press: **PRGM**

The display will show:

PRGM ?

Note: *For most accurate results, perform a Reagent Blank Correction using deionized water (see Section 1).*



3. Press: **82 ENTER**

The display will show **mg/L, PO4** and the **ZERO** icon.

Note: *For alternate forms (P, P_2O_5), press the* **CONC** *key.*

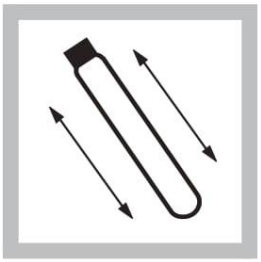
4. Insert the COD/TNT Adapter into the cell holder by rotating the adapter until it drops into place. Then push down to fully insert it.

Note: *A diffuser band covers the light path holes on the adapter to give increased performance. The band should NOT be removed.*

5. Use a TenSette Pipet to add 5.0 mL of sample to a Total and Acid Hydrolyzable Test Vial.

Note: *Adjust the pH of stored samples to 6-8 before analysis.*

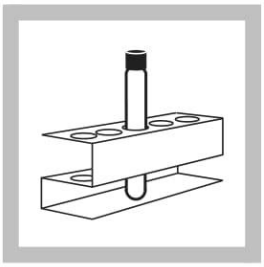
6. Using a funnel, add the contents of one Potassium Persulfate Powder Pillow for Phosphonate to the vial.



7. Cap tightly and shake to dissolve.

8. Place the vial in the DRB200 Reactor. Heat the vial for 30 minutes.

* Adapted from *Standard Methods for the Examination of Water and Wastewater.*
** Procedure is equivalent to USEPA Method 365.2 and Standard Method 4500-P B, 5 and P.E.



9. Carefully remove the vial from the reactor. Place it in a test tube rack and allow to cool to room temperature.

Note: *Vials will be hot.*

10. Use a TenSette Pipet to add 2.0 mL of 1.54 N sodium hydroxide to the vial. Cap and mix.

11. Clean the outside of the vial with a towel.

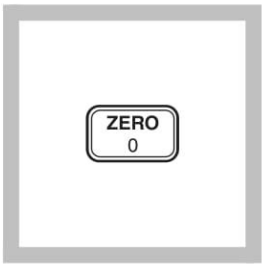
Note: *Wiping with a damp towel, followed by a dry one, will remove fingerprints or other marks.*

12. Place the sample vial in the adapter.

Push straight down on the top of the vial until it seats solidly into the adapter.

Note: *Do not move the vial from side to side as this can cause errors.*

13. Tightly cover the vial with the instrument cap.

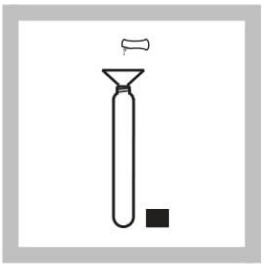


14. Press: **ZERO**

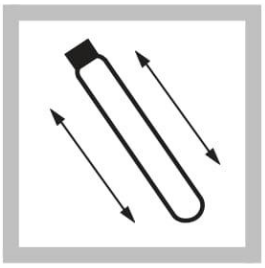
The cursor will move to the right, then the display will show:

0.00 mg/L PO4

Note: *For multiple samples, zero only on the first sample. Read the remaining samples after adding the PhosVer 3 reagent.*

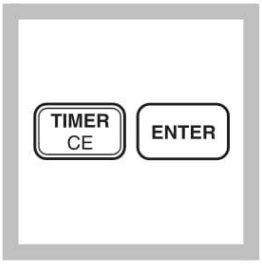


15. Remove the cap from the vial. Using a funnel, add the contents of one PhosVer 3 Phosphate Reagent Powder Pillow to the vial.



16. Cap tightly and shake for 10-15 seconds.

Note: *The powder will not completely dissolve.*



17. Press:

TIMER ENTER

A 2-minute waiting period will begin.

Note: *Read samples between 2 and 8 minutes after the addition of the PhosVer 3 reagent.*

Note: *A blue color will form if phosphate is present.*

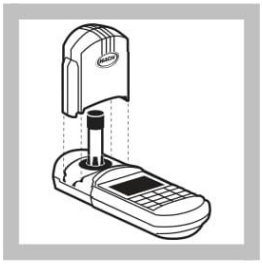
18. After the timer beeps, clean the outside of the sample vial with a towel.

Note: *Wiping with a damp towel, followed by a dry one, will remove fingerprints or other marks.*

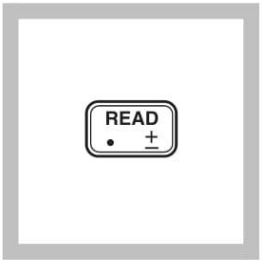
19. Place the prepared sample vial in the adapter.

Push straight down on the top of the vial until it seats solidly into the adapter.

Note: *Do not move the vial from side to side as this can cause errors.*



20. Tightly cover the vial with the instrument cap.



21. Press: **READ**

The cursor will move to the right, then the result in mg/L phosphate (PO_4^{3-}) will be displayed.

Note: *Standard Adjust may be performed using a prepared standard (see Section 1).*

IMPORTANT NOTE:

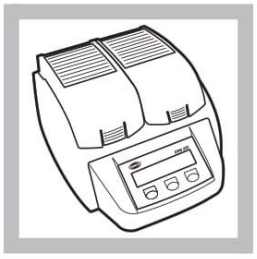
The test range for total phosphate is limited to 0 to 3.5 mg/L PO_4^{3-}. Values above 3.5 mg/L may be used to estimate dilution ratios, but should NOT be used for reporting purposes. If a value above 3.5 mg/L PO_4^{3-} is obtained, dilute the sample and repeat the digestion and the colorimetric test.

Annex 8 - HACH procedure manual, Total-nitrogen measurement

Method 10072

NITROGEN, TOTAL, HR, Test 'N Tube™ (10.0 to 150.0 mg/L N)

TNT Persulfate Digestion Method **For water and wastewater**

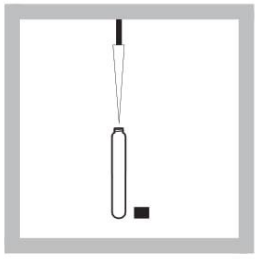


1. Turn on the DRB 200 Reactor. Heat to 103-106 °C (optimum temperature is 105 °C).

Note: *For proof of accuracy, run a 125 mg/L NH_3-N standard through digestion and analysis.*

2. Prepare a reagent blank: Using a funnel, add the contents of one Total Nitrogen Persulfate Reagent Powder Pillow to one HR Total Nitrogen Hydroxide Digestion Vial.

Note: *Wipe off any reagent that gets on the lid or the tube threads.*



3. Add 0.5 mL of organic-free water to the vial. Cap the vial and shake vigorously for about 30 seconds.

Process this reagent blank exactly the same as the sample, including digestion and color finish. Proceed to *step 6.*

Note: *Alternate water must be free of all nitrogen-containing species.*

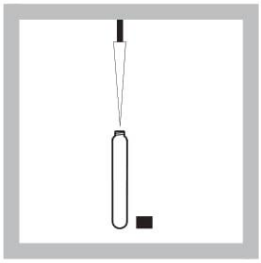
Note: *The persulfate reagent may not dissolve completely after shaking.*

Note: *One reagent blank is sufficient for each set of samples using the same lots of reagents.*

Note: *The reagent blank is stable for as long as seven days when stored in the dark; see* Blanks for Colorimetric Measurement *following this procedure.*

4. Prepare a sample: Using a funnel, add the contents of one Total Nitrogen Persulfate Reagent Powder Pillow to one HR Total Nitrogen Hydroxide Digestion Vial.

