RIGA TECHNICAL UNIVERSITY

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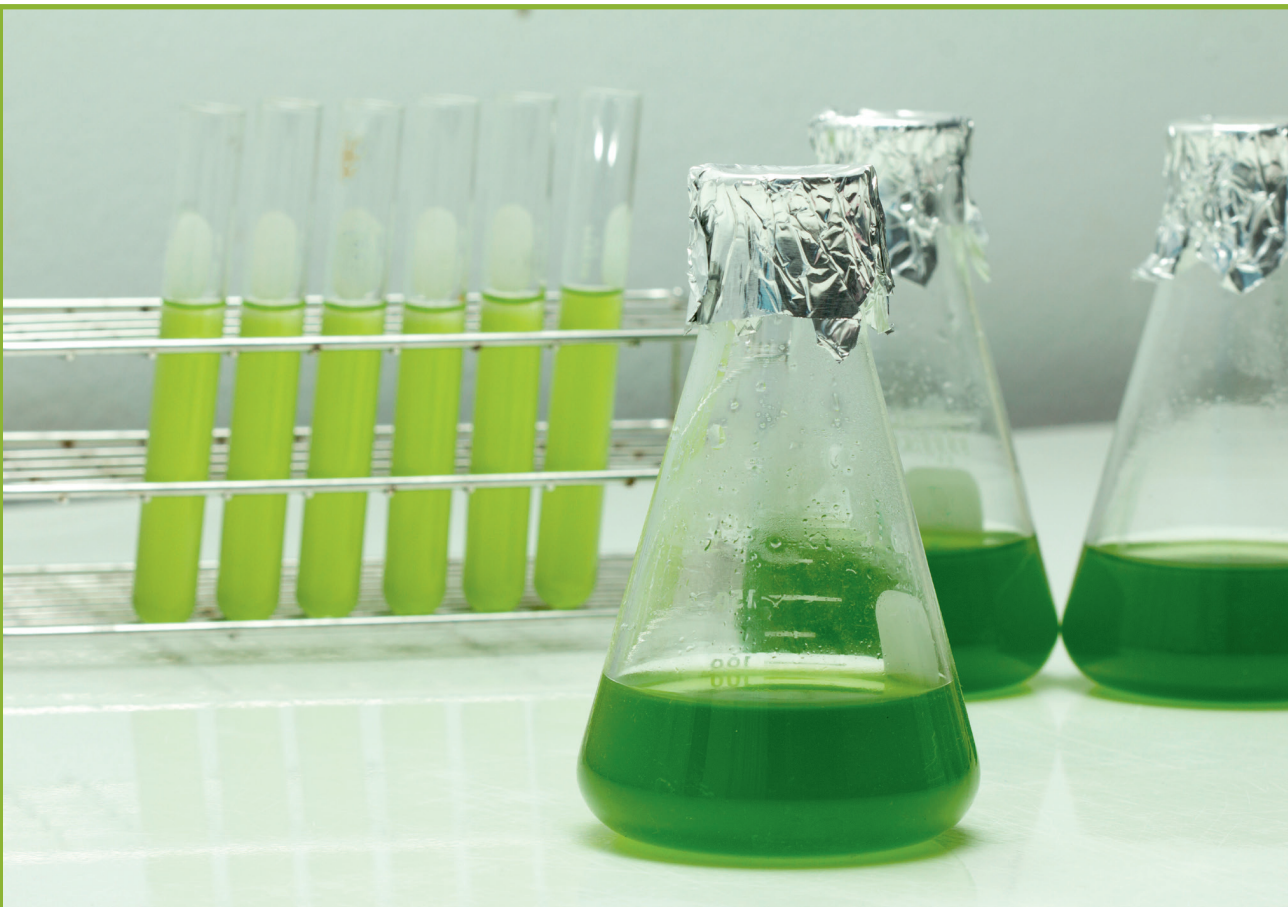
MICROALGAL TECHNOLOGY FOR MUNICIPAL WASTEWATER POST-TREATMENT

Doctoral Thesis



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RIGA TECHNICAL UNIVERSITY
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ABSTRACT

Eutrophication is a major problem affecting aquatic ecosystems globally as it deteriorates their status, degrades the services they provide and negatively affects the social, economic and mental wellbeing of the public directly dependent on the aquatic resources. Nutrient loading from wastewater treatment plants is a significant contributor to eutrophication development. Moreover, small WWTPs (PE<2000) have no legal requirement for phosphorus reduction limits before releasing the treated effluent in the surface waters. As a result, surface waters receive uncontrolled amount of phosphorus which is the limiting nutrient for phytoplankton growth and its excess concentration leads to massive algal blooms. The conventional methods for targeted phosphorus removal at the wastewater post-treatment stage involves addition of chemical precipitants which require high costs and generate excess waste material. Photosynthetic production of microalgal biomass is perceived as an eco-friendly alternative to the conventional wastewater post-treatment as it requires less energy input and can generate valuable biomass.

The Thesis "Microalgal technology for municipal wastewater post-treatment" demonstrates the potential of enhanced phosphorus removal from municipal wastewater using microalgal biomass exposed to phosphorus deficiency conditions. The study focuses on identification of appropriate microalgal species for production in municipal wastewater after P-starvation, developing a reliable P-starvation technique and identifying indicators for phosphorus deficiency status quantification. Further, the optimum initial conditions for rapid phosphorus removal and valuable algal biomass production were identified and verified during a continuous process in a sequencing batch photobioreactor.

In the first stage of Thesis development, multiple experiments in batch mode were carried out to compare the performance of different microalgal species after different periods of exposure to phosphorus deficiency conditions. The results showed that phosphorus deficiency status is established when cellular polyphosphate content is depleted. *Chlorella vulgaris* was identified as the most suitable species for biomass manipulation and production in wastewater for nutrient removal. In addition, the importance of control over pH and N/P ratio for successful performance was identified.

The second stage of the Thesis involved investigation to identify the optimum initial conditions to achieve the most efficient wastewater treatment performance in terms of rapid phosphorus removal and accumulation, biomass production and high value molecule synthesis. It was demonstrated that wastewater treatment efficiency increases with a longer biomass P-starvation period. However, optimization model suggested that a short P-starvation period for implementation in a sequencing batch reactor is a more economically sound approach. Validation of the optimization model results showed that even a period of 1 day biomass P-starvation significantly enhances phosphorus removal and polyphosphate accumulation, resulting in 101.7 and 138.0 % higher rates, respectively, comparing to the reference conditions. Lower initial biomass

concentration influences the biomass growth rate as it increases by 52.7 % and further supports the phosphorus removal rate.

The author of this study showed that the results from batch experiments and optimization model validation in sequencing batch photobioreactor provided knowledge that can be used for existing industrial scale microalgae cultivation systems as well as for microalgal based wastewater treatment system development. This Thesis improves the current understanding of targeted phosphorus removal from municipal wastewater by means of microalgal biomass cultivation. Ultimately, the obtained results show that P-starved biomass can rapidly reduce the phosphorus content in municipal wastewater to ultra-low level and significantly reduce the role of small WWTPs to eutrophication status development in surface waters.

The Thesis is written in English and consists of 58 pages, 19 figures, 3 tables, and 104 literature sources were used for the development of the Thesis.

Keywords
Microalgae, wastewater, phosphorus, *Chlorella vulgaris*, polyphosphate, post-treatment.

ANOTĀCIJA

Eitrofikācija visā pasaulē būtiski ietekmē ūdens ekosistēmas, pasliktinot to stāvokli, degradējot to sniegtos pakalpojumus, un atstājot negatīvu ietekmi uz sabiedrības sociālo, ekonomisko un mentālo labklājību. Barības vielu plūsma no notekūdeņu attīrīšanas stacijām būtiski veicina eitrofikācijas attīstīšanos. Īpaša loma eitrofikācijas veicināšanā ir mazajām notekūdeņu attīrīšanas stacijām (PE < 2000), kurām likumdošanā nav noteikts nepieciešamais fosfora samazinājuma līmenis notekūdeņos pirms tā izlaides virszemes ūdeņos. Tā rezultātā virszemes ūdeņos nonāk nekontrolēts daudzums fosfora, kas ir limitējošais barības elements fitoplanktona augšanai, un palielināta fosfora koncentrācija veicina pārmērīgu aļģu ziedēšanu. Tradicionālās metodes notekūdeņu papildu attrīšanai no fosfora ķīmisko izgulsnēšanos veicinošu vielu lietošanu, kas veido augstas izmaksas un rada papildu atkritumus. Fotosintētiskā mikroaļģu ražošana tiek piedāvāta kā videi draudzīga alternatīva tradicionālajai notekūdeņu attīrīšanai, jo tā procesā tiek patērēts mazāk enerģijas un saražota vērtīga biomasa.

Promocijas darbā "Mikroaļģu tehnoloģijas izmantošana komunālo notekūdeņu pēcattīrīšanai" demonstrēta fosfora izdalīšana no komunālajiem notekūdeņiem palielinātā ātrumā, izmantojot fosfora deficītam pakļautu mikroaļģu biomasu. Veiktajos pētījumos identificētas piemērotākās mikroaļģu sugas audzēšanai notekūdeņos pēc pakļaušanas fosfora deficītam, kā arī izstrādāta metodoloģija biomasas badināšanai un identificēti indikatori biomasas fosfora deficīta stāvokļa noteikšanai. Tika noteikti optimālie sākotnējie parametri paātrinātai fosfora izdalīšanai un augstvērtīgas aļģu biomasas ražošanai, kas tālāk pārbaudīti cikliskas plūsmas fotobioreaktorā.

Promocijas darba pirmajā daļā veikti vairāki noslēgta trauka eksperimenti, salīdzinot dažādu mikroaļģu sugu spēju attīrīt komunālos notekūdeņus pēc to pakļaušanas fosfora stresa apstākļiem dažādu periodu garumā. Rezultāti parādīja, ka fosfora deficīta stāvoklis iestājas izsīkstot aļģu šūnā uzkrātā polifosfāta rezervēm. *Chlorella vulgaris* tika identificēta kā piemērotākā suga biomasas manipulācijai un audzēšanai notekūdeņos to attīrīšanas nolūkos. Kontrolēta pH un N/P attiecība tika identificēta kā būtiska procesa sastāvdaļa sekmīgam piedāvātā notekūdeņu attīšanas procesam.

Promocijas darba otrajā daļā tika identificēti optimālie sākuma parametri, kas ļautu sasniegt efektīvāko sniegumu notekūdeņu attīrīšanā ar augstu fosfora izdalīšanas ātrumu un akumulāciju aļģu šūnās, kā arī augstu biomasas augšanas ātrumu un augstvērtīgu molekulu sintēzi. Iegūtie rezultāti parādīja augstāku notekūdeņu attīrīšanas efektivitāti, palielinoties biomasas fosfora badināšanas perioda ilgumam. Izmantotā optimizācijas modeļa rezultāti parādīja, ka pat pēc vienas dienas fosfora badināšanas perioda tiek fosfora izdalīšana no notekūdeņiem palielinājās par 101,7 %, un tā akumulācija mikroaļģu šūnās polifosfātu formā pieauga par 138,0 %. Turklāt, pie zemākas sākotnējas biomasas koncentrācijas, biomasas augšanas ātrums palielinājās par 52,7 %, kā arī papildus tika stimulēta fosfora izdalīšana.

Iegūtie rezultāti papildina esošās zināšanas par mikroaļģu audzēšanu un to izmantošanu notekūdeņu attīrīšanai, un var tikt izmantotas industriāla mēroga mikroaļģu audzēšana un turpmākā notekūdeņu attīrīšanas sistēmu attīstībā. Šie rezultāti arī papildina pieejamās zināšanas par fosfora

izdalīšanu no notekūdeņiem, izmantojot mikroaļģu kultivēšanu. Galu galā iegūtie rezultāti liecina, ka mikroaļģu biomasa pakļaušana fosfora deficītam spēj īsā laikā samazināt fosfora saturu sadzīves notekūdeņos līdz īpaši zemam līmenim, un būtiski samazināt mazo NAI lomu eitrofikācijas apstākļu veicināšanā virszemes ūdeņos.

Promocijas darbs ir uzrakstīts angļu valodā, tajā ir ievads, trīs nodaļas, secinājumi, literatūras saraksts, 19 attēlu, trīs tabulas, četri pielikumi, kopā 58 lappuses, neieskaitot pielikumus. Literatūras sarakstā ir 104 nosaukumi.

Atslēgas vārdi
Mikroaļģes, notekūdeņi, fosfors, *Chlorella vulgaris*, polifosfāti, papildu attīrīšana.

LIST OF PAPERS

Paper I

Lavrinovičs, A., Juhna, T. Review on Challenges and Limitations for Algae-Based Wastewater Treatment. *Construction Science*, 2017, 20, 17–25.

Paper II

Lavrinovičs, A., Mežule, L., Juhna, T. Microalgae Starvation for Enhanced Phosphorus Uptake from Municipal Wastewater. *Algal Research*, 2020, 52, 102090.

Paper III

Lavrinovičs, A., Murby, F., Zīverte, E., Mežule, L., Juhna, T. Increasing Phosphorus Uptake Efficiency by Phosphorus-Starved Microalgae for Municipal Wastewater Post-Treatment. *Microorganisms*, 2021, 9 (8), 1598.

Paper IV

Lavrinovičs, A., Mežule, L., Cacivkins, P., Juhna, T. Optimizing Phosphorus Removal for Municipal Wastewater Post-Treatment with *Chlorella vulgaris*. *Journal of Environmental Management*, 2022, 324, 116313.

CONTRIBUTIONS TO PAPERS

Paper I
Selected and analyzed the literature, wrote the manuscript.

Paper II
Designed and performed the experiments, analyzed the data and wrote the manuscript.

Paper III
Designed and performed the experiments, analyzed the data and wrote the manuscript.

Paper IV
Designed and performed the experiments, analyzed the data and wrote the manuscript.

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INTRODUCTION

Globally the aquatic ecosystems are central to the identities of cultures and societies. A healthy and functional aquatic ecosystem provides resources for food, living space, and welfare. However, growing areas of surface waters receive elevated loads of nutrients, mainly phosphorus and nitrogen, originating from various sources, including wastewater treatment plants (WWTP). Eventually, the eutrophication status evolves and leads to an overall degradation of the aquatic ecosystems. Therefore, wastewater treatment has always been among the main concerns for the conservation of the aquatic environment. This has challenged the scientific community to develop efficient technologies for wastewater purification and even to change the perception of it towards resource recovery rather than waste disposal.

The conventional wastewater treatment is mainly focused on the removal of organic matter and nutrients (Davis, 2010). A traditional WWTP effectively reduces nitrogen content by the nitrification-denitrification process (Ni et al., 2016) and approximately 10 to 30 % of phosphorus (to 1–2 mg L^{-1}) via solids settling or activated sludge process (Cornel and Schaum, 2009). As the natural background concentration of the total phosphorus in freshwater is usually <0.03 mg L^{-1}, total P concentration greater than 0.1 mg P L^{-1} is considered high enough to cause eutrophication (Kumar et al., 2019). Moreover, increasing concentration of available phosphorus allows plants to assimilate more nitrogen before the phosphorus is depleted. Thus, if sufficient phosphorus is available, elevated concentrations of nitrates will lead to algal blooms.

Chemical precipitation and enhanced bacterial uptake are the main methods for additional P removal used in WWTPs. Although these methods are well established and applied in the large WWTPs, their use in small WWTPs is often not practiced due to no legal requirements and economic reasons. For instance, in the European Union the regulation on municipal wastewater treatment (Directive 91/271/EEC) does not state any limits for permissible total phosphorus concentration in the effluent from WWTPs operating in small agglomerations with less than 2000 PE (Molinos-Senante, et al., 2014). In addition, the conventional methods often fail to reduce the P concentration to a level below 0.1 mg P L^{-1}. Scaling the methods for targeted P removal to small WWTPs requires high capital costs, and due to the overall system complexity, the operation is expensive (Bunce et al., 2018). As a result, the additional wastewater treatment for targeted phosphorus removal becomes detrimental as the obtained reduction rates in small scale are often insufficient to meet environmental safety standards.

Microalgae-based systems are viewed as a promising alternative to the traditional wastewater phosphorus removal and recovery methods (Borowitzka, 2013; Whitton, et al., 2015). Many studies, mostly in lab scale, have shown that various microalgal strains or mixed cultures are capable of near-complete removal of phosphorus when produced in wastewater (Cai et al., 2013). Also, there are reports on incomplete P removal rates over longer time periods and at larger operational scales (Chen et al., 2018; Osorio et al., 2020; Patel, et al., 2012) which can become a drawback for efficient microalgae-based wastewater post-treatment step. Certain conditions are

known at which algal cells can consume excess phosphorus and store it within the cell. Enhanced algal phosphorus uptake and storage can be achieved by manipulation with external phosphorus availability. The two manipulation approaches causing this phenomenon are the luxury P uptake mechanism (Powell et al., 2008; Solovchenko et al., 2019) and phosphorus starvation (Hernandez et al., 2006), which are initiated by excess phosphorus availability and phosphorus limited conditions, respectively. Both biomass manipulation approaches can increase the phosphorus uptake rate multiple times (Brown and Shilton, 2014) when compared to phosphorus consumption rates by regular biomass. An additional benefit for biomass manipulation with phosphorus availability is the enhancement of high value molecule synthesis by algal cells (Levasseur et al., 2020), thus adding value to the produced biomass and providing an opportunity for financial return.

One of the main limitations for microalgae manipulation with phosphorus availability is the reduction of biomass growth rate (Kamalanathan, 2016) which further yields less products form high value molecules. Also, it remains unknown what is the margin for optimum biomass P-starvation period. Insufficient P stress can attenuate the desired enhancement of P uptake, while overexposure to P-deficiency can damage the algal cell physiological processes and disrupt the whole treatment process. Also, there are no clear indicators for P deficiency quantification and tools for biomass P-starvation control. Thus, the reported phosphorus removal rates after microalgal biomass exposure to P deficiency are variable and the effects on similar biomass P-starvation conditions can be antipodal (Hernandez et al., 2006; Van Moorleghem et al., 2013; Wu et al., 2012). Considering these knowledge gaps, more in-depth studies on the microalgal biomass P-starvation approach is required for its successful integration in engineered systems.

This Thesis aims to develop a microalgae-based municipal wastewater post treatment method with phosphorus-starved biomass, focusing on mechanisms that gives control over the biomass phosphorus starvation process.

The Thesis outline was as follows:

First a **theoretical background** of the studied concept is provided, discussing the ability of microalgae to remove nutrients and produce valuable biomass. Conventional wastewater post-treatment is discussed and compared to microalgae-based treatment technologies. The current status, opportunities and challenges of microalgae-based wastewater treatment is discussed. The possible integration of microalgae biomass manipulation in the municipal wastewater post-treatment technology is presented. The summary of materials and methods as well as the obtained results are reported and discussed.

Paper I provide a literature review of the development and current status of microalgae-based wastewater treatment. The main advantages such as energy efficient and waste-free operation as well as valuable biomass production for potential operational cost recovery are emphasized. The challenges are mainly related to limitations for climate, biomass harvest and downstream processing and the efficiency for reduction of pollutants other than nutrients. Solutions are offered for successful microalgae use for wastewater post-treatment.

In **Paper II** three microalgal species, *Desmodesmus communis*, *Tetradesmus obliquus* and *Chlorella protothecoides* were used to study the concept of enhanced phosphorus removal from municipal wastewater. Microalgae were exposed to extended periods of phosphorus deficiency conditions and inoculated in filtered primary and secondary wastewater at batch conditions to study the nutrient uptake rate and cellular phosphorus accumulation.

In **Paper III** phosphate (PO_4) and nitrate (NO_3) removal from municipal wastewater by the microalgal species, *Chlorella vulgaris, Botryococcus braunii, Ankistrodesmus falcatus,* and *Tetradesmus obliquus* was investigated after their exposure to shorter phosphorus deficiency periods. The polyphosphate accumulation and alkaline phosphatase activity were assessed as indicators for phosphorus deficiency status in microalgal cells.

In **Paper IV** different initial phosphate and biomass concentrations with various periods of phosphorus deficiency were studied using *Chlorella vulgaris* to find the optimal combination for higher biomass growth and phosphate removal rates, polyphosphate accumulation and protein productivity at the shortest biomass phosphorus starvation period. The obtained result was verified in a sequenced batch photobioreactor with synthetic wastewater.

Finally, the **Conclusions and future outlook** sum up the obtained results and conclusions and discuss the practical aspects for implementation of the studied approach in microalgae-based municipal wastewater post-treatment. The future role of microalgae in wastewater treatment and scale-up possibilities are discussed.

1. THEORETICAL BACKGROUND AND THE RELEVANCE OF THE STUDY

1.1. Global phosphorus cycle

Phosphorus is a major element for the key biochemical processes, including the formation of genetic material (DNA, RNA) and energy transfer (ATP), as well as in structural support of organisms, provided by membranes and bone. Along with carbon and nitrogen, phosphorus is the essential nutrient for heterotrophic and photosynthetic organisms. Phosphorus is crucial to the food web as it ensures the foundation for biological productivity of the organisms.

The four major components of global phosphorus cycle are shown in Figure 1.1. They include (i) tectonic uplift and exposure of phosphorus-bearing rocks to the forces of weathering; (ii) physical erosion and chemical weathering of rocks that produce soils and provide dissolved and particulate phosphorus to rivers; (iii) riverine transport of phosphorus, including the wastewater-borne phosphorus, to lakes and the ocean; and (iv) sedimentation of phosphorus associated with organic and mineral matter and burial in sediments (Ruttenberg, 2003).

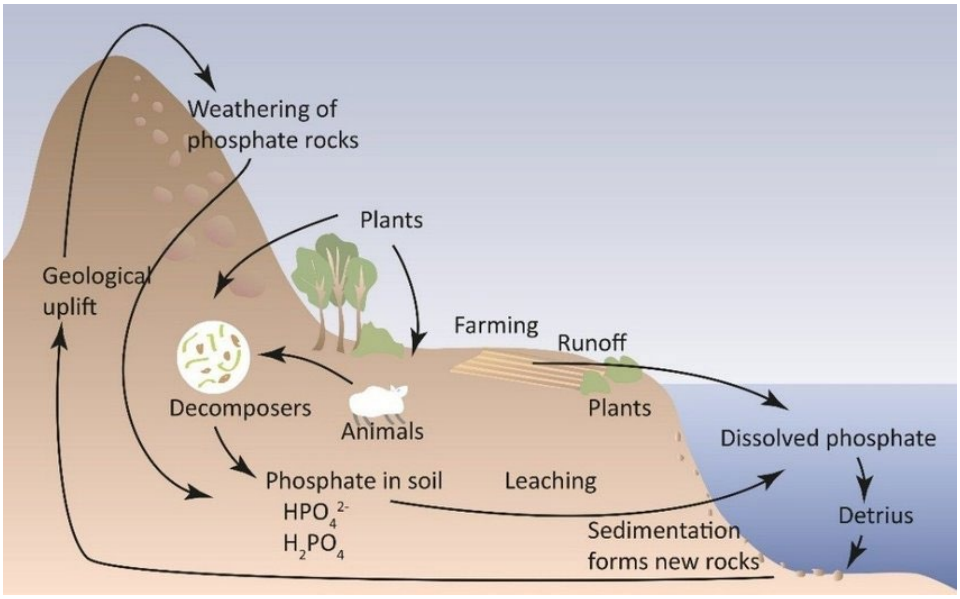

Figure 1.1. Schematic depiction of the global phosphorus cycle (Lappalainen et al., 2016).

The principal source of phosphorus is the apatite mineral pool which lies in the continental bedrock. Estimates of the global apatite pool varies between 0.8 – 4.0 billion Tg (Yuan et al., 2018). However, phosphorus becomes bioavailable only as it gets released from apatite during soil

formation process via chemical weathering. These weathering reactions are conducted as phosphorus containing minerals are exposed to naturally occurring acids derived from microbial activity. Phosphate solubilized during weathering becomes available for the uptake by terrestrial plants and is eventually returned to the soil by plant decay of litterfall. There are systematic changes occurring in the total amount phosphorus and its chemical form during the soil formation process. During the initial stages, phosphorus is present mainly as apatite. With further soil development, the primary apatite content decreases, and secondary minerals and organic-P make up an increasing fraction of soil phosphorus. In the final stages of soil development, phosphorus content is mainly formed by refractory minerals and organic phosphorus.

The phosphorus transport from continental to oceanic systems is ensured by rivers where it originates from continental rock and soils erosion. Estimations show that more than 90% of the phosphorus transported by rivers to the ocean is in the form of particulate phosphorus. The dissolved phosphorus in rivers is found in both inorganic and organic forms. The dominant form of phosphorus in river water largely depends on the geology of the catchment basin.

Upon entering the ocean phosphorus fate depends on the biogeochemical conditions in the river estuary. Net removal of phosphorus in estuaries is mainly conducted by flocculation of humic–iron complexes and biological uptake. Net phosphorus release is driven by a combination of its desorption from freshwater particles entering marine waters with high ionic strength, and flux of diagenetically mobilized phosphorus from benthic sediments.

Phosphorus as dissolved orthophosphate is consumed by photosynthetic organisms at the basis of marine food web. The marine phosphorus cycle is linked to the marine carbon and nitrogen cycles as these elements are consumed by the phytoplankton, which forms the base of marine food web. When the phosphate content is depleted, microorganisms can utilize more complex forms of phosphorus, e.g., converting the organic phosphorus species to orthophosphate via enzymatic and microbiological reactions. In the open ocean, most phosphorus associated with biogenic particles is recycled within the upper water column. A common nutrient profile in the open ocean water column is characterized by phosphorus depletion in the surface waters by photosynthetic organisms and its accumulation in deeper layers due to respiration of biogenic particles.

The general pathway for phosphorus removal from ocean systems is through the burial with marine sediments. Most phosphorus is buried in the coastal waters, where most of the terrestrial phosphorus transported by rivers is utilized for primary production. As a result, higher content of organic matter is sedimented in the coastal zone, compared to the deep sea. In addition, primary organic material is supplemented with direct terrestrial influx of particulate phosphorus. In the sediment fraction phosphorus exists mainly as calcium bound phosphorus while the remaining is bound to ferric iron, enclosed in organic matter and detrital apatite.

Microbial activity promotes phosphorus content buildup in the sediment pore waters and dissolved phosphate return to the lower water column or its incorporation in secondary authigenic minerals. This phosphorus flux from sediments is estimated to exceed the riverine input.

Reprecipitation of diagenetically mobilized phosphorus in secondary phases significantly enhances phosphorus burial efficiency, impeding return of phosphate to the water column.

The natural phosphorus cycling has been seriously altered by anthropogenic activities. The mining activities of phosphate rock for use in agriculture as fertilizer have greatly increased during the last seven decades. Consequently, with more agriculture crop and livestock food production, increasing amount of waste such as manure and sewage is generated. Also, deforestation activity increases the particulate matter content in terrestrial riverine transports to the oceans as soil becomes more prone to erosion. These actions greatly increase the phosphorus amount delivered from terrestrial to aquatic ecosystems and eventually developing in excessive nutrient availability or eutrophication.

1.2. Environmental risks by excess phosphorus loads

Human activity has an increasing impact on every aquatic ecosystem and substantially altering the growth limiting nutrient loads from terrestrial landscape to surface water. Excessive nitrogen and phosphorus load to lakes, reservoirs and coastal waters leads to their eutrophication. Phosphorus is recognized as the key limiting nutrient for primary production in freshwater ecosystems (Lepori and Capelli, 2021). Elevated concentration (>0.1 mg L^{-1}) of this nutrient is favorable for massive freshwater algae biomass production (Figure 1.2). Further it develops an adverse condition for the ecosystem status and degrades the services it provides. A healthy and functional aquatic ecosystem is crucial for economic activity and public wellbeing. It provides resources for drinking water, commercial fisheries and aquaculture, promotes the tourism and recreation businesses and adds value to the real estate properties (Dodds et al., 2009). Access to safe and clean water is important for public health, can boost economies and reduce poverty.

Algal blooms caused by eutrophication increase water turbidity which reduces the light penetration into deeper water layers. Such conditions gradually limit the photosynthetic activity and oxygen production with deeper water column. Further, the decay of dead algal biomass depletes the oxygen content in the water column. Oxygen depletion develops into hypoxic and anoxic conditions and creates the so-called *dead zones*, eventually leading to fish killings. Under eutrophic conditions the phytoplankton community shifts to cyanobacteria dominated system. The greatest concern of cyanobacteria blooms is their production of highly toxic compounds that are hazardous to humans, animals and everyone using the aquatic ecosystem services.

Figure 1.2. Algal bloom in the central Baltic Sea (Copyright: European Space Agency).

Drinking water from eutrophic sources is subjected to higher treatment costs to meet the standards for its safety and quality. Such water needs additional means of disinfection by-product treatment, taste and odor removal and prevention of health hazards caused by potential presence of toxins in treated water. In developing countries, access to healthy aquatic ecosystem, including clean water, is crucial for the community existence. Harmful algal blooms may damage the fish populations due to oxygen depletion related deaths or their exposure to toxins. This reduces the resources for commercial fisheries and further damages the business depending on commercial fishing and fish processing. Aquatic ecosystems with predominant algal and macrophyte landscape lose their appeal as tourism and recreation objects consequently affecting the related businesses. Finally, surface water eutrophication caused ecosystem deterioration seriously affects the real estate market by lowering the property value.

Over the last few decades, the possibilities to restore good ecosystem status in surface water has been extensively studied (Richter, et al., 2016). To combat eutrophication and its consequences various measures have been identified, including limiting phosphorus transport from terrestrial to aquatic ecosystems. Numerous reports show that reduction of phosphorus loads from its main sources into surface waters have greatly improved their quality (Conley et al., 2009). Estimations show that good ecosystem status restoration in the Baltic Sea requires an annual reduction of total phosphorus loads from 32 200 to 21 716 tons of phosphorus, or by 32% (HELCOM, 2015). However, a continuous effort is required to keep the anthropogenic phosphorus loads low. As wastewater of various origins is a major contributor to surface water eutrophication, it can be expected that phosphorus reduction and recovery from WWTP effluents will have a positive effect on the surface water quality and its sustainable use.

1.3. Phosphorus in municipal wastewater

Domestic wastewater is the major source of phosphorus in the municipal WWTP discharges in urbanized areas. According to HELCOM reports, municipal WWTPs delivers 4 % of the total phosphorus loads to the Baltic Sea (HELCOM, 2015).

The main source of phosphorus in domestic wastewater are human excrements and household chemicals, e.g., detergents and personal care products. Estimations show that in the 1970 and 80's the daily per capita production of domestic wastewater phosphorus was up to 5 g. However, the change of lifestyle, habits and activity have resulted in lower human input of phosphorus in domestic wastewaters, and constituting around 1.5 g of daily phosphorus inputs (Kroiss et al., 2011).

Phosphorus compound structure in domestic wastewater consists of the organic and inorganic forms. The latter exists as orthophosphate, as well as tripolyphosphates and pyrophosphates which are ingredients of detergents and toothpaste, respectively. At favorable conditions pyro- and tripolyphosphates are hydrolyzed to orthophosphate which is the form of inorganic phosphorus that is subjected for removal at the wastewater treatment process. It is also the form of phosphorus available for plant uptake, including microalgae and cyanobacteria.

To protect the receiving water bodies from degradation, both the EU Council and the associated member states have issued legislative acts for pollutant content reduction in wastewater and its allowable loads from municipal WWTPs. However, the required reduction of phosphorus content greatly depends on the agglomeration size where the WWTP operates.

The EU Urban Wastewater Treatment Directive 91/271/EEC requires phosphorus concentration reduction by at least 80 % for WWTPs operating in urban areas with more than 10 000 PE Moreover, for agglomerations with 10 000 to 100 000 PE the allowable phosphorus concentration in the WWTP effluent is 2 mg L^{-1} while for agglomerations with more than 100 000 PE it is required to reduce to 1 mg L^{-1}. However, the Article 7 of that same Directive states that the urban wastewater entering the receiving water bodies shall be subject to appropriate treatment before discharge in cases when wastewater is discharged to freshwater and estuaries from agglomerations of less than 2000 PE and discharging the wastewater to coastal waters from agglomerations of less than 10 000 PE This means that there is no legal requirement for the reduction limit for phosphorus from WWTPs operating for smaller settlements. It has been estimated that in Estonia the annual phosphorus load from small WWTPs (<2000 PE) composes 24 % of the total phosphorus discharges with wastewater (Niine et al., 2013). Moreover, a case study from Bavaria, Germany by Huber et al. (2020) showed that WWTPs with the capacity of <5000 PE makes up 36 % of the annual total phosphorus loads coming from WWTP effluents. Such a wastewater management poses a real threat to the receiving aquatic ecosystem, considering the low threshold phosphorus concentration for harmful algal bloom establishment.

1.4. Phosphorus removal from municipal wastewater

The conventional wastewater treatment typically starts with pre-treatment where coarse materials are removed. Further the treatment process undergoes three major phases (Figure 1.3) The primary physical treatment is designed to remove pollutants which will either settle or float in a clarifier tank, such as sand or grease. Primary treatment normally removes about 60 % of the suspended solids in raw sewage as well as about 35 % of the biochemical oxygen demand (BOD_5). The secondary or biological treatment further reduces the BOD_5 as well as ammonia nitrogen and phosphorus. Secondary treatment is typically achieved using the activated sludge, and the process is boosted by manipulations with oxygen availability. Tertiary or post-treatment is applied to meet specific requirements for the effluent wastewater. It is usually targeted towards removal of a specific pollutant which determines the method used. The methods include filtration or application of adsorbent such as activated carbon to remove persistent organic pollutants. It also may involve use of ferric chloride or alum to enhance the removal of phosphorous (Abu Shmeis, 2018).

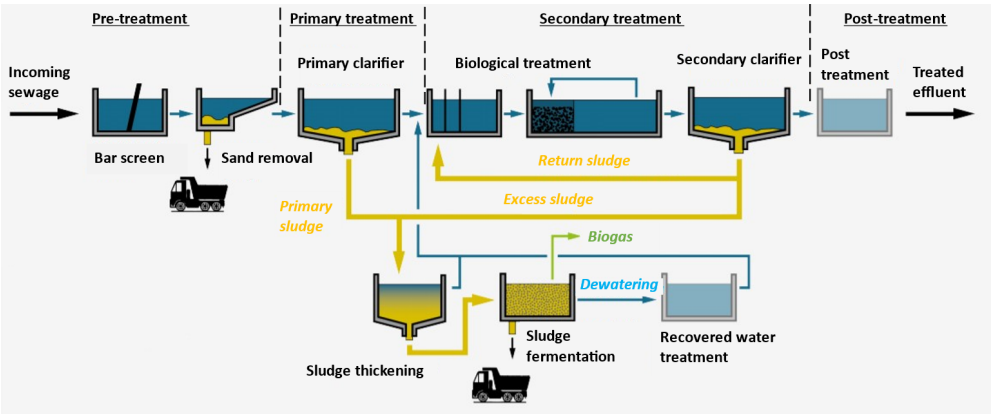

Figure 1.3. Conventional wastewater treatment plant scheme (Copyright: Jules van Lier, TU Delft)

Generally, phosphorus is removed from wastewater by transforming it from its soluble form into solids which are removed by sedimentation. The phosphorus transformation into solids can be classified in physicochemical and biological methods. Most common physicochemical process for phosphorus removal is chemical dosing of salts, e.g., trivalent metal salts, and ferric chloride, to promote phosphorus precipitation (Parsons and Smith, 2008). Commonly, the dosing of metal salts is done to either pre-treated influent, activated sludge reactors, or to the outlet from the secondary clarifier.

