FACULTY OF SCIENCE AND TECHNOLOGY

MASTER THESIS

Study programme / specialisation:
Environmental Engineering/Water Science and Technology

The spring semester, 2022

Open / Confidential

Author: Sigrid Øverli-Øyen

Sigrid Øverli-Øyen
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(signature author)

Course coordinator: Roald Kommedal

Supervisor(s): Roald Kommedal

Thesis title: **Determination of bioavailable phosphorus retention in Leikvollbekken constructed wetland by a novel algae bioassay**

Credits (ECTS): 30

Keywords:
Bioavailable phosphorus,
Constructed wetland,
Algae bioassay, phosphorus,
Microplate

Pages: 83

+ appendix: 14

Stavanger, 03.07.22

ABSTRACT

Excessive phosphorus loads from agricultural runoff contribute to freshwater eutrophication, which leads to reduced water quality and changes across all trophic levels. Constructed wetlands, specially designed to retain nutrients and particles inputs through biological, chemical and physical processes, are used worldwide to treat anthropogenic discharges. The main objective of this study was to investigate the retention of bioavailable phosphorus in Leikvollbekken constructed wetland. Leikvollbekken is located on the southwestern coast of Norway and receives nutrient rich runoff from agricultural activities. Microplate reader was used to determine bioavailable phosphorus in water samples taken from the inlet and the outlet of the constructed wetland. Water samples were simultaneously chemically analysed to determine the chemical fractions and measured for total suspended solids. Microplate reader method can determine algae growth by measuring the change of fluorescence intensity over time. Two parallel bioassays were conducted. The first bioassay was performed with inoculum from Lake Store Stokkavannet, the recipient of the constructed wetland. The parallel bioassay was conducted with a pre-grown lake water sample. Due to methodological challenges, the analysis of bioavailable phosphorus was limited to three of total ten bioassays conducted, both for pre-grown and lake water inoculum. Results from bioassays performed with pre-grown inoculum showed that the constructed wetland on average retained 34 % of the incoming bioavailable phosphorus. Phosphorus analysis conducted on the corresponding water samples showed an average retention of 1 ± 4 % (phosphate) and 34 ± 2 % (total phosphorus). Bioassays performed with lake water inoculum demonstrated an average retention of bioavailable phosphorus of 24 %. Phosphorus analysis conducted on the corresponding water samples showed an average retention of 14 ± 3 % (phosphate) and 26 ± 2 % (total phosphorus). Regression analysis indicated a moderate to strong positive relationship between chemical available and bioavailable phosphorus. The calibration of the microplate reader, using flow cytometry and direct counting, indicated a strong positive correlation between fluorescence signal and algae cell concentration (R^2 = 0.9986 for flow cytometry, R^2 = 0.9995 for direct counting). A t-test (95 % confidence level) confirmed no significant difference between the two quantification methods. Results from the statistical analysis indicated that algae cell concentration in water samples can be determined by microplate measurement. However, the study revealed that the bioassay method probably was not optimally designed for determination of bioavailable phosphorus. Analysis showed that CO_2 limitation had a substantial effect on algae growth potential. Bioassay supplied with CO_2 showed approximately 55 % (pre-grown inoculum) and 40 % (lake water inoculum) more cells compared with bioassay not supplied with CO_2. When determining bioavailable phosphorus, phosphorus must be the only limiting factor. In future studies using this method, the suggested improvements should be considered.

ACKNOWLEDGEMENT

First, I want to thank my supervisor Assoc. Prof. Roald Kommedal for giving me the opportunity to work on this project and for your guidance throughout the thesis. I greatly appreciate your guidance to develop a scientific mindset during the master's programme and the knowledge you provided that went beyond the theory in books.

To Dr. Anissa Sukma Safitri: It has been a true pleasure to work with you! Thank you for always helping me with a smile, regardless the obstacle. Your guidance, feedback and encouragement are truly appreciated.

I would also like to thank Hans Kristian Brekken and Erling Berge Monsen: Thank you so much for your assistance with the laboratory related work.

Finally, a special thanks to Magnus for supporting me through all my years of studying. Thank you for your patience, encouragement and for being the best dad for our kids.

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1. INTRODUCTION

As part of important biomolecules and involved in enzymatic reactions and regulation of metabolic pathways, phosphorus is essential for life (Madigan et al., 2015). In most freshwater systems, phosphorus is the limiting nutrient (Wetzel, 2001). However, this important growth nutrient is also primarily responsible for eutrophication in freshwater system. Eutrophication, the gradually increase of nutrients to a water body, may cause an increase in primary production (Lægreid et al., 1999). Anoxic waters, toxic algae blooms and loss of biodiversity are some of the unwanted consequences of the increase in phytoplanktonic biomass (Lægreid et al., 1999; Ødegaard, 2012). Agricultural runoff from intensive fertilisation is a common source of eutrophication. Excess phosphorus, from inorganic and organic fertilisers, may enter nearby waterways and result in eutrophication of downstream freshwater recipients (Trentman et al., 2021). Eutrophication is a global problem and the anthropogenic release of phosphorus has affected aquatic ecosystems since the 20th century (Rönspieß et al., 2021). Several technologies have been used to treat nutrient enriched water to reduce eutrophication, and constructed wetlands are one of them.

Constructed wetlands (CW) are specially designed for removal of particulate organic matter and enhanced nutrient uptake (Kadlec & Wallace, 2008). Due to low cost, maintenance and operation, CW has emerged as an accepted treatment solution to treat polluted water (Doherty et al., 2015). Rogaland is one of the county's with the most constructed wetlands in Norway, and the first constructed wetlands was built in 1994 (Hauge, 2006). Leikvollbekken CW is one of four constructed wetlands located at the edge of Lake Store Stokkavannet (location shown by red circle in Figure 1.1), on the border between the municipalities of Stavanger and Randaberg.

*Figure 1.1: The wetland location (red circle) in Stavanger/Randaberg (Norway) (*Google Maps*, 2022)*

The CW was built in 1994 to reduce agricultural runoff to Store Stokkavannet, which at present day, act as a reserve drinking water source (IVAR, 2018).

Phosphorus retention in Leikvollbekken CW has been a subject in studies from 2016, 2017, 2018 and 2021. Krahner (2017) and Luth-Hanssen (2018) focused primarily on whether the CW, in terms of chemically analysed phosphorus, can retain phosphorus. The studies provided a good picture of the total phosphorus and phosphate retention capability of the CW. However, the studies did not measure the retention of the bioavailable phosphorus (BAP), which is the fraction of total phosphorus that contribute to eutrophication (Boström et al., 1988). BAP can be determined both chemically and by algae bioassays. A chemically approach will underestimate the total BAP in water samples and is generally accepted as an inadequate estimate of BAP (Bradford & Peters, 1987; Reynolds & Davies, 2001).

The studies from 2016 and 2021 focused on the retention of BAP, by using algae bioassays. Batch culture algae bioassays were conducted in 2016, and the study revealed no clear conclusion on the BAP retention (Handley, 2016). Several methodological weaknesses were pointed out by the author, such as difficulties with determination of stationary phase of the algae, few replicates and not a suitable algae strain *(Raphidocelis subcapitata)*. Due to the unsatisfying results from the batch method, a novel algae bioassay method was tested in 2021 (Tjelta, 2021). The method used microplates and a microplate reader to determine algae growth by measuring the change in fluorescence intensity over time. The method was developed based on the work of van Wagenen et al. (2014) which demonstrated that it is possible to measure characteristic exponential growth rates of algae using microplate reader. Additionally, studies performed at University of Stavanger showed that microplate reader method could predict nutrient limited kinetic growth analysis of *Chlorella sorokiniana* (Safitri, 2021).

The main objective of this study was to evaluate the Leikvollbekken CW's retention capability of the incoming bioavailable phosphorus. This was investigated by using microplate reader method. Compared to traditional batch culture algae bioassay, which can be time-consuming and expensive (Bradford & Peters, 1987), microplate is an easy, fast and low-cost tool for use in algae bioassay (Van Wagenen et al., 2014). Additionally, the method implements more replicates than batch culture bioassays, while requiring less space.

2. THEORY

2.1 Constructed wetlands

Wetlands are areas of land covered or saturated with water, either permanently or seasonally (Mitsch & Gosselink, 2015). Wetlands are of ecologically importance, possessing high biodiversity and biogeochemical cycling of carbon and macronutrients (ibid.). Constructed wetlands (CW) are engineered treatment systems designed to remove water pollutants by the use of vegetation, soils and microorganisms (Wu et al., 2014). The treatment include contaminated water from wastewater, agricultural runoff and mine drainage (Doherty et al., 2015). Due to the low cost, low external energy requirement and minimal operation and maintenance, the CWs have become a popular alternative for water treatment and water quality improvement (Doherty et al., 2015; Wu et al., 2014). The treatment goals will affect the design and size of the constructed wetland. In general, several zones are designed to facilitate the mechanisms needed for increased nutrient and hydraulic retention time (Kadlec & Wallace, 2008).

As demonstrated in Figure 2.1 wetlands have a distinctive oxygen profile comprising of both aerobic and anaerobic compartments. The water column and the upper, thin aerobic soil layer represents a source for oxidized ions (Mitsch & Gosselink, 2015). An anaerobic soil layer exists deeper in the sediments. The reduced conditions are caused by a low oxygen diffusion rate in the water saturated soil and microbial respiration (ibid.).

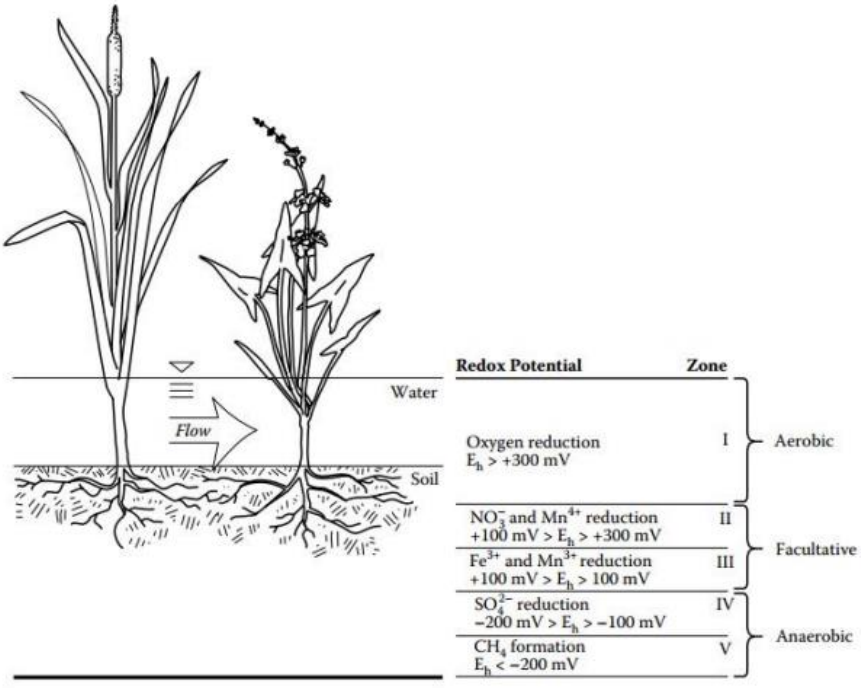

Figure 2.1: Redox potentials in a wetland's water column and soil (Kadlec & Wallace, 2008).

Oxygen is the most energetically favourable electron donor (Madigan et al., 2015). When oxygen is depleted in the wetland, a successive microbial reduction of NO_3^- , Fe^{3+}, SO_4^{2-} and CO_2 will follow. Numerous anaerobic bacteria can obtain energy by the reduction of Fe^{3+} to Fe^{2+}. The organisms in the facultative zone prefer oxygen as an electron donor, but they can switch to fermentation or anaerobic respiration if free oxygen is not available (Madigan et al., 2015). Phosphorus is mostly found in the +5 oxidation state and hence phosphorus is only indirectly affected by an alteration in redox potential (Reddy & DeLaune, 2008).

2.2 Phosphorus

Phosphorus is an essential nutrient for microorganisms and plants, and is part of important biochemical molecules such as nucleic acids, phospholipids and ATP. In addition, phosphorus is involved in controlling important enzymatic reactions and regulation of metabolic pathways (Madigan et al., 2015; Schachtman et al., 1998). Compared with the other major nutrients, such as oxygen, nitrogen, carbon, hydrogen and sulfur, phosphorus is least abundant and most commonly limits biological productivity for plants and microorganisms in freshwater environments (Trentman et al., 2021; Wetzel, 2001).

Phosphorus, in the Earth's crust, exists mainly in poorly soluble calcium phosphate minerals, such as apatite, and the atmospheric phosphorus reservoir is almost non-existing. In addition, the small fraction of available phosphorus will be rapidly fixed by either biotic or abiotic mechanisms. Living organisms play an important role in the phosphorus cycling, such as decomposition of dead biomass and bacterial solubilisation of unavailable phosphorus. However, compared to nitrogen and carbon, the phosphorus cycle is not dominated by biotic mechanisms and a rapid cycling of phosphorus does not exist (Smil, 2000). As an example, in nitrogen deficient water bodies, a supply of nitrogen can be achieved by nitrogen-fixing bacteria (Howarth et al., 1988). These bacteria convert atmospheric N_2, which is not bioavailable for other organisms, to bioavailable NH_3 (ibid.).

Phosphorus is, as mentioned above, fundamental for growth of plants and microorganisms. However, an overabundance of phosphorus in water can lead to an excessive algae growth, a process called eutrophication (Lægreid et al., 1999). Since weathering of phosphorus is rather slow, eutrophication is generally caused by anthropogenic sources, such as the use of fertilisers in agriculture and leakage of wastewater (Trentman et al., 2021; Smil, 2000). Toxic algae blooms, water anoxia, increased turbidity and loss of habitats and biodiversity are some of the undesirable consequences of eutrophication (Lægreid et al., 1999; Ødegaard, 2012). Eutrophication is most likely to occur when dissolved P concentrations exceed 0.01 mg/L, although the process is dependent more on the loading than the concentration (Smil, 2000). An important notice on the eutrophication is that

only the bioavailable fraction of phosphorus will contribute to eutrophication (Boström et al., 1988). Bioavailability of phosphorus will be further presented in section 2.5 .

2.3 Phosphorus speciation in wetlands

The phosphorus cycle shown in Figure 2.2 present different phosphorus compounds typically found in different internal compartments (sediments, water column, biota) in wetlands. The speciation of phosphorus can be difficult to estimate due to a complex phosphorus cycle and frequently rapid transformation. The retention and release mechanisms of phosphorus will be further presented in section 2.4 and 2.5.1 .

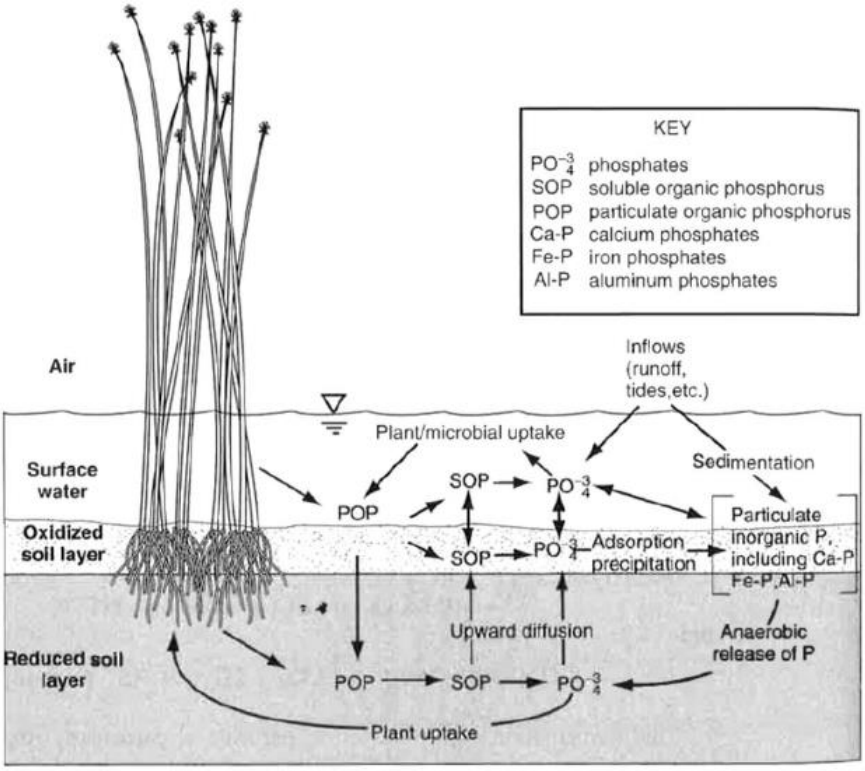

Figure 2.2: Biogeochemical phosphorus cycle in wetlands (Mitsch & Gosselink, 2015).

In wetlands, phosphorus can exist in an organic or inorganic form and be either particulate or dissolved. The classifications are usually based on analytical methods. Total phosphorus (TP) is the total amount of detectable phosphorus in a water sample. TP is determined analytically by digestion of an unfiltered sample in a strong acid to convert all phosphorus into dissolved orthophosphates, normally followed by colorimetric determination (Clesceri et al., 1998).

TP can be divided into POP, PIP, DOP and DIP (Figure 2.3). The separation between the particulate and dissolved fraction is achieved by filtration through 0.45 mm membrane filters (Clesceri et al., 1998).

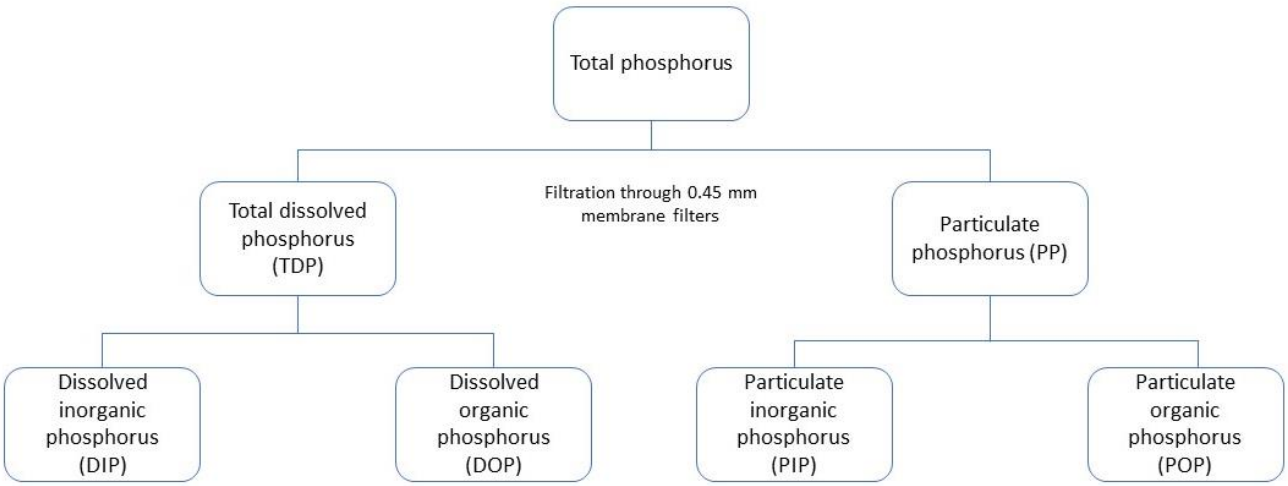

Figure 2.3: Speciation diagram of aquatic phosphorus.

POP consist of phosphorus in dead or living organisms, in addition to phosphorus associated with organic particles present in the wetland, while PIP is particulate phosphorus associated with minerals (Dodson, 2005). DOP is phosphorus associated with soluble organic compounds, which can range from simple organic compounds such as sugar phosphates, to more complex as phospholipids (Reddy & DeLaune, 2008). Tchobanoglous et al. (2014), Dodson (2005) and Reddy and DeLaune (2008) stated that DIP consists of only orthophosphates, while Kadlec and Wallace (2008) also included condensed phosphates (poly- and metaphosphates) as part of DIP.

Dissolved phosphates are the most reactive forms. In natural waters the most common species are mono- or dibasic phosphates, depending on the pH:

$$H_2PO_4^- \leftrightarrow HPO_4^{2-} + H^+ \qquad \text{Equation 2.1}$$

$$pK_a = 7.2$$

The sum of phosphoric acid and all conjugated bases are normally referred to as orthophosphates (PO_4^{3-}) in aquatic sciences (Kadlec & Wallace, 2008). The analytical ascorbic acid method (Clesceri et al., 1998), described in method section 3.3.4 , finds this form of phosphorus. However, the method may also measure minor fractions of labile dissolved organic phosphorus, which may be hydrolysed under analysis due to addition of sulfuric acid (Clesceri et al., 1998).

Orthophosphate (Figure 2.4) is readily bioavailable, meaning no further breakdown is needed for biological uptake and conversion by intracellular metabolism (Tchobanoglous et al., 2014).

Particulate and organic phosphorus need to undergo transformations before they are bioavailable (Dunne et al., 2005).

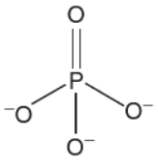

Figure 2.4: Structure of orthophosphate (Brezonik & Arnold, 2011).

Condensed phosphates are orthophosphates linked together by the sharing of oxygen atoms (Figure 2.5). Polyphosphates, such as pyrophosphate and tripolyphosphate, have a linear structure. Metaphosphates, such as trimetaphosphate, have a ring structure (Brezonik & Arnold, 2011).

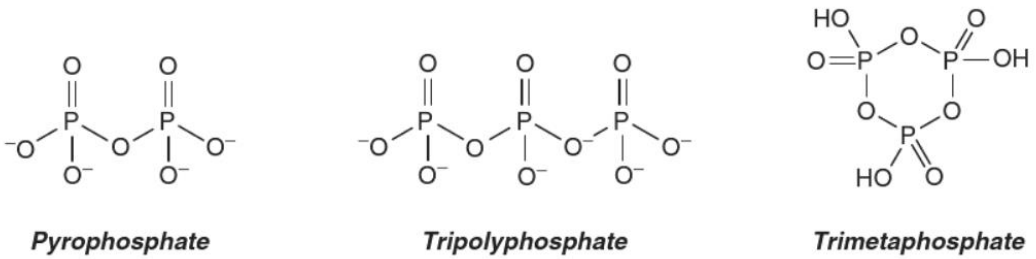

Figure 2.5: Structures of condensed phosphates. The two structures to the left are examples of polyphosphates (pyrophosphate and tripolyphosphates). The structure to the right (trimetaphosphate) is a type of metaphosphates (Brezonik & Arnold, 2011).

Based on a separation of total phosphorus into organic and inorganic fractions, most of the total phosphorus is organic (Wetzel, 2001). More than 70 % of the total organic phosphorus is in a particulate form and the remainder is either dissolved or in a colloidal state (ibid.). Even though the biggest fraction of phosphorus is organic, only a small part may be biologically available (Reddy & DeLaune, 2008). According to Wetzel (2001) the concentration of DIP is very low compared to other forms of phosphorus in the aquatic environment, and typically constitutes only five percent of TP.

Inorganic phosphorus can be categorized as (1) exchangeable P, (2), Fe and Al bound P, (3) Ca and Mg bound P and (4) residual P. Organic phosphorus can be divided into (1) easily decomposable organic P, as sugar phosphates, nucleic acids and phospholipids, and (2) slowly decomposable organic P, like inositol phosphate and phytin (Dunne et al., 2005).

Another important classification of the different forms of phosphorus is soluble reactive phosphorus (SRP). SRP is defined in different ways in the literature. Dodson (2005) defined SRP as any dissolved

inorganic or organic phosphorus readily available for uptake by algae and macrophytes, where orthophosphates is a major fraction of SRP. Kadlec et al. (2008) described SRP as the sum of orthophosphates and the fraction of DOP that are readily hydrolysed by soil enzymes. Wetzel (2001) on the other hand, defined SRP as orthophosphates. Reynolds and Davies (2001) classified SRP as the sum of orthophosphates in solution plus colloid bound phosphorus. In this thesis, SRP is defined as all soluble easily reactive phosphorus. In other words, SRP is defined as the phosphorus fraction which react with a colorimetric reagent without prior hydrolysis or digestion.

2.4 Immobilisation of phosphorus in constructed wetlands

SRP is both biologically and chemically available meaning that phosphate can quickly transform to less bioavailable forms. Both biotic and abiotic processes regulate phosphorus transformation and translocation. Biotic processes include assimilation by plants and microbes. Precipitation, sorption, sedimentation and exchange processes between soil/sediment and overlying water column are part of the abiotic processes regulating phosphorus transformation and locations (Dunne et al., 2005). The main portion of phosphorus is stored in the wetland's soils and sediments. Of the remainder, most is contained in plants and litter, while only a small fraction of phosphorus is stored in microorganisms and water. Sediment and soil phosphorus are largely in organic forms (Kadlec & Wallace, 2008). Figure 2.6 presents common phosphorus content in different internal compartments in a wetland. This subchapter presents some of the main processes which retain phosphorus in constructed wetlands.

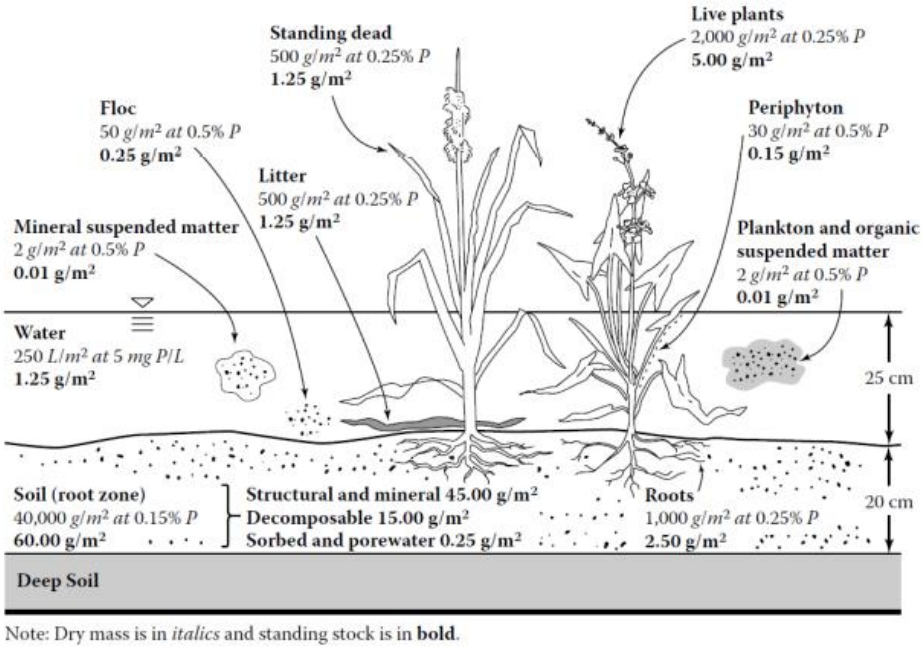

Figure 2.6: Phosphorus storage in different internal compartments in a wetland (Kadlec & Wallace, 2008).

2.4.1 Adsorption

Adsorption is the process where a substance or a material, the adsorbate, adheres and accumulate onto a solid surface, the adsorbent. Adsorption can be caused by either a chemical reaction between adsorbent and adsorbate (chemisorption) or physical attraction (physical adsorption). Physical adsorption is the most common mechanism in water. It is a rapid and reversible process caused by weak attractive forces between adsorbate and adsorbent. The reverse process is called desorption. When adsorption rate equals desorption rate the system is in equilibrium and adsorbent capacity has been reached (Crittenden et al., 2012).

The adsorption capacity is affected by the physical and chemical properties of the particles in the wetland. Both inorganic and organic phosphorus can be adsorbed (Dunne et al., 2005). Humus, clay minerals and the metallic cations aluminium, iron and calcium are the most common phosphorus adsorbents in wetland sediments (Mitsch & Gosselink, 2015; Reddy & DeLaune, 2008). Humus is decayed organic matter, derived mostly from plant litter (Cole, 1994). Clays have a sheet-like structure with repeating units of either silicon, aluminium or magnesium atoms surrounded by either oxygen or hydroxy atoms (Holden, 2012).

Adsorption capacity will increase with a high amount of positively charged clay particles and minerals (Dunne et al., 2005). pH will also alter the adsorption process (Figure 2.7). In an acidic environment, excess H^+ generate positively charged particle surfaces, in which the negatively charged phosphate ion can adsorb. Under alkaline conditions the negatively charged surfaces will preclude phosphate adsorption (Reddy & DeLaune, 2008).

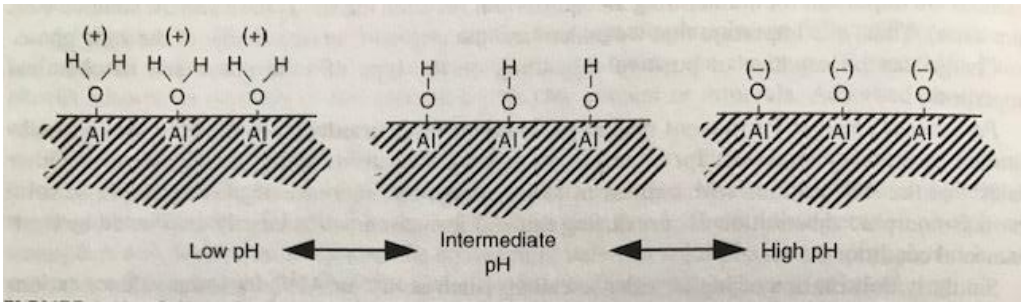

Figure 2.7: Illustration showing pH influence on aluminium solid surface (Reddy & DeLaune, 2008).

Organic phosphorus may adsorb onto organic matter and clay, where inositol phosphates will adsorb more greatly onto clay compared to the simpler sugar phosphates and nucleic acids (Dunne et al., 2005). When all sorption sites are occupied maximum sorption capacity is reached. Langmuir and Freundlich models are used to determine maximum adsorption capacity (ibid.).

2.4.2 Precipitation

Phosphorous adsorption typically occurs at low concentrations. As the phosphorus concentrations increase, precipitation may occur. The stability of the phosphate minerals is regulated by pH, redox potential (Dunne et al., 2005) and potentially available ligands, such as sulfate, fluoride and carbonate (Tchobanoglous et al., 2014; Wetzel, 2001). Phosphate has a great tendency to form complexes with calcium, iron and aluminium under aerobic conditions (Mitsch & Gosselink, 2015). The types of complexes formed are dependent on the pH. Under alkaline conditions, phosphate normally precipitate with calcium and magnesium, where hydroxyapatite ($Ca_5(PO_4)_3OH$) is a common phosphate mineral in wetlands (Kadlec & Wallace, 2008; Reddy & DeLaune, 2008). Under acidic conditions phosphates generally form complexes with aluminium or iron. Significant phosphate minerals in wetlands are strengite ($FePO_4$), vivianite ($(Fe_3(PO_4)_2$) and variscite ($AlPO_4$) (Reddy & DeLaune, 2008).

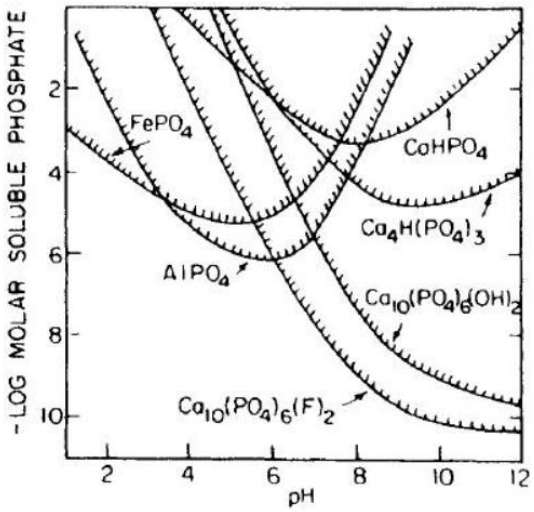

Figure 2.8: Solubility diagram of some metal phosphates in aquatic environments (Stumm & Morgan, 1995).

The impact of pH on the solubility of some phosphate minerals is demonstrated in Figure 2.8. In the lower pH range phosphate will be fixed by $FePO_4$ and $AlPO_4$. However, at very low pH the phosphate fixation by iron and aluminium will decrease. An increase in pH will decrease phosphate fixation by iron and aluminium, but increase calcium fixation of phosphate (Stumm & Morgan, 1995).

Phosphorus can also co-precipitate with other minerals, such as ferric hydroxide and calcium carbonate (Kadlec & Wallace, 2008). The latter is formed when the pH increase (Wetzel, 2001).