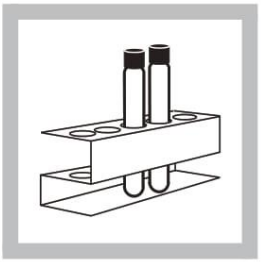
Note: *Wipe off any reagent that gets on the lid or the tube threads.*



5. Add 0.5 mL of sample to the vial. Cap the vial and shake vigorously for about 30 seconds.

Note: *The persulfate reagent may not dissolve completely after shaking.*

6. Place the vials in the Reactor. Heat for 30 minutes.



7. Using finger cots or gloves, remove the hot vials from the reactor and allow to cool to room temperature.

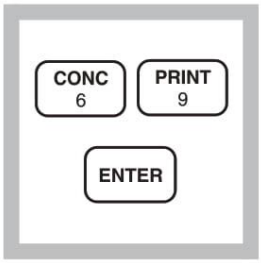
Note: *It is very important to remove the vials from the Reactor after exactly 30 minutes.*

8. Enter the stored program number for Test 'N Tube HR Total Nitrogen.

Press: **PRGM**

The display will show:

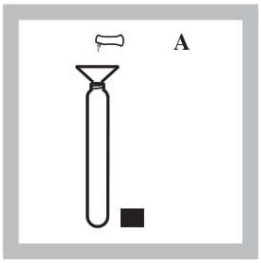
PRGM ?



9. **Press: 69 ENTER**

The display will show **mg/L, N** and the **ZERO** icon**.**

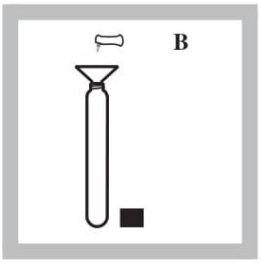
Note: *For alternate forms (NH_3, NO_3), press the* **CONC** *key.*



10. Add the contents of one Total Nitrogen Reagent A Powder Pillow to the vial containing the digested blank or sample. Cap the vial and shake for 15 seconds.

Press: **TIMER ENTER** after shaking.

A three-minute reaction period will begin.



11. After the timer beeps, add one Total Nitrogen Reagent B Powder Pillow to the vial. Cap the vial and shake for 15 seconds.

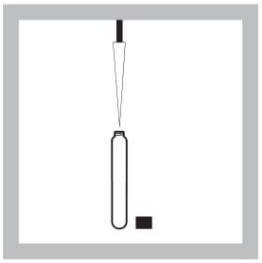
The display will show:

02:00 Timer 2

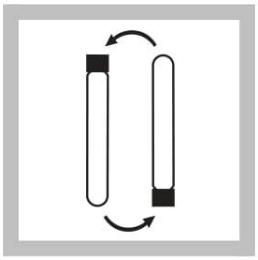
Press **ENTER** after shaking.

A two-minute reaction period will begin.

Note: *The reagent will not completely dissolve. The solution will begin to turn yellow.*

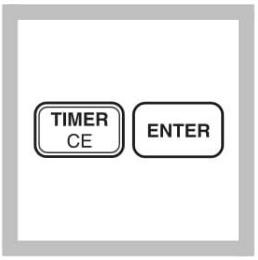


12. After the timer beeps, remove the cap from one Total Nitrogen Reagent C Vial. Add 2 mL of digested, treated sample (or reagent blank) to the vial. The vial will be warm.



13. Cap and invert slowly 10 times to mix. The vial will be warm.

Note: *Proper mixing is important for complete recovery. Hold the vial vertical with the cap up. Invert the vial and wait for all of the solution to flow to the cap end. Pause. Return the vial to the upright position and wait for all of the solution to flow to the vial bottom. This is one inversion (10 inversions = 30 seconds).*



14. The display will show: **05:00 Timer 3**

Press: **ENTER**

A five-minute reaction period will begin. Do not invert the vial again.

Note: *The yellow color will intensify.*

15. Insert the COD/ TNT Adapter into the cell holder by rotating the adapter until it drops into place. Then push down to fully insert it.

Note: *For increased performance, a diffuser band covers the light path holes on the adapter. Do not remove the diffuser band.*

16. When the timer beeps, wipe the outside of the Total Nitrogen Reagent C vial containing the reagent blank.

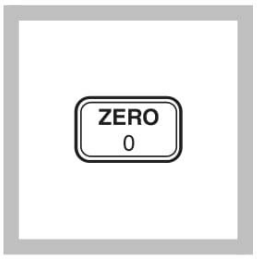
Place the vial into the adapter with the Hach logo facing the front of the instrument.

Push straight down on the top of the vial until it seats solidly into the adapter.

Tightly cover the vial with the instrument cap.

Note: *Do not move the vial from side to side during insertion, as this can cause errors.*

Note: *Wipe with a damp towel, followed by a dry one, to remove fingerprints or other marks.*



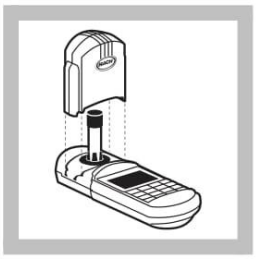
17. Press: **ZERO**

The cursor will move to the right, then the display will show:

0 mg/L N

18. Wipe the Total Nitrogen Reagent C vial containing the sample.

Note: *Wipe with a damp towel, followed by a dry one, to remove fingerprints or other marks.*



19. Place the vial into the adapter with the Hach logo facing the front of the instrument.

Push straight down on the top of the vial until it seats solidly into the adapter.

Tightly cover the vial with the instrument cap.

Note: *Do not move the vial from side to side during insertion, as this can cause errors.*

Note: *Multiple samples may be read after zeroing on one reagent blank.*

20. Press: **READ**

The cursor will move to the right, then the result in mg/L nitrogen (N) will be displayed.

Note: *Standard Adjust may be performed using a prepared standard (see* Standard Adjust *in* Section1 *of the Procedures Manual).*

Note: *If the display flashes* **Limit**, *dilute the sample and repeat the digestion and the colorimetric finish. The digestion must be repeated for accurate results; diluting and repeating the color finish does not yield complete results. Multiply the result by the dilution factor; see SECTION 1.*

Sampling and Storage

Collect samples in clean plastic or glass bottles. Best results are obtained with immediate analysis.

Preserve the sample by reducing the pH to 2 or less with concentrated sulfuric acid (at least 2 mL/L). Store at 4 °C (39 °F) or less. Preserved samples may be stored up to 28 days. Warm samples to room temperature and neutralize with 5 N sodium hydroxide before analysis. Correct the test result for volume additions; see *Correcting for Volume Additions* in *Section 1*.

Annex 9 - HACH procedure manual, nitrate measurement

Method 10020

NITRATE, High Range, Test 'N Tube (0 to 30.0 mg/L NO_3^--N)

Chromotropic Acid Method **For water and wastewater**

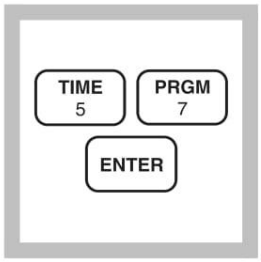
1. Enter the stored program number for Test 'N Tube nitrate nitrogen (NO_3^--N).

Press: **PRGM**

The display will show:

PRGM ?

Note: *If samples cannot be analyzed immediately, see Sampling and Storage on page 331.*



2. Press: **57 ENTER**

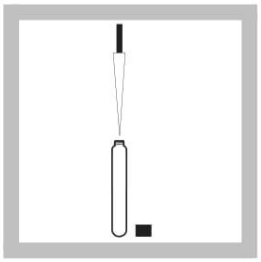
The display will show **mg/L, NO3-N** and the **ZERO** icon.

Note: *For alternate forms (NO_3) press the* **CONC** *key.*

3. Insert the COD/TNT Adapter into the cell holder by rotating the adapter until it drops into place. Then push down to fully insert it.