Typically, 90 % phosphate reduction is achieved by of chemical precipitation as additional P removal method (Thistleton, et al., 2002). However, the phosphorus recovery from precipitates is

limited. Also, the use of precipitation salts is associated with excess sludge production, raising concerns about environmental sustainability.

Phosphorus absorption by filtration media is another method for targeted phosphorus removal. Absorptive media can be produced either from naturally occurring materials, such as apatite, bauxite or limestone, industrial waste products, like fly ash, ochre or steel slag or commercial products, e.g., Filtralite^{TM} or Phoslock^{TM}. Phosphorus absorption filters are designed as separate units within the whole wastewater treatment process. However, the absorptive materials have a limited phosphorus sorption capacity. For instance, Shilton et al. (2006) used an active slag filter use for phosphorus removal from municipal wastewater, which showed a significant decrease of removal after 5 years of operation.

A less frequently used method for targeted phosphorus removal is based in ion exchange processes (Awual and Jyo, 2011). The predominant form of phosphorus in wastewater is orthophosphate which exists as anion. The principle behind ion exchange is based on the interchange between phosphate ions and the solid ion exchanger in the liquid wastewater. This process offers simultaneous phosphate removal and recovery. The most common choice for ion exchange applications in water treatment are synthetic organic resins due to their large available exchange capacities and ease of regeneration (Bektaş, et al., 2021). Guida et al., (2021) demonstrated a 95 % reduction and recovery of orthophosphate concentration in biologically treated communal wastewater using a commercial ion exchange resin. Although high P removal rates have been demonstrated at laboratory and small scales, implementation of ion exchange at a full-scale WW treatment system has been limited due to the requirement for expensive chemical addition for the recovery of P and the sensitivity of some media to pH conditions (Bunce et al., 2018).

Phosphorus can be also removed from wastewater by using microorganisms for its incorporation in cells and their removal from system as sludge. The biological methods for phosphorus removal are well established for use in municipal wastewater treatment plants. Biological phosphorus removal mainly incorporates polyphosphate accumulating microorganisms (PAOs) and is therefore considered as an environmentally friendly alternative to the chemical methods. The biochemical mechanisms behind P uptake by PAOs are mainly associated with the luxury phosphorus uptake (Khoshmanesh et al., 2002). Here, the P-removal occurs as the accumulation of P in the form of polyphosphate granules at a greater rate than necessary for their metabolic processes. The PAOs can accumulate 20 – 30 % of their dry weight as phosphorus while for regular organisms it is only 2 %. The accumulated polyphosphate is stored as an energy reserve for maintenance. It also provides a competitive advantage over ordinary heterotrophs. The growth and enhanced phosphorus uptake are promoted through manipulation with the operating conditions by circulating the sludge between anaerobic and aerobic environment (Parsons and Smith, 2008).

The enhanced biological phosphorus removal by PAOs as activated sludge is incorporated in wastewater treatment in various forms, such as membrane bioreactor (Sun, et al., 2013), biofilm reactors with fixed and moving biofilms (Yang, et al., 2010), granular activated sludge reactors

(Pronk et al., 2015) or anaerobic-anoxic reactors (Díez-Montero, et al., 2016). Still, many studies try to improve the existing methods by amending them with other processes to increase the phosphate removal rate. Wang et al. (2021) demonstrated 78.71 – 90.38 % phosphate removal from wastewater using a sequencing batch reactor operating in anaerobic-aerobic-anoxic regime over a period of 6.5 hours. Sarvajith & Nancharaiah (2022) attempted to enhance the phosphate uptake in aerobic granular sludge reactors by adding granular activated carbon and achieved 1.7 – 2.0-fold higher phosphorus removal than without added carbon. On the other hand, certain substances found in municipal wastewaters can hamper the EBPR process. For instance, Wu et al. (2021) showed that different antibiotics commonly found in wastewater inhibits phosphorus removal by PAOs due to suppression of their enzymatic activity. Interestingly, Tian et al. (2022) demonstrated that glycine had an adverse effect on the overall phosphate removal, despite previous evidence that glycine is a carbon source needed for certain PAOs.

Although the enhanced biological phosphorus removal by PAOs is fully established for use in full scale wastewater treatment and high phosphorus accumulation can be achieved, it still cannot reduce phosphorus content to an ultra-low concentration. Also, it is mainly designed for operation in a large scale, while its adjustment to small WWTPs is likely to decrease its performance efficiency (Bunce et al., 2018).

Another method for wastewater bioremediation is the use of microalgae, and it is viewed as an appropriate means for targeted phosphorus removal. Phosphorus is an essential nutrient for microalgal growth and under certain conditions it can be consumed in excess by the cells. The engineered systems for microalgae cultivation and wastewater bioremediation are well established. Photosynthesis supported biochemical activity and wide range of biomass application are viewed as the main advantages for microalgae-based wastewater treatment. However, limitations exist on its full implementation for wastewater treatment, mainly related to biomass harvest and the performance in temperate and cold climates.

1.5. Microalgae-based technologies for wastewater treatment

Nutrient removal from wastewater by microalgal production is well documented (Cai et al., 2013) and is considered as environmentally friendly and cost-effective alternative to the conventional wastewater treatment methods (Whitton et al., 2015). Being photosynthetic organism microalgae require light, inorganic carbon source and macronutrients for its biomass production. The environmental and economic benefits from microalgae use for wastewater treatment include (i) photosynthetic oxygen production instead of mechanical wastewater aeration; (ii) CO_2 sequestration form the atmosphere; (iii) nutrient, especially phosphorus uptake from the wastewater; (iv) removal of trace organic pollutants (Molinuevo-Salces et al., 2019). Additionally, the produced biomass has application possibilities as a raw material for bioenergy, food and feed production, pharmaceutical and cosmetics production or agricultural soil enrichment (Levasseur et al., 2020). Thus, the biomass value can potentially cover the wastewater treatment costs.

The mechanisms behind nutrient uptake by microalgae can be divided into direct and indirect removal. For phosphorus, the preferred form for direct cellular uptake is phosphate as H_2PO_4 or HPO_4. It is transported across the cell membrane and assimilated into nucleotides following phosphorylation for the synthesis of ribosomal RNA. A nitrogen source is therefore required for the synthesis of proteins to enable the assimilation of phosphorus. Under condition of high phosphate availability, microalgae can consume excess phosphate through a luxury uptake pathway and stores it as an acid-insoluble polyphosphate granule for use in conditions with low external phosphate concentration. A direct nutrient uptake and biomass growth promotes the alkalization of the wastewater through production of hydroxyl radicals and net uptake of protons (Larsdotter et al., 2007). Alkaline conditions promote indirect phosphorus removal through its precipitation with metal ions such as Ca, Mg and Fe at increased pH and high dissolved oxygen concentration. More than 90 % phosphorus can be removed via precipitation with pH increase to 8.5 and higher (Christensen et al., 2022).

Light and temperature are the key limiting parameters for microalgal biomass growth and productivity. Therefore, the design of bioreactors for microalgae production is focused on optimum utilization of these parameters. There are various reactor designs available for biomass growth and nutrient removal with the two major sub-categories being open and closed reactors (Figure 1.4). Open reactors are mainly represented by high-rate algal ponds (HRAPs). It is an open pond with a raceway configuration where the microalgal suspension is mixed with a paddle wheel to circulate the algal culture and prevent its settlement. Efficient light penetration is ensured by keeping the culture depth between 20 – 60 cm. Since microalgae suspension is open to the environment, mixed culture is usually produced in HRAPs. Also, the microalgal cultures in HRAPs are subjected to contamination risks and experiences constant water evaporation. The biomass concentrations usually do not exceed 1.0 g DW L^{-1}, thus long hydraulic retention time (4 – 10 days) is required for removing nutrients at the desired level (Christenson and Sims, 2011; Mehrabadi et al., 2015). Further, large HRAP installations are needed to sustain the remediation of large streams of wastewater. Overall, the employment of HRAPs for wastewater treatment requires lower capital and operational costs. However, such a design allows little control over the operational parameters, and it is limited to warm and sunny climates.

Photobioreactor is an example of a closed system for microalgae cultivation and wastewater treatment. They are available in various design configurations, such as column or tubular reactors with small diameters (e.g., 5 cm) at horizontal or vertical orientations as well as flat panel reactors. Sunlight or artificial lighting is used for PBR illumination. The suspension is mixed and circulated by air pumping. Enclosed cultures enable greater control over the biomass growth conditions. Adjustment of light, temperature, CO_2, dissolved oxygen or pH permits the growth of specific species with greater biochemical value. Also, larger biomass concentrations can be achieved than in HRAPs, exceeding 2.0 g DW L^{-1}. However, the sophisticated control over the process makes PBR expensive to construct and operate. Tredici et al. (2016) have estimated that the annual total capital and operational costs for a flat panel photobioreactor plant covering area of 1 ha would be

101 260 and 345 107 EUR, respectively. Moreover, Richardson et al. (2012) estimated that microalgae-based lipid production in PBR would be 2.5 times more costly that in HRAP.

Figure 1.4. Open (left) and closed (right) systems for microalgae production (Copyright: Green Prophet and Varicon Aqua).

The reported phosphorus removal efficiency from wastewater is highly variable as different operational parameters characterize each case. Total phosphorus removal rate is lower at colder climate and less irradiation. Larsdotter et al. (2010) performed wastewater purification with mixed microalgal species in northern Sweden and showed that in January at 11 °C and few hours of daylight with the irradiance of 70 μmol m^2 s^{-1} the phosphorus removal did not exceed 25 %. For wastewater with lower P concentration it is more likely to reach a near complete phosphate reduction. Ji et al. (2013) achieved >99.0 % phosphorus removal within 4 days in pretreated municipal wastewater with total phosphorus concentration of 1.7 mg L^{-1}. Zhou et al. (2012) used concentrated municipal wastewater with 211 mg L^{-1} phosphate and achieved 81.5 % removal at HRT of 3 - 4 days operating a 25 L semi-continuous photobioreactor. Scaling up to full wastewater treatment system, microalgal phosphorus removal rate shows further decrease. Craggs et al. (2012) demonstrated a municipal wastewater treatment in Christchurch, New Zealand at a 5 ha HRAP system. The median removal rate of dissolved reactive phosphorus varied between 14.0 to 24.4 % over a 15-month period. On the other hand, >90.0 % removal of total phosphorus is demonstrated by wastewater post-treatment facilities designed by *Clearas Water Recovery, Inc*. Their tubular photobioreactors are operating at various location across the U.S. to purify municipal wastewater with total phosphorus concentration of <2 mg L^{-1}. The different results between phosphorus removal rates in full scale HRAP and PBR show advantage of sophisticated control over the process which allows to achieve a better outcome. It also marks the possible strategy for wastewater treatment and valuable biomass production in controlled conditions for cost recovery or possible profit.

Besides the applicability for wastewater treatment, microalgae are also recognized as a source of valuable biochemical components, such as proteins, lipids, carbohydrates, pigments and minerals. Microalgae-based proteins have gained attention as a food, feed, and health product because of their nutritional value. Lipids obtained from microalgae mainly consists of triglycerides,

phospholipids, free fatty acids, and glycolipids. These compounds show high potential for alternative fuel production and are generally used for biodiesel production through extraction and transesterification. Algal carbohydrates mainly consist of cellulose, starch, glucose, and polysaccharides and are a desirable feedstock to produce value-added chemicals, such as furans, ethanol, and acetone. Pigments microalgae and cyanobacteria, such as carotenoids and phycobilins are sources for natural colorants and antioxidants for the food, cosmetics and pharmaceutical applications (Chew et al., 2017). Microalgal biomass application in agriculture and horticulture is appealing as a source of organic material and nutrients accumulated within the cell.

For many years, the research of added value product extraction from microalgae was focused on biofuel production. However, it becomes more evident that microalgae biofuel production is non-feasible due to high production costs and low financial return. Classified as a low-value product, microalgal biofuel price cannot compete with the conventional fuel as the price difference of the final product can be multifold. Recently, the research focus has shifted towards production of value-added compounds (Levasseur et al., 2020). Microalgae-based high value molecules have greater market value, and they provide natural alternatives to many synthetic products.

One of the main bottlenecks for profitable wastewater treatment with microalgae is the biomass harvest. Microalgal cells make up about 5.0 % of the whole suspension and are small in size. Therefore, methods like filtration, centrifugation or coagulation are used to harvest them. However, these methods are energy and resource intensive, making the harvest process costs to be about 20.0 – 30.0 % of the whole process (Barros et al., 2015). Such a large fraction of the total costs makes the final product more expensive, while the price for low-value products such as biofuel, becomes incompatible with conventionally produced energy. Wastewater treatment with target production of high value products for human use can be profitable. On the other hand, the presence of pathogens and hazardous substances in wastewater limits the biomass use for production products for human use or must undergo strict safety measures. Another concern for feasible microalgae biomass applications is the productivity in wastewater. Often, the municipal wastewater does not always provide the optimum combination of nutrients and microelements for high biomass productivity and valuable molecule synthesis, as the wastewater content is highly variable. Thus, it can result in lower total yields of the high value molecules. A possible strategy to overcome the low productivity of high value molecules is implementation of stress factors during the biomass production process. Stressors like pH, light or temperature can boost the synthesis of high value molecules (Shi et al., 2020). Similarly, nutrient stress boosts the biochemical processes and promotes high value molecule synthesis as well as enhanced nutrient uptake from the environment.

1.6. Microalgal biomass manipulation for enhanced phosphorus uptake and high-value molecule synthesis

Cultivation of microalgae biomass is influenced by several environmental factors, such as light and temperature as well as nutrients availability, salinity, and pH (Paliwal et al., 2017). Environmental factors not only affect the biomass productivity but also the biochemical content of the cell. The growth conditions for microalgal cells isolated from their natural environment are species specific, but under the impact of specific stressors they produce certain metabolites to overcome and adapt to the stress conditions. Such a characteristic of microalgae can be advantageous to produce desired metabolites through the abiotic stress as integrated a tool for microalgae-based wastewater treatment for a financial return.

Application of environmental stress to increase the biochemical quality of microalgal biomass is an evolving strategy for commercial biomass production. Extensive research has been carried out to assess the impact by environmental stress factors on boosting the synthesis of target microalgal metabolites (Cheng & He, 2014; Levasseur et al., 2020; Shi et al., 2020). An increase of lipid productivity and its total yield has been a major interest for microalgae biofuel production. Multiple studies have demonstrated that after microalgal biomass exposure to high light intensity, the total polar lipid content decreases, while the contents of neutral storage lipids increase (Hu et al., 2008). Moreover, in many lipids producing microalgal species lower temperature resulted in polar lipid content increase, while higher temperature boosted the accumulation of nonpolar lipids (TAG) (Renaud et al., 2002). Temperature stress at 35 °C led to an elevation in the lipid content and increased accumulation of neutral lipid in *Acutodesmus dimorphus* (Chokshi et al., 2015). Several studies have showed that the production of carotenoids, including β-carotene, astaxanthin, and lutein can be enhanced by nitrogen limitation with microalgal species like *Chlorella zofingiensis*, *Dunaliella salina*, *Neochloris oleoabundans*, and *Muriellopsis sp.* (Del Campo et al., 2000; Mulders et al., 2014; Urreta et al., 2014). Nitrogen deficiency can also promote accumulation of both lipids and astaxanthin in microalgae (Chen et al., 2015; Liu J. et al., 2016). Also, synthesis of polymers such as polyhydroxyalkanoates (PHA) and exopolysaccharides (EPS) by cyanobacteria can be enhanced under the stress of high salinity and high temperature, respectively (Paliwal et al., 2017).

A variety of effects on microalgal growth and high value substance synthesis caused by phosphorus stress have been reported. Its deficiency in a growth medium hampers the photosynthetic activity and affects the growth of microalgae. However, phosphorus has a significant impact on metabolic processes of microalgae (Fan et al., 2014). It has been demonstrated that limitation of phosphates resulted in increased lipid accumulation in microalgal cells. For example, in the freshwater microalgae *Scenedesmus sp.* (Xin et al., 2010) and *Botryococcus braunii* (Venkatesan et al., 2013), net lipid productivity increased under phosphorus deficiency conditions. Markou et al. (2012), showed that growing under phosphorus stress, the carbohydrate content increased from 11.0 to 67.0 % in *Arthrospira platensis* (Minhas, et al., 2016).

Reitan et al. (1994) showed observed unsaturated fatty acid increase in marine microalga under phosphorus limitation conations. On the other hand, elevated phosphorus content can promote the productivity of eicosapentaenoic acid (Yongmanitchai and Ward, 1991). It is suggested that phosphorus limitation can also have an indirect effect on metabolite synthesis. Perez-Garcia, et al. (2011) and Wu et al. (2021) both discussed that enhanced phosphorus assimilation caused by prior phosphorus deprivation can accelerate the synthesis of adenosine triphosphate (ATP), which is further used as an energy source for protein synthesis.

The advantages for microalgal biochemical content caused by phosphorus stress are driven by the phosphorus accumulation and storage mechanisms. Normally, phosphorous makes up ~0.03 – 0.06 % of total algal biomass (Minhas et al., 2016). With limited P availability microalgal cells contain approximately 1.0 % of P in cell dry weight (Grobbelaar 2013, 2004). Under the conditions of phosphorus deficiency followed by high phosphorus availability, microalgal cells activate the luxury P uptake mechanism, which leads to a substantial increase in cell P content by up to 4.0 – 6.0 % of the dry weigh (Powell et al. 2008, 2009; Dyhrman 2016; Schreiber et al. 2018). This extra phosphorus is stored as polyphosphate and is used as an internal phosphorus source when the external availability of phosphorus is limited. The polyphosphate acquired during luxury uptake is present in the algal cell as acid soluble and acid insoluble polyphosphate. The acid soluble polyphosphate fraction is further used for the synthesis of cell constituents, such as protein, DNA or RNA, while the acid insoluble polyphosphate provides the storage P reserves for conditions of external P limitation (Figure 1.5) (Powell et al., 2008; Su, 2021). However, luxury P uptake is thought to be a result of phosphorus availability in large quantities. This mechanism marks another advantage by phosphorus stress conditions that can be integrated in wastewater treatment with possible financial return. Several studies have demonstrated an enhanced phosphorus uptake from the environment after microalgal biomass exposure to phosphorus deficiency. Hernandez et al. (2006) showed more than two times higher phosphorus removal by *C. sorokiniana* cell after their exposure to phosphorus starvation for 72 hours. Moreover, Solovchenko et al. (2019) showed approx. 95.0 % phosphate removal within 20 hours after the polyphosphate content in *C. vulgaris* cells where entirely depleted. An enhanced phosphorus uptake by P-stressed microalgal biomass in comparison with regular biomass was also reported by Eixler et al. (2006), Wu et al. (2012) and Yewalkar-Kulkarni et al. (2017).

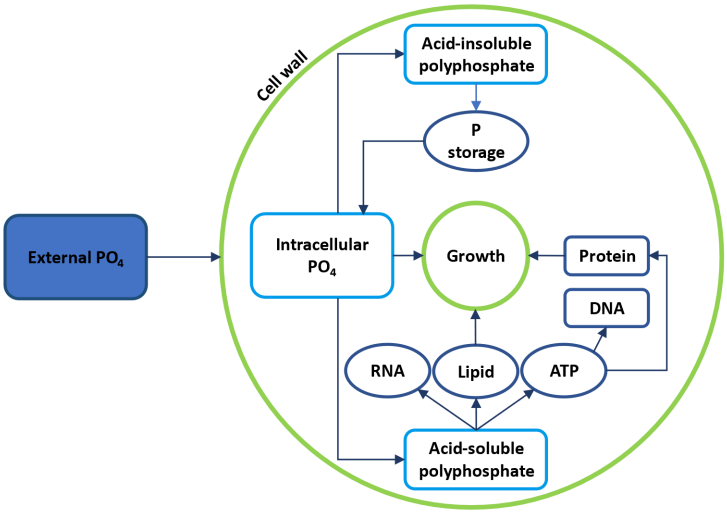

Figure 1.5. Schematic depiction of phosphorus uptake and transformation pathways within the algal cell.

Despite the promising performance, microalgal biomass exposure to phosphorus stress has limitations and knowledge gaps for its successful integration in wastewater treatment and valuable biomass production. Firstly, the reported results on biomass P-starvation effect on phosphate removal and high value molecule synthesis are obtained at experimental scale in laboratory or small volume photobioreactors. Thus, it remains unknown how P-starvation would affect the result at full scale operations and what are the additional considerations necessary for successful result in real conditions. Secondly, because phosphorus is a key nutrient for sustainable microalgal growth, its limitation and long periods of deficiency negatively affects the biomass growth and productivity. Nearly every report on enhanced phosphorus uptake and high value molecule synthesis shows a decrease in microalgal biomass production, which lowers the total yield of biomass and its produced molecules. Nevertheless, this shortcoming marks a direction for further research on biomass phosphorus deficiency effects and its possible advantages.

2. PRESENT STUDY

2.1. The main tasks of the present study

The main goal of this study was to investigate the potential of microalgae for enhanced phosphorus removal from municipal wastewater during the post-treatment step. Therefore, the main scientific question addressed in this study was: *Can microalgae rapidly reduce phosphorus content to ultra-low level in municipal wastewater after its biomass exposure to phosphorus deficiency?* According to this question, the following tasks were set to complete this study:

- To evaluate the present status of microalgae-based wastewater treatment and the associated challenges for phosphorus removal from municipal wastewater.
- To identify microalgal species that can grow in wastewaters, efficiently remove nutrients and provide a biomass for high value product extraction.
- To evaluate the biomass growth and nutrient removal rates of various microalgal species in different types of municipal wastewaters under batch conditions.
- To assess the microalgal biomass exposure to phosphorus deficiency as a technique for enhanced phosphorus uptake from wastewater.
- To identify indicators for quantification of the microalgal phosphorus deficiency status.
- To study the metabolic pathways of phosphorus for microalgal cells after its exposure to phosphorus deficiency.
- To identify the optimum conditions for municipal wastewater post-treatment in sequenced batch photobioreactor.
- To evaluate the prerequisites for scale-up of microalgae-based wastewater post-treatment with integrated biomass phosphorus starvation technique.

To obtain the goal and complete the tasks, a literature study and experiments were carried out and the results were reported in one literature review and three scientific research papers.

Although there are several operational full-scale microalgae-based wastewater treatment plants in the world, many drawbacks and challenges exist that hold back a major shift towards this technology. The main obstacles for microalgae-based wastewater treatment are its limitation to certain climatic conditions to ensure high operational performance and high costs for biomass harvesting. Therefore, one of the main objectives of the **Paper I** was to review the status of microalgae-based wastewater treatment and its major limitations for full scale use. In this paper the current solutions and possible strategies are reviewed to overcome the main challenges for microalgae-based wastewater treatment, which include biomass harvest, biomass exposure to pathogens, removal of micropollutants and operation in cold climate.

In **Paper II** the main task was to test and approbate the approach of microalgae biomass phosphorus starvation to enhance phosphorus removal from wastewater. To achieve this task, three microalgal species *Desmodesmus communis*, *Tetradesmus obliquus* and *Chlorella protothecoides* were first exposed to phosphorus deficiency conditions and then cultivated in municipal wastewater

to study the biomass growth and nutrient removal. Also, the phosphorus removal rates, and polyphosphate accumulation were compared between various phosphorus starvation periods. Finally, the main drivers behind the obtained results were discussed.

In **Paper III** the effect of 3- and 5-day phosphorus starvation periods was tested on biomass growth, nutrient uptake rates and polyphosphate accumulation by microalgal species *Botrycoccus braunii*, *Chlorella vulgaris*, *Ankistrodesmus falcatus* and *Tetradesmus obliquus.* Unlike in **Paper II**, this experiment was done under batch conditions with aeration and CO_2 supply and the illumination of blue and red spectrum. Finally, data on alkaline phosphatase activity was collected to study this enzyme as a possible indicator for microalgal biomass phosphorus deficiency status quantification.

The aim of **Paper IV** was to identify the optimum initial conditions for highest biomass growth and phosphorus removal rates at the shortest phosphorus starvation period. The study consisted of two phases. First, the microalgal specie *Chlorella vulgaris* was cultivated in a synthetic wastewater using the batch setup from **Paper III**. The obtained data on biomass growth, specific phosphate removal, polyphosphate accumulation and protein productivity were analyzed using an optimization model to determine the optimum initial conditions for highest possible gain. A different biomass starvation technique was used than in **Papers II** and **III**, focusing on biomass polyphosphate content reduction. In the second experiment phase, the obtained results for initial conditions optimization were verified in a sequencing batch photobioreactor, comparing the performance between regular and phosphorus-starved biomass.

The novelty behind this study is the demonstration of microalgae biomass P-starvation with its target application for municipal wastewater post-treatment with cost recovery option. To the author's best knowledge, no currently operating microalgae-based wastewater treatment system is supplemented with biomass manipulation with P-stress to achieve an enhanced phosphorus removal.

2.2. Summary of the materials and methods

Algal strains and cultivation conditions

In **Paper II** the photoautotrophic microalgae strains *Desmodesmus communis* (CCAP 276/4B) and *Tetradesmus obliquus* (CCAP 276/10), and the facultatively heterotrophic strain *Chlorella protothecoides* (CCAP 211/10C) from the Culture Collection of Algae and Protozoa (United Kingdom) were used. All strains were pre-grown using the synthetic growth mediums BG-11 or EG (Table 2.1).

In **Paper III** photoautotrophic microalgae strains *Botrycoccus braunii* (CCAP 807/1), *Chlorella vulgaris* (CCAP 211/11B), *Ankistrodesmus falcatus* (CCAP 202/5C) as well as *Tetradesmus obliquus* from **Paper II** were used. These strains were selected for their reported ability to grow in various types of wastewaters, reduce nutrient content, and produce valuable substances such as carbohydrates and lipids.

In **Paper IV** *Chlorella vulgaris* from **Paper III** was used as the most promising strain for phosphorus starvation induced enhanced phosphorus removal from municipal wastewater.

Table 2.1.

Chemical content of the BG-11 and EG growth mediums

BG-11 (Blue-Green medium)		**EG (Euglena Gracilis medium)**	
Ingredient	*Amount added per one liter medium*	*Ingredient*	*Amount added per one liter medium*
$NaNO_3$	150.00 g	$CaCl_2$ (1 g L^{-1} solution)	10 ml
K_2HPO_4	4.00 g	Sodium acetate trihydrate	1.00 g
$MgSO_4 \cdot 7H_2O$	7.50 g	Lab-Lemco powder	1.00 g
$CaCl_2 \cdot H_2O$	3.60 g	Tryptone	2.00 g
Citric acid	0.60 g	Yeast extract	2.00 g
Ammonium ferric citrate green	0.60 g		
$EDTANa_2$	0.10 g		
Na_2CO_3	0.20 g		
Trace metal solution:	1 ml		
H_3BO_3	2.86 g		
$MnCl_2 \cdot 4H_2O$	1.81 g		
$ZnSO_4 \cdot 7H_2O$	0.22 g		
$Na_2MoO_4 \cdot 2H_2O$	0.39 g		
$CuSO_4 \cdot 5H_2O$	0.08 g		
$Co(NO_3)_2 \cdot 6H_2O$	0.05 g		

Wastewater

In **Paper II** a wastewater from a treatment plant in Roja (Latvia) village (57.506465 N, 22.809536 E) was used. Both the incoming sewage and the effluent after biochemical oxidation and secondary settling were used. Both types of wastewaters were clarified from suspended solids before using for experiments. In **Paper III** the only effluent from the wastewater treatment plant in Roja was used.

In **Paper IV** the BG-11 growth medium was used in both batch experiment and sequencing batch photobioreactor as a synthetic wastewater to mimic the optimum N:P molar ratio for microalgae growth and focus on the phosphorus removal and its metabolic pathways in controlled conditions, possibly avoiding any biological and chemical interferences occurring in real wastewater.

Microalgal biomass starvation

In **Papers II** and **III** the biomass phosphorus starvation was done by growing the biomass in a phosphate-free (without K_2HPO_4) BG-11 growth medium. In **Paper II** microalgae biomass was exposed to P-deficit conditions for 7 and 14 days. In **Paper III** the P-deficiency periods were 3 and 5 days. In both papers, biomass grown with sufficient phosphate availability was used for reference (Figure 2.1).

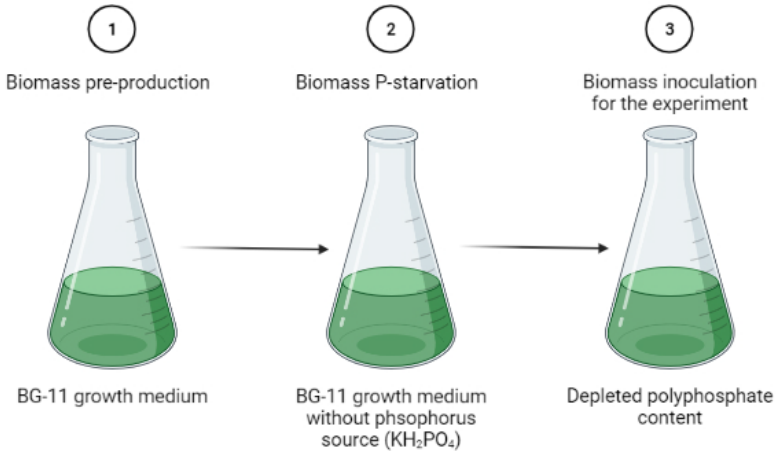

Figure 2.1. Process scheme for microalgal biomass phosphorus starvation in phosphate-free medium. Illustration prepared using BioRender™.

In **Paper IV** the phosphorus starvation status was established by algal biomass cultivation until its growth reached a stationary phase and did not show any biomass concentration increase for three consecutive days. Every next day marked a period for biomass exposure to phosphorus deficiency (Figure 2.2). The effect of biomass phosphorus starvation periods of 1, 4, 7 and 10 days were studied. Biomass that reached late exponential growth phase was used for reference.

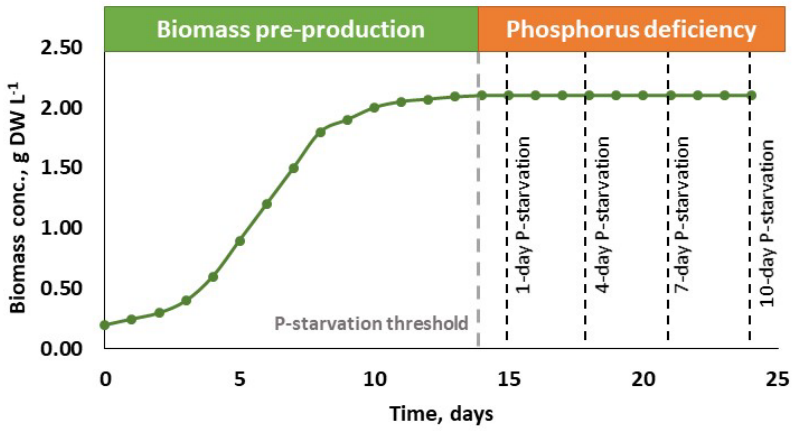

Figure 2.2. Microalgal biomass phosphorus starvation process with polyphosphate content depletion.

Batch experiment setup

In **Paper II** the microalgae growth, nutrient removal efficiency and polyphosphate accumulation was assessed under batch conditions, using 1000 ml Pyrex® bottles with a working volume of 800 ml. Bottles were placed on an orbital shaker with rotation period of 140 rpm, under white fluorescent light with the intensity of 100 µmol m^2 s^{-1} and 16:8-h lighting regime, at a room temperature (24 – 27 °C) for 10 days No additional aeration and CO_2 supply was provided (Figure 2.3).

Figure 2.3. Batch experiment setup on orbital shaker. Illustration prepared using BioRender™.

In **Papers III** and **IV** the culture mixing was provided by aeration (10 L h^{-1}) with 1.0 % (v/v) CO_2 supply. The bottles were illuminated with fluorescent light with blue-red spectrum with the intensity of 180 µmol m^2 s^{-1} at a 16:8-h lighting regime (Figure 2.4). In **Papers III** and **IV** each experiment was run for 10 days and 72 hours, respectively.

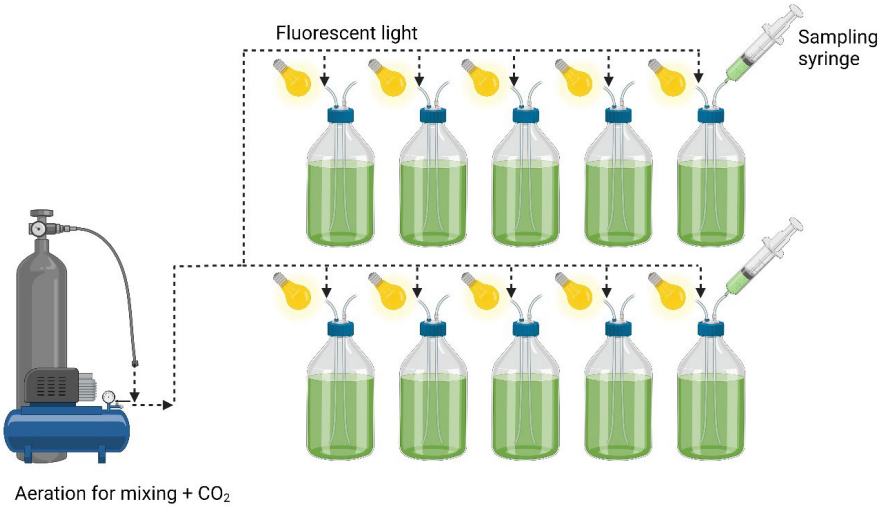

Figure 2.4. Batch experiment setup with aeration. Illustration prepared using BioRender™.

Photobioreactor configuration and operational conditions

In **Paper IV** the optimum initial biomass and phosphate concentrations and P-starvation period obtained suggested by the optimization model was verified in a sequencing batch photobioreactor (PBR). A laboratory scale PBR (Figure 2.5), manufactured by *A/S Biotehniskais centrs* (Latvia) was used for the experiment. The total PBR volume was 5.4 L, and it was operated with 4.5 L microalgal biomass suspension. The PBR was provided with air at flow rate of 0.5 L min^{-1} and 3.0 % (v/v) CO_2 supply. The biomass suspension was mixed by a magnetic stirrer at 150 rpm. LED diodes provided the photosynthetically active radiation (PAR) of 150 µmol m^{-2} s^{-1} with light/dark regime of 16:8 hours. Fresh synthetic medium (BG-11) was added when biomass concentration reached its maximum or when the studied P-starvation period was reached. Samples for biomass, nutrient, polyphosphate, APA and protein analyses were taken every 1–3 days.