Iron associated phosphate minerals are sensitive to fluctuations in redox potential, since iron is reduced under anaerobic conditions (Reddy & DeLaune, 2008). With a redox potential of approximately 300 millivolts and with pH ranging from 5 to 8 solubility of phosphorus is low.

Phosphorus solubility increase for all pH levels if the wetland soil sediment shifts from aerobic to anaerobic (from 300 mV to – 250 mV) (Dunne et al., 2005).

As presented above, the phosphorus mineral chemistry involves a great deal of complexity, hence computer models are commonly used to estimate equilibrium conditions (Kadlec & Wallace, 2008). An increase in temperature will increase the rate of both adsorption and precipitation (Reddy & DeLaune, 2008).

2.4.3 Ion exchange

Ion exchange is a reversible process where ions with the same charge are exchanged between an insoluble solid and a solution (Tchobanoglous et al., 2014). As mentioned in section 2.4.1 , an acidic environment may produce positively charged surfaces on clay particles and organic matter. This promote surface adsorption of negatively charged anions like NO_3^-, Cl^- and OH^- (Reddy & DeLaune, 2008). These anions can be replaced by phosphate in an ion exchange reducing the soluble fraction of phosphorus (ibid.).

2.4.4 Sedimentation of solids

To achieve retention, both for the PP entering the wetland and the PP formed in the wetland, the particulates need to settle. Sedimentation refers to the separation of particles that are heavier than water by gravitational settling (Tchobanoglous et al., 2014). To settle, the particles' retention time need to be greater than the hydraulic retention time (ibid). In situations with high hydraulic loadings, such as a storm event, unwanted resuspension of particles may happen (Kadlec & Wallace, 2008).

The Hjulstrøm diagram, shown in Figure 2.9, demonstrate the relationship between sediment size and the water velocity required for eroding, transporting or depositing of the sediments (Holden, 2012). As sediment grain size decrease (where clay is the smallest particle), a lower water velocity is required to keep the particles in the sediments.

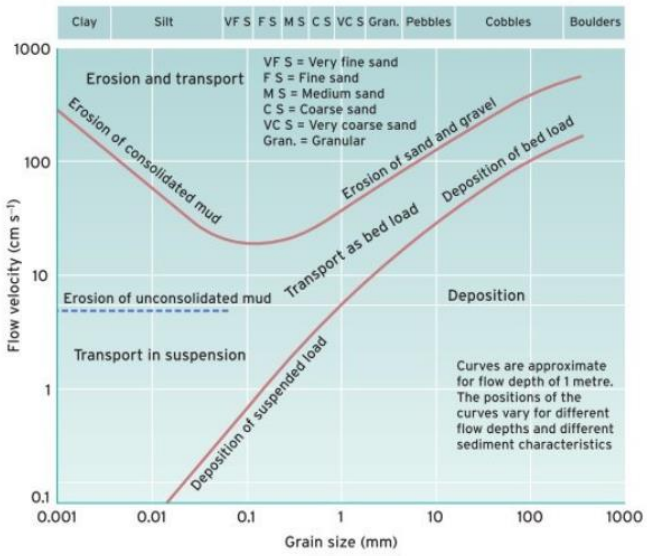

Figure 2.9: The Hjulstrøm diagram (Holden, 2012).

2.4.5 Plant and microbial uptake

Since phosphorus is an essential nutrient required for living organisms, phosphorus will also be retained by plants and microorganisms in the wetland. The season is a fundamental factor for biotic phosphorus uptake. During spring and summer, plants and microorganisms assimilate phosphorus to grow, while a release of phosphorus will happen during fall and winter due to decomposition of dead organic material (Kadlec & Wallace, 2008).

Phosphorus is readily available for biotic uptake as dissolved phosphates (Taiz & Zeiger, 2010). Plants take up phosphate primarily in the root zone which is then incorporated into organic compounds such as nucleotides and phospholipids (ibid.). Commonly, phosphorus is assimilated in excess by plants and stored for future use (Dodson, 2005). Plants store phosphorus primarily in the form of phytic acid (Figure 2.10). Phytic acid is the major form of phosphorus found in plant tissue (Taiz & Zeiger, 2010). During fall phosphorus is transported to the roots for storage, and will be utilised again when the growth season begins in spring (Reddy & DeLaune, 2008).

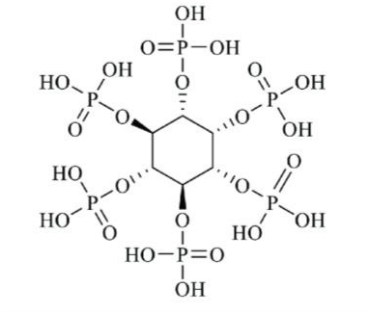

Figure 2.10: Chemical structure of phytic acid (Jatuwong et al., 2020).

As for plants, phosphorus is assimilated beyond the needs of the microorganisms, and stored for future use (Dodson, 2005). The process is commonly referred to as “luxury uptake”. Algae will assimilate phosphate at high rates if phosphate is available (ibid). Phosphate accumulation bacteria will, under aerobic conditions, take up massive amounts of phosphorus. A release of the stored phosphate will occur in an reducing environment (Henze et al., 2008). As the life cycle of microorganisms is short, the greatest portion of the phosphorus uptake is returned as particulate phosphorus and dissolved organic phosphorus (Kadlec & Wallace, 2008).

2.5 Bioavailability of phosphorus

Not all of the total phosphorus is available for biological uptake and assimilation. The fraction of TP that is available for use by plants and microorganisms are called bioavailable phosphorus (BAP) and is divided into readily available phosphorus and potentially available phosphorus (Boström et al., 1988), as shown in Figure 2.11:

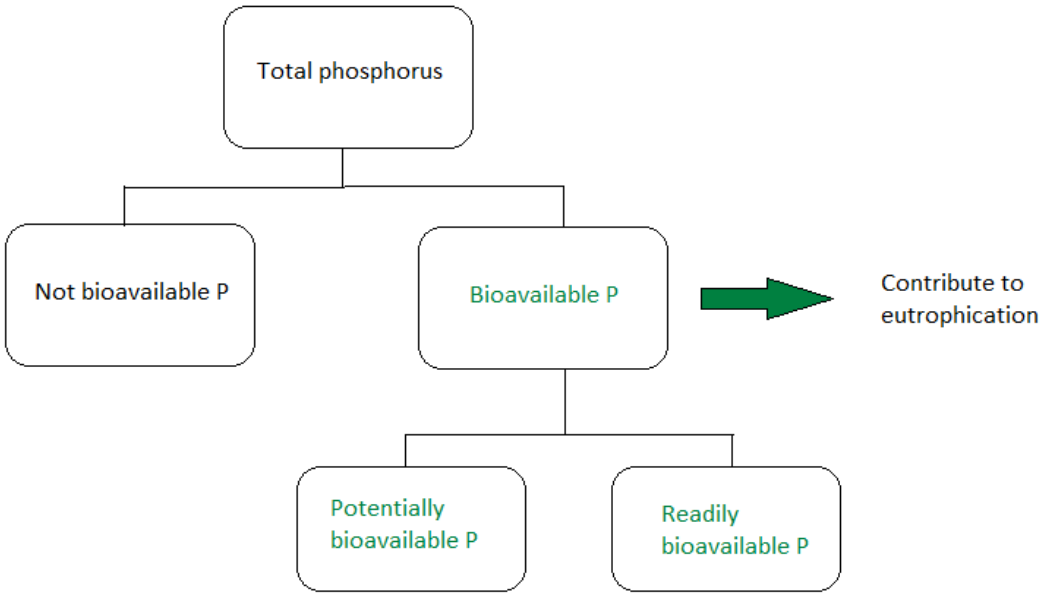

Figure 2.11: Schematic illustration of the bioavailable fraction of phosphorus.

Most of the total bioavailable phosphorus is in the inorganic form (Dunne et al., 2005). Phosphorus co-precipitated with $CaCO_3$ (apatite) is typically unavailable (ibid.). As mentioned in section 2.2 , it is the bioavailable fraction of phosphorus that will contribute to eutrophication.

As for SRP, readily and potentially bioavailable phosphorus are defined in different ways in the literature. Kadlec and Wallace (2008) defined readily bioavailable phosphorus as orthophosphates and a fraction of the condensed phosphates. Reynolds and Davies (2001) stated that readily

bioavailable phosphorus consists of dissolved orthophosphates and colloid-bound P. According to Dunne et al. (2005) potentially BAP included Fe and Al bound P, Ca and Mg bound P. Reynolds and Davies (2001) also included exchangeable P as part of potentially BAP. Søndergaard et al. (2003) defined potentially bioavailable phosphorus as redox sensitive sorbed P, iron bound P and loosely sorbed inorganic and organic P.

In this thesis, readily bioavailable phosphorus is defined as free or loosely adsorbed orthophosphate, which can directly be assimilated by plants and microorganisms without conversion. Potentially bioavailable phosphorus is defined as the phosphorus fraction that will be available due to natural occurring processes as desorption, chemical dissolution and biological p-release processes. Hence, potentially BAP includes fractions of particulate and organic phosphorus. The speciation diagram shown in Figure 2.3 can be simplified to demonstrate the bioavailable fractions of total phosphorus (Figure 2.12):

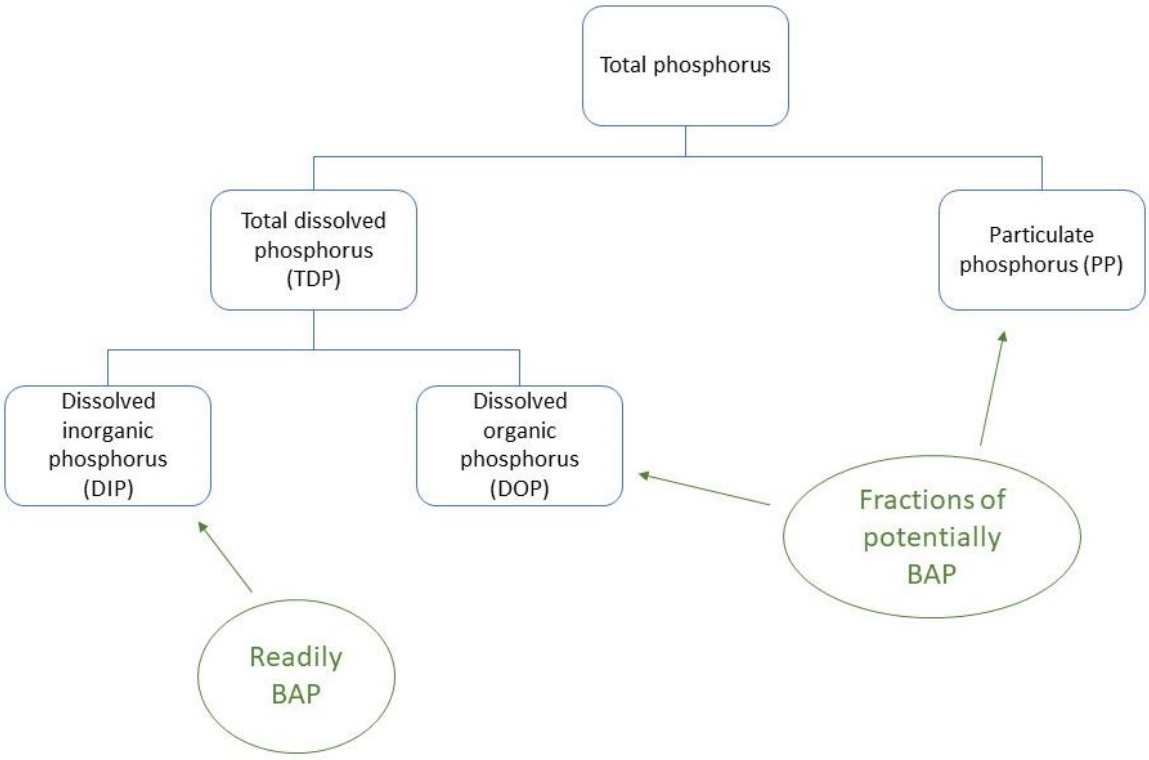

Figure 2.12: Phosphorus speciation diagram with emphasise on the bioavailable fractions.

As discussed above and shown in Figure 2.12, BAP include orthophosphates and fractions of the total dissolved phosphorus and particulate phosphorus. Hence, the total BAP cannot be determined by any of the analytical measured phosphorus fractions.

2.5.1 Potentially BAP and mechanisms for phosphorus release

A fraction of the immobilised phosphorus can be converted to a bioavailable form by chemical and biological processes. This subsection will present some of the abiotic and biotic mechanisms for phosphorus release.

2.5.1.1 Anaerobic phosphorus release

As mentioned in section 2.4.2 , phosphate is part of strengite ($FePO_{4\,(s)}$) in oxidised soils. When the oxygen and nitrate concentrations are deprived, due to microbial respiration, the redox potential is favourable for the reduction of Fe^{3+} (Madigan et al., 2015). The reduction of Fe^{3+} to Fe^{2+}, caused by iron reducing bacteria (FeRB) will release phosphate from strengite, as shown in Equation 2.2 (Snoeyink & Jenkins, 1980):

$$FePO_{4\,(s)} + e^- \leftrightarrow Fe^{2+} + PO_4^{3-} \qquad \text{Equation 2.2}$$

At lower redox potential, sulfate is reduced to sulfide by sulfate reducing bacteria (SRB) (Reddy & DeLaune, 2008). If the sulphate concentration is high during anaerobic conditions SRB may form ferrous sulfide (FeS). The microbial activity negatively affects the abundance of iron compounds that can complex with phosphate, resulting in phosphate release into sediment pore water (Wetzel, 2001). However, formation of the mineral vivianite ($Fe3(PO4)_2$) can occur since ferrous iron (Fe^{2+}) can react with phosphate (Reddy & DeLaune, 2008). The complex interactions between SRB and FeRB are shown in Figure 2.13:

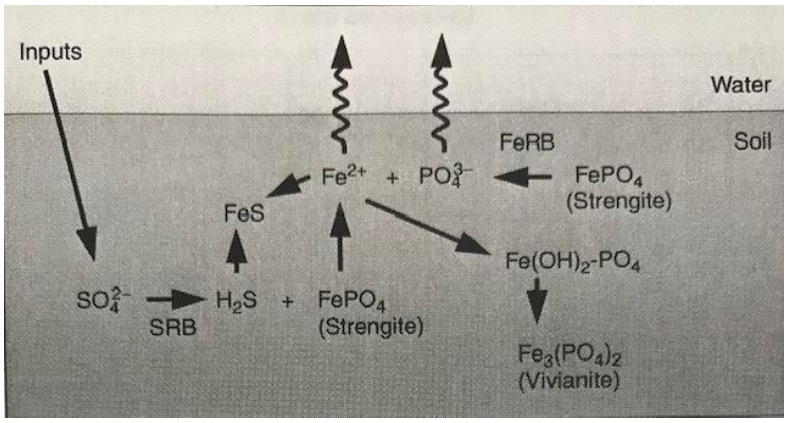

Figure 2.13: Interaction of sulfate reduction on P release (Reddy & DeLaune, 2008).

2.5.1.2 Alkaline condition phosphorus release

During intense photosynthesis, adsorped phosphorus can be released. High levels of CO_2 assimilation by algae will increase the pH due to the shift on the carbonate equilibrium, as shown by Equation 2.3 (Cole, 1994):

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3 + H^+$$ Equation 2.3

At high pH, phosphate combined with Fe and Al minerals can be replaced with OH^- ions through an ion exchange and thereby releasing phosphate. However, at high pH the released phosphate may react with Ca^{2+} and form $CaHPO_4$, hence decreasing the bioavailable phosphorus fraction (Reddy & DeLaune, 2008), shown in Equation 2.4:

$$Ca^{2+} + HPO_4^{2-} \leftrightarrow CaHPO_4$$ Equation 2.4

Under microbial decomposition of organic matter the release of organic acids can decrease the pH in alkaline soils, and hence the precipitated $CaHPO_4$ may be solubilised, releasing phosphate (Reddy & DeLaune, 2008).

2.5.1.3 Phosphate Solubilizing Microorganisms

Various soil microorganisms, commonly referred to as Phosphate Solubilizing Microorganisms (PSM), possess the ability to transform insoluble soil-bound phosphorus to soluble phosphorus. The mechanisms used by PSM can be classified into two groups: (1) production and secretion of mineral dissolving compounds and (2) secretion of phosphatases to enzymatically mineralise organic P compounds. P solubilisation and mineralisation abilities are found in a wide range of microbiological organisms, including bacteria, fungi and algae (Alori et al., 2017).

Production and excretion of organic acids, protons, hydroxyl ions and CO_2 is the primary mechanism of inorganic phosphate solubilisation, which cause a decrease in the soil pH. Some PSM can also produce inorganic acids (such as sulphuric and carbonic acids) and chelating substances. In the process of organic P mineralisation, PSM synthesise and excrete phosphatases and/or phytases (Alori et al., 2017). Phosphatases are extracellular enzymes that catalyse the hydrolysis of phospho-ester bonds in organic phosphorus compounds, thereby releasing readily bioavailable orthophosphate (Dotaniya et al., 2019). Phosphatase can either be acid or alkaline. Phytase is an enzyme that catalyses the hydrolysis of phytic acid (Alori et al., 2017).

2.5.1.4 Root-induced p-mobilisation

Plants can also directly affect the bioavailability of phosphorus, by releasing H^+, organic anions and phosphatases in the rhizosphere. The rhizosphere is the area close to the plant root system where enhanced rates of nutrient exchange between microorganisms and plants take place (Cardon & Whitbeck, 2007). An illustration of processes occurring in the rhizosphere is shown in Figure 2.14.

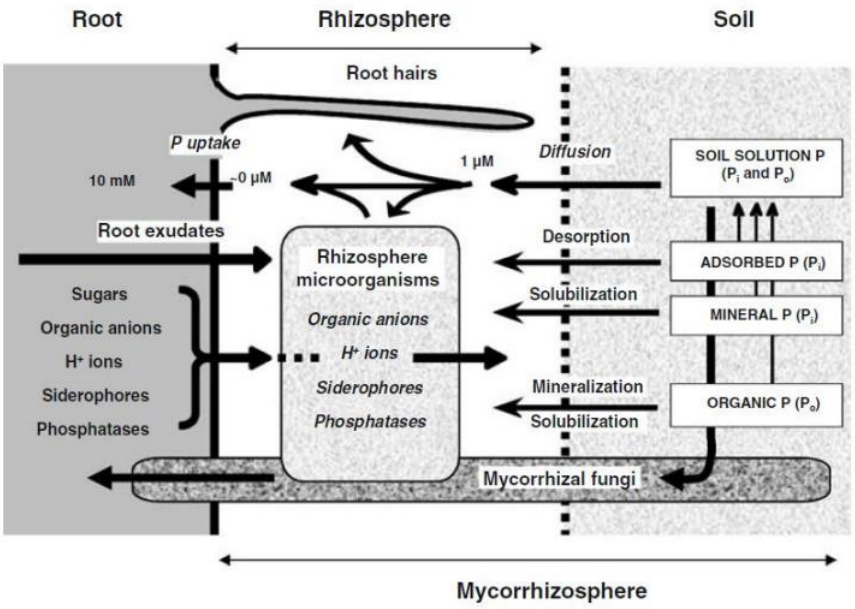

Figure 2.14: Processes affecting phosphorus availability in the rhizosphere (Richardson et al., 2009).

A release of H^+ probably increase the solubility of metal bound P or calcium phosphates (Hinsinger, 2001). The organic anions release may change the surface characteristics of soil particles and hence reduce phosphorus sorption by soils. Additionally, the release may facilitate a ligand exchange and thereby enhance phosphate desorption from adsorption sites. The release of phosphatases from the plant roots mineralise organic phosphorus, as mentioned in the previous section (Richardson et al., 2009).

The mechanisms for p-release presented in this subchapter show that the internal wetland p-load is very intricate, and that algae and microorganisms play an important role, both directly and indirectly, in regulating the pool of BAP.

2.5.2 Determination of bioavailable phosphorus

Chemical analysis and algal bioassay techniques can be applied to estimate bioavailable P in a water sample. Algae bioassays are commonly accepted as a method to determine bioavailable P (Bradford & Peters, 1987) since chemical analysis exhibit some question marks.

Disadvantages of chemical fractionation are (1) that SRP, measured by molybdate method, is not an accurate estimate of bioavailable P (Bradford & Peters, 1987) and (2) the chemical fractionation underestimates the total bioavailable P in the sample since the analytical methods cannot determine the bioavailable fraction of DOP and PP (Dijkstra et al., 2020).

During an algae bioassay, the algae growth is facilitated by creating optimal growth conditions. Phosphorus is, of course, only added as part of the samples analysed. However, these optimal growth conditions will not present an accurate measure of the bioavailable phosphorus. The conditions also favour mechanisms for phosphorus release: (1) Phosphatases will be released if phosphorus-starved algae are used and (2) the bioassays are normally conducted under 20 °C, which is a favourable temperature for p desorption. Regardless of the induced p release, an underestimation of BAP is likely to occur. The bioassays normally do not last long enough to fully reveal the BAP. In addition, the bioassays do not facilitate for the natural mechanisms responsible for p mobilisation, such as alteration of redox potential. Finally, one type of algae strain is commonly used in bioassays. This will not provide an accurate estimate of BAP, since different strains of algae and bacteria co-exist in natural waters (Ekholm, 1998).

2.6 Algae

Algae are a diverse group of predominantly aquatic photosynthetic organisms (Saber et al., 2022). Photosynthetic organisms, also called phototrophs, convert light energy into chemical energy (Madigan et al., 2015). The majority of algae are autotrophs, meaning that CO_2 is their sole source of carbon (Barsanti & Gualtieri, 2014). Some algae can use organic carbon as their carbon source (heterotrophs), while others can use both organic and inorganic carbon (mixotrophs) (ibid.). As aquatic algae can tolerate a wide range of pH, temperature, salinities and light intensities, they can grow in almost any aquatic environment (Khan et al., 2018). Some algae grow suspended in water, some grow attached to plants and on soil, while other form symbiotic relationship with other organisms (Richmond & Hu, 2013).

Chlorophyll is a light absorbing pigment found in alga (Barsanti & Gualtieri, 2014). When pigments absorb light the energy will either be utilized in the photosynthesis process, dissipate as heat or re-emit as chlorophyll fluorescens (Maxwell & Johnson, 2000).

The internal cell structure of algae is highly variable. The blue-green algae, also called cyanobacteria, are prokaryotes and have a simple cell structure. The eukaryotic algae are more complex. They have organelles such as a nucleus, commonly one or more chloroplasts, Golgi apparatus and endoplasmic reticulum, among other typical eukaryotic organelles. Algae are also morphologically diverse, and some common forms and shapes are filamentous, coccoid, flagellate and amoeboid (Richmond & Hu, 2013).

2.6.1 Factors affecting algae growth

Algae growth can be affected by both abiotic and biotic factors. To control algae growth at the laboratory, the most important parameters are light, temperature, pH, nutrient supply and mixing. The optimum of each parameter is species-specific (Barsanti & Gualtieri, 2014).

Light is the driving force of photosynthesis and is essential for algae growth. The required light intensity varies with depth and algae density. Greater depth and high cell concentration require an increase in light intensity to avoid lower layers of algae being shaded from the light by upper layers. However, photoinhibition may occur if the light intensity is too high. Usually, a light intensity of 100-200 $\mu Es^{-1}m^{-2}$ is used (5-10 % of full daylight). Light emitting in the blue or red-light spectrum is preferable since this light spectrum is optimal for effective photosynthesis. Constant illumination may retard the growth of some algae species and diurnal cycles are often applied (Barsanti & Gualtieri, 2014). According to Khan et al. (2018) several studies have found a light/dark regime of 16/8 hours to be appropriate for algae growth.

The temperature provided should ideally be approximately equal to the temperature in the algae's natural environment. For temperate climate, like in Lake Store Stokkavannet (Norway), the temperature range between 10-25 °C. Most algae species tolerate temperature between 16-27 °C, and 18-20 °C is commonly used in cultivation. Growth stagnation will occur if the temperature is under 16 °C, while a temperature higher than 35 °C will be lethal for most species (Barsanti & Gualtieri, 2014). Heat stress disturbs the algae's metabolism, as a result of alteration in structural proteins and membrane fluidity (Béchet et al., 2017).

Generally, a pH between 7 and 9 is preferable for most algae, with an optimum pH range of 8.2 to 8.7. Supply of CO_2 and aeration can be used for pH adjustment (Barsanti & Gualtieri, 2014). However, an excessive supply of CO_2 may result in acidic growth conditions and thereby supress algae growth (Tebbani et al., 2014).

Algae growth requires a sufficient supply of carbon since the biomass of algae consist of 50 % carbon (Encarnacao et al., 2015). pH determine the relative concentration of carbonaceous species in water, which exist as either CO_2 , H_2CO_3 , HCO^{3-} or CO_3^{-2} (Snoeyink & Jenkins, 1980). Autotrophs can utilize carbon in the form of CO_2, HCO_3^- and H_2CO_3, while acetate and glucose can be utilized in heterotrophic growth (Juneja et al., 2013). Generally, algae prefer CO_2 as their carbon source (Wetzel, 2001). Nitrogen is one of the most important macronutrients for algae growth. Algae dry mass contains 7-20 % nitrogen and nitrogen is essential in the synthesis of nucleic acids and proteins (Juneja et al., 2013). For algae cultivation, nitrogen sources like ammonia, nitrate, and urea are

commonly used (Richmond & Hu, 2013). Phosphorus is also important in algae nutrition. Dry algae biomass contains approximately 1 % phosphorus (Juneja et al., 2013). Phosphorus plays a crucial role in several metabolic processes and cellular functions (ibid.). Algae prefer to be supplied with orthophosphate (PO_4^{3-}) (Richmond & Hu, 2013).

Micronutrients and microelements, such as iron, sulphur, magnesium, sodium, potassium manganese, zinc, cobalt, molybdenum and copper are also required for algae growth (Tebbani et al., 2014). They are only needed in trace amounts (30-2.5 ppm for micronutrients and 4.5-2.5 ppm for microelements) (Khan et al., 2018; Juneja et al., 2013). The production of chlorophyll and cellular metabolism require iron, and protein synthesis and photosynthetic activity are dependent upon sulfur (Tebbani et al., 2014). Magnesium plays a crucial role in cellular metabolism while molybdenum is essential for nitrogen assimilation (ibid.).

Mixing is an important experimental parameter for several reasons. Firstly, a proper mixing prevent sedimentation of algae (Tebbani et al., 2014). Secondly, mixing provide a homogenous supply of nutrients in the culture and improves the gas transfer between air and the culture (Richmond & Hu, 2013). Thirdly, a proper mixing generate an equally distribution of illumination (Khan et al., 2018) and finally, the efficiency of photosynthetic processes increase by the light/dark frequency established by mixing (Richmond & Hu, 2013).

Algae growth can also be affected by biotic factors, such as a top-down control induced by grazers. Top-down control means that organisms at higher trophic levels control the biomass of organisms at lower trophic levels (Kardol et al., 2016). Some typical predators grazing on algae are rotifers, ciliates, amoeba and other zooplankton (Day et al., 2017). Many algae species have developed different defence strategies against grazers. The defence mechanisms of algae are diverse and include physiological (e.g. toxin production), morphological (e.g. colony formation, digestion-resistant cell wall) and behavioural defences (e.g. motility) (Graham & Wilcox, 2000).

2.6.2 Redfield ratio

The Redfield ratio explains the optimal relationship between carbon, nitrogen and phosphorus needed for algae growth (Shelly et al., 2010). The ratio is 106:16:1 which mean that for every phosphorus atom 16 nitrogen atoms and 106 carbon atoms are needed for optimal growth (ibid.). The nutrient ratio is not absolute, but varies among algae species and growth conditions (Dodson, 2005). The Redfield ratio can quantitatively provide information regarding a possible nutrient limitation in algae cultivation (Richmond & Hu, 2013). Through the C:N:P ratio nutrient concentrations can be correlated to enhance optimal algae growth.

Liebig's law of the minimum states that growth is determined by the scarcest resource, i.e. the limiting factor (Brezonik & Arnold, 2011). As mentioned previously, phosphorus is mainly the limiting factor for algae growth in freshwater. However, if the nitrogen and phosphorus ratio is 8:1, growth is limited by nitrogen and not phosphorus, due to the great deviation from the required nutrient ratio of 16:1.

2.6.3 Growth dynamics

The algae growth cycle consists of four main phases: lag phase, exponential phase, stationary phase and death phase (Figure 2.15).

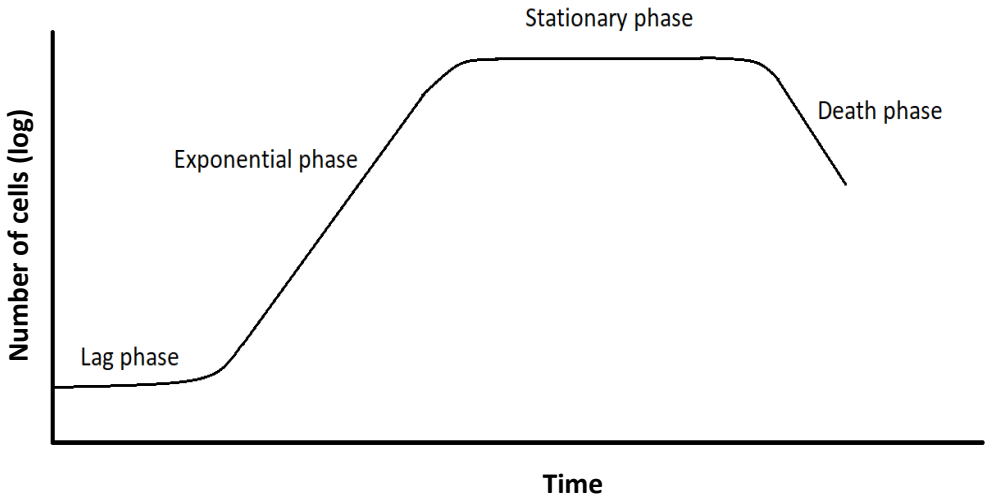

Figure 2.15: Illustration of the growth cycle of algae.

Lag phase is the period of minimal to no growth. The growth is lagged due to the cell's physiological adaptation to the environment where cells need to synthesise enzymes and cellular components necessary for survival and growth. Duration of the lag depends on inoculum, growth conditions and media. Low cell concentration and damaged cells (caused by e.g. high temperature) are reflected in an extended lag phase. Next is exponential phase, where cell doublings and the most rapid growth occurs. The specific growth rate depends on algae species and environmental factors, such as light intensity and culture medium. When nutrients, light or other factors start to limit growth, cell division slows down and the culture enter stationary phase. In stationary phase cell growth and cell death is equal, which result in a relatively constant cell concentration. The final stage is death phase where cell concentration rapidly decreases (Madigan et al., 2015).

2.7 Knowledge gaps and objective

Two previous studies have been written on the retention of bioavailable phosphorus in Leikvollbekken constructed wetland. Both studies used algae bioassay to analyse bioavailable phosphorus in water samples from inlet and outlet of the constructed wetland. Handley (2016) used batch culture bioassay for determination of BAP. Several methodologically weaknesses were identified, and the author stated that the method probably was not the best to determine bioavailable phosphorus on water samples from Leikvollbekken. Handley (2016) concluded that the constructed wetland could not retain the bioavailable phosphorus.

The main focus in the study by Tjelta (2021) was to develop an easy and effective method to measure bioavailable phosphorus. Microplate reader was used to determine the retention of bioavailable phosphorus in Leikvollbekken. Inoculum used was collected from the constructed wetlands' recipient, Lake Store Stokkavannet. Two parallel tests were conducted, one with lake water inoculum and one with a pre-grown lake water inoculum. Bioassays conducted with lake water inoculum provided inconclusive results and were not used in analysis of bioavailable phosphorus. Tjelta (2021) concluded, based on results from five bioassays performed with pre-grown inoculum, that Leikvollbekken constructed wetland retained 54 % of the incoming bioavailable phosphorus in the period from April 2021 to May 2021.

The main objective of this study is to examine Leikvollbekken constructed wetland's retention capability of the incoming bioavailable phosphorus. This will be investigated by using the microplate reader method developed in the study by Tjelta (2021). The inlet and the outlet of the CW will be measured for bioavailable phosphorus by estimating the algae growth potential. The difference in algae growth between inlet and outlet will be compared to determine if the CW retain the incoming bioavailable phosphorus. Total suspended solids and chemical phosphorus analysis will also be performed on the corresponding water samples, to determine the correlation between chemical available and bioavailable phosphorus. The study will be conducted over eight months, to get an idea of the eventually long-term retention.