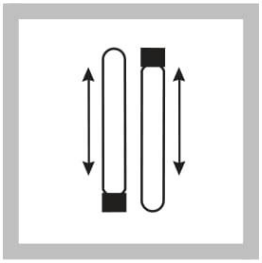
Note: *For proof of accuracy, use a 20 mg/L NO_3^--N standard in place of the sample.*

Note: *For increased performance, a diffuser band covers the light path holes on the adapter. Do not remove the diffuser band.*



4. Remove the cap from a Nitrate Pretreatment Solution Vial and add 1 mL of sample (the blank).

Note: *For most accurate results, perform a Reagent Blank Correction using deionized water (see Section 1).*



5. Cap the tube and invert 10 times to mix.

Note: *This test is technique-sensitive. Low results may occur if these instructions are not followed. Hold the vial vertical with the cap up. Invert the vial so the cap points down. Wait for all of the solution to flow to the cap end. Pause. Return the vial to the upright position. Wait for all the solution to flow to the vial bottom. This process equals 1 inversion. Do this 10 times.*

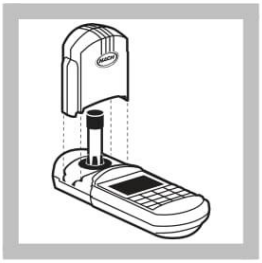
6. Clean the outside of the vial with a towel.

Note: *Wipe with a damp towel and follow with a dry one to remove fingerprints and other marks.*

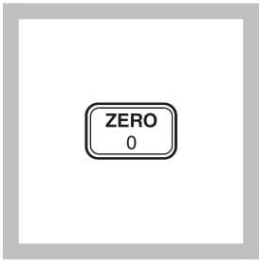
7. Place the blank in the vial adapter with the Hach logo facing the front of the instrument.

Push straight down on the top of the vial until it seats solidly into the adapter.

Note: *Do not move the vial from side to side as this can cause errors.*



8. Cover the vial tightly with the instrument cap.

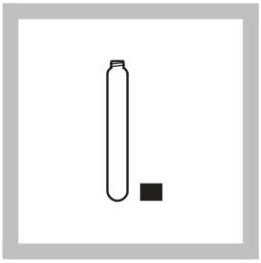


9. Press: **ZERO**

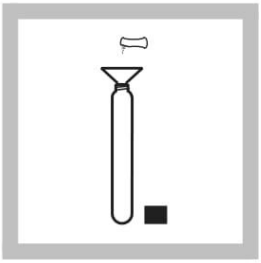
The cursor will move to the right, then the display will show:

0.0 mg/L NO3-N

Note: *If Reagent Blank Correction is on, the display may flash "limit". See Section 1.*



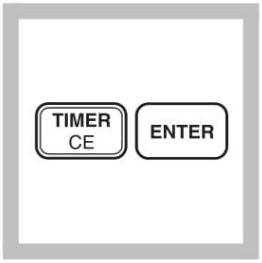
10. Remove the vial from the instrument. Remove the cap from the vial.



11. Using a funnel, add the contents of one NitraVer X Reagent B Powder Pillow to the vial. Cap. Invert 10 times to mix (this will be the prepared sample).

Note: *See Step 5 for inversion instructions*

Note: *Some solid matter will not dissolve.*



12. Press:

TIMER ENTER

A five-minute reaction period will begin. Do not invert the vial again.

Note: *A yellow color will develop if nitrate nitrogen is present.*

Note: *Complete Steps 13-16 within five minutes after the timer beeps.*

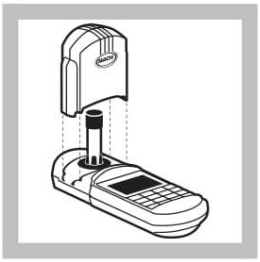
NITRATE, High Range, Test 'N Tube, continued

13. After the timer beeps, clean the outside of the vial with a damp towel and follow with a dry one to remove fingerprints and other marks.

14. Place the prepared sample in the adapter with the Hach logo facing the front of the instrument.

Push straight down on the top of the vial until it seats solidly into the adapter.

Note: *Do not move the vial from side to side as this can cause errors.*



15. Cover the vial tightly with the instrument cap.

16. Press: **READ**

The cursor will move to the right, then the result in mg/L nitrate nitrogen (NO_3-N) will be displayed.

Note: *Standard Adjust may be performed using a prepared standard (see Standard Adjust in Section 1).*

Sampling and Storage

Collect samples in clean plastic or glass bottles. Store at 4 °C (39 °F) or lower if the sample is to be analyzed within 24 to 48 hours. Warm to room temperature before running the test. For longer storage periods (up to 14 days), adjust sample pH to 2 or less with sulfuric acid, ACS (about 2 mL per liter). Sample refrigeration is still required.

Before testing the stored sample, warm to room temperature and neutralize with 5.0 N Sodium Hydroxide Standard Solution.

Do not use mercury compounds as preservatives.

Correct the test result for volume additions; see *Correction for Volume Additions* in *Section 1* for more information.

Annex 10 – Nitrogen to phosphorus calculations

NPR in WW from Rioja

The NPR was calculated by the well-known equation M=m/n, where m is the mass in gram (g), M is the Molar mass in g/mol and n is the amount of substance, (the amount of PO_4^{3-} and NO_3^{2-} molecules in our case.

The mass of the PO_4^{3-} and the NO_3^{2-} was calculated by taking the mean concentration of the WW from Roja used in the main experiment (Table 2) and multiplying that concentration with 0.8, as the reactors in the main experiment contained 0.8 litres of WW. The molar masses were calculated by using the established molar masses of each constituent, found in an online periodic table (retrieved 2020-dec.-09 from https://ptable.com/#Egenskaper). The amount of substance was calculated by dividing the mass with the molar mass for each molecule (n = m/M). Finally, the molar relationship between the nitrogen and phosphorus was obtained by dividing the amount of substance of the nitrogen with the amount of substance by the phosphorus. (NPR = $n(NO_3^{2-})/n(PO_4^{3-})$), which gives us how many NO_3^{2-}-molecules there is per PO_4^{3-}-molecule.

Table 15: Murby, F. (2020). The nitrogen to phosphorus ratio in the wastewater from Roja [Table].

NPR in the WW from Roja			
Mass PO_4^{3-} (g)	0.014	Mass NO_3^{2-} (g)	0.017
Molar mass PO_4^{3-} (g/mol)	94.97	Molar mass NO_3^{2-} (g/mol)	62.00
Amount of substance (mol)	1.5E-04	Amount of substance (mol)	2.7E-04
NPR, P=1, N=	1.831		

NPR in BG11

The NPR in the BG11 was calculated in the same way as the NPR in the WW from Roja. The concentrations of the K_2HPO_4 and $NaNO_3$ were obtained from annex 3, and they were multiplied with 1 litre to get masses. The weight of each molecule was obtained by adding the weight of the individual constituents, gained from the online periodic table (retrieved 2020-dec.-09 from https://ptable.com/#Egenskaper).

Table 16: Murby, F. (2020). The nitrogen to phosphorus ratio in the BG11[Table].

NPR in the BG11			
Mass K_2HPO_4 (g)	0.04	Mass $NaNO_3$ (g)	1.50
Molar mass K_2HPO_4 (g/mol)	174.17	Molar mass $NaNO_3$ (g/mol)	84.99
Amount of substance (mol)	2.3E-04	Amount of substance (mol)	1.8E-02
NPR, P=1, N=	76.846		

NPR in the two pre-experiments

In the two pre-experiments the BG11/WW mix was quite similar, so an approximate NPR will be calculated and used as guide. In the two pre-experiments 400 ml of BG11 was used and 500 ml of WW from Kuldiga. The WW from Kuldiga was collected at two different points in time, and the following information will be served as the basis of the calculation:

1) Nitrates were depleted in the first collection of WW from Kuldiga, and it was assumed that it was depleted in in the second collection as well.
2) In pre-experiment 2, the WW from Kuldiga was depleted of phosphorus in the second collection.
3) In pre-experiment 2, the PO_4^{3-} concentration was measured to about 10 mg/L when 500 ml of the second collected WW from Kuldiga was mixed with 400 ml BG11.