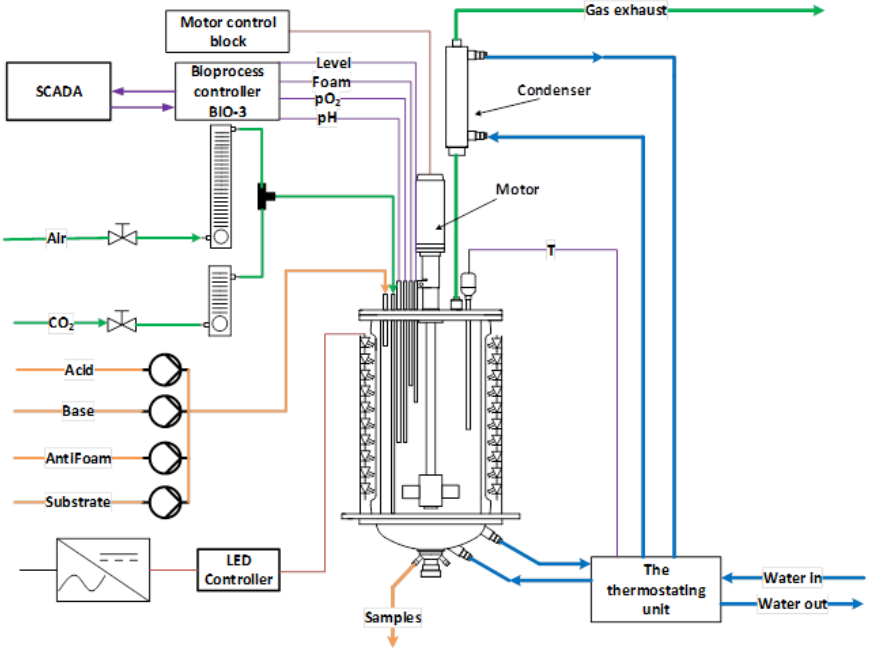

Figure 2.5. The schematic diagram of sequencing batch photobioreactor for microalgae cultivation and synthetic wastewater treatment (Copyright: Bioreactors.net).

Analytical methods

In all papers the biomass concentration, phosphate and nitrate concentrations as well as the biomass polyphosphate content was analyzed.

The biomass concentration was determined using the linear correlation between spectrophotometric light absorption at 680 nm and biomass dry weight. The light absorbance at 680 nm is proportional to the change of cell number in most unicellular organisms (Wang et al., 2016) and the maximum light absorbance for most microalgae is found at the given wavelength (Yatirajula et al., 2019). The OD_{680} values were kept below 1.200 and the samples were diluted when exceeding this value. Algae biomass growth was detected spectrophotometrically (Thermo Scientific Genesys 150 UV-Visible Spectrophotometer). To determine the algal biomass dry weight, a sample of biomass and growth medium suspension was filtered through cellulose membrane with 0.45 µm pore size. The fresh biomass sample was dried in oven for 24 hours at 80 °C and weighted on analytical scale. A growth curve was prepared for each algal strain at the beginning of each experiment. Equations describing the linear relationship between algal dry weigh biomass (g DW L^{-1}) and light absorbance value at OD_{680} for each experiment are shown in **Papers II**, **III** and **IV**.

Nutrient concentrations were measured using a pre-programmed spectrophotometric system HACH LANGE DR 3900 and commercial reagent kits. To follow the algal nutrient consumption from wastewater, concentration changes for orthophosphate, nitrate as well as total nitrogen and total phosphorus were observed. Total nitrogen and nitrate concentrations were measured using alkaline persulfate digestion method (Patton and Kryskalla, 2003) and chromotropic acid method (Cogan et al., 2015), respectively. Total phosphorus and orthophosphate concentrations were measured using the ascorbic acid method (APHA, 2018).

Polyphosphate content in algal biomass was measured following principal steps described by Mukherjee & Ray (2015). Polyphosphates were extracted from microalgal biomass through a series of physical and chemical procedures. Algal biomass suspension was concentrated to harvest enough biomass (>0.05 g fresh weight) for the extraction procedure. The algal cell walls were disrupted using an ultrasonic processing (Branson Bransonic® CPXH Digital Bath 3800) for 20 minutes at 40 kHz. The disrupted cell samples were heated at 100 °C for 2 hours. Then a mixture of chloroform and isoamyl alcohol (24:1) was added to the biomass and mixed vigorously. The suspension was centrifuged at 13 520 g for 15 minutes. Afterwards, a 0.3 ml sample of the supernatant containing polyphosphate extract was collected and mixed with 3 ml 0.2 N acetic acid and 3 ml toluidine blue solution (stock conc. 30 mg L^{-1}) for spectrophotometric light adsorption measurement at 630 nm. Biomass polyphosphate concentration (μg mg^{-1}) was calculated against a calibration curve constructed using a sodium phosphate glass Type-45 (Sigma-Aldrich) as a polyphosphate standard.

In **Papers III** and **IV** the alkaline phosphatase activity (APA) was measured to estimate the phosphorus deficiency status for microalgal biomass. APA was determined using the linear correlation between spectrophotometric light absorption at 405 nm and para-nitrophenol (p-NP, Sigma-Aldrich) standard concentration. The alkaline phosphatase activity was estimated by para-nitrophenyl phosphate disodium hexahydrate (p-NPP, Sigma-Aldrich) hydrolysis to p-NP by the enzyme released from microalgal cells. Biomass suspension sample to obtain ~10 mg DW biomass was centrifuged at 5000 rpm for 2 min, and the supernatant was discarded. The recovered cell pellet was dissolved in 3mL of demineralized water. The recovered sample was mixed with 0.5 mL Tris HCl buffer (pH 9.5) and 0.4 mL p-NPP (0.5 mg mL^{-1}). The mixture was incubated at 37 °C for 1 h in the dark. The yielded p-NP was measured spectrophotometrically at 405 nm. The hydrolyzed amount of p-NP was calculated using an equation obtained from a calibration curve constructed using a p-NP as a standard. The result was used as an indicator for the alkaline phosphatase activity. A control containing no biomass was included in the routine, and its OD_{405} reading was used as a blank. The alkaline phosphatase activity was expressed as p-NP flux from dry-weight biomass per hour.

In **Paper IV** biomass protein content was determined using the linear correlation between spectrophotometric light absorption at 595 nm and Bovine Serum Albumin (Sigma-Aldrich) standard concentration. The total protein content in algal biomass was determined following the procedure reported by Vazirzadeh *et al.* (2022). In brief, the biomass suspension sample to obtain

5 – 6 mg DW biomass was centrifuged at 2500 rpm for 10 min, and the supernatant was discarded. The recovered cell pellet was mixed with 1 ml 1M NaOH and heated at 100 °C for 2 h. The thermally processed sample was cooled to room temperature and biomass settled to the vial bottom. 50 µl of the processed sample containing no biomass was mixed with 1.5 mL Bradford reagent (Sigma-Aldrich). The solution was incubated at room temperature for 10 min. The light absorbance in the sample was measured spectrophotometrically at a wavelength of 595 nm. A solution of 50 µl 1 M NaOH mixed with 1.5 mL Bradford reagent was used as blank. The measured protein concentration to sample volume in the biomass extract (mg ml^{-1}) and the dry biomass concentration in the original suspension (g DW L^{-1}) were used to express the protein content as mg g^{-1} dry biomass.

Statistical analyses

Parametric one-way ANOVA test was used to detect the significant differences between the measured parameters among different biomass P-starvation periods. Levene's test was used to indicate the homogeneity of variance between the comparison groups. Tukey post-hoc test was used to detect pairwise differences between individual groups. For small data sets (n = 5) the difference between them was assessed using the Mann-Whitney U test. The limit of statistical significance in all tests was set to $\alpha \leq 0.05$. All results are presented as mean values from three experiment repetitions (n = 3) with standard deviation (±SD).

2.3. Results

Species identification for P-starvation and biomass production

In **Paper II** the microalgal species *D. communis*, *T. obliquus* and *C. protothecoides* were cultivated in primary and secondary municipal wastewaters. The results showed that the selected algal strains were able to grow in both types of wastewaters. Moreover, the growth rates of *D. communis* and *T. obliquus* after 7-day P-starvation in the secondary wastewater were 110.0 and 35.0 % higher, respectively, when compared to reference conditions (Figure 2.6). In contrast, the growth rate of *C. protothecoides* in both types of wastewaters decreased after P-starvation. Statistically, *D. communis* and *C. protothecoides* biomass growth showed significant difference ($p \leq 0.004$ and ≤ 0.023, respectively) between the applied biomass pre-treatments. However, due to showing higher growth rates after biomass P-starvation, *D. communis* and *T. obliquus* were identified as more suitable strains for biomass production and municipal wastewater post-treatment complemented with biomass P-starvation and were selected for further study.

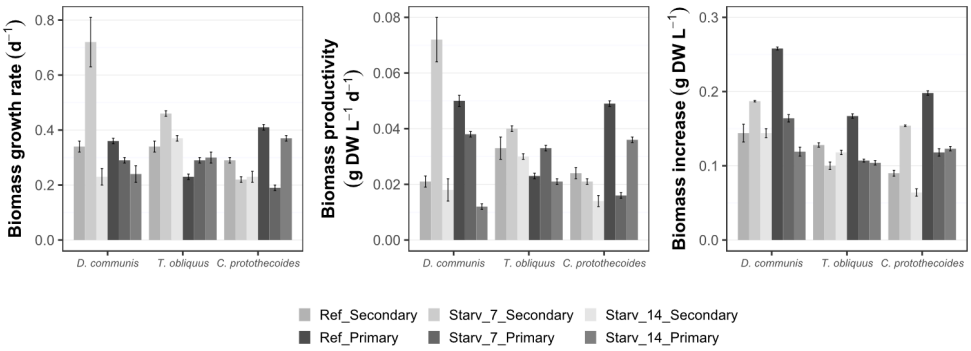

Figure 2.6. Biomass growth rate, productivity and increase (means ± SD, n = 3) for *D. communis, T. obliquus* and *C. protothecoides* obtained in primary and secondary wastewaters after 7- and 14-day P-starvation and at the reference conditions with no biomass P-starvation.

In **Paper III**, in addition to *T. obliquus*, species such as *C. vulgaris, B. braunii* and *A. falcatus* were tested for growth in secondary wastewater after 3- and 5-day P-starvation periods. All species, except *D. communis*, could produce biomass and no growth inhibition or cell death were observed due to biomass exposure to phosphorus deficiency (Figure 2.7). All the species, except *T. obliquus,* showed no significant difference for biomass growth after different P-starvation periods (p >0.05). On the other hand, the biomass concentrations obtained during its growth showed large uncertainty for *T. obliquus* as the standard deviation was more than ±50 % from the mean biomass concentration value for the biomass after its P-starvation. Also, the biomass concentrations for *B. braunii* and *A. falcatus* showed large variations with their standard deviation reaching ±30 % and ±50 %, respectively. *C. vulgaris* showed relatively more consistent biomass growth, as the standard deviation for its biomass concentration was less than ±20 % from the measured biomass concentrations.

The obtained results from **Papers II** and **III** show increase of biomass growth after its exposure to phosphorus deficiency. Tan et al. (2014) suggests that such an effect by P-starvation on biomass growth is caused by shift in cellular biosynthetic pathway which develops under nutrient deficiency conditions. In such a case the photoassimilates that are stored in the lipid bodies in the cytoplasm ensure the microalgae survival under stress conditions. Also, cellular phosphorus and energy reserves were available during the P-starvation period, which sustained the biomass growth. When inoculated in secondary wastewater with high phosphate content, optimal conditions for algal biomass growth were reestablished. Similar observations were also made by Hernandez et al., (2006).

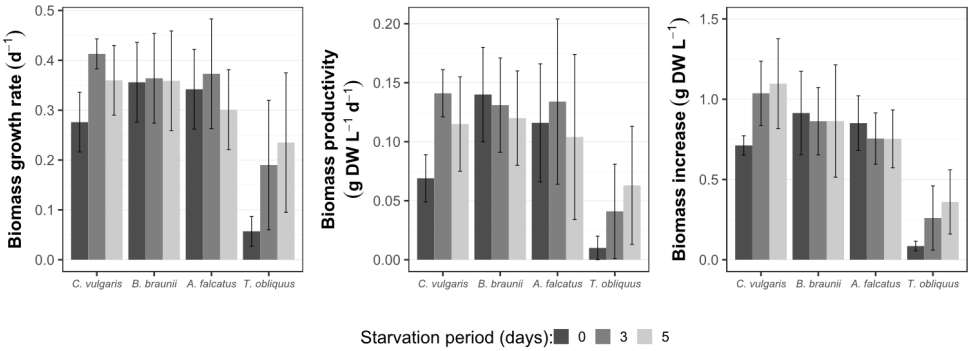

Figure 2.7. Biomass growth rate, productivity and increase (means ± SD, n = 3) for *C. vulgaris, B. braunii, A. falcatus* and *T. obliquus* in secondary wastewater after 3- and 5-day P-starvation and at the reference conditions with no biomass P-starvation.

Among all the microalgal species from **Papers II** and **III** experimentally tested for growth in real wastewaters, *C. vulgaris* showed relatively more stable biomass growth in terms of measurement errors, also when produced after its biomass exposure to P-deficiency. The ability of *C. vulgaris* to grow in various types of wastewaters have been widely reported (Wang et al., 2010) and it is often selected for commercial purposes (Görs et al., 2010). Moreover, the observed biomass growth rates, productivity, its increase as well as the response to phosphorus deficiency for *C. vulgaris* showed variance to a lesser extent than the other studied microalgal species. Considering previous reports and the results obtained in present studies, *C. vulgaris* was identified as a more appropriate selection for nutrient removal and high value molecule production at wastewater post-treatment.

Nutrient removal from municipal wastewater under batch conditions

In **Paper II** the removal of dissolved inorganic phosphorus and nitrogen from primary and secondary wastewater after biomass P-starvation was studied. All strains showed high phosphate removal capacity at the end of a 10-day experiment. However, the removal rates varied in different types of wastewaters and after different periods of P-starvation. Reference *D. communis* and *T. obliquus* nearly completely (>99.0 %) removed phosphate from secondary wastewater within 5 and 3 days, respectively (Figure 2.8). At the same conditions *C. protothecoides* could remove 93.9 % in 10 days. In primary wastewater the nutrient removal rate was lower for all three strains - 97.7, 96.6 and 84.0 % for *D. communis, T. obliquus* and *C. protothecoides*, respectively, within 10 days. Biomass exposure to P-starvation for 7-days boosted the phosphate removal by *D. communis* and *T. obliquus*, which reduced the phosphate content in secondary wastewater by 88.7 and 89.5 %, respectively, within the first 24 h after inoculation and by >99.0 % after two days. This result is likely to be related to luxury P uptake. This phosphorus uptake and storage mechanism can be triggered by biomass P-starvation, which decreases the cellular phosphorus

reserves. When re-supplied with high external phosphate, algal cells rapidly accumulate more phosphorus than needed for their growth. No distinct P-starvation effect on phosphate removal by *C. protothecoides* – complete PO_4 concentration reduction was obtained at the very end of experiment and only 27.5 % of phosphate was removed within the first 24 h.

Among the studied species, *D. communis* and *T. obliquus* showed statistically significant difference for PO_4 removal between different biomass pre-treatments with *p*-values ≤0.034 and 0.024, respectively.

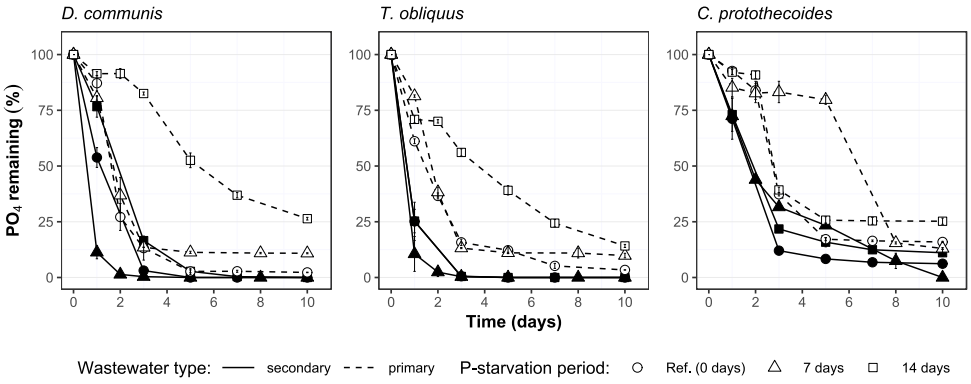

Figure 2.8. Phosphate removal rate (means ± SD, *n* = 3) for *D. communis, T. obliquus* and *C. protothecoides* obtained in primary and secondary wastewaters after 7- and 14-day P-starvation and at the reference conditions with no biomass P-starvation.

The removal of dissolved inorganic nitrogen (DIN) for all three strains at all treatments was low. The highest efficiency in 10 days growth experiment was observed for *D. communis* that had a prior 7-day P starvation period, removing 49.0 and 46.5 % from secondary and primary wastewater, respectively. 7-day P starved *T. obliquus* could remove only 41.1 % of DIN from primary wastewater, other treatments removal was below 28.0%. *C. protothecoides* could remove only between 0 and 17.1 % of DIN.

In **Paper III** the removal of phosphates and nitrates were studied in secondary wastewater after biomass exposure to shorter P-deficiency periods than in **Paper II**. All species, except *T. obliquus*, showed high nutrient removal rates, reaching more than 97.0 and 91.0 % reduction of nitrate and phosphate, respectively. Nitrate removal was not affected by prior biomass exposure to P-deficiency conditions. The maximum nitrate removal for *C. vulgaris*, *B. braunii*, and *A. falcatus* was obtained on days 5 to 6.

Higher phosphate removal rates were observed for *C. vulgaris* and *B. braunii* after both periods of prior biomass exposure to P-deficiency and reached near-complete (>99.0 %) PO_4 reduction. *A. falcatus* at the same time showed near-complete PO_4 reduction only after 3-day phosphorus

starvation. In general, the highest phosphate removal rate was recorded after 10 days of growth, except for 3-day starved *C. vulgaris* and *A. falcatus* which both reduced phosphate by 99.2 % on day 7 (Figure 2.9). Still, none of the studied microalgal species showed statistically significant difference (p-values >0.05) for the PO_4 removal at different biomass pre-treatments.

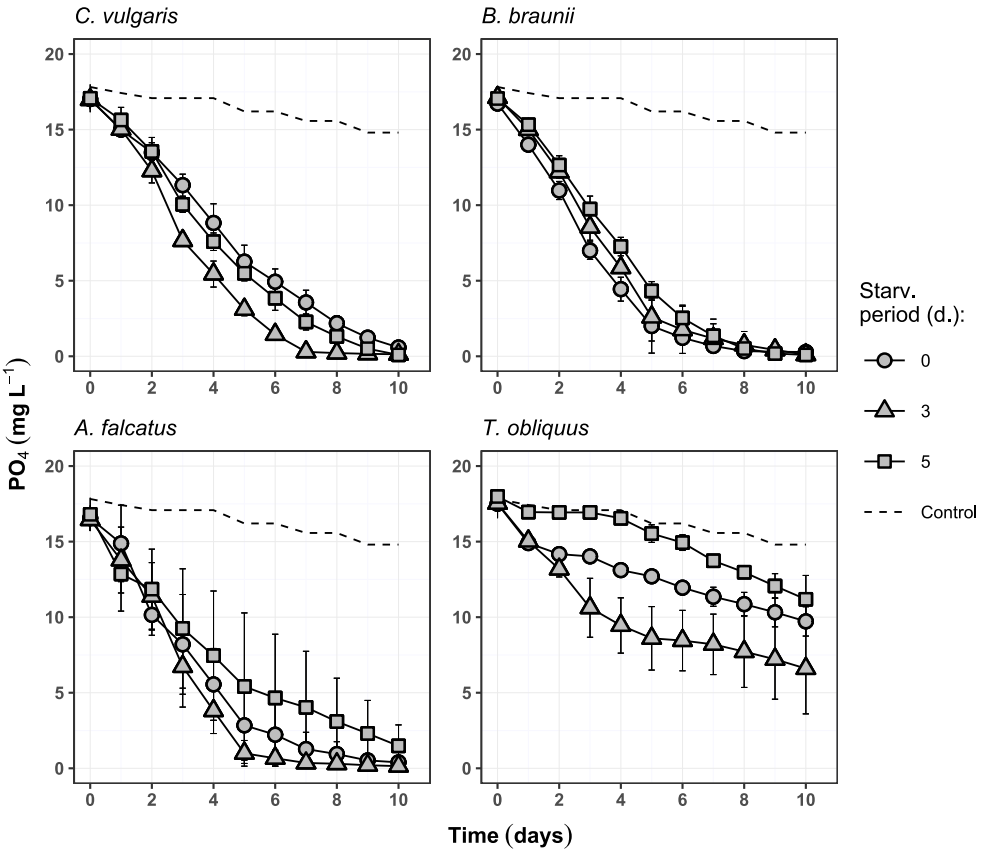

Figure 2.9. Phosphorus removal (means ± SD, n = 3) for *C. vulgaris, B. braunii, A. falcatus* and *T. obliquus* in secondary wastewater after 3- and 5-day P-starvation and at the reference conditions with no biomass P-starvation. A batch without biomass was used as a negative control.

The obtained results highlight two possible obstacles to enhance phosphate uptake by P-starved biomass. Firstly, the low N/P ratio in the wastewater as well as faster removal of nitrogen indicate the importance of nitrogen limitation. This condition makes nitrate the preferred nutrient over phosphate, omitting the possible biomass status of P-deficiency. Secondly, rapid phosphate removal by P-starved biomass in other studies was achieved at conditions where cellular

phosphorus was depleted. However, in this study to mimic practical conditions, internal biomass phosphorus reserve in the form of polyphosphate was still available after biomass exposure to P-deficiency (Figure 2.11). This condition emphasizes the prospective complexity of P-starvation implementation in pilot-scale wastewater post-treatment.

Phosphorus uptake and metabolic pathways within the algal cell under phosphorus deficiency conditions

In **Paper II** the polyphosphate accumulation in microalgal cells and its dynamics were studied as the response to biomass P-starvation and the possible involvement of luxury P uptake mechanism. All three algal strains used in the experiment could accumulate polyphosphates while growing in the wastewater. At high initial phosphate concentration, the accumulation of biomass polyphosphate occurred. When no more phosphate from the wastewater was available, the accumulated polyphosphate was used to sustain the cell doubling process and the polyphosphate reserves rapidly decreased. Higher polyphosphate accumulation for *D. communis* and *T. obliquus* was observed when biomass was growing in primary wastewater after 7 days of biomass P-starvation (Figure 2.10). For *C. protothecoides* peak value was obtained in primary wastewater without prior P-starvation. In this case the higher polyphosphate accumulation in primary wastewater is related to lower pH during the initial phase of experiment. Due to the excess organic carbon present in primary wastewater and its oxidation to CO_2 the pH remained low. This prevented pH increase and phosphate precipitation. Instead, it could be consumed directly by biomass and accumulated as polyphosphate.

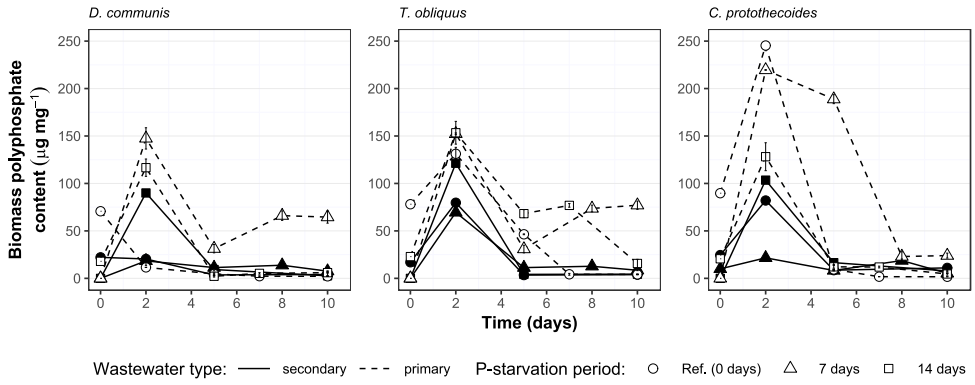

Figure 2.10. Polyphosphate accumulation (means ± SD, $n = 3$) for *D. communis, T. obliquus* and *C. protothecoides* obtained in primary and secondary wastewaters after 7- and 14-day P-starvation and at the reference conditions with no biomass P-starvation.

The polyphosphate accumulation dynamics showed no statistically significant difference between individual species, and neither were they different when compared among the biomass pre-treatments.

In **Paper III** it was observed that biomass growth phase is a major factor for successful biomass P-starvation and subsequent enhanced phosphate uptake. The initial biomass poly-P concentration and its changes over the experiment period suggest that neither of the species developed a clear P-deficiency condition during the biomass P-starvation (Figure 2.11).

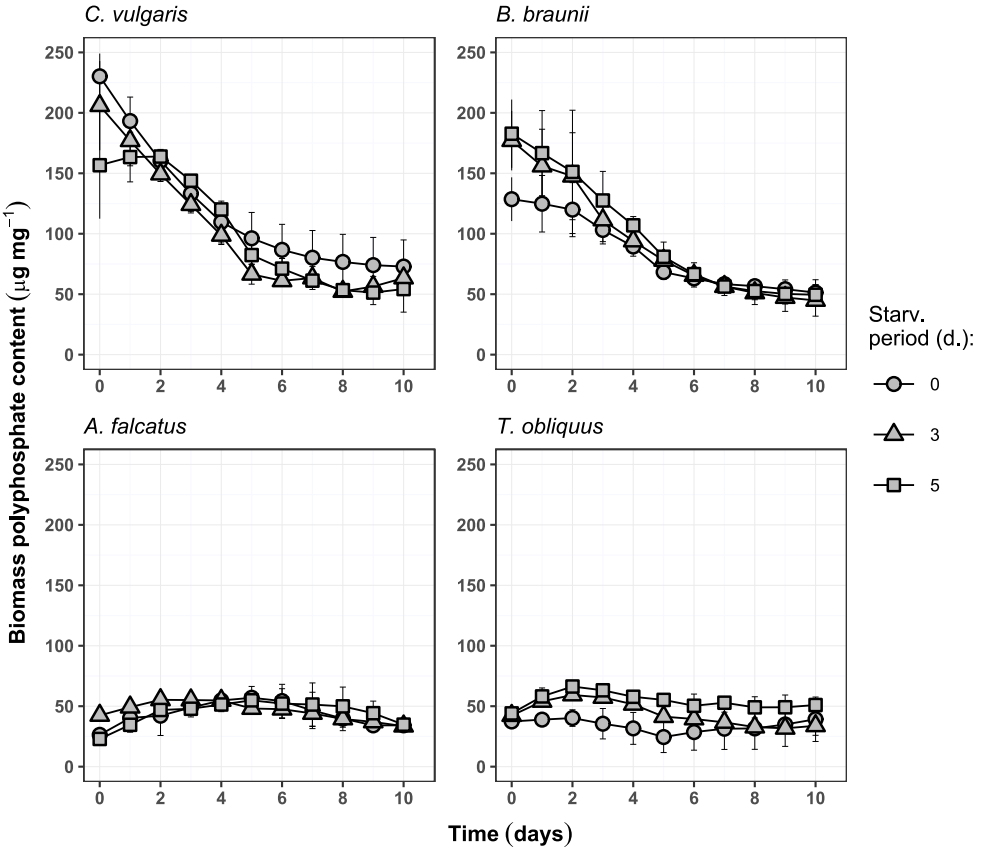

Figure 2.11. Polyphosphate accumulation (means ± SD, n = 3) for *C. vulgaris, B. braunii, A. falcatus* and *T. obliquus* in secondary wastewater after 3- and 5-day P-starvation and at the reference conditions with no biomass P-starvation.

The selected P-starvation periods were too short to reduce the poly-P content to a level where P-deficiency is induced and subsequently enhances phosphate uptake and polyphosphate storage.

Such an outcome was not in line with reports from other studies, where 3 to 5 days of P-starvation entirely depleted the cellular poly-P content (Eixler et al., 2006; Solovchenko et al., 2019). Accordingly, the result from this study indicates that before the P-starvation *C. vulgaris* and *B. braunii* were at an early stationary growth phase when poly-P was accumulated to overcome extended periods without external orthophosphate availability. Contrary, *A. falcatus* and *T. obliquus* were at the exponential growth phase, when they actively used the acquired internal P reserves for biomass production (Sanz-Luque et al., 2020). Among all studied species, only *T. obliquus* showed significantly higher (p-value <0.05) polyphosphate accumulation by P-starved biomass compared to the reference conditions where no biomass P-starvation was used.

In **Paper IV** the microalgal biomass P-starvation was started after its growth entered a stationary phase. During the experiment, biomass polyphosphate content change showed a rapid increase during the initial six hours, indicating that that majority of the inorganic phosphorus was directly incorporated into the cells instead of its adsorption on the cell surface (Yao et al., 2011). Further, the biomass polyphosphate content gradually decreases during the next days of experiment, especially for biomass with longer P-starvation period. Such dynamics indicate, that after longer biomass exposure to P-deficiency the cell incorporated inorganic phosphorus was transformed into acid soluble polyphosphate which its further used for the synthesis of cell constituents, such as protein, DNA or RNA (Powell et al., 2008; Su, 2021). Accordingly, a lesser portion of cellular P*i* was transformed into acid insoluble polyphosphate which provides the storage P reserves for conditions of external P limitation.

In **Paper IV** the protein productivity was evaluated as a response to biomass P-deficiency. The obtained result demonstrated the ability of *C. vulgaris* to synthesize protein at substantially higher rate after its biomass long-term exposure to phosphorus stress. On one hand, this observation contradicts the known metabolic pathway for cellular protein synthesis, which strongly depends on nitrogen assimilation and the available light intensity (Huang et al., 2021; Rani et al., 2020). On the other hand, it is suggested that phosphorus availability had an indirect impact on the protein synthesis. Enhanced phosphorus assimilation caused by prior P-stress condition accelerated synthesis of adenosine triphosphate (ATP), which is required as an energy source for protein synthesis. Such an involvement of phosphorus in protein synthesis have also been discussed by Perez-Garcia *et al.* (2010) and Wu *et al.* (2021) and further supports the previously discussed phosphorus assimilation pathway into acid-soluble polyphosphate.

Initial conditions for optimum performance in PBR

In **Paper IV** the microalgal specie *C. vulgaris* was used to identify the optimum biomass P-starvation period as well as the initial concentrations for biomass and phosphate to obtain a rapid phosphate removal, polyphosphate accumulation, protein productivity and biomass growth at the shortest possible period of biomass exposure to P-deficiency period.

The phosphate removal was normalized against the initial biomass concentration and presented as the specific phosphate removal rate R_{xi}. It was found that the specific phosphate removal rate

increased with longer biomass P-starvation period. In seven out of nine batches the highest R_{xi} value was obtained after 10-day P-starvation period, reaching 7.74 mg g DW h^{-1}. Such a result was obtained for batch with mid-P/low-Biomass concentration (Figure 2.12). The maximum specific P removal rates in every batch were on average 62.0 % higher than for the reference biomass in the same batch and reaching even 175.0 % increase of the R_{xi} in conditions with low-P/high-Biomass concentration after 10-day P-starvation period. However, statistically there was no significant difference observed for the specific phosphate removal rate for biomass with different P-starvation periods (p-value >0.05). Also, the effect of biomass P-starvation on the biomass growth rate was insignificant.

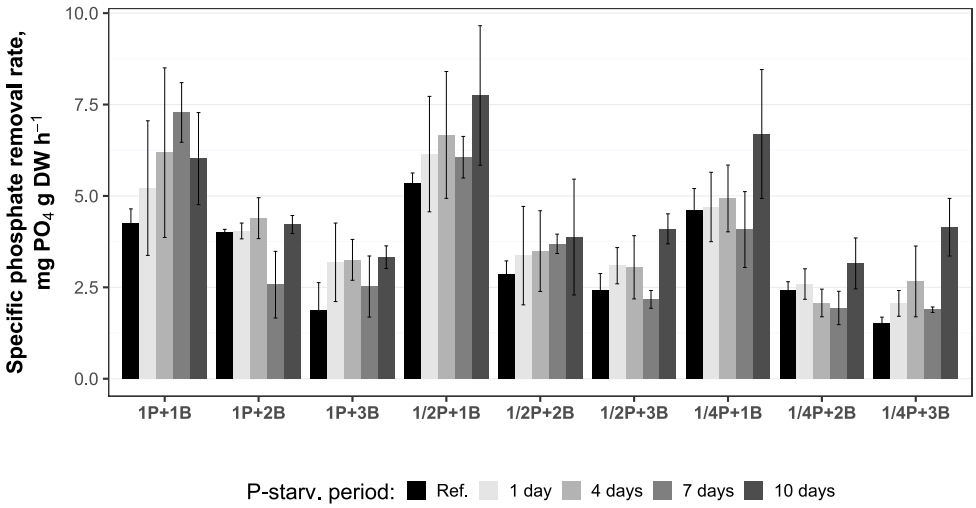

Figure 2.12. The specific phosphate removal rate by *C. vulgaris* during first 5 hours of the experiment at various initial biomass and phosphate concentrations and after different biomass P-starvation periods (means ± SD, n = 3). The number and letter combinations on the x axis refers to the initial phosphorus (P) and biomass (B) content in each batch. 1P – 22 mg P L^{-1}, 1/2P – 12 mg P L^{-1}, 1/4P – 5.5 mg P L^{-1}; 1B – 0.2 g DW L^{-1}, 2B – 0.6 g DW L^{-1}, 3B – 1.5 g DW L^{-1}.

The biomass P-starvation period had a substantial effect on the pathways and dynamics of phosphate cellular accumulation and transformation. Biomass polyphosphate accumulation rate gradually increased with longer biomass exposure to P-deficiency conditions (Figure 2.13). In all batches the 10-day starved biomass accumulated poly-P at 3.7 – 40.0 times higher rate, comparing to the reference conditions, and showing, that the biomass poly-P content was restored significantly faster after the microalgal cells have been exposed to more extensive P-deficiency status. Also, statistically polyphosphate accumulation rate was significantly higher for biomass with 10-day P-

starvation compared to other P-starvation periods (p-value <0.01), as well as for biomass with 7-day P-starvation compared to the reference biomass (p-value <0.05).

From the accumulated polyphosphate, the lesser fraction was transformed into acid insoluble polyphosphate which provides the storage P reserves for conditions of external P limitation. Polyphosphate content decrease over time indicated that it was mainly used for the synthesis of high value products, including protein.