The study by Handley (2016) used batch culture bioassay and concluded that Leikvollbekken could not retain bioavailable phosphorus. Tjelta (2021) used microplate reader method and concluded that the wetland retained BAP for a period of two months in the spring, based on bioassays conducted with pre-grown inoculum. However, Tjelta could not conclude if the CW can retain BAP by using lake water inoculum.

Hence, the specific objectives and the corresponding hypothesis of this study are as follows:

1. Can the constructed wetland retain bioavailable phosphorus?
   Hypothesis: The constructed wetland can retain bioavailable phosphorus.
2. Can microplate reader method be used as bioassay to determine bioavailable phosphorus in water samples?
   Hypothesis: Microplate reader method can be used as bioassay to determine bioavailable phosphorus in water samples.

3. METHODS

This section introduces a presentation of Leikvollbekken constructed wetland (CW), followed by sampling procedures and analytical methods. Analytical methods include analysis of total suspended solids, total phosphorus and dissolved orthophosphates. Further, chemicals and methods used in the algae bioassay are presented. At the end, an error analysis-description is given.

3.1 Sampling site – Leikvollbekken constructed wetland

Leikvollbekken constructed wetland is located northwest of Stavanger between Hålandsvatnet and Store Stokkavatnet. The area surrounding the CW consist mainly of agricultural activities and forest areas. Leikvollbekken was built in 1993/1994 to improve the water quality of Store Stokkavatnet by removing nutrients from agricultural runoff. The CW consists of two ponds connected by a stream (Figure 3.1 and Figure 3.2).

Figure 3.1: Image of Leikvollbekken at present time. The red cross indicates where the inlet test water was collected. Outlet test water was collected at the yellow cross. The blue arrows represent the direction of water from agricultural runoff through pond 1 and pond 2 in the CW and into Store Stokkavatnet (Kartverket, 2022).

Figure 3.2: To the left: Picture of pond 1. To the right: Picture of pond 2 with the sampling house (red circle) at the outlet. (Pictures taken 05.05.22).

3.2 Sampling

To provide a representable sample for average concentration water samples from inlet and outlet were collected by a portable automatic composite sampler (ISCO 6712, Teledyne) into a single collection bottle. Sampling was automatically controlled by a fixed time interval (100 mL water collected every hour for four days). The inlet sampler is connected to a car battery for power supply. The outlet sampler was connected to a refrigerator, while the inlet sampler was maintained by ambient temperature (Figure 3.3).

Figure 3.3: To the left: The portable automatic sampler at the inlet connected to a car battery for power supply. To the right: The automatic sampler at the outlet connected to a refrigerator. The outlet sampler is inside the sampling house. (Pictures taken 05.05.22).

Wetland samples were collected every 2-4 weeks, depending on the season. The first composite samples were collected from 23.09.21 to 27.09.21. The last sampling period was from 04.05.22 to 08.05.22 (Table 3.1).

Table 3.1: Sampling period and date of collected test water for each test.

Test number	Sampling period	Test water collected
1	23.09.21 – 27.09.21	27.09.22
2	07.10.21 – 11.10.21	11.10.21
3	28.10.21 – 01.11.21	01.11.21
4	29.11.21 – 03.12.21	03.12.21
5	10.01.22 – 14.01.22	14.01.22
6	06.02.22 – 10.02.22	06.02.22
7	07.03.22 – 11.03.22	11.03.22
8	31.03.22 – 04.04.22	04.04.22
9	20.04.22 – 24.04.22	24.04.22
10	04.05.22 – 08.05.22	08.05.22

On the sampling site, after approximately four days, 1 litre of inlet and outlet composite samples were collected into two separate polyethylene bottles. In addition, a freshwater sample from the surface of Lake Store Stokkavatnet, used as lake water inoculum for the algae bioassay, was collected into a 1 litre polyethylene bottle. The bottle was marked LW-I (lake water inoculum). All three samples were transported directly to the laboratory at campus without preservation and stored in a refrigerator until analysis. Analysis of TSS and PO_4^{3-} and set up of algae bioassay were performed the same day as water samples were collected. TP analysis was in general performed the day after. Occasionally TP analysis was performed the same day as PO_4^{3-}.

Test water from inlet and outlet were analysed for total suspended solids (TSS), total phosphorus (TP), orthophosphate (PO_4^{3-}) and bioavailable phosphorus (BAP), summarised graphically in Figure 3.4:

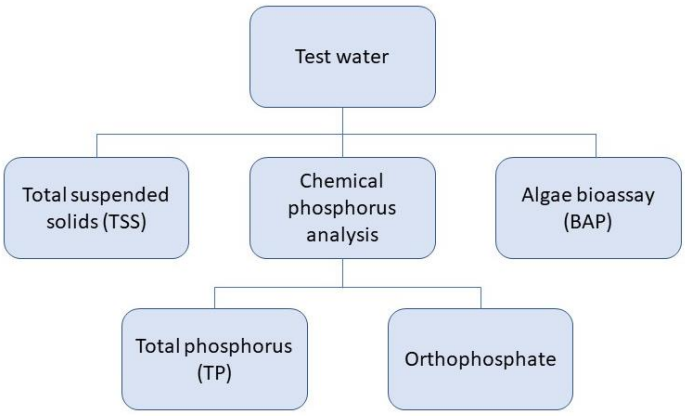

Figure 3.4: Schematic presentation of all methods performed on test water.

3.3 Analytical methods

This section describes the methods for analysis of TSS, TP and PO_4^{3-}. All analysis were performed according to Standard Methods of the Examination of Water and Wastewater (Clesceri et al., 1998) and are summarised schematically in Figure 3.5. The white boxes represent each method with the standard method abbreviation in parentheses. Boxes with sharp edges represents the parameters used for the results in the thesis.

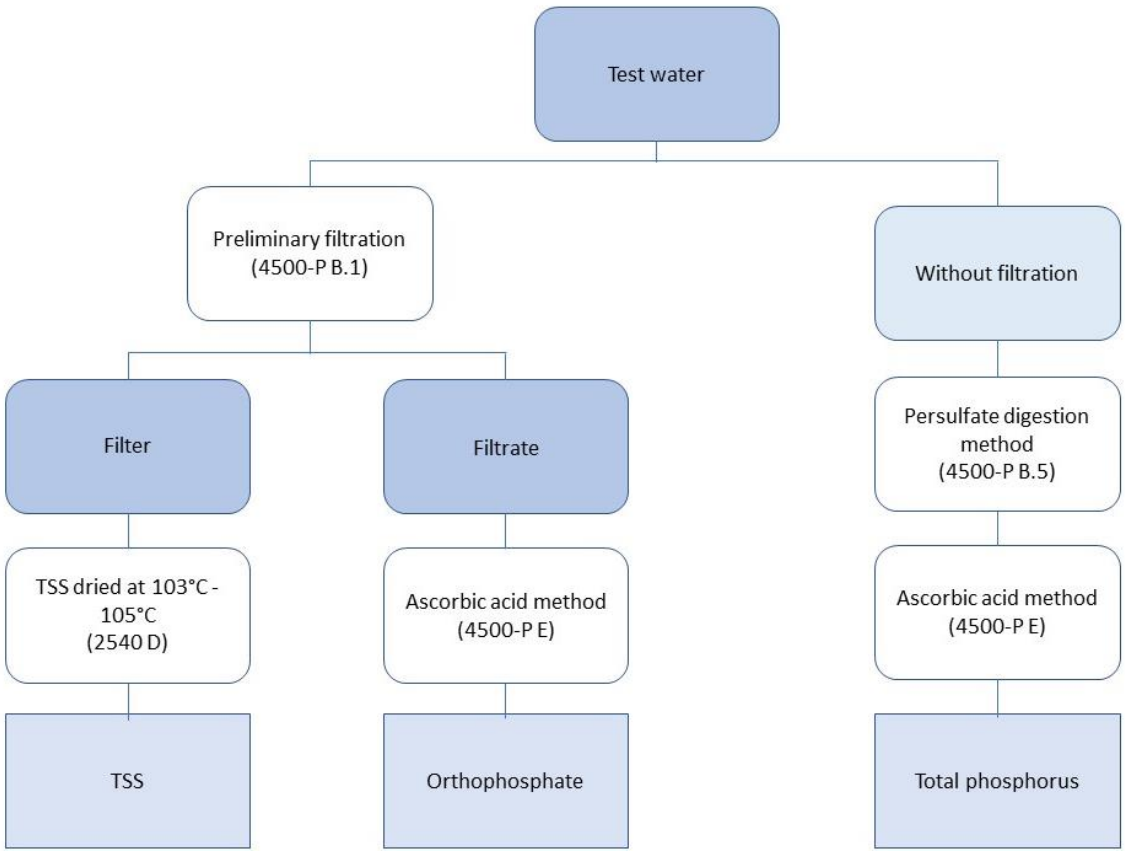

Figure 3.5: Flow diagram of the analytical methods performed on test water. White boxes represent each analytical method. Boxes with sharp edges represent the parameters used for the results.

All glassware used during analysis were washed in 0.5 N H_2SO_4 and rinsed thoroughly three times with distilled water before use.

3.3.1 Preliminary Filtration

For analysis of TSS and orthophosphate, filtration was carried out according to Standard Method 4500-P B.1, "Preliminary Filtration" (Clesceri et al., 1998). Glass fibre filters (Whatman, GE Healthcare) with 0.45 µm pore size were placed in a drying oven at 103 – 105 °C for at least 1 hour to ensure completely dry filters. The filters were then placed in a desiccator to balance temperature. Prior filtering of test water, the filters were rinsed with 0,5 N H_2SO_4 followed by distilled water.

Preliminary filtration separates dissolved forms of phosphorus from particulate forms. The filter was used for determination of TSS, while the filtrate was used for analysis of PO_4^{3-}.

3.3.2 Persulfate Digestion Method

Unfiltered sample was used for analysis of TP. As mentioned in section 2.3 , phosphorus may be present as condensed phosphates and/or organically bound phosphorus. To determine the TP content in a sample, P-containing compounds other than orthophosphates must be converted into dissolved orthophosphates by hydrolysis and digestion prior to colorimetric measurement. In test 5-10 three parallels of each sample and two distilled water blanks were digested by autoclavation (Panasonic MLS-3781L) with persulfate under acidic conditions according to Standard Method 4500-P B.5, "Persulfate Digestion Method" (Clesceri et al., 1998). In test 1-4 two parallels and one reagent blank were autoclaved. Sulfuric acid hydrolysis converts condensed phosphates into dissolved orthophosphates, while organophosphorus is converted into dissolved orthophosphates by persulfate digestion (Clesceri et al., 1998). After oxidative digestion, orthophosphate was determined by spectrophotometry, described in section 3.3.4 .

3.3.3 Total suspended solids

Total suspended solids (TSS) were analysed according to Standard Method 2540-D, "Total suspended solids dried at 103-105°C" (Clesceri et al., 1998). In tests 5-10, two parallels for test water were analysed for error analysis. One parallel of test water was analysed in tests 1-4.

3.3.4 Phosphorus analysis

Phosphorus concentrations were analysed according to Standard Method 4500-P E, "Ascorbic Acid Method" (Clesceri et al., 1998). Method E is suitable for the range of 0.01-6 mg P/L. In test 5-10 for phosphate, three parallels for test water and two parallels for reagent blanks were analysed for error analysis. In test 1-4 two parallels and one reagent blank were analysed for error analysis. Unfiltered digested samples were used for TP analysis, while filtered non-pretreated samples were used for analysis of dissolved orthophosphates. Absorbance was measured at 880 nm with a spectrophotometer (Shimadzu, UVmini-1240) using 2.5 mL disposable polystyrene cuvette (BRAND®).

A calibration curve was obtained by plotting absorbance against six known phosphorus concentrations. Calibration curve standards with concentration ranging from 1-501 µgP/L was prepared according to 4500-P.C.3e (Clesceri et al., 1998). The linear relationship determined from the calibration curve, presented in Appendix A, was used to calculate unknown phosphorus concentration in the sample.

In the ascorbic acid method orthophosphates react with ammonium molybdate and antimony potassium tartrate in acidic medium to form a yellow coloured phosphomolybdate complex (Equation 3.1). The formed phosphomolybdate complex is further reduced by ascorbic acid to form a blue coloured molybdate complex (Equation 3.2) (Brezonik & Arnold, 2011). The absorbance of the molybden blue complex is then measured by spectrophotometry at 880 nm.

$H_3PO_4 + 12(NH_4)_2MoO_4 + 2H^+ \rightarrow (NH_4)_3PO_4 \cdot 12MoO_3$ (yellow) $+ 21NH^+ + 12H_2O$ *Equation 3.1*

$(NH_4)_3PO_4 \cdot 12MoO_3$ + ascorbic acid (reducing agent) → molybden blue complex *Equation 3.2*

3.4 Algae bioassay

The following section covers the type of algae inoculum, chemicals and methods used in the algae bioassay. The section also highlights calibration of the microplate reader method.

3.4.1 Algae inoculum

Algae used as inoculum in the bioassay were taken from the surface of Lake Store Stokkavannet, the recipient of Leikvollbekken constructed wetland. In contrast to standard algae not naturally living in the recipient, a more realistic estimate of BAP will be obtained by examining algae from the recipient. Two different types of inocula were used in the bioassay. The first type of inoculum was a water sample collected from Lake Store Stokkavannet the same day as the set up of the bioassay (lake water inoculum, LW-I). The second type of inoculum used was a cultivated LW-I which was pre-grown and maintained at laboratory conditions at campus (pre grown inoculum, PG-I).

3.4.2 Chemicals and solution preparation

Solutions used for cultivation and in the bioassay are summarised in Table 3.2. Stock solutions were made at the laboratory at campus. Analytical standards of chemicals were used. All glassware used for storage of solutions were autoclaved. Stock solutions were stored in a refrigerator.

Table 3.2: Solutions used in the bioassay method.

Name of stock solution	Type of solution	For use in	Concentration in stock solution (g/L)	Concentration in PG-I culture (mg/L)	Concentration in well in microplate (mg/L)
Solution A	P source	Growth media for PG-I	K_2HPO_4: 0.35 KH_2PO_4: 0.45	K_2HPO_4: 5.83 KH_2PO_4: 7.50	Not used in bioassay
Solution B	N source	NS-P and growth media for PG-I	$NaNO_3$: 25 NH_4Cl: 21 $FeCl_3$: 0.05 EDTA: 0.2	$NaNO_3$: 1042 NH_4Cl: 875 $FeCl_3$: 2.08 EDTA: 8.3	$NaNO_3$: 313 NH_4Cl: 263 $FeCl_3$: 0.63 EDTA: 2.5
Solution C	Ca and Mg source	NS-P and growth media for PG-I	$CaCl_2$: 2.5 $MgSO_4$: 1.5	$CaCl_2$: 20.9 $MgSO_4$: 12.5	$CaCl_2$: 6.3 $MgSO_4$: 3.8
Solution D	Trace elements	NS-P and growth media for PG-I	$MnSO_4{\cdot}2H_2O$: 0.5 $MgSO_4{\cdot}7H_2O$: 3 NaCl: 1 $FeSO_4{\cdot}7H_2O$: 0.1 $CoCl_2{\cdot}6H_2O$: 0.1 $CaCl_2{\cdot}2H_2O$: 0.1 $ZnCl_2$: 0.1 $CuSO_4{\cdot}5H_2O$: 0.01 $NiCl_2{\cdot}6H_2O$: 0.02 Na_2SeO_3: 0.001 $AlK(SO_4)_2$: 0.01 H_3BO_3: 0.01 Na_2MoO_4: 0.01 $Na_2WO_4{\cdot}2H_2O$: 0.01 EDTA: 0.5	$MnSO_4{\cdot}2H_2O$: 4.2 $MgSO_4{\cdot}7H_2O$: 25 NaCl: 8 $FeSO_4{\cdot}7H_2O$: 0.8 $CoCl_2{\cdot}6H_2O$: 0.0.8 $CaCl_2{\cdot}2H_2O$: 0.8 $ZnCl_2$: 0.8 $CuSO_4{\cdot}5H_2O$: 0.08 $NiCl_2{\cdot}6H_2O$: 0.17 Na_2SeO_3: 0.01 $AlK(SO_4)_2$: 0.08 H_3BO_3: 0.08 Na_2MoO_4: 0.08 $Na_2WO_4{\cdot}2H_2O$: 0.08 EDTA: 4.2	$MnSO_4{\cdot}2H_2O$: 1.25 $MgSO_4{\cdot}7H_2O$: 7.5 NaCl: 2.5 $FeSO_4{\cdot}7H_2O$: 0.25 $CoCl_2{\cdot}6H_2O$: 0.25 $CaCl_2{\cdot}2H_2O$: 0.25 $ZnCl_2$: 0.25 $CuSO_4{\cdot}5H_2O$: 0.025 $NiCl_2{\cdot}6H_2O$: 0.05 Na_2SeO_3: 0.0025 $AlK(SO_4)_2$: 0.025 H_3BO_3: 0.025 Na_2MoO_4: 0.025 $Na_2WO_4{\cdot}2H_2O$: 0.025 EDTA: 1.25
Solution E	C source	NS-P and growth media for PG-I	$NaHCO_3$: 47	$NaHCO_3$: 2350	$NaHCO_3$: 705
POS-P	P-solution	Bioassay as positive control	P: 0.0009	Not used in growth media	P: 0.45

The growth media used for cultivation is a modified (N-source) Bushnell-Haas inorganic nutrient solution (pH 8.2) and consists of a balanced combination of solutions A, B, C, D and E (see Table 3.2 for details). Nutrient solution without P (NS-P) was used in the bioassay. Five mL of solution B, 1 mL of solution C, 1 mL of solution D and 6 mL of solution E were added to 87 mL distilled water in a 250 mL Erlenmeyer flask. The NS-P solution was homogenised before being added to the microplate. POS-P solution contains 0.9 mg P/L and was used in the bioassay as a P source for the positive controls. P concentration in POS-P were measured in Merck Spectroquant Prove 300 spectrophotometer using Merck Spectroquant Phosphate Cell Test. Two parallels were analysed and averaged.

3.4.3 Algae cultivation for pre-grown inoculum

To start a pre-grown batch, 2 mL of solution A, 5 mL of solution B, 1 mL of solution C, 1 mL of solution D and 6 mL of solution E were added to 120 mL fresh LW-I in a 250 mL Erlenmeyer flask. For final concentrations in the media, see Table 3.2. Four replicates were made. The flasks were covered with a hole-punched cork. After cultivation, all four replicates were incubated at 20-25 °C and 100 rpm in an incubator (Innova S44i Eppendorf, Germany) with photoactive radiation (PAR) at approximately 135 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a light/dark regime of 16/8 hours. In middle of January 2022 the temperature control of the incubator broke down and the PG-I batches were instead incubated at room temperature and with continuously mixing of 100 rpm on a shaker table. The shaker table was equipped with fluorescent light providing PAR at approximately 45 $\mu mol\ m^{-2} \cdot s^{-1}$. From late January 2022 until the end of all tests, PG-I batches were placed at the shaker table.

After approximately seven days, the growth media were changed to prevent excessive algae density and nutrient limitation. 20 mL of PG-I was added to 85 mL distilled water, 2 mL of solution A, 5 mL of solution B, 1 mL of solution C, 1 mL of solution D and 6 mL of solution E in a 250 mL Erlenmeyer flask. Three replicates were made.

3.4.4 Microplate reader method

The microplate reader method was developed based on previously suggested assay by van Wagenen et al. (2014). The fluorescence process is utilised as a quantification method in microplate reader analysis. Samples in a microplate are exposed to light capable of exciting chlorophyll and emitted light characteristic for the pigment is detected by a photomultiplier detection system. (Tecan, 2016). Number of fluorescent molecules are given as fluorescens intensity (FI). The relationship between algae cell concentration and FI is determined by plotting FI against known algae cell concentration.

3.4.4.1 Set-up of microplate

The microplate was made according to Table 3.3 using a 24 sterilized clear wall microplate (Corning® Costar® TC-treated, Sigma Aldrich, Germany). The first two rows represent the test for bioavailable phosphorus and contains inoculum together with nutrient solution without phosphorus (NS-P) and test water (TW) which contains the BAP of interest. PG-I was added to wells 1-6, while LW-I was added to wells 7-12. Inoculum, distilled water (DI) and NS-P were used as blanks and added to row number three. The purpose of blanks is to calibrate the test from interference in inoculum and NS-P. Wells 13-15 serve as blanks for PG-I, while wells 16-18 serve as blanks for LW-I. Row three is expected to show marginal to no growth. High blanks might confirm contamination from the analytical process. Row four serves as a positive control to confirm algae growth by adding excess of the limiting nutrient, P, together with inoculum and NS-P. Wells 19-21 act as positive control for PG-I, while wells 22-24 act as positive control for LW-I. For final concentrations of POS-P and NS-P in each well, see Table 3.2 under subsection 3.4.2 .

Table 3.3: Set up of microplate.

	1	**2**	**3**	**4**	**5**	**6**
	TW 1 mL	**TW** 1 mL	**TW** 1 mL	**TW** 1 mL	**TW** 1 mL	**TW** 1 mL
	NS-P 0.5 mL	**NS-P** 0.5 mL	**NS-P** 0.5 mL	**NS-P** 0.5 mL	**NS-P** 0.5 mL	**NS-P** 0.5 mL
Test	**PG-I** 0.5 mL	**PG-I** 0.5 mL	**PG-I** 0.5 mL	**PG-I** 0.5 mL	**PG-I** 0.5 mL	**PG-I** 0.5 mL
	7	8	9	10	11	12
	TW 1 mL	**TW** 1 mL	**TW** 1 mL	**TW** 1 mL	**TW** 1 mL	**TW** 1 mL
	NS-P 0.5 mL	**NS-P** 0.5 mL	**NS-P** 0.5 mL	**NS-P** 0.5 mL	**NS-P** 0.5 mL	**NS-P** 0.5 mL
	LW-I 0.5 mL	**LW-I** 0.5 mL	**LW-I** 0.5 mL	**LW-I** 0.5 mL	**LW-I** 0.5 mL	**LW-I** 0.5 mL
	13	14	15	16	17	18
Blank	**DI** 1 mL	**DI** 1 mL	**DI** 1 mL	**DI** 1 mL	**DI** 1 mL	**DI** 1 mL
	NS-P 0.5 mL	**NS-P** 0.5 mL	**NS-P** 0.5 mL	**NS-P** 0.5 mL	**NS-P** 0.5 mL	**NS-P** 0.5 mL
	PG-I 0.5 mL	**PG-I** 0.5 mL	**PG-I** 0.5 mL	**LW-I** 0.5 mL	**LW-I** 0.5 mL	**LW-I** 0.5 mL
	19	20	21	22	23	24
Positive control	**POS-P** 1 mL	**POS-P** 1 mL	**POS-P** 1 mL	**POS-P** 1 mL	**POS-P** 1 mL	**POS-P** 1 mL
	NS-P 0.5 mL	**NS-P** 0.5 mL	**NS-P** 0.5 mL	**NS-P** 0.5 mL	**NS-P** 0.5 mL	**NS-P** 0.5 mL
	PG-I 0.5 mL	**PG-I** 0.5 mL	**PG-I** 0.5 mL	**LW-I** 0.5 mL	**LW-I** 0.5 mL	**LW-I** 0.5 mL

To prevent excessive algae density in the wells PG-I was diluted prior to adding. Cell concentration of PG-I was determined by direct counting with microscopy and diluted within the range of 80,000-110,000 cells/mL. To avoid P-residuals from the inoculum PG-I was washed in test 8-10. The inoculum was diluted with NS-P, sentrifuged at 3000 rpm for 10 minutes, decanted and re-suspended with a vortex mixer (VWR International AS, Norway). The procedure was repeated twice before PG-I was added to the wells. Two plates were made for each test, one for inlet sample and one for outlet sample.

The first four tests were performed with PG-I maintained from July 2021. Test 5 were carried out using PG-I maintained from 4.1.22. The culture used in test 5 failed to grow due to break down of the temperature control of the incubator. A fresh LW-I was cultivated and maintained from 1.2.22 and used in test 6-8. Attempts to cultivate a freshwater sample collected on 9.3.22 failed. Still two weeks after cultivation, no growth was observed in the replicates and they were discarded. It took approximately 2.5 weeks before any growth would appear for the LW-I collected and cultivated 4.4.22 (Figure 3.6). Three replicates remained blank. In the fourth replicate growth was observed and the culture was re-cultivated and used in test 9-10.

Figure 3.6: Four replicates of LW-I collected and cultivated on 4.4.22. The picture is taken approximately 2.5 weeks after cultivation. In only one of four replicates growth is observed.

3.4.4.2 Microplate analysis

Microplates, without cover, were analysed immediately after set-up with Tecan Infinite F200 PRO microplate reader (Tecan, Switzerland) with 430 nm excitation filter and 690 nm emission filter (Tecan, Switzerland). Three measurements were made every day for six days or until stationary phase was reached. Between measurements the microplates were incubated at room temperature and with continuously mixing of 100 rpm on a shaker table equipped with continuous fluorescent light providing PAR at approximately 45 µmol $m^{-2} \cdot s^{-1}$. The microplates were covered with a plastic

cover when placed on the shaker table to prevent evaporation. To reduce heat transfer from the black bottom of the shaker table styrofoam plates were placed underneath the microplates. To determine the correlation between algae cell concentration and FI calibration curves were made. Calibration of the microplate reader method is described in subsection Calibration and cross-validation of microplate reader method.

3.4.5 Calibration and cross-validation of microplate reader method

To calibrate the microplate reader method different quantification methods can be used. In this study the microplate reader method was calibrated and cross-validated with microscopy and flow cytometry. Direct counting with microscope is a common method for cell quantification (Andersen, 2005) while flow cytometry is a rapid detection technique suitable for algal enumeration since the instrument measures cell fluorescence (Peniuk, 2015).

PG-I cultivated and maintained from 1.2.22 was used for the calibration. Cultures with six different concentrations were made (Table 3.4). The first culture was non-diluted PG-I. The other five cultures were made in five separate Erlenmeyer flasks by diluting PG-I with distilled water to 1:2, 1:5, 1:10, 1:50 and 1:100.

Table 3.4: Dilution factor of each culture for the calibration.

Culture	Dilution factor
1	1:1
2	1:2
3	1:5
4	1:10
5	1:50
6	1:100

Cell concentration in each culture was quantified using direct counting with microscope and flow cytometric enumeration and compared to microplate reader analysis.

Direct counting was done by a Neubauer counting chamber (Improved Neubauer, 0.100 mm depth, 0.0025 mm^2, Germany) (Figure 3.7) and optical microscope Visiscope Series 200 (VWR International AS, Norway).

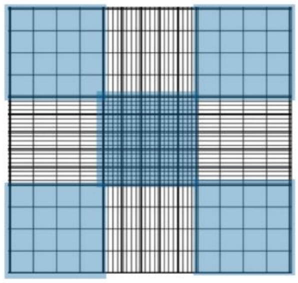

Figure 3.7: Counting chamber with counting squares (Bastidas, 2013)

The cell concentration for each culture was calculated according to Equation 3.3 (Bastidas, 2013):

$$Cell\ concentration\ (\#\frac{cells}{mL}) = \frac{number\ of\ cells\ \cdot 10\,000}{number\ of\ squares} \qquad \textit{Equation 3.3}$$

Flow cytometric enumeration was performed on Accuri^{TM} C6 Plus Flow cytometer (BD Biosciences, USA) with a flow rate of 14 μL/min with a 10 μm core size. Backflushing and cleaning, according to the manufacturers' guideline, were performed before analysis. Sample was added to a sample vial and re-suspended with a vortex mixer (VWR International AS, Norway) at 150 rpm before analysis. The cell concentration was quantified directly from the software (BD Accuri C6 Plus).

The microplate was made according to Table 3.5 using a 24 sterilized clear wall microplate (Corning® Costar® TC-treated, Sigma Aldrich, Germany). Four replicates of each culture, each replicate with a volume of 2 mL, were added to the wells. To ensure approximately equal cell concentration in the wells the Erlenmeyer flasks were homogenised prior to addition.

Table 3.5: Set up microplate for calibration.

Culture 1 2 mL	**Culture 2** 2 mL	**Culture 3** 2 mL	**Culture 4** 2 mL	**Culture 5** 2 mL	**Culture 6** 2 mL
Culture 1 2 mL	**Culture 2** 2 mL	**Culture 3** 2 mL	**Culture 4** 2 mL	**Culture 5** 2 mL	**Culture 6** 2 mL
Culture 1 2 mL	**Culture 2** 2 mL	**Culture 3** 2 mL	**Culture 4** 2 mL	**Culture 5** 2 mL	**Culture 6** 2 mL
Culture 1 2 mL	**Culture 2** 2 mL	**Culture 3** 2 mL	**Culture 4** 2 mL	**Culture 5** 2 mL	**Culture 6** 2 mL

Immediately after set-up, the coverless microplate was analysed with Tecan Infinite F200 PRO microplate reader (Tecan, Switzerland) with 430 nm excitation filter and 690 nm emission filter (Tecan, Switzerland). Statistical analysis was performed using Excel.

3.4.6 Method optimisation

Frequently throughout the test period, lack of growth was observed in positive controls. In test 10, two additional plates were made according to the procedure described in section 3.4.4.1 . The two plates were supplied with CO_2 (99.99 % concentration) to examine if CO_2 would improve the algae growth. In between measurements, two times per day, the plates were supplied with CO_2 from a CO_2-filled balloon.

3.4.7 Exclusion of data

If cell concentration in a well was beyond the upper detection limit, i.e. the well was measured as "over" that well was categorized as an outlier. The outlier was totally excluded from the calculations of average cell concentration, meaning that number of parallels decreased for the test conducted.

3.5 Error analysis

Since independent measurements were conducted in analysis of TSS, phosphorus and BAP, standard error (SE) was calculated to determine the margin of error of each measurement. SE is calculated based on the standard deviation (σ) and the sample size (n) (Equation 3.4) (Taylor, 1997):

$$SE = \frac{\sigma}{\sqrt{n}} \qquad \textit{Equation 3.4}$$

A larger sample size contributes to a smaller SE as sample size is inversely proportional to SE. Standard deviation (σ) is calculated according to Equation 3.5 (Taylor, 1997):

$$\sigma = \frac{1}{n-1} \cdot \sum_{i=1}^{n} (x_i - \bar{x})^2 \qquad \textit{Equation 3.5}$$

where n is the number of replicates, x_i is the value of the i^{th} point in the data set and $\bar{x}$ is the mean value of the replicates.

4. RESULTS

The results obtained from the experiments are presented in this chapter and is divided into four main sections. First, the result from the total suspended analysis is presented. The next section presents results obtained from the phosphorus analysis. Results from the algae bioassays are further presented in the third section. The last section is dedicated to correlations between chemical available and bioavailable phosphorus.

4.1 Total suspended solids

Inlet and outlet samples were analysed for total suspended solids from late September 2021 to the middle of May 2022. The results of the total ten analyses can be seen in Figure 4.1. TSS concentration (mg TSS/L) of inlet and outlet samples are represented by the dark and light blue bars, respectively. The date of when each analysis was conducted is shown on the horizontal axis. The error bars represent the SE between the replicates. The first four analyses were performed without parallels. For the last six analyses two parallels were analysed.