This means that the 400 ml of BG11 (in the 900 ml of solution of BG11/WW-mix) contributed with (10 mg/L★0.9 L=0.009 g) 0.009 g of PO_4^{3-}. The rest of the PO_4^{3-} came from the WW, when the PO_4^{3-} concentration was measured on other days (with WW from the first collection). Since the total PO_4^{3-} concentration usually landed somewhere around 17 mg/L (see Table 8 in the results on pre-experiments 1) which accounts for (17 mg/L ★ 0.9 L) 0.0153 g. That means that the PO_4^{3-} from the WW accounted for: 0.0153 g – 0.009 g = 0.0063 g.

The NO_3^- in the BG11/WW-mix comes solely from the BG11, according to the assumptions 1–3 above. Therefore, the concentration of 1.5 g/L (Table 16) in 400 ml of BG11 accounted for 0.6 g (1.5 g/L ★ 0.4 L = 0.6 g), meaning that the BG11/WW-mix contained to 0.6 g of $NaNO_3$.

Based on this we can calculate an approximate NPR which likely was the close to the real NPR in the two pre-experiments. The molar masses and masses for the constituents are taken from Table 15 and Table 16 above.

$n(K_2HPO_4) = m(K_2HPO_4)/M(K_2HPO_4) = 0.009/174.17 = 5.17e^{-5}$

$n(PO_4^{3-}) = m(PO_4^{3-})/M(PO_4^{3-}) = 0.0063/94.97 = 6.6 \star e^{-5}$

$n(PO_4^{3-}\text{ - tot}) = 5.17e^{-5} + 6.6 \star e^{-5} = 1.2 \star e^{-4}$

$n(NaNO_3) = m(NaNO_3)/M(NaNO_3) = 0.6/84.99 = 0.0071$

$NPR: n(NaNO_3)/n(PO_4^{3-}\text{- tot}) = 0.016/1.2 \star e^{-4} = 59.8$

The NPR is approximately 60:1

Annex 11 – Raw data

Blank

Date	Day	Strain	Starv. Days	Sample name	Biomass		PO_4, mg/L	Tot-P, mg/L	NO_3, mg/L	Tot-N, mg/L	pH
					Abs680	g DW/L					
Replicate 1											
2.11.2019	0	Control	-	NULL			17,35	21,4	22,8	28	
3.11.2019	1	Control	-	NULL			15,4				8,1
4.11.2019	2	Control	-	NULL	0,007		14,4		22,6		7,7
5.11.2020	3	Control	-	NULL			14,1				7,65
6.11.2020	4	Control	-	NULL							7,36
7.11.2020	5	Control	-	NULL	0,017	0,003	18,6	19,7	22,4	28	7,58
9.11.2019	7	Control	-	NULL			13,1		22,8		7,64
12.11.2019	10	Control	-	NULL			11,8	15,1	23,6	21	7,31
Replicate 2											
16.11.20	0	Control	-	NULL			17,5	19,5	20,6	21,8	8,41
17.11.20.	1	Control	-	NULL			18,4				7,48
18.11.20	2	Control	-	NULL			17,85		20,3		7,96
19.11.20	3	Control	-	NULL			15,9		20,4		7,46
20.11.20	4	Control	-	NULL							7,56
21.11.20	5	Control	-	NULL			15,7	17,7	20,9	23	7,74
23.11.20	7	Control	-	NULL			15,4		22,1		7,02
26.11.20.	10	Control	-	NULL			13,4	17,5	21,4	22	7,8
Replicate 3											
07.12.2020	0	Control	-	NULL			18,6	19,7	20,5	22	7,26
08.12.2020	1	Control	-	NULL			18,45		20,7		7,07
09.12.2020	2	Control	-	NULL			19,05		22,3		7,31
10.12.2020	3	Control	-	NULL			18,6		22,9		7,53
11.12.2020	4	Control	-	NULL							7,5
12.12.2020	5	Control	-	NULL			16,9	18,35	25	26	7,65
14.12.2020	7	Control	-	NULL			15,9		27,1		7,15
17.12.2020	10	Control	-	NULL			16	17,7	33	35	8,02

D. communis

Date	Day	Strain	Starv. Days	Sample name	Biomass		PO_4, mg/L	Tot-P, mg/L	NO_3, mg/L	Tot-N, mg/L	pH
					Abs680	g DW L/L					
Replicate 1											
2.11.2020	0	D. communis	0	Dc0	0,099	0,090	15,7	21,4	21,4	28	8,2
3.11.2020	1	D. communis	0	Dc0	0,209	0,188	12,65				8,14
4.11.2020	2	D. communis	0	Dc0	0,173	0,156	12,5		20,8		7,46
5.11.2020	3	D. communis	0	Dc0	0,198	0,179	12,15				7,55
6.11.2020	4	D. communis	0	Dc0	0,231	0,208					7,5
7.11.2020	5	D. communis	0	Dc0	0,24	0,216	12,5	13,6	19,6	18	7,55
9.11.2020	7	D. communis	0	Dc0	0,221	0,199	11,3		20		7,55
12.11.2020	10	D. communis	0	Dc0	0,227	0,205	8,9	12,1	20,8	19	7,36
2.11.2020	0	D. communis	1	Dc1	0,105	0,095	15,7	21,4	21,4	28	8,25
3.11.2020	1	D. communis	1	Dc1	0,225	0,203	12,55				8,2
4.11.2020	2	D. communis	1	Dc1	0,137	0,124	12,2		19,1		7,53
5.11.2020	3	D. communis	1	Dc1	0,178	0,161	11,1				7,65
6.11.2020	4	D. communis	1	Dc1	0,285	0,256					7,6
7.11.2020	5	D. communis	1	Dc1	0,243	0,219	10,9	16,5	18,8	19	7,5
9.11.2020	7	D. communis	1	Dc1	0,225	0,203	9		19,6		7,63
12.11.2020	10	D. communis	1	Dc1	0,186	0,168	5,2	7,9	20,4	20	7,23
2.11.2020	0	D. communis	3	Dc3	0,09	0,082	15,7	21,4	21,4	28	8
3.11.2020	1	D. communis	3	Dc3	0,156	0,141	13				7,97
4.11.2020	2	D. communis	3	Dc3	0,132	0,119	12,35		14,9		7,53
5.11.2020	3	D. communis	3	Dc3	0,148	0,134	11,95				7,5
6.11.2020	4	D. communis	3	Dc3	0,186	0,168					7,4
7.11.2020	5	D. communis	3	Dc3	0,206	0,186	11,2	13,1	19,2	24	7,42
9.11.2020	7	D. communis	3	Dc3	0,195	0,176	10,1		18,2		7,78
12.11.2020	10	D. communis	3	Dc3	0,138	0,125	6,9	9,6	20,6	21	7,32
2.11.2020	0	D. communis	5	Dc5	0,134	0,121	15,7	21,4	21,4	28	8,25
3.11.2020	1	D. communis	5	Dc5	0,183	0,165	13,35				8,03
4.11.2020	2	D. communis	5	Dc5	0,142	0,128	12,5		20,4		7,47
5.11.2020	3	D. communis	5	Dc5	0,146	0,132	12,45				7,56
6.11.2020	4	D. communis	5	Dc5	0,206	0,186					7,6
7.11.2020	5	D. communis	5	Dc5	0,172	0,155	11,5	13,3	19,6	28	7,43
9.11.2020	7	D. communis	5	Dc5	0,179	0,162	12		19,4		7,85
12.11.2020	10	D. communis	5	Dc5	0,161	0,145	9,3	11,9	21,2	22	7,22