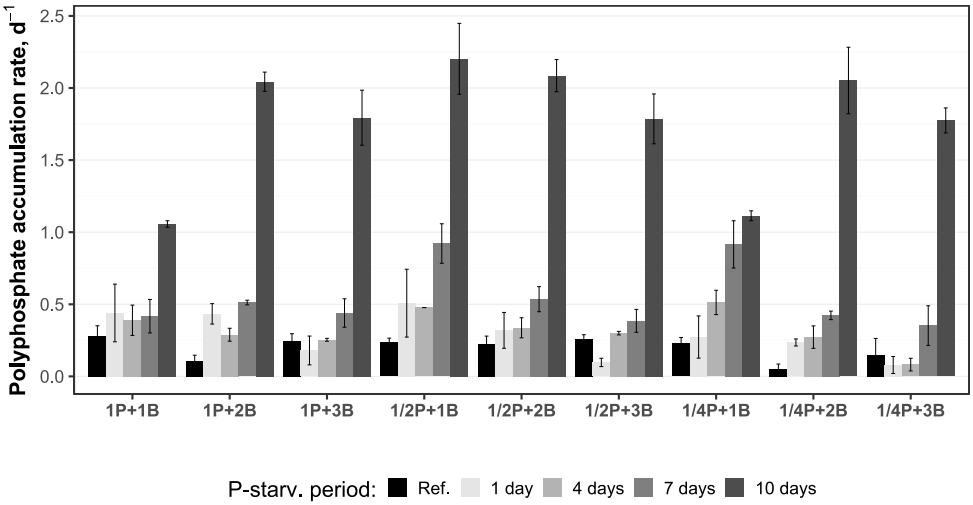

Figure 2.13. The polyphosphate accumulation rate for *C. vulgaris* at various initial biomass and phosphate concentrations and after different biomass P-starvation periods (means ± SD, n = 3). The number and letter combinations on the x axis refers to the initial phosphorus (P) and biomass (B) content in each batch. 1P – 22 mg P L^{-1},1/2P – 12 mg P L^{-1}, 1/4P – 5.5 mg P L^{-1}; 1B – 0.2 g DW L^{-1}, 2B – 0.6 g DW L^{-1}, 3B – 1.5 g DW L^{-1}.

The observed biomass protein productivity for a 3-day period was increasing with longer P-starvation periods. However, significantly higher protein productivity was obtained in batches with 10-day P-starved biomass, compared to other P-starvation periods, reaching 9.0 g DW d^{-1} for the high-P/mid-Biomass batch. In all batches the 10-day biomass P-starvation resulted in 5.7–46.8 times higher protein productivity than at the reference conditions with no prior biomass P-starvation (Figure 2.14). Also, significantly higher protein productivity was obtained in batches with 10-day P-starved biomass, comparing to other P-starvation periods (p-value <0.01).

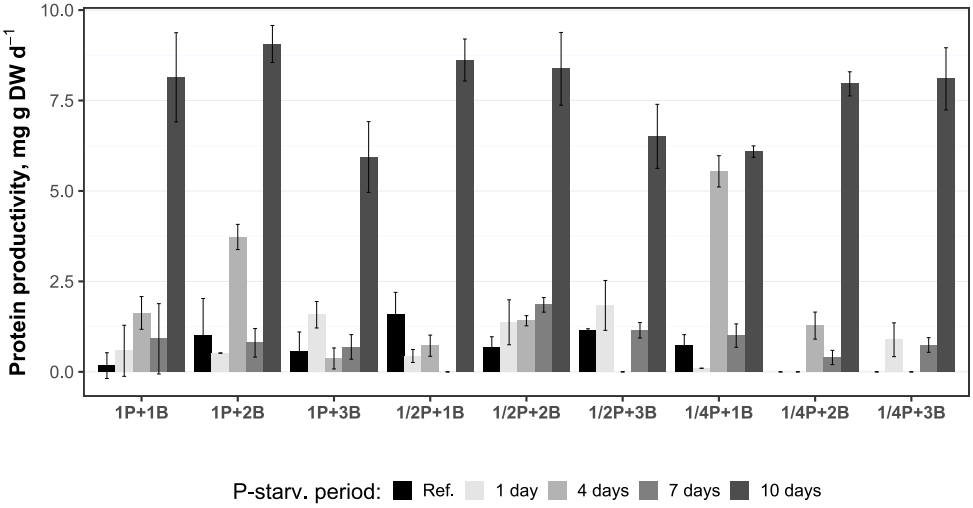

Figure 2.14. Protein productivity for *C. vulgaris* at various initial biomass and phosphate concentrations and after different biomass P-starvation periods (means ± SD, $n = 3$). The number and letter combinations on the x axis refers to the initial phosphorus (P) and biomass (B) content in each batch. 1P – 22 mg P L^{-1},1/2P – 12 mg P L^{-1}, 1/4P – 5.5 mg P L^{-1}; 1B – 0.2 g DW L^{-1}, 2B – 0.6 g DW L^{-1}, 3B – 1.5 g DW L^{-1}.

The results obtained from batch experiment series were used as input data for an optimization model aiming to find the most favorable conditions for efficient wastewater post-treatment. The model results suggested biomass P-starvation period of 1 day as the most favorable to achieve rapid phosphate removal, maintain a high biomass growth rate and ensure high polyphosphate accumulation rate and protein synthesis. To facilitate such a performance, the biomass with short P-starvation period should be inoculated at initial concentration of ~0.2 g DW L^{-1}. The initial phosphate concentration seems to have less influence on maximizing the output of the key variables and is suggested to be initially set within the margin of 10–20 mg L^{-1}.

Biomass growth, metabolic activity and nutrient removal from synthetic wastewater in a sequenced batch photobioreactor

The initial values for P-starvation period and concentrations for biomass and phosphate obtained during batch experiment in **Paper IV** were verified in a sequencing batch photobioreactor operating for 60 days. The obtained biomass growth rate was 52.7 % higher for the P-starved biomass than at the reference conditions (Table 2.2). Moreover, the P-starved biomass showed 101.7 % higher specific phosphorus removal rate than at the reference conditions, indicating that one day long biomass P-starvation was sufficient to establish a phosphorus deficiency status and

promote enhanced phosphate uptake. Also, the polyphosphate accumulation rate was 138.0 % higher for P-starved biomass than at the reference conditions. The dynamics of biomass polyphosphate content as well as the alkaline phosphatase activity further verified the P-deficiency status. The observed protein productivity for P-starved biomass was 38.7 % lower than at the reference conditions. Such an outcome agrees with the result obtained in batch experiment series where protein productivity after 1-day P-starvation did not show any significant increase comparing to the reference conditions.

Table 2.2.

Kinetic parameter values for the reference and 1-day P-starved *C. vulgaris* biomass in sequencing batch photobioreactor (means ± SD)

	Biomass growth rate, d^{-1}	**Specific PO_4 removal rate, mg g DW h^{-1}**	**Polyphosphate accumulation rate, d^{-1}**	**Protein productivity, mg g DW d^{-1}**
Reference (n = 4)	0.072 ± 0.016	0.279 ± 0.134	0.280 ± 0.066	1.682 ± 0.210
P-starved (n = 5)	0.110 ± 0.024	0.563 ± 0.082	0.666 ± 0.063	1.031 ± 0.365

Scale-up considerations

In **Paper IV** the results of initial parameter value verification show that the biomass P-starvation works well at small scale operating in a sequencing batch mode. However, the operational parameters for microalgal biomass production and wastewater post treatment in a lab scale cannot be directly translated to a pilot scale system. Operational conditions are easily controlled at a small scale, but problems can evolve in microalgae production at a large scale, leading to failures in the system. In a large scale, the key parameters are light availability and temperature control which are essential for microalgal production. Also, the uniform CO_2 supply and dissolved oxygen release are essential for successful PBR performance at a pilot scale (Díaz et al., 2020). Thus, a major consideration for a photobioreactor scale-up is the financial feasibility of its construction and operation, which further determines the design of PBR. The photobioreactor design, its geometry and its geographical location are the main factors influencing microalgae biomass productivity, nutrient removal and high value molecule synthesis. For a successful condition transfer from small to large scale modelling and simulation tools are suggested to improve photobioreactor design and operation at a large scale with the desired outcome (Benner *et al.*, 2022).

One of the major prerequisites for an efficient and cost-effective microalgae cultivation and wastewater treatment is to achieve high culture concentration, which will provide high biomass

and value-added molecule yields as well as rapid nutrient removal at high rates. However, high cellular densities can develop a thick suspension layer and leads to a cell self-shading (Ahmad et al., 2022). Socher et al. (2016) suggests that spatial light gradient caused by self-shading can be minimized by a short light path and low cell density. Photobioreactor designs such as bubble columns, tubular and stirred tank reactors are considered unfavorable for biomass production and wastewater treatment due to the curved reaction volume. The disadvantage of batch-mode cultivation is the cell concentration increase and progressing intensity of the self-shading with time. Flat panel systems with uniform illumination at continuous mode operation should allow maintaining a low and constant cell density and a negligible spatial light gradient in the reaction volume and, thus, deliver reliable results.

To facilitate wastewater post-treatment with an integrated biomass P-starvation technology on a pilot scale, the process operation requires multiple PBR units. Firstly, increasing the number of units would allow a simultaneous biomass P-starvation under sequencing batch mode. Although batch operation is not suggested for efficient light utilization by biomass, this limitation is rather minimal as the cellular P depletion begins at the biomass stationary growth phase. Further, such an approach decreases the overall hydraulic retention time for the post-treatment process. This way a PBR unit receiving the incoming effluent after secondary treatment process is always available while at the remaining units the biomass P-starvation process undergoes a certain phase of phosphorus deficiency. Moreover, such a process design reduces the microalgal cell damage by shear stress due to hydraulic transfer of the biomass suspension (Di Iaconi et al., 2005). Still, such technological design approach requires high capital and operational costs. Therefore, along with biochemical process modeling and PBR design considerations, a compact PBR design is required.

Cost evaluation of microalgae-based wastewater post treatment

In **Papers II** and **IV** it was demonstrated that microalgae biomass exposure to phosphorus deficiency can promote rapid and near-complete phosphorus content reduction in wastewater. However, such a performance comes at expenses that are often higher than the conventional post treatment methods (Table 2.3). Molinos-Senante et al. (2010) have estimated that the chemical precipitation for phosphorus removal can cost starting from 0.215 EUR m^{-3}. The cost for membrane filtration varies greatly, depending on the technology used and location-specific electricity cost. Moreover, the model estimations by Hernandez-Sancho et al. (2011) show that scale factor is important as the wastewater treatment cost increases with larger operational scale.

On the other hand, comparing the conventional systems for wastewater post-treatment and nature-based systems such as constructed wetlands and high-rate algal ponds shows that not only the conventional system is 2 to 3 times more expensive than nature-based technologies, but also the potential environmental impacts were 2- to 5-fold higher for the conventional systems due to high chemicals and electricity consumption (Garfí et al., 2017).

Table 2.3.

Price range comparison between different wastewater post-treatment methods

Wastewater post-treatment technology	Cost, EUR m^{-3}	Reference
Chemical precipitation	from 0.215	(Molinos-Senante et al., 2010)
Membrane filtration	from 0.859 to 11.828	(Clem and Mendonça, 2022) (Ozturk et al., 2003) (Hernandez-Sancho et al., 2011)
Constructed wetland	from 0.120 to 0.250	(Gikika et al., 2014)
High-rate algal pond	from 0.120 to 0.420	(Kohlheb et al., 2020) (Kit et al., 2021) (Garfí et al., 2017)
Photobioreactor	from 1.520	(Norsker et al., 2011) This study

Using microalgae for wastewater treatment becomes more expensive with the photobioreactor technology as it requires higher capital and operational costs. From the cost estimation by Norsker et al. (2011) and microalgae growth and phosphorus removal rate data obtained in this study, the projected cost for treatment of 1 m^3 wastewater would be starting from 1.52 EUR. Moreover, the operational costs for PBR can significantly differ between configuration and working environment. A study by Sarker and Salam (2019) show that indoor PBRs consumed 232 to 270 times more energy, required 25 to 57 times more capital cost, and 3.8 to 16.8 times more operating cost than for PBRs operating outdoors.

Although the cost of microalgal photobioreactor technology currently is higher than other methods, it has a greater financial return potential due to higher control over the process and the resulting variety of options for produced biomass application. The estimations by Arshiro et al. (2018) that microalgae based wastewater post treatment could cover the operational costs or even provide a profit if biomass production and its sales is integrated in the wastewater treatment process. Therefore, when putting the PBR technology for wastewater post-treatment into perspective of resource recovery and circular economy, it can become a cost-positive wastewater treatment technology.

3. CONCLUSIONS AND FUTURE OUTLOOK

The objective of this Thesis was to investigate the potential of microalgae-based municipal wastewater post-treatment with integrated biomass phosphorus starvation for enhanced phosphate removal. To achieve the goal of this Thesis, the literature study was done and P-starved microalgae potential for municipal wastewater post-treatment with a focus on dissolved inorganic phosphorus removal was examined.

In **Paper I** the current status of microalgae-based wastewater treatment was reviewed. It was emphasized that the major limitations for a successful of microalgae-based wastewater treatment in full scale are the process exposure to certain climatic conditions, expenses for microalgal biomass harvest and the presence of hazardous substances and pathogens in municipal wastewater that has a potentially harmful effect on microalgal productivity and its downstream applications. Considering that the limitations are primarily related to financial problems, microalgae biomass exposure to phosphorus deficiency was identified as a possible means to overcome the bioremediation problem at various environmental conditions and increase the biochemical value of produced biomass.

In **Paper II** it is demonstrated that microalgae biomass phosphorus starvation can enhance phosphorus removal from wastewater. The microalgal species *Desmodesmus communis*, *Tetradesmus obliquus* and *Chlorella protothecoides* could grow in both filtered incoming sewage and the effluent after biological treatment. After the exposure to phosphorus deficiency conditions for 7 days *D. communis* and *T. obliquus* could reduce the PO_4 content in secondary wastewater by 89.0 % within 24 hours. *C. protothecoides* showed the highest polyphosphate accumulation when produced in primary wastewater. This study also emphasizes the importance of pH control to avoid phosphorus precipitation and ensure its direct uptake by biomass to ensure its biochemical quality.

In **Paper III** the microalgal species *Botrycoccus braunii*, *Chlorella vulgaris*, *Ankistrodesmus falcatus* and *Tetradesmus obliquus* were exposed to 3- and 5-day phosphorus starvation periods to study the bioremediation of effluent from small wastewater treatment plant. Unlike in **Paper II**, in this study the batch conditions were supplemented with aeration and CO_2 influx for pH control. The obtained results showed that the nitrogen limitation disrupts the effect on biomass P starvation for enhanced phosphorus removal. Moreover, the results of polyphosphate accumulation emphasized the importance of biomass growth phase for efficient phosphorus starvation procedure. The accumulated polyphosphate depletion starts at the stationary phase and the phosphorus starvation period should be measured from there. In addition, the alkaline phosphatase activity was identified as a potential indicator for phosphorus deficiency status development for microalgal biomass. *Chlorella vulgaris* was identified as the most appropriate species for further study.

In **Paper IV** *Chlorella vulgaris* was produced in a synthetic wastewater to find the optimum combination of initial values for parameters such as the concentrations of biomass and phosphate as well as the P-starvation period to obtain the most rapid phosphate removal, highest rates for

biomass growth and polyphosphate accumulation and protein productivity at the shortest phosphorus starvation period. It was found the studied parameter values improved with longer biomass P-starvation period. Also, lower initial biomass concentration was favorable for phosphate removal due to higher photosynthetic activity. An optimization model was used to obtain the most efficient combination of initial values, and it suggested 1 day of P-starvation and initial biomass and phosphorus concentrations of 0.25 mg DW L^{-1} and 10–15 mg L^{-1}, respectively, for efficient performance. The optimization model results were verified in a sequencing batch reactor, where 101.7 % higher phosphate removal rate (0.563 mg g^{-1} DW h^{-1}), 52.7 % higher biomass growth rate (0.110 d^{-1}) and 138.0 % higher polyphosphate accumulation rate (0.666 d^{-1}) was obtained with P-starved biomass comparing its performance with reference conditions.

The main benefit of microalgae biomass P-starvation is the reduced reaction time for phosphorus removal to ultra-low level. Such an approach can be beneficial for municipal wastewater treatment at the additional treatment stage for targeted dissolved inorganic phosphate removal, especially for cases when standards for low PO_4 content must be met. As demonstrated in **Paper IV**, even a relatively short period of P-deficiency can double the specific phosphorus removal rate, compared to regular biomass. Sustaining an enhanced phosphorus removal rate would reduce the size of PBR hosting the reaction. Thus, the capital costs can be lowered, which is identified as one of the drawbacks for full scale operation. On the other hand, results from **Papers II** and **III** showed possible limitations and drawbacks that could hamper the efficiency of phosphorus removal by P-starved biomass. As the chemical content in municipal wastewater is variable, it might be complicated to maintain high N/P ratio which is complementary for phosphorus limiting conditions and enhanced phosphate removal. Moreover, in all experiments the biomass P-starvation effect was studied in a laboratory scale under sterile and controlled conditions, possibly excluding any organic and chemical factors that might have a negative impact on the microalgal biomass. Micro-filtered and sterile growth medium excluded presence of any other microorganisms that could outcompete the algal cells for nutrients. The content of heavy metals was at trace level, therefore no impact as hazardous substance could be assessed. The presence of certain metals can be an additional stress factor that impacts the biomass growth and high value molecule synthesis (Karcheva et al., 2022). On the other hand, the controlled conditions and exclusion of possible stressors allowed to focus this study on the mechanisms for enhanced phosphorus uptake.

Operating a microalgae-based municipal wastewater post-treatment system at a full scale is primarily regulated by light availability and temperature. However, it was not assessed during this study how major shifts from optimum temperatures and light regimes would affect the phosphate removal after biomass exposure to P deficiency. In previous studies it has been demonstrated that high nutrient removal rates can be sustained at temperatures as allow as 4 °C (Craggs et al., 1997) which potentially allows to extend the system application to higher latitudes.

Overall, the results obtained in this work approves the scientific hypothesis of this Thesis – microalgae biomass exposure to phosphorus deficiency conditions can enhance the uptake rate of

dissolved inorganic phosphorus from an aquatic environment, including municipal wastewater. The obtained results also supplement the existing knowledge on microalgae-based wastewater treatment. For instance, it was demonstrated that the phosphorus deficiency status in microalgae can be assessed using the alkaline phosphatase activity as an indicator. Such indicator would allow more control over the operating of P-starvation in full-scale wastewater post-treatment. Also, it was shown how phosphorus deficiency conditions affect its accumulation and transformation pathways within the microalgal cell, allowing to better understand the P-starvation impact on high value molecule synthesis. Ultimately, this study shows, that the proposed technology for municipal wastewater post-treatment can reduce phosphate concentration below 0.1 mg L^{-1} and possibly diminish the impact by municipal wastewaters on eutrophication status development in surface waters.

The future research for microalgae phosphorus starvation for enhanced phosphorus removal from wastewater should focus on its operation in full scale systems treating real wastewater of various origins. It is recommended to include studies on pathogen and hazardous substance presence in high value compounds derived from microalgae grown in wastewater. Such knowledge is crucial for communicating the safety and applicability of such microalgal products with the general public. Also, a better understanding of microalgal cell phosphorus deficiency status at the genetic level would not only benefit the microalgal application in controlled engineered systems but also increase the understanding of the phosphorus cycle in natural aquatic ecosystems.

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Review on Challenges and Limitations for Algae-Based Wastewater Treatment

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***Abstract* – Microalgae biomass production is recognized as a cost-effective and sustainable alternative to currently used approaches to tertiary wastewater treatment. However, such limitations, as algae biomass separation from water, process efficiency in cold climate and the algae biomass ability to reduce micropollutant content in wastewater hamper this method from full-scale use. This review discusses the identified drawbacks and offers possible improvements and modifications for wastewater phycobioremediation.**

***Keywords* – Cold climate, harvest, microalgae, micropollutants, tertiary treatment, wastewater.**

I. Introduction

The increasing global population and its economic activity lead to elevated pollution loads to natural surface waters. High nutrient concentrations in a water body promote a eutrophic ecosystem state, thus providing favorable conditions for rapid algal growth. When the algal biomass decays, dissolved oxygen content is depleted, resulting in aquatic animal death and overall deterioration of ecosystem health and the services it provides. Estimations show that the economic losses from surface water eutrophication can exceed 2 billion US dollars annually [1], affecting the real estate, recreation, and fishing industries. Among other sources, municipal wastewater treatment plants significantly contribute to freshwater eutrophication. Substantial role is played by small WWTPs. According to Regulation No. 34 by the Cabinet of Ministers of the Republic of Latvia there is no requirement for phosphorus content reduction in WWTPs that operate for less than 2000 person equivalent.

To reduce the WWTP input to eutrophication, their management must be reconsidered. Currently phosphorus content in the WWTP effluent is mostly reduced by chemical precipitation [2] at the tertiary treatment phase. However, the use of chemicals is associated with high costs and results in excess waste sludge production increasing the risk for secondary water pollution [3], [4]. These shortcomings of conventional tertiary wastewater treatment have led to introduction of more sustainable approaches. Numerous studies have demonstrated that phycoremediation, i.e. application of algae biomass for wastewater treatment, is an efficient measure for reducing nutrient concentration by up to 95 % [5]–[7]. Besides wastewater treatment, algae biomass is perceived as a raw material for bioenergy production, nutrition and perfume products as well as high value substance extraction [8]. Thus, with successful downstream processing, algal biomass not only can provide a low-cost wastewater treatment, but is also potentially profitable wastewater management approach. However, despite the promising application possibilities, certain obstacles hamper algae-based wastewater treatment from full-scale operation and from becoming a cost-effective alternative for conventional methods. Current technology for algae biomass separation from water significantly increases the total treatment costs [9]. Therefore, a lack of rapid and inexpensive biomass harvest method has been identified as the major limitation for algae-based wastewater treatment. Moreover, efficient operation with minimal energy requirement is possible at certain climate conditions. Thus, algae-based wastewater treatment is limited to finite geographical locations. Finally, the everchanging chemical content of wastewater raises new challenges, requiring treatment of wastewaters with problematic and unknown contamination [10], while their influence on algal growth is often obscure.

This review aims to point out and discuss the identified limitations and ambiguities, as well as offers possible improvements and modifications for wastewater phycobioremediation that could overcome the currently known limitations.

II. Algae-Based Wastewater Treatment

Microalgae is an autotrophic unicellular organism that can perform photosynthesis. Powered by light, algae convert water and carbon dioxide into oxygen and carbohydrates, thus providing energy for its biomass growth [11]. Also, nitrogen and phosphorus are consumed as nutrients for algae cell reproduction. The ability to produce oxygen and take up nutrients from water have made microalgae biomass cultivation prospective for cost-effective wastewater treatment.

Initially the use of microalgae for wastewater bioremediation was proposed by [12]. They demonstrated that algae biomass production in domestic sewage works as an aeration technique with low energy requirement. It resulted in biochemical oxygen demand reduction by more than 85 % in a pilot scale waste stabilization pond. Afterwards, algae-based nutrient removal and recovery from wastewater was successfully demonstrated [13], [14]. Further, wastewater was found to be a suitable growth medium for low-cost microalgae biomass cultivation for energy production and high value product extraction [15]. Present algae-based wastewater treatment studies have evolved to various scales and techniques, covering diverse contamination removal [16], [17] and optimizing algae growth conditions to reach utmost water treatment and biomass production performance [18].

Algae metabolism is the mechanism behind wastewater phycoremediation. Nutrients are taken up and transformed within the algal cell where they can be assimilated into nucleic acids and proteins for algae biomass growth [19], [20]. Nitrogen is present

in wastewater in the form of organic nitrogen, or inorganic compounds such as ammonium and nitrate. Ammonium is the preferred form for algae uptake, while nitrate within the algae cell is reduced back to ammonium and assimilated into amino acids for the synthesis of proteins [21]. Inorganic phosphorus is utilized for ribosome RNA synthesis. In addition, microalgae can consume extra phosphorus through a luxury uptake, store it in the form of polyphosphate and utilize it at low external phosphorus availability conditions [22].

[23] have estimated that there are roughly 72 500 known and described species of freshwater, marine and terrestrial algae. Still, only few of them have been tested for the tolerance in wastewater [24], [25]. Among others, *Chlorella* and *Scenedesmus* species algae are far more often used for wastewater treatment [5], [26], [27].

Reviews done by [20] and [28] show the efficiency of algae in nutrient concentration and biochemical oxygen demand reduction, and depending on the treatment setup, scale, algae species and environmental conditions, nearly complete contamination removal can be achieved. Wastewater treatment using microalgae is performed in open or closed systems. Open systems such as natural lagoons, pond and artificial ponds and reservoirs are simple to construct and maintain, and therefore are the preferred installations for algae-based wastewater treatment [29]. However, the treatment process is less controllable. Closed systems, which mostly are photobioreactors, provide more control over the treatment process, but are expensive to install and maintain. The major controllable parameters include the photosynthetic active radiation (PAR), which is considered the key factor for algae production with the optimal range of photon flux being between 30 to 400 $\mu mol\ m^{-2}\ s^{-1}$ [30]. Additional CO_2 supply can maximize the algae biomass production [31], it prevents development of alkaline pH level. Suitable pH values for algal growth are reported to be between 7 and 9 [32]. The role of temperature for algae biomass production is rather unclear and is highly variable between species and their origin [33]. Still, the optimal range is reported to be between 15–30 °C [34]. The role of nutrients is expressed by optimal molar ratio of the available nutrients. The empirical ratio between nitrogen and phosphorus is determined to be 16 : 1 [35], however, it is highly variable between species and is affected by the environmental conditions [36]. Finally, successful algae growth and resulting water treatment efficiency depend on the presence of other organisms. Although bacteria are often seen as a competitor to algae for nutrients, consortium of these two organisms is used as a source for both natural aeration and CO_2 supplementation [37]. Presence of algae grazers, such as zooplankton or rotifers, has a negative effect on algae production rate and water treatment performance.

Additional benefit to low energy wastewater treatment comes from the algae biomass suitability for added value product extraction. Due to rapid biomass production rate and high lipid content, algae have the potential to become a major raw material for biofuel production [38]. Although the estimated production costs of algae-based biofuel are uncompetitive with currently used biomass [39], diligent research is carried out on optimized algae biomass production for higher lipid yield [18]. Other high-value products derived from microalgae are β-carotene, astaxanthin, docosahexaenoic acid, eicosahexaenoic acid, phycobilin pigments and algal extracts for use in cosmetics [8], with a multi-million global market value.

Extensive research done on wastewater phycoremediation clearly demonstrates the potential of microalgae production for sustainable wastewater treatment. However, despite the massive knowledge generated throughout the past six decades, various shortcomings have been identified for this wastewater treatment approach. In further sections of this review, major obstacles that hamper a global algae-based wastewater treatment implementation are discussed.

III. Limitations for Algae-Based Wastewater Treatment

A. Algae Biomass Harvest

Algae biomass harvest has been identified as one of the major drawbacks for cost-effective wastewater treatment and its downstream processing. Main factors that complicate algae harvest include very small portion (up to 0.05 %) of algae dry weigh in the total suspension, microscopic size of a single cell, negative cell surface charge that prevents them from forming larger and easily harvestable particles as well as rapid growth rate [9]. These aspects significantly increase the total costs of both algae biomass harvest and its application for wastewater treatment. It is estimated that cost of algae biomass harvest can constitute up to 30 % of the total production expenses [40], which is due to high energy consumption, which, depending on the chosen method, varies between 0.1 and 15 $kWh\ m^{-3}$ [41]. Moreover, up to 90 % of the total inventory costs are attributable to the harvest and dewatering devices [42]. Although various algae biomass harvest techniques have been developed and are widely used, each of them has its advantages and drawbacks, which encourages research on finding more economically feasible, universal and simpler methods.

Physical and Chemical Methods

One of the simplest algae biomass harvest methods is sedimentation, which offers an inexpensive solution for algae biomass harvest. It is done using gravitational force, where algal biomass is separated from liquid due to differences in their densities. Still, the difference between algae cell and water density is relatively small, making the process rather slow. Moreover, sedimentation rate is affected by a series of biotic and abiotic factors. Different sedimentation rates are suggested for various algal functional groups, reaching 3.6 $m\ d^{-1}$ for the wastewater tolerant *Chlorella sp.* [43]. The water temperature affects its viscosity, so algae sedimentation rate is likely to increase at higher temperatures. Also, elevated pH levels promote algae cell floc formation leading to more rapid sedimentation. As sedimentation is a relatively slow process, it is often coupled with other harvest techniques or is modified for more rapid performance.

Centrifugation can be viewed as a derivation of sedimentation, where instead of gravitational force centrifugal force is employed. Centrifugation provides simple and rapid algae biomass separation from the liquid, and is efficient for all algae species and cell sizes [44]. However, due to large investment and operating expenses, it becomes cost-inefficient for large scale

algae-based wastewater treatment systems [45], [46], leading to fourfold increase in total treatment costs [47].

To neutralize the negative surface charge of algae cells and promote formation of larger particles that easily settle, electrolytes and synthetic polymers are used. Usually, aluminum sulfate and ferric chloride are used to stimulate flocculation [45], and their use can result in more than 90 % of algal biomass recovery [46]. On the other hand, use of chemical flocculants can lead to secondary pollution and it limits the downstream processing of algae biomass. In addition, for large scale algae-based wastewater treatment systems, flocculant use for algae biomass harvest becomes economically detrimental, and it is likely to become more expensive than precipitant use for tertiary treatment alone.

Certain algae species can form flocs naturally under environmental stress, such as elevated pH level or changes in nutrient and dissolved oxygen concentrations [48]. Manipulation of these parameters in controlled environment offer a relatively cheap flocculation method. However, it is still viewed as slow and unreliable flocculation technique, with limited possibilities for application [49].

Another method for neutralizing the algae cell surface charge and promoting their flocculation is the use of electrophoresis [45]. Although this method does not require addition of chemicals, flocculation using electric field is associated with high energy consumption and maintenance costs [48], especially for large scale performance.

Flotation can be viewed as the next step after flocculation. Most commonly dissolved air floatation is used. It employs decompression of pressurized fluid that generates microscopic bubbles and promotes easily harvestable algae mat formation [50]. Although this method has been recognized as effective for large scale use, it is still an energy-intensive process [51].

Filtration is another simple and effective algae harvest method, which gives up to 90 % recovery of algal biomass [52]. There are several types and designs available for filters, but their application is mainly determined by the variable algae cell size. In addition, the algae cell diameter is inversely proportional to the expenses of this method. Macrofiltration methods with lower energy requirement are applicable for macroscopic filamentous algae species, such as *Spirulina* [53]. For species like *Chlorella* and *Scenedesmus* the cell diameter varies between 5 μm and 20 μm, therefore membrane microfiltration is applied for their harvest [54]. However, microfiltration is associated with slow performance and is not suitable for large scale. Micro and ultrafiltration methods are also related to high performance and maintenance costs, due to rapid membrane clogging that needs to be frequently changed as well as high energy demand due to micro-pore membrane pressure resistance [45].

Different harvest approach is offered by immobilization of algae cells into a polymeric matrix, which provides a convenient and cost-effective alternative for conventional algae biomass harvest methods [55]. The immobilization matrix can be either synthetic or naturally derived polymer and must meet certain requirements such as phototransparency, non-toxicity and stability in the algae growth medium [56]. Besides simple biomass harvest, immobilization matrix has the advantage of hyperconcentrated culture use and protection of algae cells against hazardous bacteria or natural grazers. Among other natural immobilization matrixes like agar, alginate or collagen, chitosan obtained from chitin is a frequently used material [56], [57]. The natural properties of chitosan include considerable uptake rate of nutrients which can be done in parallel with immobilized algae biomass hyper-concentrate. [56] obtained 70 % nitrate and 94 % phosphate uptake using immobilized *Scenedesmus sp.* cells. However, various studies show that the choice of material for immobilization matrix can play an important role in overall wastewater treatment efficiency. [58] used alginate bed as immobilization matrix and found that free-cell cultures show better wastewater treatment performance over immobilized cultures. [59] used *Chlorella vulgaris* and *Azospirillum brasilense* bacteria as algae growth-promoting organism co-immobilized in alginate bed to treat synthetic wastewater. Co-immobilized culture showed 32 % higher removal rate of ammonium than single algae culture alone. Phosphorus reduction was not observed, though. The existing studies show that immobilized algae cultures are a good solution for biomass harvest, and thus, can significantly reduce harvest costs. Still, its performance mostly has been studied in bench-scale under controlled conditions. Thus, assessment of pollutant removal efficiency and economic viability of immobilization polymer use on pilot-scale is required.

Biological Methods

A perspective, yet undeveloped technique for microalgae biomass separation from water is the application of natural algae predators for biofiltration. In natural ecosystems algae cells are consumed by filter feeding organisms from higher trophic levels of the food-web, which include zooplankton, mussels and certain filter feeding fish species. Such an approach for wastewater treatment is attractive due to low energy consumption under optimal conditions for biomass cultivation. Contaminant removal is done by algae in accordance with the previously described mechanism. Electrical energy may be required for aeration, additional CO_2 feeding, mixing and water pumping. Algae harvest performed by zooplankton and fish basically require food resource which is ensured by the filterable algae biomass. Although the provisional energy consumption of such wastewater treatment approach would be relatively low, it requires trained personnel for regular monitoring and maintenance, as this treatment method is a living system not mechanical process.

The potential use of filter feeders for algal biomass removal has been studied in lab-scale systems [60] and mezocosms [61], [62]. [60] used a chain of *Scenedesmus sp*. algae, *Daphnia sp*. zooplankton and *Notemigonus crysoleucas, Pimephales promelas* and *Notropis lutrensi* filter feeding fish as a tertiary water treatment system. Each of the organism groups was isolated forming an artificial aquatic food-web (AAFW). It was reported that such a system could remove up to 78 % of nitrogen and 98 % of phosphorus compounds from secondary treated domestic wastewater. Similar results were achieved by [61] and [62]. However, they excluded filter-feeding fish from the treatment system. [63] showed that continuous light regime promotes algal biomass growth and thus increases nutrient removal efficiency.

The latest study, using AAFW for water treatment, demonstrated reduction of TN and TP concentration by 28 % and 47 % respectively in a eutrophied subtopic river [64].

Although proved to be efficient on a laboratory scale and demonstrating significant pollutant reduction rates in outdoor conditions, such systems still need to be tested under various climate conditions. The influence of seasonal temperature variations, fluctuating water inflow rate, as well as natural light regime are location-specific. Thus, similarly to other wastewater bioremediation systems [65], [66] variable response to performance efficiency can be expected in different climate zones. Also, biotic factors, such as species selection for AAFW organisms is likely to affect performance. For instance, zooplankton preference for green microalgae species can reduce algae harvest efficiency, especially when open cultivation systems with mixed algae strains are used.

Another important factor affecting AAFW based wastewater treatment efficiency is the content of waste stream. Besides nutrients and organic matter, that are the target parameters in conventional wastewater treatment, micropollutants such as heavy metals, coliform bacteria and a variety of pharmaceuticals, personal care products, prescription and illicit drugs and other substances, their compounds and residuals, all together classified as emerging contaminants, are present in wastewater. Variable efficiency in reduction of these pollutants has been demonstrated by algae-based wastewater treatment systems [67]. Yet, it is unclear how these micropollutants would affect the filter-feeding organisms in the AAFW and its overall water treatment performance.

B. Removal of Micropollutants

Along with nutrients and organic compounds, wastewater contains a large variety of micropollutants that often pass the conventional wastewater treatment and are released in natural water bodies. Substances like heavy metal ions, pathogenic bacteria as well as compounds and residuals of pharmaceuticals, personal care products, household chemistry, drugs and others can cause adverse effects on aquatic organism development and human health [68]. For the reduction of their content advanced treatment is used, which, however, is associated with high energy demands and performance costs. Although several studies have demonstrated the applicability of algae for micropollutant removal from wastewater [16], [69], [70], limitations and knowledge gaps exist to rely on algae biomass production as an effective means for micropollutant content reduction.