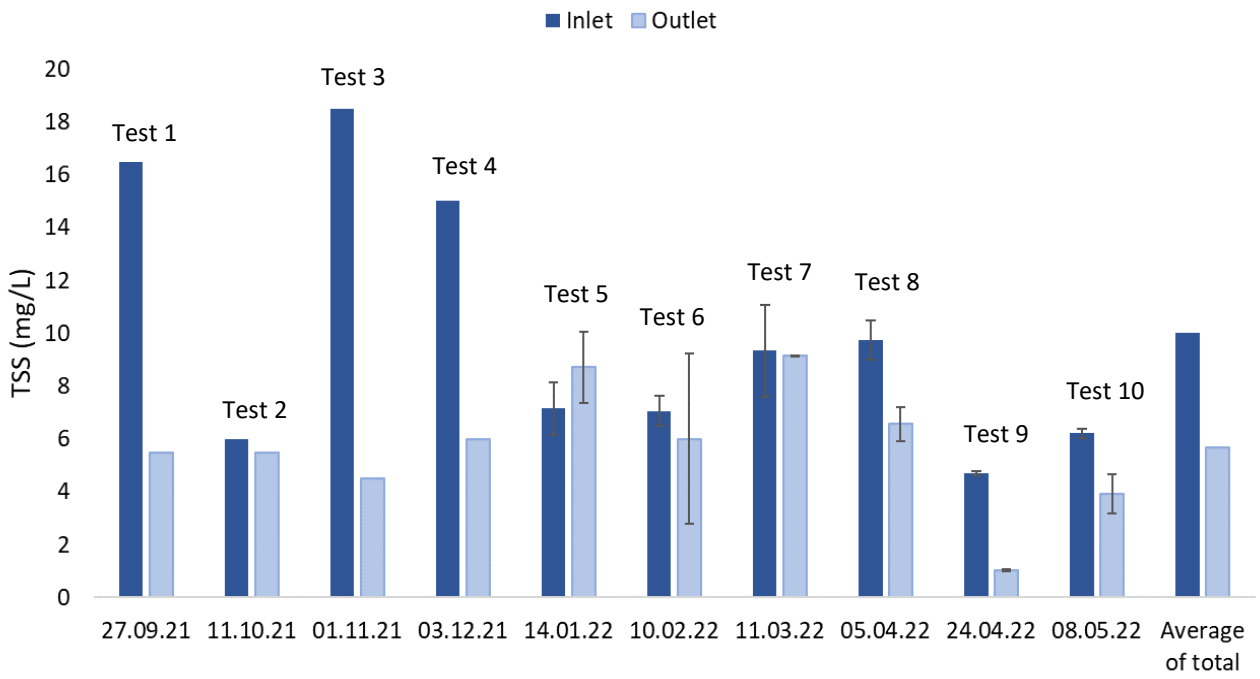

Figure 4.1: Inlet (dark blue bars) and outlet (light blue bars) concentrations of TSS plotted against time. SE are represented by the error bars. The date of when each test was conducted is shown on the horizontal axis.

Inlet and outlet TSS concentration varied during the analysis period. The highest TSS inlet concentration can be seen in the first four analysis, except test 2 (conducted on 11.10.21). The first four analysis were performed without any parallels and the uncertainty was higher compared to the

six lasts analyses. For the total ten analysis conducted, average TSS inlet concentration (10 mg/L) was higher than average TSS outlet concentration (6 mg/L). Since the first four analysis were performed without parallels, the average TSS concentration is given without SE.

All tests, except analysis performed on 14.01.22, showed a positive retention of TSS in the CW. Analysis conducted on 27.09.21, 01.11.21 and 24.04.22 showed the highest retention, with a retention of 67 %, 76 % and 78 ± 1 %, respectively. TSS analyses performed on 14.01.22 showed a retention of -22 ± 25 %, meaning that the CW released TSS into Store Stokkavannet.

4.2 Phosphorus

As for TSS, TP and PO_4^{3-} concentrations of the inlet and outlet samples were analysed for from late September 2021 to the middle of May 2022. The results are presented in Figure 4.2. Dark blue bars represent the TP content in inlet samples, while TP content in outlet samples is shown by the light blue bars. PO_4^{3-} concentrations of inlet and outlet samples are indicated by the dark and light green bars, respectively. The horizontal axis displays the date of when each analysis was performed. Occasionally analysis of TP was performed the day after PO_4^{3-} analysis. To simplify the graphical presentation in Figure 4.2 both analyses are presented as having been performed on the same day. The SE between replicates is represented by error bars. Two replicates were analysed in the first four analyses. In the six lasts analyses, three replicates were used.

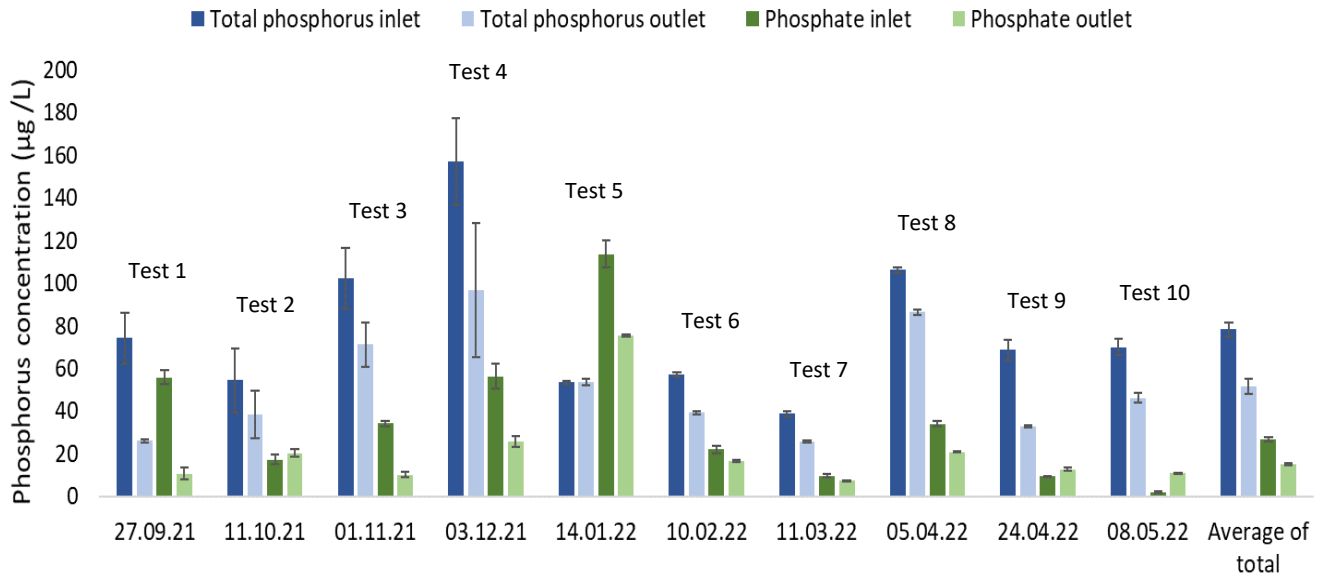

Figure 4.2: Results of TP and phosphate concentration in inlet and outlet samples analysed throughout the thesis. The error bars represent the SE between the replicates. The date of when each test was conducted is shown on the horizontal axis.

TP and PO_4^{3-} concentration of both inlet and outlet samples varied throughout the analysis period. The results from analysis of PO_4^{3-} on 14.01.22 were incorrect since the PO_4^{3-} concentration of both inlet and outlet samples were higher than the TP concentration. The sulfuric acid used were tested, and concentration of stock solution were found to be incorrect. According to Standard methods of Water and Wastewater (Clesceri et al., 1998), water sample should be filtered immediately after collection if phosphate analysis are to be performed. Consequently, the phosphate analysis was not repeated.

Analysis performed on 03.12.21 showed the highest phosphorus content, both for inlet and outlet samples (TP inlet 157±20 µg/L, TP outlet 97±31 µg/L, PO_4^{3-} inlet 57±6 µg/L, PO_4^{3-} outlet 26±3 µg/L). Based on all ten analyses, the average TP and PO_4^{3-} inlet concentrations (TP 78 ± 3 µg/L, PO_4^{3-} 26.8 ± 0.8 µg/L) were higher than the average TP and PO_4^{3-} outlet concentration (TP 52 ± 4 µg/L, PO_4^{3-} 15.2 ± 0.5 µg/L).

The phosphorus retention during the test period is presented in Figure 4.3. The dark and light blue bars represent the percentage retention of TP and PO_4^{3-}, respectively. The phosphorus concentration retained, positive or negative, is highlighted on the top of each bar. SE are represented by the error bars. As mentioned earlier, the result of phosphate analysis performed on 14.01.22 was incorrect and not shown in Figure 4.3.

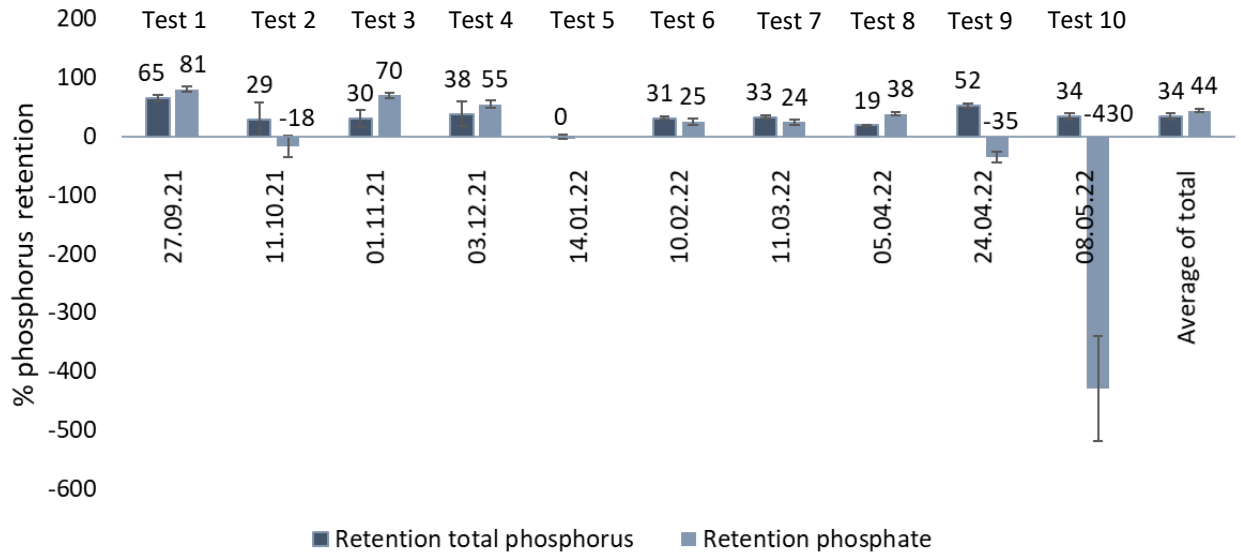

Figure 4.3: Percentage retention of TP (dark blue bars) and PO_4^{3-} (light blue bars) plotted against time. The two bars to the right represent average phosphorus retention. SE are represented by error bars.

The degree of phosphorus retention varied throughout the test period. Figure 4.3 demonstrates a positive retention of TP in nine of ten analyses. Test 5 revealed a marginal negative retention of TP with an outlet concentration of 54 ± 2 µg P/L versus an inlet concentration of 53.7 ± 0.7 µg P/L. The highest positive retention of TP was measured on 27.09.21 (65 ± 6 %).

The phosphate analysis revealed a positive retention in six tests. As for TP, the greatest positive PO_4^{3-} retention was measured on 27.09.21 (81 ± 5 %). Analyses performed on 11.10.21, 24.04.22 and 08.05.22 showed a negative phosphate retention (-18 ± 18 %, -35 ± 9 % and -430 ± 90 %). Throughout the test period the CW demonstrated a positive phosphorus retention. In Figure 4.3 the two bars to the right represents the average phosphorus retention. In average, 34 ± 5.2 % of the incoming TP and 44 ± 2.5 % of the incoming PO_4^{3-} were retained by the CW.

4.3 Algae bioassay

This section is divided into four subsections: (1) Results from algae bioassay with pre-grown inoculum, (2) Results from algae bioassay with lake water inoculum, (3) Method calibration and cross-validation and (4) Method optimisation.

Each type of inoculum had six parallels of test water, three parallels of blanks and three parallels of positive controls. The wells which represented the test for bioavailable phosphorus contained algae from the inoculum together with algae and other microorganisms from test water. Blanks and positive controls contained only algae from inoculum. Low blank values were expected. Blanks were used to calibrate the test wells from interference in inoculum and NS-P. The difference between test well value and blank value gave the adjusted cell concentration. Positive controls were added 1 mL of 0.9 mg P/L. With a concentration of 0.45 mg P/L in each well positive controls were expected to have the highest measured values.

4.3.1 Results from algae bioassay with pre-grown inoculum

Pre-grown inoculum (PG-I) was a cultivated and maintained water sample taken from the surface of Store Stokkavannet. Due to similar growth conditions in tests and cultivation, such as temperature and light intensity, a short lag phase and fast growth rates were expected to be observed in the tests. In addition, the growth conditions for PG-I in the cultivation flasks were not phosphorus limited.

The results of all parameters from the ten tests performed with PG-I are shown in Figure 4.4. Average cell concentration (cells/mL) are plotted against time (hours). The vertical axis is displayed on a log scale. The period of when each test was conducted is highlighted on the individual plot. Composite water samples used in tests were collected on the same day as the test started.

The test parameters are presented as coloured dots in the graphs, where blue is test water inlet, green is positive control inlet, grey is blank inlet, red is test water outlet, yellow is positive control outlet and orange is blank outlet. Standard error is represented by error bars.

During the tests some wells were measured “over” and excluded in calculations of average cell concentration. The outliers are listed below:

- Test 1: One well of inlet test water and one well of inlet blank
- Test 6: Three wells of inlet test water, one well of inlet blank, five wells of outlet test water and two wells of outlet blank
- Test 9: Two wells of outlet positive control

In test 6 cell concentration was too dense in the beginning of the test. The culture should have been more diluted before it was added to the wells.

As shown in Figure 4.4 growth was observed for inlet and outlet test water in all ten tests. In test 1, 2, 6 and 7 growth were observed in inlet and outlet blanks. No growth appeared in inlet and outlet blanks in test 3, 4 and 5, but the positive controls in these tests did not grow either. In test 8, 9 and 10, PG-I was washed before the tests were performed. For these tests, no growth was measured in blanks, and the positive controls showed the highest growth of all parameters. The last three tests were the only tests which produced the expected and desired results.

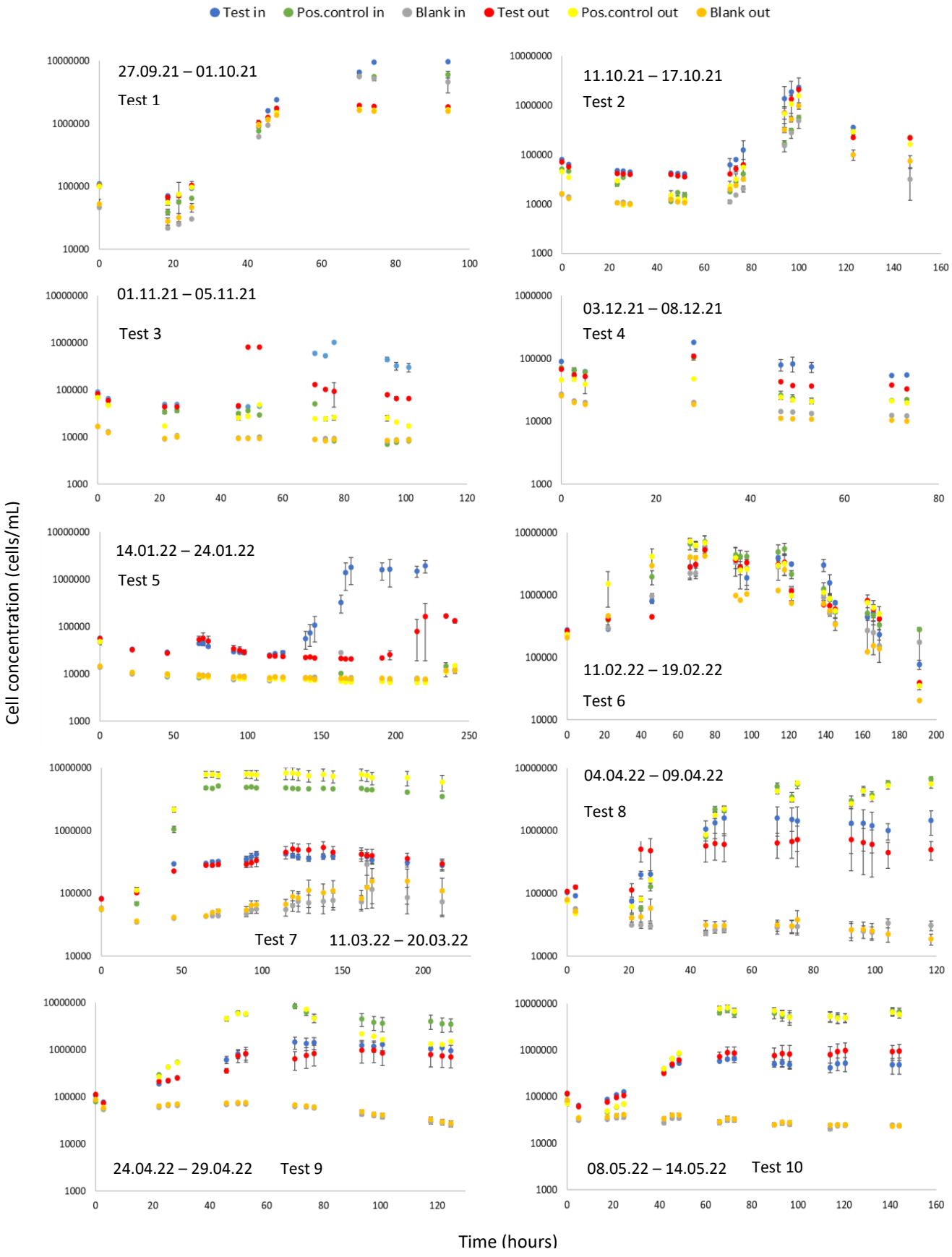

Figure 4.4: Average cell concentration of all test parameters in tests performed with PG-I plotted against time (hours). The vertical axis is displayed on a log scale. Standard error is represented by the error bars.

Growth curves from the total ten tests performed with PG-I can be seen in Figure 4.5. Adjusted average cell concentration (cells/mL) are plotted against time (hours). The growth curves are displayed on a log scale. Blue and red dots represent adjusted average cell concentration in inlet and outlet test water, respectively. Standard error is represented by error bars.

Lag phase was a common denominator for pre-grown algae in tests 1-5. Of these five tests, tests 1 and 2 had the most similar growth pattern. In test 1, a stationary phase was observed for both inlet and outlet pre grown algae at hour 48. Then the cell concentration slightly decreased for the outlet pre-grown algae until the end of the test. In contrast to the outlet pre-grown algae, growth was observed after hour 48 for inlet pre-grown algae. Test 2 showed a longer lag-phase than test 1 and a more consistent exponential growth phase. Stationary phase was reached at hour 100 for both inlet and outlet pre-grown algae. Then cell concentration drastically decreased, both for inlet and outlet.

Tests 3 and 4 showed no data points in the exponential growth phase. In test 3 at hour 46, outlet pre-grown algae were in the lag-phase. Three and six hours later the highest cell concentration of the test was measured and the estimated stationary phase was reached at this point. Then, cell concentration decreased throughout the test. A similar pattern can be seen for inlet pre-grown algae in the same test. Due to logistical challenges in test 4, only one measurement represented the stationary phase. Stationary phase was reached at hour 28 for both inlet and outlet. Test 4 showed the shortest time to reach stationary phase of all tests. The first four tests were performed with the same PG-I culture. The culture used in these tests was maintained from July 2021.

A different PG-I culture was used in test 5. This culture was maintained from the beginning of January 2022. The temperature in the incubator reached over 30 °C due to break down of the temperature control system. Consequently, the culture failed to grow properly. Test 5 possessed the longest lag-phase of all tests conducted with PG-I. A first stationary phase was observed for inlet and outlet pre-grown algae, around hour 70. Then, cell concentration decreased for both test waters. A second exponential growth phase began at hour 140 for inlet pre-grown algae and the second stationary phase was reached at hour 170. Outlet pre-grown algae had a longer second lag-phase and the second exponential growth phase began at hour 196. After 220 hours, the second stationary phase was reached. Test 5 had the longest test run of all the tests performed with pre-grown algae.

The high number of excluded data points in test 6 resulted in frequently negative adjusted average cell concentration. Consequently, these data points are not shown in Figure 4.5 since only positive values can be interpreted on a log scale. In addition, high number of excluded data points provide a lack of consistency between the data points as no growth curve can be seen in Figure 4.5.

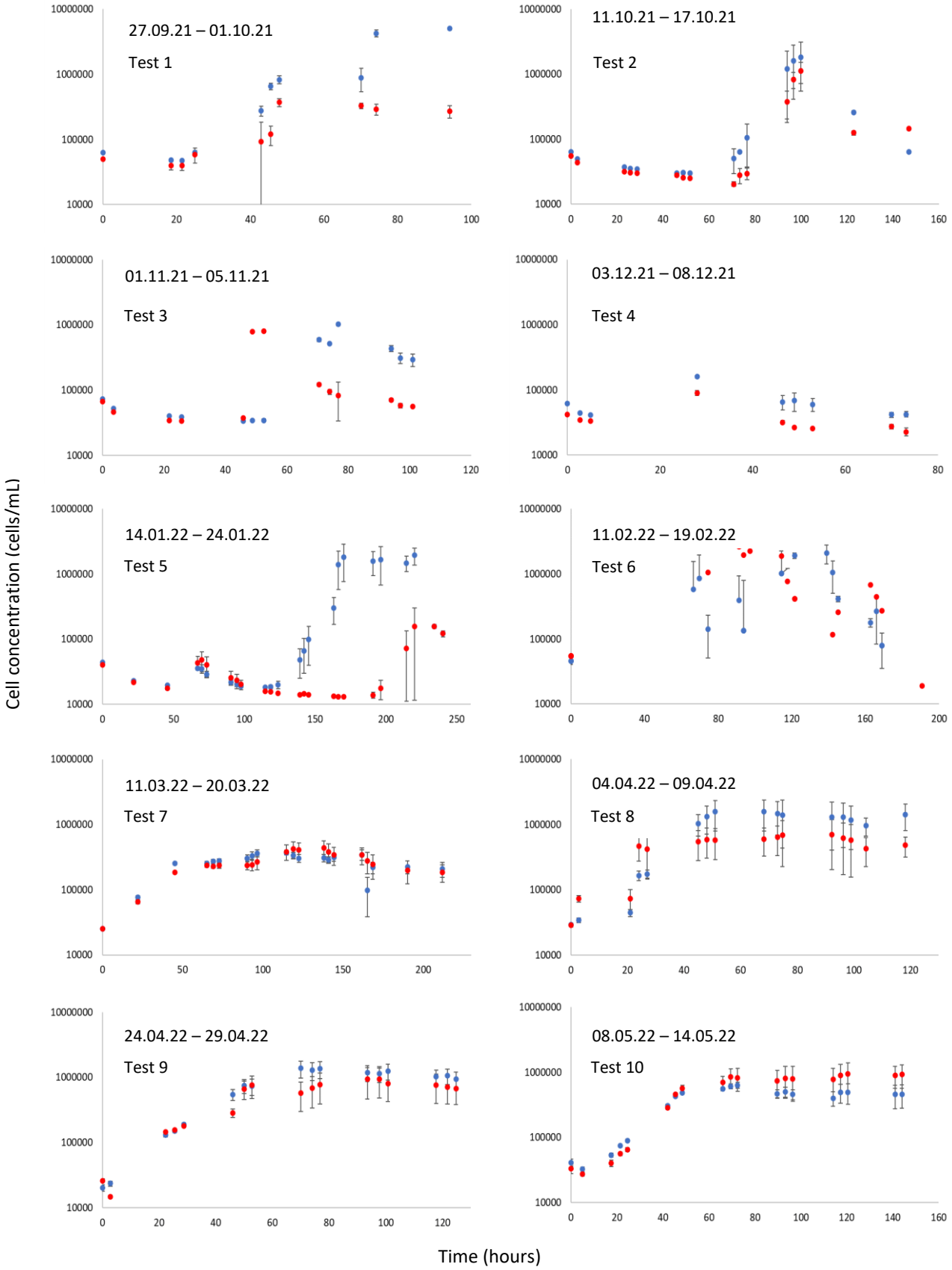

Figure 4.5: Pre-grown inoculum growth curves with adjusted average cell concentration plotted against time (hours). The vertical axis is displayed on a log scale. Blue and red dots represent adjusted average cell concentration with inlet and outlet test water, respectively. Standard error is represented by the error bars.

Growth curves in tests 7, 8, 9 and 10 had a similar growth pattern with a short or non-excisting lag phase and a steep exponential growth phase. The pre-grown algae in test 7 reached stationary phase around 65 hours, both for inlet and outlet. As mentioned earlier, minimal growth was observed in inlet and outlet blanks in test 7. The exponential growth phase in test 8 was less smooth than in tests 7, 9 and 10. An estimated stationary phase was reached after 48 hours for outlet and after 51 hours for inlet in test 8. In test 9, pre-grown algae inlet reached stationary phase after approximately 70 hours. A decrease in cell concentration was observed at hour 70 for outlet pre-grown algae. Then, growth increased again and stationary phase was estimated to be at hour 93. In test 10, the growth curves were almost similar for inlet and outlet pre-grown algae. Estimation of stationary phase was at hour 66 for inlet pre-grown algae and hour 70 for outlet pre-grown algae.

Estimated net growth (cells/mL) for all ten tests performed with PG-I can be seen in Figure 4.6. The blue bars represent net growth of inlet pre-grown algae, while net growth of outlet pre-grown algae is shown by the red bars. The date of each test run is shown on the horizontal axis. Standard errors are represented by the error bars. Net growth represents an estimation of the bioavailable phosphorus fraction of the test water. Higher net growth should correspond to higher levels of phosphorus in the corresponding water samples.

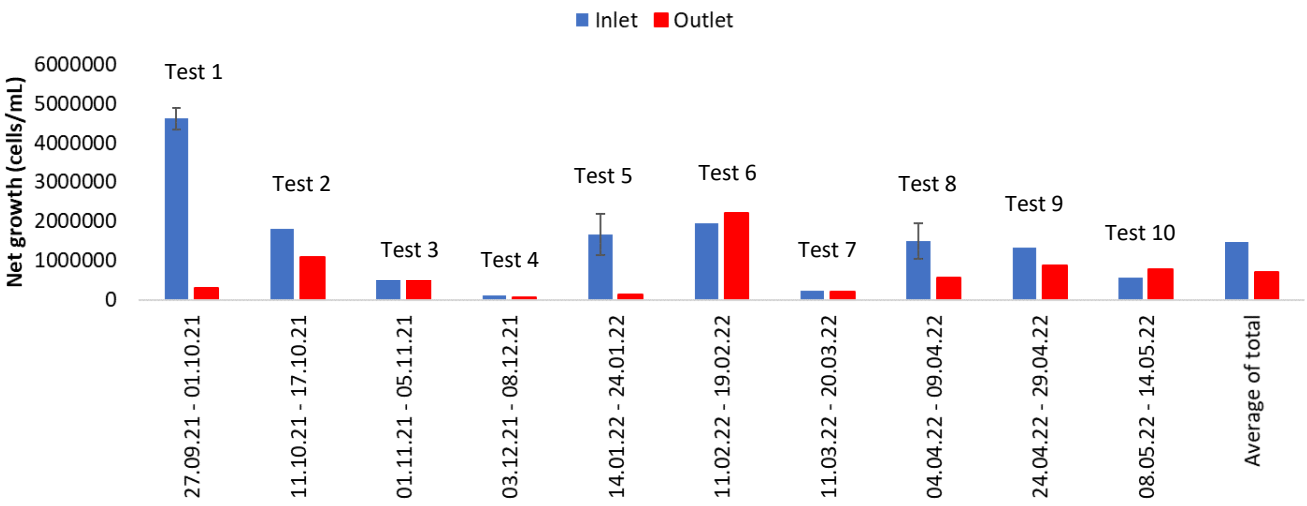

Figure 4.6: Estimated average net growth presented for the ten tests performed with PG-I. Blue bars represent net growth of inlet pre-grown algae. Net growth of outlet pre-grown algae is represented by the red bars. The date of each test run is shown on the horizontal axis. Standard error is represented by the error bars.

Net growth was calculated as the difference between cell concentration in stationary phase and at the end of the lag-phase. Cell concentration in stationary phase was estimated by taking an average

of the first data points in the stationary phase. Number of data points averaged depends on the characteristics of the growth curves. Same procedure was done for estimation of cell concentration at the end of lag-phase. For tests where no lag-phase exist or only one measurement represents the stationary phase, no average or SE can be calculated. This explain why only a few bars in Figure 4.6 is shown with SE.

As for the total suspended solids and phosphorus analysis, the net growth varied throughout the test period. As can be seen in Figure 4.6 the lowest net growth for inlet and outlet pre-grown algae was measured in test 4. The net growth did not correspond with either results of TSS or phosphorus analysis. Results from TSS analysis of test 4 showed the third highest inlet TSS concentration and an approximately average outlet TSS concentration, as shown in Figure 4.1. The results of test 4 from phosphorus analysis showed the highest TP concentration and the highest phosphate concentration in both inlet and outlet water samples (Figure 4.2). Overall, the average inlet net growth was higher than the average outlet net growth, corresponding with the results from both TSS and phosphorus analysis where average inlet concentrations were higher than average outlet concentrations.

The retention of pre-grown algae in the total ten tests conducted throughout the test period is shown in Figure 4.7. Percentage retention is given on the vertical axis. The date of each test run is shown on the horizontal axis. The percentage cell concentration retained, positive or negative, is highlighted on the top of each bar. SE are represented by the error bars.

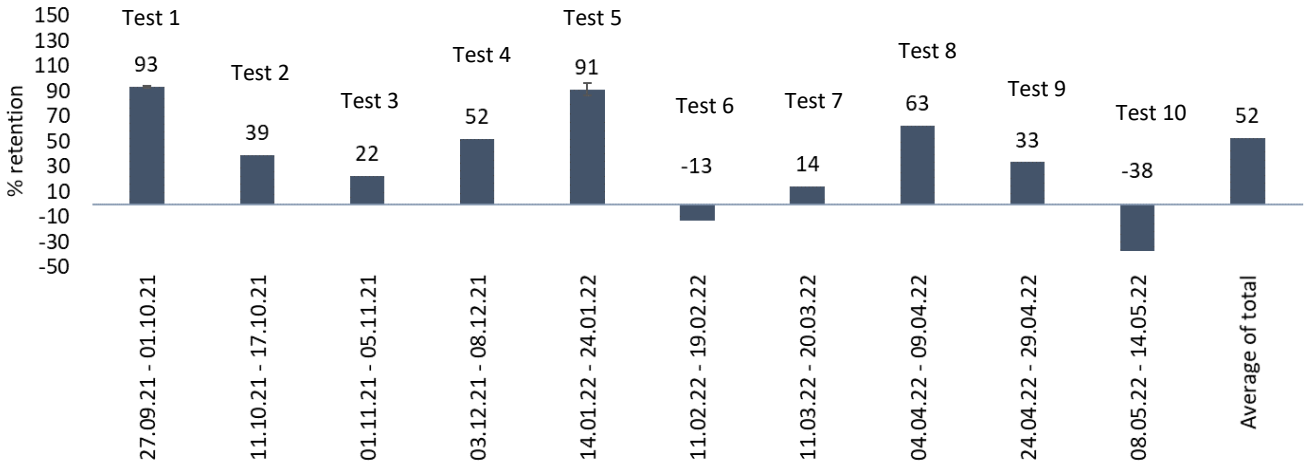

Figure 4.7: Percentage retention of cell concentration of pre-grown algae plotted against time. The bar to the right represents the retention of the average cell concentration. The date of each test run is shown on the horizontal axis SE are represented by error bars.

The degree of pre-grown algae retention varied throughout the test period. Figure 4.7 shows a positive retention of pre-grown algae in eight of ten tests. A positive algae retention means that the inlet contained more cells than the outlet.

The positive pre-grown algae retention in tests 1, 2, 3, 4, 7, 8 and 9 corresponded with a positive retention of total phosphorus (Figure 4.3) for the same test runs. Test 5 demonstrated a negative TP retention, not corresponding to the positive pre-grown algae retention in the same test. The positive pre-grown algae retention corresponded with the phosphate analysis in tests 1, 3, 4, 7 and 8. In tests 2 and 9, the pre-grown algae retention did not correspond, since the phosphate retention was negative for tests 2 and 9.

The right bar in Figure 4.7 (Average of total) demonstrated that, in average, the inlet contained 52 % more cells than the outlet. This means that the CW retained 52 % of the incoming BAP throughout the test period, given that the results from the bioassays provided a correct estimation of cell concentration.

4.3.2 Results from algae bioassay with lake water inoculum

Lake water inoculum was taken from the surface of Store Stokkavannet the same day as the tests were conducted. The LW-I culture was adapted to a P-limited and relatively cold environment (compared to room temperature at the lab). In addition, they lived under a natural dark/light regime. The differences between their natural growth conditions and the growth conditions at the lab, gave an expected decrease in cell concentration in the beginning of the tests and a longer lag phase, compared to PG-I. In addition, lower cell density, compared to PG-I, was expected for LW-I, especially in the colder seasons.