T. obliquus

Date	Day	Strain	Starv. Days	Sample name	Biomass		PO_4, mg/L	Tot-P, mg/L	NO_3, mg/L	Tot-N, mg/L	pH
					Abs680	g DW/L					
Replicate 1											
2.11.2020	0	T. obliquu	0	To0	0,335	0,182	16,2	21,4	21,6	28	8,42
3.11.2020	1	T. obliquu	0	To0	0,404	0,222	10,45				8,34
4.11.2020	2	T. obliquu	0	To0	0,375	0,205	10,65		21,8		7,85
5.11.2020	3	T. obliquu	0	To0	0,412	0,227	10,1				7,54
6.11.2020	4	T. obliquu	0	To0	0,463	0,256					7,7
7.11.2020	5	T. obliquu	0	To0	0,312	0,169	9,5	10,7	22,4	23	7,53
9.11.2020	7	T. obliquu	0	To0	0,51	0,284	10,6		21,6		7,82
12.11.2020	10	T. obliquu	0	To0	0,513	0,286	9,7	6,9	22,6	28	7,44
2.11.2020	0	T. obliquu	1	To1	0,287	0,154	16,2	21,4	21,6	28	8,47
3.11.2020	1	T. obliquu	1	To1	0,332	0,180	11,4				8,25
4.11.2020	2	T. obliquu	1	To1	0,338	0,184	12,55		21,3		7,63
5.11.2020	3	T. obliquu	1	To1	0,362	0,198	10,35				7,6
6.11.2020	4	T. obliquu	1	To1	0,376	0,206					7,7
7.11.2020	5	T. obliquu	1	To1	0,328	0,178	9,2	11,6	22,4	22	7,56
9.11.2020	7	T. obliquu	1	To1	0,406	0,223	7,5		21,8		7,57
12.11.2020	10	T. obliquu	1	To1	0,408	0,224	4,9	7,3	22,4	23	7,51
2.11.2020	0	T. obliquu	3	To3	0,267	0,142	16,2	21,4	21,6	28	8,12
3.11.2020	1	T. obliquu	3	To3	0,286	0,153	12,55				8,4
4.11.2020	2	T. obliquu	3	To3	0,285	0,153	14,55		21		7,63
5.11.2020	3	T. obliquu	3	To3	0,333	0,181	11,5				7,72
6.11.2020	4	T. obliquu	3	To3	0,385	0,211					7,7
7.11.2020	5	T. obliquu	3	To3	0,307	0,166	10,8	12,2	21,8	22	7,53
9.11.2020	7	T. obliquu	3	To3	0,379	0,208	10,3		20,8		7,66
12.11.2020	10	T. obliquu	3	To3	0,393	0,216	6,9	10,4	21,6	19	7,45
2.11.2020	0	T. obliquu	5	To5	0,314	0,170	16,2	21,4	21,6	28	8,51
3.11.2020	1	T. obliquu	5	To5	0,441	0,244	9,65				8,5
4.11.2020	2	T. obliquu	5	To5	0,645	0,362	6,7		11,3		7,9
5.11.2020	3	T. obliquu	5	To5	0,907	0,515	4,15				7,8
6.11.2020	4	T. obliquu	5	To5	1,095	0,624					7,7
7.11.2020	5	T. obliquu	5	To5	0,978	0,556	0,15	2,6	0,8	4	7,56
9.11.2020	7	T. obliquu	5	To5	1,243	0,710	0,21		0,5		7,66
12.11.2020	10	T. obliquu	5	To5	1,322	0,756	0,03	4,7	0,6	8	7,3
Replicate 3											
07.12.2020	0	T. obliquu	0	To0	0,254	0,135	17,85	19,7	20,4	22	8,1
08.12.2020	1	T. obliquu	0	To0	0,305	0,165	16,75		20,2		7,13
09.12.2020	2	T. obliquu	0	To0	0,271	0,145	15,7		19,7		7,55
10.12.2020	3	T. obliquu	0	To0	0,348	0,190	15,8		20,2		7,46
11.12.2020	4	T. obliquu	0	To0	0,275	0,147					6,9
12.12.2020	5	T. obliquu	0	To0	0,265	0,141	14,55	15,7	20,5	30	7,12
14.12.2020	7	T. obliquu	0	To0	0,303	0,163	12,9		21,1		7,02
17.12.2020	10	T. obliquu	0	To0	0,324	0,176	11	12,8	21,8	27	7,75
07.12.2020	0	T. obliquu	1	To1	0,229	0,120	18,4	19,7	20,1	22	7,41
08.12.2020	1	T. obliquu	1	To1	0,303	0,163	15,75		19,5		7,42
09.12.2020	2	T. obliquu	1	To1	0,254	0,135	15,1		18,8		7,63
10.12.2020	3	T. obliquu	1	To1	0,358	0,195	13,9		18		7,73
11.12.2020	4	T. obliquu	1	To1	0,388	0,213					7,16
12.12.2020	5	T. obliquu	1	To1	0,433	0,239	7,3	8,3	15	15	7,29
14.12.2020	7	T. obliquu	1	To1	0,675	0,380	1,75		11,1		7,06
17.12.2020	10	T. obliquu	1	To1	1,06	0,604	0,76	0,84	11,9	12	7,71
07.12.2020	0	T. obliquu	3	To3	0,221	0,116	18,2	19,7	20,4	22	7,62
08.12.2020	1	T. obliquu	3	To3	0,345	0,188	14,2		17,9		7,51
09.12.2020	2	T. obliquu	3	To3	0,547	0,305	9,4		12,1		7,56
10.12.2020	3	T. obliquu	3	To3	0,768	0,434	4,95		7,9		7,64
11.12.2020	4	T. obliquu	3	To3	0,86	0,487					7,22
12.12.2020	5	T. obliquu	3	To3	0,956	0,543	0	1,6	5	5	7,55
14.12.2020	7	T. obliquu	3	To3	1,2	0,685	0		1,5		7,15
17.12.2020	10	T. obliquu	3	To3	1,325	0,758	0	0,31	0,9	2	7,71
07.12.2020	0	T. obliquu	5	To5	0,235	0,124	17,95	19,7	20,6	22	7,25
08.12.2020	1	T. obliquu	5	To5	0,294	0,158	16,6		20,4		7,52
09.12.2020	2	T. obliquu	5	To5	0,216	0,113	17,05		20,1		7,43
10.12.2020	3	T. obliquu	5	To5	0,329	0,178	15,8		20,7		7,87
11.12.2020	4	T. obliquu	5	To5	0,241	0,127					7,28
12.12.2020	5	T. obliquu	5	To5	0,201	0,104	14,95	16,6	21,2	22	7,32
14.12.2020	7	T. obliquu	5	To5	0,273	0,146	13,9		22,1		7,26
17.12.2020	10	T. obliquu	5	To5	0,32	0,173	12,75	14,7	24	23	7,87