Heavy Metals

With growing industrialization, heavy metal ions are becoming more common pollutant in the urban sewage [71], thus increasing the load to natural aquatic ecosystems. Unlike organic contaminants, heavy metals are not biodegradable and tend to accumulate in living organisms. It is known that many heavy metal ions are toxic or carcinogenic, causing dysfunction of the organism containing them. Therefore, heavy metal ions, such as zinc, copper, nickel, mercury, cadmium, lead and chromium are of major concern when it comes to wastewater treatment. Methods for heavy metal content reduction in water include chemical precipitation, ion exchange, adsorption, membrane filtration, coagulation and flocculation, floatation and electrochemical treatment [72]. Still, more economical and sustainable ways for heavy metal content reduction are being searched for, and algae application seems to be a promising alternative [73].

The ability of algae to remove metals from wastewaters have been broadly studied. It is known that algae cells are capable to adapt to toxic environment and take part in heavy metal uptake. Both living and dead cells were found to contribute to metal sorption in wastewater [74]. [73] studied the capability of *Cladophora fracta* to remove metals from stock solutions and achieved 85–99 % removal of Cu, Zn, Cd and Hg. Gao et al. (2016) used *Chlorella vulgaris* for domestic wastewater treatment in membrane photobioreactor and achieved complete reduction of Fe and Mn ions, while Cu, Zn and Al ions were reduced by 65 %, 80 % and 93 %, respectively. [75] studied metal removal from textile wastewater using lab-scale algae pond system under different flow and light conditions. They achieved 98 % reduction of chromium (Cr) concentration regardless the loading rate and light regime, while for zinc (Zn) the removal rate was higher (80 %) at high loading rate under continuous 24 h lighting. For other metals (Pb, Cd and Cu) the removal rates were between 20 % and 30 %.

Despite the evident efficiency of algae-based metal-ion removal from wastewater, there are still ambiguities regarding efficiency for certain metal ions. The abovementioned studies show significant contrast in removal rate between different metal ions at equal conditions. Yet, the key factors for unequal metal ion removal remains unclear. In addition, algae biomass use for metal-ion reduction in wastewater clearly limits its further application due to the negative effects of metal ion accumulation in living organisms.

Coliforms

Coliform bacteria are pathogenic microorganisms that are found in gastrointestinal tract of all warm-blooded animals. They are used as an indicator for fecal contamination in water. Although considered to be a harmless organism, certain strains can cause gastroenteritis [76]. Due to the negative effects on human health, coliform content in the effluent wastewater becomes relevant when it is reused, for instance, in public swimming pools.

To prevent outbreaks of waterborne diseases, advanced tertiary wastewater treatment for coliform removal is performed. Common methods for coliform and other bacteria removal are chlorination, sand filtration or ultraviolet disinfection [77]. Although chlorination is the most widely used water sterilization method, certain shortcomings of this method, such as formation of toxic and potentially cancerogenic by-products have been identified [78]. Therefore, development of novel and sustainable treatment methods is required.

There are several studies proposing application of microalgae for coliform content reduction in wastewater. It has been demonstrated that in open algae-based wastewater treatment systems photosynthetic growth of algae develops adverse conditions for pathogenic organisms [79], [80], forming high pH and dissolved oxygen concentration. Also, auxiliary effect is

given by received light intensity [81], [82]. However, certain limitations are known for algae-based coliform removal. A study by [83] showed that algae decay can produce significant amount of dissolved organic carbon, which promotes bacterial survival. Such conditions can be formed by extended hydraulic retention time, insufficient mixing and algae sedimentation. In addition, it was observed that elevated algae cell density reduces light attenuation, thus lowering their contribution to coliform neutralization [84]. Also, it is hypothesized that algae are promoting decay of fecal coliforms and producing a neutralizing substance. This phenomenon is attributed to observations in natural eutrophic lake in tropical climate [85] as well as in laboratory experiments with different types of wastewater [69]. Still, this mechanism is not fully understood, thus a relevant topic for further research remains open.

Additional research topic regarding algae-based coliform removal from wastewater is the influence of climate on outdoor treatment facility performance. Depending on the latitude, earth surface receives different solar radiation. Also, location-specific temperature is likely to influence algae growth and resulting coliform inactivation. However, to support these assumptions, detailed case studies are required.

Emerging Contaminants

Over the last decades there has been an increasing concern about the content of emerging contaminants (ECs) in wastewater. They include a vast variety of organic and inorganic micropollutants, such as pharmaceuticals, prescription and illicit drugs, personal care products, nanomaterials, perfluorinated compounds and other substances [86]. With effluent wastewater, ECs are delivered to natural waters and can also be found in drinking water. Although these pollutants are mostly present in trace concentrations ($\mu g\ l^{-1}$), they are known to have adverse effect on aquatic and terrestrial organisms as well as on human health [87], [88].

The main pathways of ECs to the wastewater include domestic use of personal care products and household chemicals, use of prescription and illicit drugs and subsequent excretion of their residuals, disposal of expired medicine as well as waste disposal from pharmaceutical industry, chemical labs or hospitals. Despite the awareness of EC presence, there are no legal regulations for their removal from wastewaters. In addition, conventional wastewater treatment plants mostly are not designed for EC removal, which results in continuous EC loads to natural aquatic ecosystems [87].

Several advanced wastewater treatment methods have been applied for ECs removal. However, the success of their removal largely depends on the chemical properties of certain micropollutants. A study by [89] showed that advanced treatment methods like coagulation, flocculation and lime softening could not sufficiently reduce the total EC content, mainly due to pollutant competition for sorption surface. [90] demonstrated that more substantial EC reduction can be achieved by methods used for drinking water preparation, with powder activated carbon and chlorination being more effective than others. Still, the removal rate was variable between certain substances. In addition, chlorine by-product formation from its reaction with ECs makes it unsuitable for this purpose. Among others, membrane filtration technology such as reverse osmosis and nanofiltration have shown the most promising results with nearly complete EC removal [91]. Still, the main disadvantage of membrane filtration is its high consumption of energy.

Bioremediation has been proposed as an energy-efficient technique for EC content reduction in wastewater. Different studies on removal of ECs like pharmaceuticals, personal care products and pesticides have been done in constructed wetlands [92], [93], all showing highly variable substance-specific removal rates. Removal of ECs is also studied in algae-based wastewater treatment systems. A comprehensive review by [67] shows that similarly to constructed wetlands, EC removal rate in HRAPs varies from insignificant to complete reduction. Also, results from studies conducted in closed algae reactors [94] indicate that removal efficiency of emerging contaminants depends on chemical properties of each substance individually, and is largely determined by treatment system scale, operational regime and design as well as climatic conditions. Thus, it can be concluded that the unpredictable and selective performance makes algae production rather unsuitable for emerging contamination reduction, and likewise is the applicability of AAFW.

C. Algae-Based Wastewater Treatment Efficiency in Cold Climate

The microalgae and aquatic vegetation use for wastewater treatment in higher latitudes is limited by the short vegetation season as well as low temperatures and shorter daylight hours in seasons other than summer. Moreover, low temperature becomes a serious concern if filter feeding organism use is considered for algal biomass harvest. Due to seemingly less efficient performance determined by low temperature and PAR, algae use for contaminant removal from wastewater in cold and temperate climates has not been extensively investigated. Still, the existing studies highlight certain conditions for successful wastewater phytoremediation in cold climate. Operation under low temperature also becomes a challenge if biofiltration is to be applied for algal biomass harvest.

The potential for successful algae-based wastewater treatment at low temperature is shown in a study by [33], which indicates the importance of algal strain origin. Their results demonstrated that *Clamydomonas sp.* isolated in cold climate zone lost productivity and the resulting nutrient uptake rate at temperature above its natural environment. Further, a study by [95] showed that at cold climate conditions phosphorus removal rate was significantly affected by the available PAR, while the role of temperature was minor. The importance of sufficient light availability over temperature and origin of algal strain was also shown by [96]. They used marine cyanobacteria (*Oscillatoria sp.*) and diatom (*Phaeodactylum tricornutum*) strains from temperate climate in a mesoscale corrugated raceway and achieved continuously complete removal of both ammonium and orthophosphate even at temperatures as low as 4 °C. [97] demonstrated the capability of polar cyanobacteria (*Phromidium sp.*) strain in nutrient content reduction in growth medium at 5 °C and constant illumination of 225 $\mu mol\ m^2 s^{-1}$. However, due to the low temperature, reduced nutrient removal pace was observed.

The abovementioned studies that used microalgae showed reduced biomass growth rate at lower temperatures. Still, considerable nutrient removal rates were observed. An indication for causes of such relation can be found in a study by [95]. During their experiment, most of the phosphorus was reduced by algal luxury uptake and stored as polyphosphate which is not used for biomass production. However, it is not clear whether this is a temperature driven mechanism. In addition, reduced biomass growth also points to indirect nitrogen compound removal, while direct utilization of nitrogen would result in protein synthesis and algal biomass production [20].

Despite the promising contamination removal rates in experimental scale studies, algal bioremediation efficiency show decrease with system scale-up. In a comprehensive study by [98], it was conformed that at temperature below 10 °C and PAR below 200 μmol m^2 s^{-1} substantial reduction of both BOD and COD (90 % and 65 % respectively) can be achieved in a pilot-scale HRAP. However, a lower reduction of total nitrogen and phosphorus by 47 % and 20 %, respectively, was observed.

Even though nutrient reduction by algae has been successfully demonstrated at low temperatures, it is apparent that outdoor treatment facilities cannot be used during winter at negative temperatures, when most biological processes stop and no contaminant removal takes place. As a solution, greenhouse treatment plants can be used. The performance of such an approach was studied by [99], who used a hybrid consisting of conventional wastewater treatment plant and AAFW for additional treatment. This setup showed reduction of total nitrogen, total phosphorus and heavy metal concentrations by 39 %, 28 % and 47–98 %, respectively. Additionally, pathogen content was reduced close to bathing standards. However, it was concluded that the estimated energy costs required to run such a system in cold climate cannot compete with conventional wastewater treatment, unless valuable biomass is produced for profit. Similar study was conducted by [100], who achieved higher nutrient reduction rate. However, the resulting energy consumption led to the same conclusions.

IV. Conclusions and Recommendations

The acquired knowledge on algae-based wastewater treatment marks a promising alternative for conventional wastewater treatment as cost-effective and sustainable technology. Still, certain limitations should be overcome before implementing microalgae as a key organism for the wastewater treatment process. Algae biomass harvest is recognized as the major obstacle for competitive wastewater treatment performance. All the currently known harvest methods have their drawbacks related to economically detrimental large-scale use and downstream processing limitations. Although understudied, artificial aquatic food-web for wastewater treatment is an attractive alternative for existing harvest techniques due to its low energy consumption and sustainable performance under optimal conditions. Nevertheless, certain limitations exist also to this harvest approach. Firstly, its applicability in colder climate, where additional energy is necessary for optimal performance conditions makes this method noncompetitive with conventional wastewater treatment. Similarly to arctic algal strains, the use of filter feeding organism species originating from cold climate might result in satisfactory performance under low temperatures. However, this assumption needs to be confirmed in a case study. Another consideration for testing different species is related to AAFW application in open systems, where natural shift of dominant algal specie is likely to develop. Thus, biofiltration stage of the AAFW system might lose it efficiency due to grazer preference of food.

Since algae-based wastewater treatment is viewed as a raw material source in bioenergy production and valuable substance extraction, biofiltration is not applicable as a biomass harvest method. Because of biofiltration, valuable substances produced by algae are used for filter feeder metabolism. To cover the performance costs and gain profit, AAFW for wastewater treatment is recommended to be oriented on high-valued organism aquaculture.

Even though algae-based wastewater treatment leads to efficient reduction of coliforms, the mechanism and its drivers are still not completely clear. Also, not all metal ions and emerging contaminants can be reduced using algae. Therefore, if filter feeding organisms are used for algae harvest, they can be exposed to harmful and toxic substances, which can limit the use of aquaculture products and ultimately collapse the AAFW system. Thus, before the impact of microcontaminants on AAFW organisms and their further biomass use is investigated, this wastewater treatment method has limited applicability with respect to wastewater origin and its chemical content.

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Microalgae starvation for enhanced phosphorus uptake from municipal wastewater

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ABSTRACT

Three microalgal species, *Desmodesmus communis*, *Tetradesmus obliquus* and *Chlorella protothecoides* were studied for enhanced phosphorus removal from municipal wastewater. Microalgae were first exposed to phosphorus deficit conditions for 7 and 14 days and then inoculated in filtered primary or secondary wastewater from a small municipal wastewater treatment plant at ambient temperature and CO_2 concentration. *D. communis* and *T. obliquus* strains showed higher biomass growth rates in secondary wastewater after 7-day starvation period, while *C. protothecoides* grew better in the same effluent without starvation. All strains were able to achieve nearly complete (> 99.9%) removal of dissolved inorganic phosphorus (DIP). Moreover, *D. communis* and *T. obliquus* showed ~89% DIP reduction within 24 h after 7-day phosphorus starvation. Dissolved inorganic nitrogen removal for all algal strains did not exceed 50% for any of the treatments. All three strains were able to store excess phosphorus within their cells as polyphosphate and the highest Poly-P content was observed in cultures grown in primary wastewater. Poly-P concentration in *C. protothecoides* reached 250 μg mg^{-1}. At the same time no correlation between Poly-P and P removal was observed. The efficiency of nutrient (P) removal from municipal wastewater to ultra-low levels (< 0.1 mg L^{-1}) is closely linked to algae starvation and auxiliary factors, like environmental pH and N/P ratio.

1. Introduction

Elevated nutrient, mainly phosphorus and nitrogen, loads from wastewater treatment plant (WWTP) effluents to surface waters leads to eutrophication and overall degradation of the aquatic ecosystems and subsequent economic losses. It is estimated that the financial loss from freshwater eutrophication and its consequences exceeds a billion US dollars annually [1], mainly affecting real estate and recreation industries. In conventional WWTP the nitrification-denitrification process effectively reduces nitrogen content [2] and approximately 10 to 30% of phosphorus (to 1–2 mg L^{-1}) via solids settling or activated sludge process [3]. However, phosphorous concentrations greater than 0.1 mg PL^{-1} are usually considered high enough to cause eutrophication [4]. Thus, development of wastewater post-treatment (viz reclamation) facilities to remove phosphorus to ultra-low concentrations are needed. Use of microalgae is proposed as an alternative for conventional wastewater post-treatment with activated sludge. This approach can be cost effective, and also allow phosphorus recovery, production of valuable products and capturing greenhouse gases [5].

It has been demonstrated that several microalgae strains can effectively reduce phosphorus content in various types of wastewater [6,7]. Moreover, algae have the capability of taking up P in the form of inorganic orthophosphate (P*i*) and certain organic forms in large excess when available. The phosphorus is then stored in the cells as polyphosphate granules. There are two pathways known to be involved in this phenomenon: 1) when exposed to high external P concentration, the polyphosphate accumulation is executed by the luxury P uptake mechanism [8,9] and results in 3- to 10-fold increase in cell P content [10,11]; 2) when P-limited algae are suddenly re-supplied with phosphorus, enhanced phosphorus uptake is observed and excess P as polyphosphate is stored in the cells. This process is referred as phosphorus overshoot or over-compensation [12] or phosphorus starvation [13], and is the focus of this study. Wu et al. [29] have demonstrated that P-starved algal biomass actively consumes phosphorus even after 26 days, with an average phosphorus uptake rate of approximately 6.15 mg P d^{-1}. Solovchenko et al. [8,9] showed that the P uptake rate increased 10 times for pre-starved algae re-fed with inorganic P. Increased phosphorus uptake efficiency by P-starved algae also has been

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demonstrated by Hernandez et al. [13].

In addition to enhanced P uptake, phosphorus and also nitrogen deficit can increase the algal cell lipid, carbohydrate and protein content [14]. Therefore, manipulation with nutrient availability can supplement the wastewater treatment process as an approach for high quality biomass production, added value compound extraction and remove phosphorus to ultra-low level without using chemicals.

Although the benefits of algal biomass P-starvation are well demonstrated, there are many uncertainties on how to control the process in engineered systems such as wastewater treatment plants (WWTP) There still is a need for systematic screening of different microalgal strains under relevant environmental, biological, and process conditions. This would reveal the potential triggers for enhanced P uptake and allow control over the technological process.

This paper investigates the short-term dynamics of phosphate removal from municipal wastewater by P-starved algal biomass in the laboratory scale. The study demonstrates the effect of different phosphorus starvation periods on algal biomass phosphate uptake rate and biomass polyphosphate accumulation and presents findings on biochemical dynamics of specific algae species after manipulation with phosphorus availability and discusses their applicability for wastewater post-treatment.

2. Methods

2.1. Microalgae strain and culturing conditions

The photoautotrophic microalgae strains *Desmodesmus communis* (CCAP 276/4B) and *Tetradesmus obliquus* (CCAP 276/10), and the facultatively heterotrophic strain *Chlorella protothecoides* (CCAP 211/10C) were used in the study.

Algae cells were pre-cultured in a sterile BG-11 growth medium [15]. Every two weeks half of the culture volume was replaced by newly prepared growth medium. The cell culturing was done in a 1000 ml Pyrex® bottles with constant 10 L h^{-1} aeration. The culture was kept under white fluorescent light with the photosynthetically active radiation of 100 μmol m^2 s^{-1} and 16:8-hour lighting regime. The ambient temperature was between 22–25 °C and the pH ~8.

For algal biomass phosphorus starvation, a phosphate-free (without K_2HPO_4) BG-11 growth medium was used. Microalgae that have been exposed to P-deficit conditions for 7 and 14 days were used for the experiments and biomass grown with sufficient phosphate availability was used as a reference. The periods of 7 and 14 days of phosphorus starvation were selected for near-complete to total depletion of cellular polyphosphate.

2.2. Wastewater source

For the experiment a wastewater from a treatment plant (WWTP) in Roja (Latvia) village (57.506465 N, 22.809536 E) was used. The plant provides service to two villages (Roja and Rude) with total population of ~3000 inhabitants. It also receives sewage from local fish processing factory, which operates seasonally and is generally responsible for high N and P concentrations. Both primary (after primary settling) and secondary (after biochemical oxidation and secondary settling) wastewaters were used for the experiment. Prior use the wastewater was double filtered through membrane filters with pore sizes of 0.45 and 0.2 μm to remove bacterial pollution and microparticles. The chemical content of both wastewater types after the filtration is shown in Table 1.

2.3. Experimental setup

The experiment was conducted in batch regime, in a 1000 ml Pyrex® bottles with a working volume of 800 ml, at a room temperature (24–27 °C) for 10 days. The average initial biomass concentration was 0.05 g DW L^{-1}. The bottles were placed on an orbital shaker with rotation period of 140 rpm and under white fluorescent light with the intensity of 100 μmol m^2 s^{-1} and 16:8-h lighting regime. No additional aeration and CO_2 supply was provided. Sampling was performed daily for the first five days and two more samples were taken on the 7th and 10th day. Each time a 50–100 mL sample was taken to detect the biomass change, nutrient concentration, the amount of cellular polyphosphate, pH and temperature.

Table 1
Characteristics of wastewater used for the experiment. NH_4 – dissolved ammonium, NO_{2+3} – sum of dissolved nitrite and nitrate, PO_4 – dissolved phosphate, EC – electrical conductivity, BOD – biochemical oxygen demand, COD – chemical oxygen demand.

Parameter		Primary WW	Secondary WW
Total nitrogen	mg N L^{-1}	110	40
NH_4	mg N L^{-1}	68	0.5
NO_{2+3}	mg N L^{-1}	21	32
Total phosphorus	mg P L^{-1}	36	36
PO_4	mg P L^{-1}	30	30
pH		8.3	8.2
EC	μS/cm	1700	1600
BOD	mg O_2 L^{-1}	530	5.3
COD	mg L^{-1}	970	42
N/P ratio		3:1	1:1

2.4. Analytical procedures

2.4.1. Nutrient analysis

To follow the algal nutrient consumption rate, the concentrations of total dissolved nitrogen (DIN) and phosphate (DIP) were measured. For DIN concentration measurement persulfate digestion method was used. For the DIP concentration measurement, the ascorbic acid method was used. Measurements were done using a spectrophotometer (DR 3900, HACH LANGE, USA) and appropriate reagent sets. The percentage nutrient removal was obtained using Eq. (1):

$$R_{N,P} = \frac{(C_0 - C_i)}{C_0} \times 100 \quad (1)$$

where $R_{N,P}$ is the removal (%) of nitrogen and phosphorus, C_0 is the initial nutrient concentration and C_i is the nutrient concertation at the end (or specific day) of experiment.

2.4.2. Determination of biomass change

Algae biomass growth was monitored using a UV–Visible spectrophotometer (Genesys 150, Thermo Fisher Scientific, USA). Light absorbance was measured at OD_{680} nm which is proportional to the change of cell number in most unicellular organisms [16]. The OD_{680} values were kept bellow 1.2. and the samples were diluted appropriately when exceeding this value. The dry weight of algal biomass was used for making growth curves. The relationship between algal dry weigh biomass (g L^{-1}) and light absorbance value at OD_{680} of *D. communis*, *T. obliquus* and *C. protothecoides* microalgae can be described by the Eqs. (2), (3) and (4), respectively:

$$DW_{D.communis} = 0.5555 \times OD_{680} - 0.0024;\ R^2 = 0.998 \quad (2)$$

$$DW_{T.obliquus} = 0.3166 \times OD_{680} - 0.0067;\ R^2 = 0.997 \quad (3)$$

$$DW_{C.protothecoides} = 0.4923 \times OD_{680} - 0.0060;\ R^2 = 0.996 \quad (4)$$

The specific biomass growth in the exponential phase was estimated by Eq. (5):

$$\mu(day^{-1}) = \ln\left(\frac{N_2}{N_1}\right)/(t_2 - t_1) \quad (5)$$

where N_1 and N_2 are the measured dry biomass at time t_1 and t_2, respectively. The biomass productivity (P) was calculated after the Eq.

(6):

$$P = (DW_i - DW_0)/(t_i - t_0) \quad (6)$$

where DW_i and DW_0 are dry biomass ($g\,L^{-1}$) at time t_i and t_0 (initial time), respectively.

2.4.3. Polyphosphate detection and quantification

For visual detection of intracellular polyphosphate granules microalgal cell sample was stained with 200 μg ml^{-1} 4′,6-diamidino-2-phenylindole (DAPI) and observed under fluorescent microscope. Stained cells and polyphosphate granules were observed using epifluorescent microscope (DM6000B, Leica, Germany) equipped with digital camera (DFC400 C, Leica, Germany). To detect poly-p granules fluorescent filter set with excitation wavelength at 370 nm and emission at 526 nm was used. DAPIstained polyphosphate granules present in the algal cells show green to bright yellow fluorescence at 526 nm [17].

Polyphosphate quantification was done following the protocol of Mukherjee & Ray [18]. In brief, the polyphosphates were extracted from microalgal biomass through a series of physical and chemical procedures. At first the algal cells were sonicated for 5 min at 30 kHz (amplitude 65%). Afterwards the sonicated cells were put into boiling (100 °C) waterbath for 2 h. Then a mixture of chloroform and isoamylalcohol (24:1) was added to the biomass and mixed vigorously. The suspension was centrifuged at 13,520*g* for 15 min. Afterwards the supernatant was collected and mixed with 0.2 N acetic acid and toluidine blue solution (stock conc. 30 mg L^{-1}) for spectrophotometric light adsorption measurement at 630 nm. Biomass polyphosphate concentration (μg mg^{-1}) was calculated against a calibration curve constructed using a sodium phosphate glass Type-45 (Sigma-Aldrich) as a polyphosphate standard.

2.5. Data analysis

Levene's test indicated no equal variances for the biomass growth and phosphate consumption data. The non-parametric Kruskall-Wallis *H* test was used to compare the impact on different P-starvation periods and wastewater types on algal biomass growth and phosphate consumption for each algal strain. Dunn's *post-hoc* test was used to detect pairwise differences between the treatments. Due to small population size ($n = 5$) the difference in polyphosphate accumulation was tested using Mann-Whitney *U* test. The limit of statistical significance in all tests was set to $\alpha \leq 0.05$. Statistical analyses were conducted using IBM SPSS v.23 software. The obtained results were visualized using the R v.3.3.2 software.

3. Results and discussion

3.1. Biomass growth of the selected algal species

All three of the selected algal strains were able to grow in both primary and secondary wastewater and after the exposure to 7- and 14-day P-deficit period, presented typical biomass growth curves (Fig. 2). *D. communis* and *T. obliquus* showed higher growth rates and productivity in the secondary wastewater. *C. protothecoides* grew better in the primary wastewater (Fig. 1).

D. communis and *T. obliquus* growth rates after 7-day P starvation in the secondary wastewater demonstrated 110 and 35% increase, respectively, when compared to reference conditions. In contrary, the growth rate of *C. protothecoides* in both types of wastewater decreased after P-starvation. The biomass productivity for *D. communis* and *T. obliquus* in the secondary wastewater increased by 248 and 19%, respectively, compared to the reference conditions, and it was higher than in primary wastewater. The productivity of *C. protothecoides* was the highest (0.049 g DW L^{-1} d^{-1}) in primary wastewater with no prior P-starvation. All strains had the highest biomass increase in the primary wastewater without biomass starvation. Statistically, *D. communis* and *C. protothecoides* biomass growth showed significant difference ($\chi^2 \geq 17.08$, $p \leq 0.004$ and $\chi^2 \geq 13.07$, $p \leq 0.023$, respectively) between the applied biomass pre-treatments.

The results of biomass growth suggest, that *D. communis* and *T. obliquus* are more suitable strains as biomass producers for municipal wastewater post-treatment complemented with biomass P-starvation. Their response to 7-day P deficiency was positive and it did not slow down or inhibit the growth of these strains. Such an effect can be explained by a shift in cellular biosynthetic pathway under nutrient deficiency conditions - photoassimilates are stored in the lipid bodies in the cytoplasm, ensuring the microalgae survival under stress conditions [19]. Also, cellular phosphorus and energy reserves were available during the P-starvation period, so the biomass growth was not interrupted. When inoculated in secondary wastewater with high phosphate content, optimal conditions for algal biomass growth were re-established. Similar observations were also made by Hernandez et al. [13]. After 14-day P-starvation the cellular P and energy resources were depleted, so the biomass growth was inhibited. After inoculation in wastewater it recovered slowly, and the resulting biomass productivity and increase was lower.

For *Chlorella protothecoides* the exposure to P-deficit conditions resulted in 25% lower biomass production, when compared to the reference conditions. This indicates the importance of cellular P reserves for *C. protothecoides* biomass production. Moreover, in this study *C. protothecoides* showed higher biomass production in primary wastewater, where higher amount of carbon and nitrogen were available, than in the secondary wastewater (Table 1). This result suggests that *C. protothecoides* could grow heterotrophically consuming the organic compounds present in the primary wastewater, and it is in line with the findings by Zhou et al. [20]. It also implies the importance of nitrogen availability for *C. protothecoides* biomass production. This can be related to *C. protothecoides* naturally high lipid productivity [21], which requires both N and P to sustain the lipid production and biomass growth [22].

3.2. Nutrient removal

All three strains showed high phosphate removal capacity at the end of the experiment. However, the removal rates varied in different types of wastewater and after different periods of P-starvation.

Reference *D. communis* and *T. obliquus* nearly completely (> 99.0%) removed phosphate from secondary wastewater within 5 and 3 days, respectively. At the same conditions *C. protothecoides* could remove 93.9% in 10 days. In primary wastewater the nutrient removal rate was lower for all three strains - 97.7, 96.6 and 84% for *D. communis*, *T. obliquus* and *C. protothecoides*, respectively, within 10 days (Fig. 4). High phosphate removal rates from municipal wastewaters by these strains have also been reported by Samorì et al. [28] and Tejido-Nuñez et al. [23]

Biomass exposure to P-starvation for 7-days boosted the phosphate removal by *D. communis* and *T. obliquus*, which reduced the phosphate content in secondary wastewater by 88.7 and 89.5%, respectively, within the first 24 h after inoculation and by > 99.0% after two days. This result is likely to be related to luxury P uptake [12]. This phosphorus uptake and storage mechanism can be triggered by biomass P-starvation, which decreases the cellular phosphorus reserves. When resupplied with high external phosphate, algal cells rapidly accumulate more phosphorus than needed for their growth.

Different algal strains respond differently to P-starvation. Results from this study showed no distinct P-starvation effect on phosphate removal by *C. protothecoides* – complete PO_4 concentration reduction was obtained at the very end of experiment and only 27.5% of phosphate was removed within the first 24 h. This is could be caused by too long P-starvation period. Similar effect on *Chlorella* sp. P-starvation was also reported by Hernandez et al. [13] - P-deficit for 3 days increased the P removal rate from wastewater two times, while longer period of P-

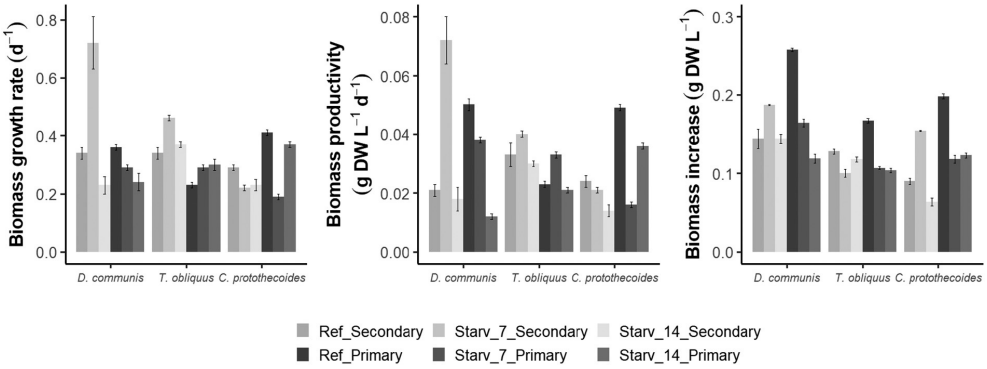

Fig. 1. Biomass growth parameters for *D. communis*, *T. obliquus* and *C. protothecoides* in batch under six different treatments within ten days (means ± SD, $n = 3$).

deficit (5d) hampered the biomass growth and the phosphate removal decreased.

The enhanced phosphate concentration decrease in the secondary wastewater was also contributed by its precipitation due to increasing pH [24]. The rapid biomass growth of *D. communis and T. obliquus* resulted in consumption of the dissolved CO_2 in wastewater which increased the pH of the medium from 8.0 to 11.5 as no CO_2 was added to the batch to control the pH. This assumption was confirmed by the phosphate content change in the negative control (Fig. 3) - without biomass, but with increasing pH, the phosphate concentration in wastewater was decreasing. On the other hand, certain portion of phosphate had to be consumed for biomass growth before it could establish alkalinity conditions that favored phosphate precipitation. Thus, it can be concluded that during the initial phase of experiment (until day 2–3, depending on the treatment) phosphate was removed via biomass uptake, while at the later growth stage the phosphate was mainly precipitated.

In the primary wastewater phosphate concentration was reduced slower and to a lesser extent. This can be explained by the high organic carbon content in the primary wastewater characterized by BOD and COD concentrations (Table 1). The photosynthetic oxygen production provided conditions for organic carbon oxidization to CO_2, which kept the pH < 9 during the initial 2–3 days of experiment (Fig. S1).

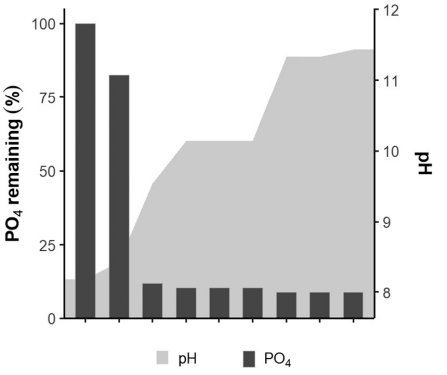

Fig. 3. Relation of the phosphate content to pH changes in the control batch.

Although no data were obtained to support this presumption, COD oxidation to CO_2 is known as one of the key processes in biological wastewater treatment [25]. In the secondary wastewater the organic carbon content was significantly lower, therefore less CO_2 could be

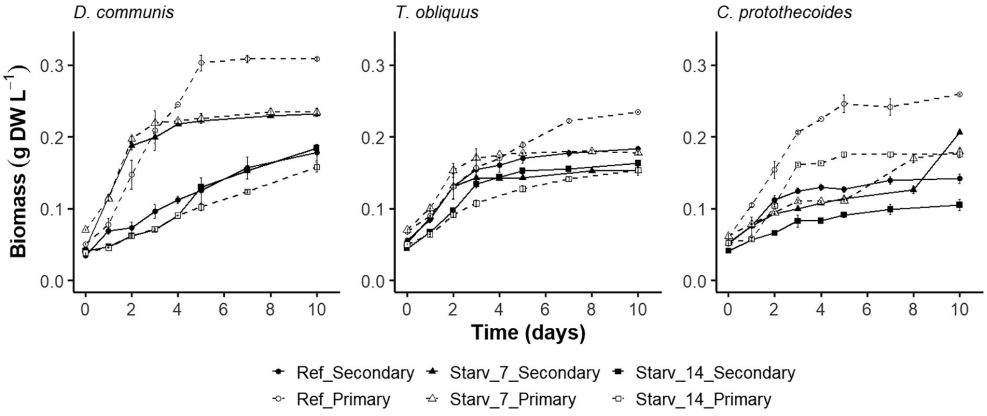

Fig. 2. Biomass growth under different treatments (means ± SD, n = 3). *Ref_Secondary* – reference conditions with secondary wastewater; *Starv_7_ Secondary* – algal biomass starvation for 7 day period with secondary wastewater; *Starv_14_ Secondary* – algal biomass starvation for 14 day period with secondary wastewater; *Ref_Primary* – reference conditions with primary wastewater; *Starv_7_ Primary* – algal biomass starvation for 7 day period with primary wastewater; *Starv_14_ Primary* – algal biomass starvation for 14 day period with primary wastewater.

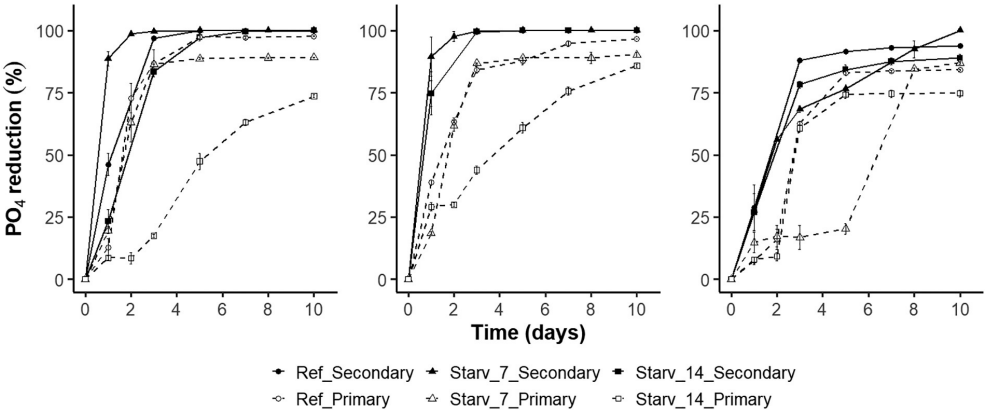

Fig. 4. Phosphate reduction rate (%) under different treatments (means ± SD, n = 3). *Ref_Secondary* – reference conditions with secondary wastewater; *Starv_7_ Secondary* – algal biomass starvation for 7 day period with secondary wastewater; *Starv_14_ Secondary* – algal biomass starvation for 14 day period with secondary wastewater; *Ref_Primary* – reference conditions with primary wastewater; *Starv_7_ Primary* – algal biomass starvation for 7 day period with primary wastewater; *Starv_14_ Primary* – algal biomass starvation for 14 day period with primary wastewater.

produced, and more phosphate precipitated due to high pH.