The results of all parameters from the ten tests performed with LW-I are shown in Figure 4.8. Average cell concentration (cells/mL) are plotted against time (hours). The vertical axis is displayed on a log scale. The period of when each test was conducted is highlighted on the individual plot. Composite water samples used in tests were collected on the same day as the test started. The test parameters are presented as coloured dots in the graphs, where blue is test water inlet, green is positive control inlet, grey is blank inlet, red is test water outlet, yellow is positive control outlet and orange is blank outlet. Standard error is represented by error bars.

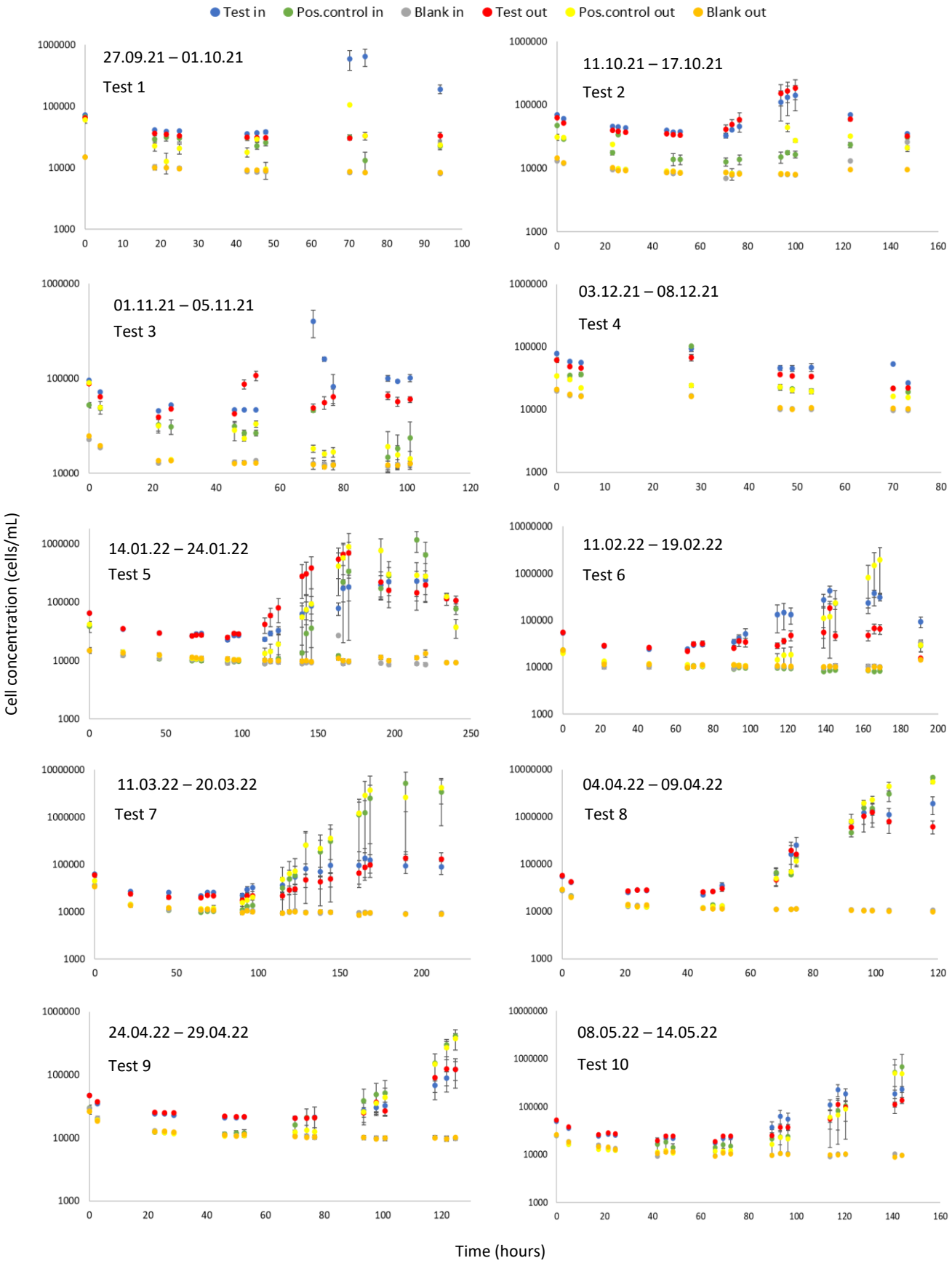

Figure 4.8: Average cell concentration of all test parameters in tests performed with LW-I plotted against time (hours). The vertical axis is displayed on a log scale. Standard error is represented by the error bars.

Some wells were also measured “over” in the tests performed with LW-I and excluded in the calculations of average cell concentration. The outliers were three wells of inlet test water in test 1.

As can be seen in Figure 4.8 growth was observed for inlet and outlet test water in all ten tests. No growth appeared in blanks, except a marginal growth at the end of test in inlet blanks in test 2 and 6. Minimal or no growth was observed in inlet and outlet positive controls in tests 1-3, while growth was observed only in either inlet or outlet positive controls in test 4 and 6. In tests 5 and 7-10 growth was observed in both inlet and outlet positive controls with the highest standard errors shown in test 5 and 7.

Growth curves from the total ten tests performed with LW-I are presented in Figure 4.9. Adjusted average cell concentration (cells/mL) are plotted against time (hours). The growth curves are displayed on a log scale. Blue and red dots represent adjusted average cell concentration in inlet and outlet test water, respectively. Standard error is represented by error bars.

No growth was observed in outlet lake water algae in test 1. In all other tests, growth was observed for both inlet and outlet lake water algae. An initial decrease was observed for all ten tests. Moreover, all tests, except test 4, showed a lag phase. The lag-phases were longer compared to the lag phases observed for pre-grown algae. The longest lag phases are shown for test 5, 6 and 7, which were performed in January, February and March. The lag phases in these tests lasted approximately 100 hours, except for inlet lake water algae, which showed a lag phase of approximately 70 hours. Test 9, conducted in end of April, also had one of the longest lag phases (approximately 80 hours).

Test 2-4 showed a common pattern in which the cell concentration drastically decreased after stationary phase was reached. The tests were conducted during fall and early winter. The stationary phase consisted of only one or two data points. Also, tests 3 and 4 showed no data points in the exponential phase. In test 3, after the last data point of the lag phase, the next data point showed that stationary phase was reached. As mentioned previously, test 4 had some logistical challenges and hence explained some of the lack of data points before stationary phase. The radical change in the algae population was also observed in test 2-4 performed with pre grown algae.

Some tests showed higher standard errors than others. A general trend was that standard error increased when the cell concentration increased, meaning greater standard errors were observed in exponential and stationary phase. The highest standard errors are shown in test 5 and 6 which were conducted in January and February.

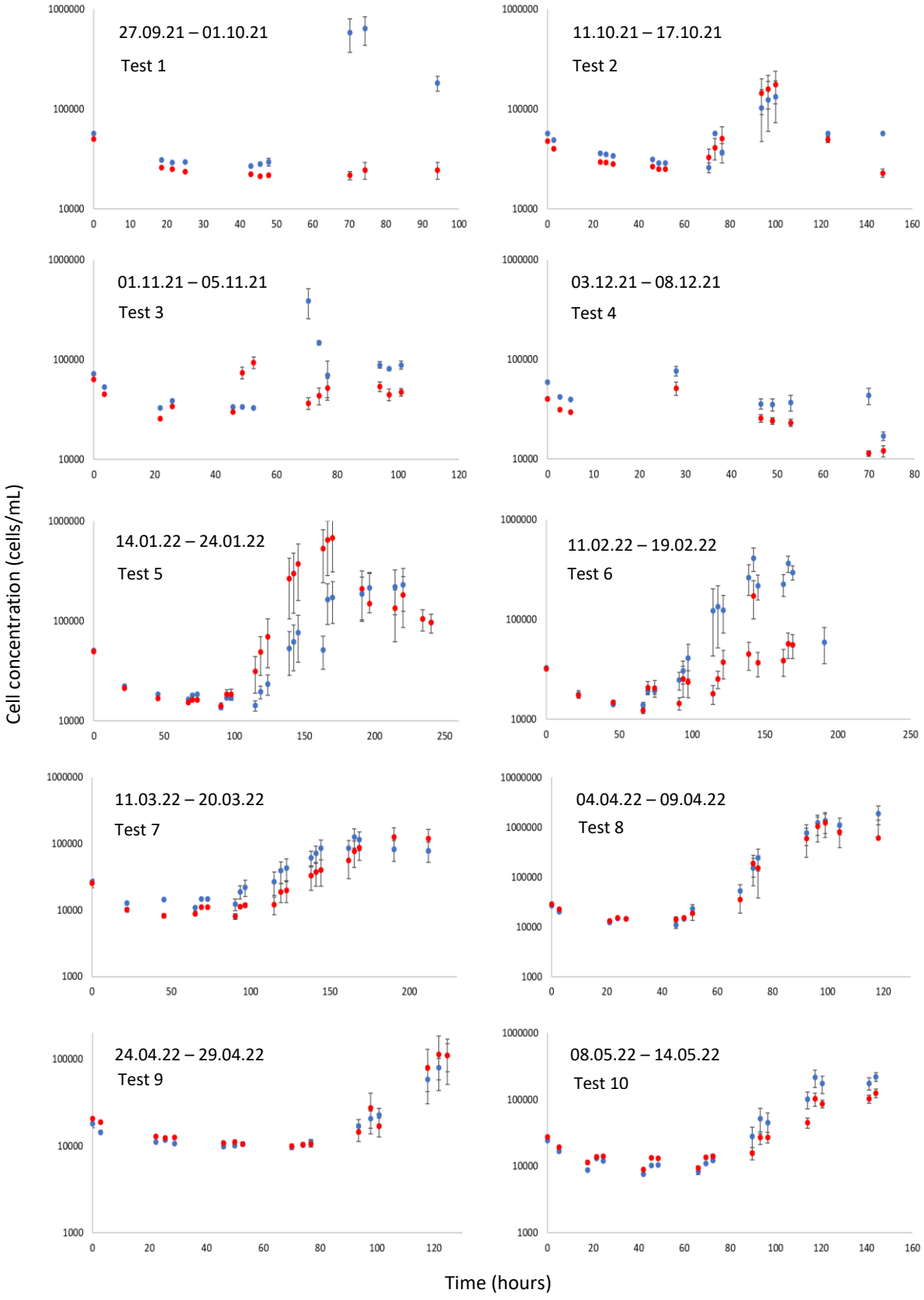

Figure 4.9: LW-I growth curves with adjusted average cell concentration plotted against time (hours). The vertical axis is displayed on a log scale. Blue and red dots represent adjusted average cell concentration with inlet and outlet test water, respectively. Standard error is represented by the error bars.

The time it took for the lake water algae to reach stationary phase was the shortest for the tests performed during fall and early winter (early December). Lake water algae in tests 4 and 2 reached stationary phase at hour 28 and hour 100, respectively. Lake water algae in tests 1 and 3 reached stationary phase around hour 70. In January, February and March the lake water algae reached stationary phase around hour 160-200. Stationary phase was reached after a shorter time for the tests performed in April and May, where stationary phase was reached at around hour 100-120.

Estimated net growth (cells/mL) for all ten tests conducted with LW-I can be seen in Figure 4.10. The blue bars represent net growth of inlet lake water algae, and the red bars represent net growth of outlet lake water algae. The date of each test run is displayed on the horizontal axis. Standard errors are represented by the error bars. As mentioned previously, net growth represents an estimation of the bioavailable phosphorus fraction of the test water. Higher net growth should correspond to higher levels of phosphorus in the corresponding water samples.

Net growth was calculated in the same way as for pre-grown algae and was the difference between cell concentration in stationary phase and at the end of the lag-phase (see section 4.3.1).

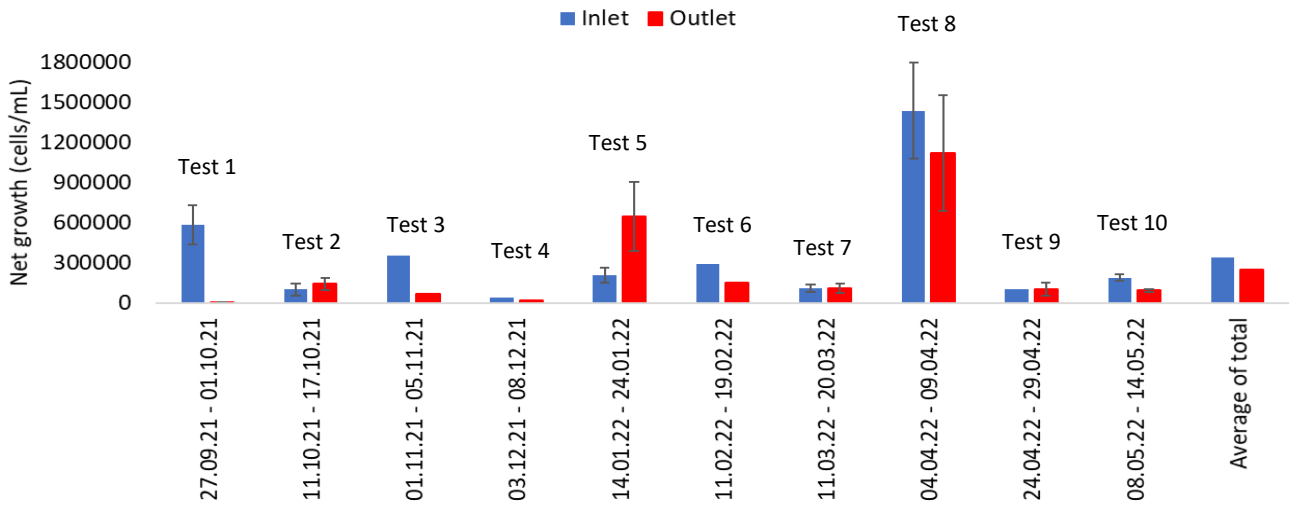

Figure 4.10: Estimated average net growth presented for the ten tests performed with LW-I. Blue bars represent net growth of inlet lake water algae. Net growth of outlet lake water algae is represented by the red bars. The date of each test run is shown on the horizontal axis. Standard error is represented by the error bars.

Net growth for lake water algae varied throughout the test period. Compared to phosphorus analysis, the highest net growth should be in test 4. This is not the case, as the highest net growth was seen in test 8, both for inlet and outlet. Net growth in test 4 was the lowest for outlet, and second lowest for inlet. In general, the average inlet net growth was higher than the average outlet

net growth. This corresponded to the results from both TSS and phosphorus analysis where average inlet concentrations were higher than average outlet concentrations.

Figure 4.11 present the retention of lake water algae for all ten tests. Percentage retention is given on the vertical axis. The date of each test run is shown on the horizontal axis. The percentage cell concentration retained, positive or negative, is highlighted on the top of each bar. SE are represented by the error bars. Due to lack of standard error in inlet or outlet net growth, some of the bars in Figure 4.11 are shown without standard error.

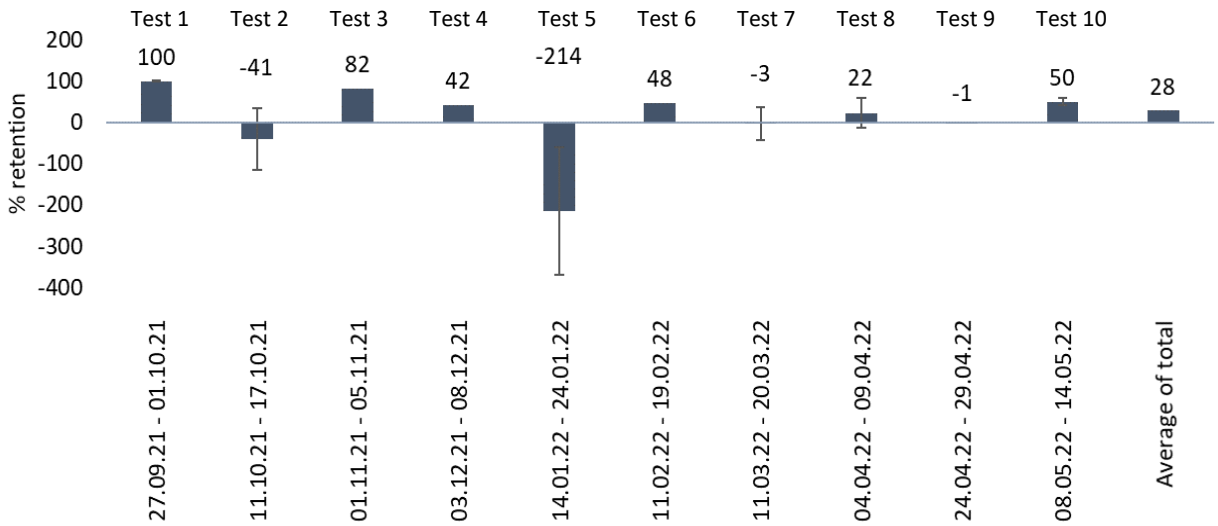

Figure 4.11:Percentage retention of cell concentration of lake water algae plotted against time. The bar to the right represents the retention of the average cell concentration. SE are represented by error bars.

A positive retention of lake water algae was seen in six of ten tests. In test 1, all of the incoming bioavailable phosphorus was retained by the CW. This corresponded quite well with TP (65 % retention) and phosphate (81 % retention) retention in the same test. The positive lake water retention in tests 1, 3, 4, 6 and 8 corresponded with a positive retention of both TP and phosphate. Tests 2 and 9 showed a negative lake water algae retention, corresponding to a negative phosphate retention in the same tests. Test 10 did not correlate well, since lake water algae retention was positive (50 %), TP retention was positive (34 %) and phosphate retention was negative (-430 %). The average lake water algae retention was positive (28 %), meaning that the CW retained on average 28 % of the incoming bioavailable phosphorus throughout the test period. The statement is based upon that the results from the bioassays provided a correct estimation of cell concentration.

4.3.3 Method calibration and cross-validation

The results from the calibration of microplate reader method are presented in this section. Three parallels from six cultures with different cell concentration were quantified by flow cytometry and direct counting and compared to microplate reader analysis. The mean and standard error of cell concentration from flow cytometric enumeration and direct counting are shown in Table 4.1.

Table 4.1: Cell quantification using flow cytometry and direct counting. Six different cultures, each with three replicates, were quantified (mean±standard error)

Method	Cell concentration (cells/mL)					
	1	2	3	4	5	6
Flow cytometry	2446352±110	1033613±973	495545±143	244295±150	63449±102	37391±115
Direct counting	2426667±56877	1068889±22471	485926±9102	242963±1614	61111±642	37037±370

4.3.3.1 Calibration of microplate reader method

The calibration curves of microplate fluorescence signal using flow cytometric enumeration (R^2= 0.9986) and direct counting (R^2= 0.9995) are presented in Figure 4.12. Standard errors are represented by error bars. Both methods indicated that more than 99 % of the variance of cell concentration can be explained by the variance of microplate fluorescence signal.

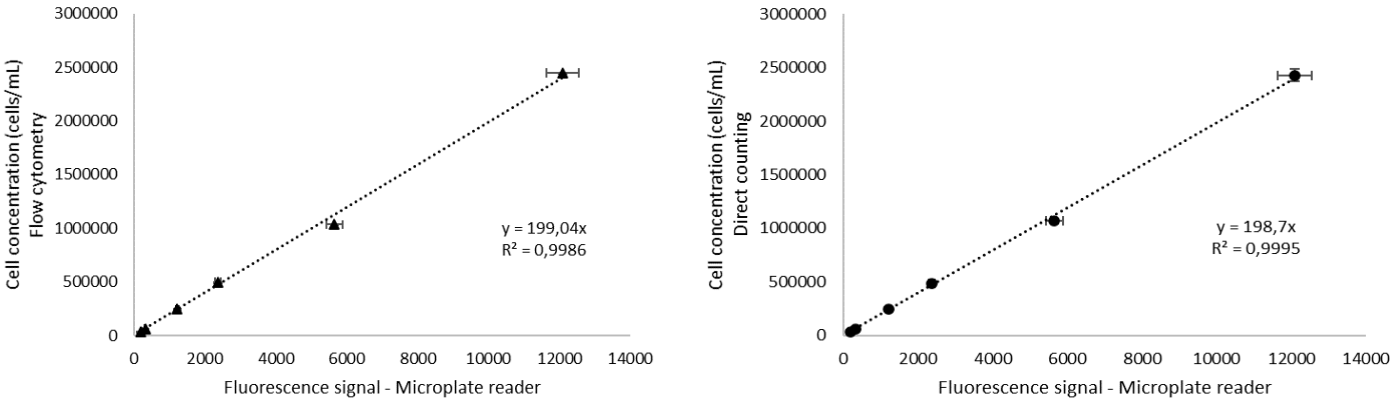

Figure 4.12: Calibration curve of microplate fluorescence signal using flow cytometric enumeration (triangle) and direct counting (circle) with error bars showing standard errors.

The calibrations were carried out to estimate the calibration factor needed for the conversion of fluorescence detection signal to algal cell concentration (Equation 4.1). The regression model estimated a calibration factor of 199.04 and 198.7 from flow cytometric enumeration and direct counting, respectively.

Cell concentration (cells/mL) = calibration factor · fluorescence detection signal *Equation 4.1*

4.3.3.2 Cross-validation

To cross-validate the microplate reader method statistical analysis with Excel was performed. Correlations between flow cytometric enumeration and direct cell counting were estimated by regression analysis and a 95 % two-tailed confidence analysis. The linear relationship between flow cytometric enumeration and direct cell quantification is shown in Figure 4.13. The R^2 value of 0.9998 indicated a significant correlation between the two quantification methods.

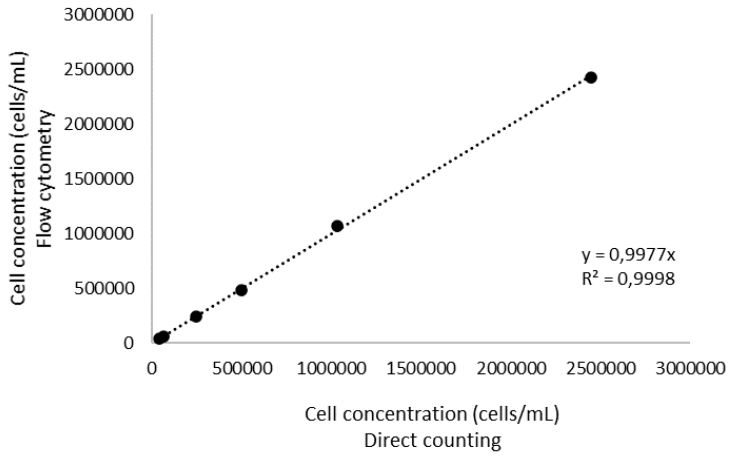

Figure 4.13: Flow cytometric enumeration plotted against direct cell quantification.

The t-test with 95 % confidence level, shown in Table 4.2, confirmed no significant difference ($p > 0.05$) between flow cytometric enumeration and direct counting.

Table 4.2: P-value of 95 % two-tailed confidence analysis of flow cytometric enumeration and direct cell quantification from six different cell concentration with three replicates.

Method	Two-tailed p-value (t-test)					
	1	2	3	4	5	6
Flow cytometry **Direct counting**	0.76	0.25	0.41	0.48	0.06	0.40

4.3.4 Method optimisation

The growth curves from the CO_2 test are shown in Figure 4.14 where adjusted cell concentration (cells/mL) are plotted against time (hours). Growth curves from test 10 are also presented in the figure for comparison. The top two graphs show the test supplied with CO_2. The bottom two graphs show the growth curves from test 10. The graphs on the left represent pre-grown algae, while the graphs on the right represents lake water algae. The growth curves are displayed on a log scale. Blue and red dots represent adjusted average cell concentration in inlet and outlet test water, respectively. Standard error is represented by error bars. The results from the test supplied with CO_2 showing all parameters can be found in Appendix E.

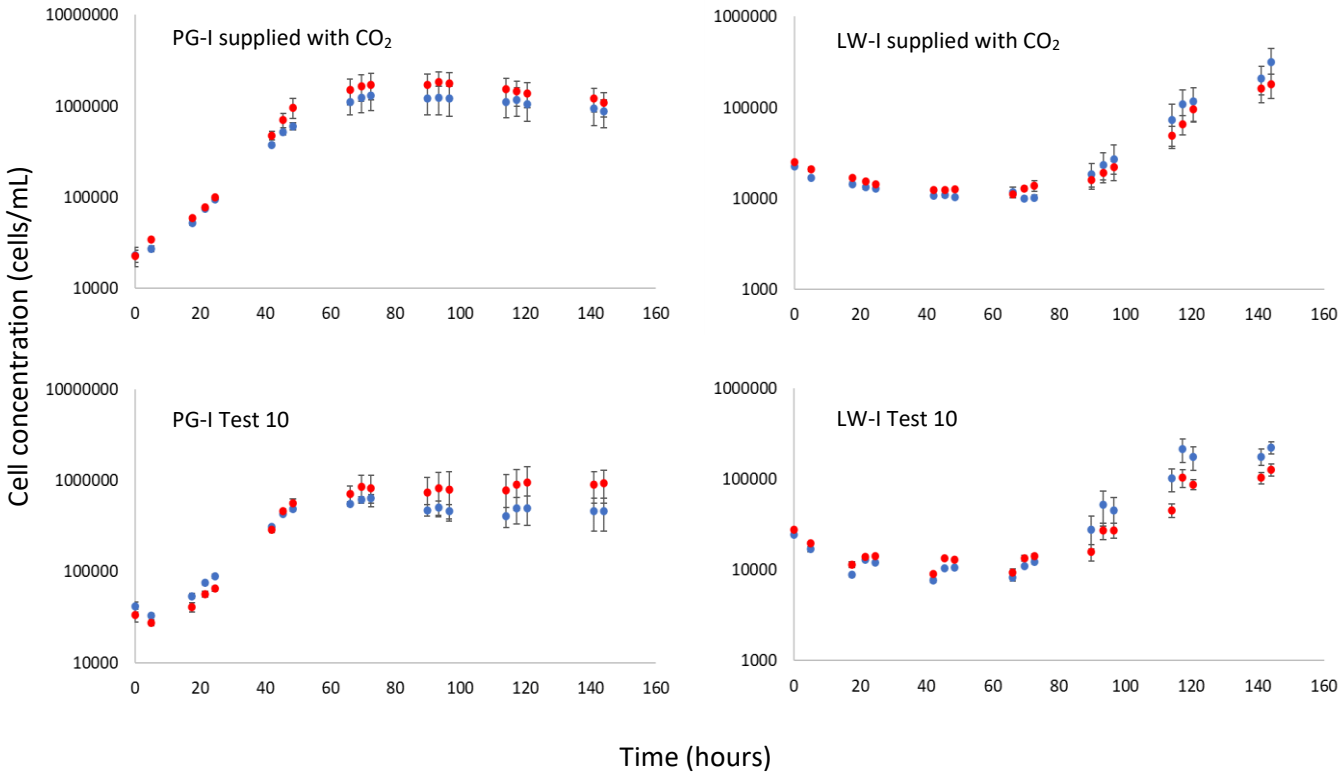

Figure 4.14: Growth curves with adjusted average cell concentration plotted against time (hours). The vertical axis is displayed on a log scale. The top two graphs are the test supplied with CO_2. The bottom two graphs are the growth curves from test 10. The graphs on the left represent pre grown algae, while the graphs on the right represents lake water algae. Blue and red dots represent adjusted average cell concentration with inlet and outlet test water, respectively. Standard error is represented by the error bars.

Smoother growth curves are seen for tests supplied with CO_2. A smoother stationary phase reduces the uncertainty of the estimated algae growth potential, which is preferable. The fluctuation in cell concentration was more evident for lake water algae. A distinct pattern appeared for the lag-phase in test 10 with LW-I. The lag phase began at around hour 20, which was the first data point in day number 2 in the test run. The first data point for day number 3 was around hour 42, and the first data point for day number 4 was around hour 66. For the lag-phase, the lowest cell concentration was always at the day's first measurement. In the two subsequent measurements the cell concentration increased. The next day the cell concentration decreased. This pattern was not observed for LW-I supplied with CO_2.

No lag phase was observed for pre-grown algae supplied with CO_2. The algae went straight to exponential phase. For pre-grown algae in test 10, a decrease in cell concentration was observed prior to the exponential phase.

The estimated net growth for inlet and outlet pre grown algae supplied with CO_2 was 1229710 cells/mL and 1731372 cells/mL, respectively. This means that the CO_2 supply provided 54 % more cells in inlet and 55 % more cells in outlet compared to test 10, which were not supplied with CO_2. For LW-I supplied with CO_2, the estimated net growth for inlet and outlet was 306649 cells/mL and 159043 cells/mL. Compared to estimated inlet and outlet net growth in test 10, CO_2 supply provided 39 % more cells in inlet and 41 % more cells in outlet.

4.4 Chemical available versus bioavailable phosphorus

This chapter presents correlations between chemical available and bioavailable phosphorus. Tests 8-10 were applied in correlation analysis for PG-I with the following justification: 1) no growth in blanks and 2) highest growth in positive controls. Tests 7, 8 and 10 were applied in the correlation analysis for LW-I, with the following justification: 1) no growth in blanks, 2) highest growth in positive controls and 3) the stationary phase was assumable reached for both inlet and outlet.

The correlations are presented in Figure 4.15 and Figure 4.16. Net growth (cells/mL) is plotted against TP concentration, phosphate concentration and TSS concentration in three separate plots. Inlet (triangle) and outlet (circle) are plotted separately in each plot.

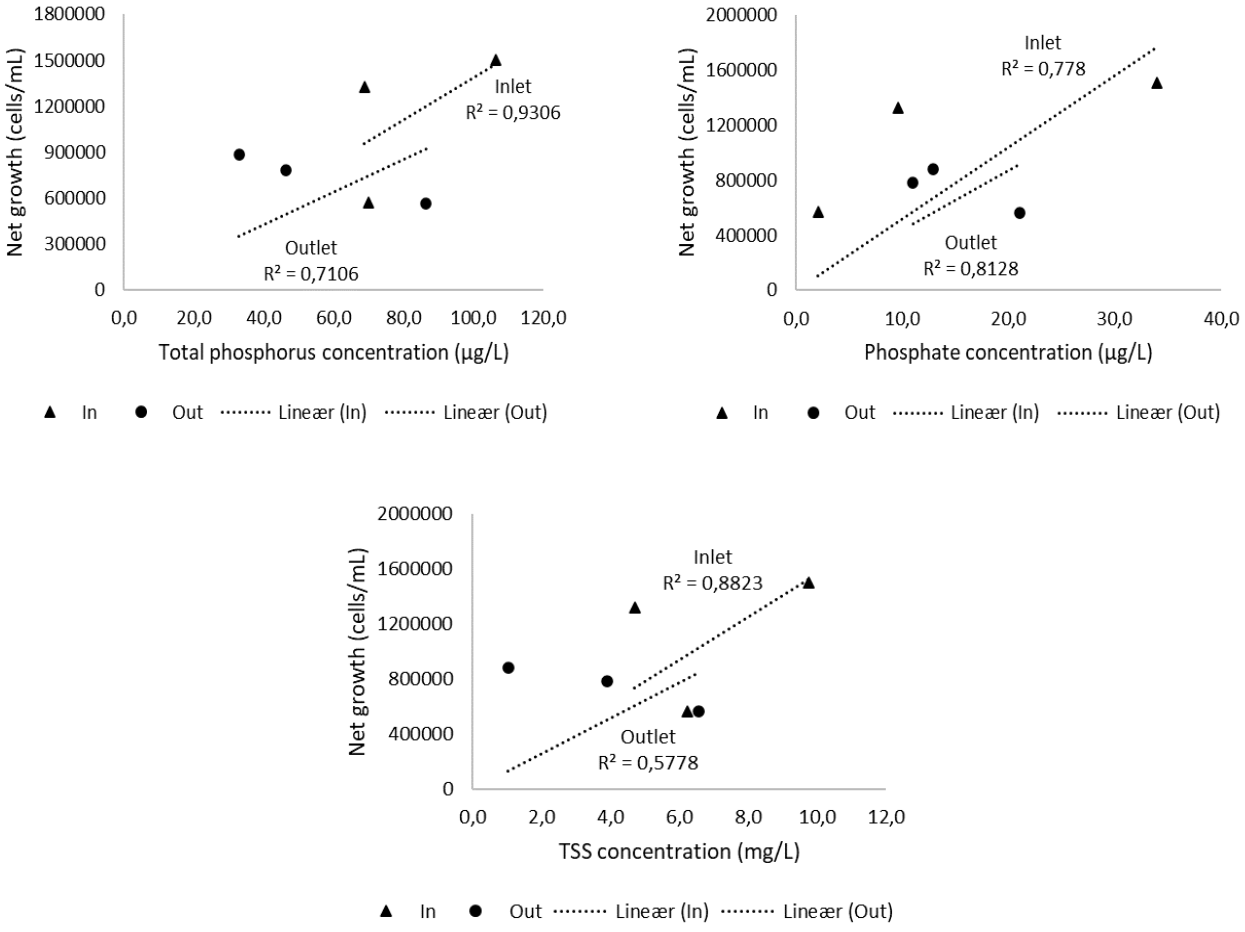

Figure 4.15: Correlation plots between pre-grown algae net growth and TP concentration (upper left), phosphate concentration (upper right) and TSS concentration (bottom). Inlet correlations presented by triangles and outlet correlations presented by circles.