C. vulgaris

Replicate 1											
Date	Day	Strain	Starv. Days	Sample name	Biomass		PO_4, mg/L	Tot-P, mg/L	NO_3, mg/L	Tot-N, mg/L	pH
					Abs680	g DW/L					
2.11.2020	0	C. vulgaris	0	Cv0	0,39	0,154	16,1	21,4	21,8	28	8,44
3.11.2020	1	C. vulgaris	0	Cv0	0,396	0,156	11,9				8,39
4.11.2020	2	C. vulgaris	0	Cv0	0,509	0,202	10,85		14,4		7,48
5.11.2020	3	C. vulgaris	0	Cv0	0,626	0,250	8,85				7,62
6.11.2020	4	C. vulgaris	0	Cv0	1,135	0,457					7,55
7.11.2020	5	C. vulgaris	0	Cv0	1,082	0,436	3,92	9,7	1	2	7,57
9.11.2020	7	C. vulgaris	0	Cv0	1,634	0,661	1,83		0,7		7,91
12.11.2020	10	C. vulgaris	0	Cv0	2,144	0,869	0,08	2,2	0,6	3	7,66
2.11.2020	0	C. vulgaris	1	Cv1	0,392	0,155	16,1	21,4	21,8	28	8,22
3.11.2020	1	C. vulgaris	1	Cv1	0,379	0,149	13,6				8,47
4.11.2020	2	C. vulgaris	1	Cv1	0,483	0,192	8,95		10,9		7,81
5.11.2020	3	C. vulgaris	1	Cv1	0,782	0,314	4,3				7,52
6.11.2020	4	C. vulgaris	1	Cv1	1,366	0,552					7,55
7.11.2020	5	C. vulgaris	1	Cv1	1,788	0,724	1,52	9,5	0	0	7,76
9.11.2020	7	C. vulgaris	1	Cv1	1,97	0,798	0,22		0		7,95
12.11.2020	10	C. vulgaris	1	Cv1	2,198	0,891	0,07	6	0	0	7,55
2.11.2020	0	C. vulgaris	3	Cv3	0,359	0,141	16,1	21,4	21,8	28	8,49
3.11.2020	1	C. vulgaris	3	Cv3	0,418	0,165	12,8				8,45
4.11.2020	2	C. vulgaris	3	Cv3	0,698	0,279	10,2		11,7		7,7
5.11.2020	3	C. vulgaris	3	Cv3	0,931	0,374	6,55				7,56
6.11.2020	4	C. vulgaris	3	Cv3	1,3	0,525					7,7
7.11.2020	5	C. vulgaris	3	Cv3	1,856	0,751	2,52	7,6	0	0	7,7
9.11.2020	7	C. vulgaris	3	Cv3	1,988	0,805	0,28		0		7,68
12.11.2020	10	C. vulgaris	3	Cv3	2,266	0,918	0,14	1,9	0	0	7,53
2.11.2020	0	C. vulgaris	5	Cv5	0,395	0,156	16,1	21,4	21,8	28	8,49
3.11.2020	1	C. vulgaris	5	Cv5	0,393	0,155	14,45				8,46
4.11.2020	2	C. vulgaris	5	Cv5	0,575	0,229	11,4		12,7		7,54
5.11.2020	3	C. vulgaris	5	Cv5	0,92	0,370	8				7,66
6.11.2020	4	C. vulgaris	5	Cv5	1,164	0,469					8
7.11.2020	5	C. vulgaris	5	Cv5	1,696	0,686	3,88	6,6	0	0	7,8
9.11.2020	7	C. vulgaris	5	Cv5	1,774	0,718	1,54		0		7,99
12.11.2020	10	C. vulgaris	5	Cv5	2,166	0,878	0,05	2,6	0	0	7,73
Replicate 2											
16.11.20	0	C. vulgaris	0	Cv0	0,303	0,118	17,6	19,6	20,8	22	9,07
17.11.20.	1	C. vulgaris	0	Cv0	0,293	0,114	16,85				7,82
18.11.20	2	C. vulgaris	0	Cv0	0,41	0,162	13,8		11,7		8,06
19.11.20	3	C. vulgaris	0	Cv0	0,786	0,315	10,3		4,7		7,85
20.11.20	4	C. vulgaris	0	Cv0	0,971	0,391					7,89
21.11.20	5	C. vulgaris	0	Cv0	1,252	0,505	6,34	9,2	0	7	7,35
23.11.20	7	C. vulgaris	0	Cv0	1,63	0,659	3,62		0		7,07
26.11.20.	10	C. vulgaris	0	Cv0	2,24	0,908	0,49	4	0	1	7,95
16.11.20	0	C. vulgaris	1	Cv1	0,335	0,131	17,8	19,8	20,6	21,8	8,84
17.11.20.	1	C. vulgaris	1	Cv1	0,312	0,122	16,05				7,54
18.11.20	2	C. vulgaris	1	Cv1	0,588	0,234	12,6		9,5		8,23
19.11.20	3	C. vulgaris	1	Cv1	0,871	0,350	8,6		2,9		7,57
20.11.20	4	C. vulgaris	1	Cv1	1,206	0,486					7,59
21.11.20	5	C. vulgaris	1	Cv1	1,83	0,741	5,62	7,9	0,4	10	5,54
23.11.20	7	C. vulgaris	1	Cv1	2,25	0,912	1,14		0,4		7,65
26.11.20.	10	C. vulgaris	1	Cv1	2,672	1,084	0,13	4	0,7	7	7,55
16.11.20	0	C. vulgaris	3	Cv3	0,307	0,120	17,3	19,3	21,2	22,4	8,87
17.11.20.	1	C. vulgaris	3	Cv3	0,32	0,125	15,75				7,85
18.11.20	2	C. vulgaris	3	Cv3	0,64	0,256	11,8		9,6		8,1
19.11.20	3	C. vulgaris	3	Cv3	1,128	0,455	6,4		4,5		7,69
20.11.20	4	C. vulgaris	3	Cv3	1,664	0,673					7,86
21.11.20	5	C. vulgaris	3	Cv3	1,868	0,756	3,28	6,2	0	0	7,4
23.11.20	7	C. vulgaris	3	Cv3	2,288	0,927	0,16		0		7,51
26.11.20.	10	C. vulgaris	3	Cv3	3,42	1,389	0,08	3,1	0	0	7,59
16.11.20	0	C. vulgaris	5	Cv5	0,332	0,130	16,8	18,8	21	22,2	8,94
17.11.20.	1	C. vulgaris	5	Cv5	0,303	0,118	16,3				7,63
18.11.20	2	C. vulgaris	5	Cv5	0,586	0,234	14,05		13,3		7,91
19.11.20	3	C. vulgaris	5	Cv5	1,057	0,426	9,05		0,6		7,99
20.11.20	4	C. vulgaris	5	Cv5	1,524	0,616					7,96
21.11.20	5	C. vulgaris	5	Cv5	1,786	0,723	5,8	8	0,5	13	7,51
23.11.20	7	C. vulgaris	5	Cv5	2,236	0,906	2,74		0,4		7,94
26.11.20.	10	C. vulgaris	5	Cv5	3,256	1,322	0,09	2,8	0,7	7	7,62