The obtained results on phosphate removal highlights *D. communis* and *T. obliquus* strains to be appropriated for municipal wastewater post-treatment supplemented with P-starvation. However, an increase in pH and subsequent phosphate precipitation is an undesirable side effect for algae-based wastewater treatment. To fulfill the purpose of algal use for wastewater treatment (i.e., no waste and high-value biomass production), pH must be controlled and kept at neutral level.

Statistically significant difference in PO_4 removal between different biomass pre-treatments was identified for *D. communis* and *T. obliquus* algae (p-value ≤ 0.034 and 0.024, respectively).

The removal of dissolved inorganic nitrogen (DIN) for all three strains at all treatments was low (Table 2). The highest efficiency in 10 days growth experiment was observed for *D. communis* that had a prior 7-day P starvation period, removing 49.0 and 46.5% from secondary and primary wastewater, respectively. 7-day P starved *T. obliquus* could remove only 41.1% of DIN from primary wastewater, other treatments removal was below 28%. *C. protothecoides* could remove only between 0 and 17.1% of DIN. Most likely, the inorganic nitrogen could not be removed through nitrification-denitrification process due to lack of the bacterial species involved and inappropriate oxygen conditions within the batch.

3.3. Biomass polyphosphate accumulation

All three algal strains used in the experiment could accumulate polyphosphates while growing in the wastewater. It was confirmed visually, using fluorescence microscopy (Fig. 5) and quantitively by Poly-P granule extraction. Changes in the biomass polyphosphate content followed a similar pattern in all three selected strains (Fig. 6) – an increase during the first two days, gradual decrease during the next three days and a slight increase or no change until the 10th day of experiment. Only for *D. communis* biomass grown in both types of wastewater with no prior P-starvation did not follow the common polyphosphate formation pattern. The observed dynamics of biomass polyphosphate concentration change is another indicator to involvement of the luxury P uptake mechanism in phosphate removal. At high initial phosphate concentration, the accumulation of biomass polyphosphate occurred. When no more phosphate from the wastewater was available, the accumulated polyphosphate was used to sustain the cell doubling process and the polyphosphate reserves rapidly decreased.

All three strains showed higher polyphosphate accumulation when

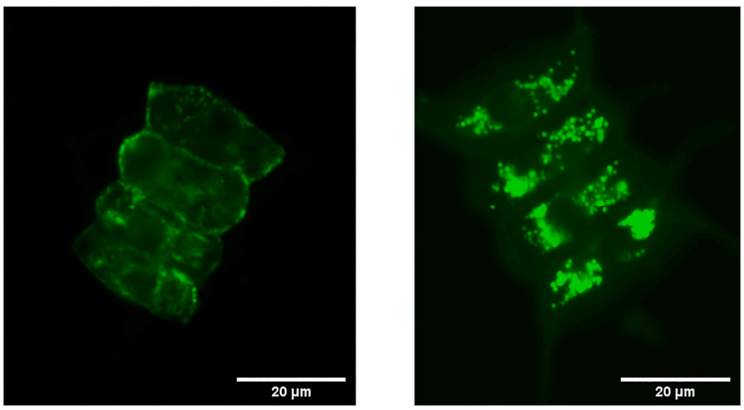

Fig. 5. *Desmodesmus communis* cell without polyphosphate granules (left) and with Poly-P granules stored in the cell (right).

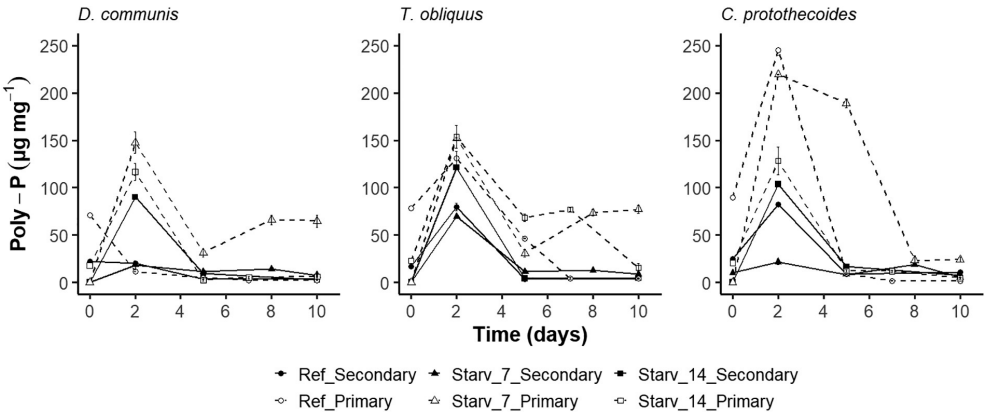

Fig. 6. Polyphosphate (Poly-P) accumulation in algal biomass under different treatments (means ± SD, $n = 3$). *Ref_Secondary* – reference conditions with secondary wastewater; *Starv_7_ Secondary* – algal biomass starvation for 7 day period with secondary wastewater; *Starv_14_ Secondary* – algal biomass starvation for 14 day period with secondary wastewater; *Ref_Primary* – reference conditions with primary wastewater; *Starv_7_ Primary* – algal biomass starvation for 7 day period with primary wastewater; *Starv_14_ Primary* – algal biomass starvation for 14 day period with primary wastewater.

grown in primary wastewater. The peak value for biomass polyphosphate content was similar for *D. communis* and *T. obliquus*, reaching 160.8 and 152.3 µg mg^{-1}, respectively, grown in primary wastewater after 7 days of biomass P-starvation. The highest biomass polyphosphate content in *C. protothecoides* (245.3 µg mg^{-1}) was obtained in primary wastewater without prior P-starvation.

Higher polyphosphate accumulation for all three strains grown in primary wastewater corresponds to slower pH increase during the initial phase of experiment (Fig. S1). Such pH dynamics can be explained by the excess organic carbon present in primary wastewater and its oxidation to CO_2. This prevented pH increase and phosphate precipitation during the first 2–3 days of experiment. Subsequently, phosphate could be consumed by biomass and accumulated as polyphosphate. In addition, due to lower growth rates of *D. communis* and *T. obliquus* in primary wastewater, less of the accumulated Poly-P was used for new cell production. In contrary, for *C. protothecoides* the chemical composition of primary wastewater (N/P ratio and carbon content) was more favorable for biomass growth and Poly-P accumulation [26].

The biomass Poly-P content of *D. communis* and *T. obliquus* grown in secondary wastewater after 7-day P-starvation showed no difference from the result obtained at reference conditions. This is a further indication for phosphate removal mechanism switch from biomass uptake initially and phosphate precipitation after day 2–3. Thus, the cellular polyphosphate was simultaneously accumulated and used for production of new cells, supporting the high biomass growth rate.

There were no significant differences observed for biomass polyphosphate formation among the treatments for individual species and neither were they different among each other.

From the obtained results on biomass polyphosphate accumulation dynamics it is hard to point out a suitable strain for wastewater treatment and high-quality biomass production. Direct phosphate uptake by biomass would produce biomass rich in polyphosphate which is a source for synthesis of all vital cell molecules, such as amino acids, sugars, lipids [27]. Therefore, these results further emphasize the importance of pH control during algae-based wastewater treatment.

3.4. Algal biomass P-starvation in pilot-scale systems

The main advantage to algal biomass phosphorus starvation is the resulting rapid phosphate reduction to ultra-low (< 0.1 mg P L^{-1}) concentration. This makes it appropriate for post-treatment of the

Table 2
Nutrient removal rates for *D. communis*, *T. obliquus* and *C. protothecoides* in batch under six different treatments within ten days (% ± SD, $n = 3$).

Strain	Treatment	DIP removal (10 days), %	DIP removal (24 h), %	DIN removal (10 days), %
Desmodesmus communis	*Ref_Secondary*	> 99.0 ± 0.00	48.0 ± 1.84	17.3 ± 2.51
	Starv_7_ Secondary	> 99.0 ± 0.00	88.7 ± 2.36	49.0 ± 2.20
	Starv_14_ Secondary	> 99.0 ± 0.00	23.4 ± 3.85	27.9 ± 2.69
	Ref_Primary	97.7 ± 0.14	11.7 ± 4.40	20.5 ± 2.01
	Starv_7_ Primary	89.1 ± 0.19	19.4 ± 0.85	46.5 ± 3.29
	Starv_14_ Primary	73.6 ± 0.49	9.8 ± 0.93	9.8 ± 2.40
Tetradesmus obliquus	*Ref_Secondary*	> 99.0 ± 0.00	77.3 ± 3.34	25.5 ± 2.36
	Starv_7_ Secondary	> 99.0 ± 0.00	89.5 ± 6.47	27.4 ± 3.57
	Starv_14_ Secondary	> 99.0 ± 0.00	74.7 ± 4.30	28.1 ± 3.36
	Ref_Primary	96.6 ± 0.01	38.8 ± 0.59	18.5 ± 2.96
	Starv_7_ Primary	90.2 ± 0.83	18.6 ± 0.43	41.1 ± 1.10
	Starv_14_ Primary	85.9 ± 0.57	29.1 ± 1.32	10.1 ± 3.24
Chlorella protothecoides	*Ref_Secondary*	93.9 ± 0.33	35.7 ± 3.97	0.0 ± 0.00
	Starv_7_ Secondary	> 99.0 ± 0.00	27.5 ± 0.66	17.1 ± 0.33
	Starv_14_ Secondary	88.9 ± 1.33	26.9 ± 6.14	3.0 ± 0.32
	Ref_Primary	84.0 ± 0.29	7.2 ± 0.97	14.5 ± 7.32
	Starv_7_ Primary	87.0 ± 0.35	14.8 ± 3.36	11.6 ± 1.90
	Starv_14_ Primary	74.8 ± 1.14	7.9 ± 0.96	13.4 ± 6.06

effluent from small (PE < 4000) WWTPs, where phosphorus concentrations remain relatively high (> 6 mg P L^{-1}) and often has no requirement for phosphorus content reduction limits. Algae-based phosphorus removal system supplemented with nutrient starvation approach could provide not only enough phosphorus content reduction but also an economical alternative to chemical post-treatment. The obtained results show that algae P-starvation can be an effective means for rapid and near-complete inorganic P removal from wastewater. Considering the purpose and desired outcome of using this approach, it could work in a sequenced batch mode. Still, the expected hydraulic retention time of such a system is too long for practical introduction in full scale. Thus, the algae P-starvation period tested in this study (7 days) is far too long for pilot applications and should be decreased, despite its promising performance on phosphate removal.

Another consideration is the control over the process in pilot scale systems. Multiple PO_4 removal pathways can be involved, if biomass P-starvation is applied. This study show that pH must be controlled and kept at neutral level to avoid phosphate precipitation and stimulate its uptake by algal cells. This would result in more valuable biomass production and possible economic return from biomass downstream processing. Also, the biomass production and quality are affected by the availability and amount of carbon and inorganic nitrogen. Results in this study show, that higher carbon content and N/P ratio can promote polyphosphate accumulation in *C. prototthecoides*, which subsequently results in higher production of high-value products extractable from the biomass Smachetti et al. [14]. Therefore, the carbon and inorganic nitrogen concentrations and N/P ratio are other parameters that needs deliberate control in full-scale systems for successful application of algal biomass P-starvation.

4. Conclusions

This study investigated the effect of phosphorus starvation on three microalgal species, *Desmodesmus communis*, *Tetradesmus obliquus* and *Chlorella prototthecoides*, and their biomass growth, phosphate reduction rate and biomass polyphosphate accumulation in municipal wastewater. No single strain can be emphasized as more effective for municipal wastewater treatment, however, *D. communis* and *T. obliquus* demonstrated more rapid PO_4 removal. Seven days of algal P-starvation had a positive effect on phosphate reduction, reaching ~89% PO_4 removal in secondary wastewater within 24 h for *D. communis* and *T. obliquus*. Biomass polyphosphate accumulation was higher for *C. prototthecoides*, especially in primary wastewater, reaching 245 μg g^{-1}.

Nevertheless, both PO_4 removal and biomass Poly-P accumulation are highly dependent on the pH and N/P ratio and organic carbon concentration in the growth medium. Change in pH was directly affected by biomass growth, and indirectly by organic carbon concentration in wastewater. As a result of high pH, majority of phosphate precipitates, disabling its involvement in biomass quality improvement. Therefore, no control over pH changes makes P-starvation inoperative in full scale algae-based wastewater treatment systems. The N/P ratio in wastewater is also a concern for biomass growth and nutrient removal efficiency. Since its fluctuation during wastewater treatment cannot be avoided, inconsistent results must be expected when using algae for wastewater treatment in pilot conditions.

Polyphosphate content in the algal cells did not show to be an indicative parameter for P-starvation efficiency as higher phosphate uptake rate by P-starved algae did not correlate to its biomass Poly-P concentration. This means that polyphosphates are constantly in use for new cell production. However, multiple auxiliary factors, such as pH, N/P ratio and organic carbon can affect the cellular Poly-P content.

To advance the P-starvation towards use in engineered systems, a feasible balance is needed between the key process parameters – starvation period, biomass production and nutrient reduction rates and cellular chemical content.

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CRediT authorship contribution statement

AL designed the experiments and methodology, performed formal data analysis and visualization, wrote the original draft, worked on funding acquisition; **LM** designed the methodology, edited and reviewed the draft, worked on funding acquisition, administrated the project; **TJ** supervised the experiment, edited and reviewed the draft, worked on funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Statement of informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

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Increasing Phosphorus Uptake Efficiency by Phosphorus-Starved Microalgae for Municipal Wastewater Post-Treatment

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Abstract: Four microalgal species, *Chlorella vulgaris*, *Botryococcus braunii*, *Ankistrodesmus falcatus*, and *Tetradesmus obliquus* were studied for enhanced phosphorus removal from municipal wastewater after their exposure to phosphorus starvation. Microalgae were exposed to phosphorus starvation conditions for three and five days and then used in a batch experiment to purify an effluent from a small WWTP. After 3-day P-starvation, *C. vulgaris* biomass growth rate increased by 50% and its PO_4 removal rate reached > 99% within 7 days. *B. braunii* maintained good biomass growth rate and nutrient removal regardless of the P-starvation. All species showed 2–5 times higher alkaline phosphatase activity increase for P-starved biomass than at the reference conditions, responding to the decline of PO_4 concentration in wastewater and biomass poly-P content. The overall efficiency of biomass P-starvation on enhanced phosphorus uptake was found to be dependent on the species, N/P molar ratio in the wastewater, as well as the biomass P content.

Keywords: microalgae; phosphorus starvation; municipal wastewater; nutrient removal; polyphosphate; alkaline phosphatase activity

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1. Introduction

Globally, phosphorus (P) is a major nutrient causing eutrophication of aquatic ecosystems. Among the many phosphorus sources, effluents from wastewater treatment plants (WWTPs) provide significant loading of P into the surface waters. Recovery of P from wastewaters is lately gaining more attention [1]. Chemical precipitation and enhanced biological (bacterial) uptake are the main methods for additional P removal used in WWTPs. Although these methods are well established and applied in the large WWTPs, their use in small WWTPs is often not practiced due to legislative acts. For instance, in the European Union the regulation on municipal wastewater treatment (Directive 91/271/EEC) does not state any limits for permissible P concentration in the effluent from WWTPs operating in small agglomerations with less than 2000 p.e. [2]. Furthermore, these methods still cannot reduce the P concentration to ultra-low level (<0.1 mg P L-1). Scaling the alternative P removal methods to small WWTPs results in high capital and operational costs and overall system complexity [3], and is rather detrimental as the obtained phosphorus reduction rates in small scale are often insufficient and fail to meet environmental safety standards.

A promising alternative to the traditional wastewater P removal and recovery methods is the use of microalgae systems [4,5]. Many studies, mostly in lab scale, have shown that various microalgal strains or mixed cultures are good candidates for wastewater post-treatment as they can reduce the phosphorus concentrations in wastewaters to ultra-low levels [6]. Still, there are often reports on incomplete P removal rates over longer

time periods [7–9] which can be viewed as a drawback for efficient microalgae-based wastewater post-treatment step. On the other hand, certain conditions are known at which algal cells can consume more phosphorus than their production requires. Enhanced algal phosphorus uptake (EAPU) and storage of excess phosphorus in algal cells can be achieved by manipulation with external phosphorus availability [10,11]. The two approaches known to cause this phenomenon are the luxury P uptake mechanism [12,13] and phosphorus starvation [14], which are initiated by excess phosphorus availability and phosphorus limited conditions, respectively. Both biomass manipulation approaches can result in ten times higher phosphorus uptake rate [15] when compared to regular biomass phosphorus consumption. In addition to more efficient phosphorus uptake, biomass exposure to nutrient stress is known to promote valuable substance production by algal cells [16], thus adding value to the produced biomass and providing an opportunity for financial return.

The existing knowledge on enhanced phosphorus uptake induced by algal biomass starvation shows it as a promising feature for algae-based wastewater post-treatment technologies. Though, several ambiguities still need to be specified before proceeding with EAPU implementation into full scale algae-based wastewater post-treatment systems: (1) it is unclear whether biomass manipulation for EAPU is limited to specific strains; (2) more information is needed for indicators that characterize the status of biomass phosphorus deficiency, and (3) it should be clarified under which biochemical conditions the EAPU is more efficient, and what are the physical and biochemical conditions that hampers this process.

This paper investigates the impact of various P-starvation periods on phosphate removal efficiency from municipal wastewater. In the batch experiments we compared the performance of different microalgal species in terms of their biomass growth and nutrient (phosphate and nitrate) removal rates to estimate their applicability for wastewater treatment. Biochemical parameters such as polyphosphate accumulation and alkaline phosphatase activity were assessed for quantitative characterization of algal biomass P-starvation.

2. Materials and Methods

2.1. Microalgae Strain Selection and Culturing Conditions

The photoautotrophic microalgae strains *Tetradesmus obliquus* (CCAP 276/10), *Botrycoccus braunii* (CCAP 807/1), *Chlorella vulgaris* (CCAP 211/11B), and *Ankistrodesmus falcatus* (CCAP 202/5C) were used in this study. These strains were selected for their reported ability to grow in various types of wastewaters, reduce nutrient content, and produce valuable substances such as carbohydrates and lipids.

Algae cells were pre-cultured in a sterile BG-11 growth medium [17]. Every two weeks half of the culture volume was replaced by freshly prepared growth medium. The cell culturing was done in a 1000 mL Pyrex® bottles with constant 10 L h^{-1} aeration. Tubular (T5) fluorescent lamps with the blue-red spectrum were used as a light source. The provided photosynthetically active radiation was 180 µmol m^2 s^{-1} at a 16:8-h lighting regime. The ambient temperature was between 25 and 27 °C and the pH ~7.5.

For the phosphorus starvation, algal biomass was prepared by washing it with demineralized water. At first it was centrifuged (4000× *g*, 2 min) and the growth medium supernatant was replaced by demineralized water. Afterwards the biomass was vortexed and centrifuged again. After the centrifugation, the supernatant was discarded, and biomass was inoculated in a phosphate-free (without K_2HPO_4) BG-11 growth medium. Microalgae that have been exposed to P-deficit conditions for 3 and 5 days were used for the experiments and biomass produced with sufficient phosphate availability was used as a reference.

2.2. Wastewater Source

Wastewater from a treatment plant (WWTP) in Roja (Latvia) village (57.506465 N, 22.809536 E) was used for the research. The plant provides service to two villages (Roja and Rude) with a total population of ~3000 inhabitants. It also accepts sewage from three

local fish processing factories that accounts for 21.4% of the total 30,846 m^{-3} of sewage the plant received in 2019.

Secondary wastewater after biochemical oxidation and secondary settling was double filtered through membrane filters with pore sizes of 0.45 and 0.2 µm to remove bacterial pollution and microparticles prior all experiments. The chemical content of the wastewater after the filtration is shown in Table 1.

Table 1. Characteristics of wastewater used for the experiment.

Parameter	Unit	Value
Total nitrogen	mg N L^{-1}	24.5
NH_4	mg N L^{-1}	0.5
NO_{2+3}	mg N L^{-1}	21.3
Total phosphorus	mg P L^{-1}	20.1
PO_4	mg P L^{-1}	17.5
pH		8.2
EC	µS/cm	1600
BOD	mg O_2 L^{-1}	5.3
COD	mg L^{-1}	74

2.3. Experimental Setup

The experiments were conducted in batch regime, in 1000 mL Pyrex® bottles with working volume of 800 mL, at room temperature (25–27 °C) for 10 days. The algae-wastewater suspensions were mixed by aeration (10 L h^{-1}) and supplemented with 1% (*v*/*v*) CO_2. The bottles were illuminated with fluorescent light with blue-red spectrum with the intensity of 180 µmol m^2 s^{-1} at a 16:8-h lighting regime. Sampling was performed daily, each time taking a 70 mL sample to detect the biomass change, nutrient concentration, the amount of cellular polyphosphate, alkaline phosphatase activity, pH, and temperature.

2.4. Analytical Procedures

2.4.1. Nutrient Analysis

To follow the algal nutrient consumption from wastewater, concentration changes for orthophosphate, nitrate as well as total nitrogen and total phosphorus were observed. Measurements were done using a spectrophotometer (HACH LANGE DR 3900, Loveland, CO, USA) and commercial reagent sets for each parameter analysis. Total nitrogen and nitrate concentrations were measured using alkaline persulfate digestion method [18] and chromotropic acid method [19], respectively. Total phosphorus and phosphate concentrations were measured using the ascorbic acid method [20]. The percentage nutrient removal was obtained using Equation (1):

$$R_{N,P} = \frac{(C_0 - C_i)}{C_0} \times 100 \quad (1)$$

where $R_{N,P}$ is the removal (%) of nitrogen and phosphorus, C_0 is the initial nutrient concentration, and C_i is the nutrient concertation at the end (or specific day) of experiment.

2.4.2. Determination of Biomass Concentration Change

Algae biomass growth was detected spectrophotometrically using an UV-Visible spectrophotometer (Thermo Scientific Genesys 150, Waltham, MA, USA). Light absorbance was measured at 680 nm which is proportional to the change of cell number in most unicellular organisms [21]. The OD_{680} values were kept bellow 1.2. and the samples were diluted appropriately when exceeding this value. The dry weight of algal biomass was used for making growth curves. The relationship between algal dry weigh biomass (g L^{-1}) and light absorbance value at OD_{680} was described by the individual equations constructed for each algal species (Table 2).

Table 2. Calibration curves: optical density (OD_{680}) to biomass dry weight (g L^{-1}).

Species	Equation	R^2
T. obliquus	y = 0.5817x − 0.0129	0.997
C. vulgaris	y = 0.4076x – 0.0052	0.999
B. braunii	y = 0.5183x – 0.0054	0.989
A. falcatus	y = 0.5421x – 0.003	0.992

The specific biomass growth in the exponential phase was estimated by Equation (2):

$$\mu\left(\text{day}^{-1}\right) = \frac{\ln(N_2/N_1)}{(t_2 - t_1)} \quad (2)$$

where N_1 and N_2 are the measured dry biomass at time t_1 and t_2, respectively. The biomass productivity (Pr) was calculated according to the Equation (3):

$$\text{Pr} = \frac{(DW_i - DW_0)}{(t_i - t_0)} \quad (3)$$

where DW_i and DW_0 are dry biomass (g L^{-1}) at time t_i and t_0 (initial time), respectively.

2.4.3. Polyphosphate Detection and Quantification

Polyphosphate content in algal biomass was measured following the protocol of Mukherjee and Ray [22]. Briefly, the polyphosphates were extracted from microalgal biomass through a series of physical and chemical procedures. Suspension samples were concentrated to harvest enough biomass (>0.05 g fresh weight) for the extraction process. At first the algal cells were disrupted using an ultrasonic processor (Cole-Palmer Instruments, 130-watt, Vernon Hills, IL, USA), treating the sample for five minutes at 30 kHz. Afterwards the disrupted cell samples were heated at 100 °C for 2 h. Then a mixture of chloroform and isoamyl alcohol (24:1) was added to the biomass and mixed vigorously. The suspension was centrifuged at 13,520 g for 15 min. Afterwards the supernatant was collected and mixed with 0.2 N acetic acid and toluidine blue solution (stock conc. 30 mg L^{-1}) for spectrophotometric light adsorption measurement at 630 nm. Biomass polyphosphate concentration (µg mg^{-1}) was calculated against a calibration curve constructed using a sodium phosphate glass Type-45 (Sigma-Aldrich, St. Louis, MO, USA) as a polyphosphate standard.

2.4.4. Alkaline Phosphatase Activity Detection

The alkaline phosphatase (AP) activity was estimated by using a method which involves para-nitrophenylphosphate disodium hexahydrate (p-NPP, Sigma-Aldrich, St. Louis, MO, USA) hydrolysis to para-nitrophenol (p-NP). Total of 3 mL sample was mixed with 1 mL Tris HCl buffer (pH 9.5) and 0.4 mL p-NPP (0.5 mg mL^{-1}). The mixture was incubated at 37 °C for 1 h in the dark. The yielded p-NP was measured spectrophotometrically at 405 nm. p-NP amount was calculated against a calibration curve that was constructed using a p-NP as a standard. The result was used as an indicator for AP activity. A control containing no biomass was included in the routine, and its OD_{405} reading was subtracted from the hydrolyzed value. AP activity was expressed as p-NP flux from dry-weight biomass per hour.

2.5. Determination of Nutrient Uptake Kinetics

Nutrient uptake rates and biomass nutrient consumption for NO_3-N and PO_4-P were estimated for the biomass exponential growth rate period, from experiment day 1–5.

The biomass nutrient consumption $V_{N(P)}$ (mg *N(P)* g^{-1} DW) was calculated as nutrient concentration change over biomass concentration increase:

$$V_{N(P)} = \frac{cN(P)_0 - cN(P)_i}{b_i - b_0} \quad (4)$$

where $cN(P)_0$ is the initial nutrient concentration, $cN(P)_i$ is the nutrient concentration at a specific time, b_0 is the initial biomass concentration, b_i is the biomass concentration at a specific time.

The nutrient uptake rate k (d^{-1}) was calculated as biomass nutrient consumption over biomass growth rate:

$$k = \frac{V_{N(P)}}{\mu} \quad (5)$$

where $V_{N(P)}$ is the biomass nutrient consumption from Equation (4) and μ is the biomass growth rate from the Equation (2).

2.6. Data Analysis

Parametric one-way ANOVA test was used to detect the significant differences among different biomass P-starvation periods on algal biomass growth, nutrient consumption, biomass polyphosphate accumulation, and APA for each algal specie. Tukey post-hoc test was used to detect pairwise differences between the treatments. The limit of statistical significance in all tests was set to $\alpha \leq 0.05$. Results are presented as mean values ($n = 3$). Statistical analyses were conducted using IBM SPSS Statistics version 23 software (Armonk, NY, USA). The obtained results were visualized using R: A Language and Environment for Statistical Computing, version 4.0.5 (Vienna, Austria).

3. Results and Discussion

3.1. Algal Growth and Biomass Production

All species used for the experiment could produce biomass in secondary wastewater. No negative effect (e.g., growth inhibition or cell death) on the biomass growth were observed due to biomass exposure to phosphorus deficiency (Figure 1). Differences in biomass growth rate (μ), biomass productivity (Pr), and biomass increase were observed among the species and applied P-starvation periods (Figure 2). *C. vulgaris* showed higher μ after its biomass exposure to P-deficiency. Comparing to the reference conditions, the biomass growth rate after 3- and 5-day starvation was 49.7 and 30.3% higher, respectively. Moreover, the productivity of *C. vulgaris* reached 104.9% after 3-day starvation. Unlike other species, after 3- and 5-day P-starvation *C. vulgaris* showed 45.5 and 55.0% higher biomass increase respectively, than in the reference batch. The concentration change of P-starved biomass showed no statistically significant difference ($p > 0.05$) from the reference biomass.

The observed enhanced biomass growth of *C. vulgaris* as a response to P-starvation is partly in line with the observations by Hernadez et al. [14], who obtained a significant biomass growth increase for *C. vulgaris* in domestic wastewater after P-starvation for 3 days. Moreover, rapid biomass growth of *C. vulgaris* after 3-day P-starvation was demonstrated by Solovchenko et al. [23]. Their given explanation behind such an outcome is a rapid P*i* uptake by starved biomass after phosphorus re-feeding, further synthesis of phosphorus to polyphosphate that is a source of energy and metabolic processes for cells, and its intensive consumption for new cell production.

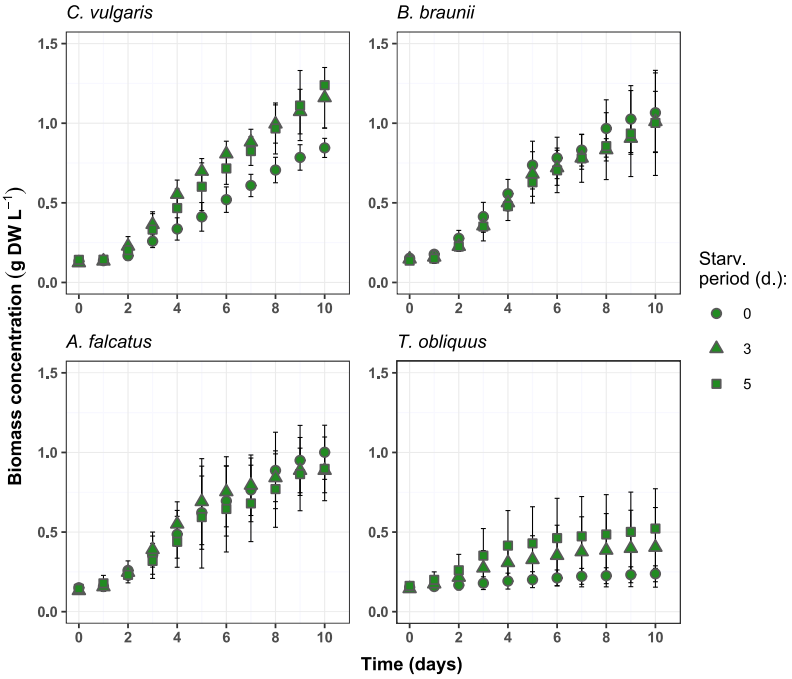

Figure 1. Biomass concentration changes over time in the experiment batches for *C. vulgaris*, *B. braunii*, *A. falcatus* and *T. obliquus* (means ± SD, $n = 3$).

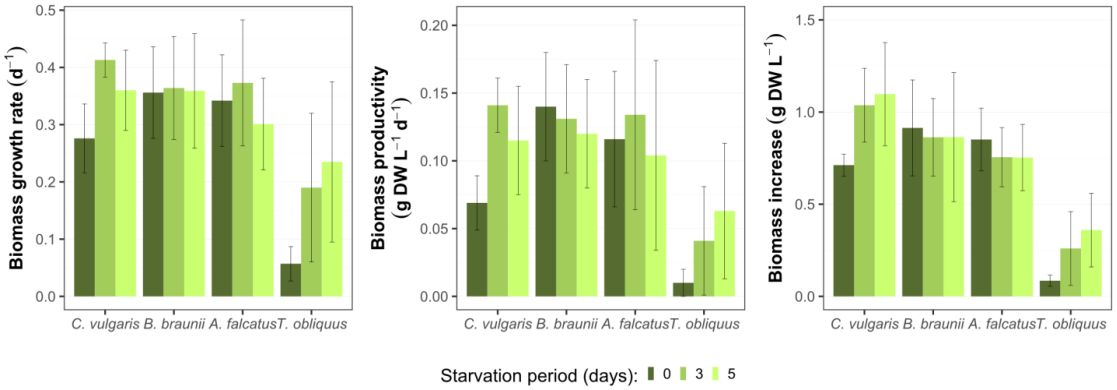

Figure 2. Biomass growth parameters for *C. vulgaris*, *B. braunii*, *A. falcatus*, and *T. obliquus* in batch conditions after various P-starvation periods within ten days (means ± SD, $n = 3$).

For *B. braunii* biomass concentration change at both P-starvation periods did not show significant difference ($p > 0.05$) from the reference batch. The biomass growth rate and productivity were 6.0 and 10.0% higher, respectively, for both starvation periods. The observed biomass increases for 3- and 5-day starved biomass was 3.3 and 7.3% lower than in the reference batch. Other studies show inconsistent results for *B. braunii* biomass production in wastewater. For instance, Aravantinou et al. [24] demonstrated more than 10 times lower biomass growth rate of *B. braunii*, than obtained in this study. Álvarez-Díaz et al. [25] argued that *B. braunii* is unsuitable for production in municipal wastewater due to its low

growth rate and productivity. On the other hand, Ruangsomboon [26] showed that *B. braunii* biomass production increased with higher phosphate concentration, and the rates were comparable to the present study. This specie has also shown good productivity in industrial wastewaters with high carbon content [27,28]. Thus, different studies suggest that *B. braunii* is a nutrient demanding specie and phosphorus stress is rather adverse for its biomass production. On the other hand, the results from present study show that the selected *B. braunii* strain can tolerate phosphate stress and grows well in wastewater after exposure to different P-starvation periods.

The growth rate of *A. falcatus* biomass increased by 8.8% after 3-day P-starvation but was 12.0% lower than the reference after 5-day P-starvation. The biomass productivity of *A. falcatus* was 12.8% higher for 3-day starved and 15% lower for 5-day starved, while the biomass increase was 11.3% lower for both starvation periods than in the reference batch. No significant difference ($p > 0.05$) was observed between the growth of P-starved and reference biomasses. Compared to other species *A. falcatus* showed less productive performance for all biomass growth parameters. A study by Álvarez-Díaz et al. [25] showed, that in terms of biomass growth rate and productivity *A. falcatus* was outperformed by *C. vulgaris* and *B. braunii*. Compared to the reference conditions, *A. falcatus* reached slightly higher biomass growth rate and productivity after 3 days of P-starvation. A possible reason for such a behavior by *A. falcatus* is its high lipid productivity, which is boosted under P-deficiency conditions. Being a key substance for algal cell metabolism, excessively accumulated lipids can enhance cell-doubling process. A similar outcome was observed by Álvarez-Díaz et al. [29] where *A. falcatus* showed increased growth rate under P-deficiency and produced more lipids than under regular conditions. On the other hand, a large standard deviation was obtained for *A. falcatus* growth rate and productivity, so the present result is rather ambiguous and cannot be genuinely related to phosphorus stress conditions.