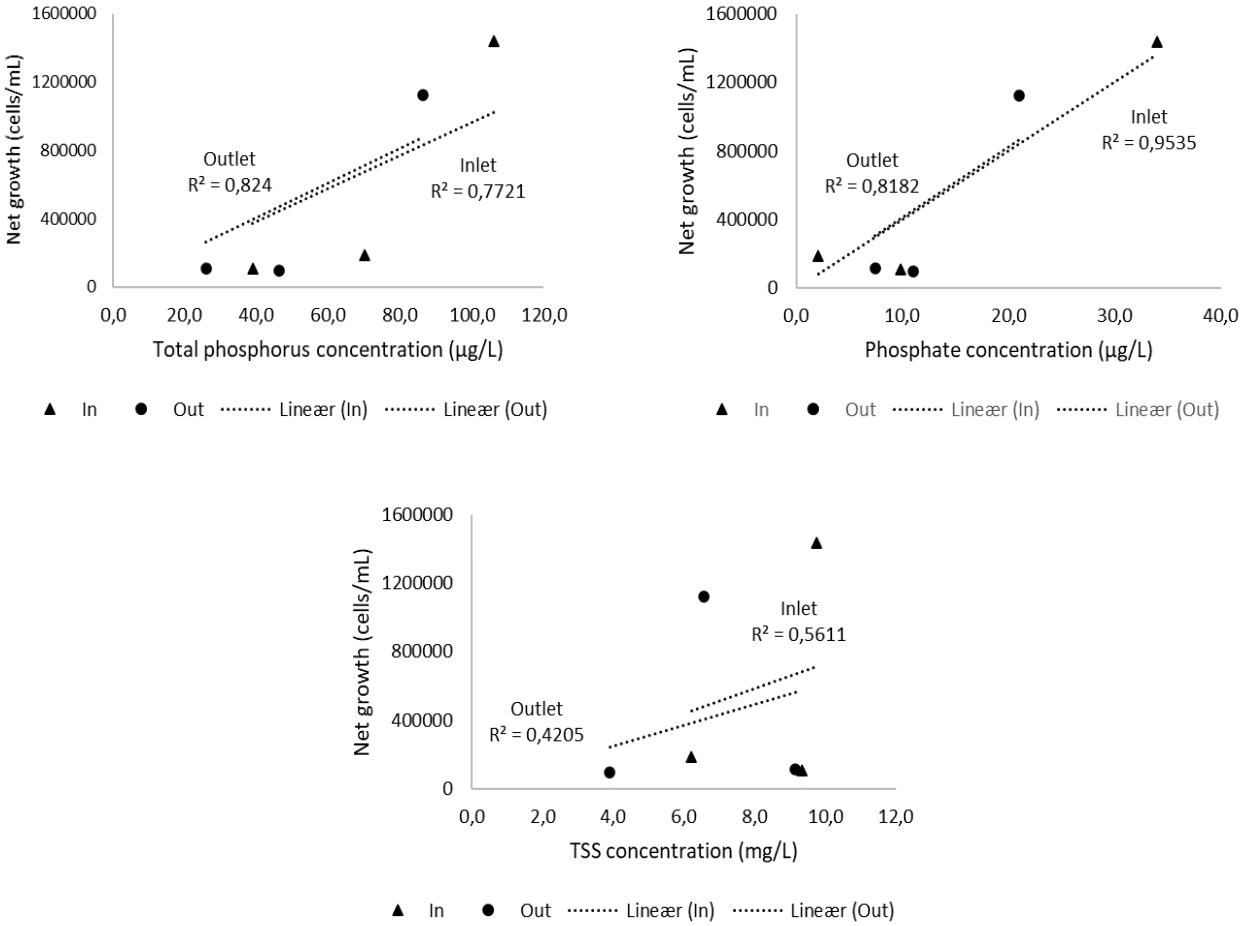

Figure 4.16: Correlation plots between lake water algae net growth and TP concentration (upper left), phosphate concentration (upper right) and TSS concentration (bottom). Inlet correlations presented by triangles and outlet correlations presented by circles.

The estimated R^2 values for all plots indicated a moderate to strong positive correlation between TSS and BAP, and chemical available and BAP. In general, the weakest correlation was between net growth and TSS.

For LW-I, the highest correlation was between net growth and phosphate concentration. R^2 value of 0.95 (inlet) and 0.82 (outlet) indicated a strong positive correlation between chemical and BAP. For pre-grown algae, plotted against phosphate concentrations, the R^2 value of inlet was lower (0.78) than lake water algae. However, the R^2 value of pre-grown algae outlet showed an equal correlation (0.82) with outlet lake water algae. The TSS plots demonstrated a moderate correlation with net growth of lake water algae and pre-grown algae. For LW-I the R^2 value was estimated to 0.53 (inlet) and 0.56 (outlet). For PG-I the R^2 value was estimated to 0.88 (inlet) and 0.58 (outlet). For the TP correlation plots, the R^2 values ranged between 0.71 and 0.93. In general, the R^2 values of TP and phosphate plots were quite similar.

5. DISCUSSION

Results obtained during this thesis are discussed in this chapter. This chapter is divided into 4 subchapters: (1) TSS and phosphorus analysis, (2) Algae bioassay, (3) Estimation of bioavailable phosphorus and (4) Recommendations and further research.

5.1 Total suspended solids and chemical phosphorus analysis

Phosphates can be part of suspended particles by adsorption, precipitation and exchange processes. To retain the phosphorus bound suspended solids, they need to settle in the sediments in the CW. The retention of suspended solids in a wetland is controlled by particle size, water flow velocity and hydraulic retention time (Mereta et al., 2020). Total suspended solids analysis (Figure 4.1) showed that the average TSS retention was 43 %. A positive TSS retention was observed in nine of ten analyses. As CW is regarded as efficient sediment traps (Kadlec & Reddy, 2001), the results are consistent with theory. In test 5, the outlet TSS concentration (9±1 mg/L) was higher than the inlet TSS concentration (7±1 mg/L), probably caused by resuspension of the sediments. This means that in this period (10.01.22 – 14.01.22) the CW released suspended solids to Store Stokkavannet. Regression analysis indicated a moderate to strong positive correlation between TP and TSS concentration (R^2 = 0.87 for inlet and R^2 = 0.74 for outlet). The moderate to strong correlation could indicate that, statistically, a significant fraction of the TSS were associated with phosphorus. The regression plot can be found in Appendix A.

Chemical phosphorus analysis showed an average positive TP and phosphate retention of 34 % and 43 % during the thesis period (Figure 4.3). TP was retained in nine of ten analyses. The analysis conducted in January 2022 showed a marginal negative TP retention of -0.1 % (Figure 4.3). In general, the highest phosphate retention was in the fall and early winter, except the analysis performed in October 2021 where a negative retention of phosphate was observed (-18 %) (Figure 4.3). The negative phosphate retention may be due to a storm event, which can cause wash out of phosphate and particles. TP analysis conducted on the same water samples (in October 2021) showed a relatively low TP concentration compared to the other analyses performed in the fall and early winter (September – December). Late winter and early spring (January – May) showed a lower phosphate retention compared to fall and early winter. Negative phosphate retention was observed for the two lasts analyses, conducted on 24.04.22 and 08.05.22. The greatest period of phosphorus retention was expected to be in spring, since spring is a period of increased growth for plants and algae. At the same time, a higher external phosphate load is expected since spring is the period when farmers start to fertilize their crops. Phosphate analysis, on the other hand, showed an inlet

concentration of 9.6 ± 0.3 µP/L (24.04.22) and 2.1 ± 0.3 µP/L (08.05.22). The phosphate concentrations are the two lowest concentrations measured throughout the thesis period. To compare, average inlet phosphate concentration is 26.8 ± 0.8 µP/L. The negative phosphate retention in spring may indicate that the CW does not function as it should be. Phosphate is the most reactive form of phosphorus and should rapidly be taken up by plants and microorganisms or be immobilised by adsorption and precipitation.

Phosphorus analysis is sensitive of contamination from other sources containing phosphorus. The contamination may come from detergents containing phosphorus, dust and from the analyst (e.g. contaminated hands). The highest standard errors can be seen for the first four analyses, especially for the TP analysis (Figure 4.2). For the last six analyses the standard errors were relatively low, both for TP and phosphate (Figure 4.2). Three replicates were analysed in the last six tests, providing a lower degree of uncertainty.

5.2 Algae bioassay

This subsection first discusses results obtained from bioassays performed with pre-grown inoculum and lake water inoculum. Further, a discussion on the calibration and cross-validation of the microplate reader method is given. The last subsection discusses aspects regarding the bioassay methodology.

5.2.1 Algae bioassay performed with pre-grown inoculum

It was expected that no growth would appear in blanks since blanks were not added any source of phosphorus. Nevertheless, growth was observed in inlet and outlet blanks for test 1, 2, 6 and 7. Most likely, growth in blanks was caused by P-residuals in inoculum. All wells, included blanks, contained algae and NS-P. If phosphorus is present, growth might occured. In test 8-10 PG-I was washed, as mentioned in Methods sections 3.4.4.1 , before being added to the microplates. No growth was observed in inlet and outlet blanks for these tests, indicating that P-residuals in inoculum caused the growth in blanks for test 1, 2, 6 and 7 (Figure 4.4).

In test 7 minimal growth was observed in inlet and outlet blanks, compared to high growth in blanks for test 1, 2 and 6. In test 7, PG-I was transferred to a new media one week before the test was conducted. In test 6 PG-I was transferred to a new media the day before the test was conducted. This can explain why minimal growth in blanks was observed in test 7 and high growth was observed in test 6 (Figure 4.4).

The test well values were subtracted from blank values to estimate the real cell growth. If blank values are high, the estimation of cell growth will be falsely low. As an example, growth was observed in blanks in test 1. A minimal difference was observed in outlet test wells and blanks. The minimal difference provided a very low adjusted cell concentration. A larger difference in cell concentration was observed in inlet test wells and blanks. Because of the greater difference between outlet test wells and blanks, the calculated adjusted cell concentration will be falsely lower for the outlet test well.

In test 3-5, no growth appeared in blanks and positive controls. However, growth was observed in test wells (Figure 4.4). Positive controls were added 0.9 mg P/L and were expected to show the highest growth of all test parameters. Phosphorus concentration in a positive control well was 0.45 mg P/L, which was significantly higher than the highest phosphorus concentration measured in this thesis (157 µP/L) (Figure 4.2). In test 5, PG-I was transferred to a new media the day before the test was conducted. With reference to tests 1, 2, 6 and 7, growth was expected in blanks and positive controls, but this did not happen. The culture used in test 5 failed to grow properly due to malfunction of the temperature control system of the incubator. This was probably the reason why positive controls and blanks did not grow, although the PG-I was transferred to a new media the day before the test. It is uncertain when PG-I was transferred to a new media in test 3 and 4. A possible explanation for the lack of growth in positive controls in test 3 and 4 is that the temperature control system of the incubator was unstable prior to the break down. If the temperature frequently reached 30 ° C this would have a negative impact on the growth potential. As mentioned in theory section 2.6.1 , the optimal growth temperature for most algae species range between 16-27 °C. The growth in the test wells can be explained by the beneficial interaction between pre-grown algae and the bacteria from the test water. As mentioned in theory section 2.5.1.3 , bacteria possess the ability to mobilize bioavailable phosphorus and hence promote algae growth. Positive controls and blanks did not contain bacteria from test water, hence lacking this beneficial interaction. However, the positive controls contained 0.45 mg/L of phosphate, which is, according to theory, considered readily bioavailable. And as previously mentioned, phosphorus concentration in positive controls was significantly higher compared to test wells. This may indicate that the interaction between bacteria and algae promote other beneficial growth conditions in addition to phosphorus mobilisation. Moreover, growth of algae from test water might be an explanation why growth was observed in test wells and not in blanks and positive controls.

As mentioned above, the malfunction of the temperature control system of the incubator contributed to a minimal grow of the culture in test 5. The low growth potential produced a

distinctive pattern, as can be seen in Figure 4.4. The cell concentration decreased twice during the test run. After the first decrease in cell concentration, minimal growth was observed. At this point, the cell concentration was approximately equal to the start cell concentration. Then, cell concentration decreased again. It seems like the culture struggled to adapt because of their low growth potential. The time they reached exponential phase and stationary phase was by far the longest compared to the other tests conducted with pre-grown algae. Inlet pre-grown algae reached exponential phase and stationary phase at around hour 100 and 170, respectively. Outlet pre-grown algae reached exponential phase and stationary phase at around hour 160 and 220, respectively.

Test 2-4 were performed during fall and early winter (September – early December) and showed a common pattern not observed for the other tests conducted during the thesis period (Figure 4.5). After stationary phase was reached, which consisted of only one or a few data points, the cell concentration drastically decreased. The significant change in the population might be a result of a top-down control, which is a mechanism to keep a population down to restore balance in an ecosystem. A top-down control is caused by grazers and virus, as mentioned in theory section 2.6.1 . It is reasonable to think that grazers are dominant in the fall, as a natural consequence of algal blooms earlier in the summer. Since test 2-4 were conducted during fall and early winter, it is reasonable to think that the drastic decrease in algae population was caused by a top-down control. The possible presence of grazers and virus generated an ambiguity whether a decline in algae population was caused by a top-down control or a bottom-up control, like phosphorus limitation.

Attempts to cultivate a freshwater sample collected on 9.3.22 failed. Two weeks after cultivation no growth was observed, and the replicates were discarded. The lack of growth might be caused by an abundance of diatoms in the collected water sample. Diatoms require silicon (Si) to grow (Barsanti & Gualtieri, 2014). Silicon is involved in building their outer cell wall. Considering the lack of silicon in the nutrient media, it is reasonable to think that the failure in cultivation from 9.3.22 was caused by a domination of diatoms in the culture.

Net growth (Figure 4.6) compared with phosphorus concentration for the corresponding test show a various correlation throughout the test period. The highest TP and phosphate concentrations were measured in test 4, which gave an expectation of the greatest net growth for pre-grown algae in test 4. Yet, the estimated net growth in test 4 was the lowest for all tests, both for inlet and outlet (Figure 4.6). The low net growth in test 4 was probably caused by a top-down control. A greater relationship was observed for test 7. The lowest TP concentrations, both inlet and outlet, were measured in test 7 (Figure 4.2). The phosphate concentrations for the same test were one of the lowest throughout the test period and net growth for the corresponding test was the second lowest.

No consistency was found throughout the test period when comparing percentage retention of algae (Figure 4.7) and phosphorus (TP and phosphate) in the corresponding test. As an example, in test 3, 22 % of the cells were retained compared to 30 % retention of TP and 70 % retention of phosphate. Theoretically, phosphate is readily bioavailable, and a fraction of TP is potentially bioavailable. This means that the retention of algae should be higher than 22 %. As mentioned previously, a top-down control probably happened during test 3. It makes no sense to compare retention of algae and phosphorus in either test 5 or test 6. The phosphate analysis in test 5 was incorrect, and the bioassay in test 6 had great numbers of outliers, giving no consistency between the data points.

It was challenging to compare net growth with the corresponding phosphorus concentration or to compare retention since the bioassays had some challenging aspects. In some tests growth in blanks were observed, in some tests lack of growth was observed in positive controls, and a top-down control probably affected the net growth in tests 1-4. The decision was made to only compare the bioassays conducted in test 8-10 with the corresponding phosphorus and TSS concentration. For these tests no growth was measured in blanks, while the greatest growth response was measured in positive controls. Also, a long stationary phase was obtained in these tests.

5.2.2 Algae bioassay performed with lake water inoculum

The growth curves of lake water algae (Figure 4.9) were very different from the growth curves of pre-grown algae (Figure 4.5). The most prominent features were initial decrease in cell concentration, longer lag phases and that the stationary phase was reached much later. The main reason is that the lake water algae was not acclimatised prior to the test, as lake water inoculum was collected the same day as the set-up. The lake water inoculum was adapted to a phosphorus limited and relatively cold environment, at least when compared to room temperature at the laboratory. In addition, the algae were exposed to a natural dark/light regime. The lake water algae had to adapt to several simultaneously stressors resulting in initial decline in population and long lag phase. Moreover, low initial abundance of lake water algae, compared to PG-I, provided an additional challenge.

As shown in Figure 4.9, which show growth curves of lake water algae, the highest standard errors are found in test 5 and 6. High standard errors reflect uneven growth response in the wells and happened probably because of a poorly adapted algae population. Tests 5 and 6 were conducted in January and February. Winter temperature, less light and probably less nutrients (due to winter stagnation) would provide low initial algae population in the inoculum. Lake water algae was never counted directly with microscope. The calculated adjusted cell concentration, estimated from the calibration curve, showed an initial cell concentration of approximately 50,000 cells/mL (test 5) and 32,000 cells/mL (test 6). The highest initial lake water cell concentration measured was in November

(72,000 cells/mL) and the lowest initial lake water cell concentration measured was in late April (18,000 cells/mL). Compared to this, the initial cell concentration in tests 5 and 6 were in the middle range. Therefore, probably more significant, the great difference between the natural (winter) conditions and the growth condition at the laboratory provided an extra stressful adaptation period and hence a lower growth potential. The greater difference between the natural growth condition and test conditions during winter was also reflected in the long lag-phases (approximately 100 hours for inlet and outlet in test 5 and outlet in test 6).

Both or one of the positive controls struggled to grow in test 1-4 and 6 (Figure 4.8). Growth was observed in test wells in the corresponding tests. As mentioned earlier, the test wells contained algae and other microorganisms from test water. The positive controls did not. The bacterial interaction probably enhanced growth potential and that is why growth was observed in test wells. Additionally, most likely a fraction of the observed growth derived from the growth of algae from the test water. Exponential growth was observed in outlet positive control at the end of test 6. At the last data point, a minimal growth was observed for outlet positive control. This indicated a growth potential, but the growth response was delayed compared to test wells.

In test 5 and 7-10, growth was observed in both inlet and outlet positive controls. As shown in Figure 4.8, the highest standard errors for the positive controls are found in test 5-7. Test 5-7 were conducted in January, February and March. Only three parallels, low algae density and stressful growth conditions increased the probability of uneven growth potential and growth response for the positive controls, and thereby an increase in standard errors.

Minimal growth was observed at the end of test in inlet blanks in test 2 and 6. The growth might be caused by contamination of POS-P (0.9 mg P/L) during pipetting when the microplates were made. POS-P was used a P-source for positive controls in the bioassays.

The growth curves in tests 1-4, presented in Figure 4.9, showed a stationary phase with only one data point, followed by a drastic decrease in cell concentration. This pattern was also observed for the corresponding tests conducted with pre-grown algae. The significant change of the algae population was probably caused by a top-down control, as previously discussed in subsection 5.2.1 .

The same challenges appeared for lake water algae when comparing net growth with the corresponding phosphorus concentration, or when comparing cell concentration retention with phosphorus retention. To give an example, the high TP and phosphate concentration in test 4 (Figure 4.2) should provide an expected high growth response for lake water algae. As for pre-grown algae, the estimated net growth for lake water algae in test 4 was the lowest for all tests, both for inlet and outlet (Figure 4.10). The similar inconsistency when comparing algae and phosphorus retention was

also found for lake water inoculum. As for pre-grown algae, just a few of the tests conducted with lake water algae showed expected and desired results and the decision was made to only compare the bioassays conducted in test 7, 8 and 10, with the corresponding phosphorus and TSS concentration (Figure 4.2). For these tests no growth was measured in blanks, the greatest growth response was measured in positive controls and a stationary phase was reached in these tests. The same applies for test 5, but since phosphate analysis in test 5 was incorrect, test 5 was excluded for comparison.

5.2.3 Calibration and cross-validation

The microplate reader method was calibrated using flow cytometry and direct counting. Direct counting is a simple low-cost method commonly used for cell quantification. Despite its simplicity the method is time-consuming and subjectivity of the analyst will influence the results. A more accurate and precise cell counting is achieved by flow cytometric enumeration. More cells can be analysed increasing the statistical significance of the data. As presented in Table 4.1, the standard errors are higher for direct counting compared to flow cytometry, indicating that the mean value calculated from microscopy was less reliable as a representative number of the data set. However, the flow cytometry instruments are expensive compared to equipment required for direct counting with microscope. In addition, a proper cleaning and control of the instrument are central to avoid signals from non-target particles as detritus and contamination.

The calibration factors were estimated to 199.04 and 198.7 from flow cytometric enumeration and microscopy, respectively (Figure 4.12). The coefficient of determination of both calibrations (R^2 = 0.9986 for flow cytometry and R^2 = 0.9995 for direct counting) indicated a reliable relationship between fluorescence signal and cell concentration. Since microscopy was used to count cell concentration of pre-grown algae prior testing, the calibration factor from direct counting (198.7) was used for the conversion of fluorescence detection signal to algae cell concentration.

To cross-validate the microplate reader method statistical analysis were performed. The linear relationship between flow cytometric enumeration and direct cell quantification estimated a R^2 value of 0.9998 (Figure 4.13), which indicated a significant correlation between the two quantification methods. Moreover, the t-test with 95 % confidence level confirmed no significant difference between flow cytometric enumeration and direct counting (Table 4.2). The results from the cross-validation indicated that microplate measurement is a suitable method for determination of algae cell concentration in a water sample.

5.2.4 Methodology

The algae bioassay was conducted with inoculum collected from Lake Store Stokkavannet. Using inoculum from the constructed wetland's recipient provides some advantages. Firstly, the inoculum represents the actual algae growth potential of the recipient. The algae from Store Stokkavannet have an interaction with algae and other microorganisms in the inoculum and test water. Secondly, the inoculum is easily available. The downside is the uncertainty of the type of algae present in the inoculum. Different types of algae demand a slightly different nutrient media. In addition, the uncertainty of type of algae provides a question mark on the growth phase of the algae. Thirdly, the algae in Store Stokkavannet are probably selected for phosphorus limited growth. The cultivation of pre-grown algae in phosphorus enriched environment changes the premisses.

A general trend for all tests was the high standard error, especially when the algae were in the exponential phase and stationary phase. High standard error reflects uneven growth response. Algae growth is the process of increasing in cell size and number, with an emphasize on *process*. Growth is a complex process, and it is difficult to micromanage the processes going on in each well. The population in one well might be in exponential phase, while the population in another well is still in lag-phase. The populations are not in sync, hence high standard error.

Some wells were measured «over», meaning that the measured cell concentration was beyond the upper detection limit. These were categorized as outliers and were excluded from the calculations of average cell concentration. A great proportion of outliers were excluded in test 6 conducted with pre-grown algae, due to a too high initial cell concentration, which should be avoided. Great deviations between wells were not defined as outliers. The reason was that it is difficult to know if the deviations was caused by the algae's growth potential, random errors or other causes.

In determination of BAP, it is crucial that phosphorus is the only limiting factor during the experiment. If other factors limit the growth, you cannot be sure if growth declines due to phosphorus limitation or for example CO_2 limitation. A weakness of the methodology is that the algae did not get continuously supply of CO_2. The growth curves from the test supplied with CO_2 (Figure 4.14) demonstrated, both for pre-grown algae and lake water algae, smoother growth curves and a higher estimated net growth compared to test 10, which was not supplied with CO_2. The decrease in cell concentration for pre-grown algae in test 10, versus no decrease in cell concentration in the test supplied with CO_2, indicated that CO_2 might be a limiting factor. The greater fluctuation in cell concentration for lake water algae in test 10 compared to the test supplied with CO_2, also indicated that CO_2 might be a limiting factor. For pre-grown algae supplied with CO_2, approximately 55 % more cells were measured compared to test 10. For lake water algae supplied with CO_2,

approximately 40 % more cells were measured compared to test 10. The only difference in growth conditions for the two tests was the supply of CO_2. This also indicated that CO_2 might be a limiting factor during experiments. The results are consistent with theory, which states that most algae obtain their carbon from CO_2. To prevent CO_2 limitation, the microplates should be placed in a CO_2 incubator.

A potentially top-down control was observed for tests 1-4 for both pre-grown algae and lake water algae. Again, when testing for BAP, the growth should be limited by phosphorus, nothing else. The test water could be examined with a microscope prior experiment to rule out if growth can be limited by grazers.

When estimating the algae's growth potential, it is optimal with several, not fluctuating, points at the end of lag phase and at stationary phase. Due to few data points or fluctuating data points, it was sometimes challenging to estimate the net growth.

The methodology has the potential to better mimic the algae's natural conditions and thereby provide more accurate results. The constant illumination and room temperature during testing are not natural for the lake water algae. Hence, a natural dark/light cycle should be installed on the illumination source. During testing, vapor was observed on the covers of the microplates. It is possible that the styrofoam plate did not isolate the heat properly from the shaker table. Fluorescent lamp was the illumination source during the experiment. LED should replace the fluorescent lamp, since LED give off less heat.

When the growth conditions at the laboratory better mimic the natural growth conditions, the LW-I will more quickly adapt to the new environment at the lab. As a result, a shorter lag phase can be expected. This further decreases the time it takes before the algae reaches stationary phase. At stationary phase it is assumed that phosphorus is the limiting factor (given that the methodology is optimally designed for BAP determination). The difference between the highest cell concentration and the lowest cell concentration measured in each test represent the algae growth potential, aka the BAP. An accurate estimation of BAP is dependent on the certainty that stationary phase is reached. Optimal, the tests should show some data points with decreasing cell concentration after the stationary phase, to be sure that stationary phase is actual reached. In this experiment, it often took too much time before the LW-I reached stationary phase. And if they did show a plateau after exponential growth phase, in only a few tests a decrease in cell concentration was observed. This leaves a higher degree of uncertainty when estimating BAP. More quickly adapted LW-I gives shorter test runs and less uncertainty in the estimated BAP.

5.3 Estimation of bioavailable phosphorus

The main objective of this thesis was to examine if the CW could retain the incoming bioavailable phosphorus. Unfortunately, due to methodological challenges, only about a third of the tests conducted were applied in the analysis of bioavailable phosphorus. The analysis was based upon tests 8-10 conducted with pre-grown algae, and tests 7, 8 and 10 conducted with lake water algae. These tests provided expected and desired results. For the tests conducted with pre-grown algae no growth was measured in blanks, while the greatest growth response was measured in positive controls. Also, a long stationary phase was obtained in these tests. For the tests conducted with lake water algae the tests showed no growth in blanks, the greatest growth response was measured in positive controls and a stationary phase was reached.

The fraction of bioavailable phosphorus in a water sample can be estimated by the growth potential of algae. Net growth, which reflect the algae's growth potential, was calculated as the difference between average cell concentration in stationary phase and average cell concentration at the end of the lag-phase.

The results from test 8-10 performed with pre-grown algae (Figure 5.1) show that the highest inlet net growth was measured in test 8, with 1504269 ± 451373 cells/mL. The result correlated well with phosphorus concentrations for test 8-10 (Figure 4.2), since both inlet TP and phosphate concentrations were highest in test 8.

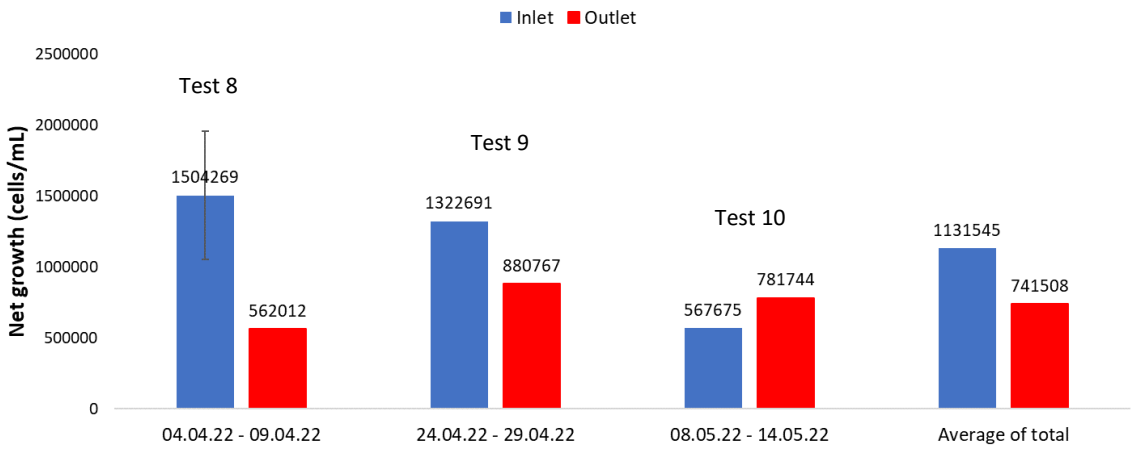

Figure 5.1: Estimated average net growth presented for tests 8-10 performed with PG-I. Blue bars represent net growth of inlet pre grown algae. Net growth of outlet pre grown algae is represented by the red bars. The date of each test run is shown on the horizontal axis. Standard error is represented by the error bars.

The lowest inlet net growth was measured in test 10, with 567675 cells/mL (Figure 5.1). With reference to phosphorus analysis, this also correlated well. Phosphorus analysis showed that

phosphate inlet concentration was lower in test 10 (2.1 ± 0.3 µgP/L) compared to test 9 (9.6 ± 0.3 µgP/L). Moreover, TP inlet concentrations were approximately equal in test 9 and 10.

Since the highest outlet net growth was measured in test 9 (880767 cells/mL) the outlet phosphorus concentration should be highest in test 9. However, the highest outlet phosphorus concentrations, for both TP and phosphate, was measured in test 8 (Figure 4.2). The difference between outlet net growth in tests 9 and 10 are relatively small (20% difference), which is reflected in the minimal difference in outlet phosphate concentration (test 9: 12.9 ± 0.8 µgP/L and test 10: 11.0 ± 0.3 µgP/L).

As shown in Figure 4.15, significant positive correlations were found between net growth and TP concentration (R^2 values of 0.93 and 0.71), and net growth and phosphate concentration (R^2 values of 0.78 and 0.81). According to theory, which states that phosphate is readily bioavailable and only a fraction of TP is potentially bioavailable, the highest correlation should be between phosphate and net growth. However, the most significant correlation was between inlet TP concentration and net growth (R^2 = 0.93). In addition, the R^2 values from the TP versus net growth and phosphate versus net growth were relatively similar. The correlation between TSS and net growth provided R^2 values of 0.88 (inlet) and 0.58 (outlet). The R^2 value of 0.88 is higher than R^2 values from the phosphate correlation plot. This was not expected since only a fraction of TSS is potentially bioavailable. The R^2 value of 0.58 indicated a moderate positive correlation between TSS and net growth, and this moderate correlation is more consistent with theory. The results from the correlation plots demonstrated that the methodology might not be optimal.

The average inlet and outlet net growth in test 8-10 conducted with pre-grown algae were estimated to 1131545 cells/mL and 741508 cells/mL, respectively (Figure 5.1). The result demonstrated a retention of bioavailable phosphorus, since the inlet consisted of 34 % more algae. Phosphorus analysis performed in test 8-10 showed an average phosphate retention of 1 ± 3.6 % and an average TP retention of 34 ± 2 %. A phosphate retention of 1 % and an algae retention of 34 % indicated that the algae utilize other forms of phosphorus than phosphate. The statement is further supported by the positive TP retention. The results are consistent with theory, which states that a fraction of DOP and fraction of PP is potentially bioavailable.

The results from test 7, 8 and 10 performed with lake wate algae (Figure 5.2) showed a significantly higher net growth in test 8 compared to test 7 and 10. The result corresponded well with phosphorus analysis performed on the corresponding water sample, since the highest TP and phosphate concentrations were measured in test 8 (Figure 4.2).