C. vulgaris

Replicate 3											
07.12.2020	0	C. vulgaris	0	Cv0	0,323	0,126	17,35	19,7	20,4	22	7,36
08.12.2020	1	C. vulgaris	0	Cv0	0,33	0,129	18,3		19,9		7,35
09.12.2020	2	C. vulgaris	0	Cv0	0,358	0,141	17,55		18		7,74
10.12.2020	3	C. vulgaris	0	Cv0	0,53	0,211	14,8		14,6		7,73
11.12.2020	4	C. vulgaris	0	Cv0	0,612	0,244					7,49
12.12.2020	5	C. vulgaris	0	Cv0	0,736	0,295	8,525	10,4	3,8	4	7,33
14.12.2020	7	C. vulgaris	0	Cv0	1,26	0,508	5,24		0,8		7,36
17.12.2020	10	C. vulgaris	0	Cv0	1,875	0,759	1,16	2,5	0,8	2	7,88
07.12.2020	0	C. vulgaris	1	Cv1	0,288	0,112	17	19,7	20,7	22	7,5
08.12.2020	1	C. vulgaris	1	Cv1	0,281	0,109	16,75		19,7		7,46
09.12.2020	2	C. vulgaris	1	Cv1	0,347	0,136	13,95		17		7,37
10.12.2020	3	C. vulgaris	1	Cv1	0,534	0,212	11,25		12,3		7,63
11.12.2020	4	C. vulgaris	1	Cv1	0,752	0,301					7,26
12.12.2020	5	C. vulgaris	1	Cv1	1,068	0,430	4,36	6,6	0,8	3	7,9
14.12.2020	7	C. vulgaris	1	Cv1	1,84	0,745	1,46		0,8		7,13
17.12.2020	10	C. vulgaris	1	Cv1	2,57	1,042	0,08	1,5	0,8	3	8,15
07.12.2020	0	C. vulgaris	3	Cv3	0,281	0,109	17,55	19,7	20,5	22	7,57
08.12.2020	1	C. vulgaris	3	Cv3	0,285	0,111	16,5		19,9		7,62
09.12.2020	2	C. vulgaris	3	Cv3	0,376	0,148	14,8		16,2		7,57
10.12.2020	3	C. vulgaris	3	Cv3	0,66	0,264	10		7,4		7,73
11.12.2020	4	C. vulgaris	3	Cv3	1,144	0,461					7,32
12.12.2020	5	C. vulgaris	3	Cv3	1,452	0,587	3,5	5,1	0,7	2	7,83
14.12.2020	7	C. vulgaris	3	Cv3	2,08	0,843	0,44		0,8		7,42
17.12.2020	10	C. vulgaris	3	Cv3	2,89	1,173	0,17	1,6	0,8	4	8,07
07.12.2020	0	C. vulgaris	5	Cv5	0,357	0,140	18,3	19,7	20,3	22	7,64
08.12.2020	1	C. vulgaris	5	Cv5	0,336	0,132	16,15		20,4		7,73
09.12.2020	2	C. vulgaris	5	Cv5	0,37	0,146	15,2		19		7,31
10.12.2020	3	C. vulgaris	5	Cv5	0,506	0,201	13,1		16,2		7,84
11.12.2020	4	C. vulgaris	5	Cv5	0,788	0,316					7,5
12.12.2020	5	C. vulgaris	5	Cv5	0,98	0,394	6,8	10	4,1	6	8,39
14.12.2020	7	C. vulgaris	5	Cv5	2	0,810	2,54		0,8		7,66
17.12.2020	10	C. vulgaris	5	Cv5	3,735	1,517	0,14	1,7	1	4	8,45

A. Falcatus

Date	Day	Strain	Starv. Days	Sample name	Biomass		PO_4, mg/L	Tot-P, mg/L	NO_3, mg/L	Tot-N, mg/L	pH
					Abs680	g DW/L					
Replicate 1											
2.11.2020	0	A. falcatus	0	Af0	0,278	0,148	15,7	21,4	21,6	28	8,57
3.11.2020	1	A. falcatus	0	Af0	0,32	0,170	12,35				8,45
4.11.2020	2	A. falcatus	0	Af0	0,593	0,318	8,8		15,8		7,55
5.11.2020	3	A. falcatus	0	Af0	0,88	0,474	4,9				7,81
6.11.2020	4	A. falcatus	0	Af0	1,177	0,635					8
7.11.2020	5	A. falcatus	0	Af0	1,58	0,854	0,32	3	0	0	7,73
9.11.2020	7	A. falcatus	0	Af0	1,786	0,965	0,16		0		7,97
12.11.2020	10	A. falcatus	0	Af0	2,164	1,170	0,08	1,5	0	0	7,72
2.11.2020	0	A. falcatus	1	Af1	0,257	0,136	15,7	21,4	21,6	28	8,5
3.11.2020	1	A. falcatus	1	Af1	0,3	0,160	11,6				8,36
4.11.2020	2	A. falcatus	1	Af1	0,46	0,246	7,55		16,9		7,7
5.11.2020	3	A. falcatus	1	Af1	0,681	0,366	6,8				7,69
6.11.2020	4	A. falcatus	1	Af1	0,874	0,471					7,65
7.11.2020	5	A. falcatus	1	Af1	1,276	0,689	4,72	7,5	1,2	0	7,45
9.11.2020	7	A. falcatus	1	Af1	1,442	0,779	0,57		0,7		7,9
12.11.2020	10	A. falcatus	1	Af1	1,676	0,906	0,53	2,9	0,5	2	7,79
2.11.2020	0	A. falcatus	3	Af3	0,226	0,120	15,7	21,4	21,6	28	8,51
3.11.2020	1	A. falcatus	3	Af3	0,291	0,155	11,6				8,31
4.11.2020	2	A. falcatus	3	Af3	0,522	0,280	9,15		14,1		7,73
5.11.2020	3	A. falcatus	3	Af3	0,922	0,497	4,05				7,57
6.11.2020	4	A. falcatus	3	Af3	1,285	0,694					7,8
7.11.2020	5	A. falcatus	3	Af3	1,928	1,042	0,14	2,7	0	2	7,72
9.11.2020	7	A. falcatus	3	Af3	1,786	0,965	0,1		0		7,93
12.11.2020	10	A. falcatus	3	Af3	1,908	1,031	0,07	1,35	0	0	7,87
2.11.2020	0	A. falcatus	5	Af5	0,312	0,166	15,7	21,4	21,6	28	8,26
3.11.2020	1	A. falcatus	5	Af5	0,427	0,228	10,4				8,45
4.11.2020	2	A. falcatus	5	Af5	0,529	0,284	9,2		14,8		7,74
5.11.2020	3	A. falcatus	5	Af5	0,788	0,424	5,3				7,52
6.11.2020	4	A. falcatus	5	Af5	1,109	0,598					7,8
7.11.2020	5	A. falcatus	5	Af5	1,86	1,005	0,54	2,3	0	0	7,7
9.11.2020	7	A. falcatus	5	Af5	1,698	0,917	0,32		0		7,83
12.11.2020	10	A. falcatus	5	Af5	2,028	1,096	0,09	1,9	0	0	7,95
Replicate 2											
16.11.20	0	A. falcatus	0	Af0	0,288	0,153	17,4	19,4	25	26,2	9
17.11.20.	1	A. falcatus	0	Af0	0,274	0,146	17,55				7,81
18.11.20	2	A. falcatus	0	Af0	0,374	0,200	11,5		18		7,97
19.11.20	3	A. falcatus	0	Af0	0,439	0,235	12,8		15,2		7,64
20.11.20	4	A. falcatus	0	Af0	0,627	0,337					7,89
21.11.20	5	A. falcatus	0	Af0	0,725	0,390	5,36	7,5	0,6	13	7,66
23.11.20	7	A. falcatus	0	Af0	1,046	0,564	2,38		5,6		7,39
26.11.20.	10	A. falcatus	0	Af0	1,54	0,832	0,69	5,7	1	1	7,61
16.11.20	0	A. falcatus	1	Af1	0,251	0,133	17,1	19,1	24,2	25,4	8,91
17.11.20.	1	A. falcatus	1	Af1	0,265	0,141	15,65				7,78
18.11.20	2	A. falcatus	1	Af1	0,429	0,230	14		16,3		8,01
19.11.20	3	A. falcatus	1	Af1	0,562	0,302	8,2		12,2		7,61
20.11.20	4	A. falcatus	1	Af1	0,788	0,424					7,98
21.11.20	5	A. falcatus	1	Af1	0,748	0,402	0,76	2,7	0,5	5	7,65
23.11.20	7	A. falcatus	1	Af1	1,14	0,615	0,5		2,6		7,42
26.11.20.	10	A. falcatus	1	Af1	1,348	0,728	0,2	2,4	0,7	5	7,55
16.11.20	0	A. falcatus	3	Af3	0,271	0,144	17,2	19,2	23,8	25	8,94
17.11.20.	1	A. falcatus	3	Af3	0,294	0,156	15,95				7,89
18.11.20	2	A. falcatus	3	Af3	0,401	0,214	13,6		15,7		8,15
19.11.20	3	A. falcatus	3	Af3	0,527	0,283	9,4		13		7,66
20.11.20	4	A. falcatus	3	Af3	0,755	0,406					7,56
21.11.20	5	A. falcatus	3	Af3	0,774	0,417	1,84	4,3	0,7	9	7,61
23.11.20	7	A. falcatus	3	Af3	1,118	0,603	0,6		3,1		7,52
26.11.20.	10	A. falcatus	3	Af3	1,376	0,743	0,2	1,95	0,7	1	7,51
16.11.20	0	A. falcatus	5	Af5	0,229	0,121	17,9	19,9	24,6	26,8	8,97
17.11.20.	1	A. falcatus	5	Af5	0,24	0,127	15,3				7,61
18.11.20	2	A. falcatus	5	Af5	0,334	0,178	14,5		14,7		7,94
19.11.20	3	A. falcatus	5	Af5	0,4	0,214	13,2		15,9		7,76
20.11.20	4	A. falcatus	5	Af5	0,523	0,281					7,65
21.11.20	5	A. falcatus	5	Af5	0,503	0,270	10,28	12,4	0,6	12	7,61
23.11.20	7	A. falcatus	5	Af5	0,82	0,442	7,74		8,4		7,32
26.11.20.	10	A. falcatus	5	Af5	1,292	0,697	2,86	4,3	0,7	1	7,92