T. obliquus biomass growth showed considerable improvement after P-starvation. Its biomass growth rate increased by 232 and 310% after 3- and 5-day P-starvation, respectively, comparing to the reference conditions. The biomass productivity was 316 and 541% higher and biomass increase was 205 and 323% after 3- and 5-day P-starvation compared to the reference conditions. However, it remains unclear what caused such an improvement in biomass growth. At all growth parameters *T. obliquus* showed markedly worse performance than the other species. Biomass concentration change of *T. obliquus* after 3- and 5-day starvation significantly differed ($p < 0.05$) from the reference conditions. However, the biomass growth values for *T. obliquus* show large uncertainty as the standard deviation is more than $\pm$50% from the mean biomass growth value for all treatments.

The observed biomass growth rates, productivity, and increase as well as the response to phosphorus deficiency emphasize *C. vulgaris* and *B. braunii* as more appropriate for nutrient removal at wastewater post-treatment than the other studied species. The ability of *C. vulgaris* to grow in various types of wastewaters have been widely reported [30] and it is often selected for commercial purposes [31]. *B. braunii* is known as a carbohydrate producer and has a potential for bioenergy production [32]. If high biomass productivity can be achieved, *B. braunii* is a suitable candidate for resource recovery from wastewater. Both *A. falcatus* and *T. obliquus* are often viewed as a potential species for high economic return due to their lipid productivity [33,34]. However, lower growth rate and productivity obtained in the present study deems them less appropriate for wastewater post-treatment with possible economical return.

3.2. Nutrient Removal

All species, except *T. obliquus*, showed high nutrient removal rates, reaching more than 97 and 91% reduction of nitrate and phosphate, respectively (Table 3). Nitrate removal was not affected by prior biomass exposure to P-deficiency conditions and showed no statistically significant difference ($p > 0.05$) among the treatments for any of the species. The maximum nitrate removal for *C. vulgaris*, *B. braunii*, and *A. falcatus* was obtained on days 5–6 (Figure 3).

Table 3. Nutrient removal rates and biomass uptake kinetics (mean values, $n = 3$).

	Starvation Period (Days)	Nutrient Removal (%)		Biomass Nutrient Consumption (V), (mg N(P) g^{-1} DW)		Nutrient Uptake Rate (k), (d^{-1})	
		NO_3-N	PO_4-P	NO_3-N	PO_4-P	NO_3-N	PO_4-P
C. vulgaris	0	97.7	96.6	57.70	31.70	209.07	114.87
	3	98.9	99.2	29.75	21.12	72.04	51.14
	5	98.1	99.4	34.76	22.12	96.68	61.53
B. braunii	0	99.3	97.3	29.19	15.63	72.77	38.96
	3	99.0	99.1	28.17	16.79	66.26	39.50
	5	98.6	99.2	29.23	15.06	68.72	35.40
A. falcatus	0	98.7	97.7	44.32	25.95	129.40	75.77
	3	98.4	99.2	35.31	23.88	94.74	64.08
	5	98.7	91.2	49.87	17.89	165.47	59.37
T.obliquus	0	10.2	44.5	32.20	111.56	563.02	1.10
	3	10.8	62.4	12.50	49.29	65.66	2.02
	5	3.6	37.8	2.97	5.67	12.65	0.36

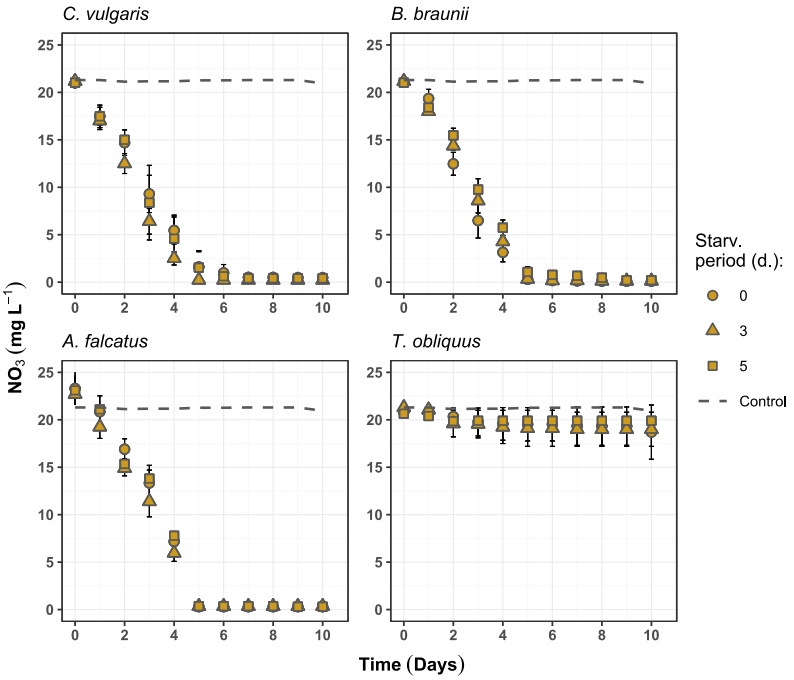

Figure 3. Nitrate concentration changes over time in the experiment batches for *C. vulgaris*, *B. braunii*, *A. falcatus*, and *T. obliquus* (means ± SD, $n = 3$).

Higher phosphate removal rates were observed for *C. vulgaris* and *B. braunii* after prior biomass exposure to P-deficiency and reached near-complete (>99%) PO_4 reduction. *A. falcatus* at the same time showed near-complete PO_4 reduction only after 3-day phosphorus starvation. In general, the highest phosphate removal rate was recorded after 10 days of growth, except for 3-day starved *C. vulgaris* and *A. falcatus* which both reduced phosphate by 99.2% on day 7 (Figure 4). *T. obliquus* could reduce PO_4 content by 62.4% after 3-day P-starvation at the end of the experiment. This treatment showed significantly higher ($p < 0.05$) PO_4 reduction than the 5-day starved biomass. All other species showed no statistically significant differences ($p > 0.05$) among the treatments in PO_4 removal.

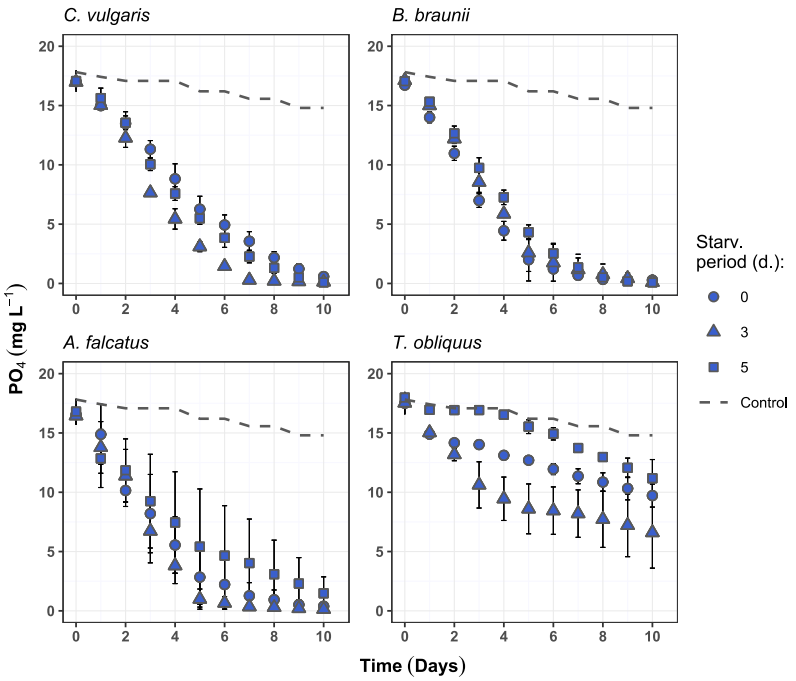

Figure 4. Phosphate concentration changes over time in the experiment batches for *C. vulgaris*, *B. braunii*, *A. falcatus*, and *T. obliquus* (means ± SD, $n = 3$).

Although slight increase in phosphate removal for certain species was observed after biomass P-starvation, it can be viewed as underperformance. Similar studies, where biomass P-starvation is performed to enhance the phosphate uptake, show near complete phosphate reduction in the range from within few hours to two days [10,23]. The obtained results highlight two major bottlenecks behind slow phosphate uptake by P-starved biomass. First, the low N/P ratio in the wastewater as well as faster removal of nitrogen indicate the importance of nitrogen limitation. This results in the preference for nitrate over phosphate, omitting the possible biomass status of P-deficiency. Second, rapid phosphate removal by P-starved biomass in other studies was achieved at conditions where cellular phosphorus was depleted. However, in this study to mimic practical conditions, internal biomass phosphorus reserve in the form of polyphosphate was still available after biomass exposure to P-deficiency (Figure 5). This condition emphasizes the prospective complexity of P-starvation implementation in pilot-scale wastewater post-treatment—complete biomass polyphosphate depletion requires long exposure to P-deficiency.

Evidently, the phosphate concentration decreases slightly faster in batches with 3-day P-starved *C. vulgaris* and *A. falcatus* biomass during its exponential growth period (Figure 3). However, when assessing the phosphate removal efficiency in terms of PO_4 reduction per daily produced biomass, the P-starvation effect on PO_4 uptake becomes less distinct. The calculated biomass nutrient consumption (V) and nutrient uptake rates (k) show that *C. vulgaris* and *A. falcatus* removed phosphate more efficiently without prior biomass P-starvation (Table 3). Both V and k represent nutrient reduction at biomass exponential growth phase, which was observed from day 1 to 5. *C. vulgaris* biomass consumed at least 30% more phosphate than the previously starved biomass. The biomass phosphate uptake rate was 45–55% higher by *C. vulgaris* and 15–22% higher for *A. falcatus* for the reference biomass. Although it was observed that the biomass production of P-starved cells increased, the produced biomass to P-uptake ratio was lower than at the reference

conditions, indicating biomass growth inhibition. Such a condition can be observed after biomass P-deficiency [35] and is associated to cell respiratory metabolism repression [36].

In terms of phosphate and nitrate removal, all species used in this study have their benefits. *C. vulgaris* and *A. falcatus* show indications of enhanced phosphate uptake after P-starvation. Thus, they seem more appropriate for application in pilot-scale systems for wastewater post-treatment. However, the N/P ratio must be adjusted and kept at optimum (around 16/1) to achieve rapid phosphate removal. *B. braunii* seemingly retains its removal efficiency after exposure to P-deficiency, but it is unknown whether P-starved biomass inoculated in the medium with optimum N/P ratio would improve the biomass phosphate consumption and uptake rate.

3.3. Biomass Poly-P Content and AP Activity as Indicators for P-Deficiency

It is a generally accepted knowledge that microalgae primarily assimilate the inorganic form of phosphorus for their growth [37,38]. Therefore, low availability or complete lack of inorganic phosphorus from external sources is viewed as the major driver of algal P-deficiency. On the other hand, microalgae can temporarily survive from its internal P reserves. The internal P is extensively acquired after temporary P deprivation and is stored within the cell as polyphosphate granules [39]. Another alternative phosphorus source is the dissolved organic phosphorus, that can be enzymatically hydrolyzed to the bioavailable inorganic P [40]. Thus, fluctuations in cellular poly-P content and high extracellular alkaline phosphatase (AP) activity indicate on P-deficiency and can be applied to monitor P-starvation.

The obtained results on the biomass polyphosphate content show inconsistency among the species (Figure 5). *A. falcatus* and *T. obliquus* showed increase in biomass poly-P content during the first half of the experiment period. For *A. falcatus* the 3- and 5-day starved biomass increased its poly-P content by 23 and 58%, respectively, compared to its initial content. For *T. obliquus* the poly-P content during experiment increased by 28 and 33% for 3- and 5-day starved biomass, respectively, and was accumulating 5–6 times more poly-P than the reference biomass. However, this observed increase of poly-P after P-starvation is many orders of magnitude lower than reported in other studies [10,23,41]. The initial poly-P content for *C. vulgaris* biomass was 230, 205, and 156 μg mg^{-1} for the reference, 3- and 5-day starved biomass, respectively. Still, the poly-P content showed a declining dynamic indicating to a constant consumption of internal P reserves. Only 5-day starved *C. vulgaris* biomass showed 4.4% accumulation during first two days, followed by poly-P content reduction until the end of the experiment period. Similar biomass poly-P content change was observed for *B. braunii* at all treatments—high initial biomass poly-P content and its decrease during the experiment period. Among all species, only *T. obliquus* showed significantly higher ($p < 0.05$) poly-P accumulation by P-starved biomass compared to the reference conditions.

The observed initial biomass poly-P concentration and its changes over the experiment period suggest that neither of the species developed a clear P-deficiency condition during the biomass P-starvation that might resemble full-scale situation. Thus, the selected P-starvation periods were too short to reduce the poly-P content to a level where P-deficiency is induced and subsequently enhances phosphate uptake and polyphosphate storage. Such an outcome is not in line with reports from other studies, where 3–5 days of P-starvation have entirely depleted the cellular poly-P content [23,41]. It has been previously demonstrated that the cellular poly-P accumulation and consumption rate can be affected by the biomass growth phase [42]. Accordingly, the result from this study indicates that before the P-starvation *C. vulgaris* and *B. braunii* were at an early stationary growth phase when poly-P was accumulated to overcome extended periods without external orthophosphate availability. Contrary, *A. falcatus* and *T. obliquus* were at the exponential growth phase, when they actively used the acquired internal P reserves for biomass production.

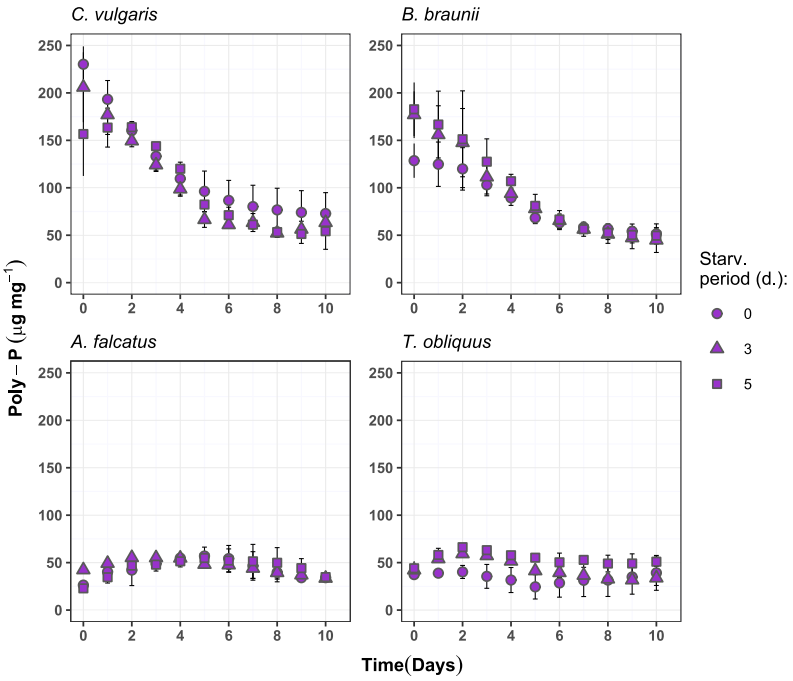

Figure 5. Biomass polyphosphate content change over time in the experiment batches for *C. vulgaris*, *B. braunii*, *A. falcatus* and *T. obliquus* (means ± SD, $n = 3$).

The results on biomass poly-P content dynamics suggests that it is not directly related to external P availability. It also depends on the cell growth phase as well as light intensity, temperature, and pH [12,42]. Therefore, the biomass poly-P content alone is not a reliable indicator for biomass P-deficiency.

The observed alkaline phosphatase (AP) activity partly supports the incomplete P-deficiency status for all species. The P-starved biomass for all four species showed lower AP activity than the reference biomass. Concurrently, the P-starved biomass showed more rapid increase in AP activity relative to its initial value. For all species, the AP activity dynamics for P-starved biomass significantly differed ($p < 0.05$) from the reference biomass.

Differences in AP activity change during the experiment period was observed among the species. For *C. vulgaris* the AP activity developed differently from other species, showing increase during the first two days, followed by decline and a steadily low value until the end of the experiment (Figure 6). For *B. braunii*, *A. falcatus*, and *T. obliquus* an increase in AP activity was observed for the P-starved biomass while for the reference biomass it mostly decreased or showed no change.

The initially low AP activity for P-starved biomass indicate on phosphorus availability from internal reserves during the exposure to P-starvation. Under such condition there was no need for enhanced release of enzymes to hydrolyze organic P to a bioavailable inorganic form. On the other hand, for all species the AP activity showed increase by P-starved biomass as the available phosphorus resource was decreasing. Moreover, a multifold increase of AP activity during the biomass exponential growth phase relative to its initial value was observed for the P-starved biomass by all species (Figure 7). This indicates that P-starved algal cells are potentially more sensitive to bioavailable phosphorus decrease and releases the phosphatase to compensate P-deficiency.

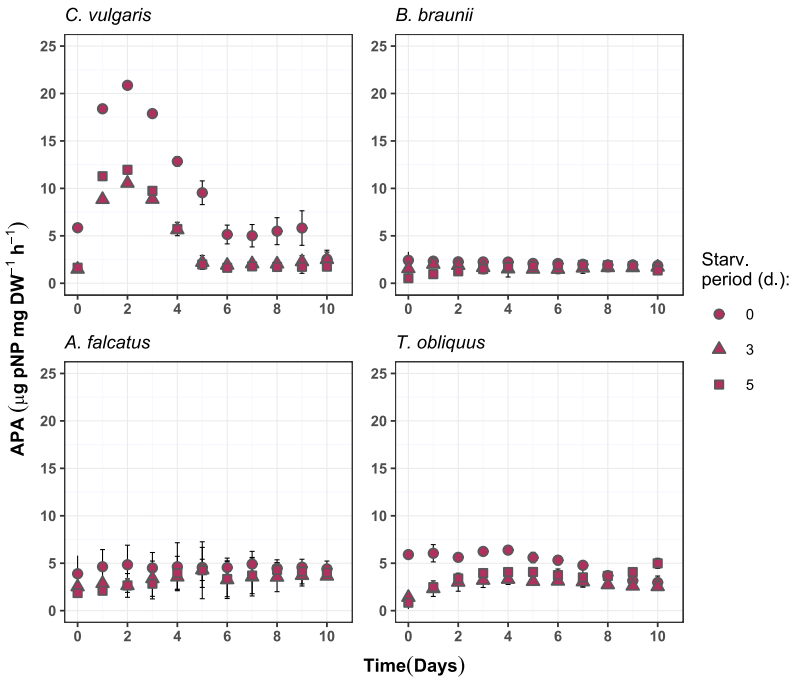

Figure 6. Alkaline phosphatase activity change over time in the experiment batches for *C. vulgaris*, *B. braunii*, *A. falcatus*, and *T. obliquus* (means ± SD, $n = 3$).

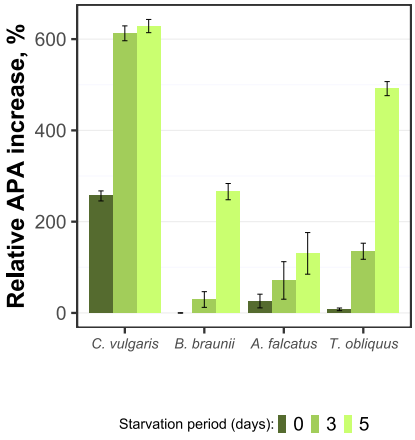

Figure 7. Relative increase of alkaline phosphatase activity for *C. vulgaris*, *B. braunii*, *A. falcatus*, and *T. obliquus* (means ± SD, $n = 3$).

AP activity is commonly used as an indicator for algal P-deficiency in natural aquatic ecosystems [43–45]. However, this study shows that AP activity also detects P-deficiency in laboratory algal species grown in real wastewater. Thus, AP activity has potential application in engineered systems for algae-based wastewater treatment supplemented with biomass P-starvation for enhanced phosphorus uptake. The monitoring of AP activity gives more control over the manipulation with algal P-deficiency in wastewater post-

treatment phase. It would allow a prompt detection of P-deficiency status development and optimize the overall hydraulic and biochemical performance of phosphorus removal. This way high phosphorus removal rates can be achieved in short time periods, and ultimately increase the efficiency of small WWTPs performance.

4. Conclusions

Performance of four different microalgal species, *Chlorella vulgaris*, *Botryococcus braunii*, *Ankistrodesmus falcatus*, and *Tetradesmus obliquus* was compared after their exposure to phosphorus starvation. *C. vulgaris* showed the best performance in terms of biomass growth and nutrient uptake after exposure to P-starvation. Its biomass growth rate increased by nearly 50% after 3-day P-starvation. Its PO_4 removal rate increased after 3-day P-starvation, reaching > 99% within 7 days. *B. braunii* showed promising performance as it maintained good biomass growth rate and nutrient removal regardless of the P-starvation. Lower performance was observed for other species.

The estimated biomass phosphate consumption for P-starved biomass showed no change or even a decrease when compared to the reference biomass. This indicates that biomass P-starvation was hindered and did not take the anticipated effect on phosphorus removal rate.

An enhanced phosphorus uptake was hampered by low N/P ratio, which created nitrogen-limited conditions and made nitrate the preferred nutrient for biomass consumption. Thus, there is a need of strict control over N/P ratio if algae-based wastewater post-treatment is supplemented with P-starvation. In addition, the previously accumulated polyphosphate ensured biomass survival during P-starvation, and it is another factor behind weak P-deficiency condition. The biomass phosphorus starvation did not affect the nitrate removal.

All species showed more rapid alkaline phosphatase activity development at the biomass exponential growth phase, compared to the reference conditions. The AP activity increase is relatable to the decline of PO_4 concentration and biomass poly-P content. AP activity can be a good indicator to detect and quantify P-deficiency in algae-based wastewater treatment system supplemented with P-starvation for enhanced phosphorus removal.

The results from the present study show that biomass P-starvation can supplement algae-based wastewater post-treatment for rapid phosphate removal. However, its efficiency relies (not limited to) on strict control over physical and chemical parameters (e.g., pH, N/P molar ratio) in wastewater and accurate monitoring of biochemical indicators (e.g., cellular P content and enzymatic activity).

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Research article

Optimizing phosphorus removal for municipal wastewater post-treatment with *Chlorella vulgaris*

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ABSTRACT

The microalgal species *Chlorella vulgaris* was cultivated in batch conditions to identify the optimum set of initial conditions for the best biomass growth rate, phosphate removal, polyphosphate accumulation, and protein productivity. To study the effect of phosphorus deficiency caused stress, the microalgal biomass was exposed to phosphorus deficiency conditions for periods varying between 1 and 10 days and inoculated at different initial biomass and phosphate concentrations. A 10-day period of phosphate deficiency, supported by low initial biomass concentration (~0.25 mg DW L^{-1}), increased the phosphate removal by 62–175% when compared to the reference conditions. A 10-day period of biomass P-deficiency also boosted the polyphosphate accumulation and protein productivity, increasing them up to 40 and 46.8 times, respectively, if compared to reference conditions. At the same time, optimization algorithm model results suggested one-day biomass P-starvation with low initial biomass concentration as the optimum combination to achieve the highest performance while the initial phosphate concentration had less impact. The initial conditions suggested by the optimization model were validated in a sequencing batch photobioreactor, giving 101.7 and 138.0% more phosphate removal and polyphosphate accumulation, compared to the reference conditions. The obtained results present microalgae exposure to phosphorus stress as a supplementary tool for wastewater post-treatment targeted on rapid phosphorus removal.

1. Introduction

The excessive worldwide use of phosphorus facilitates eutrophication of aquatic ecosystems and deterioration of their ecological status. Effluents from municipal wastewater treatment plants (WWTPs) contribute to a significant amount of phosphorus loading into natural waters despite controlled sewage treatment (Carey and Migliaccio, 2009). Technologies for phosphorus removal in large WWTPs are well established and include chemical precipitation and enhanced bacterial uptake. Furthermore, the traditionally used treatment technologies often generate excess waste or require high capital and operational costs (Bunce et al., 2018). On the other hand, in specific cases, there are no strict requirements for phosphorus reduction limits for municipal wastewater. For instance, legislative acts of the European Union (Directive 91/271/EEC) require phosphorus reduction to appropriate levels in small WWTPs (<2000 p. e.).

Microalgae-based wastewater treatment is often viewed as a nature-friendly and cost-effective alternative to conventional wastewater treatment methods. The microalgae's ability for near-complete phosphate removal in wastewater makes it a promising organism for use in the wastewater post-treatment stage (Whitton et al., 2015). Moreover, microalgal cells produce high-value molecules, such as macronutrients for food and energy production and pigments for pharmaceutical or cosmetic applications (Levasseur et al., 2020). Although microalgae-based technology poses environmental and economic advantages over conventional wastewater post-treatment, the phosphorus removal rate must be maintained at high levels (>90%) to meet the discharge standards for good ecosystem status in the receiving waters (Bunce et al., 2018). Considering this necessity, the microalgal phosphorus famine or starvation technique is gaining attention as an approach to enhance phosphorus removal. Multiple studies have shown that microalgal biomass exposure to phosphorus deficiency conditions depletes the intracellular P reserves and subsequently boosts the phosphorus removal multiple times (Lavrinovičs et al., 2020), and also

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promotes high-value molecule synthesis (Paliwal et al., 2017). Such manipulation with biomass phosphorus availability could decrease the phosphorus removal period from up to two days to a few hours which becomes compatible with the conventional methods. In addition, exposure to phosphorus stress conditions could increase the biomass value in terms of phosphorus recovery and high-value molecule extraction. Unfortunately, one of the main observed drawbacks of microalgae manipulation with phosphorus availability is the resulting reduction of biomass growth rate (Kamalanathan et al., 2016) which subsequently lowers the total yields of high-value molecules. Furthermore, the margin for optimum P-starvation remains rather obscure. Insufficient P stress can attenuate the desired enhancement of P removal, while overexposure to P-deficiency can damage the algal cell physiological processes and disrupt the whole treatment process. Up till now, variable P-starvation periods and process techniques have been reported (Lavrinovičs et al., 2020; Solovchenko et al., 2019), as well as indicators for P deficiency quantification, have been offered (Eixler et al., 2006). Nevertheless, the obtained results on phosphorus removal rates are variable and the reported effects on similar biomass P-starvation conditions can be antipodal (Hernandez et al., 2006; Wu et al., 2012). Thus, a profound investigation of the microalgal biomass P-starvation approach is required for its successful integration into the engineered systems.

This study aims to find the optimum initial conditions that would result in a trade-off between the highest possible phosphate uptake and accumulation rates as well as biomass growth rate at the shortest biomass P-starvation period. Additionally, protein productivity induced by P-starvation was assessed as a possible improvement of biochemical quality of *C. vulgaris*. An innovative optimization algorithm based on prior research on evolutionary algorithms (Narica et al., 2017) was used to identify the most optimum initial settings for efficient wastewater post-treatment and resource recovery. These algorithms are well-suited for solving complex non-linear and multi-objective problems with many constraints as they can efficiently search a large space of potential solutions. The modeled optimum initial conditions were verified in a sequencing batch photobioreactor. The presented approach for wastewater post-treatment with P-starved biomass aims to significantly reduce the reaction time for phosphate removal without notable biomass productivity loss and maintain high yields of valuable molecules synthesized by the algal cells.

2. Materials and methods

2.1. Algal strain and growth conditions

The photoautotrophic microalgae strain *Chlorella vulgaris* (CCAP 211/11B) was used in this study. It has been extensively reported that *C. vulgaris* can grow in various types of wastewaters and reduce the ambient nutrient content and is often used for protein production (Cai et al., 2013 and references therein).

The microalgal biomass was pre-cultured in a sterile BG-11 growth medium (Stanier et al., 1971). The cell cultivation was performed in a laboratory-scale photobioreactor with the total and working volumes of 6.2 and 4.5 L, respectively. Aeration rate was set at 0.5 L min^{-1} and supplemented with 1% (v/v) CO_2 influx. Fluorescent LED lights were used as a light source. The provided photosynthetically active radiation was 140 μmol m^2 s^{-1} at a 16:8-h lighting regime. The ambient temperature and pH were 25–27 °C and ~7.2, respectively.

To establish the phosphorus starvation status for algal biomass, it was cultivated until its growth reached a stationary phase and did not show any biomass concentration increase for three consecutive days, as suggested by Solovchenko et al. (2019). The longer phosphorus starvation periods were determined by the number of days since the defined threshold period for the establishment of phosphorus starvation status. Biomass at its late exponential growth phase was used for the reference conditions.

2.2. Experimental setup

2.2.1. Conditions for batch experiment

The batch experiment series were conducted in 1000 mL Pyrex® bottles with working volume of 500 mL, at a room temperature (25–27 °C). The pH varied from 7.4 at the beginning of the experiment to 8.2 at its end. The algae suspensions were mixed by aeration (10 L h^{-1}) and supplemented with 1% (v/v) CO_2. The bottles were illuminated with fluorescent light with blue-red spectrum with the PAR of 180 μmol m^2 s^{-1} at a 16:8-h lighting regime. Each experiment was run for 72 h. Phosphate sampling was done hourly for the first 5 h and daily for the remaining 3 days of experiment period. Biomass concentration, temperature and pH was measured daily. Polyphosphate and protein content was measured in the inoculant biomass, 6 h from the experiment initiation and daily for the remaining period. Every batch test was replicated three times.

The studied initial conditions included biomass concentration, phosphate concentration and biomass P-starvation periods. The initial parameter values and their combination designations are given in Table 1.

2.2.2. Sequencing batch photobioreactor conditions

The data from batch experiment series were used for modeling the optimum initial conditions, using an industrial AI platform *xT SAAM* (see Section 2.4.). The model results were validated in a laboratory-scale photobioreactor (Fig. 1) operating at a sequencing batch mode. The working volume of PBR was 4.5 L. Aeration rate was set at 0.5 L min^{-1} and supplemented with 2% (v/v) CO_2 influx. The photosynthetically active radiation was set at 140 μmol m^2 s^{-1} at a 16:8-h lighting regime, provided by fluorescent LED lights. The biomass suspension was mixed by magnetic propeller at 150 rpm. The ambient temperature and pH were set at 25–27 °C and ~7.2, respectively.

For the reference conditions biomass was produced until a late exponential growth phase. Afterwards, approx. half of the biomass suspension was discarded and replaced with fresh BG-11 growth medium. For the P-starvation conditions biomass was produced until its growth rate was in the stagnation phase for three consecutive days plus an additional period obtained from the optimization model result. The experiment was run for 60 days, containing four and five sequencing batch cycles for the reference and P-starvation conditions, respectively.

Phosphate sampling for both conditions was done after 1, 3 and 5 h at the beginning of each cycle and daily for the remaining experiment period until its complete reduction. Biomass concentration, polyphosphate content, alkaline phosphatase activity and protein content was measured daily or every two days.

2.3. Analytical procedures

2.3.1. Biomass concentration

Algae biomass growth was detected spectrophotometrically (Thermo Scientific Genesys 150 UV–Visible Spectrophotometer). Light absorbance was measured at 680 nm which is proportional to the change of

Table 1
Denotations for experiment batches with individual initial parameter value combinations.

		Initial phosphate concentration (P), mg P-PO_4 L^{-1}		
		22	12	5.5
Initial biomass concentration (B), g DW L^{-1}	**0.2**	1P+1B	1/ 2P+1B	1/ 4P+1B
	0.6	1P+2B	1/ 2P+2B	1/ 4P+2B
	1.5	1P+3B	1/ 2P+3B	1/ 4P+3B

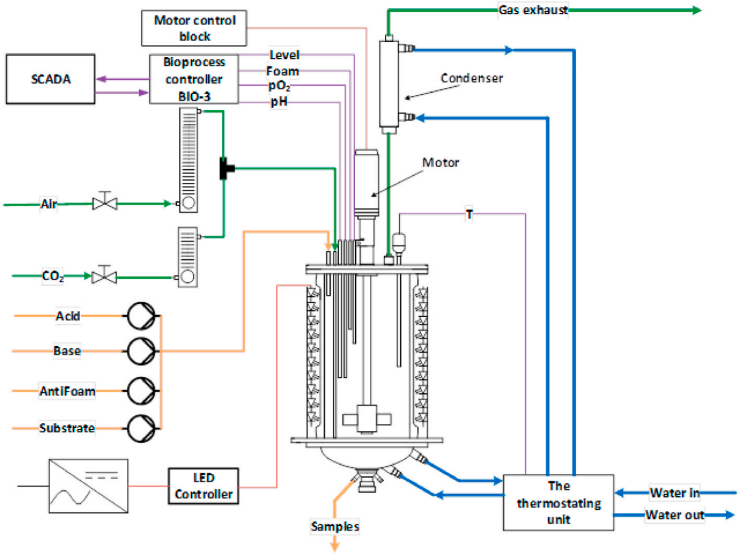

Fig. 1. The schematic diagram of sequencing batch photobioreactor for microalgae cultivation and synthetic wastewater treatment (© Bioreactors.net).

cell number in most unicellular organisms (Wang et al., 2016) and the maximum light absorbance for most microalgae is found at this wavelength (Santos-Ballardo et al., 2015; Yatirajula et al., 2019). The OD_{680} values were kept bellow 1.2. and the samples were diluted appropriately when exceeding this value. The dry weight of algal biomass was used for making growth curves. The relationship between algal dry weigh biomass (g L^{-1}) and light absorbance value at OD_{680} was described by Equation (1):

$$Biomass\ conc._{c.vulgaris} = 0.4065 \times OD_{680} - 0.0051, R^2 = 0.997 \quad (1)$$

The specific biomass growth in the exponential phase was estimated by Equation (2):

$$\mu\left(\mathrm{day}^{-1}\right) = \frac{\ln\left(N_2/N_1\right)}{\left(t_2 - t_1\right)} \quad (2)$$

where N_1 and N_2 are the measured dry biomass at time t_1 and t_2, respectively.

2.3.2. Phosphorus concentration and removal rate

To follow the algal phosphorus consumption from the medium, concentration changes for phosphate were observed. Measurements were done using HACH LANGE DR 3900 spectrophotometer and commercial reagent sets for the phosphate analysis, based in the Ascorbic acid method.

The phosphorus removal was characterized by the specific phosphate removal rate, described as milligrams of phosphate utilized by per gram of dry weight biomass per hour and calculated by Equation (3):

$$R_{xi} = \frac{cN(P)_0 - cN(P)_i}{t_i - t_0} \times b_0 \quad (3)$$

where R_{xi} is the specific phosphorus removal rate (mg P g^{-1} DW h^{-1}); S_0 is the initial substrate concentration, S_i is the substrate concentration at a specific time t_i, t_0 is the initial time, and b_0 is the initial biomass concentration. The specific phosphorus removal rate was calculated for entire experiment period or to the timestep where no significant change in PO_4 concentration was observed.