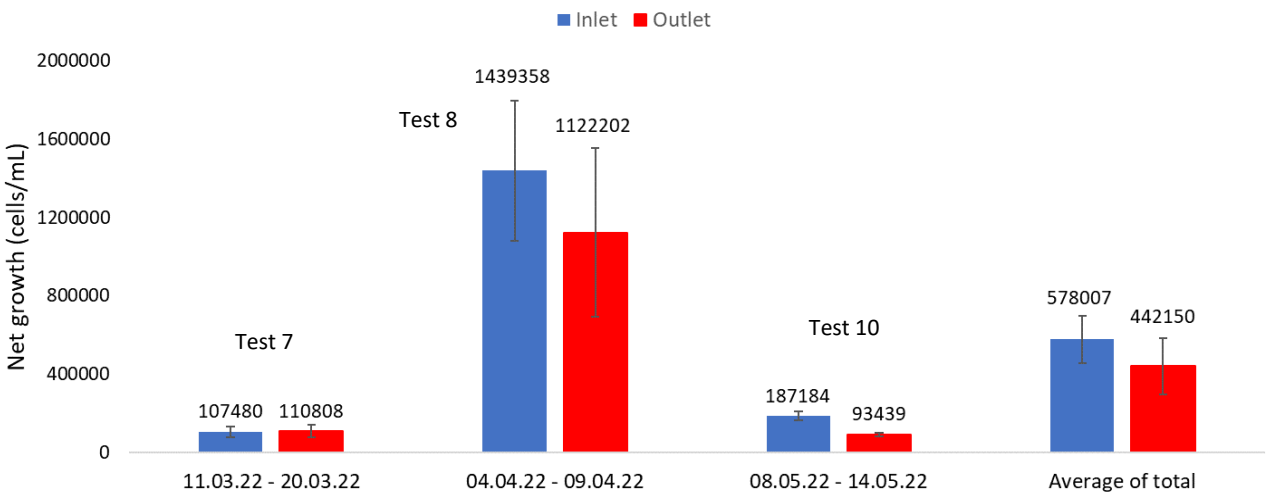

Figure 5.2: Estimated average net growth presented for tests 7, 8 and 10 performed with LW-I. Blue bars represent net growth of inlet pre grown algae. Net growth of outlet pre grown algae is represented by the red bars. The date of each test run is shown on the horizontal axis. Standard error is represented by the error bars.

For test 7 and 10, the highest inlet net growth was measured in test 10. This is consistent with TP analysis, but not with phosphate analysis on the corresponding water samples. The highest outlet net growth for tests 7 and 10 were measured in test 7. This was not consistent with either TP or phosphate analysis conducted on the same water samples. According to phosphorus analysis performed on water samples in test 7 and 10, the highest outlet net growth should be measured in test 10 (Figure 4.2).

Significant positive correlations were also found between lake water net growth and TP concentration (R^2 values of 0.77 and 0.82), and lake water net growth and phosphate concentration (R^2 values of 0.95 and 0.82) (Figure 4.16). Compared to pre-grown algae, a greater correlation between net growth and phosphate was found, and a lower correlation between net growth and TP was found for lake water algae. The degree of correlation between these parameters is more consistent with the theory. Also, a more moderate correlation between TSS and lake water net growth (R^2 values of 0.56 and 0.42) was found for lake water algae compared to pre-grown algae. The correlations found between lake water net growth, phosphorus and TSS better suits the theory and may indicate that the algae bioassays conducted with lake water were better at estimating bioavailable phosphorus.

The average inlet and outlet net growth in test 7, 8 and 10 conducted with lake water algae were estimated to 578007 ± 119926 cells/mL and 442150 ± 143803 cells/mL, respectively (the two bars to the right in Figure 5.2). This means the CW retained, on average, 24 % of the bioavailable phosphorus in the water samples collected in test 7, 8 and 10. Phosphorus analysis performed on the

corresponding tests showed an average phosphate retention of 14 ± 3 % and an average TP retention of 26 ± 2 %. The results show (1) a positive TP retention and (2) a lower phosphate retention compared to the retention of algae. This indicated, once again, that algae utilized other forms of phosphorus in addition to phosphate.

5.4 Recommendations and further research

With reference to the discussion in subsection 5.2.4 a summary of recommendations for the methodology are listed below:

- The microplates should be placed in a CO_2 incubator during experiment to prevent CO_2 limitation.
- The test water should be examined with a microscope for determination of presence of grazers. A top-down control, caused by grazers, will affect the growth response of algae.
- The illumination source should be provided by LED tubes. LED gives off less heat compared to fluorescent tubes. In addition, LED provide a more optimal spectrum of light.
- A dark/light cycle can be applied on the illumination source.
- Conducting the experiment with two parallels of microplates provides a doubling of parallels of each test parameter. This might be preferable regarding the high standard errors.
- As discussed in subsection 5.2.1 , the pre-grown algae should be washed prior to the experiment to prevent growth in blanks caused by P-residuals from inoculum.

The tests were performed from late September to middle of May. To get a better idea of the annual change of bioavailable phosphorus, the experiment should be conducted over a year. In addition, a comparison should be made between Leikvollbekken CW and another constructed wetland to analyse any difference in performance. The bioassays in this thesis were conducted with unfiltered test water. To further examine the speciation of bioavailable phosphorus, the bioassays can also be performed with filtered water and compared with bioassays performed with unfiltered water.

6. CONCLUSION

The hypotheses in this study were that (1) the CW can retain bioavailable phosphorus (BAP) and (2) microplate reader method can be used as bioassay to determine bioavailable phosphorus.

Due to methodological challenges, only three of ten bioassays were applied in the estimation of BAP. The results from test 8, 9 and 10 conducted with pre-grown algae demonstrated that the CW retained, on average, 34 % of the incoming BAP. Phosphorus analysis performed on the corresponding water samples showed an average phosphate and TP retention of 1 ± 4 % and 34 ± 2 %, respectively. Based on test 7, 8 and 10 conducted with lake water algae the CW retained, on average, 24 % of the incoming BAP. Phosphorus analysis performed on the corresponding water samples showed an average phosphate and TP retention of 14 ± 3 % and 26 ± 2 %, respectively. A lower phosphate retention, compared to algae retention, indicated the algae utilised other forms of phosphorus in addition to phosphate. The results were consistent with theory, which states that a fraction of DOP and fraction of PP is potentially bioavailable.

Results from the calibration and cross-validation of microplate reader method indicated that microplate measurements can be used to determine algae cell concentrations in water sample. The calibration, using flow cytometric enumeration and direct counting with microscope, indicated a significant positive relation between fluorescence signal and algae cell concentration (R^2 = 0.9986 for flow cytometry, R^2 = 0.9995 for direct counting). The statistical analysis from the cross-validation of the microplate reader method indicated a significant positive correlation between the two quantification methods (R^2 = 0.9998). No significant difference between the two quantifications method was confirmed by a t-test with 95 % confidence level.

The hypotheses were confirmed since the following conclusions can be made:

- Leikvollbekken constructed wetland can retain bioavailable phosphorus.
- Microplate reader method can be used as bioassay to determine bioavailable phosphorus in water samples.

Several methodological weaknesses were identified. It is crucial that phosphorus is the only limiting factor in determination of BAP. The bioassay supplied with CO_2 measured approximately 55 % (pre-grown algae) and 40 % (lake water algae) more cells compared to the bioassay not supplied with CO_2. Moreover, the correlation plots of net growth and phosphorus concentrations did not indicate the strongest positive correlation between net growth and phosphate. To improve the determination of BAP, further development and optimisation of the method should be provided.

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APPENDIX

Appendix A: Total suspended solids and phosphorus concentrations in water samples and TP/TSS correlation plot

The measured total phosphorous, phosphate and total suspended solids concentrations with standard error are given in table A.1.

Table A.1: Concentration of TP, phosphate and TSS in water samples.

Test	Date	TP (µgP/L)					Phosphate (µgP/L)					TSS (mg/L)				
		n	In	SE	Out	SE	n	In	SE	Out	SE	n	In	SE	Out	SE
1	27.09.21	2	74	12	26.4	0.8	2	56	3	11	3	1	17	-	6	-
2	11.10.21	2	55	15	39	11	2	17	2	20	2	1	6	-	6	-
3	01.11.21	2	102	14	71	10	2	34	1	10	1	1	19	-	5	-
4	03.12.21	2	157	20	97	31	2	57	6	26	3	1	15	-	6	-
5	14.01.22	3	53.7	0.7	54	2	3	114	6	75.6	0.5	2	7	1	9	1
6	10.02.22	3	57	1	39.4	0.8	3	22	2	16.6	0.4	2	7.1	0.6	6	3
7	11.03.22	3	39	1	26.0	0.5	3	10.4	0.3	12.7	0.2	2	9	2	9.159	0.002
8	05.04.22	3	106	1	86	1	3	34	1	21.0	0.4	2	9.8	0.7	6.6	0.7
9	24.04.22	3	69	5	32.9	0.4	3	9.6	0.3	12.9	0.8	2	4.7	0.1	1.02	0.05
10	08.05.22	3	70	4	46	2	3	2.1	0.3	11.0	0.3	2	6.2	0.2	3.9	0.7

The correlation plot between TP concentration and TSS concentration is given in figure A.1. The R^2 value from the plot (R^2 = 0.87 for inlet, R^2 = 0.74 for outlet) were used to indicate the relationship between TP concentration and TSS concentration measured in water samples throughout the thesis period.

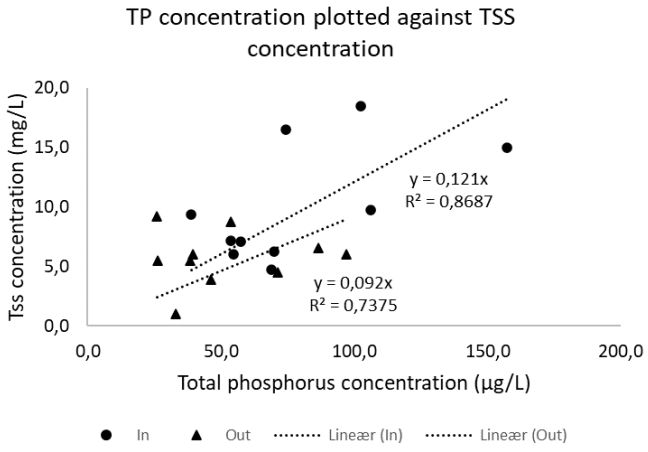

Figure A.1: Calibration curve for phosphorus analysis.

Appendix B: Calibration curve for phosphorus analysis

The calibration curve obtained from standard phosphorus solutions of 1, 10, 20.2, 100.4, 201 and 501 µgP/L is given in figure B.1. The calibration factor obtained from the curve (1541.5) was used to calculate unknown TP and phosphate concentrations in water samples throughout the thesis period.

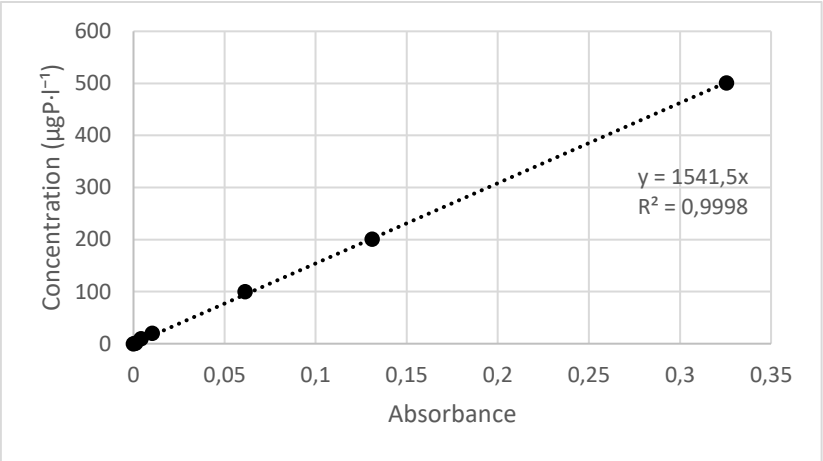

Figure B.1: Calibration curve for phosphorus analysis.

Appendix C: Average cell concentrations for test water, blank and positive controls from algae bioassay

Tables C.1 – C.20 show the average cell concentrations for test water, blank and positive control from the algae bioassays. Standard error and number of replicates (n) are also shown in the tables. The results from pre-grown algae (PG-I) and lake water algae (LW-I) are shown in the same table. Results from inlet and outlet in the same test are separated in two tables.

Table C.1: Test 1 – Inlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
27.09.21	0	5	109155	3766	3	71723	1606	2	46292	1391	3	14967	239	3	106426	9740	3	67419	7501
28.09.21	18,50	5	70293	1450	3	41325	526	2	21755	894	3	10331	866	3	38875	4120	3	29074	4041
	21,50	5	72637	1924	3	39272	265	2	25232	1589	3	9934	199	3	56491	3434	3	31524	4671
	25,00	5	93737	4065	3	39339	89	2	30299	894	3	9868	434	3	64836	701	3	30067	3756
29.09.21	43,00	5	898113	49727	3	35696	239	2	621372	4669	3	8676	289	3	759090	29918	3	30597	3009
	45,58	5	1606646	78856	3	36623	989	2	950982	26524	3	8543	500	3	1249101	5346	3	22782	2718
	47,92	5	2381736	117203	3	38080	2530	2	1553082	9338	3	8609	289	3	1757656	10566	3	25563	2887
30.09.21	70,17	5	6530095	349831	3	594302	215136	2	5644399	130994	3	8278	350	3	5687877	162660	3	31193	3212
	74,25	5	9535567	497264	3	647035	203955	2	5252106	483786	3	8411	289	3	5575292	225826	3	13113	4868
01.10.21	94,17	5	9677146	222997	3	189541	30832	2	4635204	1575334	3	8013	403	3	6044773	705995	3	22517	2868

Table C.2: Test 1 – Outlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
27.09.21	0	6	102519	3381	6	65167	397	3	52716	9350	3	14967	132	3	101062	8912	3	60134	3951
28.09.21	18,50	6	67584	5809	6	35961	286	3	27683	3506	3	9934	229	3	54902	5213	3	22517	2868
	21,50	6	71723	6283	6	35067	280	3	32054	4580	3	9934	115	3	74373	42319	3	12583	701
	25,00	6	104638	15046	6	33345	214	3	46028	7198	3	9603	66	3	96625	3108	3	20464	1781
29.09.21	43,00	6	1025387	91523	6	31358	188	3	932869	33709	3	9139	229	3	955916	66493	3	17881	8742
	45,58	6	1262346	40131	6	30497	152	3	1142079	28384	3	9139	303	3	1173073	69657	3	29206	8261
	47,92	6	1738781	53736	6	30862	233	3	1367647	72534	3	9007	239	3	1485729	13213	3	9404	350
30.09.21	70,17	6	1949912	30788	6	30332	2051	3	1621427	34201	3	8676	175	3	1689773	104784	3	104784	1205
	74,25	6	1876731	55009	6	32882	4806	3	1585334	4149	3	8345	344	3	1643216	57882	3	32879	2606
01.10.21	94,17	6	1855406	56965	6	32882	4806	3	1585334	4149	3	8345	344	3	1648382	37522	3	23643	2215

Table C.3: Test 2 – Inlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
11.10.21	0	6	79671	528	6	70101	810	3	15828	132	3	13047	239	3	50730	1177	3	47286	1732
	2,75	6	63909	570	6	60597	747	3	13908	303	3	11921	199	3	46094	2452	3	29007	1352
12.10.21	23,25	6	47716	157	6	45564	406	3	10530	115	3	9537	115	3	25299	2517	3	17616	1434
	25,75	6	46127	357	6	44637	446	3	10861	66	3	9404	175	3	34504	781	3	34173	639
	28,75	6	45034	400	6	43213	294	3	10265	175	3	9338	199	3	10199	132	3	9470	265
13.10.21	46,00	6	42749	385	6	39769	511	3	12583	861	3	8609	66	3	11259	132	3	8742	115
	48,75	6	41425	1448	6	37286	244	3	11126	199	3	8345	344	3	16954	1149	3	13908	3007
	51,75	6	40763	1305	6	37418	279	3	10795	265	3	8477	239	3	15100	1950	3	13775	2363
14.10.21	70,83	6	61690	20863	6	32716	2840	3	11192	672	3	6888	66	3	17881	607	3	12649	1800
	73,42	6	79902	528	6	40729	810	3	15100	1618	3	7749	115	3	28477	1177	3	8212	1732
	76,58	6	124672	66889	6	45431	8084	3	20265	2016	3	8411	239	3	41061	2129	3	13709	2342
15.10.21	93,92	6	1364104	1033048	6	110234	54801	3	151394	31908	3	8013	265	3	168150	5887	3	15033	3014
	96,75	6	1882294	1191633	6	132321	64455	3	277688	52127	3	8013	175	3	312722	28765	3	17683	1352
	100,00	6	2313397	1283937	6	140235	59528	3	483852	40163	3	7881	175	3	560211	28992	3	16557	1941
16.10.21	123,00	6	359710	11043	6	70101	810	3	100466	24154	3	13047	239	3	294378	41606	3	23378	2278
17.10.21	147,00	6	224177	528	6	35398	810	3	32186	596	3	26160	7751	3	220998	1177	3	30862	1732

Table C.4: Test 2 – Outlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
11.10.21	0	6	71326	271	6	62352	522	3	16093	115	3	14437	239	3	45961	2862	3	30928	3707
	2,75	6	56889	369	6	52154	528	3	12914	229	3	12119	115	3	34901	1149	3	30266	1339
12.10.21	23,25	6	42021	168	6	39670	419	3	10596	239	3	10199	66	3	30067	239	3	24040	1209
	25,75	6	40498	140	6	38213	311	3	10133	229	3	9139	115	3	9603	66	3	9934	303
	28,75	6	39835	216	6	37352	286	3	9868	239	3	9272	331	3	9735	115	3	9603	289
13.10.21	46,00	6	40200	1010	6	35100	512	3	12517	574	3	8609	369	3	15166	3474	3	9007	369
	48,75	6	36888	239	6	33875	323	3	11192	239	3	8874	132	3	12649	2021	3	9007	403
	51,75	6	35796	248	6	33511	239	3	10662	239	3	8477	331	3	12186	1660	3	8543	459
14.10.21	70,83	6	40299	1619	6	41359	6826	3	19934	927	3	8609	66	3	23312	5988	3	8609	403
	73,42	6	51756	7339	6	48842	9921	3	23709	589	3	8013	66	3	32120	8195	3	8212	350
	76,58	6	61856	6047	6	58942	15845	3	32186	2967	3	8146	199	3	55167	24381	3	8345	115
15.10.21	93,92	6	695115	173997	6	152553	56916	3	316100	37952	3	8080	175	3	680347	256482	3	8212	369
	96,75	6	1350262	232610	6	167057	59211	3	521469	63263	3	8146	229	3	1074263	481586	3	44107	6656
	100,00	6	2091438	404871	6	183249	62977	3	971082	142310	3	8013	175	3	1572287	588265	3	27285	1782
16.10.21	123,00	6	225535	8969	6	59207	3584	3	100466	24154	3	9470	175	3	294378	41606	3	31921	1515
17.10.21	147,00	6	219674	7397	6	32286	2165	3	74969	20300	3	9470	175	3	163050	8718	3	21126	1359

Table C.5: Test 3 – Inlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
01.11.21	0	6	90144	449	6	95442	628	3	16890	526	3	22784	331	3	70340	3909	3	52457	3380
	3,5	6	65505	697	6	71731	1579	3	13048	239	3	18744	66	3	48284	1323	3	49013	2975
02.11.21	21,75	6	48946	1509	6	45734	746	3	9074	289	3	12849	239	3	33911	3109	3	32322	4505
	25,75	6	49013	383	6	52589	289	3	10664	175	3	13777	132	3	36230	2832	3	30997	5395
03.11.21	45,75	6	43416	483	6	46761	196	3	9604	175	3	13048	738	3	31726	1893	3	31130	2766
	48,75	6	43747	995	6	46562	293	3	9538	397	3	12916	607	3	36031	1752	3	26692	1793
	52,5	6	44376	1658	6	46628	339	3	10001	289	3	13578	566	3	29540	1264	3	26626	1878
04.11.21	70,5	6	598915	35806	6	398592	129442	3	8809	66	3	12717	1698	3	50867	229	3	45900	1732
	74	6	527880	18962	6	160086	8189	3	9273	239	3	12584	976	3	8610	175	3	12121	1000
	76,75	6	1026517	38708	6	82262	27867	3	9273	239	3	12584	962	3	8213	175	3	12187	1264
05.11.21	94	6	442008	45263	6	100575	6904	3	8014	132	3	11856	1528	3	7087	175	3	14836	4703
	97	6	320536	57372	6	93389	3270	3	8743	115	3	12386	1558	3	7749	115	3	18148	7131
	101	6	302289	64242	6	101436	8765	3	8809	66	3	12717	1698	3	8345	199	3	23513	11439

Table C.6: Test 3 – Outlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
01.11.21	0	6	83288	395	6	88090	822	3	16691	414	3	24705	403	3	68485	1320	3	90342	1084
	3,5	6	58948	836	6	64445	403	3	12452	265	3	19473	414	3	47423	2880	3	49609	7488
02.11.21	21,75	6	43714	1370	6	39243	528	3	9273	175	3	13644	350	3	17022	672	3	31792	5223
	25,75	6	43780	302	6	47820	132	3	10001	239	3	13578	66	3	10134	199	3	13975	66
03.11.21	45,75	6	46297	1109	6	42522	295	3	9273	175	3	12717	229	3	25500	2602	3	28613	6638
	48,75	6	802682	20080	6	87097	9664	3	9339	199	3	12849	66	3	27421	1690	3	23314	1628
	52,5	6	815034	18352	6	106867	12730	3	9339	229	3	12849	369	3	48152	1558	3	33249	2525
04.11.21	70,5	6	130678	6288	6	48980	5066	3	8942	199	3	12319	229	3	24308	239	3	18148	1562
	74	6	102562	8131	6	55504	8502	3	8345	0	3	11723	229	3	24374	2696	3	16095	1293
	76,75	6	92031	49534	6	64313	12604	3	8942	229	3	12253	175	3	25831	2929	3	16757	2005
05.11.21	94	6	78652	3880	6	65902	5851	3	8478	132	3	12187	331	3	25235	3343	3	19141	8216
	97	6	66101	4976	6	56961	6027	3	8478	175	3	12121	911	3	20731	781	3	15565	4066
	101	6	64677	2194	6	60140	4188	3	8677	132	3	12651	764	3	17221	1237	3	14306	2712

Table C.7: Test 4 – Inlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
03.12.21	0	6	89018	2888	6	78983	2280	3	26957	672	3	19936	175	3	71466	934	3	61928	5775
	2,7	6	65074	606	6	58981	742	3	21062	304	3	16823	289	3	65505	6434	3	34773	1732
	5	6	61564	405	6	56000	723	3	20201	175	3	16426	289	3	61133	3903	3	36627	2679
05.12.21	28	6	180022	7545	6	93422	8813	3	20201	175	3	16426	289	3	104715	9896	3	102728	4748
06.12.21	46,5	6	79546	16121	6	46132	4275	3	14240	403	3	10200	239	3	26162	3753	3	22718	1035
	49	6	82692	21548	6	45535	4899	3	14174	239	3	10266	403	3	23380	3058	3	21393	781
	53	6	73519	13503	6	47125	6813	3	13313	115	3	10067	350	3	21261	2189	3	19738	846
07.12.21	70	6	54179	3704	6	53152	8007	3	12452	289	3	9670	369	3	21592	1369	3	21725	3233
	73,17	6	54576	4089	6	26692	1757	3	12253	265	3	9670	175	3	22453	716	3	19075	698
	77,17	6	49311	3034	6	34011	4877	3	12319	344	3	9405	434	3	19870	2417	3	15962	1886
08.12.21	93,5	6	29308	3470	6	15201	1125	3	11790	239	3	9273	289	3	18148	3805	3	3805	2103

Table C.8: Test 4 – Outlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
03.12.21	0	6	67757	771	6	61299	1851	3	25831	698	3	20930	66	3	46297	1241	3	34574	1987
	2,7	6	54643	205	6	48748	802	3	20135	175	3	17486	500	3	46628	2493	3	30202	1690
	5	6	52192	428	6	46098	1048	3	18744	350	3	16360	66	3	38945	11436	3	22056	1032
05.12.21	28	6	109153	7747	6	67624	7767	3	18744	350	3	16360	66	3	47556	1648	3	24175	1453
06.12.21	46,5	6	43118	2020	6	36461	2160	3	11260	542	3	10730	199	3	24440	1130	3	22851	2516
	49	6	37356	896	6	34276	1906	3	10929	199	3	10134	199	3	21857	414	3	20466	2106
	53	6	36263	573	6	33779	1828	3	10796	239	3	10597	331	3	20599	403	3	19671	1803
07.12.21	70	6	37919	2347	6	21890	714	3	10465	369	3	10465	764	3	21195	1330	3	16293	2371
	73,17	6	32918	3098	6	22354	1598	3	10067	369	3	10266	738	3	19738	1320	3	15565	1155
	77,17	6	19837	1036	6	18512	1465	3	9935	397	3	10399	1002	3	17353	1035	3	15565	1090
08.12.21	93,5	6	24738	3296	6	16260	762	3	9935	414	3	11193	1778	3	17751	1383	3	14969	2632

Table C.9: Test 5 – Inlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
14.01.22	0	6	57921	234	6	65405	1210	6	13909	304	3	14969	1703	3	48085	6557	3	38747	8304
15.01.22	22	6	33183	487	6	34673	510	6	10134	115	3	12386	66	3	10399	66	3	12319	115
16.01.22	46	6	28812	574	6	29374	301	6	9074	132	3	10929	115	3	8677	175	3	10664	132
17.01.22	67	6	44178	1885	6	26593	542	6	8677	66	3	10266	239	3	8279	175	3	10001	66
	70	6	44840	5367	6	28646	195	6	9405	66	3	10730	199	3	8942	115	3	10465	175
	74	6	38184	3348	6	28845	188	6	9405	175	3	10465	66	3	8478	66	3	9935	115
18.01.22	91	6	29573	1766	6	22586	494	6	7683	239	3	9008	175	3	7948	0	3	9405	175
	95	6	29076	1749	6	26758	284	6	8610	132	3	9736	115	3	8412	175	3	9670	331
	98	6	28116	1252	6	27156	270	6	9008	369	3	10134	344	3	8544	115	3	9736	115
19.01.22	115	6	25434	651	6	23115	1712	6	7286	175	3	8942	229	3	7749	304	3	8942	500
	119	6	27056	1164	6	29010	2843	6	8478	239	3	9538	397	3	8345	115	3	9803	764
	124	6	28381	2525	6	32819	5310	6	8478	66	3	9405	265	3	7948	115	3	10796	1756
20.01.22	139	6	56133	22740	6	62160	24777	6	8279	239	3	8942	115	3	7882	239	3	13512	4868
	142	6	75109	36514	6	70870	30066	6	8412	239	3	9273	403	3	7882	66	3	29010	20269
	146	6	107298	59315	6	85607	37580	6	8478	66	3	9273	132	3	7617	132	3	35567	26528
21.01.22	163	6	330339	133393	6	78288	18900	6	28547	1434	3	26758	927	3	10266	175	3	12054	672
	166	6	1416201	825152	6	172637	71423	6	7882	175	3	8809	239	3	7219	331	3	220425	200249
	170	6	1829596	1054193	6	180983	77343	6	8345	0	3	9471	239	3	7219	66	3	337591	315515
22.01.22	191	6	1601091	636401	6	195918	87990	6	8014	331	3	9008	331	3	7087	66	3	171213	49686
	196	6	1666762	975079	6	222908	93116	6	8014	239	3	8544	526	3	7021	66	3	282022	112374
23.01.22	215	6	1493727	394097	6	229399	104649	6	8147	397	3	8809	403	3	7484	369	3	1156699	448117
	220	6	1947359	570611	6	239400	104989	6	7816	239	3	8610	403	3	7219	289	3	649683	415980
24.01.22	234	6	169094	11226	6	114186	25305	6	11790	239	3	9273	289	3	14571	2627	3	125049	17736
	240	6	133526	13464	6	105675	20482	6	11790	239	3	9273	289	3	12121	1846	3	77294	15698

Table C.10: Test 5 – Outlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
14.01.22	0	6	55470	437	6	64346	1257	3	14770	289	3	14903	639	3	48417	6332	3	41661	2027
15.01.22	22	6	32885	613	6	34905	315	3	10929	304	3	13644	369	3	10862	265	3	14108	115
16.01.22	46	6	27950	934	6	29341	339	3	10134	229	3	12518	229	3	9736	199	3	12187	132
17.01.22	67	6	53252	10560	6	26526	358	3	9604	239	3	11326	199	3	9140	115	3	10862	132
	70	6	57292	16334	6	27255	288	3	9140	199	3	11127	344	3	8875	66	3	10664	132
	74	6	49311	13751	6	27255	443	3	9140	229	3	11061	265	3	8610	66	3	10664	175
18.01.22	91	6	34408	6260	6	24871	1056	3	8677	66	3	10796	289	3	8412	132	3	10399	66
	95	6	32223	5803	6	28878	2074	3	8942	229	3	10399	403	3	8014	239	3	10266	175
	98	6	28944	3398	6	28613	2355	3	8743	115	3	10200	289	3	7948	115	3	10332	344
19.01.22	115	6	24308	469	6	41495	12516	3	8412	132	3	10200	464	3	7882	132	3	13247	3115
	119	6	24275	501	6	58782	20477	3	8677	66	3	9935	414	3	8014	289	3	14373	4638
	124	6	23480	350	6	79480	35370	3	8544	304	3	9935	229	3	7551	304	3	19208	9188
20.01.22	139	6	22453	363	6	275531	161334	3	8279	175	3	9803	464	3	7816	66	3	55172	42045
	142	6	22751	260	6	307985	179260	3	8279	132	3	9869	350	3	7352	0	3	74579	60448
	146	6	22056	251	6	383359	213397	3	7882	239	3	9604	369	3	7352	115	3	93455	76901
21.01.22	163	6	21327	480	6	544504	290508	3	8080	132	3	10862	652	3	7153	199	3	412303	282342
	166	6	21095	421	6	655147	361674	3	8080	175	3	9935	752	3	6756	115	3	572124	381046
	170	6	21029	264	6	693198	373508	3	7948	115	3	9736	414	3	6756	0	3	883685	597169
22.01.22	191	6	22056	1439	6	220888	107078	3	8147	304	3	11326	911	3	6888	66	3	764002	436336
	196	6	25566	5711	6	159192	78841	3	8014	175	3	9935	716	3	6623	66	3	298911	197444
23.01.22	215	6	80606	61414	6	145084	71829	3	7948	115	3	11193	781	3	6557	115	3	286724	183066
	220	6	164259	145025	6	194958	95847	3	7749	115	3	13180	1858	3	6623	239	3	279902	177575
24.01.22	234	6	169094	11226	6	114186	25305	3	11790	239	3	9273	289	3	11193	2413	3	125049	17736
	240	6	133526	13464	6	105675	20482	3	11790	239	3	9273	289	3	15035	1227	3	37289	13247

Table C.11: Test 6 – Inlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
11.02.22	0	3	276193	5791	6	54278	710	5	229995	7253	3	21460	1198	3	235923	7044	3	23115	2014
12.02.22	22,17	3	285929	11661	6	27851	1501	5	299242	12717	3	10067	632	3	399519	68187	3	12452	265
13.02.22	46,00	3	803145	73334	6	24076	509	5	966974	86136	3	10001	239	3	1960109	502347	3	10796	175
14.02.22	66,50	3	2833992	961840	6	23976	958	5	2249185	495061	3	10134	344	3	6560809	1306957	3	9736	199
	69,67	3	3102634	1083837	6	29706	1288	5	2243820	433067	3	10332	199	3	6115589	1141997	3	10664	265
	74,50	3	5931394	63995	6	30070	1672	5	5699809	1059657	3	10664	175	3	7196450	1660959	3	10399	369
15.02.22	91,25	3	3797289	533736	6	34011	5057	5	3402340	731415	3	9405	289	3	4395112	1274149	3	9074	66
	93,83	3	2791006	665733	6	40899	7844	5	2655427	291493	3	10465	175	3	4135013	1046594	3	9803	66
	97,33	3	1888312	651817	6	50900	15435	5	2683841	83057	3	10067	66	3	4116203	890591	3	9538	115
16.02.22	114,33	3	3900547	1232840	6	132897	79476	5	2874096	23943	3	10200	239	3	4935443	1437740	3	9604	239
	117,75	3	3322661	1261047	6	145150	83129	5	3360712	948296	3	10001	239	3	5513726	1168820	3	9471	66
	121,50	3	3160456	170491	6	133162	50548	5	1247538	119121	3	9803	175	3	2148808	320343	3	9471	132
17.02.22	138,92	3	3011895	666614	6	272186	89439	5	908158	98853	3	9736	229	3	1249889	324525	3	8147	344
	142,25	3	1558735	544766	6	421178	109156	5	508672	53450	3	10134	607	3	852489	197796	3	8544	229
	145,17	3	761948	40607	6	228273	60425	5	348719	80076	3	10664	542	3	536291	33952	3	8743	199
18.02.22	162,75	3	448002	26378	6	236056	55057	5	270133	146144	3	10332	526	3	514103	118905	3	8544	115
	165,92	3	516223	183256	6	373291	68118	5	249567	138693	3	10465	464	3	473105	93719	3	8080	66
	169,00	3	234135	43620	6	306362	49555	5	155582	72724	3	10067	566	3	333154	54386	3	8279	66
19.02.22	190,75	3	77096	13106	6	92561	23443	5	177141	112762	3	33183	4997	3	283015	19544	3	28613	7709