B. braunii

Replicate 1											
Date	Day	Strain	Starv. Days	Sample name	Biomass		PO_4, mg/L	Tot-P, mg/L	NO_3, mg/L	Tot-N, mg/L	pH
					Abs680	g DW/L					
2.11.2020	0	B. braunii	0	Bb0	0,329	0,165	15,65	21,4	21,6	28	8,5
3.11.2020	1	B. braunii	0	Bb0	0,313	0,157	10,9				8,42
4.11.2020	2	B. braunii	0	Bb0	0,495	0,251	9		15,5		7,66
5.11.2020	3	B. braunii	0	Bb0	0,786	0,402	5,2				7,72
6.11.2020	4	B. braunii	0	Bb0	1,215	0,624					8,1
7.11.2020	5	B. braunii	0	Bb0	1,724	0,888	1,13	3,2	0	0	7,83
9.11.2020	7	B. braunii	0	Bb0	1,684	0,867	0,14		0		7,86
12.11.2020	10	B. braunii	0	Bb0	2,232	1,151	0,09	1,65	0	0	7,91
2.11.2020	0	B. braunii	1	Bb1	0,327	0,164	15,65	21,4	21,6	28	8,1
3.11.2020	1	B. braunii	1	Bb1	0,356	0,179	12,8				8,42
4.11.2020	2	B. braunii	1	Bb1	0,516	0,262	10,1		13,8		7,6
5.11.2020	3	B. braunii	1	Bb1	0,8	0,409	6,3				7,73
6.11.2020	4	B. braunii	1	Bb1	1,157	0,594					7,9
7.11.2020	5	B. braunii	1	Bb1	1,844	0,950	2,12	4,8	0	0	7,65
9.11.2020	7	B. braunii	1	Bb1	1,804	0,930	0,14		0		7,81
12.11.2020	10	B. braunii	1	Bb1	2,032	1,048	0,12	2,05	0	0	8,19
2.11.2020	0	B. braunii	3	Bb3	0,288	0,144	15,65	21,4	21,6	28	8,47
3.11.2020	1	B. braunii	3	Bb3	0,317	0,159	11,85				8,36
4.11.2020	2	B. braunii	3	Bb3	0,42	0,212	10,7		15,9		7,47
5.11.2020	3	B. braunii	3	Bb3	0,674	0,344	7,4				7,65
6.11.2020	4	B. braunii	3	Bb3	0,998	0,512					7,85
7.11.2020	5	B. braunii	3	Bb3	1,696	0,874	2,24	4,3	0	0	7,76
9.11.2020	7	B. braunii	3	Bb3	1,686	0,868	0,46		0		7,94
12.11.2020	10	B. braunii	3	Bb3	2,098	1,082	0,13	1,35	0	0	8,25
2.11.2020	0	B. braunii	5	Bb5	0,279	0,139	15,65	21,4	21,6	28	8,6
3.11.2020	1	B. braunii	5	Bb5	0,279	0,139	12,65				8,42
4.11.2020	2	B. braunii	5	Bb5	0,376	0,189	11,8		17,7		7,72
5.11.2020	3	B. braunii	5	Bb5	0,57	0,290	10,75				7,52
6.11.2020	4	B. braunii	5	Bb5	0,916	0,469					7,65
7.11.2020	5	B. braunii	5	Bb5	1,432	0,737	5,02	7,1	1	1	8,02
9.11.2020	7	B. braunii	5	Bb5	1,522	0,783	1,22		0,7		7,77
12.11.2020	10	B. braunii	5	Bb5	1,762	0,908	0,2	3	0,6	1	8,12
Replicate 2											
16.11.20	0	B. braunii	0	Bb0	0,266	0,132	16,1	18,1	20,8	22	9,25
17.11.20.	1	B. braunii	0	Bb0	0,362	0,182	16				8,06
18.11.20	2	B. braunii	0	Bb0	0,676	0,345	12,55		8,8		7,94
19.11.20	3	B. braunii	0	Bb0	1,021	0,524	7,3		0,8		7,7
20.11.20	4	B. braunii	0	Bb0	0,724	0,370					7,95
21.11.20	5	B. braunii	0	Bb0	1,552	0,799	4,7	9,4	0,3	3	7,74
23.11.20	7	B. braunii	0	Bb0	1,784	0,919	1,9		0,6		7,49
26.11.20.	10	B. braunii	0	Bb0	2,556	1,319	0,75	3,4	0,8	1	7,35
16.11.20	0	B. braunii	1	Bb1	0,307	0,154	17,7	19,7	20,8	22	9,13
17.11.20.	1	B. braunii	1	Bb1	0,3	0,150	17,8				7,58
18.11.20	2	B. braunii	1	Bb1	0,661	0,337	12,05		8,7		8,14
19.11.20	3	B. braunii	1	Bb1	1,022	0,524	7,05		0,7		7,8
20.11.20	4	B. braunii	1	Bb1	0,708	0,362					7,84
21.11.20	5	B. braunii	1	Bb1	1,526	0,786	4,3	6,6	0,5	2	7,75
23.11.20	7	B. braunii	1	Bb1	1,74	0,896	2,06		0,5		7,5
26.11.20.	10	B. braunii	1	Bb1	2,46	1,270	0,07	3	0	0	7,45
16.11.20	0	B. braunii	3	Bb3	0,249	0,124	17,2	19,2	21	22,2	8,91
17.11.20.	1	B. braunii	3	Bb3	0,238	0,118	16,6				7,81
18.11.20	2	B. braunii	3	Bb3	0,48	0,243	13,35		13,1		7,71
19.11.20	3	B. braunii	3	Bb3	0,802	0,410	8,25		5,2		7,77
20.11.20	4	B. braunii	3	Bb3	0,578	0,294					7,96
21.11.20	5	B. braunii	3	Bb3	1,25	0,642	5,4	8,4	0,4	4	7,59
23.11.20	7	B. braunii	3	Bb3	1,496	0,770	3,04		0,6		7,51
26.11.20.	10	B. braunii	3	Bb3	2,324	1,199	0,17	3,1	0,9	8	7,75
16.11.20	0	B. braunii	5	Bb5	0,219	0,108	17,3	19,3	20,8	22	8,84
17.11.20.	1	B. braunii	5	Bb5	0,25	0,124	16,4				7,73
18.11.20	2	B. braunii	5	Bb5	0,528	0,268	13,25		12,5		8,02
19.11.20	3	B. braunii	5	Bb5	0,943	0,483	8,65		4,4		7,5
20.11.20	4	B. braunii	5	Bb5	0,668	0,341					7,67
21.11.20	5	B. braunii	5	Bb5	1,374	0,707	6,26	10	0,4	2	7,33
23.11.20	7	B. braunii	5	Bb5	1,87	0,964	2,82		0,6		7,82
26.11.20.	10	B. braunii	5	Bb5	2,788	1,440	0,08	3,3	0	1	7,82