2.3.3. Polyphosphate content and accumulation rate

Polyphosphate content in algal biomass was measured following the protocol of Mukherjee and Ray (2015). In brief, the polyphosphates were extracted from microalgal biomass through a series of physical and chemical procedures. Algal biomass suspension was concentrated to harvest enough biomass (>0.05 g fresh weight) for the extraction procedure. The algal cells were disrupted using an ultrasonic processing (Branson Bransonic® CPXH Digital Bath 3800) and treating the sample for 20 min at 40 kHz. Afterwards the disrupted cell samples were heated at 100 °C for 2 h. Then a mixture of chloroform and isoamyl alcohol (24:1) was added to the biomass and mixed vigorously. The suspension was centrifuged at 13 520 g for 15 min. Afterwards the supernatant containing polyphosphate extract was collected and mixed with 0.2 N acetic acid and toluidine blue solution (stock conc. 30 mg L^{-1}) for spectrophotometric light adsorption measurement at 630 nm. Biomass polyphosphate concentration (μg mg^{-1}) was calculated against a calibration curve constructed using a sodium phosphate glass Type-45 (Sigma-Aldrich, St. Louis, MO, USA) as a polyphosphate standard. The change of biomass polyphosphate content over time was expressed as an accumulation rate, following Equation (4):

$$\mu\mathrm{Poly-P}\left(\mathrm{day}^{-1}\right) = \frac{\ln\left(Pp_i/Pp_0\right)}{\left(t_2 - t_1\right)} \quad (4)$$

where $\mu Poly\text{-}P$ is the polyphosphate accumulation rate, Pp_1 and Pp_2 are the measured biomass polyphosphate content at time t_1 and t_2, respectively.

2.3.4. Total protein content measurement

The total protein content in algal biomass was determined following the procedure reported by Vazirzadeh et al. (2022). In brief, the biomass suspension sample to obtain 5–6 mg DW biomass was centrifuged at 2500 rpm for 10 min, and the supernatant was discarded. The recovered cell pellet was mixed with 1 mL 1M NaOH and heated at 100 °C for 2 h. The sample was cooled to room temperature and biomass settled to the vial bottom. 50 μl of the processed sample was mixed with 1.5 mL Bradford reagent (Sigma-Aldrich). The solution was incubated at room temperature for 10 min. The light absorbance in the sample was measured spectrophotometrically at a wavelength of 595 nm. A solution

of 50 μl 1 M NaOH mixed with 1.5 mL Bradford reagent was used as blank. The standard curve was prepared using bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA). The measured protein concentration to sample volume in the biomass extract and the dry biomass concentration in the original suspension were used to express the protein content as mg g^{-1} dry biomass. Biomass protein content change over time was expressed as protein productivity, following Equation (5):

$$\text{ProtProd}\left(mg\ d^{-1}\right) = \frac{Prot_i - Prot_0}{(t_2 - t_1)} \quad (5)$$

where ProtProd is the protein accumulation rate, $Prot_i$ and $Prot_0$ are the total protein content in biomass at time t_1 and t_2, respectively.

2.3.5. Alkaline phosphatase activity measurement

The alkaline phosphatase activity was estimated by para-nitrophenyl phosphate disodium hexahydrate (p-NPP, Sigma-Aldrich, St. Louis, MO, USA) hydrolysis to para-nitrophenol (p-NP) by the enzyme released from microalgal cells. Biomass suspension sample to obtain ~10 mg DW biomass was centrifuged at 5000 rpm for 2 min, and the supernatant was discarded. The recovered cell pellet was dissolved in 3 mL of demineralized water. The recovered sample was mixed with 0.5 mL Tris HCl buffer (pH 9.5) and 0.4 mL p-NPP (0.5 mg mL^{-1}). The mixture was incubated at 37 °C for 1 h in the dark. The yielded p-NP was measured spectrophotometrically at 405 nm. p-NP amount was calculated against a calibration curve constructed using a p-NP as a standard. The result was used as an indicator for AP activity. A control containing no biomass was included in the routine, and its OD_{405} reading was used as a blank. AP activity was expressed as p-NP flux from dry-weight biomass per hour.

2.4. AI platform for initial conditions optimization

An industrial artificial intelligence (AI) based platform *xT SAAM* (https://www.x-t.ai/) was used to optimize initial conditions using augmented evolutionary algorithms. The platform recognizes an optimization problem as a black-box problem, which is defined by its inputs and outputs. Inputs set up boundaries of the decision space, whereas outputs are related to the optimization objectives. Once problem's inputs and outputs are set up, the platform learns from achieved outputs and iteratively suggests new input configurations to improve said outputs.

Experimental data from the batch experiment series describer in Section 2.2.1. consisting of records with related input and output values was imported into the AI platform. Output information was used to rank each experimental record based on optimization objectives related to each output. The process of ranking produced score values, which were calculated as a weighted sum of min-max scaled output values. The augmented evolutionary algorithms then used score values as a fitness criterion to produce a multitude of novel input configurations for the next iteration. These input configurations were then visualized as a density heatmap showing regions of interest (ROIs). The ROIs were used to determine the preferred input configuration for the initial conditions (see Fig. 6).

2.5. Statistical analyses

Parametric one-way ANOVA test was used to detect the significant differences among different biomass P-starvation periods on algal biomass growth rate, specific phosphate removal rate, biomass polyphosphate accumulation, and protein productivity in the batch experiment series. Levene's test was used to indicate the homogeneity of variance between the comparison groups. Tukey post-hoc test was used to detect pairwise differences between individual groups. The limit of statistical significance in all tests was set to $\alpha \leq 0.05$. Results are presented as mean values ($n = 3$). Statistical analyses were conducted using IBM SPSS Statistics version 23 software. The obtained results were visualized using R: A Language and Environment for Statistical Computing, version 4.0.5. In the *xT SAAM* platform, post-optimization cross-validated data modeling and visualization of predicted model outputs were made (Fig. 6).

3. Results and discussion

3.1. Microalgal biomass growth

Positive growth rates were observed for the *Chlorella vulgaris* biomass when produced with different initial biomass concentrations, under different initial phosphate concentrations and after exposure to various P-starvation periods (Fig. 2). Each of the studied initial parameters had its effect on the biomass growth rate.

The highest microalgal biomass growth rate was observed for the reference batch with a combination of the lowest initial biomass concentration and high initial phosphate supply (1P+1B) reaching 0.317 d^{-1}. With every additional biomass augmentation step, the growth rate decreased by 23–84% when compared between batches with constant initial phosphate concentration. There are two main drivers behind decreasing biomass growth rates with gradually higher initial biomass concentration. Firstly, the light attenuation due to the self-shading effect lowered the photosynthetic efficiency as a function between irradiance and biomass productivity (Ahmad et al., 2022). Secondly, under conditions with lower initial biomass concentration, the dissolved phosphate was distributed between fewer cells, resulting in a larger substrate quantity consumed by a single cell. Thus, more substrate was available for cellular metabolic processes, and it could enhance the cell doubling process. The batches with lower initial biomass concentration showed significantly higher (p-value <0.01) growth rates than those with medium and high initial biomass.

The reduction of the initial phosphate content had a variable effect on the biomass growth rate. The P-starved batches showed 47% lower to 88% higher growth rates compared to the reference batch at constant initial biomass concentration. Although reduced initial phosphate concentration provided less substrate for uptake and biomass production, the amount of phosphate available in batches with low phosphate was still enough to sustain conditions for undisrupted biomass production. Thus, it was presumed that the initial phosphate concentration had no substantial effect on the biomass growth rate. In addition, no statistically significant difference was observed between the biomass growth rate at different initial phosphate concentrations (p-value ≥0.087).

The observed effect of *C. vulgaris* biomass exposure to extended P-deficiency periods on its biomass growth rate was rather ambiguous. No clear impact of P-starvation on biomass growth rate could be observed for the batches with medium phosphorus concentration (1/2P) as it was highly variable between P-starvation periods. In other cases, the μ value gradually decreased with longer P-starvation periods for batches with a lower initial biomass concentration (1B). This observation is in line with other studies that report biomass growth reduction due to phosphorus stress (El-Sheek and Rady, 1995; Kamalanathan et al., 2016).

An opposite trend with an increasing μ value was observed as a combined effect of high initial biomass concentration (3B) and gradually longer biomass P-starvation period. For the batches with low initial phosphate concentration a strong P limitation status developed due to high N/P ratio and low cellular phosphorus content. Thus, it is suggested that a luxury phosphate uptake mechanism was stimulated, promoting enhanced phosphate incorporation in cells which subsequently resulted in a greater biomass growth rate. Similar observations were made by Yao et al. (2011) and Singh et al. (2018). However, it remains unclear why a similar trend was observed in batches with optimum N/P ratio (1P). Statistically, there was no significant difference between the biomass growth rate after exposure to different P-starvation periods (p-value ≥ 0.956).

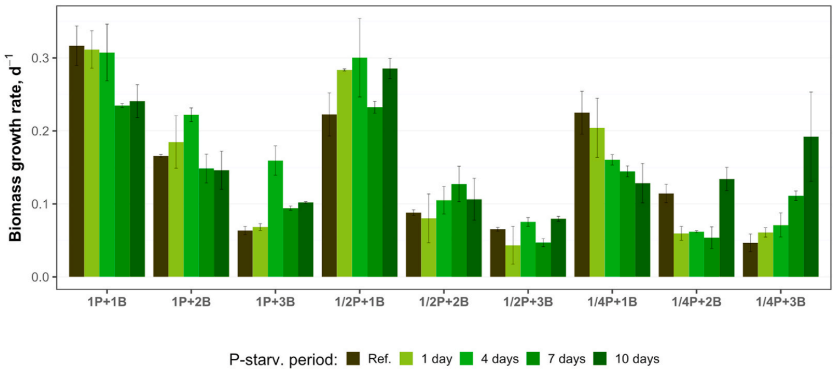

Fig. 2. The growth rate of *C. vulgaris* at various initial biomass and phosphate concentrations and after different biomass P-starvation periods (means ± SD, n = 3). The number and letter combinations on the x axis refers to the initial phosphorus (P) and biomass (B) content in each batch. See Table 1 for specific concentrations.

3.2. Phosphorus uptake, storage, and transformation

3.2.1. Effect of initial biomass and P-PO_4 concentrations on phosphate removal

In all batches and at all of the P-starvation periods the most rapid phosphate removal was observed during the initial 5 h of the experiment, and in many cases, a near-complete phosphate removal was achieved at the initial experimental phase (Fig. S1). In the batches with low initial PO4 the phosphate concentration was reduced by 92.4–98.4% within 24 h. Initial conditions with medium phosphate and high biomass resulted in 99.2% phosphate reduction within 5 h, while in the 1/4P+3B batch it took only 2 h for near-complete phosphate reduction. To eliminate the impact of higher initial biomass concentration on phosphate removal, the phosphate removal was normalized against the initial biomass concentration and presented as the specific phosphate removal rate R_{xi}. The specific phosphate removal rate was compared for the initial 5 h of the experiment when the most rapid PO_4 reduction was observed.

The obtained R_{xi} values were in a similar range when compared among the different initial phosphate concentrations and at a constant initial biomass concentration (Fig. 3). No statistically significant difference was observed for R_{xi} at different initial phosphate concentrations (p-value ≥0.188). Yet, it was found that lower biomass concentration resulted in higher R_{xi} value when compared at constant initial phosphate concentration. The reduced initial biomass density provided greater light diffusion and ensured higher specific phosphate removal due to elevated cell culture activity (Ruiz-Marin et al., 2010; Iasimone et al., 2018). In this study R_{xi} was exceeding 4 mg P g^{-1} DW h^{-1} in all batches with low initial biomass concentration. In contrary, higher cell density caused the self-shading effect, which further reduced the cell activity and lowered the specific phosphate removal rate, as it varied between 1.50 and 4.39 mg P g^{-1} DW h^{-1}. Also, a statistically significant difference was observed between the specific phosphate removal rate obtained for batches with low initial biomass concentration and the ones with medium and high initial biomass (p-value <0.01).

3.2.2. Effect of biomass P-starvation on phosphate removal

The biomass P-starvation effect on phosphate removal was assessed by the specific phosphate removal rate. In most batches the R_{xi} increased with longer P-starvation period. In seven out of nine batches the highest R_{xi} value was obtained after 10-day P-starvation period, reaching 7.74 and 6.69 mg g^{-1} DW h^{-1}, for the 1/2P+1B and 1/4P+1B batches, respectively. For the 1P+1B batch the maximum R_{xi} value was obtained after 7-day P-starvation, reaching 7.28 mg g^{-1} DW h^{-1}. The obtained maximum specific P removal rates in every batch were on average 62% higher than for the reference biomass in the same batch and reaching even 175% increase of the R_{xi} in the 1/4P+3B batch after 10-day P-starvation period. However, no statistically significant difference was

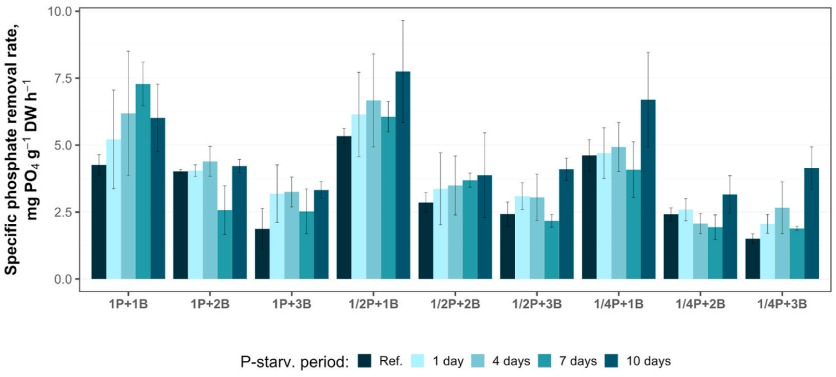

Fig. 3. The specific phosphate removal rate by *C. vulgaris* during first 5 h of the experiment at various initial biomass and phosphate concentrations and after different biomass P-starvation periods (means ± SD, n = 3). The number and letter combinations on the x axis refers to the initial phosphorus (P) and biomass (B) content in each batch. See Table 1 for specific concentrations.

found for the R_{xi} after different biomass P-starvation periods (p-value ≥0.302). High difference between the specific P-removal rate at reference conditions and the maximum obtained R_{xi} was additionally supported by phosphorus-limited conditions that were present at mid-P and especially at low-P batches. According to the initial conditions tested, the specific phosphate removal rate mainly depended on the P-starvation period and the initial biomass concentration.

An enhanced phosphate removal by P-starved biomass after it is refed with phosphorus has been reported previously and refers to the shortage of the intracellular phosphorus pool as the major driver (Lavrinovičs et al., 2020; Van Moorleghem et al., 2013). In the present study, the initial intracellular polyphosphate content became gradually lower with a longer biomass P-starvation period (Table S1) and promoted an increase of phosphorus uptake per biomass unit over time. The mechanisms behind such a performance is discussed by Grossman and Aksoy (2015), suggesting that microbial cells activate high-affinity P transporter synthesis when the available P*i* decreases below a dissociation constant which ensures a rapid restoration of intracellular P pool and its further survival. Moreover, Levy et al. (2011) proposed a dual P-transporter synthesis system for microorganisms, that prolongs the preparation for a P-starvation response by switching from low-affinity to high-affinity P transporter activation when biomass growth limitation takes action. Presence of such a system could explain the gradual increase of R_{xi} with longer biomass P-starvation period observed in the present study, providing phosphorus compensation with a P transport rate adjusted to the actual extent of P-deficiency. In this study, an impeded biomass growth was used as the main indicator for P-deficiency status establishment. Thus, it is suggested that longer exposure to P-deficiency status developed a gradually stronger activity of high-affinity P transporters. However, to uphold this proposition a gene expression profile assessment for microalgal cells under different gradients of P-depletion would be necessary.

Although the highest specific phosphate removal rates were mostly obtained after 10-day P-starvation period, relatively high R_{xi} values were observed for one-day P-starved biomass (2.06–6.14 mg g^{-1} DW h^{-1}) or even without prior biomass P-starvation (1.5–5.3 mg g^{-1} DW h^{-1}). Such specific phosphate removal rates are substantially higher than those reported by Ji et al. (2013), Ruiz-Marin et al. (2010) or Wang et al. (2014). If biomass P-starvation is done for the sole purpose of enhanced phosphorus removal from the environment, shorter exposure to P-deficiency can be enough to achieve the goal. However, biomass exposure to ambient stress including P-deficiency is often induced to promote the productivity of high value molecules (Paliwal et al., 2017) and is suggested for microalgal biomass manipulation.

3.2.3. Polyphosphate accumulation

The observed short-term polyphosphate content change showed a rapid increase during the initial 6 h (Fig. S2), which indicate that majority of P*i* was directly incorporated into the cells instead of its adsorption on the cell surface (Yao et al., 2011). Moreover, the biomass poly-P content observed at the final day of experiment was gradually decreasing with longer biomass P-starvation period. Such dynamics indicate, that after longer biomass exposure to P-deficiency the cell incorporated P*i* was transformed into acid soluble polyphosphate which its further used for the synthesis of cell constituents, such as protein, DNA or RNA (Powell et al., 2008; Su, 2021). Accordingly, lesser portion of cellular P*i* was transformed into acid insoluble polyphosphate which provides the storage P reserves for conditions of external P limitation.

The obtained biomass polyphosphate accumulation rate gradually increased with longer biomass exposure to P-deficiency conditions. In all batches the 10-day starved biomass accumulated poly-P at 3.7–40.0 times higher rate, comparing to the reference conditions (Fig. 4). The polyphosphate accumulation rate was significantly higher for biomass with 10-day P-starvation compared to other P-starvation periods (p-value <0.01), as well as for biomass with 7-day P-starvation compared to the reference biomass (p-value ≤0.022). No statistically significant difference was observed for polyphosphate accumulation at different initial biomass (p-value ≥0.845) and phosphate concentrations (p-value ≥0.807).

The obtained value shows, that the biomass poly-P content was restored significantly faster after the microalgal cells have been exposed to more extensive P-deficiency status. This observation coincides with previously reported P-stress impact on the kinetics of microalgal phosphorus uptake and transformation (Singh et al., 2018; Yao et al., 2011). Such an outcome poses biomass P-starvation as a possible strategy for enhanced phosphate removal in algae-based wastewater treatment systems and improvement of algal biomass quality as a raw material for downstream processing.

3.2.4. Biomass protein synthesis

The biomass protein productivity for a 3-day period for *C. vulgaris* biomass was increasing with longer P-starvation periods (Fig. 5). However, significantly higher protein productivity was obtained in batches with 10-day P-starved biomass, comparing to other P-starvation periods (p-value <0.01). The protein productivity after 10-day biomass P-starvation varied between 5.9 mg g DW d^{-1} in the high-P/high-Biomass batch and 9.0 g DW d^{-1} for the high-P/mid-Biomass batch. In all batches the 10-day biomass P-starvation resulted in 5.7–46.8 times higher protein productivity than at the reference conditions with no

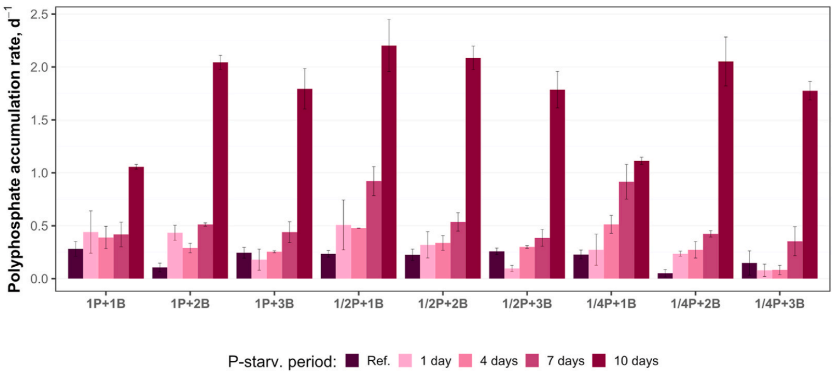

Fig. 4. Polyphosphate accumulation rate for *C. vulgaris* at various initial biomass and phosphate concentrations and after different biomass P-starvation periods (means ± SD, n = 3). The number and letter combinations on the x axis refers to the initial phosphorus (P) and biomass (B) content in each batch. See Table 1 for specific concentrations.

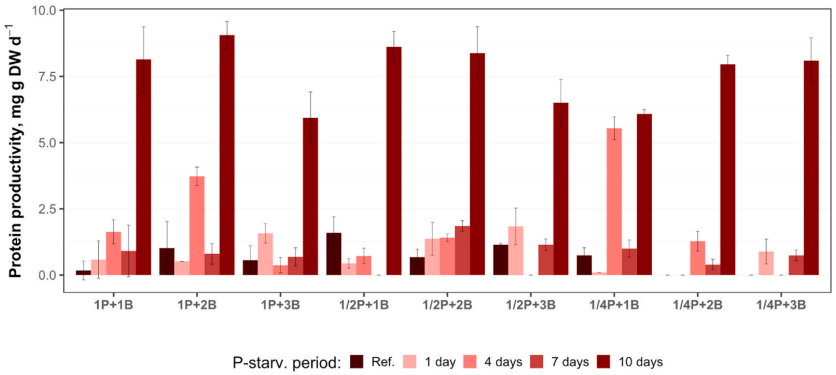

Fig. 5. Protein productivity for *C. vulgaris* at various initial biomass and phosphate concentrations and after different biomass P-starvation periods (means ± SD, n = 3). The number and letter combinations on the x axis refers to the initial phosphorus (P) and biomass (B) content in each batch. See Table 1 for specific concentrations.

prior biomass P-starvation. No significant difference was observed for protein productivity at different initial biomass and phosphate concentrations (p-values ≥0.763 and ≥ 0.899, respectively).

The obtained result of protein productivity demonstrated the ability of *C. vulgaris* to synthesize protein at substantially higher rate after its biomass long-term exposure to phosphorus stress. On one hand, this observation contradicts the known metabolic pathway for cellular protein synthesis, which strongly depends on nitrogen assimilation and the available light intensity (Huang et al., 2021; Rani et al., 2020). On the other hand, it is suggested that phosphorus availability had an indirect impact on the protein synthesis. An enhanced phosphorus assimilation caused by prior P-stress condition accelerated synthesis of adenosine triphosphate (ATP), which is required as an energy source for protein synthesis. Such an involvement of phosphorus in protein synthesis have also been discussed by Perez-Garcia *et al.* (2011) and Wu et al. (2021) and further supports the previously discussed phosphorus assimilation pathway into acid-soluble polyphosphate.

The phosphate uptake and its metabolic utilization after algal biomass exposure to P-deficiency conditions show possible benefits and a strategy for algae-based wastewater post-treatment and high-value biomass production. Biomass P-starvation and cellular poly-P content depletion can significantly enhance the inorganic phosphorus removal form wastewater and boost the production of valuable cell metabolites, such as protein. On the other hand, biomass exposure to extended periods of P-starvation would complicate the technical performance in a full-scale water bioremediation and biomass production process and increase the capital and operational costs. Thus, a compromise between biomass P-starvation period and the resulting biomass quality indicators should be identified.

3.3. AI-based data analysis for the optimization of initial conditions

A heatmap generated by an AI-based platform *xT SAAM* showing regions of interest (ROI) that identify the optimized initial conditions for efficient algae-based wastewater treatment allowed to determine the preferred conditions with highest probability of success (Fig. 6, yellow color). Short biomass P-starvation period of 1 day is the most favorable to achieve a rapid phosphate removal, maintain a high biomass growth rate and ensure high polyphosphate accumulation rate and protein synthesis. To facilitate such a performance, the biomass with short P-starvation period should be inoculated at initial concentration of ~0.2 g DW L^{-1} (Fig. 6B and C). The initial phosphate concentration seems to have less influence on maximizing the output of the key variables and is suggested to be initially set within the margin of 10–20 mg L^{-1} (Fig. 6A and C).

The ROIs above show the preferred next optimization step, where the densities of suggested input configurations are the highest (Fig. 6, bright yellow color). To validate the ROIs suggestion, the final experiment was set up and included P-starvation period of one day, initial biomass of 0.25 g DW L^{-1} and initial PO_4 of 14 mg L^{-1}. Separately, the experimental source data, from which ROIs were produced, was used to train a regression model to further test the validity of its suggestions. Score,

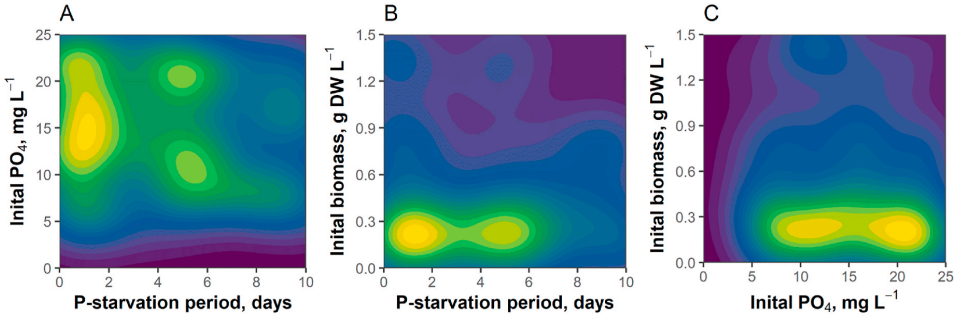

Fig. 6. AI-based platform analysis and comparison of ROIs for *C. vulgaris* to model the optimal initial conditions for efficient phosphate removal, biomass production and value-added product synthesis (A – P-starvation period and initial phosphate concentration; B – P-starvation period and initial biomass concentration; C –Initial phosphate concentration and initial biomass concentration).

previously discussed in subsection 2.4, was selected as a modeling target, since it represents all important experimental outputs to be optimized simultaneously.

For this, a random forest regressor and a linear regressor with polynomial features were used. The latter showed a better performance with a cross-validated R^2 score of 0.6 at a standard deviation of 0.2 (see Figs. S4a and S4b). The final intercept and feature coefficients are shown in Equation (6):

$$PredictedScore = 54 - 4.7a + 3.9b - 75.9c + 0.7a^2 - 0.1ab + 0.9ac - 0.1c^2 - 0.3bc + 32.4c^2 \quad (6)$$

where a is P-starvation period (days), b is Initial biomass (g DW L^{-1}), and c is Initial PO_4 (mg L^{-1}). The model outputs were then visualized (Fig. 7) and compared to ROIs.

Clearly, the results of a cross-validated model display some resemblance with suggestions achieved in ROIs. Still, it is important to state that neither ROIs nor model results could guarantee the optimized outcome for suggested optimal initial conditions before performing the validation experiment in real life as discussed in the next subsection.

3.4. Validation of the optimization model results in a sequencing batch photobioreactor

The ROI optimization model results were validated in a photobioreactor operating in a sequencing batch mode. The initial values of biomass and phosphate concentrations and P-starvation period, as suggested by the optimization model output, gave the anticipated result for all studied parameters, except for protein productivity, which might be due to the slight change (upscaling) of the final validation bioreaction process. Also, the driving factors for the results in the batch experiment series were largely present in the sequencing batch photobioreactor experiment.

The obtained biomass growth rate was 52.7% higher for the P-starved biomass than at the reference conditions (Table 2). As suggested by ROI model output, the initial biomass concentration was set lower and, thus, allowed greater light penetration and resulted in higher photosynthetic efficiency. With the P-starved biomass phosphate was removed at 101.7% higher rate than at the reference conditions. This result shows that the biomass P-starvation for one day was sufficient to establish a phosphorus deficiency status and promote enhanced phosphate removal. The development of P-deficiency was indicated by polyphosphate content decrease at the end of each batch cycle (Fig. S3).

Table 2
Kinetic parameter values for the reference and 1-day P-starved *C. vulgaris* biomass in sequencing batch photobioreactor (means ± SD).

	Biomass growth rate, d^{-1}	Specific PO_4 removal rate, mg g^{-1} DW h^{-1}	Polyphosphate accumulation rate, d^{-1}	Protein productivity, mg g DW d^{-1}
Reference (n = 4)	0.072 ± 0.016	0.279 ± 0.134	0.280 ± 0.066	1.682 ± 0.210
P-starved (n = 5)	0.110 ± 0.024	0.563 ± 0.082	0.666 ± 0.063	1.031 ± 0.365

Moreover, the measured alkaline phosphatase activity showed moderate increase when the biomass polyphosphate content was at a decline (Fig. S3), indicating that the cell seeks to obtain substrate from external source using enzymatic hydrolysis of the organic phosphorus (Ghyoot et al., 2014; Wang and Xu, 2016).

The observed protein productivity for P-starved biomass was 38.7% lower than at the reference conditions. Such an outcome is not in compliance with the optimization model output nor with the regression model prediction, as discussed further in this subsection. It rather agrees with the result obtained in batch experiment series where protein productivity after 1-day P-starvation did not show any significant difference from the reference conditions. It can be hypothesized that the polyphosphate accumulation rate achieved in the photobioreactor experiment was insufficient to provide enough material for energy transfer molecules and further protein synthesis.

Overall, the optimum initial values predicted by the ROI model were favorable for efficient microalgae-based wastewater post-treatment and allowed to sustain enhanced phosphate removal and polyphosphate accumulation for an extended period.

The achieved validation results were used to retrain the polynomial regression model. Interestingly, the cross-validated R^2 score fell from 0.6 to 0.5. The reason for model quality decrease could mean any of the following: either an important inexplicable training point was found, or there occurred an unknown discrepancy within the setup of a photobioreaction process, or a confounding or stochastic parameter exists, or a process is more non-linear than previously expected. Since the polynomial regression model was cross-validated and thus not overfitted, the validation experiment does look like an anomaly that doesn't align with polynomial regression model's expected outcome. Still, a better model would need more high-quality training data, which could explain why optimization is more important than modeling when number of available data points is low. Therefore, optimization such as

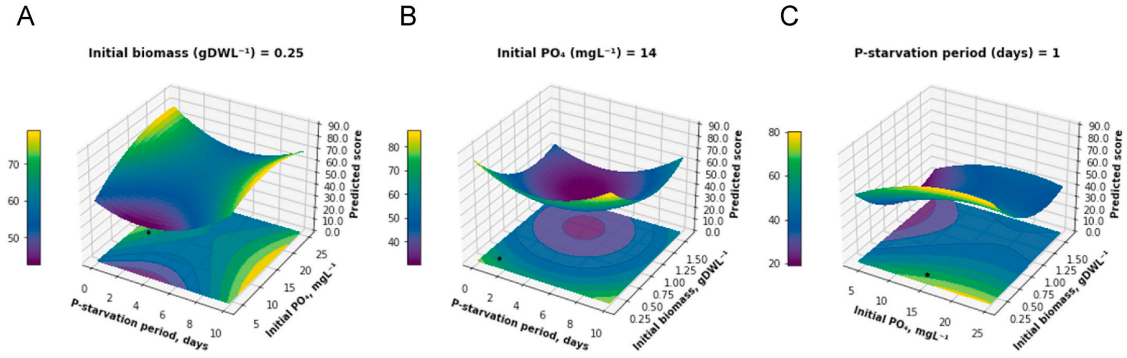

Fig. 7. Surface plots for model's predicted score values at varying input configurations (A – P-starvation period and initial phosphate concentration at constant initial biomass selected from ROIs; B – P-starvation period and initial biomass concentration at constant initial phosphate concentration selected from ROIs; C – Initial phosphate concentration and initial biomass concentration at constant P-starvation period selected from ROIs). Black markers on charts show preferred initial conditions as selected from ROIs.

ROI could be used to further gather more informative data points that could lead to better regression modeling results and better overall understanding of a complex photo-bioreaction process. It was also observed that if experimental points 11, 25, 37, 43, 45 together with the validation experiment (point 46) were completely omitted from regression model training and testing, the final model could achieve cross-validated R^2 of 0.73 at a standard deviation of 0.15 (see Figs. S5a and S5b). Finally, by using the same logic and completely omitting points 11, 25, 37, 43, 45, 46 from cross validated training and testing an even better regression model was found. This regression model was based on gradient boosting and achieved cross-validated R2 of 0.75. Interestingly, filled contour plots for gradient boosting regression model's predicted score values at varying input configurations achieved comparably similar results to those from ROI model's density heatmaps (see Fig. S6), further validating the initial optimal conditions selected by ROI model.

3.5. Application in pilot-scale systems

The results form Section 3.4. show that the biomass P-starvation works well in a laboratory scale column photobioreactor operating in a sequencing batch mode. However, to facilitate wastewater post-treatment with an integrated biomass P-starvation technology in a pilot scale, the process operation requires multiple units. Firstly, increasing the number of units would allow a simultaneous biomass P-starvation under sequencing batch mode. Further, such approach decreases the overall hydraulic retention time for the post-treatment process. This way a unit to receive the incoming effluent after secondary treatment process is always available while at the remaining units the biomass P-starvation process undergoes a phosphorus deficiency certain phase. On the other hand, such a technological design approach requires high capital and operational costs. Secondly, it has been argued that maintaining the same surface-to-volume ratio is a critical measure for successful process transfer from laboratory to pilot scale (Norsker et al., 2019). Setting up multiple units for the wastewater post-treatment process would allow maintaining the surface-to-volume ratio used for the initial process validation.

Scaling up the wastewater post-treatment process from laboratory to pilot scale involves considerations for the most economically viable design solutions. It can result in a completely different photobioreactor design for the process implementation at a pilot scale. The most impactful parameters on biomass growth and nutrient removal are temperature and lighting regimes which in a natural environment do not comply with the artificial laboratory conditions. To avoid a disparity between laboratory and full scale conditions, Benner et al. (2022) suggests that the scale-up of algae-based wastewater post-treatment supplemented with biomass P-starvation engages a process modeling approach where the microalgae growth kinetics is combined with the physics of light transport in suspensions and fluid dynamics. Present study shows that the suggested optimization model for initial parameter values can further support a successful scale-up of the process that has been validated in laboratory conditions.

4. Conclusions

Microalgal species *C. vulgaris* is capable of enhanced phosphate removal from wastewater after its biomass exposure to phosphorus deficiency conditions. After 10-day P-starvation, the specific phosphate removal rate reached 7.8 mg g DW h^{-1}. The enhanced phosphate removal is mainly driven by depletion of cellular P reserves and activation of P transporters. *C. vulgaris* can also accumulate polyphosphate at higher rate after 10-day P-starvation, reaching up to 40-fold increase compared to reference conditions. The gradual reduction of accumulated polyphosphate indicates that majority of polyphosphate is utilized for cell metabolite synthesis, including protein. Biomass protein productivity is thought to be indirectly related to P-deficiency as it accelerates the synthesis of adenosine triphosphate (ATP) – an energy source for protein synthesis. The protein productivity by *C. vulgaris* biomass shows a substantial increase after 10-day P-starvation when compared to reference conditions. The effect biomass P-starvation is further supported by lower initial biomass concentration which allows higher light availability and increases cellular activity. The initial phosphate concentration had a lesser impact on the overall *C. vulgaris* biochemical performance. The validation of modeled optimum initial conditions in sequencing batch photobioreactor shows that microalgae exposure to phosphate deficiency for one day is enough to sustain a rapid phosphate removal and polyphosphate accumulation. This approach can be a good strategy for improved algae-based municipal wastewater post-treatment.

Credit author statement

AL designed the experiments and methodology, performed formal data analysis and visualization, wrote the original draft, worked on funding acquisition; **LM** edited and reviewed the draft, worked on funding acquisition, administrated the project; **PC** designed the regression models, wrote the original draft on optimization and modeling content; **TJ** edited and reviewed the draft, worked on funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Linda Mezule reports financial support was provided by Latvian Council of Sciences.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.jenvman.2022.116313.

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