Table C.12: Test 6 – Outlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
11.02.22	0	5	263278	-	3	55172	1134	1	208834	-	3	23049	980	3	232015	6927	3	20002	239
12.02.22	22,17	5	414091	-	3	28911	812	1	462971	-	3	11525	459	3	1500980	858699	3	13379	265
13.02.22	46,00	5	449658	-	3	26096	640	1	2968975	-	3	11326	115	3	4177601	1290163	3	11657	66
14.02.22	66,50	5	2789748	-	3	22188	884	1	4105937	-	3	9935	115	3	7321499	870838	3	11061	175
	69,67	5	3048853	-	3	31163	3242	1	3921941	-	3	10399	239	3	6221827	819256	3	10465	239
	74,50	5	5315026	-	3	31726	3992	1	4251584	-	3	11193	66	3	6869456	1908522	3	10730	229
15.02.22	91,25	5	3604418	-	3	25599	2007	1	975418	-	3	11260	175	3	3914787	1917223	3	11127	795
	93,83	5	2788158	-	3	35965	8705	1	835931	-	3	10664	66	3	2498785	1248685	3	10796	891
	97,33	5	3291863	-	3	33978	7200	1	1039201	-	3	10465	132	3	2574423	1157390	3	10664	738
16.02.22	114,33	5	3064749	-	3	28712	3873	1	1178092	-	3	10730	304	3	2948311	1240379	3	14439	4708
	117,75	5	3292658	-	3	35932	5009	1	2527861	-	3	10664	66	3	3174100	1145082	3	17817	7488
	121,50	5	1155838	-	3	47489	11844	1	736978	-	3	10266	66	3	991248	175044	3	18347	8613
17.02.22	138,92	5	695847	-	3	55272	13990	1	724063	-	3	10200	66	3	1093380	94580	3	110014	84434
	142,25	5	674984	-	3	182903	70995	1	558546	-	3	10465	66	3	881433	94835	3	119551	103985
	145,17	5	593914	-	3	46695	10028	1	337989	-	3	10001	175	3	562917	136417	3	236784	192062
18.02.22	162,75	5	809305	-	3	47291	11611	1	124386	-	3	8809	691	3	754596	247301	3	811358	674348
	165,92	5	598087	-	3	66929	16235	1	154589	-	3	10067	175	3	624382	192775	3	1482964	1279092
	169,00	5	413097	-	3	65505	15178	1	138891	-	3	9869	66	3	497412	148790	3	1965540	1589628
19.02.22	190,75	5	39541	-	3	15300	543	1	20466	-	3	14373	1084	3	34971	4877	3	29341	8128

Table C.13: Test 7 – Inlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
11.03.22	0	6	83586	1127	6	62756	1477	3	58153	517	3	35832	2737	3	54841	414	3	35302	3254
12.03.22	22,00	6	112232	3232	6	26493	499	3	34839	1028	3	13777	464	3	68618	4234	3	14041	403
13.03.22	45,25	6	296560	14125	6	25500	472	3	40270	781	3	10929	115	3	1052249	123576	3	10730	304
14.03.22	65,00	6	298679	15444	6	21592	428	3	43184	632	3	10597	132	3	4744426	265301	3	9803	239
	69,00	6	317754	19881	6	25665	480	3	43780	1591	3	10995	265	3	4691373	235031	3	10266	66
	72,75	6	320172	21888	6	25467	549	3	43913	2233	3	10730	229	3	5113611	216745	3	10067	239
15.03.22	90,25	6	352726	37050	6	22056	2483	3	47158	4423	3	9736	414	3	4899942	165203	3	10995	1764
	93,25	6	385577	47806	6	29573	4160	3	53980	6005	3	10664	289	3	4910473	133349	3	12982	3245
	96,50	6	419224	45083	6	32454	6004	3	56696	8916	3	10465	369	3	4806752	139717	3	13512	3875
16.03.22	114,75	6	425748	29868	6	36130	10349	3	55636	12155	3	9206	66	3	4787412	168239	3	31461	21250
	119,00	6	398923	36442	6	49112	14176	3	63783	12023	3	9736	344	3	4661105	213741	3	48814	26374
	122,50	6	380411	40925	6	53682	15526	3	74579	23915	3	10332	229	3	4576723	195617	3	57822	31809
17.03.22	138,00	6	386173	41039	6	70042	15922	3	74910	27468	3	9206	350	3	4711906	117957	3	182473	141969
	129,00	6	358256	34442	6	81268	20897	3	70340	22717	3	9670	434	3	4607919	81301	3	255131	205509
	144,25	6	392234	43330	6	95707	28671	3	77162	22927	3	9803	566	3	4633684	93224	3	310038	247098
18.03.22	161,75	6	417204	62534	6	94548	26738	3	73718	18820	3	9471	175	3	4730186	41023	3	1127755	942944
	165,25	6	386306	58807	6	135977	40295	3	288645	154578	3	9604	331	3	4415975	156709	3	1222734	1006376
	168,50	6	336929	45323	6	124287	36612	3	116571	58712	3	9339	526	3	4459888	160729	3	2475934	2201993
19.03.22	190,25	6	312390	51074	6	92131	28947	3	86236	38749	3	9074	289	3	4081629	192422	3	5107848	3809996
20.03.22	212,00	6	281293	53340	6	88090	26916	3	73387	31182	3	9206	517	3	3502154	101802	3	3341935	2684550

Table C.14: Test 7 – Outlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
11.03.22	0	6	81500	836	6	59511	3616	3	56365	1155	3	34110	577	3	56497	1978	3	45171	7287
12.03.22	22,00	6	101734	4821	6	23612	859	3	36230	464	3	13512	500	3	111603	12041	3	14240	289
13.03.22	45,25	6	228306	4721	6	20201	619	3	41727	827	3	11922	115	3	2149272	198031	3	12054	175
14.03.22	65,00	6	279472	14849	6	19870	683	3	43913	1000	3	10995	66	3	7799041	832439	3	11458	542
	69,00	6	280035	19128	6	22254	513	3	49410	1928	3	11193	350	3	7854942	801774	3	11392	861
	72,75	6	289671	24598	6	21691	371	3	52059	4015	3	10597	434	3	7617959	902050	3	11790	1558
15.03.22	90,25	6	290268	35100	6	17552	772	3	54510	6725	3	9471	175	3	7968002	1118927	3	15697	5565
	93,25	6	307621	48638	6	21791	577	3	65306	10068	3	10465	369	3	7851829	1204984	3	17419	7287
	96,50	6	335571	68713	6	21791	927	3	66101	10496	3	9935	199	3	7725191	1345099	3	20201	10465
16.03.22	114,75	6	451612	102170	6	21393	3696	3	67094	12891	3	9206	175	3	8234327	1754764	3	47820	37591
	119,00	6	512944	119108	6	28878	5897	3	88885	21057	3	9935	199	3	8263536	1784795	3	62922	52294
	122,50	6	494498	112583	6	29971	6965	3	84779	22014	3	10001	434	3	7906737	1662530	3	71797	61467
17.03.22	138,00	6	540795	127543	6	43085	13277	3	101734	40068	3	9935	397	3	7804340	1706621	3	216119	202917
	129,00	6	494266	120304	6	47092	14773	3	111073	51440	3	9538	397	3	7474432	1584655	3	253276	238597
	144,25	6	454096	105816	6	49576	16939	3	109550	49989	3	9736	500	3	7281891	1575953	3	350706	334934
18.03.22	161,75	6	423032	100458	6	64743	26768	3	82262	29939	3	8345	344	3	7849577	1673169	3	1189220	1157116
	165,25	6	401573	99738	6	86170	33209	3	124982	67217	3	9339	397	3	7522716	1491167	3	2878567	2833243
	168,50	6	401208	98373	6	96932	31542	3	157834	89349	3	9405	434	3	6911515	1554130	3	3711584	3659015
19.03.22	190,25	6	355673	75391	6	134818	48395	3	155715	88518	3	8942	115	3	6919131	1767680	3	2631848	2467699
20.03.22	212,00	6	295964	55454	6	128062	45879	3	109219	64172	3	8743	304	3	5887216	1629718	3	4168594	2283038

Table C.15: Test 8 – Inlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
04.04.22	0	6	104450	1347	6	54709	1776	3	74910	1698	3	27884	2066	3	75374	691	3	27950	175
	2,75	6	91104	2879	6	41429	636	3	56894	764	3	21195	66	3	50139	1344	3	21393	1172
05.04.22	21,00	6	75771	5385	6	25235	429	3	31063	691	3	12849	265	3	40998	2352	3	13048	289
	24,00	6	198236	28801	6	28447	95	3	31461	3599	3	13512	304	3	58285	4815	3	13048	66
	27,00	6	204098	26965	6	27652	220	3	30202	3231	3	12982	175	3	127830	17626	3	12518	0
06.04.22	45,00	6	1064933	376461	6	22619	1649	3	23049	1909	3	11657	350	3	797847	17900	3	12121	414
	48,00	6	1342086	599264	6	26361	739	3	26162	2775	3	11922	304	3	2175434	250217	3	13843	927
	51,00	6	1599104	773061	6	34938	5315	3	26560	3115	3	11922	304	3	2081714	135774	3	13048	289
07.04.22	68,25	6	1602946	782070	6	63783	16672	3	28679	6346	3	11061	289	3	5117519	653548	3	67094	16689
	73,00	6	1504258	790153	6	163828	86745	3	29805	7186	3	11061	239	3	3483873	603624	3	60339	4164
	74,75	6	1426070	963460	6	256687	114468	3	29474	7976	3	11326	414	3	5446632	738399	3	139620	26850
08.04.22	92,25	6	1322481	892818	6	787150	350094	3	26162	6804	3	10796	289	3	2993813	580309	3	467939	91703
	96,25	6	1320626	848349	6	1234854	537989	3	24705	5561	3	10531	596	3	4344576	534509	3	1565955	311573
	99,00	6	1197631	759558	6	1366526	609946	3	23910	5222	3	10597	350	3	3745495	437514	3	1507140	408504
	104,25	6	1001448	297451	6	1117257	407482	3	33779	5723	3	10531	304	3	5756008	504864	3	3082433	969188
09.04.22	118,25	6	1462266	617266	6	1905665	784342	3	30534	5258	3	10531	752	3	6701621	559971	3	6774875	515267

Table C.16: Test 8 – Outlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
04.04.22	0	6	108060	2188	6	57656	3908	3	79083	2371	3	29275	239	3	73585	1858	3	29540	2278
	2,75	6	126307	8604	6	42853	1380	3	52457	2555	3	20069	199	3	47887	2149	3	20267	827
05.04.22	21,00	6	114253	27377	6	27123	943	3	40469	5540	3	14041	350	3	60802	12561	3	13578	289
	24,00	6	510195	193999	6	28282	725	3	42191	10127	3	12916	304	3	81467	8790	3	12452	175
	27,00	6	476814	266422	6	28447	687	3	58352	22085	3	13777	403	3	163795	6028	3	12452	350
06.04.22	45,00	6	578349	265117	6	25798	1315	3	31196	5606	3	11591	239	3	853152	33275	3	12121	199
	48,00	6	622726	284664	6	26427	979	3	30136	5330	3	11326	115	3	1783465	231391	3	12916	414
	51,00	6	607360	288039	6	30335	3970	3	30534	5725	3	11458	369	3	2212392	261549	3	13048	1028
07.04.22	68,25	6	634615	274409	6	46529	11486	3	31063	6054	3	11260	132	3	4252577	375182	3	50602	12970
	73,00	6	670877	307357	6	196912	98273	3	30335	6818	3	11127	199	3	3176153	332468	3	69611	28864
	74,75	6	726447	460913	6	163828	70126	3	38084	14747	3	11326	526	3	5721103	293969	3	115908	44433
08.04.22	92,25	6	726944	497350	6	606962	231049	3	26427	8816	3	10531	414	3	2711328	455491	3	800430	327600
	96,25	6	643424	445079	6	1048805	569592	3	26825	7720	3	10332	199	3	4373321	758908	3	1942955	724709
	99,00	6	606101	424436	6	1245584	644404	3	25235	7565	3	10332	414	3	3384523	474802	3	2302602	922005
	104,25	6	451778	202287	6	807616	363224	3	22321	5804	3	10134	229	3	5298733	686454	3	4467770	1367628
09.04.22	118,25	6	501850	164620	6	623322	190843	3	18810	3771	3	10001	66	3	5553996	788581	3	5576184	1541561

Table C.17: Test 9 – Inlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
24.04.22	0	6	106172	2384	6	47125	1757	3	86037	2030	3	29076	3843	3	79679	1396	3	29606	3606
	2,75	6	77559	2073	6	35071	554	3	54046	2233	3	20731	1434	3	54113	1041	3	19473	397
25.04.22	22,25	6	190222	7222	6	24275	269	3	59941	1672	3	13114	344	3	293745	4305	3	12386	132
	25,50	6	217080	9288	6	24241	246	3	65107	2153	3	12518	397	3	428199	10989	3	12187	175
	28,75	6	253210	12073	6	22851	103	3	65107	2617	3	12121	459	3	547286	16295	3	11856	239
26.04.22	46,00	6	615043	107559	6	20897	119	3	68684	2358	3	11061	239	3	4499297	517057	3	11591	662
	50,00	6	814902	179085	6	20996	483	3	71863	3681	3	10862	517	3	6091745	572795	3	11856	1227
	52,75	6	801887	207469	6	21460	592	3	69810	2706	3	10929	526	3	5657784	498176	3	11856	1624
27.04.22	70,00	6	1444284	409866	6	20499	614	3	62855	3659	3	10796	589	3	8340433	881732	3	16161	5833
	74,00	6	1350696	399967	6	20996	604	3	60935	4281	3	10730	803	3	6304022	953207	3	20930	7740
	76,75	6	1415771	403661	6	21228	850	3	58484	3989	3	10266	764	3	4705547	967702	3	21062	9941
28.04.22	93,50	6	1227999	325077	6	27089	3258	3	42985	3904	3	10134	752	3	4458696	1352141	3	38680	20997
	97,75	6	1190755	323291	6	30497	4661	3	39670	3621	3	9934	827	3	3817106	1268665	3	49140	24310
	100,75	6	1286983	336232	6	32451	4692	3	37617	3436	3	9868	920	3	3609420	1245630	3	51591	29478
29.04.22	117,75	6	1056547	277196	6	68445	16220	3	29272	3091	3	10133	716	3	4045059	1349484	3	152586	60682
	121,75	6	1086680	277172	6	89572	22023	3	28080	3018	3	9802	1002	3	3534319	1180066	3	297821	40679
	124,75	6	964459	260905	6	121129	39820	3	25895	3204	3	9868	764	3	3455111	1071001	3	409016	41491

Table C.18: Test 9 – Outlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
24.04.22	0	6	112365	1535	6	47125	551	3	86302	1237	3	26361	1126	1	87825	-	3	26295	2407
	2,75	6	73055	690	6	37389	672	3	58285	2369	3	18545	265	1	60007	-	3	19274	698
25.04.22	22,25	6	210423	2703	6	25500	160	3	64578	3556	3	12651	350	1	271424	-	3	12651	132
	25,50	6	224531	3907	6	25169	475	3	68154	3606	3	12783	175	1	427602	-	3	12121	304
	28,75	6	250660	4740	6	24838	508	3	71002	2764	3	12319	115	1	531920	-	3	11790	175
26.04.22	46,00	6	357660	43025	6	21989	566	3	73122	2803	3	11127	115	1	4574273	-	3	10796	132
	50,00	6	740323	211281	6	21824	551	3	75307	3231	3	10730	229	1	5875559	-	3	10730	304
	52,75	6	834010	284849	6	21758	275	3	74844	3860	3	11260	350	1	5820519	-	3	10862	239
27.04.22	70,00	6	638224	271318	6	20565	525	3	66896	4556	3	10597	464	1	10292461	-	3	12319	1020
	74,00	6	749331	348901	6	20632	532	3	63518	3965	3	10266	289	1	7155584	-	3	13180	2749
	76,75	6	838448	385263	6	20963	927	3	60736	3442	3	10399	350	1	4586990	-	3	12651	1822
28.04.22	93,50	6	988665	471637	6	24838	3364	3	47887	5070	3	10266	566	1	2186892	-	3	25897	9707
	97,75	6	978466	451434	6	37120	13537	3	42584	4152	3	9735	303	1	1914282	-	3	35829	13418
	100,75	6	850814	386850	6	26921	4099	3	40862	4119	3	10066	289	1	1633547	-	3	44173	16883
29.04.22	117,75	6	793561	361832	6	90300	49830	3	33113	4323	3	9934	500	1	1340295	-	3	148348	68119
	121,75	6	740712	323221	6	123745	70780	3	29338	3525	3	9735	639	1	1293407	-	3	269013	96542
	124,75	6	699685	285705	6	121327	60245	3	27550	3217	3	10133	344	1	1478974	-	3	379876	134476

Table C.19: Test 10 – Inlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
08.05.22	0	6	114385	5135	6	49410	817	3	73122	9759	3	24970	589	3	78950	4534	3	25301	746
	5,00	6	64776	1884	6	35667	1081	3	31726	2504	3	18744	175	3	35037	806	3	16625	66
09.05.22	17,50	6	87031	3449	6	24606	402	3	33249	2923	3	15830	920	3	46761	1205	3	14770	814
	21,50	6	110543	3445	6	26791	338	3	35700	3124	3	13777	517	3	59941	2413	3	13048	464
	24,50	6	125976	4489	6	25467	378	3	36826	3659	3	13379	239	3	69214	2737	3	12319	304
10.05.22	42,00	6	336035	15330	6	16989	400	3	27950	2975	3	9339	229	3	403891	15336	3	16625	5796
	45,50	6	464163	18863	6	21956	176	3	34839	3019	3	11657	239	3	659883	26256	3	18413	7783
	48,50	6	519667	23982	6	22023	140	3	34971	2819	3	11525	199	3	844011	35908	3	13975	2764
11.05.22	66,00	6	582290	32751	6	17651	756	3	27487	2418	3	9405	132	3	6326542	610139	3	14174	4638
	69,50	6	645080	53197	6	21923	434	3	31527	3100	3	10995	66	3	7226057	909876	3	15962	5234
	72,50	6	664950	68871	6	23049	598	3	31130	3047	3	10862	331	3	6073332	1041290	3	15167	5237
12.05.22	89,75	6	497644	68318	6	37621	10890	3	25102	2527	3	9803	239	3	6164071	1407756	3	21393	11858
	93,25	6	532781	89121	6	62491	21525	3	27354	2961	3	10597	132	3	5608771	1439999	3	23579	12857
	96,50	6	487212	80875	6	55934	17250	3	25963	2467	3	10597	265	3	5208987	1794098	3	24043	13519
13.05.22	114,00	6	424059	100530	6	109848	28359	3	20334	1822	3	9140	0	3	5370530	1386278	3	59345	45239
	117,25	6	518044	159095	6	224564	62604	3	24109	2123	3	10266	239	3	5062081	1323544	3	82461	66068
	120,50	6	520163	172836	6	183996	50459	3	24241	2087	3	10067	239	3	4995053	1114030	3	105576	84616
14.05.22	141,00	6	482874	180080	6	186447	35883	3	24374	2278	3	10399	175	3	6887869	1326046	3	520528	448400
	144,00	6	484994	182061	6	230956	34377	3	23844	1990	3	9803	331	3	6618035	1336148	3	686310	556803

Table C.20: Test 10 – Outlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
08.05.22	0	6	117962	5353	6	53483	546	3	84712	4194	3	26096	1330	3	71201	1893	3	26096	1205
	5,00	6	62193	1851	6	37819	568	3	34706	2434	3	18347	632	3	35965	414	3	16558	175
09.05.22	17,50	6	76731	4896	6	26328	839	3	36230	3537	3	14903	414	3	48880	1277	3	12849	239
	21,50	6	96105	3781	6	28414	340	3	39674	3795	3	14571	66	3	61266	1756	3	12651	175
	24,50	6	105808	4585	6	27089	289	3	40932	3723	3	13048	434	3	69479	2358	3	12452	239
10.05.22	42,00	6	321298	17904	6	19638	322	3	33580	3420	3	10664	331	3	397599	16506	3	11392	566
	45,50	6	498472	27346	6	24738	319	3	39872	4455	3	11392	652	3	664916	27211	3	12054	876
	48,50	6	606333	63274	6	24473	382	3	40204	4233	3	11458	265	3	874280	44901	3	10862	434
11.05.22	66,00	6	735058	163128	6	18645	817	3	28944	3165	3	9339	115	3	7735722	1121585	3	11790	1035
	69,50	6	887957	277578	6	24341	923	3	34243	3842	3	10862	478	3	8075300	1165826	3	11922	1590
	72,50	6	861232	317661	6	24473	901	3	32984	3775	3	10399	350	3	6832564	1066036	3	11988	2066
12.05.22	89,75	6	763670	335317	6	25268	3178	3	24970	2424	3	9604	289	3	7035106	1022371	3	16558	6127
	93,25	6	844806	416968	6	37422	5637	3	28547	2424	3	10531	526	3	6025445	1151913	3	22851	6701
	96,50	6	826857	439780	6	37455	5125	3	27686	2581	3	10200	434	3	5078640	1211583	3	21327	10502
13.05.22	114,00	6	802814	381442	6	55239	7944	3	24308	2407	3	9935	229	3	5286811	1187837	3	60140	25816
	117,25	6	923127	426892	6	112762	23035	3	24904	1869	3	10001	369	3	4807348	1111252	3	67889	35279
	120,50	6	981147	452179	6	97363	11503	3	25235	1821	3	10399	517	3	4981541	1029840	3	88885	38811
14.05.22	141,00	6	929452	341379	6	111901	14388	3	23182	1672	3	8942	500	3	6518089	1186658	3	505095	238790
	144,00	6	956972	367717	6	135381	19395	3	23645	1821	3	9670	369	3	5957423	1160810	3	486219	219554

Appendix D: Calibration and cross-validation of microplate reader method

The results from the microplate reader, flow cytometer and microscope used for the calibration and cross-validation are given in table D.1.

Table D.1: Results from microplate reader, flow cytometer and direct counting.

Dilution	Microplate (FI)			Flow cytometer (cells/mL)			Microscope (cells/mL)		
	n	Mean	SE	n	Mean	SE	n	Mean	SE
1	4	12096	456	3	2446352	110	3	2426667	56877
2	4	5659	236	3	1033613	973	3	1068889	22471
5	4	2373	81	3	495545	143	3	485926	9102
10	4	1227	32	3	244295	150	3	242963	1614
50	4	319	4	3	63449	102	3	61111	642
100	4	192	5	3	37391	115	3	37037	370

Appendix E: Supplemental material from algae bioassay supplied with CO_2

Tables D1 and D2 show the average cell concentrations for test water, blank and positive control for the algae bioassay supplied with CO_2. Standard error and number of replicates (n) are also shown in the tables. The results from pre-grown algae (PG-I) and lake water algae (LW-I) are shown in the same table. Results from inlet and outlet are separated in two tables.

Table D.1: Algae bioassay supplied with CO_2 – Inlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
08.05.22	0	6	109815	3523	6	51033	1450	3	86964	3254	3	28348	814	3	81401	2060	3	24506	976
	5,00	6	64114	1767	6	34773	637	3	36958	1721	3	17883	526	3	37024	4050	3	15896	229
09.05.22	17,50	6	94316	3020	6	28613	645	3	41859	3278	3	14240	175	3	52523	6016	3	13909	115
	21,50	6	118955	3687	6	26493	542	3	43582	3659	3	13247	66	3	64578	7467	3	13180	132
	24,50	6	139885	5198	6	25102	298	3	44906	3303	3	12253	175	3	76168	8887	3	12319	115
10.05.22	42,00	6	420913	18006	6	22420	214	3	43979	3711	3	11657	132	3	471515	50369	3	11591	350
	45,50	6	560202	32645	6	21758	470	3	42654	3369	3	10929	304	3	753537	69447	3	10995	403
	48,50	6	643324	56991	6	21228	195	3	43118	3712	3	10929	229	3	984823	85515	3	11525	716
11.05.22	66,00	6	1158156	316767	6	22817	1589	3	41330	4685	3	11127	115	3	7984031	491829	3	12916	2292
	69,50	6	1266944	383339	6	20565	472	3	38680	4646	3	10531	304	3	8059669	685440	3	12717	2886
	72,50	6	1344669	417896	6	20532	656	3	38415	5234	3	10332	304	3	7303483	791746	3	13843	3312
12.05.22	89,75	6	1255950	416779	6	28712	5820	3	35236	5339	3	10200	175	3	4706938	934528	3	26493	15575
	93,25	6	1265553	430930	6	33613	8614	3	34839	6071	3	10266	331	3	4852188	1123110	3	34375	22360
	96,50	6	1240650	435798	6	37223	11474	3	33448	6409	3	10067	175	3	4043346	996055	3	53185	23898
13.05.22	114,00	6	1146433	367368	6	82759	35752	3	29010	6953	3	9339	304	3	3060245	696197	3	139355	99966
	117,25	6	1211474	410283	6	119021	46479	3	33249	8804	3	9670	239	3	3212250	611015	3	315337	143466
	120,50	6	1077351	365153	6	127599	47583	3	32852	9899	3	9736	199	3	2798888	535689	3	382365	298537
14.05.22	141,00	6	973464	326007	6	219630	71483	3	37753	15946	3	9471	464	3	3811728	534640	3	1283470	545713
	144,00	6	909185	294901	6	326299	131567	3	34905	14654	3	9008	403	3	3464931	517118	3	1408319	736502

Table D.2: Algae bioassay supplied with CO_2 – Outlet.

Date	Duration (h)	Cell concentration (cells/mL)																	
		Test water (PG-I)			Test water (LW-I)			Blank (PG-I)			Blank (LW-I)			Pos.control (PG-I)			Pos.control (LW-I)		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
08.05.22	0	6	115908	5487	6	53086	666	3	93190	5075	3	27818	526	3	85375	2135	3	24904	577
	5,00	6	68055	1456	6	38349	1056	3	33779	1960	3	17353	764	3	38482	2220	3	15697	199
09.05.22	17,50	6	98853	2198	6	31063	705	3	39872	2355	3	14174	239	3	56232	3976	3	13379	289
	21,50	6	118922	2837	6	28116	491	3	42124	3042	3	12717	115	3	69942	3698	3	12253	350
	24,50	6	142104	3788	6	26493	409	3	42323	2782	3	12121	229	3	80540	5055	3	12187	289
10.05.22	42,00	6	519501	53260	6	24076	80	3	42257	3115	3	11591	289	3	472509	29068	3	11723	607
	45,50	6	746052	126630	6	23645	178	3	42456	3681	3	11193	239	3	747576	43142	3	11591	764
	48,50	6	1007310	239656	6	23480	292	3	41528	3333	3	10929	0	3	964689	46012	3	11657	830
11.05.22	66,00	6	1544263	479401	6	21691	512	3	39541	3648	3	10531	115	3	7752810	517240	3	15035	3079
	69,50	6	1700706	538588	6	23182	757	3	38945	3981	3	10266	66	3	8242076	670132	3	16890	4025
	72,50	6	1755548	549682	6	24208	1832	3	38084	3918	3	10465	175	3	7747379	754546	3	19075	5221
12.05.22	89,75	6	1750613	539449	6	26328	2563	3	32918	2912	3	10399	132	3	4961936	890433	3	37289	13716
	93,25	6	1858706	550497	6	29176	3072	3	31593	3730	3	10134	199	3	4837881	905925	3	46496	17150
	96,50	6	1808004	546359	6	32223	3669	3	31461	3707	3	10134	304	3	4345105	834254	3	55967	21747
13.05.22	114,00	6	1572611	455761	6	58252	13647	3	29010	3926	3	9471	589	3	3924391	706178	3	138229	46669
	117,25	6	1474321	442989	6	74546	15665	3	28414	3447	3	9273	175	3	3795634	664390	3	182605	54707
	120,50	6	1407988	420803	6	105874	27214	3	26825	3253	3	9339	500	3	4028046	784331	3	227048	82407
14.05.22	141,00	6	1238630	351865	6	171842	49994	3	26891	3975	3	9538	229	3	4581161	823131	3	734991	249858
	144,00	6	1106792	327871	6	188633	54408	3	25566	3843	3	8743	304	3	4096863	776298	3	935347	265214

The results of all parameters from the algae bioassay supplied with CO_2 performed with PG-I and LW-I are shown in figure D.1 and D2, respectively. The average cell concentration (cells/mL) are plotted against time (hours). The vertical axis is displayed on a log scale.

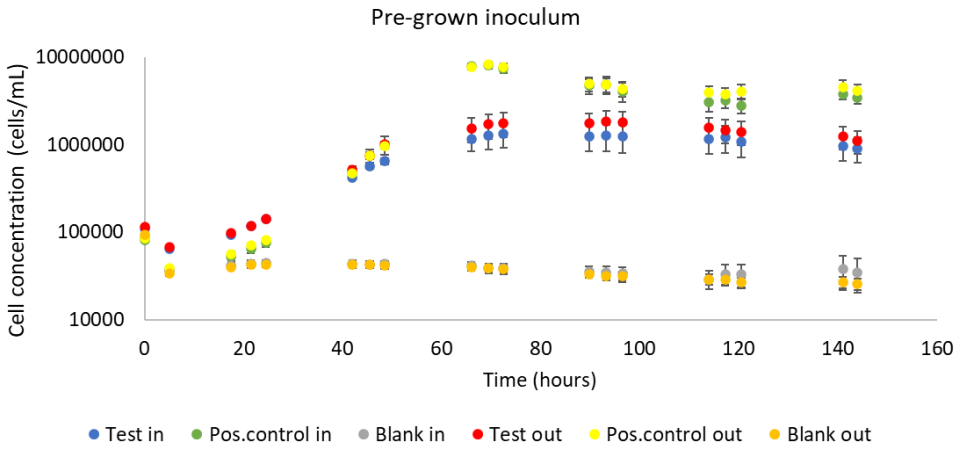

Figure D.1: Average cell concentration of all test parameters of PG-I supplied with CO_2 plotted against time (hours). Standard error is represented by the error bars.

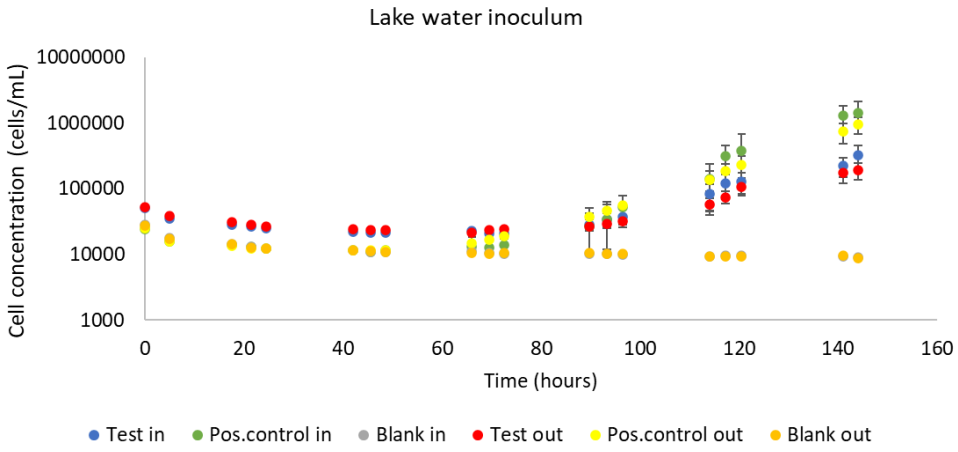

Figure D.2: Average cell concentration of all test parameters of LW-I supplied with CO_2 plotted against time (hours). Standard error is represented by the error bars.